Università degli Studi di Pavia
Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

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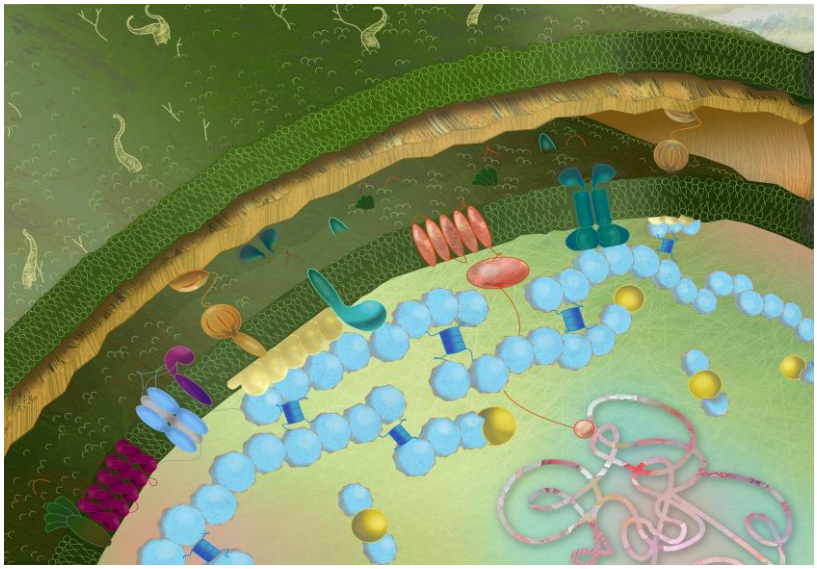

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Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
XXXIII Ciclo – A.A. 2017-2020

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Cover image: representation of the *Escherichia coli* division machinery.
Created by Ornella Trespidi

A tutta la mia famiglia e in particolare a Diletta

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Abstract

Burkholderia cenocepacia has emerged as an important opportunistic pathogen for patients suffering from cystic fibrosis. This bacterium is able to establish aggressive infections in the lungs, colonizing the viscous mucus covering the airways epithelium. Moreover, *B. cenocepacia* shows extremely high levels of drug resistance, making its eradication almost impossible. Bacterial divisome is considered a valuable pool of druggable targets, and this was confirmed by the characterization of C109, a potent inhibitor of the FtsZ GTPase activity, effective against *B. cenocepacia* and a broad-spectrum of Gram-positives and -negatives. To find more putative cellular targets, the division mechanism of *B. cenocepacia* was explored, starting from the characterization of the division cell wall (*dcw*) operon. The cluster transcriptional organization was assessed by identifying the transcription units and the transcription start site, as well as the promoter and the binding site of MraZ, the *dcw* transcriptional regulator. Moreover, the FtsZ interactome was dissected in *B. cenocepacia*, finding significant differences from the most studied microorganisms. Finally, the biofilm inhibitory potential and the cytotoxicity of the C109 nanosuspension were tested in an *in vivo*-like three-dimensional lung epithelial cell model. This work provides an overview of the *B. cenocepacia* division pathway, which will be essential for the target-based design of new drugs, and validate the antimicrobial activity of the C109 in a physiologically relevant model.

Abbreviations

CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
Bcc	*Burkholderia cepacia* complex
QS	Quorum sensing
RND	Resistance nodulation division family
dcw	Division cell wall operon
C109	Compound 10126109
TPGS	D-α-tocopheryl polyethylene glycol 1000 succinate
TPGS109	C109 nanocrystals stabilized with TPGS
5'-RACE	5'-rapid amplification of cDNA ends
IMAC	Immobilized metal-ion affinity chromatography
EMSA	Electrophoretic mobility shift assay
6-Fam	6-carboxyfluorescein
BACTH	Bacterial adenylate cyclase two-hybrid system
MBP	Maltose binding protein

1. Introduction

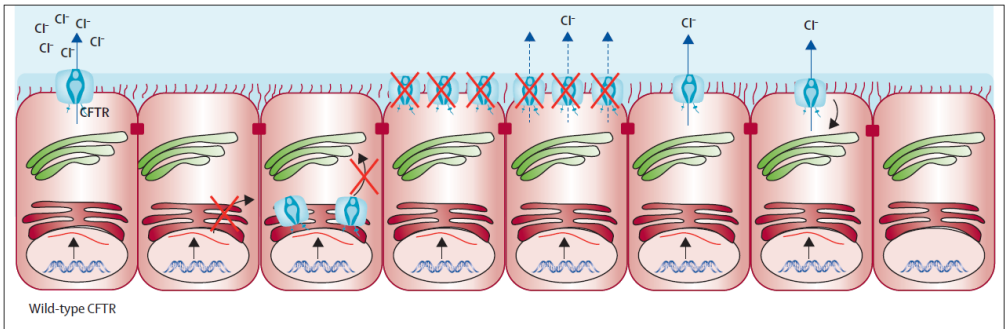
1.1 Cystic fibrosis

Cystic fibrosis (CF) is the most common genetic disease among Caucasians, caused by mutations on both alleles of the gene *CFTR* (cystic fibrosis transmembrane conductance regulator). This condition leads to the formation of a defective protein in the whole body, affecting multiple organ systems, in particular lungs and pancreas. Historically, CF was first described by Dorothy Andersen in 1938, since until that time CF symptoms had been associated to celiac disease (Andersen, 1938). Since then, the understanding of the disease increased constantly; in 1946 it was recognized to be genetic with an autosomal recessive transmission pattern (Andersen and Hodges, 1946), while, few years later, in 1950s, CF started to be associated with high sweat chloride concentration and lung infections, not only with mucus abnormalities (Di Sant'Agnese *et al*., 1953). However, the characterization of the chloride transport defect as the main cause of CF and finally the identification of the *CFTR* gene occurred only in the 1980s (Kerem *et al*., 1989; Riordan *et al*., 1989; Rommens *et al*., 1989). At present, more than 100,000 people worldwide suffer from CF, but thanks to the improvements of symptomatic treatments, the life expectancy in developed countries has increased from few months in 1950s to more than 40 years now (Burgel *et al*., 2015). The first characterization of the CFTR gene and protein paved the way to the subsequent studies on the molecular pathology of CF, which result essential to develop a cure for this life-shortening disorder.

1.1.1 CFTR defects in CF

The *CFTR* gene is located on the long arm of the chromosome 7 (7q31.2) and consists of 189kb divided in 27 exons encoding a protein of 1480 amino acids (Bear *et al*., 1992). It is classified as member of the ATP-binding cassette transporter family (ABC), being its activity dependent on the ATP hydrolysis. However, unlike most proteins of this family, which actively transport substrates against their gradient, it uses the chemical energy of the ATP hydrolysis to conduct anions down their electrochemical gradient

forming a channel pore. The mature channel is composed of two transmembrane domains forming an anion-selective pore, as well as two cytoplasmic ATP-binding domains and a regulatory (R) domain that allows the channel to open only after phosphorylation by cAMP-dependent protein kinase (Csanády *et al*., 2019). In healthy individuals, CFTR, after the proper folding of the cytosolic domains in the endoplasmic reticulum and glycosylation at the Golgi, is transferred to the cell membrane, where it functions as chloride/bicarbonate channel. In CF patients more than 2000 mutations of *CFTR* have been identified so far (http://www.genet.sickkids.on.ca), although only about 200 of them are demonstrated to cause the disease. Among those, the most are missense mutations, but also a lower percentage of frameshift, splicing, nonsense as well as deletions or insertions and promoter mutations have been characterized. However, the deletion of the phenylalanine 508 (Phe508Δ) remains the most common in CF, being present in roughly 85% of the patients worldwide (De Boeck *et al*., 2014b). This multitude of mutations has been organized in 7 classes (Figure 1) according to their effect on the protein, since different mutations can cause the same defect. In particular, they can affect the production, folding, trafficking, channel gating and permeation or even the half-life of CFTR at the surface (De Boeck and Amaral, 2016).



	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable	No mRNA
Mutation examples	GLy542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	Ala455Glu, 3272-26A→G, 3849+10 kg C→T	c. 120del23, rPhe508del	dele2,3(21 kb), 1717-1G→A
Corrective therapy	Rescue synthesis	Rescue traffic	Restore channel activity	Restore channel activity	Correct splicing	Promote stability	Unrescuable
Drug (approved)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides, correctors, potentiators? (no)	Stabilisers (no)	Bypass therapies (no)

Figure 1. Classes of *CFTR* gene mutations and approved therapies. Class I mutations decrease the production of full-length CFTR protein; Class II mutations includes the Phe508Δ and impair the protein folding and trafficking to the cell membrane; Class III mutations affect the regulation of the channel gating; Class IV mutations decrease the anionic conductance of the CFTR; Class V mutations lead to the formation of aberrant proteins due to abnormal splicing variants; Class VI mutations reduce the lifetime of the CFTR protein in the cell membrane; Class VII mutations cause the complete ablation of the CFTR mRNA, for this reason they are considered pharmacologically unrescuable (De Boeck and Amaral, 2016).

1.1.2 Pathophysiology of CF

In humans, CFTR channel is known to be expressed mostly at the apical part of the epithelia, regulating the luminal pH and maintaining the correct surface hydration. Therefore, the impairment of its function in CF causes abnormalities in the mucus that physiologically covers the mucosal surfaces of the human body. This compromises the normal functions of multiple

organs as pancreas, liver, gut and in particular lungs (Elborn, 2016). One of the most common CF symptoms is the pancreatic insufficiency that is often present at birth or soon after. Over years, the severity of the disease increases, and many patients show also biliary cirrhosis and intestinal obstruction, which causes a severe deficiency in nutrient absorption. Moreover, the abnormal inflammatory response, elicited by the obstruction of the pancreatic ducts, destroys the pancreatic islets function, leading to CF-related diabetes (Davis, 2006). Currently, corrective targeted pharmacological therapies are available, reducing the impact of these dysfunctions on patients' quality of life. These include the administration of exogenous pancreatic enzymes, insulin replacement and a high energy diet to manage the consequences of the metabolic alterations (Cystic Fibrosis Foundation, 2019). Furthermore, CF patients present defects in sweat glands, causing an excessive salt loss and, in male, in vas deferens, leading to infertility (Elborn, 2016).

Nevertheless, the most affected organ is the lung, and respiratory failure remains the leading cause of death, accounting for 66.5% (European Cystic Fibrosis Society, 2019) and 59.3% (Cystic Fibrosis Foundation, 2019) of deaths, in Europe and USA respectively, excluding the transplant-related mortality. In lung, the homeostasis of the airway epithelium surface strongly depends on the balance between chloride/bicarbonate secretion and sodium absorption. Defective CFTR protein causes an electrolyte imbalance, reducing the chloride transport towards the epithelium surface and increasing the sodium adsorption mediated by the epithelial Na^+ channel (Figure 2). Indeed, the absence of WT CFTR is thought to promote the overactivation of this channel, leading to surface liquid dehydration, even though experiments in different models have given conflicting results (Csanády *et al*., 2019). This condition causes the impairment of the major innate defense of the lung, the mucociliary clearance. This mechanism is the airway's first line of defense that through the ciliary beating is able to remove the inhaled particles and pathogens trapped into the mucus layer covering the epithelium (Knowles and Boucher, 2002). Mucociliary clearance relies on two anatomic features: the airway surface liquid (ASL) and the ciliated apical surface of the epithelium. In healthy people, the beating of the cilia creates a current moving the mucus towards the nasopharynx, where it is expectorated. This is possible because the ASL is composed of two phases: the upper one, which consists of viscous mucus able to trap particulates; and the lower one, named periciliary layer (PCL), that is more fluid, in order to

facilitate the movement of the cilia (Knowles and Boucher, 2002). In CF, the dehydration of the mucus decreases the thickness of the PCL and increases its viscosity, impairing the airway clearance (Figure 2) and allowing the establishment of opportunistic infections (Lyczak *et al*., 2002). Moreover, the lower bicarbonate secretion decreases the ASL pH, resulting in the reduction of antimicrobial peptides activity (Shah *et al*., 2016). Among these, the key antibacterials β-defensin-3 and LL-37, highly effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, were demonstrated to decrease their individual and synergistic activity in acidic environments (Abou Alaiwa *et al*., 2014), representing another important defense mechanism of the airways impaired in CF.

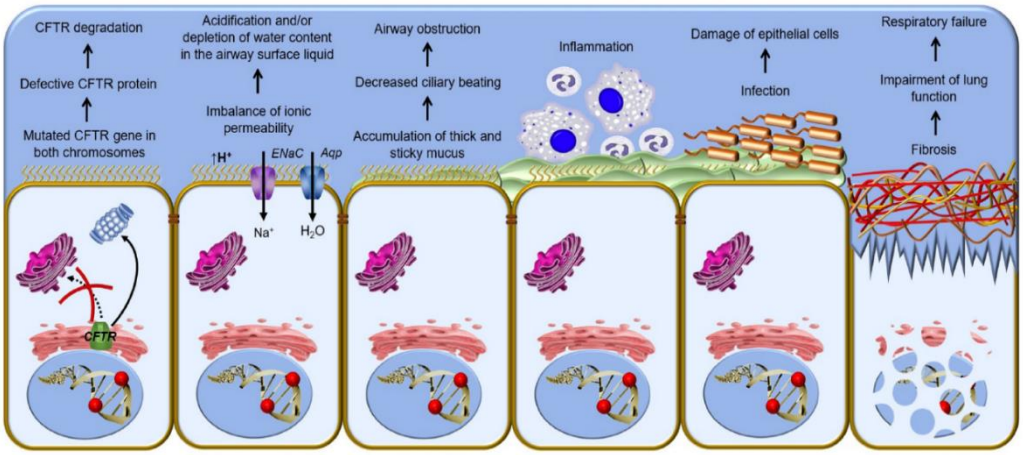


Figure 2. Lung pathology of CF. The production of a defective CFTR channel causes a dramatic imbalance of ionic permeability and water absorption, impairing the mucociliary clearance of the lung epithelial surface. This condition leads to an increased susceptibility to infections and to an uncontrolled inflammation that eventually causes respiratory failure. Aqp, aquaporin; ENaC, epithelial Na^+ Channel (Lopes-Pacheco, 2016).

Besides this, CF patients show a defective inflammatory response. This abnormal reaction is detected early in life, often shortly after birth, and in most cases leads to an uncontrolled chronic lung inflammation. Over years, this condition damages the airways architecture and compromises the respiratory function (Pillarisetti *et al*., 2011). In the early stage, the

inflammation seems independent to any infection and is characterized by the presence of a high number of neutrophils in the lower respiratory tract, likely attracted by interleukin (IL)-8. Indeed, this cytokine is present in abnormally high concentrations in CF lungs, as well as other pro-inflammatory mediators, such as IL-6, IL-1β and IL-17, further increasing inflammation and neutrophil infiltration in lungs (Armstrong *et al*., 2005; Tan *et al*., 2011). On the contrary, anti-inflammatory molecules such as IL-10 (Armstrong *et al*., 2005) and lipoxins (Karp *et al*., 2004) have been found to be reduced in CF lung, causing an imbalance in the inflammatory response. However, this increased immune activation is not coupled with a more efficient bacteria clearance, since the phagocytic capacity of CF neutrophils results impaired (Alexis *et al*., 2006). Moreover, the release of elevated quantities of neutrophil elastase, a broad-spectrum serine protease, by activated neutrophils is associated to a fast decline in pulmonary functions and the onset of bronchiectasis. Indeed, an excess of this enzyme, usually involved in bacterial killing, leads to the degradation of fibronectin and elastin as well as triggers the activation of the metalloprotease 9, causing the disruption of the lung tissue (Cohen-Cymberknoh *et al*., 2013). Nevertheless, other immune cells are involved in this pathological process as well, such as eosinophils (Keown *et al*., 2019), lymphocytes (Tan *et al*., 2011) and monocytes (Tarique *et al*., 2019), although probably with a less prominent role. Finally, using CF epithelial cell cultures, it was demonstrated that the airway epithelium itself could produce an increased quantity of pro-inflammatory mediators, promoting the uncontrolled inflammation of lungs (Cigana *et al*., 2007).

The administration of anti-inflammatory therapies is thought to be a good way to reduce this pathological condition. For this reason, several therapies have been tested, but only few of them resulted to have a beneficial effect. Indeed, the use of potent anti-inflammatory drugs can reduce the effectiveness of the immune response against the pathogens and so worsen the lung infections (Konstan *et al*., 2014). Nowadays, Azithromycin and inhaled corticosteroids are the most used in CF, reducing the rate of lung function decline (Cystic Fibrosis Foundation, 2019). Nevertheless, other promising molecules, including protease inhibitors and antioxidants, are currently undergoing clinical trials and, in the future, they could broaden the therapeutic spectrum for this pathology (De Boeck and Amaral, 2016).

1.1.3 CFTR modulator therapies

The improvements in symptomatic therapeutic regimens have led to the substantial increasing of life expectation for CF patients seen in the last 30 years. Despite this, the disease remains incurable and the quality of life of these people is still low. The introduction of the CFTR modulators could revolutionize the therapeutic approach for CF, directly targeting the channel protein defects. Indeed, these molecules are able to increase or even restore the correct expression and maturation of the protein CFTR, although, until now, only in patients with specific mutations. These compounds are grouped in 5 classes, based on their effect on the protein: potentiators, able to restore the channel gating and conductance; correctors, used to rescue the protein folding, maturation and trafficking; stabilizers, which increase the protein stability at the plasma membrane; read-through agents, able to rescue the protein synthesis; amplifiers, used to increase the abundance of the protein (Lopes-Pacheco, 2020).

The first modulator that reached the market was ivacaftor (Kalydeco®), a potentiator approved in 2012 by both FDA and EMA, which is administered to patients with a gating mutation in at least one allele (De Boeck *et al*., 2014a). Currently, ivacaftor is also used in combination with first- and second-generation correctors, lumacaftor/ivacaftor (Orkambi®) and tezacaftor/ivacaftor (Symdeko®), combining the two effects to increase the quantity of active channel at the membrane in patients homozygous for Phe508∆ or heterozygous for Phe508∆ but with a residual function mutation in trans (McNamara *et al*., 2019; Walker *et al*., 2019). Finally, the most recent modulator approved is the corrector elexacaftor that is administered in combination with tezacaftor/ivacaftor in the first triple combination (Trikafta®), further increasing the therapeutic activity of the older combination (Taylor-Cousar *et al*., 2019). Although they do not fully restore the CFTR function, these modulators are proving to be effective in decreasing the severe CF symptoms in adults and preventing the rapid deterioration of lungs and pancreas in younger patients, giving high expectations for the future development of improved and broader spectrum therapies.

Nowadays, approximately 80% of CF patients are eligible for CFTR modulators, but the individuals with rare or unique mutations remain without therapeutic options. However, even though the molecules currently available belong only to two of the five classes described above, several new potential

modulators are being tested in clinical trials, and within the next years some of them could be approved, increasing the eligibility for these therapies (Lopes-Pacheco, 2020).

1.2 CF-associated lung infections

The human respiratory system is conventionally divided into upper airways that, in healthy individuals, are colonized by numerous commensal microorganisms, and lower airways, kept sterile by mucociliary clearance and innate immunity. Differently, in CF patients the detection of pathogens in the lower respiratory tract secretions starts early in childhood and persists for their entire life. Indeed, defective CFTR protein causes the dehydration of the airway mucus, blocking the mucociliary clearance mechanism (Lyczak *et al*., 2002). Moreover, in these patients, both innate and adaptive immune system are dysregulated in lung, leading to a chronic activation that nevertheless is unable to clear the infections (Hartl *et al*., 2012). Taken together, these factors lead to microenvironment alterations in CF airways. In particular, lungs are characterized by a thickened hypoxic mucus layer that promotes the slow growth of bacteria in biofilms, as well as interfering with the normal migration of immune cells and the diffusion of antibacterial molecules (Lyczak *et al*., 2002). This condition facilitates the establishment of persistent opportunistic infections.

Using different molecular techniques and culture methodologies, viruses (Billard *et al*., 2017) and fungi (King *et al*., 2016) are being increasingly detected and isolated from CF lungs, but bacteria are still the most prevalent microorganisms isolated from sputum. In particular, *Staphylococcus aureus* and *Haemophilus influenzae*, usually considered non-pathogenic (Verhagen *et al*., 2013), are the most prevalent during childhood, instead *Pseudomonas aeruginosa* and the emerging pathogens *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and non-tuberculous mycobacteria are mostly acquired by adults (Figure 3). These bacterial species have different prevalence depending on country and age, but overall *P. aeruginosa* and *S. aureus*, including Methicillin-resistant *S. aureus* (MRSA), remain the most prevalent infections in CF (Cystic Fibrosis Foundation, 2019; European Cystic Fibrosis Society, 2019). However, besides them, the species of the *Burkholderia cepacia* complex (Bcc) are enumerated among the most lethal

pathogens for these patients. Indeed, despite infecting only a small percentage of CF individuals (around 3%) it is demonstrated that they lead to a faster deterioration of the respiratory function, strongly reducing patients' survival rate (Jones *et al*., 2004).

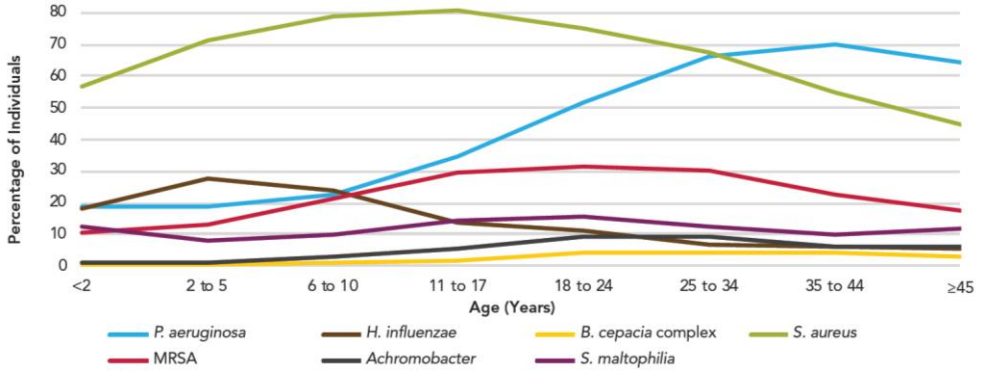


Figure 3. Prevalence of respiratory microorganisms in CF patients in US by age cohort (Cystic Fibrosis Foundation, 2019).

1.2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, motile, non-lactose fermenter environmental bacillus, easily cleared by host defenses of healthy people. Unfortunately, it is also associated with invasive and fulminant infections in immunocompromised individuals, as well as chronic lung infections in CF patients, which persist months to decades (Faure *et al*., 2018). Although it is still the most common bacterium isolated in adults with CF, the data show a constant decrease in *P. aeruginosa* prevalence since the late 90s (Cystic Fibrosis Foundation, 2019). This is probably due to the implementation of aggressive antibiotic treatments to eradicate initial acquisition and decrease the probability of poor outcome. Indeed, the establishment of *P. aeruginosa* chronic infection can cause accelerated respiratory function decline and premature death, representing a major threat for CF patients (Kerem *et al*., 2014).

In Europe, in 2017, the prevalence of *P. aeruginosa* chronic infections in all patients ranged between 14 - 62% (European Cystic Fibrosis Society, 2019), showing a significant variability between countries. This difference is likely

due to several factors, as different environmental distribution of the bacterium, different infection control protocols, as well as country-dependent inclusion criteria.

P. aeruginosa is commonly acquired by CF patients from the environment, even though cases of patient-to-patient transmission have been reported (Knibbs *et al*., 2014), showing initially the characteristics of an acute infection. Over time, host-pathogen interactions evolve, and the bacterium decreases the production of the virulence factors necessary for the initial colonization, mainly by accumulating mutations in global regulators (Winstanley *et al*., 2016). Moreover, during this transition it starts the overproduction of the exopolysaccharide alginate, switching from the non-mucoid to the mucoid phenotype. Alginate, and the other two exopolysaccharides produced by *P. aeruginosa*, Psl and Pel, are fundamental for the establishment and maintenance of its biofilm structure and promote the persistence of the infection by increasing host immune evasion (Faure *et al*., 2018).

The intrinsic *P. aeruginosa* high resistance to antibiotics, in particular β-lactams, results in a limited panel of effective therapies available and, after the transition to the mucoid phenotype, the infection is considered almost impossible to eradicate (Cohen-Cymberknoh *et al*., 2016). Currently, tobramycin is the most used antibiotic against this bacterium in CF patients: the inhalable formulation is used to control chronic infections, whereas intravenous tobramycin is used in case of exacerbations (Langton *et al*., 2017). Moreover, aztreonam, amikacin, ciprofloxacin, and colistin have been validated as good alternatives to tobramycin (Hansen and Skov, 2015; Langton *et al*., 2017; Caceres Guido *et al*., 2019).

1.2.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-motile and non-spore forming coccus, usually found as a commensal in the anterior nares and skin of humans. However, it is notoriously also the leading cause of acute and chronic infections involving bloodstream, skin, soft tissue and respiratory tract (Tong *et al*., 2015). Often *S. aureus* is associated with nosocomial acquired infections, forming persistent biofilms on medical implants (Suresh *et al*., 2019). In CF, it is usually the first respiratory infection acquired, and the most prevalent during childhood, reaching more than 80% of prevalence in some European countries (European Cystic Fibrosis Society, 2019) and

in the United States (Cystic Fibrosis Foundation, 2019). In adults, the prevalence decreases, although a significant percentage of patients carries this infection (European Cystic Fibrosis Society, 2019; Cystic Fibrosis Foundation, 2019).

The pathogenicity of *S. aureus* chronic colonization in CF lung is still debated, even though a recent study demonstrated that in certain conditions it is correlated to a more severe lung disease. In particular, the high bacterial density in sputa, the appearance of small-colony variants, and the coinfections with pathogens as *P. aeruginosa* and *S. maltophilia* were found as critical risk factors for worse lung deterioration (Junge *et al*., 2016). Instead, the presence of MRSA infections is clearly associated with respiratory function decline, frequent hospitalization, increased administration of antibiotics, and higher risk of premature death (Ren *et al*., 2007; Dasenbrook *et al*., 2010). The methicillin resistance of these strains is given in most cases by the expression of an altered penicillin binding protein, encoded by the gene *mecA*, although some MRSA strains show *mecA*-independent resistance mechanisms (Hryniewicz and Garbacz, 2017). The threat of MRSA is particularly serious in USA, since the prevalence of these infections dramatically increased in the last 20 years, and today more than 20% of young adults with CF harbor the pathogen (Cystic Fibrosis Foundation, 2019).

Currently, there are no specific international guidelines for *S. aureus* treatment in CF; nevertheless, the administration of antibiotic prophylaxis is considered the best strategy, in particular for MRSA strains, to prevent the onset of severe symptoms (Akil and Muhlebach, 2018). Concerning this, in the United Kingdom the randomized registry trial CF START (www.cfstart.org.uk) is ongoing, with the objective to assess the safety and efficacy of flucloxacillin as a long-term prophylaxis agent for infants with CF.

1.2.3 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is a Gram-negative, motile, ubiquitous, rod-shaped bacterium, isolated from diverse environmental sources, including extreme natural and man-made niches (Hayward *et al*., 2010). Although this bacterium does not infect healthy individuals, it is enumerated among the most dangerous emerging nosocomial pathogens. Indeed, *S. maltophilia* can infect immunocompromised people, causing numerous clinical syndromes, including endocarditis, meningitis, pneumonia, and urinary tract infections.

Its ability to adhere and form recalcitrant biofilms on abiotic surfaces, such as medical devices, as well as contaminate disinfectants, make this bacterium a severe threat for hospitalized patients (Brooke, 2012). This microorganism is also known as CF pathogen, causing chronic infections in lungs, and its prevalence in these patients has increased over years (Cystic Fibrosis Foundation, 2019). This trend has been correlated with the increasing administration of antibiotics. In particular, the extensive use of antipseudomonal agents seems to promote lung colonization by *S. maltophilia* (Talmaciu *et al*., 2000). Although the effect of its infection on lung function is still not completely clarified, a recent cohort study demonstrated a correlation between *S. maltophilia* acquisition and acceleration in respiratory decline in CF individuals (Barsky *et al*., 2017). Moreover, a serious issue for these patients is the misidentification of this bacterium as part of the Bcc (McMenamin *et al*., 2000) or as *P. aeruginosa* (Kidd *et al*., 2009), leading to the administration of ineffective treatments.

Regarding the therapies against *S. maltophilia*, currently there is no consensus for the management of these infections and the role of antibiotic treatments remains unclear (Amin and Waters, 2016). However, the development of a potential therapy results challenging, since the bacterium exhibits several intrinsic and acquired antibiotic and stress resistance mechanisms (Sanchez, 2015).

1.2.4 *Achromobacter xylosoxidans*

Achromobacter xylosoxidans is a Gram-negative non-fermenting, motile rod commonly found in soil and aquatic environments. It is also known as multidrug resistant opportunistic pathogen correlated with numerous human infections and CF. Indeed, it is able to colonize CF lungs causing infections with a prevalence that varies between 3 and 30% (Pereira *et al*., 2011; Cystic Fibrosis Foundation, 2019). *A. xylosoxidans* is prevalently acquired from the environment, although cases of patient-to-patient transmission have been reported (Pereira *et al*., 2011).

The clinical impact of these infections on CF patients is not completely understood yet, even though two recent Canadian studies tried to elucidate this point. The first study analyzed a cohort of patients attending an adults CF clinic for 29 years, proving that there was no worsened long-term prognosis associated with *Achromobacter* infections. Most patients were able to clear the infection, even when classified as persistent. However,

incident infections were associated with a greater risk of pulmonary exacerbations (Edwards *et al*., 2017). The second study was performed on a larger population of both adults and children and confirmed the absence of long-term detrimental effects on pulmonary functions in case of chronic infection but evidenced a greater risk of death or transplantation (Somayaji *et al*., 2017).

Currently, no consensus data are available regarding the antibiotic treatment against *A. xylosoxidans* initial infections or exacerbations in CF patients. Nevertheless, it is reported that the early treatment with combinations of inhaled and intravenous antibiotics successfully delays or prevents the establishment of chronic infections (Wang *et al*., 2013).

1.2.5 Non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) are commonly found in the environment, both in water sources and in soil. Most of them are non-pathogenic to healthy humans, but they are reported as opportunistic pathogens in immunocompromised or CF patients. The species mainly associated with lung infections are the NTM of the *Mycobacterium avium* complex (MAC) (*Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium chimaera*) and the subspecies of the *Mycobacterium abscessus* complex (MABSC) (*Mycobacterium abscessus* subsp *abscessus*, *Mycobacterium abscessus* subsp *massiliense* and *Mycobacterium abscessus* subsp *bolletii*) (Floto *et al*., 2016). In CF patients NTM acquisition is correlated with age, since the prevalence increases constantly over years, from 10% in 10 years old children to around 30% in 40 years old adults (Rodman *et al*., 2005). However, a correlation between age and the prevalent NTM species isolated has been demonstrated. Indeed, MABSC bacteria infect prevalently young patients with a severe CF phenotype, whereas MAC bacteria are often isolated from adults with a less severe disease (Catherinot *et al*., 2013).

Concerning transmission, the cross-infection between patients is considered unlikely, as confirmed by a recent study. In this paper, 48 patients, from four CF centers in Italy, carrying *M. abscessus* infections, were followed for 10 years, periodically analyzing the strain harbored by each individual. The unchanged presence of a variety of different *M. abscessus* strains demonstrated a very low risk of inter-human transmission (Tortoli *et al*., 2017). Currently, NTM infections are increasingly monitored since they can

cause a severe inflammatory condition termed “NTM pulmonary disease”, leading to progressive lung damage (O'Connell *et al*., 2012). For this reason, the increasing prevalence of these infections reported in US and Europe (Cystic Fibrosis Foundation, 2019; European Cystic Fibrosis Society, 2019) represents a major threat for CF patients’ health. In response to this challenge, a joint effort of the European and US CF societies led to the development of international guidelines for the screening, diagnosis and management of NTM pulmonary infections in these individuals (Floto *et a*l., 2016). Thanks to this, now CF clinics have a standard protocol to follow for the management of these infections, even though it is fundamental to consider the strain specific antibiotic resistance, which is known to be intrinsically very high in NTM (Waters and Ratjen, 2016).

1.3 The genus *Burkholderia*

The genus *Burkholderia* was named after the American plant pathologist W. H. Burkholder, who published the first report describing *Burkholderia cepacia* as the phytopathogen causing sour skin of onion (Burkholder, 1950). Initially, due to its similarity to the pseudomonads, this Gram-negative bacterium was classified as *Pseudomonas cepacia*, until Yabuuchi and colleagues (1992) proposed the creation of the new genus *Burkholderia.* At that time, the novel genus contained only seven species and *B. cepacia* was the type species. Since then, more than one hundred species have been ascribed to this genus which includes a very heterogeneous group of bacteria isolated from virtually every ecological niche. The extraordinary variety of environments in which *Burkholderia* can be found indicates an incredible adaptability to different conditions, implying a great metabolic versatility. This makes them interesting for several biotechnological applications as: the degradation of toxic xenobiotics, producing a variety of hydrolytic enzymes; the production of antimicrobial and antifungal secondary metabolites; the stimulation of plant growth (Figure 4) (Chiarini *et al*., 2006; Compant *et al*., 2008). However, some of them can be pathogenic for eukaryotic hosts, as human, animals and plants (Figure 4), making their application as biocontrol agents hazardous and, accordingly, severely restricted (Eberl and Vandamme, 2016). Therefore, a major effort to discriminate between pathogenic and beneficial *Burkholderia* has been made.

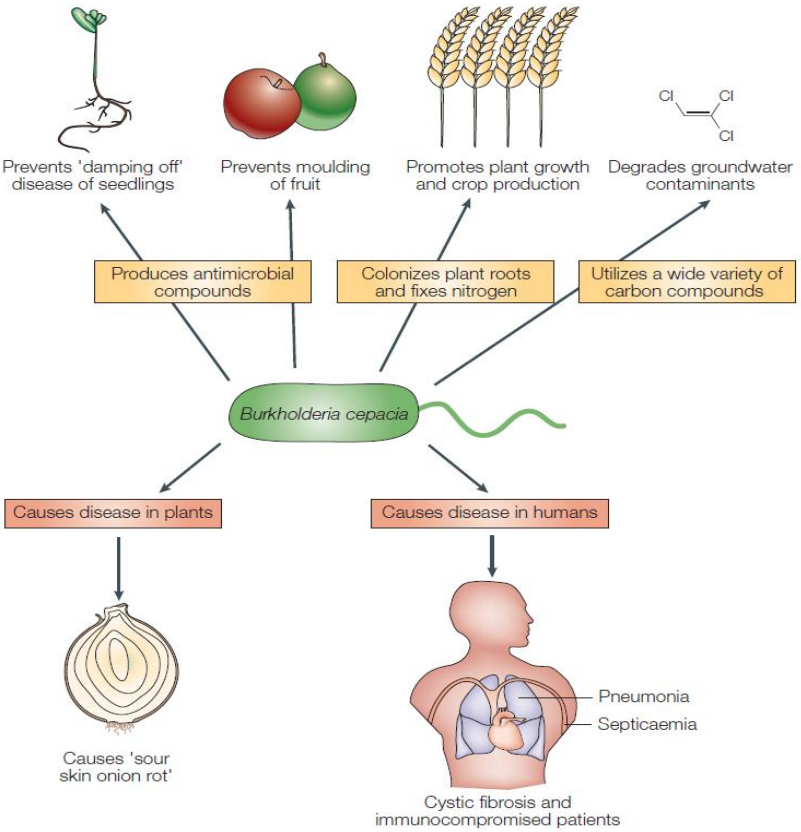


Figure 4. *Burkholderia cepacia* complex beneficial effects and human health threat. Bcc bacteria have a high biotechnological potential, producing antimicrobial compounds, increasing crop production, and degrading toxic pollutants. However, they are also known as opportunistic pathogens, limiting their use as biocontrol agents (Mahenthiralingam *et al*., 2005).

This eventually led to the recent subdivision of the genus in *Burkholderia sensu stricto*, which contains the most pathogenic species, and in other six genera: *Paraburkholderia* (Sawana *et al*., 2014), *Caballeronia* (Dobritsa and Samadpour, 2016), *Robbsia* (Lopes-Santos *et al*., 2017), *Mycetohabitans* and *Trinickia* (Estrada-de Los Santos *et al*., 2018), *Pararobbsia* (Lin *et al*., 2020), which encompasses primarily environmental species. Currently,

Burkholderia sensu stricto includes 32 validly named species and among them there are plant pathogens, as *Burkholderia glumae* (Ham *et al*., 2011), *Burkholderia gladioli* (Lee *et al*., 2016) and *Burkholderia plantarii* (Wang *et al*., 2016), but also the human and animal pathogens of the *Burkholderia cepacia* complex and *Burkholderia pseudmallei* complex (Tuanyok *et al*., 2017; Vandamme *et al*., 2017). This complex comprises *Burkholderia mallei* and *Burkholderia pseudomallei*, classified as category B bioterrorism agents by the US Centers for Disease Control and Prevention (Larsen and Johnson, 2009).

1.3.1 *Burkholderia cepacia* complex

Burkholderia cepacia complex (Bcc) is a cluster of non-spore-forming, aerobic Gram-negative bacilli, with remarkable genetic and phenotypic similarities. To date, in 2020, Bcc is composed of 24 closely related species (Table 1), even though the name of two species, *B. paludis* and *B. aenigmatica*, is still awaiting the official validation. Originally, some of these bacteria were known as different genomovars (phenotypically similar but genotypically distinct bacteria) of *Burkholderia cepacia*, but, after a molecular polyphasic approach, they were finally classified as separate species with official binomial names (Vermis *et al*., 2002).

Species name	Reference
Burkholderia cepacia (genomovar I)	Yabuuchi et al., 1992
Burkholderia multivorans (genomovar II)	Vandamme *et al*., 1997
Burkholderia cenocepacia (genomovar III)	Vandamme *et al*., 2003
Burkholderia stabilis (genomovar IV)	Vandamme *et al*., 2000
Burkholderia vietnamiensis (genomovar V)	Gillis *et al*., 1995
Burkholderia dolosa (genomovar VI)	Vermis *et al*., 2004
Burkholderia ambifaria (genomovar VII)	Coenye *et al*., 2001a
Burkholderia anthina (genomovar VIII)	Vandamme *et al*., 2002
Burkholderia pyrrocinia (genomovar IX)	Vandamme *et al*., 2002
Burkholderia ubonensis (genomovar X)	Yabuuchi *et al*., 2000
Burkholderia latens	Vanlaere *et al*., 2008
Burkholderia diffusa	Vanlaere *et al*., 2008
Burkholderia arboris	Vanlaere *et al*., 2008
Burkholderia seminalis	Vanlaere *et al*., 2008
Burkholderia metallica	Vanlaere *et al*., 2008
Burkholderia contaminans	Vanlaere *et al*., 2009
Burkholderia lata	Vanlaere *et al*., 2009
Burkholderia pseudomultivorans	Peeters *et al*., 2013
Burkholderia stagnalis	De Smet *et al*., 2015
Burkholderia territorii	De Smet *et al*., 2015
Burkholderia paludis	Ong *et al*., 2016
Burkholderia catarinensis	Bach *et al*., 2017
Burkholderia puraquae	Martina *et al*., 2018
Burkholderia aenigmatica	Depoorter *et al*., 2020

Table 1. *Burkholderia* species currently included in the *Burkholderia cepacia* complex.

The identification of the Bcc species has always been problematic since the classically used 16S rRNA or the *recA* gene sequence analysis approaches are not able to discriminate between the single species, due to their very high gene sequence identities (16S rRNA: 98%–100%; *recA*: 94%–95%) (Coenye *et al*., 2001b). Phenotypic analysis and biochemical tests are reported to lead often to misidentifications; for this reason, several different approaches have been tested for their reliability and accuracy (Vandamme and Dawyndt, 2011). Among them, the *recA*-restriction fragment length polymorphism, which relies on the use of species-specific PCR primers and the analysis of a specific portion of the gene *recA*, is one of the best techniques available for this purpose, allowing the identification of most Bcc species (Payne *et al*., 2005). In more recent years, matrix-assisted laser desorption ionization time-of-flight mass spectrometry has been used for the identification of these species, giving promising results, although the accuracy is lower compared to the *recA* gene sequencing (Fehlberg *et al*., 2013). Finally, the most powerful taxonomic tool available is the multilocus sequence analysis, based on the study of seven housekeeping genes, that can discriminate the individual Bcc members at the strain level (Baldwin *et al*., 2005).

These methods allow the fast and unambiguous identification of these bacteria that is fundamental for an early diagnosis and treatment since most species of the complex have been characterized as human opportunistic pathogens. In particular, they are known to establish aggressive lung infections in patients with underlying medical conditions, as chronic granulomatous disease, or CF. Moreover, hospitalized and immunocompromised patients can be infected by Bcc bacteria, mainly due to their ability to contaminate disinfectants, intravenous solutions, nebulizer solutions, mouthwashes, or invasive medical devices (Tavares *et al*., 2020).

1.3.2 Bcc species as CF pathogens

The first report of *P. cepacia* in CF dates back to 1984, when Isles *et al*. (1984) described an increasing number of patients infected by this bacterium. This report pointed out the greater pulmonary function impairment caused by *P. cepacia* compared to *P. aeruginosa*, as well as the very high levels of antibiotic resistance. Since then, reports and studies concerning *Burkholderia* infections in CF have increased over years, raising our understanding of the mechanisms involved in the pathogenicity. Today, Bcc infections are particularly feared by CF patients being often associated to poor prognosis. Indeed, despite their relatively low prevalence, infecting

around 3% of CF population, upon chronic infection establishment, they cause a significant lung inflammation, leading to fast respiratory function deterioration (Mahenthiralingam *et al*., 2005). Moreover, in more than 10% of the cases, patient develops a fatal clinical syndrome characterized by high fever, severe progressive respiratory failure, leukocytosis, and elevated erythrocyte sedimentation, known as “cepacia syndrome” (Jones *et al*., 2004). This already serious condition is also worsened by the intrinsic resistance to almost every existing antibiotic, making the infection extremely hard to treat and eradicate (Rhodes and Schweizer, 2016; Scoffone *et al*., 2017). Concerning the prevalence of the Bcc species infecting CF patients, *B. cenocepacia* and *B. multivorans* are the most prevalent, although *B. vietnamiensis*, *B. cepacia*, and *B. contaminans* are increasingly isolated (Kenna *et al*., 2017). In recent years, *B. multivorans* accounts for the majority of *Burkholderia* species isolated from CF patients, as reported in USA (LiPuma, 2010), UK (Kenna *et al*., 2017) and Spain (Medina-Pascual *et al*., 2012), with the exception of Italy (Teri *et al*., 2018), Australia (Ramsay *et al*., 2013) and Canada (Cystic Fibrosis Canada, 2019). However, *B. cenocepacia* used to be the most common until 15 years ago and still remains one of the most serious.

1.4 *Burkholderia cenocepacia*

1.4.1 *B. cenocepacia* epidemiology

Representing a major threat for CF patients, this species has been the most studied; by *recA* sequence analysis it was subdivided into four phylogenetic subgroups: IIIA, IIIB, IIIC, IIID (Vandamme *et al*., 2003). This classification allowed the discrimination between the most pathogenic strains, IIIA, IIIB (Drevinek and Mahenthiralingam, 2010) and IIID (Manno *et al*., 2004), and the environmental strain IIIC (Balandreau *et al*., 2001), even though it is known that they frequently overlap (LiPuma *et al*., 2002; Baldwin *et al*., 2007).

Due to its clinical relevance, most of the research was focused on the molecular pathogenesis of the epidemic *B. cenocepacia* Electrophoretic Type-12 (ET-12), a highly transmissible lineage belonging to genomovar IIIA (Drevinek and Mahenthiralingam, 2010). ET-12 lineage emerged from Canada during 1980s, causing multiple outbreaks in CF population and also

spreading to Europe, particularly in UK. The spreading of this infection was mainly due to UK CF patients attending summer camps in Canada and caused increased mortality among CF patients during 1990s (Mahenthiralingam *et al*., 2005). The evidence of patient-to-patient transmission of *B. cenocepacia* strains have increased the awareness of the threat that they represent for CF people, and resulted in the application of stringent infection control measures to prevent further outbreaks. This has limited *B. cenocepacia* spreading among patients, but probably contributed to the increasing infection prevalence of environmental strains, such as *B. multivorans* (Saiman and Siegel, 2004).

Unfortunately, ET-12 is not the only epidemic lineage characterized, since the CZI clone, a *B. cenocepacia* IIIA strain isolated in Czech Republic, was described as its European counterpart (Drevinek *et al*., 2005). This strain is positive for the marker of transmissible *B. cenocepacia* strains (BCESM), but negative for the presence of the cable pilin subunit gene *cblA*, reported in ET-12 strains (Mahenthiralingam *et al*., 1997). After a more detailed characterization, CZI resulted the same strain type as another Canadian epidemic strain, the RAPD 01 (Speert *et al*., 2002). This raised the possibility of an intercontinental spread of this clone, even though no direct epidemiological link between Czech and Canadian patients was found (Drevinek *et al*., 2005).

In USA, instead, the main epidemic strains characterized belong to the *B. cenocepacia* IIIB subgroup, and they are known as Midwest clone and PHDC strain. The first was originally reported in the late 1980s as the cause of several infections in the midwestern region of USA (LiPuma *et al*., 1988; Coenye and LiPuma, 2002). Instead, PHDC strain takes its name from the two cities in the mid-Atlantic part of USA where it was first described, and it was reported to be endemic in different CF centers for at least two decades (Chen *et al*., 2001). Moreover, this strain was isolated also in Europe, representing the second transatlantic clone affecting European CF patients (Coenye *et al*., 2004). Surprisingly, PHDC was found also in agricultural soil in New York state, demonstrating for the first time that the natural environment can be the source of highly pathogenic *Burkholderia* strains (LiPuma *et al*., 2002).

1.4.2 *B. cenocepacia* genome

B. cenocepacia J2315 was isolated from a CF patient in Edinburgh in 1989 (Govan *et al*., 1993), and is one of the best characterized members of the ET-12 epidemic lineage. This strain is notorious for the extremely high transmissibility and virulence and caused many devastating outbreaks in CF centers in Canada and Europe (Govan *et al*., 1993). The critical clinical impact of this bacterium has increased the interest in better understanding the factors mediating its pathogenicity and its genetics, becoming the first Bcc bacterium entirely sequenced (Holden *et al*., 2009).

Interestingly, *B. cenocepacia* J2315 possesses one of the largest genomes among Gram-negative bacteria, accounting for 8.06-Mb, that reflects its extreme complexity. It shows a high G+C content and is composed of three circular chromosomes and the plasmid pBCJ2315 (Holden *et al*., 2009). Through an accurate genome annotation, it has been predicted that the four replicons encode 3.537, 2.849, 776 and 90 initial coding sequences respectively, of which 126 are partial genes or pseudogenes. It has also been noticed a distinct partitioning of functions between chromosomes, since chromosome 1 contains mainly genes encoding for core cellular functions, such as cell division and metabolism, while chromosomes 2 and 3 contain genes encoding for accessory functions and a consistent number of coding sequences with unknown functions (Holden *et al*., 2009). Concerning the genetic basis of the high virulence of this bacterium, several virulence factors have been identified, demonstrating to be well equipped with functions correlated with CF lung long term colonization. Moreover, fourteen DNA regions have been predicted as genomic islands deriving from horizontal genetic transfer, since they are not present in other less virulent *B. cenocepacia* strains (Holden *et al*., 2009). The best characterized is the *cenocepacia* island (cci), a 44-kb pathogenicity island that contains the *Burkholderia cepacia* epidemic strain marker (BCESM), as well as genes involved in arsenic resistance, antibiotic resistance, ion and sulfate family transporter, stress response, in addition to metabolism and various regulators that include an N-acyl-homoserine lactone-dependent quorum sensing system (Baldwin *et al*., 2004). Nevertheless, it has been shown that, besides the acquisition of new functions, gene loss can have an important role in infection as well, in particular in the persistence. Indeed, as in *P. aeruginosa* (Winstanley *et al*., 2016), the inactivation of genes essential for the acute infection may give an advantage to the establishment of the chronic

lung infection, thus facilitating the transmission to other patients (Holden *et al*., 2009).

1.4.3 B. *cenocepacia* pathogenicity and virulence factors

B. cenocepacia infections are notoriously the most feared by CF patients, since they are associated to reduced survival and to a higher risk of developing cepacia syndrome than the other Bcc species. The severity of these infections is well described in a UK report that compared the mortality rate over a period of 5 years in *B. cenocepacia* or *P. aeruginosa* infected CF populations. Indeed, it was shown that 33.3% and 14.7% of the patients died respectively within that time span, highlighting a dramatically higher risk of death for *B. cenocepacia* infected individuals (Jones *et al*., 2004). Moreover, *B. cenocepacia* chronic infection is often considered a contraindication for lung transplantation, which is the final treatment option for end-stage CF, since the post-transplant survival within the first year for these patients has been reported to be eight times lower than the non-infected patients (Alexander *et al*., 2008).

The extreme virulence of *B. cenocepacia* is due to its ability to colonize CF lung and adapt to its stressful environment, taking advantage of the production of several virulence factors. These include secreted, membrane-associated, or cytosolic proteins, that are essential for the bacterium to exert its pathogenicity towards the host, mediating adherence, cellular invasion, and intracellular survival. In CF lung, *B. cenocepacia* is initially entrapped within the thick mucus layer and, through specific proteins, adheres to mucin, the main component of the airway mucus. This interaction is mediated by AdhA, the 22-kDa adhesin associated with the cable pilus, a combination commonly found in strains belonging to the ET-12 lineage (Sajjan *et al*., 1992). The expression of these proteins is probably one of the factors that makes this lineage so virulent, since the increased binding ability to mucin is associated to persistent infections and cepacia syndrome (Sajjan *et al*., 1992). Moreover, AdhA and cable pilus are involved in the interaction with cytokeratin 13, a protein expressed selectively during tissue repair after an epithelial injury. This condition is hypothesized to be relevant in CF, knowing that the airway epithelium undergoes several injury and healing cycles, thus giving a further advantage to *B. cenocepacia* initial adhesion (Sajjan *et al*., 2000).

To establish the infection, secreted proteins are known as important virulence factors for this bacterium: ZmpA and ZmpB are the best characterized zinc metalloproteases. These are involved in the degradation of antimicrobial peptides, lactoferrin, immunoglobulins and some components of the extracellular matrix *in vitro*, promoting the persistence of the infection (Kooi and Sokol, 2009).

In CF lung microenvironment, the amount of freely available iron is limited, for this reason the expression of siderophore, molecules specialized in iron chelation and uptake, results fundamental in these conditions for *B. cenocepacia*. Concerning this, different studies demonstrated that ornibactin is the most relevant siderophore for this bacterium, while pyochelin is expressed, but in smaller amounts (Thomas, 2007).

During the infection, to ensure its persistence, *B. cenocepacia* is able to evade certain host defenses by producing the lipopolysaccharide (LPS), a complex glycolipid located on the outer membrane of Gram-negatives. Interestingly, its LPS is characterized by a unique feature, containing the positively charged 4-amino-4-deoxy-L-arabinose residues, which reduce the net charge on the external membrane and thus decrease its susceptibility to the cationic antimicrobial peptides (Loutet *et al*., 2006).

The activation of the innate immune system is normally involved in the clearance of respiratory pathogens, but *B. cenocepacia* can deregulate this mechanism. Indeed, it can stimulate an exaggerated pro-inflammatory cytokine response by interacting with different cellular receptors. This effect is mediated by the interaction of the toll-like receptor 4 with the LPS of *B. cenocepacia*, which is demonstrated to be one of the most potent stimulators of pro-inflammatory cytokines among Bcc and CF pathogens (Zughaier *et al*., 1999; De Soyza *et al*., 2004). Recently, the trimeric autotransporter adhesin BcaA has been demonstrated to be involved in this mechanism, by binding specifically the tumor necrosis factor receptor 1, and stimulating the production of the pro-inflammatory molecule IL-8 (Mil-Homens *et al*., 2017). Taken together these virulence mechanisms lead to an abnormal recruitment of neutrophils and, accordingly, to inflammation-related lung injury (Cohen-Cymberknoh *et al*., 2013).

B. cenocepacia is not only able to persist in the CF airways lumen, but it is demonstrated to invade the lung epithelium through different mechanisms (McClean and Callaghan, 2009). In this process, flagellum and secreted

lipases result crucial, increasing dramatically the invasion capability of *B. cenocepacia* in *in vitro* models (Tomich *et al*., 2002; Mullen *et al*., 2007).

Moreover, after the internalization of the bacterium within vesicles, it can survive intracellularly both in epithelial cells and in macrophages, avoiding the recognition by host defenses during *in vivo* chronic infections. Its ability to survive within cells is mediated by several factors (Figure 5), as the type IV secretion system that blocks the fusion of the vacuole containing the bacterium with the lysosome, impairing the normal endosomal pathway (Sajjan *et al*., 2008). A prominent role in this mechanism is played also by the catalase/peroxidase KatA and KatB (Lefebre *et al*., 2005), the periplasmic superoxide dismutase SodC (Keith and Valvano, 2007), and the *B. cenocepacia* melanin-like pigment (Keith *et al*., 2007). Indeed, these increase the oxidative stress resistance of the bacterium, facilitating its survival within the phagosome.

Finally, the alternative sigma factors RpoE and RpoN exert the function of main regulators of the intraphagosomal survival and are necessary to delay the phagolysosomal fusion (Figure 5) (Flannagan and Valvano, 2008; Saldías *et al*., 2008). In addition to the above-mentioned virulence determinants, *B. cenocepacia* possesses also different quorum sensing systems and it can form biofilms.

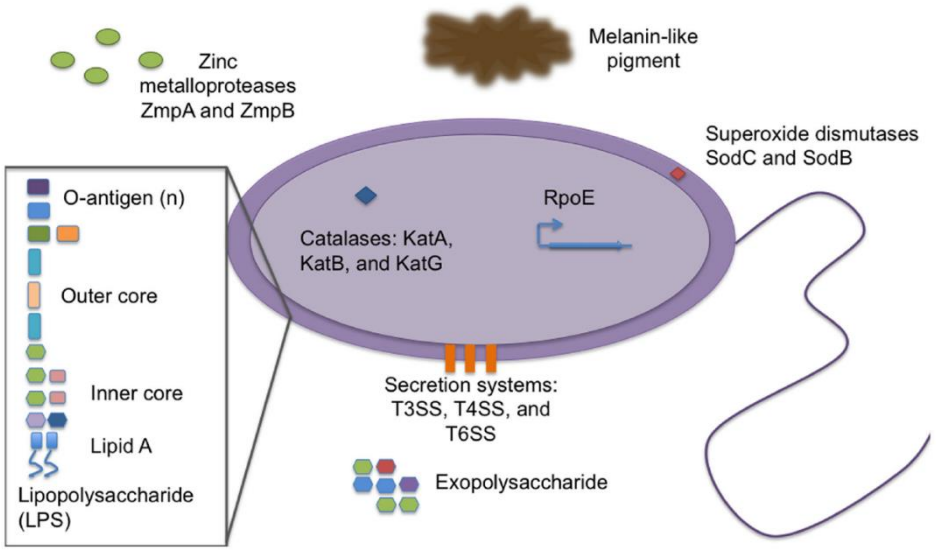


Figure 5. Virulence determinants used by *Burkholderia cenocepacia* for intraphagosomal survival and interactions with phagocytes. Cartoon of the virulence factors involved in persistence of *B. cenocepacia* in host phagocytes (Porter and Goldberg, 2011).

1.4.3.1 Quorum sensing

Quorum sensing (QS) is a mechanism of cell-to-cell communication by which a bacterial population can coordinate its pattern of gene expression based on cell density. This process is mediated by soluble autoinducer molecules produced and secreted by bacteria in the extracellular environment, until the threshold concentration is reached. At that point, the inducer triggers a collective alteration of gene expression (Papenfort and Bassler, 2016).

Each *B. cenocepacia* strain encodes a classical LuxIR-like QS system, CepIR, while some epidemic strains are provided with an additional system, CciIR, encoded on the *cci* pathogenicity island (Lutter *et al*., 2001; Malott *et al*., 2005). The CepIR system mediates the production of N-octanoyl-homoserine-lactone (C8-HSL) and smaller quantities of N-hexanoyl-homoserine-lactone (C6-HSL, Figure 6) by the synthase CepI, regulating

various functions in *B. cenocepacia* through the activation of the transcriptional regulator CepR, the receptor of the acyl-homoserine-lactone (AHL) molecule (Lutter *et al*., 2001). Instead, the other AHL-based QS system, CciIR involves the production of C6-HSL, and a lower amount of C8-HSL (Figure 6), by CciI and the subsequent activation of the cognate receptor CciR (Malott *et al*., 2005). Besides these, the bacterium possesses CepR2, an orphan regulator that is not coupled with an AHL synthase (Malott *et al*., 2009). The complex array of signaling systems of *B. cenocepacia* comprehends also an AHL-independent QS system, the *Burkholderia* diffusible signal factor system (BDSF), which is very similar to the one described in *Xanthomonas campestris* (Boon *et al*., 2008). Indeed, the diffusible signal molecule of this system is the *cis*-2-dodecenoic acid (Figure 6), produced by a bifunctional crotonase DfsA (Spadaro *et al*., 2016), which activates the receptor RpfR (Yang *et al*., 2017). Recently, a novel QS signal was discovered, the diazeniumdiolate compound valdiazen (Figure 6). This molecule was demonstrated to positively regulate the transcription of the operon responsible of its biosynthesis, besides influencing the expression of more than 100 genes in *B. cenocepacia* H111 (Jenul *et al*., 2018).

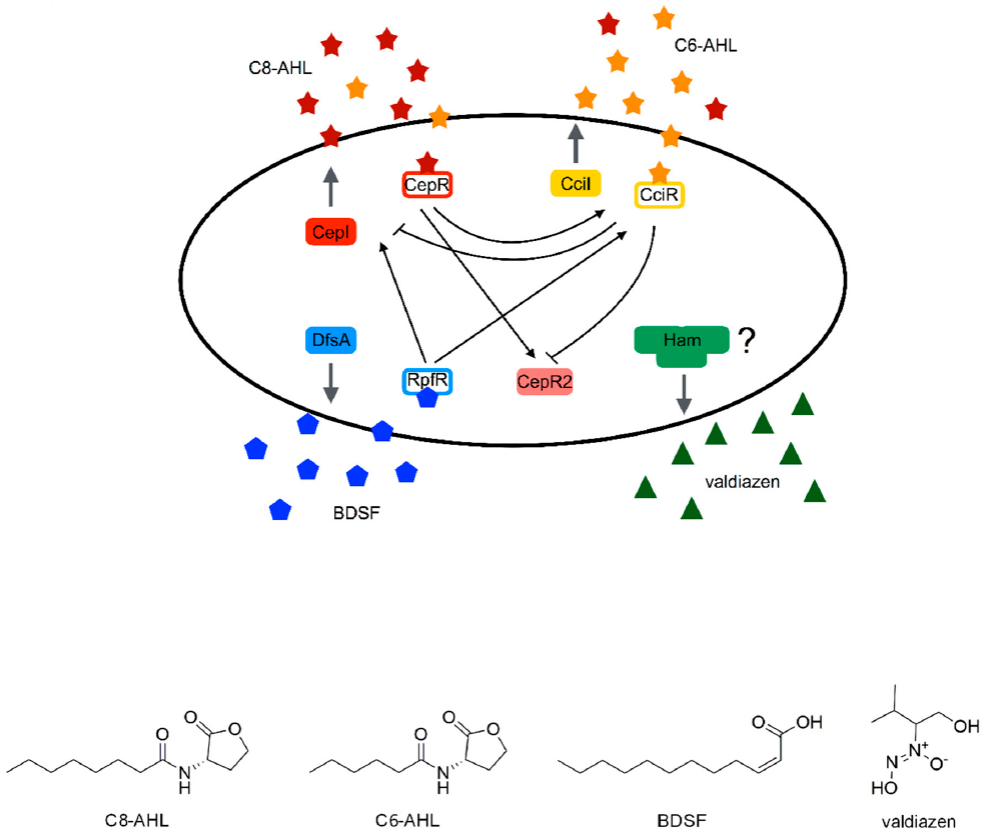


Figure 6. QS systems of *Burkholderia cenocepacia* and their interactions. The systems CepIR, CciIR, BDSF, and the orphan receptor CepR2 form a complex interactome, whereas the interactions of the Ham system are not characterized yet. C8-HSL, N-octanoyl-homoserine-lactone; C6-HSL, N-hexanoyl-homoserine-lactone; BDSF, *Burkholderia* diffusible signal factor and valdiazen. Arrows indicate positive regulation, T-bars negative regulation. (Scoffone *et al*., 2019)

Taken together, these systems are demonstrated to interact each other, forming a complex regulon (Figure 6) known to control hundreds of genes (O'Grady *et al*., 2009; Udine *et al*., 2013; Jenul *et a*l., 2018). Among those, there are several virulence factors, including toxins, lipases, proteases, and siderophores, but also genes involved in biofilm formation and swarming

motility, demonstrating that QS is a major determinant of the *B. cenocepacia* pathogenesis (Subramoni and Sokol, 2012). For this reason, QS is considered a good target for the development of novel antivirulence compounds (Scoffone *et al*., 2019).

1.4.3.2 Biofilm

Biofilms are complex communities of microorganisms adhered to a surface and embedded within a matrix of polymeric compounds composed of exopolysaccharides, DNA, and proteins. It is known that biofilm formation is a multistep process including adhesion, aggregation, maturation, dispersion (Figure 7).

The initial step is triggered by environmental signals, which lead to the reversible attachment of planktonic cells to a biotic or abiotic surface. Then, during growth, bacteria interact through intercellular communication systems, as QS, that eventually activate the coordinate production of the polysaccharide, trapping nutrients and bacteria, and leading to the irreversible attachment of the cells. After that, microorganisms form microcolonies that are progressively covered by the matrix until the complete maturation of the biofilm and the subsequent dispersion (Rabin *et al*., 2015).

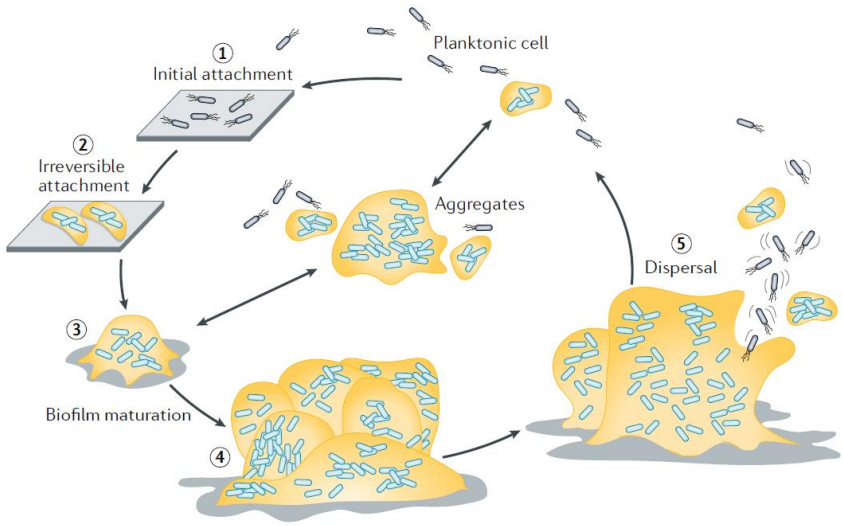


Figure 7. Schematic representation of the biofilm formation ad dispersion steps (Rumbaugh and Sauer, 2020).

The biofilm organization is known to lead to an increased resistance of the bacterial population to environmental stresses, antibiotics, and host defenses. Indeed, the thick matrix can protect bacteria from immune system recognition, as well as limit the penetration of the antibacterial molecules and promoting the formation of persisters, a subpopulation of dormant bacteria extremely resistant to antibiotics (Rabin *et al*., 2015).

B. cenocepacia is thought to form biofilm in CF lung, including mixed biofilms with *P. aeruginosa* (Tomlin *et al*., 2001), although this has been demonstrated only *in vitro*. Biofilm formation is a tightly regulated process that involves several genes in *B. cenocepacia* (Huber *et al*., 2002) and is modulated by multiple gene regulation systems, including QS (Suppiger *et al*., 2013), the alternative sigma factor RpoN indirectly activated by c-di-GMP (Fazli *et al*., 2017), the LysR-type regulator ShvR (Subramoni *et al*., 2011), and the hybrid sensor kinase-response regulator AtsR (Aubert *et al*., 2008). Moreover, it is affected by other factors as iron availability (Berlutti *et al*., 2005), exopolysaccharide synthesis, and motility (Cunha *et al*., 2004). Concerning the advantages given by the biofilm lifestyle, several studies compared the levels of antimicrobial resistance in planktonic and sessile *B. cenocepacia*. These studies show conflicting results, since some of them report no major differences in the resistance (Rose *et al*., 2009; Peeters *et al*., 2009), while others demonstrate an increased tolerance to antimicrobials (Caraher *et al*., 2007; Coenye *et al*., 2011), making further investigations necessary.

1.4.4 *B. cenocepacia* antibiotic resistance

Bacteria are defined resistant when able to grow in the presence of therapeutic levels of antibiotics. It is reported that two types of resistance exist: intrinsic and acquired. The first is an innate characteristic of the bacterium, independent of antibiotic selective pressure or heterologous DNA acquisition. Conversely, the second involves the selection of a bacterial subpopulation with genetic mutations that give a growth advantage in the presence of a specific antibiotic or resistance genes acquired through horizontal gene transfer.

B. cenocepacia shows extremely high levels of intrinsic resistance, being insensitive to high concentrations of aminoglycosides, quinolones, polymyxins, and β-lactams (Drevinek and Mahenthiralingam, 2010). Nevertheless, it can acquire resistance also through mutations in the drug

cellular target, as reported for the resistance to levofloxacin (Tseng *et al*., 2014), besides the acquisition of large genomic islands by horizontal gene transfer (Patil *et al*., 2017). The mechanisms used by *B. cenocepacia* to defend itself from antibiotics are the same ones shared by most Gram-negative bacteria. Indeed, through the formation of structured biofilms, it can protect itself from hostile environments (Tomlin *et al*., 2001), whereas the appearance of persisters during the antibiotic treatment prevents the complete eradication of the bacterial population (Chiarelli *et al*., 2020). Moreover, the bacterium can inactivate several β-lactams by expressing β-lactamases as AmpC, an enzyme able to hydrolyze expanded-spectrum cephalosporins, AmpD, an important cell wall recycling protein, and PenB, a class A penicillinase (Hwang and Kim, 2015). However, the most important resistance mechanisms of *B. cenocepacia* are the reduced permeability of the cell envelope, due to the presence of the LPS on the outer membrane (Moffatt *et al*., 2019), and the overexpression of efflux pumps.

1.4.4.1 Efflux-pumps

Overexpression of efflux-pumps is the leading mechanism responsible for multidrug resistance in *B. cenocepacia* since, due to their broad substrate specificity, a single type of efflux pump can extrude many drugs. In general, it is reported that Gram-negatives can express up to five different types of efflux pumps (Figure 8): the major facilitator superfamily (MFS), the ATP-binding cassette family (ABC), the small multidrug resistance family (SMR), the multidrug and toxic compound extrusion family (MATE), and the resistance nodulation division family (RND) (Li *et al*., 2015).

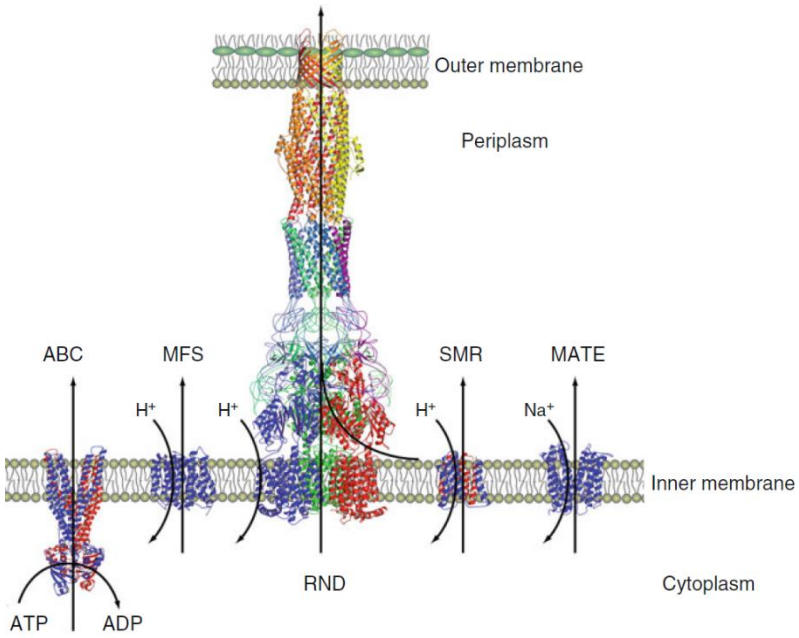


Figure 8. Crystal structure of the five major families of efflux pumps from different bacteria. From the left: Sav1866 from *S. aureus* (ABC), EmrD (MFS), AcrB-AcrA-TolC (RND), EmrE (SMR) from *Escherichia coli*, and NorM from *Vibrio cholerae* (MATE) (Murakami, 2016).

In *B. cenocepacia* J2315, the efflux pumps of the RND family are well characterized and were initially identified as 16 open reading frames organized in operons (Buroni *et al*., 2014). Concerning the protein structure, these transporters are composed of three subunits (Figure 8): one in the inner membrane, one in the outer membrane, while the third part is the periplasmic adaptor which connects the other two (Li *et al*., 2015). RND efflux pumps have been largely demonstrated to be important mediators of the intrinsic antibiotic resistance in *B. cenocepacia*, besides influencing other virulence determinants as QS and biofilm formation (Buroni *et al*., 2009; Bazzini *et a*l., 2011). Moreover, it was reported that RNDs are differentially expressed depending on bacterial physiological state of growth. Indeed, the efflux of the same molecules is mediated by different pumps in planktonic and sessile cells (Coenye *et al*., 2011; Buroni *et al*., 2014). Finally, it was

demonstrated that RNDs, MFS and ABC transporters are involved in adaptive mechanisms promoting long-term colonization of *B. cenocepacia* in CF lungs (Mira *et al*., 2011), and represent an important resistance mechanism in clinical isolates (Tseng *et al*., 2014). Recently, it was shown that RND efflux pump systems also mediate the resistance to promising novel experimental compounds (Scoffone *et al*., 2014, 2015; Perrin *et al*., 2018), representing a real challenge for the development of new anti-*Burkholderia* antimicrobials.

1.5 Canonical and alternative treatments against *B. cenocepacia*

Antibiotic therapy in CF is currently used for the prevention, control, or eradication of respiratory infections. Over the last decades the implementation of aggressive antibiotic treatments in CF therapies has led to decreased morbidity and increased longevity, in particular when started early in life. Indeed, it is demonstrated that the early treatment of lung infections in CF children within the first two years of life leads to improved respiratory function and decreases the risk of early inflammation (Ramsey *et al*., 2014).

However, the setup of standard antibiotic treatment in CF is challenging, due to the variety of pathogens usually found colonizing patients' lung, their difficult identification, as well as their natural or acquired antibiotic resistance. For these reasons, there is no consensus therapy for CF patients, and the treatment is often based on the antimicrobial susceptibility test, although this method is not always able to predict the clinical outcome of the therapy (Somayaji *et al*., 2019). Moreover, the optimal duration of intravenous antibiotic therapies, which are standard of care for lung exacerbations or pathogen eradication, is not clearly defined yet and ranges from 10 to 21 days (Abbott *et al*., 2019).

B. cenocepacia shows one of the most worrisome resistance profiles among CF pathogens, being resistant to most available antibiotics (Scoffone *et al*., 2017). So far, no standard guidelines have been described for *B. cenocepacia* chronic infection eradication, making its complete elimination from CF lungs virtually impossible (Regan and Bhatt, 2019). Indeed, several issues are still a matter of debate, including the duration of therapies, the use of mono vs. combined antibiotic therapy and the inconsistency between *in*

vitro and *in vivo* susceptibility data (Gautam *et al*., 2015). However, current treatment strategies are based on the use of double or triple antibiotic combinations and the administration of trimethoprim–sulfamethoxazole is the most used. Other therapeutic strategies include the administration of different combinations of ceftazidime, meropenem, and penicillins, after an *in vitro* susceptibility assessment (Avgeri *et al*., 2009). Moreover, aztreonam, doripenem, and tobramycin were tested in different trials against *B. cenocepacia* infections within the last years, even though none of them gave unambiguous results on the efficacy (Scoffone *et al*., 2017).

Since canonical antibiotic treatments are often poorly effective against *B. cenocepacia*, the need for new treatments that go beyond classic antibacterial compounds is urgent and alternative therapies must be considered. Currently, some promising approaches, that can be used alone or in combination with antibiotics, are reported in literature (Scoffone *et al*., 2017). Among those, anti-biofilm agents as OligoG (Powell *et al*., 2014), imidazoles (Van den Driessche *et al*., 2017), cysteamine (Fraser-Pitt *et al*., 2016), and antimicrobial peptides (de la Fuente-Núñez *et al*., 2014) are being investigated, as well as molecules able to modulate the host immune response in lungs as immunosuppressors and corticosteroids (Gilchrist *et al*., 2012) or IFN-γ (Assani *et al*., 2014). Moreover, natural compounds, as plant nanoparticles (Amato *et al*., 2016), fish oils (Mil-Homens *et al*., 2016), and glycopolymers (Narayanaswamy *et al*., 2019) have been considered as alternative therapies. Finally, QS inhibitors are currently studied as antivirulence compounds. The few QS inhibitors described active against *B. cenocepacia* target the CepIR system and are analogs of the acyl-homoserine lactone signal molecule (Brackman *et al*., 2012) or inhibitor of the synthase CepI, as the diketopiperazines (Scoffone *et al*., 2016). The latter class of molecules was demonstrated to impair the production of a wide range of virulence factors, both *in vitro* and *in vivo* in a *C. elegans* model of infection, and in a later study its mechanism of action was elucidated using a site directed mutagenesis approach on the target protein CepI (Buroni *et al*., 2018).

1.6 Bacterial cell division

Binary fission is the process by which bacteria achieve cell division, leading eventually to the formation of two daughter cells genetically identical to the mother cell. Although this mechanism is extensively studied, there are still several aspects that are not completely elucidated. Indeed, bacterial division is an extremely complex process to investigate, integrating DNA replication, chromosome segregation and septum formation (Thanbichler, 2010). It is known that each of these steps involves many proteins acting in concert to carry out a successful division.

In particular, the components of the divisome (Figure 9), the huge protein complex assembling at the midcell during division, are considered particularly important. This is confirmed by several studies performed on *Caulobacter crescentus* (Osorio *et al*., 2017), *Bacillus subtilis* (Errington and Wu, 2017) and *Escherichia coli* (Du and Lutkenhaus, 2017), which demonstrated the essential role of the divisome in the septation. Indeed, this dynamic structure is responsible for the constriction and the peptidoglycan synthesis at the division site.

In *E. coli*, the formation of the Z-ring, a circular structure composed of Filamenting temperature-sensitive mutant Z (FtsZ) polymers at the midcell, primes the events of the early phase of the divisome assembly. Initially, the Z-ring structure is stabilized and anchored to the plasma membrane by interactions with the proteins FtsA and ZipA. FtsA is an actin-like ATPase, able to form polymers tethered to the membrane by its C-terminal amphipathic domain (Szwedziak *et al*., 2012). The protein is well known for its structural role in the divisome, nevertheless it is also demonstrated to interact with many division proteins and to be involved in the regulation of the divisome activity (Liu *et al*., 2015). Instead, ZipA is a bitopic membrane protein that, in contrast to FtsA, is enumerated among the non-strictly essential component of the divisome (Krupka *et al*., 2018). Both proteins are known to bind the conserved C-terminal peptide (CCTP) of FtsZ monomers, and to interact each other (Vega and Margolin, 2019). In this phase, the Z-ring structure is further stabilized by the proteins ZapA, ZapC and ZapD which cross-link FtsZ polymers (Caldas *et al*., 2019; Bhattacharya *et al*., 2015; Schumacher *et al*., 2017). Subsequently, FtsEX, an ABC transporter-like complex controlling the peptidoglycan hydrolysis at the septum, localizes to the forming divisome through the direct interaction of FtsE with the FtsZ

CCTP (Du *et al*., 2019). The recruitment of this complex concludes the first stage of the process.

After a physiological delay, the late phase occurs, leading finally to the formation of the mature divisome (Figure 9). In this phase, the DNA translocase FtsK (Chen and Beckwith, 2001; Männik *et al*., 2017) is recruited simultaneously with FtsQ, FtsL, FtsB, FtsW, FtsI and FtsN. FtsBLQ is a complex of bitopic membrane proteins acting as a protein scaffold with regulatory activity over the division (Boes *et al*., 2019). FtsW is a peptidoglycan glycosyltransferase (Taguchi *et al*., 2019) that collaborates with FtsI, the divisome peptidoglycan transpeptidase, in the biosynthesis of the septal peptidoglycan (Wang *et al*., 1998). Instead, FtsN is the trigger of the divisome activation and its recruitment starts the septation (Pichoff *et al*., 2018).

Among the above-mentioned proteins, the most important component of this structure is FtsZ, the prokaryotic homolog of the tubulin, fundamental for the division of most prokaryotes and archaea. As its eukaryotic counterpart, it shows GTPase activity which allows its polymerization and the formation of dynamic polymers through head-to-tail interactions (Du *et al*., 2018). These functions are fundamental for the assembly of the Z-ring, the molecular scaffold of the divisome. Given the essential role of FtsZ in bacterial division, the interactions with regulatory proteins result fundamental for a successful division. Among these, the best characterized are the proteins preventing the Z-ring formation in wrong places, such as: MinCD, that prevents its formation at the cell poles (Arumugam *et al*., 2014), and SlmA, that blocks its assembly over the nucleoid (Schumacher *et al*., 2016). Moreover, SulA, a protein induced by the SOS response, is demonstrated to bind FtsZ (Figure 9) and to inhibit its polymerization when DNA damage occurs, blocking the division until the DNA is completely repaired. SulA is demonstrated to directly interact with the T7 loop of FtsZ (Cordell *et al*., 2003) thus blocking the polymerization by both sequestrating the monomers and capping the formed filaments (Vedyaykin *et al*., 2020). However, besides their structural role, FtsZ polymers are demonstrated also to mediate other fundamental functions for the division, as generating the force that leads to mid cell constriction (Erickson and Osawa, 2017), as well as guiding the synthesis of septal wall through a treadmilling behavior (Baranova *et al*., 2020). Finally, for its essential role in the division and its extreme conservation in prokaryotes,

FtsZ is considered an ideal molecular target for the development of a new generation of antibiotics (Buroni *et al*., 2020).

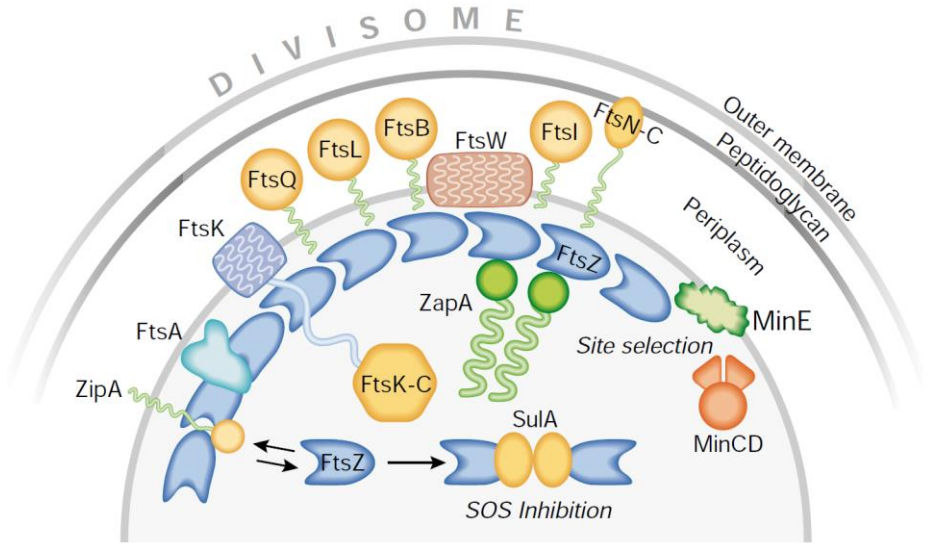


Figure 9. Cartoon of a transverse section of the divisome in *Escherichia coli*. The key components of the structure and their interactions are represented, including some modulators of the FtsZ polymerization (Vicente and Löwe, 2003).

1.6.1 The division cell wall operon (*dcw*)

In rod-shaped bacteria, FtsZ and the other proteins recognized as the absolutely essential for the optimal division, FtsL, FtsI, FtsW, FtsQ, FtsA, are encoded by genes clustered in the division cell wall (*dcw*) operon (Du and Lutkenhaus, 2017). This cluster contains genes involved in bacterial division and cell wall biosynthesis known as *fts* (filamenting temperature sensitive), *mra* (murein region a) and *mur* genes (Dewar and Dorazi, 2000). The operon is present in many bacteria and surprisingly shows a conserved gene composition and order, even in phylogenetically distant microorganisms sharing the same shape, in particular bacilliform bacteria (Tamames *et al*., 2001). On the contrary, this pattern is less conserved in cocci, showing a

more heterogeneous distribution of the *dcw* genes within the genome (Massidda *et al*., 2013).

The conservation of the *dcw* structure in rods is due to a constant positive selection of this trait since there is a clear correlation between the gene organization and the maintenance of the correct cell shape (Tamames *et al*., 2001). The selective advantage of this organization is postulated by the genomic channeling theory, according to which the genomic arrangement could facilitate the co-transcription of related genes. Accordingly, this leads to the compartmentalization of their translation in specific subcellular locations, facilitating the assembly of multiprotein complexes directly at the division site (Mingorance *et al*., 2004). In *E. coli*, the operon in composed of 16 tightly packed genes with the same transcriptional orientation (Figure 10) (Dewar and Dorazi, 2000).

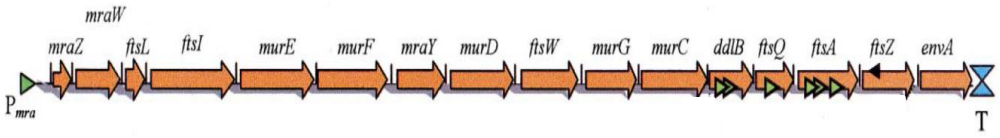


Figure 10. Representation of the *dcw* cluster of *E. coli*. The 16 genes are drawn to scale and their transcriptional direction is indicated. Promoters of the cluster are in green and the only transcriptional terminator is represented in light blue after *envA* (Dewar and Dorazi, 2000).

The transcription of these genes is finely regulated by several promoters and control regions, which contribute to the modulation of the expression of the genes in different growth conditions or different times during the cell cycle. The best characterized promoter is the P_{mra} (mraZp1), localized upstream of the first gene *mraZ* (Figure 10). Its presence is demonstrated to be essential for the transcription of the first nine genes, from *mraZ* to *ftsW* (Hara *et al*., 1997), but also to affect the expression of the gene *ftsZ*, although it is located 17 kb downstream (Mengin-Lecreulx *et al*., 1998). However, despite the relatively large distance between the promoter and *ftsZ*, this result can be confirmed by the fact that the only transcriptional terminator identified in the operon is located after *envA*, the last gene of the cluster (Mengin-Lecreulx *et al*., 1998).

At the distal part of the operon, in the *ddlB-ftsA* region, other six promoters (Figure 10) were experimentally demonstrated to be active and regulate the levels of expression of *ftsZ* (Flärdh *et al*., 1997). However, the six promoters account only for the 33% of the total expression of *ftsZ* (Flärdh *et al*., 1998), whereas the remaining part originates from distal promoters upstream of *ddlB* (de la Fuente *et al*., 2001). Interestingly, the complex regulatory function of the *ddlB-ftsA* region seems to be a conserved characteristic among bacteria, since it was characterized also in other microorganisms, such as *Bacillus subtilis* (Real and Henriques, 2006), *Bacillus mycoides* (Santini *et al*., 2013), and surprisingly in the coccus *Neisseria gonorrhoeae* (Francis *et al*., 2000), which shows a *dcw* organization very similar to bacilli.

The fine regulation of the expression of *ftsZ* is known to be essential in each step of the division; for this reason, several molecular mechanisms are involved in this process. In particular, in *E. coli*, the activity of the six promoters proximal to *ftsZ* is modulated by several factors, as the protein SdiA (Wang *et al*., 1991), as well as by the bacterial growth phase (Smith *et al*., 1993), and alternative sigma factors (Sitnikov *et al*., 1996). More recently, the transcriptional regulator controlling the activity of the *dcw* operon promoter P_{mra} was identified as the protein MraZ (Eraso *et al*., 2014).

1.6.2 MraZ

The characterization of the real function of MraZ was challenging and took many years to be achieved. Indeed, the gene *mraZ* is localized at the 5' of the *dcw* operon in many microorganisms, including *E. coli* (Figure 10) (Eraso *et al*., 2014), and for this reason it was thought to have a role in PG synthesis and cell division (Mingorance *et al*., 2004). Even the resolution of its crystal structure in *Mycoplasma pneumoniae* (Chen *et al*., 2004) and *E. coli* (Adams *et al*., 2005) did not shed light on its cellular function. However, its presence in mycoplasmas (Fisunov *et al*., 2016), that usually lack cell walls, suggested additional or alternative functions. This was confirmed by the fact that the N-terminal end of MraZ is similar to the N-terminal of the transition state regulator AbrB from *B. subtilis* and the antidote protein of the MazE/F addiction module, MazE, from *E. coli*, characterized as DNA-binding proteins (Bobay *et al*., 2005). These two are functionally closely related proteins, grouped in a family of transcriptional regulators, characterized by a dimeric N-terminal region containing the so-called "looped-hinge helix fold". Thus, due to their homology in the N-terminal domain, they were clustered in the same superfamily and, by inference, MraZ was hypothesized to bind DNA

(Bobay *et al*., 2005). Finally, almost ten years later, Eraso *et al*. (2014) experimentally proved the DNA binding ability of MraZ, showing that it is the transcriptional regulator of the *dcw* operon in *E. coli*. Indeed, they demonstrated its role as transcriptional repressor of the first 11 genes of the cluster, also regulating its own expression, as already reported for AbrB and MazE (Eraso *et al*., 2014; Strauch *et al*., 1989; Zhang *et al*., 2003). Moreover, its DNA binding site (DBS) was identified within the 38 bp region located between the promoter P_{mra} and the first codon of the gene *mraZ*, as three TGGGN direct repeats separated by 5 nucleotides (Figure 11).

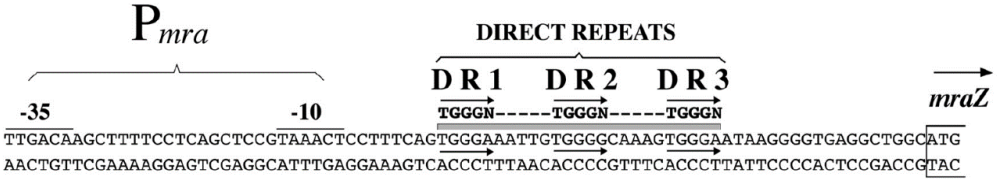


Figure 11. Representation of the MraZ binding site in *E. coli*. The DNA sequence is composed of three direct repeats (DR1, DR2, DR3) in the intergenic region between the *dcw* promoter P_{mra} and the gene *mraZ*. The interaction of MraZ with its binding site leads to the transcriptional repression of up to 11 *dcw* genes (Eraso *et al*., 2014).

Probably, each direct repeat is recognized and bound by an octamer (8-mer) since, as demonstrated in *Mycoplasma gallisepticum* that shares with *E. coli* a very similar DBS, the stoichiometry of the binding of MraZ to its DBS depends on the protein concentration, varying from an 8-mer (low MraZ concentrations) to a 24-mer (high MraZ concentrations) (Fisunov *et al*., 2016). In the absence of DNA, the free MraZ in solution forms a toroidal dodecameric structure, both in *E. coli* (Adams *et al*., 2005) and in *M. gallisepticum* (Fisunov *et al*., 2016), but when it binds the DNA it forms an octameric ring with a central pore of a size that can contain the DNA molecule (Fisunov *et al*., 2016).

In *E. coli*, MraZ activity is known to be dependent on MraW, a protein encoded by the gene *mraW*, located directly downstream of *mraZ.* MraW is characterized as a 16S rRNA methyltransferase and antagonizes the toxic

effect on the cell caused by the overproduction of MraZ (Eraso *et al*., 2014). However, the molecular mechanism responsible for this inhibition is not clear, although it can rely on direct interactions of the two proteins or a MraW-mediated post translational modification (Eraso *et al*., 2014). Regarding its transcriptional regulator activity, it was shown that MraZ controls a bigger regulon, influencing the transcription of genes outside the *dcw* cluster. Indeed, in stationary phase it modulates the expression of approximately 2% of the *E. coli* genome, whereas during the early logarithmic phase it can influence the transcription of about 23% of the genes through overexpression (Eraso *et al*., 2014). Finally, it was demonstrated that the important function of MraZ is conserved also in phylogenetically distant prokaryotes, as in *M. gallisepticum*, where it controls the transcription of the *dcw* genes, although in this case with an opposite role, activating the transcription (Fisunov *et al*., 2016).

1.7 The 10126109 compound (C109)

The characterization of new therapeutic agents against *B. cenocepacia* infections is the main research topic of our laboratory, focusing the attention on the study of new antimicrobials and anti-virulence molecules. In recent years, through the screening of a collection encompassing more than 500 newly synthesized compounds, we characterized a particularly promising molecule, named 10126109 (C109). This compound belongs to the 2,1,3-benzothiadiazol-5-yl family and was synthesized by Dr. Vadim Makarov of the Federal Research Centre “Fundamentals of Biotechnology” of the Russian Academy of Sciences (Moscow, Russia). This molecule showed a minimal inhibitory concentration (MIC) of 8 µg/ml against *B. cenocepacia* J2315 and the bactericidal activity was achieved using a 2X concentration of the MIC (Scoffone *et al*., 2015). Moreover, C109 resulted active also against all the Bcc members, including more than 50 *Burkholderia* clinical isolates (Scoffone *et al*., 2015). Its spectrum of activity was further explored against several Gram-positive and -negative pathogens, demonstrating to have a potent inhibitory activity towards many of the most worrisome opportunistic pathogens, such as some microorganisms of the ESKAPE group, as well as *Mycobacterium abscessus* (Hogan *et al*., 2018).

Besides the assessment of the C109 antimicrobial potential, we focused the attention on the identification of its molecular target. Initially, the screening of low-frequency spontaneous mutants resistant to the C109 led only to the isolation of resistant bacteria overexpressing the RND-9 efflux pump, demonstrating once more the central role of efflux systems in *B. cenocepacia* drug resistance (Scoffone *et al*., 2015). This result also proved that C109 targets an essential protein of the bacterium. Thus, in collaboration with Prof. Silvia Cardona of the University of Manitoba (Winnipeg, Canada), a high-throughput approach was used, taking advantage of a high-density transposon mutant library of *B. cenocepacia* in which transposons contain an outward rhamnose-inducible promoter (P_{rhaB}). This library was enriched for mutants expressing essential genes under the control of P_{rhaB}. By growing the knockdown mutant library under sensitizing conditions (low rhamnose) in the presence of the C109, the hypersusceptible mutants became depleted and their relative abundances were determined by Illumina sequencing. The identified hypersusceptible mutants were characterized by phenotypical and molecular analysis, identifying the putative molecular target as a protein expressed in the *dcw* cluster. Finally, through biochemical assays, the C109 cellular target was characterized as the essential division protein FtsZ (Hogan *et al*., 2018). Indeed, the compound inhibits its GTPase activity, preventing the formation of the FtsZ polymers and, consequently, the Z-ring assembly (Hogan *et al*., 2018).

Since C109 showed high potential as prospective broad-spectrum antibiotic, but low solubility in water, a nanosuspension of C109 nanocrystals stabilized with D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) embedded in hydroxypropyl-β-cyclodextrin was developed in collaboration with Prof. Francesca Ungaro of the University of Napoli “Federico II” (Napoli, Italy). This was designed for aerosol delivery and optimized to improve its penetration in mucus and biofilm layers usually found in CF lung microenvironment (Costabile *et al*., 2020). As expected, the C109 nanosuspension showed enhanced solubility and diffusion properties, maintaining the same *in vitro* antimicrobial and biofilm inhibitory activity of the pure compound. Moreover, it showed no cytotoxicity towards human bronchial epithelial cells in therapeutic concentrations and was active in combination with piperacillin in a *Galleria mellonella in vivo* model of infection (Costabile *et al*., 2020). Finally, more than 50 C109-derivatives were synthetized and tested against Gram-negative species, but unfortunately none of them showed improved properties compared to the starting molecule (Chiarelli *et al*., 2020).

2. Aims of the work

Respiratory failure caused by chronic lung infections is the leading cause of death for individuals affected by cystic fibrosis (CF). *Burkholderia cepacia* complex bacteria are enumerated among the most dangerous opportunistic pathogens affecting these patients and, in particular, *Burkholderia cenocepacia* infections are often associated with poor prognosis. This bacterium, indeed, shows extremely high levels of antibiotic resistance towards nearly each class of antibiotics and the eradication of its chronic infections is considered impossible to achieve, even through the administration of aggressive antibiotic treatments. Given the limited panel of therapies available against *B. cenocepacia*, there is an urgent need for the development of new antimicrobials able to overcome the serious problem of drug-resistant strains, improving the CF patients' outcome.

In recent years, we characterized the compound C109, a newly synthesized benzothiadiazole derivative with bactericidal activity and a very low minimal inhibitory concentration (MIC) against *B. cenocepacia* (Scoffone *et al*., 2015). This molecule resulted active against *B. cenocepacia* biofilms, Bcc CF isolates and a broad range of Gram-positive and -negative pathogens (Hogan *et al*., 2018). Moreover, it was demonstrated that C109 exerted its antimicrobial activity by impairing the divisome formation, blocking the GTPase activity of the essential protein FtsZ (Hogan *et al*., 2018). To overcome the poor solubility of this promising antibacterial compound, a PEGylated nanoparticles formulation for inhalation was developed, showing the same bactericidal activity and an improved penetration of the synthetic CF mucus (Costabile *et al*., 2020). Taken together, these data validated the protein FtsZ as a promising molecular target in *B. cenocepacia*. However, this also demonstrated the high potential of the division proteins as druggable targets, in particular the essential proteins encoded in the division cell wall (*dcw*) operon.

For this reason, the first aim of the research was the characterization of the *dcw* cluster in *B. cenocepacia* J2315, analyzing the organization of the transcription units and the presence of promoter and terminator sequences. Furthermore, the DNA binding activity of the protein MraZ, the conserved

dcw transcriptional regulator, was tested using a specific fragment in order to characterize the regulator of the *B. cenocepacia* cluster. In addition, the interactions of FtsZ with the key components of the bacterial divisome were investigated using a bacterial adenylate cyclase two-hybrid system, as well as co-sedimentation assays, giving new insights into *B. cenocepacia* division mechanisms.

Moreover, I spent two months in the laboratory of Prof. Tom Coenye at Ghent University (Ghent, Belgium), testing the C109 antimicrobic potential in an innovative physiologically relevant model. In particular, the biofilm inhibitory activity against *B. cenocepacia* J2315 and the cytotoxicity of the water-soluble C109 nanosuspension (TPGS109) in a CF three-dimensional lung epithelial cell model were evaluated. This experiment provided preliminary results for the future experimentation *in vivo* of the TPGS109 using mouse models.

3. Materials and methods

3.1 Strains and culture conditions

Strain	Genotype	Source
E. coli		
DH5α	F⁻, \80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*, *endA1*, *recA1*, *hsdR17*($r_k^-m_k^+$), *supE44, thi-1*, Δ*gyrA96, relA1*	Laboratory stock
BL21(DE3)	F⁻, *ompT, hsdS*$_B$($r_B^-m_B^-$), *gal, dcm* (DE3)	Laboratory stock
TOP10	F⁻⁻, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80*lacZ*ΔM15, Δ*lacX*74, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K λ⁻ *rps*L(Str^r), *end*A1, *nup*G	Laboratory stock
XL1Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [*F´ proAB lacI*^{q}*ZΔM15 Tn10* (Tet^r)].	Laboratory stock
BTH101	F⁻, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* (Str^r), *hsdR2*, *mcrA1*, *mcrB1*	Euromedex
B. cenocepacia		
J2315	Wild type strain	Laboratory stock
K56-2	Wild type strain	Laboratory stock

Table 2. Bacterial strains used in this work.

Escherichia coli and *Burkholderia cenocepacia* strains were cultured in Luria-Bertani (LB) medium (Difco, BD, USA) with shaking at 200 rpm at 37°C or in LB agar plate at 37°C. *E coli* cultures for BACTH complementation assay were grown in LB with shaking at 30°C or in LB agar at 30°C. For plasmid and strain selection, the following antibiotics were used: Kanamycin (BioChemica) 50 µg/ml (*E. coli*); Gentamicin (Sigma) 20 µg/ml (*E. coli*), 500 µg/ml (*B. cenocepacia*); Ampicillin (BioChemica) 100 µg/ml (*E. coli*); Chloramphenicol (Sigma) 34 µg/ml (*E. coli*); Streptomycin (Sigma) 100 µg/ml (*E. coli*). For the BACTH complementation assay, agar plates were supplemented with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 40 µg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

3.2 RNA extraction and reverse transcription (RT)-PCR

The total RNA *of B. cenocepacia* J2315 was extracted following the Direct-zol RNA Miniprep Kit protocol (Zymo Research) with a preliminary step to increase the cell lysis efficiency. In this step, $5x10^8$ bacterial cells, harvested in mid-exponential phase (OD_{600}=0.6), were resuspended in TRI Reagent® (Zymo Research) and mixed with zirconia beads (Ambion) in a 2 ml bead beating tube. Subsequently, they were disrupted through 2 cycles of 4 min at the maximum speed of bead beating using the Minilys personal homogenizer (Bertin instruments). After the extraction, RNA was treated twice with the DNA-free™ DNA Removal Kit (Invitrogen), to completely remove DNA contaminations, and the quality of the sample was assessed using agarose gel electrophoresis. Finally, the retrotranscription was performed following the ProtoScript® II Reverse Transcriptase (NEB) protocol using 0.5 µg of total RNA as template and the reverse primers listed in Table 3.

3.3 Analysis of the *dcw* transcription units by PCR

To characterize the transcription units of the *dcw* cluster of *B. cenocepacia* J2315, the cDNAs obtained were used as templates to amplify by PCR specific fragments using primers annealing on two consecutive *dcw* genes. All the primers used for reverse transcription PCR and cDNA PCR are listed in Table 3. The results were verified by agarose gel electrophoresis and the amplicons obtained were purified and sequenced. The specificity of the results was validated using PCR negative controls (using RNA as template for the cDNA amplification) and positive controls (amplification of the genomic DNA using the cDNA primers).

Primer	Sequence 5'- 3'	Expected fragment
mraZRTfor1	GTGTTCCAAGGGGCGTCGGC	661bp
mraWRTrev1	GCATCCTTGATGCCCTGCGCCGTCT	
mraWRTfor1	AAGTCGTACTAGACGCGGCA	503bp
ftsLRTrev1	TCGAGATCGGCTGCATTTTCAGCGA	
ftsLRTfor1	GCCTCAATATCTTCCTGCTG	509bp
ftsIRTrev1	CCTGCTTCTGATAGAACGCG	
ftsIRTfor1	GGGTAGCGGCGCAAAGCATA	514bp
murERTrev1	CGGGCATCCCGGTGCCGAGCGT	
murERTfor1	GGCTCGAGTCGGTCAATGGC	561bp
murFRTrev1	ATCAGGCGGGCGGCTTCGCCG	
murFRTfor1	AAGATGGAGCGCGTGGTCGA	512bp
mraYRTrev1	ACAGCCCGATCACCGATTGC	
mraYRTfor1	ACCTGCTGTTCCCGCACATC	571bp
murDRTrev1	CCTCGCGGGTATCGGCAATA	
murDRTfor1	TCGACATGTTCACGAACTAC	529bp
ftsWRTrev1	GTTCACGCCCTTGCCGACAT	
ftsWRTfor1	ACGATGCTCGTGTGGCTGTC	581bp
murGRTrev1	GGTGCCGCCTGCCATCACCA	
murGRTfor1	AGGCTACCGACGAAGTCGCG	568bp
murCRTrev1	GAACGACGCATCCGACTCGT	
murCRTfor1	CTGGCGCTGGCGGAGTTCAA	544bp
ddlRTrev1	GAACAACACTGCCACCTTGC	
ddlRTfor1	CGCGACGCACGACAAGATCG	512bp
ftsQRTrev1	CGCCAACACGAGCAGCAGCA	
ftsQRTfor1	GGCAGGCATGCGGTTCCTGAC	557bp
ftsARTrev1	CCCGTCACGATGTGCACCTTC	
ftsARTfor1	AAGACATCAAGGTCGGCTACGGGAT	616bp
ftsZRTrev1	TGGTCTCGGTTTCCAGCATT	
ftsZRTfor1	GAAGAAGCAGCAGTCGGCAC	581bp
BCAL3456RTrev1	AATCCGAAGATCACCACCCG	
BCAL3456RTfor1	CAAATTCGAAGTGAGCGATG	573bp
lpxCRTrev1	TCGACATACAGGTTGTCGAT	

Table 3. Primers used for the transcription analysis of the *dcw* cluster and expected length of the PCR fragments.

3.4 β-galactosidase activity assay

The activity of the putative promoter sequences of the *B. cenocepacia* J2315 *dcw* operon was measured by β-galactosidase activity assay. To this aim, two overlapping fragments, containing the putative promoter located in the non-coding region upstream of the cluster, were obtained by PCR using dcwPROMOrev2 coupled with dcwPROMOfor1_2 or dcwPROMOfor2_2 primers (Table 4) and genomic DNA as template. Moreover, in order to assess the presence of active promoters in the distal part of the operon, four fragments, covering the entire *ddl-ftsA dcw* region, were amplified by PCR using genomic DNA and the primers murCPROMOfor1-ddlPROMOrev1, ftsQPROMOfor1-ftsQPROMOrev1, ftsAPROMOfor1-ftsAPROMOrev1, ftsAPROMOfor2-ftsAPROMOrev2 (Table 4).

Primer	Sequence 5'- 3'	Restriction site
dcwPROMOfor1_2	GGTATCGATAAGCTTACGCCCGTCTGGGCCGTCTT	*Hind*III
dcwPROMOrev2	TCTAGCTAGAAGCTTTTTCCGCTCTCCCGTTCAGG	*Hind*III
dcwPROMOfor2_2	GGTATCGATAAGCTTATTCGTCAAAAAAGCGGGCC	*Hind*III
murCPROMOfor1	GGTATCGATAAGCTTCATTCAACAGAAGGCATGAC	*Hind*III
ddlPROMOrev1	TCTAGCTAGAAGCTTACGTCGTTCCGTTCCTGCGT	*Hind*III
ftsQPROMOfor1	GGTATCGATAAGCTTATGTGGAACAACGTTCGCCA	*Hind*III
ftsQPROMOrev1	TCTAGCTAGAAGCTTAAAGTGCTCTTGCGTGTGAT	*Hind*III
ftsAPROMOfor1	GGTATCGATAAGCTTATGAGCAAAGACTACAAGGA	*Hind*III
ftsAPROMOrev1	TCTAGCTAGAAGCTTGATGTCGACCAGCACCACGC	*Hind*III
ftsAPROMOfor2	GGTATCGATAAGCTTGGCGGCGGCACGACGGACAT	*Hind*III
ftsAPROMOrev2	TCTAGCTAGAAGCTTTGTTGCCTCCGTCAAGAGAA	*Hind*III

Table 4. Primers used for promoter sequences cloning into pSU11 vector. The restriction site is underlined.

The fragments were purified and independently cloned upstream of a promoterless *lacZ* reporter gene into the pSU11(Table 9) shuttle vector (Jenul *et al*., 2018), linearized with *Hind*III and dephosphorylated using alkaline phosphatase (Roche), following the In-fusion HD Cloning Kit protocol (Takara).

The resulting plasmids (Table 9) were mobilized from *E. coli* DH5α to *B. cenocepacia* K56-2 by triparental mating (Craig *et al*., 1989). The β-galactosidase assay was carried out as described before (Stachel *et al*., 1985) with minor changes. Bacteria were grown overnight in liquid LB, diluted 1/50 in fresh LB and cultured for additional six hours. Then, the precise OD_{600} was measured and 1 ml of culture was harvested by centrifugation, resuspended in the same volume of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 pH=7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM Dithiothreitol (DTT)) and permeabilized with 25 µl of chloroform and 25 µl of 0.1% SDS. The obtained cell suspension was vortexed for 10 seconds and 500 µl were diluted 1:2 in Z-buffer and incubated at 28°C for 10 min. Then, 200 µl of *o*-nitrophenyl-β-D-galactoside (ONPG) solution (4 mg/ml in Z-buffer) were added to the sample that was subsequently vortexed and further incubated at 28°C to allow the reaction to proceed. When the sample turned yellow, the reaction was stopped adding 500 µl of 1 M Na_2CO_3. Before the spectrophotometric quantification, the sample was centrifuged at 15000 *x g* for 10 min at room temperature and 1 ml of cell debris-free supernatant was used to measure the absorbance at 420 nm (A420) and 550 nm (A550). Finally, the calculation of the promoter activity in Miller Units (MU) was performed using the Miller formula:

$$Miller\ Units = 1000 * \frac{[A420 - (1{,}75 * A550)]}{T * V * OD600}$$

T= reaction time (min)

V= volume of cell extract in the reaction (ml)

3.5 5'-rapid amplification of cDNA ends (5'-RACE)

The transcription start site of the *dcw* operon of *B. cenocepacia* J2315 was assessed by 5' rapid amplification of cDNA ends (5'-RACE). The RNA extraction, DNase treatment and reverse transcription were carried out as described above, using the oligonucleotide mraZRACErev1 (Table 5) for the RT-PCR.

Then, to degrade the RNA of the resulting cDNA-RNA heteroduplex, the sample was treated with ribonuclease H (Promega) by adding 2 U of enzyme

directly in 20 µl of RT-PCR mix and incubating at 37°C for 30 min. After that, the single strand cDNA was purified and a tail of polyadenosine was added to the 3' end by terminal deoxynucleotidyl transferase (Promega) reaction, according to the manufacturer's instructions. The polyadenylated cDNA was amplified by PCR using the polyT universal forward primer RA1 (Table 5) and the reverse primer mraZRACErev2 (Table 5). The reaction product was used as template for the nested PCR performed with the primers RA2 and mraZRACErev3 (Table 5), designed to amplify a fragment within the first product sequence. The resulting amplified fragment was purified and sequenced to identify the *dcw* cluster transcription start site.

Primer	Sequence 5'- 3'
mraZRACErev1	CATTCCCAACAACATGACTT
RA1	GACCACGCGTATCGATGTCGAC$(T)_{16}$
mraZRACErev2	CGGAAACAGCAACAGGCAGC
RA2	GACCACGCGTATCGATGTCGAC
mraZRACErev3	GTCACAGTCACCCGTCCTTC

Table 5. Primers used for the 5'-RACE experiment.

3.6 Expression and purification of *B. cenocepacia* MraZ

The gene *mraZ* (*BCAL3471*) of *B. cenocepacia* J2315 was amplified by PCR using the primers mraZpET28aFOR-mraZpET28aREV (Table 6) and genomic DNA as template. These primers were designed following the In-fusion HD Cloning Kit protocol instructions (Takara) to allow the recombination of the amplicon into the pET28a (Novagen) digested with *Bam*HI/*Hind*III. Moreover, the PreScission protease (GE Healthcare) cleavage site was added to the forward primer to remove the 6-histidine tag from the protein. The amplified fragment was purified and cloned into the linearized pET28a (Novagen) vector by recombination according to the In-fusion HD Cloning Kit protocol, obtaining the pET28a-MraZ (Table 9).

Primer	Sequence 5'- 3'	Restriction site
mraZpET28aFOR	ATGGGTCGCGGATCCCTGGAAGTTCTGTTCCAGGGGCCCaTGT TCCAAGGGGCGTCGGC	*Bam*HI
mraZpET28aREV	TGCGGCCGCAAGCTTTCAGAACGTGAAATTCTTCA	*Hind*III
sulApBADM41for	TTCAGGGCGCCATGGggCTGGAAGTTCTGTTCCAGGGGCCCCA CCCCGCCCTCGCC	*Nco*I
sulApBADM41rev	ACGGAGCTCGAATTCTCAGGCGACGGCGCC	*Eco*RI
ftsASUMOfor	GAGAACAGATTGGTGGTATGAGCAAAGACTACAAGGA	
ftsASUMOrev	ATACCTAAGCTTGTCTTCAGAAGTTGCTCAGGAACC	
ZipASUMOFor	GAGAACAGATTGGTGGTTGGCAGGGCGCGAAAGTGCGGCGC	
ZipASUMORev	ATACCTAAGCTTGTCTTTACTGGCTGAAGAGGCGGCGCGTGAC	

Table 6. Primers used for gene cloning into plasmids for recombinant protein expression. The restriction site is underlined and the prescission protease sequence is in blue.

For the optimal expression of the recombinant MraZ protein, pET28a-MraZ was transformed in *E. coli* BL21(DE3) strain. Three liters of LB supplemented with Kanamycin were inoculated 1/50 with the overnight starter culture and incubated at 37°C with shaking until OD_{600}=0.6 was reached. At this point, the protein expression was induced with 0.5 mM of IPTG and the culture was incubated overnight at 18°C. Cells were harvested by centrifugation, resuspended in buffer A (25 mM Tris-HCl pH=7.5, 300 mM NaCl, 5 mM $MgCl_2$, glycerol 5%), supplemented with 1 mM of the non-specific protease inhibitor Phenylmethanesulfonyl fluoride (PMSF, Sigma), and lysed by sonication. The lysate was centrifuged at 50000 *x g* for 30 minutes at 4°C and the protein was purified from the cell-free extract using immobilized metal affinity chromatography (IMAC) on a HiTrap TALON crude column (1 ml, GE Healthcare), taking advantage of the MraZ 6-histidine tag interaction with the cobalt bound to the resin. The purified protein was eluted with 250 mM of imidazole, dialyzed against the buffer B (50 mM Tris-HCl pH=7.5, 300 mM NaCl, 5 mM $MgCl_2$, glycerol 5%, 1 mM DTT), and digested with PreScission protease (GE Healthcare) to remove the 6-histidine tag. After that, the MraZ protein was further purified in the same buffer on a nickel nitrilotriacetic acid resin (Qiagen) packed in a column. Finally, the protein was quantified by spectrophotometric analysis, and 5 ml of MraZ concentrated 1 mg/ml were stored at -80°C.

3.7 Electrophoretic mobility shift assay (EMSA)

The DNA binding site (DBS) of the protein MraZ was characterized by electrophoretic mobility shift assay (EMSA). The entire intergenic region upstream of *mraZ*, containing the putative DBS, was amplified by PCR using mraZBS1for-mraZBS1rev and mraZBS2for-mraZBS2rev primers (Table 7) and genomic DNA as template. The primers were labeled with 6-carboxyfluorescein (6-Fam) to be easily detectable on gel using the ChemiDoc XRS+ System (Bio-Rad) and were designed to amplify the two DNA probes that consisted in two overlapping fragments of 250 bp covering the whole intergenic region. After PCR, the amplicons BS1-6-Fam and BS2-6-Fam were sequenced to confirm their sequences.

Primer	Sequence 5'- 3'
mraZBS1for	GATTGGCGCCGGGGTGGCGT
mraZBS1rev	GGAGCGGCCCGCTTTTTTGA
mraZBS2for	AAGTTGCACTAGCTCATTCA
mraZBS2rev	TTTCCGCTCTCCCGTTCAGG
mraZBSNSfor	ACACGAACTCGCGGCGGATC
mraZBSNSrev	TTGCGGCAGCGGCATGTCCT

Table 7: Primers used for the EMSA experiment.

The binding reaction was carried out incubating 0.34 pmoles of labeled DNA probes with increasing quantities of MraZ (250, 300, 350, 400 pmoles) in a reaction volume of 25 µl in binding buffer (20 mM Tris-HCl pH=7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT, 150 ng/µl BSA, 50 ng/µl deoxyribonucleic acid from herring sperm) for 30 minutes at 37°C. After the incubation, samples were loaded on agarose gel 1.5% and the electrophoretic run was performed in TBE 0.5X for 1 hour at 100 V at 4°C in the dark, after an initial equilibration of the gel for 30 min at 60 V in TBE 0.5X. The gel was then visualized using the ChemiDoc XRS+ System (Bio-Rad). The specificity of the binding was assessed by repeating the experiment in the same conditions, but adding a 20X excess of specific (non-labeled BS2 fragment) or non-specific (non-labeled 250 bp fragment amplified by PCR from the gene *cepI* of *B. cenocepacia* J2315 with mraZBSNSfor and mraZBSNSrev (Table 7) primers) DNA competitors in the sample.

3.8 Characterization of the interactors of the protein FtsZ

3.8.1 Bacterial adenylate cyclase two-hybrid system (BACTH)

In order to characterize the proteins interacting with FtsZ in *B. cenocepacia* J2315, the bacterial adenylate cyclase two-hybrid system (BACTH, Euromedex) was used. The system requires non-reverting adenylate cyclase (AC) deficient *E. coli* strain (BTH101), since the detection of the protein-protein interaction i*n vivo* relies on the reconstitution of the AC activity. This can be obtained using specific vectors, pKT25 and pKNT25 (low copy-number), pUT18 and pUT18C (high copy-number), which express respectively the fragments T25 and T18 of the *Bordetella pertussis* AC that are not active when physically separated. Thus, the fusion of the C- or N-terminus of the two subunits with interacting proteins leads to the heterodimerization of these hybrid proteins, to the restoration of the AC activity and, consequently, to the production of cAMP. This molecule binds and activates the catabolite activator protein, which is the global regulator of carbon utilization in *E. coli*, including *lac* and *mal* operons. Therefore, bacteria able to utilize lactose or maltose as the unique carbon source can be easily distinguished on indicator or selective media.

3.8.2 Construction of the recombinant plasmids

The genes coding for FtsZ (*BCAL3457*), FtsA (*BCAL3458*), FtsE (*BCAL0454*), FtsQ (*BCAL3459*), FtsI (*BCAL3468*), FtsN (*BCAL0678*), ZipA (*BCAL2097*), ZapA (*BCAL2667*), and SulA (*pBCA006*) of *B. cenocepacia* J2315 were amplified by PCR using the proper couples of primers listed in Table 8 and genomic DNA as template. Each fragment was purified and inserted by recombination, following the In-fusion HD Cloning Kit protocol (Takara), into each of the four plasmids of the BACTH system, obtaining the plasmids listed in Table 10. These vectors were previously digested with the corresponding restriction enzymes listed in Table 8. The resulting recombinant vectors expressed hybrid proteins in which the polypeptides of interest were fused to the C- or N-termini of the T25 and T18 fragments of adenylate cyclase, respectively.

Primer	Sequence 5'- 3'	Restriction site
FtsZpUT18pKNT25For	GATTACGCCAAGCTTGATGGAATTCGAAATGCTGGA	*HindIII*
FtsZpUT18pKNT25Rev	GGTACCCGGGGATCCTCGTCAGCCTGCTTGCGCAGGAAAG	*BamHI*
FtsZpUT18CFor	GACTCTAGAGGATCCCATGGAATTCGAAATGCTGGA	*BamHI*
FtsZpUT18CRev	GAATTCGAGCTCGGTACCCGGTCAGCCTGCTTGCGCAGGAAAG	*KpnI*
FtsZpKT25For	GACTCTAGAGGATCCCATGGAATTCGAAATGCTGGA	*BamHI*
FtsZpKT25Rev	CTTAGTTACTTAGGTACCCGGTCAGCCTGCTTGCGCAGGA	*KpnI*
FtsApUT18pKNT25For	GATTACGCCAAGCTTGATGAGCAAAGACTACAAGGATC	*HindIII*
FtsApUT18pKNT2Rev	TTCGAGCTCGGTACCCGGAAGTTGCTCAGGAACC	*KpnI*
FtsApUT18CpKT25For	GACTCTAGAGGATCCCATGAGCAAAGACTACAAGGATC	*BamHI*
FtsApUT18CRev	GAATTCGAGCTCGGTACCCGGAAGTTGCTCAGGAACCATTCC	*KpnI*
FtsApKT25Rev	GTTACTTAGGTACCCGGAAGTTGCTCAGGAACCATTCC	*KpnI*
FtsEpUT18pKNT25For	GATTACGCCAAGCTTGATGATCCGCCTCGAACGCATCGAC	*HindIII*
FtsEpUT18pKNT25Rev	GTACCCGGGGATCCTCGAACGCCGGCACGCCTTGCGCGAG	*BamHI*
FtsEpUT18CpKT25For	GACTCTAGAGGATCCCATGATCCGCCTCGAACGCATCGAC	*BamHI*
FtsEpUT18CRev	CGAGCTCGGTACCCGGAACGCCGGCACGCCTTGCGCGAG	*KpnI*
FtsEpKT25Rev	GTTACTTAGGTACCCGGAACGCCGGCACGCCTTGCGCGAG	*KpnI*
FtsQpUT18pKNT25For	GATTACGCCAAGCTTGATGTGGAACAACGTTCGCCAAC	*HindIII*
FtsQpUT18pKNT25Rev	GTACCCGGGGATCCTCCTTCTTGCGCTTGTCGGTATCG	*BamHI*
FtsQpUT18CpKT25For	GACTCTAGAGGATCCCATGTGGAACAACGTTCGCCAAC	*BamHI*
FtsQpUT18CRev	CGAGCTCGGTACCCGCTTCTTGCGCTTGTCGGTATCG	*KpnI*
FtsQpKT25Rev	GTTACTTAGGTACCCGCTTCTTGCGCTTGTCGGTATCG	*KpnI*
FtsIpUT18pKNT25for	GATTACGCCAAGCTTGATGAAGCCGTCCCAGAAGCGC	*HindIII*
FtsIpUT18pKNT25rev	GTACCCGGGGATCCTCTCGAACTACTCCTGGTGAATTAC	*BamHI*
FtsIpUT18CFor	CAGGTCGACTCTAGAGATGAAGCCGTCCCAGAAGCGC	*XbaI*
FtsIpUT18CRev	GTTATATCGATGAATTCGATCGAACTACTCCTGGTGAATTAC	*EcoRI*
FtsIpKT25For	GGGTCGACTCTAGAGATGAAGCCGTCCCAGAAGCGC	*XbaI*
FtsIpKT25Rev	AACGACGGCCGAATTCTCATCGAACTACTCCTGGTGAATTAC	*EcoRI*
FtsNpUT18pKNT25For	GATTACGCCAAGCTTGGTGCTGGGCCTGATCGTCGGCCTCG	*HindIII*
FtsNpUT18pKNT25Rev	GTACCCGGGGATCCTCCTGCTTCGTGAAGCGGATCACC	*BamHI*
FtsNpUT18CpKT25For	GACTCTAGAGGATCCCGTGCTGGGCCTGATCGTCGGCCTCG	*BamHI*
FtsNpUT18CRev	CGAGCTCGGTACCCGCTGCTTCGTGAAGCGGATCACC	*KpnI*
FtsNpKT25Rev	GTTACTTAGGTACCCGCTGCTTCGTGAAGCGGATCACC	*KpnI*
ZipApUT18pKNT25For	GATTACGCCAAGCTTGATGGACGAGTTGACACTCGGTTTG	*HindIII*

ZipApUT18pKNT25Rev	CGGGGATCCTCTAGAGTCTGGCTGAAGAGGCGGCGCGTGA	*XbaI*
ZipApUT18CpKT25For	GACTCTAGAGGATCCCATGGACGAGTTGACACTCGGTTTG	*BamHI*
ZipApUT18CRev	GAATTCGAGCTCGGTACCCGCTGGCTGAAGAGGCGGCGCGTGAC	*KpnI*
ZipApKT25Rev	GTTACTTAGGTACCCGCTGGCTGAAGAGGCGGCGCGTGAC	*KpnI*
ZapApUT18pKNT25For	GATTACGCCAAGCTTGATGAGCACCAAGCAGATCGAAGTCT	*HindIII*
ZapApUT18pKNT25rev	GTACCCGGGGATCCTCCTGCGTCTCGTGCTGTGCGAGC	*BamHI*
ZapApUT18CpKT25For	GACTCTAGAGGATCCCATGAGCACCAAGCAGATCGAAGTCT	*BamHI*
ZapApUT18CRev	CGAGCTCGGTACCCGCTGCGTCTCGTGCTGTGCGAGC	*KpnI*
ZapApKT25Rev	GTTACTTAGGTACCCGCTGCGTCTCGTGCTGTGCGAGC	*KpnI*
SulApUT18pKNT25For	GATTACGCCAAGCTTGATGCACCCCGCCCTCGCCCATCCTG	*HindIII*
SulApUT18Rev	CGGTACCCGGGGATCCTCGGCGACGGCGCCGGCGATCGTGGCC	*BamHI*
SulApKNT25Rev	GTACCCGGGGATCCTCGGCGACGGCGCCGGCGATCGTGGCC	*BamHI*
SulApUT18CpKT25For	GACTCTAGAGGATCCCATGCACCCCGCCCTCGCCCATCCT	*BamHI*
SulApUT18CRev	GTTATATCGATGAATTCGAGGCGACGGCGCCGGCGATCGTGGCC	*EcoRI*
SulApKT25Rev	GTTACTTAGGTACCCGGGCGACGGCGCCGGCGATCGTGGCC	*KpnI*
pUT18pKNT25CheckFor	CTTTATGCTTCCGGCTCG	
pUT18CheckRev	GTTCGCGATCCAGGCCGC	
pKNT25CheckRev	GCGTAACCAGCCTGATGCG	
pUT18CCheckFor	GTCACCCGGATTGCGGCG	
pUT18CCheckRev	GTGTCGGGGCTGGCTTAAC	
pKT25CheckFor	GCAGTTCGGTGACCAGCGG	
pKT25CheckRev	GCAAGGCGATTAAGTTGGG	

Table 8. Primers used for the construction of the BACTH recombinant plasmids. The restriction site is underlined.

3.8.3 BACTH complementation assay

BACTH complementation assay was performed using various combinations of recombinant pUT18, pUT18C, pKT25 and pKNT25 (Table 10), carrying the divisome genes, co-transformed in BTH101 cells. The transformants were plated onto LB-IPTG-XGal medium containing Kanamycin and Ampicillin and incubated at 30°C for 48 h. The interactions between different hybrid proteins were evaluated in solid media (LB-IPTG-XGal and

MacConkey-Lactose) and in liquid culture quantifying the β-galactosidase activity. The β-galactosidase assay was performed as previously described, on strains grown in LB broth in the presence of 0.5 mM IPTG, Ampicillin and Kanamycin at 30°C for 18 h. A level of β-galactosidase activity at least four-fold higher than the control (BTH101 cells transformed with empty pUT18/pKT25) was considered to indicate an interaction.

3.9 Expression and purification of *B. cenocepacia* SulA

The protein pBCA006 of *B. cenocepacia* J2315, the putative SulA, was expressed using the following protocol. The gene *pBCA006* was amplified by PCR using the primers sulApBADM41for-sulApBADM41rev (Table 6) and genomic DNA as template. The amplified fragment was inserted by recombination, following the In-fusion HD Cloning Kit protocol instruction (Takara), into the pBADM-41 plasmid digested with *Nco*I/*Eco*RI, obtaining the pBADM-41-SulA (Table 9). This plasmid allows the inducible expression of SulA fused with the *E. coli* Maltose Binding Protein (MBP).

The pBADM-41-SulA vector was co-transformed by electroporation with the pG-Tf2 plasmid (Chaperone plasmid set, Takara), expressing the *E. coli* chaperones GroEL-GroES and tig, into *E. coli* TOP10 competent cells. Upon induction, the mild overexpression of the GroEL-ES complex in this strain assisted the correct folding of MBP-SulA, increasing the quantity of soluble protein produced. Conversely, without this chaperone, the intrinsic instability of SulA led to the accumulation of the recombinant protein within inclusion bodies. For the expression of MBP-SulA, cells were grown in 3 L of LB supplemented with Ampicillin and Chloramphenicol, and 5 ng/ml of Tetracycline to induce the chaperone expression, until OD_{600}=0.6 was reached. Then, the protein expression was induced with 0.2% of L-arabinose overnight at 18°C. After that, cells were collected by centrifugation, resuspended in buffer C (50 mM Tris-HCl pH=7, 5 mM $MgCl_2$, glycerol 5%) supplemented with 1 mM of PMSF and lysed by sonication. Subsequently, the lysate was centrifuged at 50000 *x g* for 30 min and a solution of ATP pH=7 was added to the supernatant obtaining a final concentration of 5 mM, in order to facilitate the detachment of GroEL from MBP-SulA. After 15 min of incubation at 4°C, the extract was loaded on a MBPTrap HP (5 ml, GE Healthcare), eluting MBP-SulA complexed with GroEL with 10 mM of

maltose in buffer C. To eliminate the chaperone contamination, the eluate was further purified using cation exchange chromatography. A column of SP Sepharose Fast Flow resin (GE Healthcare) was used, and the protein was eluted in buffer C with a (0 - 1 M) KCl gradient. The fractions containing exclusively MBP-SulA were pooled, desalted using a HiPrep 26/10 Desalting column (GE Healthcare), and eluted in buffer D (20 mM Tris-HCl pH=7.9, 50 mM KCl, 1 mM EDTA, glycerol 10%). Finally, the protein was quantified through spectrophotometric analysis and 6 aliquots concentrated 1 mg/ml were stored at -80°C.

3.10 Expression and Purification of *B. cenocepacia* FtsA

For the expression of the FtsA protein of *B. cenocepacia* J2315, the gene *ftsA* (*BCAL3458*) was amplified by PCR, using the primers ftsASUMOfor and ftsASUMOrev (Table 6), with genomic DNA as template. The PCR product was purified and inserted into the pETSUMO (Invitrogen) plasmid, using the In-fusion HD Cloning Kit protocol (Takara).

Then the pETSUMO-FtsA (Table 9) was transformed into *E. coli* BL21(DE3) competent cells by electroporation. Three liters of culture were grown in LB supplemented with Kanamycin and the protein expression was induced at OD_{600}=0.6 with 0.5 mM of IPTG overnight at 20°C. Cells were harvested by centrifugation, resuspended in buffer E (50 mM Tris-HCl pH=7.5, 300 mM NaCl, 5 mM $MgCl_2$, glycerol 5%) containing 1 mM of PMSF and lysed by sonication. The lysate was clarified by centrifugation at 50000 *x g* for 30 minutes and the supernatant was applied on a HisTrap HP nickel column (1 ml, GE Healthcare), eluting the protein with 250 mM imidazole in buffer E. Then, the purified FtsA was dialyzed overnight against buffer B and the SUMO protease was used to remove the SUMO protein. A further purification step was carried out by size exclusion chromatography, using a HiLoad 16/60 Superdex-75 column (GE Healthcare) in buffer D, obtaining the purified FtsA that was concentrated to 5 mg/ml and stored at -80°C.

3.11 Expression and purification of *B. cenocepacia* ZipA

To express the soluble fragment of the protein ZipA of *B. cenocepacia* J2315, the gene *zipA* (*BCAL2097*) was amplified by PCR, using the primers ZipASUMOFor and ZipASUMOrev (Table 6), with genomic DNA as template. The amplicon obtained was purified and cloned into the pETSUMO (Invitrogen) plasmid, using the In-fusion HD Cloning Kit protocol (Takara).

Subsequently, the pETSUMO-ZipA (Table 9) was transformed into *E. coli* BL21(DE3) competent cells by electroporation. The culture was grown in 3 L of LB supplemented with Kanamycin and the protein production was induced at OD_{600}=0.6 with 0.5 mM of IPTG overnight at 18°C. Cells were harvested by centrifugation, resuspended in buffer E containing 1 mM of PMSF and lysed by sonication. The lysate was clarified by centrifugation at 50000 *x g* for 30 minutes and the supernatant was loaded on a HisTrap HP nickel column (1 ml, GE Healthcare), eluting the protein with 250 mM imidazole in buffer E. Then, the purified protein was dialyzed overnight against buffer B and the SUMO protease was used to remove the SUMO protein. A further purification step was carried out by size exclusion chromatography, using a HiLoad 16/60 Superdex-75 column (GE Healthcare) in buffer D, obtaining the purified ZipA that was concentrated to 10 mg/ml and stored at -80°C.

3.12 Co-sedimentation assay of FtsZ with ZipA or SulA

The polymerization reactions were carried out in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, adding 12 µM FtsZ, 12 µM ZipA or SulA and 2 mM GTP or GDP. The reaction mixtures were incubated for 10 min at 30°C and 300 rpm to allow the polymerization to occur. Then, samples were ultracentrifuged at 350,000 *x g* for 10 min at 25°C, and the supernatants were immediately separated from the pellets, which contained the protein polymers. Supernatant and pellet samples were analyzed by SDS-PAGE on 12% polyacrylamide gels. The quantification of FtsZ polymerization was performed as previously described (Król and Scheffers, 2013), using densitometry, by calculating the percentage of the relative intensity of the bands.

3.13 Co-sedimentation assay of FtsZ with FtsA and vesicles

To allow FtsA interactions with membranes via its C-terminal amphipathic helix in the assay, lipid vesicles were added to the reaction. Vesicles from *E. coli* total lipid extract (Avanti Polar Lipids) were prepared in 25 mM PIPES (pH 6.8), 300 mM KCl and 10 mM $MgCl_2$ by sonication. The reaction mixtures contained 2 mg/ml of vesicles with 12 µM of FtsZ and FtsA, 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, and 2 mM GTP, GDP or ATP. The reactions were incubated for 10 min at 30°C and 300 rpm to allow the polymerization to occur, then samples were centrifuged at 20,000 *x g* at 20°C for 25 min. Supernatants were immediately removed for analysis and the pellets were resuspended in the same volume of 25 mM PIPES (pH 6.8), 300 mM KCl and 10 mM $MgCl_2$ and solubilized with SDS gel loading buffer. Finally, samples were analyzed by SDS–PAGE on 12% polyacrylamide gels.

3.14 Biofilm inhibition assay of *B. cenocepacia* J2315 in three-dimensional (3-D) lung epithelial cell model

The biofilm inhibitory activity of the D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) 109 nanosuspension was tested in a 3-D lung epithelial model. This model was validated as physiologically relevant using the human adenocarcinoma alveolar epithelial cell line A549 (ATCC® CCL-185™), demonstrating that cells grown in these conditions show *in vivo*-like characteristics, normally not observed in conventional monolayers (Carterson *et al*., 2005). Indeed, they form lung epithelium-like architectures, they have apical-basal orientation, barrier function, and they produce mucus. Moreover, they show a decreased expression of cancer markers, reflecting a phenotype more similar to the parental tissue (Carterson *et al*., 2005).

In this work, the 3-D model of human CF bronchial epithelial cell line CFBE41o- (Ehrhardt *et al*., 2006) was used. Cells were cultured in minimum essential medium (MEM) (Thermo Fischer) supplemented with 2 mM L-glutamine (Thermo Fischer), 10% of heat-inactivated fetal bovine serum (FBS) (Thermo Fischer), and 100 U/ml penicillin, 100 µg/ml streptomycin, following the protocol described for A549 cells (Carterson *et al*., 2005; Crabbé *et al*., 2017). After 14 days of incubation in the rotating-wall vessel (Synthecon), in a state of constant free fall in the growth medium, CFBE41o-

cells formed fully differentiated 3-D aggregates using the type I collagen-coated dextran beads as growth scaffold. At this point, the culture medium was changed with LHC-8 w/o Gentamicin (Thermo Fisher) and $2.5x10^5$ cells in 250 µl per well were seeded in 48-wells plate. Then, a culture of *B. cenocepacia* J2315 grown for 20 h in LB at 37°C was resuspended in LHC-8 medium and added to the 3-D cells at a multiplicity of infection (MOI) of 15:1. Biofilm formation was determined in the absence (negative control) or presence of different concentrations of TPGS109 (16-24 µg/ml), in the presence of ciprofloxacin (16 µg/ml) as antibiotic control, and in the presence of the nanoparticles carrier only (TPGS 64 µg/ml). Plates were incubated statically for 1 h at 37°C, 5% CO_2 and, subsequently, cultures were rinsed once with Hanks' Balanced Salt Solution (HBSS) to limit the quantity of bacteria not associated with host cells. After that, fresh medium containing the same concentrations of TPGS109 and the control antibiotic was added to the wells and further incubated for 16 h in the same conditions. The day after, 3-D aggregates were transferred in new wells, to avoid the inclusion of bacteria adhered to the bottom of the wells, and then rinsed twice with HBSS. Subsequently, *B. cenocepacia* biofilm and the cells where disrupted adding 200 µl of 1% Triton-X100 and vigorously pipetting several times. The suspensions, containing both adhered and intracellular bacteria, were serially diluted, plated onto LB agar plates and incubated at 37°C. After 48 h, colonies were counted and CFU/ml were determined.

3.15 Cytotoxicity assessment in 3-D lung epithelial cell model

Cytotoxicity of the TPGS109 to 3-D aggregates of CFBE41o- cells was assessed by measuring the lactate dehydrogenase (LDH) enzymatic activity in the culture supernatant. 3-D cell cultures were generated as described above and seeded in a 48-wells plate ($2.5x10^5$ cells per well in 250 µl). The cytotoxicity was determined using the same conditions of the biofilm inhibition assay, in the presence of the same TPGS109 concentrations. After 17 h of incubation at 37°C, 5% CO_2, supernatants were collected and centrifuged at 3700 rpm for 15 min to eliminate cell debris. Finally, the LDH activity was quantified using the LDH activity assay kit (Sigma-Aldrich) following the manufacturer's instructions. Culture media of cells incubated in LHC-8 alone were used as negative (cell viability) control (Ct-), while supernatants of cells disrupted with 1% Triton-X100 were used as positive

(death) control (Ct+). The cytotoxic effect was assessed using the following formula:

$$Cytotoxicity\ (\%) = \frac{(LDH\ activity\ compound) - (LDH\ activity\ Ct-)}{(LDH\ activity\ Ct+) - (LDH\ activity\ Ct-)} * 100$$

3.16 Metabolic transformation of C109 in CFBE41o- cultures

To verify if CFBE41o- cells can metabolize the C109 compound, cells were grown as monolayer in a T175 flask (Thermo Fisher), with the same medium used for the 3-D cultures, incubated at 37°C, 5% CO_2. Once cells reached confluence, the monolayer was rinsed twice with PBS and 80 ml of LHC-8 medium w/o Gentamicin (Thermo Fisher) and 16 µg/ml C109 were added to the culture. The flask was further incubated at 37°C, 5% CO_2 and 20 ml of supernatant were collected after 0, 10, 30, 60 min of incubation. These samples were subjected to chloroform extraction (10 ml x 3) and the organic phases were evaporated. The obtained residues were resuspended in 500 µl of chloroform and 5 µl of each sample were analyzed by thin layer chromatography (Silica gel 60 F_{254}, Sigma Aldrich) using chloroform or chloroform/methanol 99:1 as eluents. Metabolites were visualized under UV light at a wavelength of 254 nm.

Plasmid	Description	Source
pSU11	*E. coli-Burkholderia* shuttle vector containing *lacZ* reporter gene downstream of the MCS, Gm^r	Jenul *et al*., 2018
pSU11-161	pSU11 containing the *dcw* predicted promoter sequence in a 161 bp fragment	This study
pSU11-289	pSU11 containing the *dcw* predicted promoter sequence in a 289 bp fragment	This study
pSU11-ftsQp	pSU11 containing the DNA sequence of the gene *ddl*	This study
pSU11-ftsAp	pSU11 containing the DNA sequence of the gene *fts*Q	This study
pSU11-ftsZp1	pSU11 containing the first half of the *ftsA* gene sequence	This study
pSU11-ftsZp2	pSU11 containing the second half of the *ftsA* gene sequence	This study
pET28a	Expression vector IPTG inducible, Kan^r	Novagen
pET28a-MraZ	pET28a containing MraZ coding sequence	This study
pBADM-41	Expression vector for toxic or unstable proteins, containing MBP fusion, controlled by *araBAD* promoter, Amp^r	Laboratory collection
pBADM-41-SulA	pBADM-41 containing SulA coding sequence	This study
pETSUMO	Expression system incorporating a SUMO fusion, IPTG inducible, Kan^r	Invitrogen
pETSUMO-FtsA	pETSUMO containing FtsA coding sequence	This study
pETSUMO-ZipA	pETSUMO containing ZipA coding sequence	This study

Table 9. Plasmids used for promoter characterization and expression of recombinant proteins.

Plasmid	Description	Source
pUT18	Derivative of pUC19. A multicloning site sequence (MCS)allows construction of in-frame fusions at the N-terminal end of the T18 polypeptide under the control of a lac promoter, Amp^r	Karimova *et al*., 1998
pUT18C	Derivative of pUC19. A MCS allows construction of in-frame fusions at the C-terminal end of the T18 polypeptide under the control of a lac promoter, Amp^r	Karimova *et al*., 1998
pKT25	Derivative of pSU40. A MCS allows construction of in-frame fusions at the C-terminal end of the T25 polypeptide under the control of a lac promoter, Kan^r	Karimova *et al*., 1998
pKNT25	Derivative of pSU40. A MCS allows construction of in-frame fusions at the N-terminal end of the T25 polypeptide under the control of a lac promoter, Kan^r	Karimova *et al*., 1998
pUT18FtsZ	pUT18 containing the T18 fused to the C-terminal end of FtsZ	This study
pUT18CFtsZ	pUT18C containing the T18 fused to the N-terminal end of FtsZ	This study
pKT25FtsZ	pKT25 containing the T25 fused to the N-terminal end of FtsZ	This study
pKNT25FtsZ	pKNT25 containing the T25 fused to the C-terminal end of FtsZ	This study
pUT18FtsA	pUT18 containing the T18 fused to the C-terminal end of FtsA	This study
pUT18CFtsA	pUT18C containing the T18 fused to the N-terminal end of FtsA	This study
pKT25FtsA	pKT25 containing the T25 fused to the N-terminal end of FtsA	This study
pKNT25FtsA	pKNT25 containing the T25 fused to the C-terminal end of FtsA	This study
pUT18FtsE	pUT18 containing the T18 fused to the C-terminal end of FtsE	This study
pUT18CFtsE	pUT18C containing the T18 fused to the N-terminal end of FtsE	This study
pKT25FtsE	pKT25 containing the T25 fused to the N-terminal end of FtsE	This study
pKNT25FtsE	pKNT25 containing the T25 fused to the C-terminal end of FtsE	This study
pUT18FtsQ	pUT18 containing the T18 fused to the C-terminal end of FtsQ	This study
pUT18CFtsQ	pUT18C containing the T18 fused to the N-terminal end of FtsQ	This study
pKT25FtsQ	pKT25 containing the T25 fused to the N-terminal end of FtsQ	This study
pKNT25FtsQ	pKNT25 containing the T25 fused to the C-terminal end of FtsQ	This study
pUT18FtsI	pUT18 containing the T18 fused to the C-terminal end of FtsI	This study
pUT18CFtsI	pUT18C containing the T18 fused to the N-terminal end of FtsI	This study
pKT25FtsI	pKT25 containing the T25 fused to the N-terminal end of FtsI	This study
pKNT25FtsI	pKNT25 containing the T25 fused to the C-terminal end of FtsI	This study
pUT18FtsN	pUT18 containing the T18 fused to the C-terminal end of FtsN	This study
pUT18CFtsN	pUT18C containing the T18 fused to the N-terminal end of FtsN	This study
pKT25FtsN	pKT25 containing the T25 fused to the N-terminal end of FtsN	This study
pKNT25FtsN	pKNT25 containing the T25 fused to the C-terminal end of FtsN	This study
pUT18ZipA	pUT18 containing the T18 fused to the C-terminal end of ZipA	This study
pUT18CZipA	pUT18C containing the T18 fused to the N-terminal end of ZipA	This study

pKT25ZipA	pKT25 containing the T25 fused to the N-terminal end of ZipA	This study
pKNT25ZipA	pKNT25 containing the T25 fused to the C-terminal end of ZipA	This study
pUT18ZapA	pUT18 containing the T18 fused to the C-terminal end of ZapA	This study
pUT18CZapA	pUT18C containing the T18 fused to the N-terminal end of ZapA	This study
pKT25ZapA	pKT25 containing the T25 fused to the N-terminal end of ZapA	This study
pKNT25ZapA	pKNT25 containing the T25 fused to the C-terminal end of ZapA	This study
pUT18SulA	pUT18 containing the T18 fused to the C-terminal end of SulA	This study
pUT18CSulA	pUT18C containing the T18 fused to the N-terminal end of SulA	This study
pKT25SulA	pKT25 containing the T25 fused to the N-terminal end of SulA	This study
pKNT25SulA	pKNT25 containing the T25 fused to the C-terminal end of SulA	This study

Table 10. Plasmids used for BACTH complementation assays.

4. Results

4.1 Characterization of the *dcw* operon of *B. cenocepacia* J2315

4.1.1 Identification of the *dcw* cluster in *B. cenocepacia* genome

It is known that in rod-shaped bacteria the genes coding for the key proteins of cell division are clustered in the *dcw* (division cell wall) operon (Tamames *et al*., 2001). This is thought to facilitate the coordinated expression of these proteins during binary fission. In order to localize this cluster in *B. cenocepacia* J2315, the *Burkholderia* genome database (https://www.burkholderia.com, Winsor *et al*., 2008) was used. The *dcw* cluster was identified as a DNA region of 19.158 bp (3803412 - 3784254) on the negative strand of the chromosome 1, encompassing 17 genes transcribed with the same polarity (Figure 12). These show the same arrangement found in the *dcw* operon of *E. coli*, with the insertion of the gene *BCAL3456*, a thioredoxin reductase, between *ftsZ* and *lpxC* (*envA*) (Figure 12).

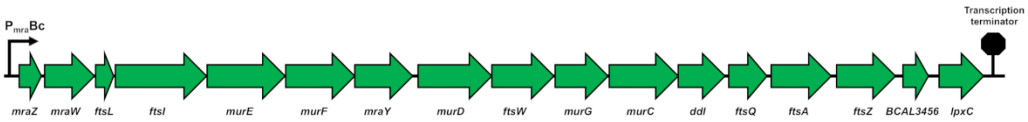


Figure 12. Representation of the *dcw* cluster of *B. cenocepacia* J2315. The genes are drawn to scale with the arrows indicating the transcriptional orientation. The promoter P_{mra}Bc is represented by the black arrow at the 5' of the cluster, whereas the transcription terminator is found downstream of the last gene *lpxC*.

4.1.2 Study of the transcriptional organization of the *dcw* operon

According to previous reports (Dewar and Dorazi, 2000; Francis *et al*., 2000; Real and Henriques, 2006), the *dcw* operon is usually subdivided in many

transcriptional units, controlled by several promoters and transcription factors. To determine the transcriptional organization of the cluster in *B. cenocepacia*, firstly its whole DNA sequence was analyzed using the bacterial rho-independent transcription terminator prediction software TransTermHP (http://transterm.ccb.jhu.edu/query.php, Kingsford *et al*., 2007). A single transcription terminator site (3784225 - 3784210) was predicted downstream of the gene *lpxC*, with no other intermediate terminators within this sequence. Then, to verify whether the transcription can proceed continuously along the entire cluster, RT-PCR experiments were carried out. The total RNA of *B. cenocepacia* J2315 was extracted from bacteria in mid-log phase and retrotranscribed using the reverse primers listed in Table 3. Each primer was designed to anneal at the 5'-end of a different *dcw* gene. The cDNAs obtained were used as templates for PCR reactions containing the same reverse primers coupled with forward primers (Table 3) annealing at the 3'-end of the upstream gene. This allowed the analysis of the co-transcription of every gene pair combination from *mraZ* to *lpxC*. The expected fragment length for each primer pair is reported in Table 3. Every fragment of the gene pairs within the *mraZ-ftsZ* region were successfully amplified (data not shown), demonstrating that each gene is transcribed together with the flanking genes, and thus the transcription can proceed from *mraZ* to *ftsZ*. This implies the formation of a single polycistronic mRNA of about 17 kb. Conversely, none of the expected amplicons of the *ftsZ-lpxC* region were obtained, demonstrating that the last two genes of the cluster are transcribed as single mRNAs. These results suggest the presence of a different mechanism to block the transcription, since no rho-independent transcription terminator sequences were found downstream of *ftsZ*.

4.1.3 Characterization of the *dcw* cluster promoter sequence and transcription start site

In *E. coli*, it is reported that the transcription of the first 9 genes of the *dcw* cluster starts 38 bp upstream of the *mraZ* gene and is controlled by the P_{mra} promoter (Hara *et al*., 1997). For this reason, to identify the promoter of the first 15 *dcw* genes in *B. cenocepacia*, a bioinformatic analysis of the non-coding region upstream of the *mraZ* gene (3803413 - 3803798) was carried out using the BDGP Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and the default parameters for predictions of promoters in prokaryotes. This search allowed the

identification of a 50 bp putative promoter sequence starting 141 bp upstream of the first translated codon of *mraZ* (Figure 13).

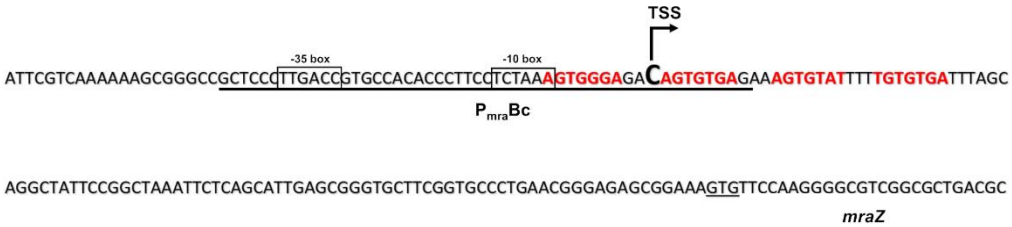


Figure 13. Representation of the P_{mra}Bc promoter sequence. The P_{mra}Bc sequence is underlined in bold, and its -10 and -35 boxes are indicated. The transcription star site (TSS), the repeats of the putative MraZ binding site (highlighted in red) and the first *mraZ* translated codon (underlined) are also present.

To experimentally demonstrate its promoter activity, two overlapping fragments were amplified using the primers dcwPROMOfor1_2 and dcwPROMOrev2, and dcwPROMOfor2_2 and dcwPROMOrev2 (Table 4). These fragments were 289 bp and 161 bp long and both contained the putative promoter sequence. After the amplification, the fragments were cloned within the MCS of the pSU11 vector, upstream of a promoterless *lacZ* gene, obtaining the pSU11-161 and pSU11-289 (Table 9). The plasmids were mobilized from *E. coli* to *B. cenocepacia* K56-2 by triparental mating and the expression of *lacZ* was assessed by β-galactosidase activity assay on cells in early stationary phase. The tested fragments showed comparable activities (empty pSU11: 2.2±0.92; pSU11-161: 101.8±4.7 MU; pSU11-289: 87.7±5.6 MU) (Figure 14A), demonstrating that the sequence has promoter activity and the 161 bp fragment contains the minimal transcription promoter P_{mra}Bc (Figure 13) of the *dcw* operon in *B. cenocepacia*.

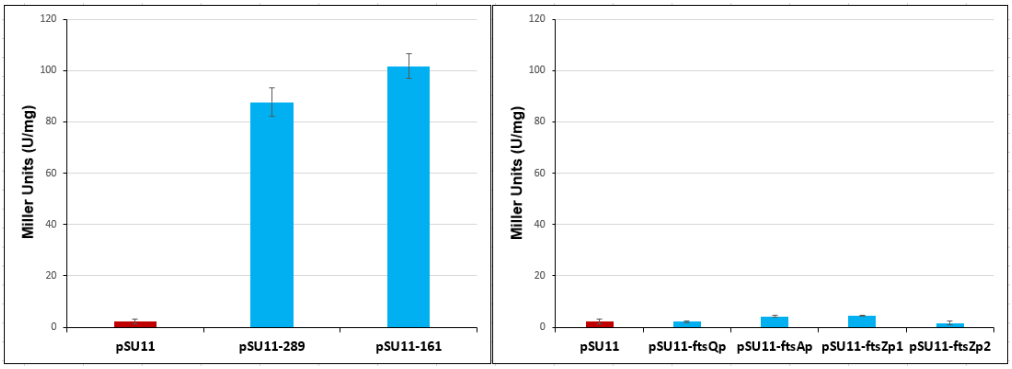


Figure 14. Charts showing the promoter activities of the tested fragments expressed in Miller units. A) The 289 bp- (pSU11-289) and 161 bp- (pSU11-161) fragments show a strong promoter activity, containing the promoter of the first 15 *dcw* genes of P_{mra}Bc. B). The four fragments covering the *ddl-ftsA* DNA region show a promoter activity comparable to the negative control (empty pSU11 vector, red bars), proving the absence of additional promoters within the *dcw* operon. Results are expressed as mean of 3 experiments and the error bars represent the standard deviation (SD).

The expression of FtsZ is known to be finely regulated in many microorganisms (Flärdh *et al*., 1997; Real and Henriques, 2006; Santini *et al*., 2013) since it was demonstrated that its intracellular concentration strongly influences bacterial division (Palacios *et al*., 1996). Thus, the presence of additional promoters in the *dcw* region upstream of *ftsZ* of *B. cenocepacia* was assessed. The *ddl-ftsA* region was analyzed using the BDGP Neural Network Promoter Prediction software as previously described. Within this sequence, no putative promoters were found by bioinformatic analysis, but nevertheless it was experimentally tested using *lacZ* fusions. Four consecutive fragments were amplified by PCR: ftsQp, using murCPROMOfor1-ddlPROMOrev1; ftsAp, using ftsQPROMOfor1-ftsQPROMOrev1; ftsZp1, using ftsAPROMOfor1-ftsAPROMOrev1; ftsZp2, using ftsAPROMOfor2-ftsAPROMOrev2 (Table 4). In this way, the whole *ddl-ftsA* region was amplified and tested (Figure 15).

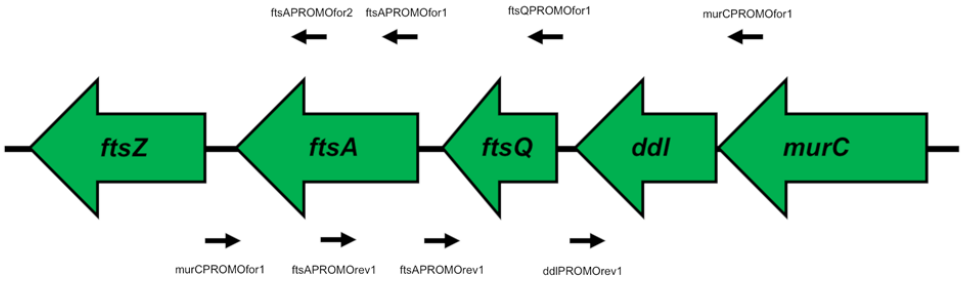


Figure 15. Magnified view of the distal genes of the *dcw* cluster. The primers used for the amplification of the *ddl-ftsA* fragments are depicted as black arrows.

The amplified fragments were cloned upstream of the promoterless *lacZ* gene into the pSU11 vector, creating the pSU11-ftsQp, pSU11-ftsAp, pSU11-ftsZp1, pSU11-ftsZp2 plasmids (Table 9). Their promoter activities were assessed by β-galactosidase activity assay using *B. cenocepacia* K56-2. The experiment was performed using cells in early stationary phase since, in *E. coli*, it is demonstrated that promoters of this region increase their activity once the growth rate decreases (Flärdh *et al*., 1997). In these conditions, the tested fragments showed no promoter activity (empty pSU11: 1.93±0.55 MU; pSU11-ftsQp: 2.20±0.38 MU; pSU11-ftsAp: 4.22±0.49 MU; pSU11-ftsZp1: 4.38±0.21 MU; pSU11-ftsZp2: 1.63±0.71 MU) (Figure 14B), confirming the bioinformatic prediction. Consequently, these results demonstrated that, in standard laboratory culture conditions, the *dcw* cluster transcription starts only from P_{mra}Bc, and *ftsZ* expression is exclusively controlled by this promoter.

Consequently, the transcription start site (TSS) of the *dcw* polycistronic mRNA was identified using 5'-RACE as described in Materials and methods. The TSS resulted to be a cytosine localized 100 bp upstream of the first codon of *mraZ*, at the 3'-end of the P_{mra}Bc promoter sequence (Figure 13).

4.2 Characterization of MraZ, the transcription factor of the *dcw* cluster

4.2.1 Expression and purification of MraZ

The MraZ protein of *B. cenocepacia* J2315 was expressed in the conditions described in Materials and methods, using the pET28a-MraZ vector (Table 9) transformed into *E. coli* strain BL21(DE3). The purification was achieved by immobilized metal affinity chromatography (IMAC) and the soluble fraction of the his-tagged MraZ was eluted using 250 mM of imidazole (Figure 16).

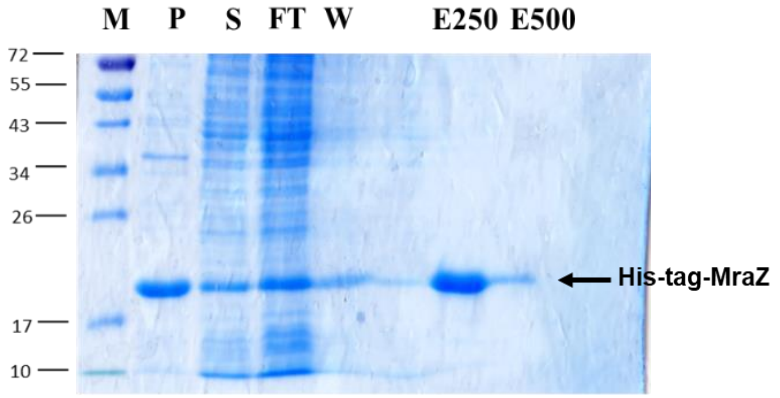


Figure 16. SDS-PAGE analysis of the fractions collected during the purification of MraZ. The soluble protein, indicated by the arrow, was eluted with 250 mM of imidazole (E250). M, molecular weight marker (Thermo Fisher); P, pellet; S, supernatant; FT, flow-through; W, wash; E250, elution with 250 mM imidazole; E500, elution with 500 mM imidazole.

After that, the his-tag tail was removed from the protein using the PreScission protease and MraZ was further purified by IMAC. Finally, 5 mg of pure protein were obtained from 3 liters of culture and used for the electrophoretic mobility shift assay.

4.2.2 Electrophoretic mobility shift assay (EMSA) of MraZ

To characterize the DNA binding site (DBS) of MraZ, two fragments, overlapping for 137 bp and covering the whole intergenic region upstream of *mraZ*, were amplified using 6-carboxyfluorescein (6-Fam) labeled primers. In particular, fragment BS1-6-Fam, containing the 5' half of the region from -386 bp to -136 bp upstream of the *mraZ* gene, was obtained using mraZBS1for and mraZBS1rev (Table 7); instead, fragment BS2-6-Fam, containing the 3' half of the region from -250 bp to -1 bp upstream of *mraZ*, was amplified with mraZBS2for and mraZBS2rev (Table 7). The two probes were chosen according to the fact that the MraZ DBS consists of repeated sequences localized within this DNA region in *E. coli*, *B. subtilis*, and mycoplasmas (Fisunov *et al*., 2016). Both probes were 250 bp long and were used for EMSA in the presence of different MraZ concentrations, proving that the protein is able to bind BS2-6-Fam (Figure 17A-B), but not BS1-6-Fam (data not shown). The specificity of the binding reaction was confirmed by adding a 20X excess of non-labeled BS2 fragment or a 20X excess of a non-specific 250 bp-competitor in the binding reaction (Figure 17C-D).

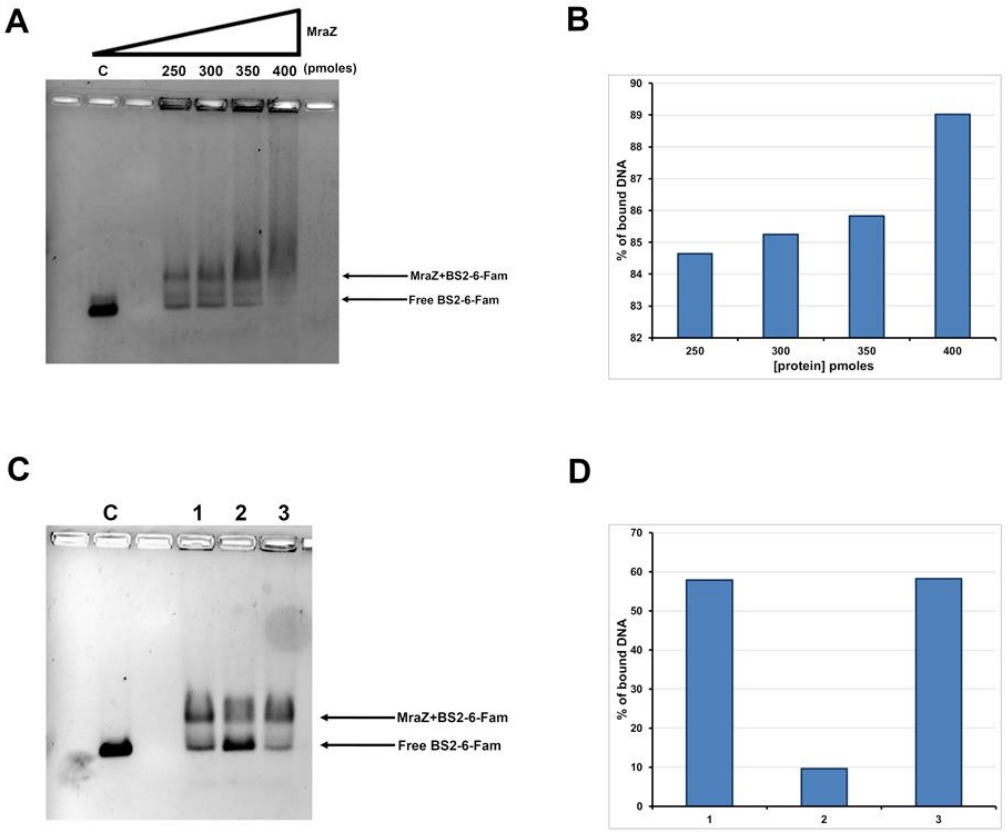


Figure 17. Electrophoretic mobility shift assay of the MraZ protein and relative quantification of the bound DNA. A) The gel shows the band shift using a constant BS2-6-Fam probe amount (C, 0.34 pmoles) and increasing quantities of MraZ. B) The bars indicate the relative quantification of the percentage of the bound DNA obtained by densitometry analysis in function of the protein concentration. C) Demonstration of the specificity of the binding of MraZ to the fragment BS2, using a fixed amount of protein (300 pmoles) and BS2-6-Fam (C, 0.34 pmoles) (lane 1), a 20X excess of non-labeled BS2 fragment which competes for the binding of MraZ (lane 2), or a 20X concentration of non-specific, non-labeled competitor (a 250 bp *cepI* fragment) (lane 3). D) The bars indicate the relative quantification of the percentage of the bound DNA of the corresponding lanes obtained by densitometry analysis.

Finally, to find a putative MraZ DBS in *B. cenocepacia*, the BS2 fragment sequence was analyzed, searching for a repeated GTG motif, known to be a conserved in evolutionary distant bacteria (Fisunov *et al*., 2016). The sequence was identified as series of four repeats of seven nucleotides separated by a 3-nucleotide spacer region (Figure 13). In particular, it is localized downstream of the P_{mra}Bc -10 box, as reported in *E. coli* (Eraso *et al*., 2014), and it overlaps with the *dcw* operon TSS (Figure 13).

4.3 Study of the FtsZ interactors in *B. cenocepacia* J2315 *in vivo* and *in vitro*

4.3.1 Complementation analysis of cell division protein using a Bacterial Adenylate Cyclase Two Hybrid (BACTH) assay

Since the division mechanism of *B. cenocepacia* has never been studied before, and the proteins involved in the formation of the divisome can only be deduced from homology comparison, the Bacterial Adenilate Cyclase Two Hybrid (BACTH) assay was used to identify FtsZ interactors in this bacterium. The proteins FtsA, FtsE, FtsQ, FtsI, FtsN, ZipA, ZapA, SulA were selected for the analysis, comparing the sequence of the *B. cenocepacia* proteins with the *E. coli* well characterized homologues, and cloned into the four plasmids of the BACTH system (pUT18, pUT18C, pKT25, pKNT25). The obtained recombinant vectors (Table 10) expressed the inserted proteins fused at the C- or N-terminus of either the T18 or the T25 fragment of the *Bordetella pertussis* adenylate cyclase. Each protein was tested for the interaction with FtsZ in every possible combination, using the four different constructs (Table 10), co-transforming the plasmids in *E. coli* strain BTH101 (*cya*$^-$). The functional complementation efficiencies between the hybrids were initially evaluated using β-galactosidase activity in solid media; then only pairs of vectors showing a significant complementation efficiency were further tested quantifying the β-galactosidase activity in liquid medium. BTH101 cells co-transformed with empty pUT18 and pKT25 vectors were used as negative controls. Levels of β-galactosidase activity at least four-fold higher than that measured for the negative control were considered indicative of an interaction.

The first interaction assessed was between two FtsZ monomers, co-transforming BTH101 cells with both vectors expressing FtsZ, and obtaining a β-galactosidase activity value five-fold higher than the control. This interaction (Figure 18A-B) indicates a head to tail interaction of the monomers, known to be necessary for the GTPase activity and the polymerization of FtsZ (Schumacher *et al*., 2020).

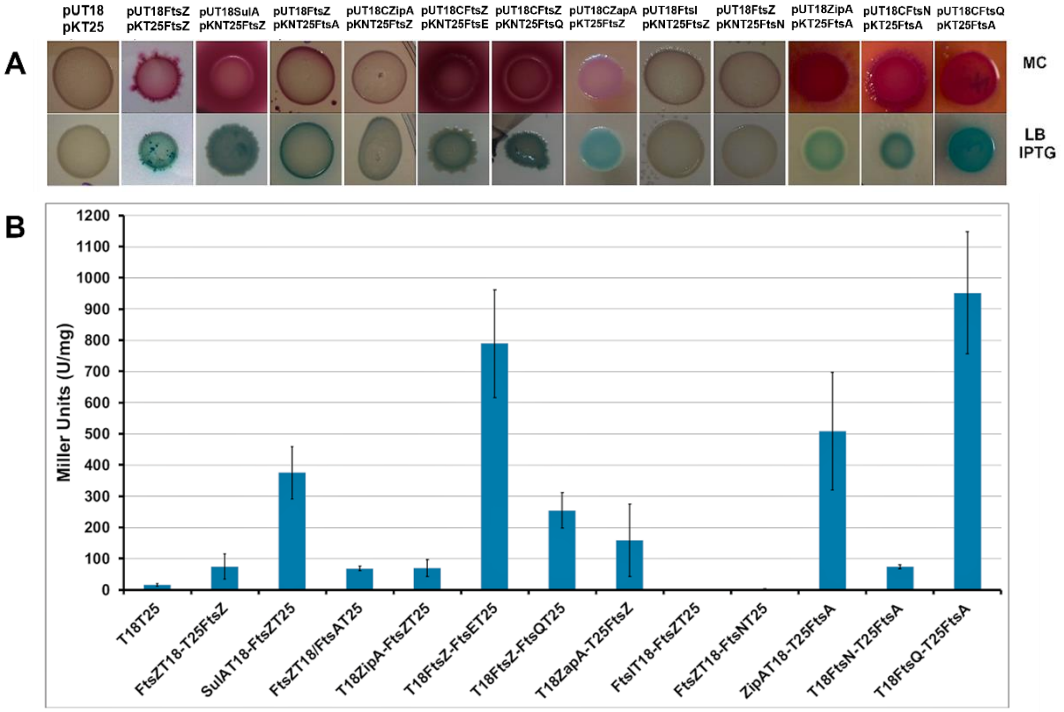


Figure 18. BACTH analysis of interactions between division proteins. A) *E. coli* strain BTH101 was co-transformed with two-hybrid plasmids (Table 10) expressing different hybrid proteins, as indicated. Transformants were spotted onto LB plates containing IPTG and X-Gal, or on MacConkey agar plates (MC) and incubated at 30°C for 40 h. Blue coloration on LB-IPTG-X-Gal or pink coloration on MC indicates a positive interaction. B) The efficiencies of functional complementation between the indicated hybrid proteins were quantified by measuring β-galactosidase activities in suspensions of *E. coli* BTH101 cells carrying the corresponding plasmids. These results represent the mean value of at least three independent experiments and error bars indicate the SD. The *E. coli* BTH101 harboring the empty vectors was used as control.

The SulA protein blocks FtsZ polymerization in *E. coli* and in *Pseudomonas aeruginosa* in case of DNA damage (Cordell *et al*., 2003). Using the BACTH assay, the putative SulA of *B. cenocepacia* was demonstrated to interact with FtsZ using its N-terminal part (Figure 18A-B), since the β-galactosidase activity resulted twenty-five-fold higher than the control. These results confirmed the involvement of the protein in the cell division of *B. cenocepacia*.

FtsA is demonstrated to have a key role in the bacterial division, directly interacting with FtsZ to anchor the Z-ring to the membrane (Szwedziak *et al*., 2012). The BACTH assay showed that the co-transformed strain containing *B. cenocepacia* FtsZ and FtsA had a β-galactosidase activity five-fold higher than the control strain (Figure 18A-B). This combination of the two hybrid proteins indicates a physical interaction between the N-terminus of FtsA and FtsZ.

ZipA, a protein involved in the first steps of cell division, has been described to help the tethering of FtsZ to the membrane (Liu *et al*., 1999). The results obtained indicate that *B. cenocepacia* FtsZ interacts with the C-terminus of ZipA, since the β-galactosidase activity of the BACTH assay is 4.5-fold higher than the control (Figure 18A-B).

The last early phase protein analyzed was ZapA (Caldas *et al*., 2019). The two-hybrid assay showed a β-galactosidase activity ten-fold higher than the control in BTH101 strains carrying the plasmids expressing *B. cenocepacia* FtsZ and ZapA (Figure 18A-B), demonstrating the interaction of FtsZ with the C-terminus of ZapA.

Regarding the late proteins of the divisome assembly, the interaction between FtsZ and FtsE, FtsQ, FtsI and FtsN was evaluated. Results showed that BTH101 cells expressing FtsZ and FtsE had a β-galactosidase activity fifty-fold higher than the control. This demonstrates a physical interaction between FtsZ and the N-terminal region of FtsE, as reported in *E. coli* (Du *et al*., 2019). Conversely, the proteins FtsZ and FtsQ were hypothesized to interact in *E. coli* only in a work by Descoteaux and Drapeau (1987), but a further confirmation of this result was never achieved. Here, through BACTH assay, it was demonstrated the interaction between the C-terminal part of FtsZ and the N-terminal part of FtsQ. Indeed, the BTH101 strain transformed with the vectors expressing the two proteins of *B. cenocepacia* showed a β-galactosidase activity sixteen-fold higher than the control strain. Instead, the

two-hybrid assays performed on clones expressing FtsZ together with FtsI or FtsN did not show any significant β-galactosidase activity (Figure 18A-B).

Since the proteins FtsA and ZipA physically interact in *E. coli* (Vega and Margolin, 2019), this was investigated also in *B. cenocepacia* using the BACTH assay. The experiment revealed that the strain expressing the two proteins had a β-galactosidase activity thirty-two-fold higher than the control strain (Figure 18A-B). This result demonstrated that FtsA is able to interact with the N-terminal part of ZipA. In *E. coli* FtsA interacts also with FtsN to connect early and late divisome proteins (Busiek *et al.*, 2012). Here, it was shown that *B. cenocepacia* FtsA and FtsN interact each other with their C-termini, having a β-galactosidase activity five-fold-higher than the control (Figure 18A-B). Finally, the interaction between FtsA and FtsQ was also tested, revealing that the early division protein FtsA of *B. cenocepacia* interacts also with the late protein FtsQ (Figure 18A-B), as previously described in *E. coli* (Tsang and Bernhardt, 2015).

4.3.2 Expression and purification of SulA

The SulA protein of *B. cenocepacia* J2315 was expressed in the conditions described in Materials and methods, using the pBADM-41-SulA vector (Table 9) co-transformed with the pG-Tf2 plasmid (containing the *E. coli* GroES-GroEL-tig chaperones) in *E. coli* strain TOP10. The first step of purification was achieved using the column MBPTrap HP and the soluble fraction of MBP-SulA complexed with GroEL was eluted with 10 mM of maltose (Figure 19).

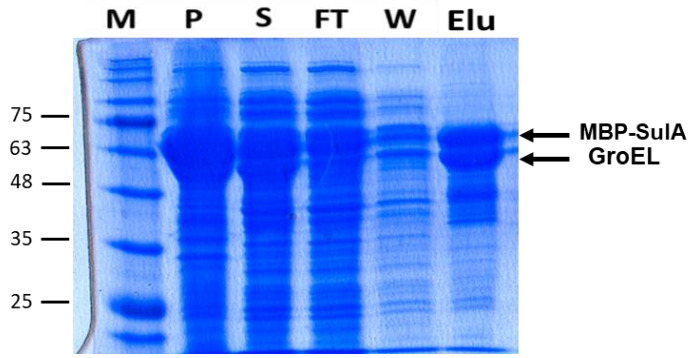


Figure 19. SDS-PAGE analysis of the fractions collected during the purification of SulA. The soluble protein was eluted with 10 mM of maltose (Elu). M, molecular weight marker (PanReac); P, pellet; S, supernatant; FT, flow-through; W, wash; Elu, elution with 10 mM maltose.

Then, to eliminate the GroEL chaperone, the MBP-SulA protein was further purified using cation exchange chromatography and eluted with a (0 - 1 M) KCl gradient (Figure 20).

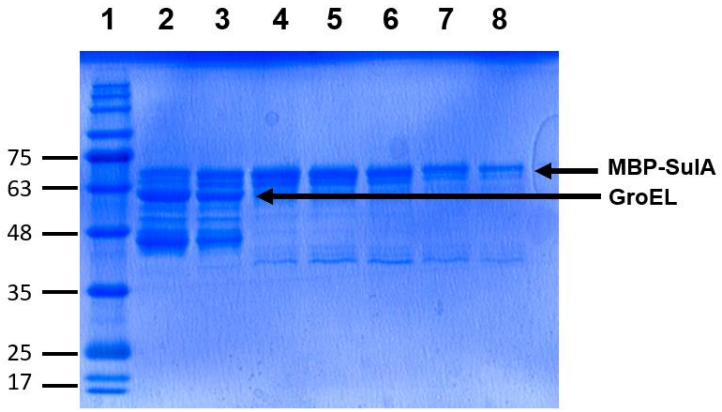


Figure 20. SDS-PAGE analysis of the fractions collected after the ion exchange chromatography of SulA. Fractions starting from the left: 1) molecular weight markers (PanReac); 2,3) elution of GroEL and contaminant proteins; 4-8) fractions containing MBP-SulA without the chaperone GroEL.

The fractions containing the pure MBP-SulA were pooled and concentrated, obtaining 6 mg of protein from 3 liters of culture, which were used for the co-sedimentation assay with FtsZ.

4.3.3 SulA inhibits FtsZ polymerization *in vitro*

To confirm the physical interaction between FtsZ and SulA, demonstrated *in vivo* using the BACTH assay, and its regulatory function in cell division, a co-sedimentation assay was carried out. To this aim, *B. cenocepacia* SulA was expressed and purified as described above, whereas *B. cenocepacia* FtsZ was obtained as described by Hogan *et al*. (2018). In order to investigate the effect of SulA on FtsZ polymerization, the assay was performed in the absence and in the presence of SulA. In the presence of its co-factor GTP, FtsZ alone is able to polymerize, allowing the recovery of a good amount of protein in the pellet fraction (Figure 21).

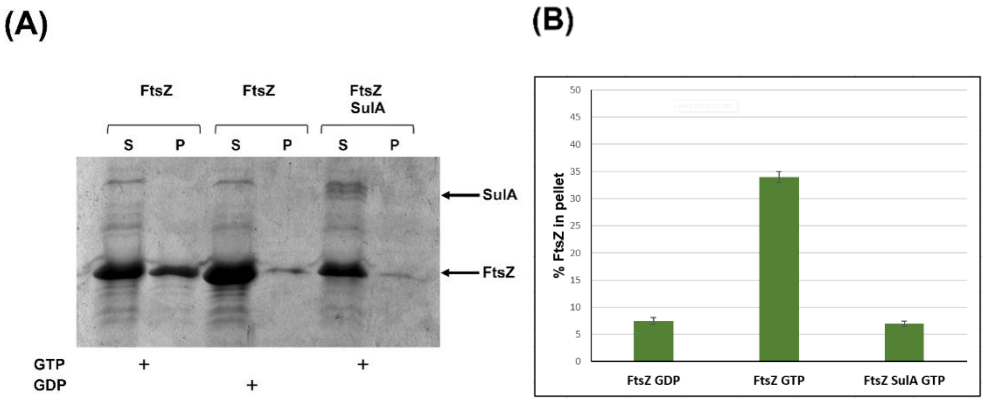


Figure 21. Effect of SulA on FtsZ *in vitro* polymerization. FtsZ was induced to polymerize in the presence or absence of SulA. (A) The polymeric FtsZ was collected by sedimentation and the amount of FtsZ in the pellets (P) was estimated by Coomassie-blue staining of the SDS-PAGE. S, supernatant. (B) The bars indicate the relative quantification of FtsZ percentage in the pellet obtained by densitometry analysis. Data are the average ± SD of three independent experiments.

When SulA was added to the polymerization assay, in an equimolar concentration of FtsZ and in the presence of GTP to start the reaction, FtsZ was recovered only in the supernatant fraction. This indicates that FtsZ was not able to polymerize. As a control, FtsZ in the presence of GDP (no polymerization) was recovered only in the supernatant (Figure 21A-B). These results demonstrated that SulA blocks FtsZ polymerization *in vitro*, confirming its role as regulator of FtsZ activity in *B. cenocepacia*.

4.3.4 Expression and purification of FtsA

The FtsA protein of *B. cenocepacia* J2315 was expressed in the conditions described in Materials and methods, using the pETSUMO-FtsA vector (Table 9) transformed into *E. coli* strain BL21(DE3). The purification was achieved by IMAC and the soluble fraction of the SUMO-FtsA fusion was eluted using 250 mM of imidazole (Figure 22).

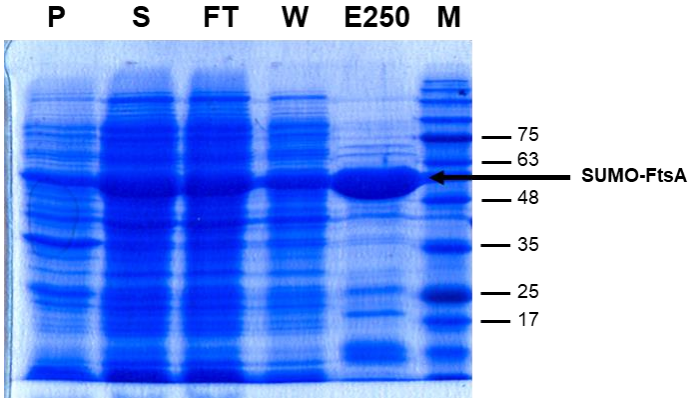


Figure 22. SDS-PAGE analysis of the fractions collected during the purification of FtsA. The soluble protein was eluted with 250 mM of imidazole (E250). P, pellet; S, supernatant; FT, flow-through; W, wash; E250, elution with 250 mM imidazole; M, molecular weight marker (PanReac).

After that, the SUMO protein was removed from FtsA using the SUMO protease, and the native protein was further purified by size exclusion chromatography (Figure 23).

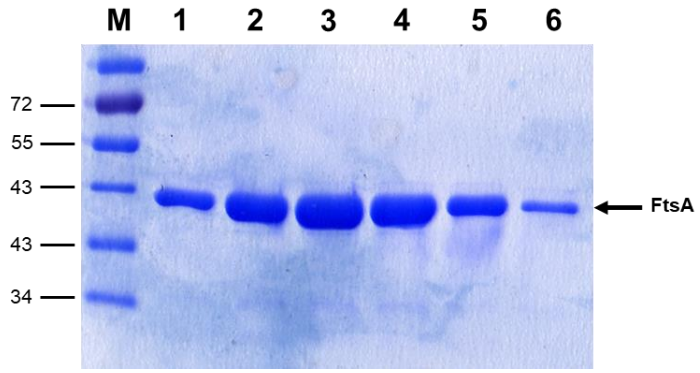


Figure 23. SDS-PAGE analysis of the fractions collected during the size exclusion chromatography of FtsA. Fractions 1-6 contain exclusively the protein FtsA. M, molecular weight markers (PanReac).

Finally, 5 mg of pure protein were obtained from 3 liters of culture and used for the co-sedimentation assay with FtsZ.

4.3.5 FtsA-FtsZ interaction *in vitro*

FtsA and FtsZ in *B. cenocepacia* interact *in vivo* as highlighted by BACTH assay. To better study this interaction *in vitro*, the two proteins were expressed and purified as described above and used for co-precipitation assays in the presence of vesicles that allow FtsA to interact with the membranes via its C-terminal amphipathic helix (Szwedziak *et al*., 2012). The liposomes co-sedimentation showed that the presence of lipids did not affect FtsZ polymerization, since about half of the protein was found in the pellet fraction (Figure 24).

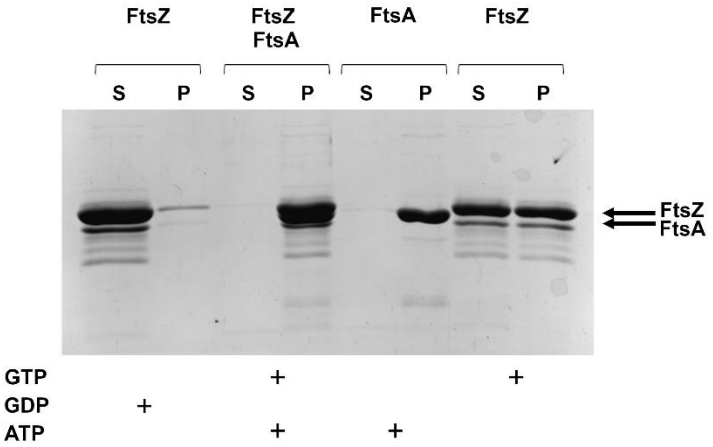


Figure 24. *In vitro* reconstitution of the FtsZ/FtsA interaction. FtsZ was induced to polymerize in the presence or absence of FtsA and GTP or GDP. The co-sedimentation was carried out in the presence of vesicles. The polymeric FtsZ and FtsA were collected by sedimentation and the amount of proteins in the pellet (P) was estimated by Coomassie-blue staining of SDS-PAGE. S, supernatant.

In the presence of ATP and vesicles, FtsA polymerized completely (Figure 24), while in absence of ATP, the protein was recovered only in the supernatant (data not shown). In the presence of both FtsZ and FtsA and their cofactors (GTP and ATP), all proteins were recovered in the pellet fraction (Figure 24). This result indicated that FtsA interaction with FtsZ increased the sedimentation of FtsZ in the pellet.

4.3.6 Expression and purification of ZipA

The ZipA protein *of B. cenocepacia* J2315 was expressed in the conditions described in Materials and methods, using the pETSUMO-ZipA vector (Table 9) transformed in *E. coli* strain BL21(DE3). The purification was achieved by IMAC and the soluble fraction of the SUMO-ZipA fusion was eluted using 250 mM of imidazole (Figure 25).

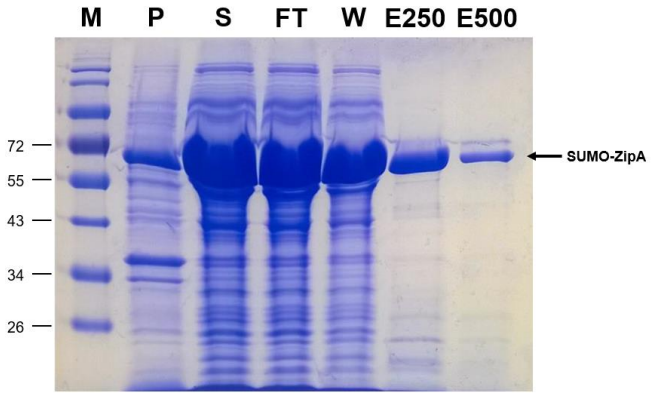


Figure 25. SDS-PAGE analysis of the fractions collected during the purification of ZipA. The soluble protein was eluted with 250 mM and 500 mM of imidazole (E250 and E500). M, molecular weight marker (PanReac); P, pellet; S, supernatant; FT, flow-through; W, wash; E250, elution with 250 mM imidazole; E500, elution with 500 mM imidazole.

Then, the SUMO protein was removed from ZipA using the SUMO protease, and the native protein was further purified by size exclusion chromatography (Figure 26).

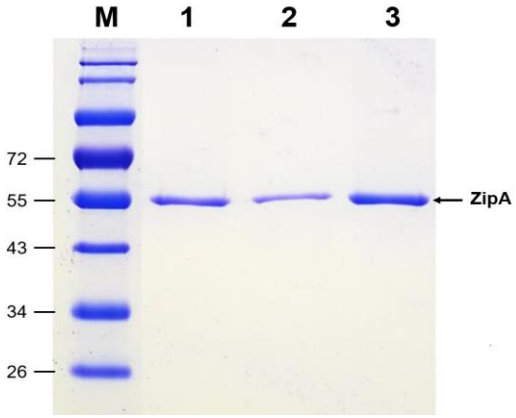


Figure 26. SDS-PAGE analysis of the fractions collected during the size exclusion chromatography of ZipA. Fractions 1-3 contain exclusively the protein ZipA. M, molecular weight markers (PanReac).

Finally, 20 mg of pure protein were obtained from 3 liters of culture and used for the co-sedimentation assay with FtsZ.

4.3.7 ZipA-FtsZ interaction *in vitro*

To confirm the physical interaction, shown by BACTH assay, between *B. cenocepacia* FtsZ and ZipA, the proteins were expressed and purified, and a co-sedimentation assay was set up (Figure 27).

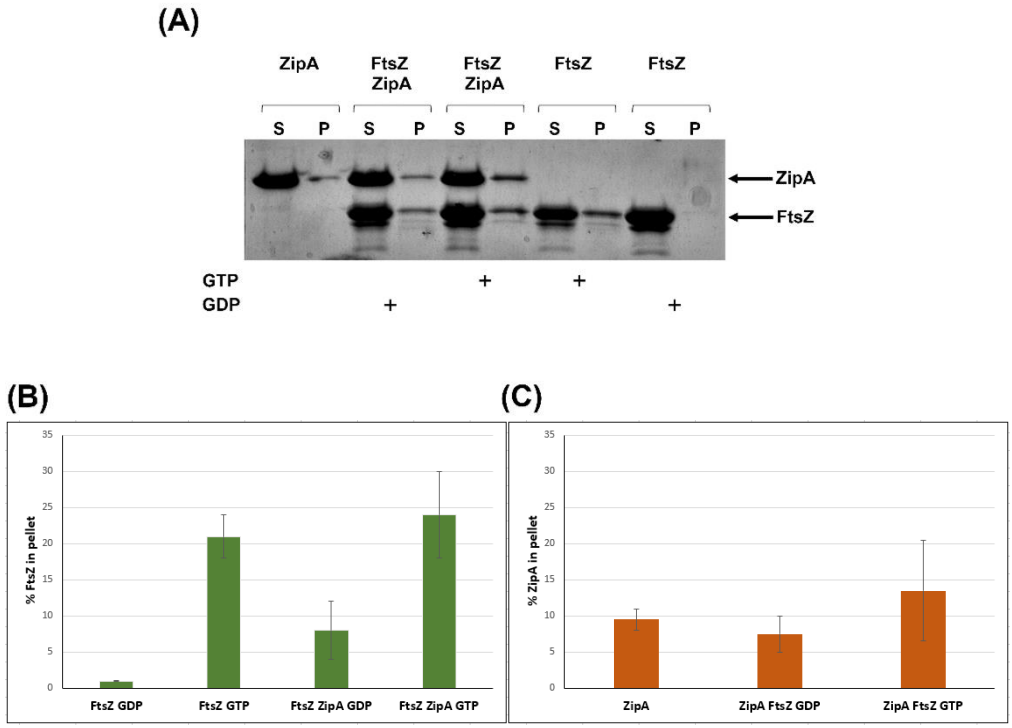


Figure 27. *In vitro* reconstitution of the FtsZ/ZipA interaction. FtsZ was polymerized in the presence or in the absence of ZipA with GTP or GDP. (A) The polymeric FtsZ was collected by sedimentation and the amount of FtsZ in the pellets (P) was estimated by Coomassie-blue staining of the SDS-PAGE. S, supernatant. (B) and (C) The bars indicate the relative quantification of FtsZ (B) and ZipA (C) percentage in the pellet obtained by densitometry analysis. Data are the average ± SD of three independent experiments.

In the presence of ZipA and GTP the quantity of the polymerized FtsZ increased. Interestingly, also when the polymerization is not induced (using GDP in the reaction), FtsZ is present in the polymerized fraction together with ZipA (Figure 27). Their interaction was confirmed also by the fact that, in the presence of FtsZ and GTP, the quantity of ZipA in the polymerized fraction increased (Figure 27). These results confirmed that *B. cenocepacia* ZipA interacts with FtsZ in its monomeric and filamentous form.

4.4 Biofilm inhibitory activity of TPGS109 against *B. cenocepacia* J2315 in the 3-D CFBE41o- cell model

The development of the water-soluble nanoparticles formulation of the FtsZ inhibitor C109 increased the compound solubility and dissolution rate in biological fluids, allowing its experimentation in *in vivo* models, as *Galleria mellonella*, avoiding the use of solvents (Costabile *et al*., 2020). However, this nanosuspension was optimized for aerosol delivery, thus the best way to test its activity will be the aerosolization in a mouse model of chronic *B. cenocepacia* lung infection. In order to obtain preliminary data on the activity and the toxicity of the D-α-tocopheryl polyethylene glycol 1000 succinate C109 (TPGS109), the compound was tested against *B. cenocepacia* J2315 in a 3-D lung epithelial cell model.

The experiment was carried out as described in Materials and methods, and the TPGS109 biofilm inhibitory activity was measured after 17 h of incubation. The best results were obtained treating the infected CFBE41o- aggregates with 20 μg/ml (70 μM) or 24 μg/ml (84 μM) of TPGS109, obtaining a significant reduction, in both cases, of about 0.9 Log-unit (8-fold) in the number of biofilm-forming bacteria compared to the untreated control (Figure 28).

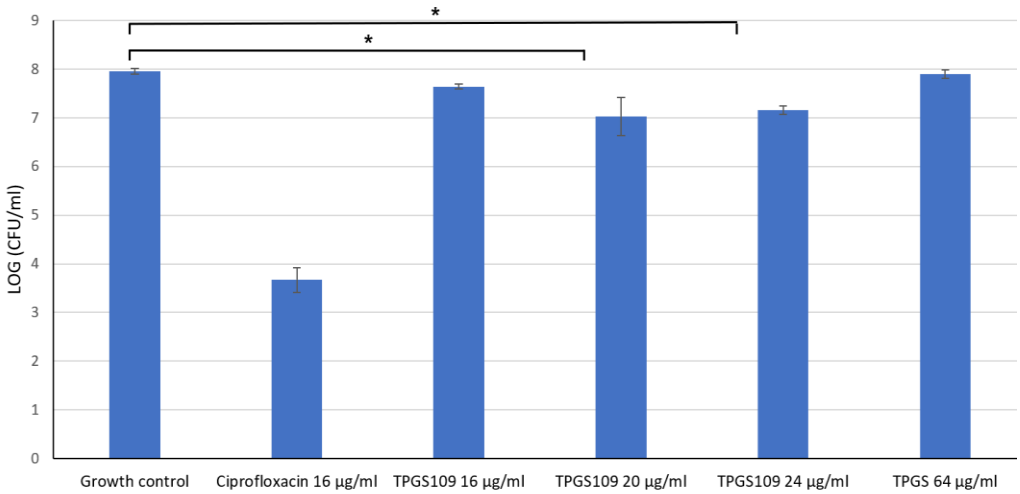


Figure 28. Biofilm-inhibitory activity of TPGS109 against *B. cenocepacia* J2315 in the 3-D lung epithelial cell model after 17 h of incubation. Growth control represents the untreated bacteria, ciprofloxacin was used as antibiotic control and TPGS as the nanoparticle carrier only control. Data are reported as mean ± standard error of at least three independent experiments. *p < 0.05 (One-way ANOVA test).

Instead, the lower concentration tested, 16 µg/ml (56 µM), resulted in the reduction of 0.3 Log unit (2-fold) of the CFU obtained after the biofilm disruption, but this was not considered statistically significant according to the one-way ANOVA test (Figure 28). The carrier TPGS alone (64 µg/ml) did not show any effect on the *B. cenocepacia* biofilm formation.

4.4.2 Cytotoxicity of TPGS109 in the 3-D CFBE41o- cell model

The effect of the tested concentration of TPGS109 on cell viability was assessed measuring the enzymatic activity of the cytosolic LDH released in the supernatant by dead cells as described in Materials and methods. As shown in Figure 29, the toxicity of the nanosuspension on the CFBE41o- increases proportionally with the concentration used, reaching the 45% of dead cells in the presence of 24 µg/ml TPGS109 (Figure 29).

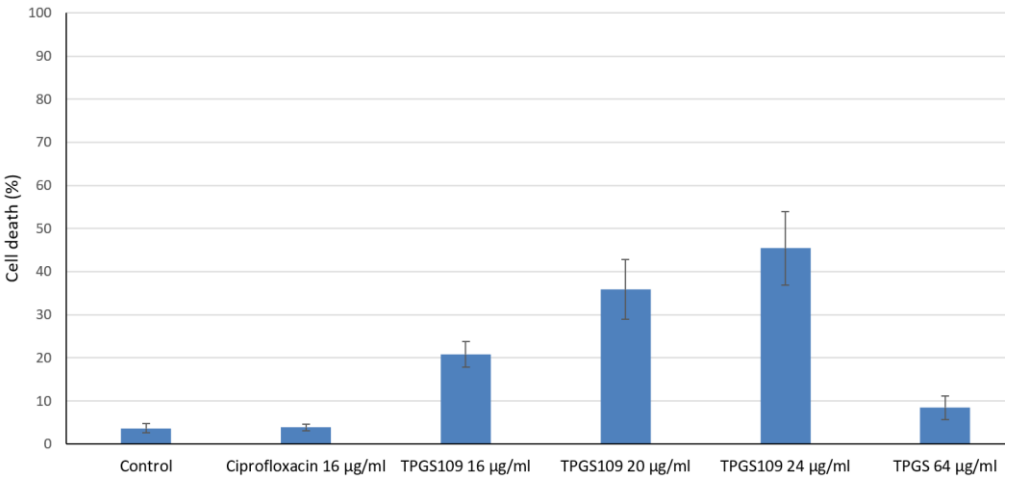


Figure 29. Cytotoxicity of TPGS109 to 3-D lung epithelial cells after 17 h of incubation. Cell viability was evaluated by LDH-release assay. Control represents untreated 3-D cells. Data are reported as mean ± standard error of at least three independent experiments.

Moreover, observing the treated 3-D aggregates using optical microscopy, it was noticed a change in cell morphology using 24 µg/ml TPGS109, compared to the control, with the appearance of round-shaped cells, as well as a significant detachment of the cells from the beads (Figure 30D).

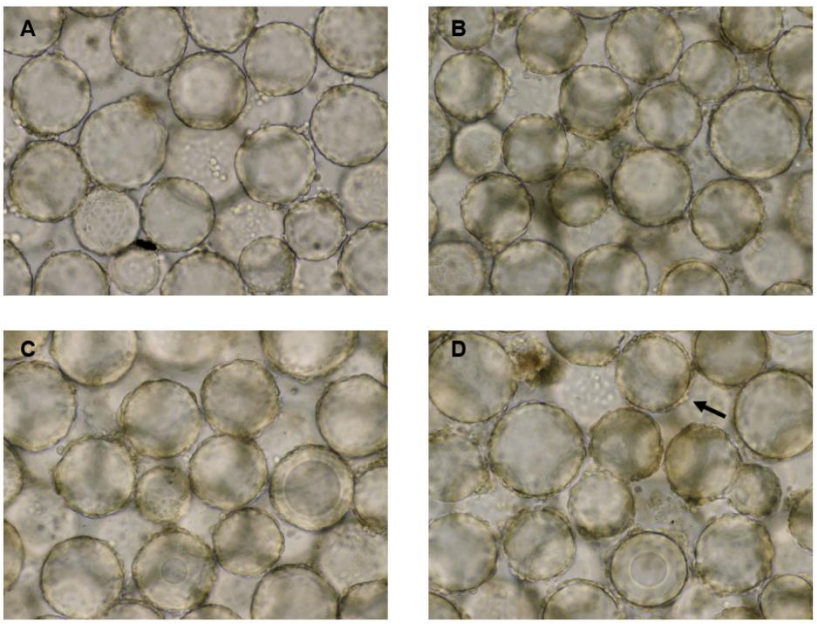


Figure 30. Effect of TPGS109 on cell adhesion to microcarrier beads after 17h of incubation. The 3-D aggregates phenotype was evaluated by optical microscopy. Images were obtained at a magnification of 1200×. A) Untreated control. B) Cells treated with 16 µg/ml TPGS109. C) Cells treated with 20 µg/ml TPGS109. D) Cells treated with 24 µg/ml TPGS109. The black arrow indicates round-shaped cells detaching from the surface of microcarrier beads.

Considering these toxicity data, it is clear that the best concentration of TPGS109 to achieve a significant biofilm inhibition with a small effect on cell viability in this model is 20 µg/ml.

4.4.3 Metabolic transformation of C109 in CFBE41o- cultures

Despite the good biofilm inhibitory activity against *B. cenocepacia* J2315 demonstrated in the 3-D CFBE41o- cell model, TPGS109 showed an activity 1000 folds higher in the same assay conditions but in the absence of the cells (data not shown). This was probably due to the metabolization of the C109 by the cells, as already reported in *B. cenocepacia* cultures (Chiarelli *et al*., 2020). For this reason, the C109 stability over time in CFBE41o-

cultures was tested and the C109 metabolites obtained were visualized by TLC, using chloroform as eluent. As shown in Figure 31A, the quantity of compound resulted strongly decreased after 60 min of incubation with cells, with the consequent appearance of the metabolite M1 (Figure 31A). Moreover, using a slightly more polar eluent (chloroform/methanol 99:1) it was possible to identify also other two metabolites (M2 and M3) that resulted enriched after 60 min compared to the 30 min incubation (Figure 31B).

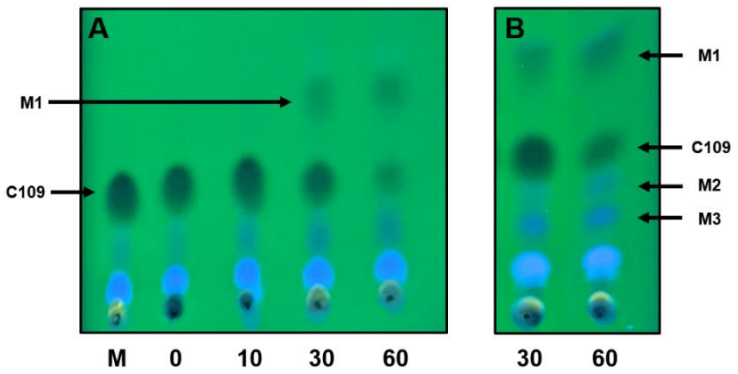


Figure 31. TLC analysis of the C109 metabolites produced by CFBE41o- cells in different time spans. A) Visualization of the C109 metabolization in medium only (M) after 10 min of incubation and in the presence of cells after 0, 10, 30, 60 min (TLC eluent: chloroform). B) Comparison of the C109 degradation after 30 and 60 min of incubation with cells (TLC eluent: chloroform/methanol 99:1). Both TLCs (A and B) were visualized under UV light. M1, C109 metabolite 1; M2, C109 metabolite 2; M3, C109 metabolite 3.

This analysis highlighted the presence of different (at least 3) products of degradation of the C109 upon incubation with CFBE41o- cells, which probably do not retain the same biofilm inhibitory activity of the raw C109.

5. Discussion and future perspectives

The shortage of therapeutic options for the treatment of *B. cenocepacia* lung infections represents a serious threat for cystic fibrosis patients and requires a major effort to find new effective treatments. Since this pathogen shows intrinsic and acquired resistance to almost all currently used antibiotics that hit the classical cellular targets, it is fundamental to focus the research on the development of novel molecules with innovative molecular targets that can prevent the easy insurgence of resistance.

Bacterial division is widely considered a valuable pool of new drug targets, given the essentiality and the high conservation among bacteria of most proteins involved in this pathway (Buroni *et al*., 2020). We demonstrated that our most promising molecule, C109, is an inhibitor of FtsZ polymerization, which represents the first step of the bacterial divisome assembly, exerting its antimicrobial activity on a broad range of pathogens (Hogan *et al*., 2018). For this reason, the work reported in this thesis is focused on the characterization of the *B. cenocepacia* division mechanism, studying the genetics and the protein interactions involved in this process, with the aim of characterizing further essential druggable targets in this cystic fibrosis pathogen.

Since the genes encoding the key components of the divisome are often clustered in the division cell wall (*dcw*) operon, first of all this was identified in the *B. cenocepacia* genome. The cluster encompassed 17 tightly packed genes on the chromosome 1 and showed the same conserved genetic arrangement described in Gram-positive and -negative bacilli (Tamames *et al*., 2001). This characteristic is thought to be fundamental for a successful division in rod-shaped bacteria. Indeed, it facilitates the synthesis of long polycistronic mRNAs that allow the co-translation of interacting proteins in the same cellular compartment. Consequently, the ordered assembly of protein complexes required for the septation is promoted (Mingorance *et al*., 2004). The results obtained in this work confirmed the presence of a long polycistronic transcription unit also in *B. cenocepacia*, since the first 15 genes of the operon, from *mraZ* to *ftsZ*, resulted to be co-transcribed in a single mRNA, and no transcriptional terminators were found within this

region. The transcription of this long mRNA was demonstrated to start 100 bp upstream of the *mraZ* gene, and to be controlled by the promoter P_{mra}Bc. This was found in the intergenic regulatory region at the 5' of the operon, as its analogue P_{mra} in *E. coli* (Hara *et al*., 1997), which similarly controls the formation of a long transcript that includes the whole operon (de la Fuente *et al*., 2001).

However, the *E. coli dcw* cluster contains a few other promoters, modulating the formation of shorter transcription units (Dewar and Dorazi, 2000), which are absent in *B. cenocepacia*. In particular, the absence of additional promoters upstream of *ftsZ*, in the *ddl-ftsA* region is peculiar, since the fine regulation of its expression is fundamental to achieve a successful division, as reported in *E. coli* (Palacios *et al*., 1996). This result suggests the involvement of post-transcriptional control mechanisms that can replace the regulatory functions ascribed to the distal promoters found in other bacilli, such as *E. coli* (Flärdh *et al*., 1997), *Bacillus subtilis* (Real and Henriques, 2006), and *Bacillus mycoides* (Santini *et al*., 2013).

Within this work, the transcriptional regulator of the operon was also characterized as MraZ, the protein encoded by the first gene of the cluster. In *B. cenocepacia*, it specifically binds a conserved series of four repeats of seven nucleotides that partially overlap to the P_{mra}Bc sequence, regulating the *dcw* transcription as well as its own transcription, as already reported in even very distantly related microorganisms (Eraso *et al*., 2014; Maeda *et al*., 2016; Fisunov *et al*., 2016). Indeed, MraZ is an extremely conserved transcription factor with essential functions in the division, since it is present also in mycoplasmas (Fisunov *et al*., 2016), the simplest self-replicating organisms with a minimal genome.

This first part of the thesis provided an overview of the transcriptional arrangement of the *dcw* operon of *B. cenocepacia*, evidencing important regulatory mechanisms involved in the control of the division gene expression in this poorly characterized bacterium.

This work also provided a better knowledge of the interactions among proteins involved in the septation of *B. cenocepacia*. In particular, the *B. cenocepacia* homologues of the best studied FtsZ interactors of *E. coli* were characterized. Their interactions with *B. cenocepacia* FtsZ was assessed using the BACTH system. Firstly, the interaction between two FtsZ monomers was tested, confirming that, also in *B. cenocepacia*, the two

monomers interact head to tail. This is known to be essential for the constitution of the GTPase active site and, accordingly, for FtsZ polymerization during the divisome assembly (Schumacher *et al*., 2020).

Then, the SOS response protein SulA was demonstrated to interact both *in vivo* and *in vitro* with FtsZ by two-hybrid and co-sedimentation assays, respectively. These results confirmed what already reported in other microorganisms (Cordell *et al*., 2003; Dajkovic *et al*., 2008) and validated the regulatory function of SulA in this bacterium, that consists in the inhibition of FtsZ polymerization by capping the forming polymers (Vedyaykin *et al*., 2020).

Regarding the early division proteins, the interaction of FtsZ with the N-terminal part of FtsA was demonstrated by BACTH assay. Since FtsA is reported to form polymers stabilized by the interaction of its C-terminal amphipathic domain with the membranes (Szwedziak *et al*., 2012), the co-sedimentation assay with FtsZ was performed in the presence of vesicles. The results revealed that FtsA of *B. cenocepacia* is able to polymerize in these conditions, anchoring FtsZ polymers to the membrane and increasing the quantity of FtsZ found in the pellet fraction. Interestingly, in this experiment FtsA showed a higher affinity for FtsZ compared to the same experiment performed with *E. coli* proteins (Loose and Mitchison, 2014), evidencing possible differences in their interaction mechanism that will be further investigated through mutational studies and pull-down assays.

The second early protein characterized was ZipA, a known interactor of FtsZ in *E. coli* (Liu *et al*., 1999; Ohashi *et al*., 2002). In *B. cenocepacia*, ZipA was demonstrated to interact with the C-terminal part of FtsZ by BACTH system, and this was further evaluated *in vitro* using a co-sedimentation assay. This showed that the presence of ZipA causes an increased sedimentation in the pellet fraction of FtsZ, even when polymerization is not induced. This result suggests that ZipA could stabilize the spontaneous formation of FtsZ polymers which occurs in the absence of GTP.

Some late divisome proteins were also investigated, starting from FtsE, an ABC-transporter-like protein involved in the maturation of the divisome and in cell constriction (Schmidt *et al*., 2004; Du *et al*., 2019). Through BACTH assay, the physical interaction between the C-terminal domain of FtsZ and the N-terminal region of FtsE was validated in *B. cenocepacia*, as already

described in *E. coli* using different approaches (Corbin *et al*., 2007; Du *et al*., 2019).

FtsQ is known to be an essential component of the division machinery in *E. coli* and, upon the formation of the complex FtsQLB, it acts as a sensor that modulates the divisome activation (Tseng and Bernhardt, 2015). In this thesis, it was demonstrated the *in vivo* physical interaction between FtsQ and FtsZ in *B. cenocepacia*, evidencing a difference in the FtsZ interactome between this pathogen and *E. coli*. Indeed, in the latter, FtsQ is demonstrated to interact only with other divisome proteins, such as FtsI, FtsN, FtsW, and FtsK (Grenga *et al*., 2010). This interesting new interaction is worth to be further studied *in vitro*, recombinantly expressing the cytoplasmic part of FtsQ to characterize the exact contact site with FtsZ.

Instead, the other late division proteins tested, FtsI and FtsN, were confirmed not to be part of the FtsZ interactome in *B. cenocepacia*, similarly to *E. coli*.

Even though the early and late proteins are recruited at the mid-cell in two distinct phases, protein-protein interactions between components of the two groups were reported, demonstrating a molecular and functional interconnection among them (Alexeeva *et al*., 2010). This was confirmed also in *B. cenocepacia*, where FtsA interacts with ZipA, FtsQ, and FtsN, which are recruited in three different stages of the divisome assembly.

In conclusion, these results confirmed the involvement of the described proteins in the division of *B. cenocepacia* and gave a complete overview of their interactions. This will be useful as starting point for the future characterization of divisome assembly in this bacterium. Most important, this knowledge will be exploited towards the design of new antibacterials targeting division proteins or essential protein-protein interactions.

The second part of my thesis was focused on the FtsZ inhibitor C109. It is well established that C109 has a potent antimicrobial activity *in vitro* (Hogan *et al*., 2018), however there is a lack of data about its activity *in vivo*, since it was tested only in a *Galleria mellonella* infection model (Costabile *et al*., 2020). To validate its efficacy also in physiologically relevant conditions, in this thesis C109 was tested in an *in vivo*-like 3-D lung epithelial cell model that mimics the complex microenvironment of biofilm-associated infections. The water-soluble nanosuspension of C109 (TPGS109) was tested for its biofilm inhibitory potential against *B. cenocepacia* J2315 infecting 3-D

CFBE41o- aggregates, finding an 8-fold reduction of the biofilm forming bacteria following the treatment with 20 µg/ml (70 µM) of TPGS109. At this concentration it resulted mildly toxic for these cells, confirming the cytotoxicity data already reported for CFBE41o- cells (Costabile *et al*., 2020). Unfortunately, lower concentrations of TPGS109 were not active in this model since the compound was demonstrated to be almost completely metabolized by the cells after 1h. Similar results were already reported after 2h of incubation of C109 in *B. cenocepacia* cultures, leading to the formation of at least six molecules that unfortunately did not retain any antimicrobial effect (Chiarelli *et al*., 2020). However, it is not excluded that metabolites obtained after C109 transformation by CFBE41o- could still have at least a partial antimicrobial activity. This hypothesis will be explored after the identification of these molecules.

To conclude, the last part of this thesis demonstrated the efficacy of the TPGSC109 in an *in vivo*-like model of infection, obtaining a significant decrease of the biofilm formation at micromolar concentration, slightly affecting cell viability. In order to further decrease the cytotoxicity, lower concentrations of nanosuspension will be tested in combination with meropenem and piperacillin, taking advantage of their additive effect highlighted *in vitro* (Hogan *et al*., 2018) and *in vivo* (Costabile *et al*., 2020).

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Burkholderia cenocepacia Infections in Cystic Fibrosis Patients: Drug Resistance and Therapeutic Approaches

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Burkholderia cenocepacia is an opportunistic pathogen particularly dangerous for cystic fibrosis (CF) patients. It can cause a severe decline in CF lung function possibly developing into a life-threatening systemic infection known as cepacia syndrome. Antibiotic resistance and presence of numerous virulence determinants in the genome make *B. cenocepacia* extremely difficult to treat. Better understanding of its resistance profiles and mechanisms is crucial to improve management of these infections. Here, we present the clinical distribution of *B. cenocepacia* described in the last 6 years and methods for identification and classification of epidemic strains. We also detail new antibiotics, clinical trials, and alternative approaches reported in the literature in the last 5 years to tackle *B. cenocepacia* resistance issue. All together these findings point out the urgent need of new and alternative therapies to improve CF patients' life expectancy.

Keywords: ***Burkholderia cenocepacia*, resistance, epidemiology, antibiotics, new therapies**

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INTRODUCTION

Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex (Bcc) (Vanlaere et al., 2008; De Smet et al., 2015; Ong et al., 2016; Weber and King, 2017), a group of 22 Gram negative related bacterial species widespread in the environment. It can infect cystic fibrosis (CF) patients causing a severe decline in lung function which can further develop into a life-threatening systemic infection known as cepacia syndrome. CF is the commonest autosomal recessive disease in Europe affecting 1 in 2500 newborns, nevertheless it is considered rare according to the European Union definition (i.e., disease affecting less than five individuals per 10,000 people) (Farrell, 2008). More than 2,000 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are responsible for the disease. The CFTR encodes a chloride ion channel which is expressed on the surface of cells in many tissues, including lungs, gut, and pancreas; its malfunctioning causes the production of viscous secretions which are particularly dangerous in the lungs where opportunistic infections consequently occur (Knowles and Durie, 2002). It is noteworthy, that chronic respiratory infections and inflammation are the leading cause of death in CF (Ciofu et al., 2013).Moreover, colonization of immunocompromised individuals has also been reported (Ganesan and Sajjan, 2012).

Bcc bacteria, particularly *B. cenocepacia*, are naturally resistant to different classes of antibiotics used in clinical practice (Mahenthiralingam et al., 2005; Burns, 2007) and their pathogenicity is

promoted by several virulence determinants (Loutet and Valvano, 2010; Sousa et al., 2011). These characteristics, together with the ability to adapt to environmental changes, make the treatment of *B. cenocepacia* infections particularly challenging. In fact, it has been shown that during long-term colonization, *B. cenocepacia* can undergo transcriptional reprogramming in response to host immune response, antimicrobial therapy, nutrient availability, and oxygen limitation (Mira et al., 2011). Consequently, a genomic approach as well as the construction of mutant libraries revealed crucial in identifying essential genes responsible for antimicrobial resistance, virulence, and adaptation (Wong et al., 2016; Gislason et al., 2017).

From an epidemiological perspective, a recent study by Salsgiver et al. (2016) reported that from 1995 to 2012 the prevalence of Bcc infections decreased from 3.6 to 3.0% in the United States (Salsgiver et al., 2016). This was ascribed to the combination of new antimicrobial therapies with CFTR correctors and potentiators, as well as the introduction of new guidelines detailing prevention and eradication strategies (Saiman et al., 2014).

Although *Burkholderia* species are relatively rare amongst CF patients, they still cause serious challenges. Indeed, despite therapeutic advances, respiratory failure remains the major cause of premature death and lung transplant the best option to treat the most severe cases (Ramos et al., 2017). However, a main post-transplant complication is represented by infection with multidrug resistant bacteria and Bcc was recognized as a significant contributor to CF morbidity and mortality associated to increased post-transplant death rate (Alexander et al., 2008; Chaparro and Keshavjee, 2016). In this context, studying the resistance profile, the mechanisms underlying resistance, as well as the epidemiology of CF pathogens seems essential to improve management of these infections.

In this review, we describe the clinical distribution of *B. cenocepacia* resistant strains reported in the last 6 years and the methods for identification and classification of epidemic strains. All together these data point out the urgent need of new and alternative therapies to treat Bcc infections and improve CF patients' life quality and expectancy. Finally, we summarize new antibiotics, clinical trials, and alternative approaches reported in the literature in the last 5 years to tackle *B. cenocepacia* resistance to antimicrobials.

EPIDEMIOLOGY OF *B. cenocepacia* STRAINS

The frequency of Bcc infections in CF patients is quite variable. *B. cenocepacia* and *Burkholderia multivorans* are the most commonly isolated in Australia, New Zealand, and several European countries. *B. multivorans* is currently the most prevalent in the United States and Canada[1,2]. Moreover, *Burkholderia gladioli*, a non-Bcc member, is increasingly isolated and has become the third most common *Burkholderia* species in the United States, but it is not common in European CF patients[2,3].

It is worth mentioning that following to the recommendations for infection prevention and control in CF, published in 2003 and updated in 2013 (Saiman et al., 2014), strategies to reduce the risk of patient-to-patient transmission and acquisition from the environment were implemented and a progressive decrease in Bcc prevalence was subsequently observed (Salsgiver et al., 2016). Transmission of Bcc infection occurs as a consequence of both direct and indirect contact (e.g., *via* infectious droplets) between patients. Initially, social contacts during summer camps, very popular among CF patients until 1998, were the most likely cause of Bcc cross-infections. Nevertheless, a *B. cenocepacia* strain isolated from soil in 2000 was shown to be indistinguishable from clinical isolates by different typing methods, thus demonstrating that acquisition of Bcc from the environment could occur, and explaining why the incidence of Bcc infection in CF patients has not been eliminated (LiPuma et al., 2002).

Several single-center or multicenter studies have suggested that poor outcomes might be a consequence of the infecting Bcc species (Murray et al., 2008). Indeed, the worst outcome was observed with *B. cenocepacia* which leads to an excessive mortality rate among CF patients (Corey and Farewell, 1996; Alexander et al., 2008; De Soyza et al., 2010; Gilljam et al., 2017). Despite advanced lung disease, several *B. cenocepacia* infected patients are not considered for lung transplant (Ramos et al., 2016).

From the end of the 1990s, epidemiological studies demonstrated that *B. cenocepacia*, at that time known as Bcc genomovar III, was the most prevalent Bcc pathogen in CF patients. Using *recA* sequence analysis, the species was then subdivided into four phylogenetic clusters IIIA – IIID, however, almost all clinical isolates belong to the IIIA and IIIB subgroups. Among them, the ET-12 strain (ST28) and the Czech strain (ST32), which spread within CF patients in Canada and Europe, belong to the *B. cenocepacia* IIIA group, while the *B. cenocepacia* strains dominant in the United States, such as the Midwest clone and the PHDC strain, are part of the subgroup IIIB (Drevinek and Mahenthiralingam, 2010). *B. cenocepacia* IIIC group is exclusively environmental, while IIID strains have been isolated from CF patients only in Sweden, Argentina, and in Italy (Manno et al., 2004; Campana et al., 2005).

These studies suggest that chronic *B. cenocepacia* infections resulted from the colonization of few clonal bacterial strains. Nevertheless, CF patients infected with genotypically similar *Burkholderia* strains can manifest different outcomes. Indeed, rapid evolution of *Burkholderia* species was demonstrated during infections or *in vitro* under stress conditions (Drevinek et al., 2010; Sass et al., 2011), suggesting that mutations can occur and accumulate in clonal lineages as a response to suboptimal growth conditions. For instance, an epidemic *B. cenocepacia* clone prevalent in the Serbian CF population (i.e., ST856) was

[1]http://www.cysticfibrosis.ca/uploads/Registry%20Report%202015/2015%20Registry%20Annual%20Report%20EN.pdf

[2]https://www.cff.org/Our-Research/CF-Patient-Registry/2015-Patient-Registry-Annual-Data-Report.pdf

[3]https://www.ecfs.eu/sites/default/files/general-content-files/working-groups/ecfs-patient-registry/ECFSPR_Annual%20Repor

shown to be subjected to variations in virulence and genotype as a consequence of the lung adaptation (Malešević et al., 2017).

It is noteworthy that, thanks to the prevention and control strategies introduced in the late '90s, novel Bcc infections occurring in Canada, the United States and many European countries are presently caused by non-epidemic *B. cenocepacia* strains or by non-clonal *B. multivorans* strains or Bcc species other than *B. cenocepacia*. The presence of non-clonal isolates of various Bcc species in CF patients, also when strict control measures are undertaken, suggests an acquisition from environmental sources rather than cross-infections. Further studies are now necessary to analyze so far unexplored environmental niches of *Burkholderia* species and then implement appropriate prevention measures (Zlosnik et al., 2015).

Epidemiological Methods

The analysis of Bcc epidemiology, particularly of *B. cenocepacia*, in CF patients is paramount as highlighted by the several methods used for genotyping. Indeed, transmissibility markers were identified in *B. cenocepacia* epidemic strains, such as the *cblA* pilin gene (Sun et al., 1995) and the *B. cepacia* epidemic strain marker (BCESM) belonging to a pathogenicity island (Baldwin et al., 2004), or the IS1363 insertion sequence (Liu et al., 2003). However, even if some epidemic lineages are associated to these genetic markers, others were not, thus genotyping studies are still required for a full understanding of cross-transmission and for epidemiological surveillance.

Among molecular typing methods, the most used are ribotyping (LiPuma et al., 1988; Dasen et al., 1994), pulsed-field gel electrophoresis (PFGE) (Tenover et al., 1995), random amplified polymorphic DNA (RAPD) (Mahenthiralingam et al., 1996), repetitive elements PCR (rep-PCR) (van Belkum et al., 1996) and multilocus sequence typing (MLST) (Coenye and LiPuma, 2002; Urwin and Maiden, 2003; Spilker et al., 2009). Macro-restriction of chromosomal DNA followed by PFGE was considered the gold standard in bacterial typing for a long time, and was widely applied to the molecular epidemiology of Bcc (Coenye et al., 2002). However, as PFGE is quite a laborious technique and typing results are difficult to compare between different laboratories, MLST replaced it as preferred genotyping method also for CF pathogens (Saiman et al., 2014). MLST differentiates bacterial isolates by comparing the sequence of seven housekeeping gene fragments and then characterizing strains by the resulting allelic profile. For each housekeeping gene, sequence variants are designated as distinct alleles and each allele profile defines a specific sequence type (ST). Compared to other genotyping methods, MLST offers several advantages, primarily yielding unambiguous and reproducible results that can be easily and reliably compared between different laboratories. MLST profiles of more than 2000 Bcc isolates are freely available online[4] (Jolley and Maiden, 2010), however this technique is quite expensive and time consuming, thus limiting its application for routine use in clinical microbiology laboratories or in genotyping large collection of isolates in national surveillance programs.

For the above reasons, alternative methods were developed to allow routine or large-scale analysis, such as the SNaPBcen assay targeting only single nucleotide polymorphisms in MLST genes instead of analyzing full sequences (Eusebio et al., 2013); the PCR assays based on MLST and specific for particularly globally distributed epidemic strain (Dedeckova et al., 2013); or multilocus variable-number tandem-repeat analysis (MLVA) (Segonds et al., 2015).

Evolution and adaptation of pathogens during chronic infections are of great importance for choosing the appropriate therapeutic strategy, however, the underlying molecular bases for Bcc are still poorly understood. As a consequence, the current genotyping methods currently available are not sufficient to assess the genetic diversity of Bcc strains and predict the clinical outcome (Lee et al., 2017). By contrast, a genomic approach could provide more insights in Bcc evolution during chronic lung infections. A recent study describing the whole genome sequence analysis of 215 *B. cenocepacia* isolates, collected from 16 CF patients at different times of infection, demonstrated a considerable phenotypic and genotypic variability within single patients and confirmed that distinct lineages could follow distinct evolution patterns during chronic lung infection (Lee et al., 2017). Similar results were previously achieved by comparative genomic analysis of clinical isolates of *Pseudomonas aeruginosa* and *Burkholderia dolosa* from CF patients, thus confirming that these bacteria are indeed capable of accumulating different mutations at different stages of chronic infections (Lieberman et al., 2011; Markussen et al., 2014; Lee et al., 2017).

Genome-based taxonomic and phylogenetic analyses that have emerged as present identification standards can provide more accurate genotyping data of clinical isolates and also allow the identification of strains that have rapidly evolved after introduction of novel determinants by horizontal gene transfer (Juhas et al., 2009). For instance, a recent genome-based analysis of Bcc clinical isolates in India identified a previously unknown *B. cenocepacia* clone characterized by a novel genomic island (i.e., BcenGI15), very similar to that found in *Burkholderia pseudomallei* strain EY1, and so demonstrated transfer of genomic islands also between different pathogenic species within the *Burkholderia* genus (Patil et al., 2017).

RESISTANCE MECHANISMS

Antibiotic resistance could be intrinsic or acquired. The first one is independent of antibiotic selective pressure and horizontal gene transfer, instead it is the result of inherent structural or functional characteristics. On the other hand, bacteria can acquire resistance to antibiotics, such as mutations in drug targets or transfer of resistance genes through phage-mediated transduction and mobile plasmids. Moreover, tolerance to antibiotics plays an important role in protecting bacteria during

[4]http://pubmlst.org/bcc/

infections. This phenomenon is related to bacteria adaptation to environment, such as planktonic or sessile (biofilm) growth and presence of persister cells, and it can be due or not to mutations in target genes.

The four main mechanisms of antibiotic resistance are: prevention of access to target due to (1) reduced permeability of the cell envelope or to (2) increased efflux activity; (3) mutation in antibiotic target; (4) enzymatic modification or inactivation of the drug (hydrolysis or transfer of a chemical group). In addition, (5) the ability to form biofilms greatly enhance antibiotic resistance traits.

Reduced Permeability of the Cell Envelope

In Gram negative bacteria the cell envelope is composed of an inner membrane, a periplasmic space and an outer membrane containing lipopolysaccharide (LPS) (Hamad et al., 2012). LPS comprises lipid A, core oligosaccharide (OS) and a polymer composed of glycan monomers called O-antigen (O-Ag). The lipid A-core OS and O-Ag are synthetized independently on the cytoplasmic side of the inner membrane, then they are joined in the periplasmic side and finally the complete LPS molecule is transferred to the outer membrane surface by a group of conserved proteins forming the lipopolysaccharide transport machinery (LPT) (Hamad et al., 2012). LPS undergoes further modifications, such as addition or removal of sugars, phosphates, or acyl groups allowing bacterial survival in stress conditions such as presence of antimicrobial peptides (Raetz et al., 2007). Among LPS alterations, the cationic substitution of phosphate groups by the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N), decreases the net negative charge of lipid A. *B. cenocepacia* is extremely resistant to antimicrobial peptides like polymyxin B. In *Burkholderia* spp. L-Ara4N is the main constituent of the lipid A and of the OS portion of the LPS (Olaitan et al., 2014). Deletion of the OS region leads to an increased binding of polymyxin B to *B. cenocepacia* cells and so increased sensitivity to polymyxin B (Ortega et al., 2009).

In addition, another important polymyxin B resistance determinant in *B. cenocepacia* is the alternative sigma factor RpoE involved in controlling the expression of a group of genes which are part of the extra-cytoplasmic stress response (Loutet and Valvano, 2011).

Finally, there are two other minor determinants of antimicrobial peptide resistance in *B. cenocepacia*. Two secreted zinc metalloproteases, ZmpA and ZmpB, are involved in the degradation of different antimicrobial peptides *in vitro*, however, *B. cenocepacia* knocked-out strains for one or both proteases did not show increased sensitivity. Loutet et al. (2011) demonstrated that antimicrobial peptide resistance could be found in a deep-rough LPS *B. cenocepacia* mutant in which the LPS molecule is truncated through the disruption of the ADP-L-glycero-D-manno-heptose synthesis.

Overexpression of Efflux Pumps

In Gram negative bacteria efflux mechanisms play a major role in antibiotic resistance. Up to five families of transporters can be involved: the major facilitator superfamily (MFS), the ATP-binding cassette family (ABC), the small multidrug resistance family (SMR), the multidrug and toxic compound extrusion family (MATE), and the resistance nodulation division family (RND) (Li et al., 2015).

In *B. cenocepacia* 16 open reading frames encoding RND efflux pumps were identified. This type of transporters catalyzes substrate efflux via an H^+ antiport mechanism. Genes are usually organized as operons and encode multimeric structures composed of an RND protein placed in the inner cellular membrane, an outer membrane protein (OMP) in the outer membrane and a membrane adaptor protein in the periplasmic region that connects the first two proteins (Li et al., 2015).

Different studies highlighted the key role of RND efflux pumps in drug resistance of *B. cenocepacia*, showing that in particular RND-3, RND-4, and RND-9 protect the bacterium from different compounds (Buroni et al., 2009, 2014; Bazzini et al., 2011). For example, *B. cenocepacia* cells treated with chlorhexidine overexpress several genes coding for drug resistance determinants such as RND efflux systems (Coenye et al., 2011). Analysis of RND mutants confirmed that some efflux transporters are involved in chlorhexidine efflux during planktonic growth (RND-1 and RND-4), while some others (RND-3 and RND-9) during sessile growth (Coenye et al., 2011).

Moreover, in order to better clarify the role of each RND efflux pumps in *B. cenocepacia*, MIC differences for certain antibiotics were evaluated using knock-out mutants for the 16 RND efflux systems, and analyzed during both planktonic and sessile growth. During planktonic growth RND-3 efflux pump was demonstrated to be crucial for resistance to ciprofloxacin and tobramycin, while RND-4 is important for extrusion of ciprofloxacin, tobramycin, minocycline, and chloramphenicol (Buroni et al., 2014). On the other hand, RND-8 and RND-9 play a key role in protection against tobramycin only during sessile growth (Buroni et al., 2014).

Mira et al. (2011) described the adaptive mechanisms that promote long-term colonization of *B. cenocepacia* in CF lungs by DNA microarrays transcriptomic analysis of two clonal variants isolated during long-term infection. Among the up-regulated genes in the most resistant strain, *mdtABC* and *bpeA*, were identified, respectively encoding RND-6 and RND-4 efflux pumps, *BCAM0201*, encoding an efflux system of the MFS; and *BCAM2188*, encoding a component of an ABC-transporter (Mira et al., 2011).

RND-4 and RND-9 were also involved in the resistance to two novel anti-*Burkholderia* experimental compounds (Scoffone et al., 2014, 2015).

A further study showed that in Bcc clinical isolates, RND-3 is the most up-regulated among the RND efflux systems due to mutations in its transcriptional regulator (Tseng et al., 2014).

Other efflux systems are also involved in *Burkholderia* antibiotic resistance: for example, the overexpression of *fsr* (fosmidomycin resistance gene) results in the upregulation of an

efflux pump which leads to fosmidomycin resistance (Messiaen et al., 2011).

Mutations in Antibiotic Cellular Target

Drug target modification is not the main resistance mechanism described in *B. cenocepacia*. A recent work analyzed the roles of the class 1 integron, the quinolone resistance-determining regions (QRDRs) of topoisomerases II and IV, in Bcc clinical isolates (Tseng et al., 2014). Levofloxacin resistance is due to accumulation of mutations in the QRDR genes encoding topoisomerases and efflux pump activation (Pope et al., 2008; Nikaido and Pages, 2012). Among 66 Bcc clinical isolates, 6 levofloxacin resistant strains were identified carrying single-base mutations in the QRDR region of the DNA gyrase subunit A (*gyrA* gene) at codon 81 (Gly81Asp), 83 (Thr83Ile), and 87 (Asp87His), respectively. No mutations were found in the QRDR region of the DNA topoisomerase IV subunit A (*parC* gene) (Tseng et al., 2014).

Antibiotic Modification

This mechanism is commonly used by bacteria to achieve resistance to β-lactams and aminoglycosides.

β-lactams, such as penicillins, cephalosporins, clavams, carbapenems, and monobactams are inactivated by periplasmic β-lactamases through hydrolysis of the β-lactam ring (Bush and Fisher, 2011). Several β-lactamases were identified in *B. cenocepacia*: AmpC, hydrolysing expanded-spectrum cephalosporins; AmpD, a cell wall recycling enzyme (Holden et al., 2009); PenB, a Class A penicillinase extremely conserved across the Bcc (Poirel et al., 2009). β-lactams block the cell wall recycling system (Cho et al., 2014), but bacteria can detect them using PenR (AmpR), a transcriptional regulator that normally binds a precursor of peptidoglycan (i.e., UDP-MurNAc-pentapeptide) and represses the expression of *ampC*. PenR becomes a transcriptional activator after binding peptidoglycan degradation products like 1,6-anhydroMurNAc-peptides. Mutations in *ampD* have a key role in controlling expression of *ampC* and *penB* (Lee et al., 2015; Vadlamani et al., 2015). In a recent study, *ampD* mutations induced by ceftazidime were demonstrated to cause overexpression of PenB and AmpC β-lactamases encoding genes, hence causing a reduction to ceftazidime, cefotaxime, and meropenem susceptibility (Hwang and Kim, 2015).

A therapeutic strategy could be to block the activity of β-lactamases using β-lactamase inhibitors; a large number of class A enzymes are blocked by clavulanic acid, sulbactam or tazobactam, while class C (AmpC β-lactamases) and some class D enzymes are inhibited by avibactam (Drawz and Bonomo, 2010; Lagacé-Wiens et al., 2014). No inhibitors effective against class B β-lactamases have been described yet (Livermore et al., 2011; Aktaş et al., 2012). In this context, a recent work evaluated the effect of β-lactamase inhibitors on Bcc treated with β-lactam antibiotics (Everaert and Coenye, 2016). In *B. cenocepacia* LMG 16656 the authors did not observe any difference in the MIC for ceftazidime and cefepime when sulbactam, tazobactam, or avibactam were present. A possible explanation could be that in *B. cenocepacia* LMG 16656 the majority of the β-lactamases belong to the metallo-β-lactamase family (Winsor et al., 2008), so no effective inhibitors are available yet (Everaert and Coenye, 2016).

Biofilm Formation and Persister Cells

When chronic infections are established in CF patients, bacterial cells are able to form biofilm, a matrix of extracellular polymeric molecules composed of DNA, polysaccharides, and proteins. In this context, they can further develop antibiotic resistance due to decreased antibiotic diffusion inside the biofilm matrix, reduction of nutrient availability resulting in metabolic changes that decrease antibiotic susceptibility, and, finally, phenotypic differentiation with appearance of persister cells which play key role in long-term infections (Mulcahy et al., 2010). Persister cells are a sub-population of cells that survive antibiotic treatment but, in contrast to resistant bacteria, this group does not express a specific resistance mechanism and their tolerance derives from physiological processes rather than genetic mutations (Allison et al., 2011). This kind of sub-populations is present in both sessile and planktonic cultures, but they are more difficult to eradicate after biofilm formation.

Persister cells are not mutated, but they are phenotypic variants of the wild type. After antibiotic treatment, these cells neither grow nor die and, and after drug removal, they start again to grow causing symptoms of infection. Van Acker et al. (2013) demonstrated that *B. cenocepacia* biofilms contain tolerant persister cells after tobramycin treatment.

Molecular mechanisms at the base of persistence are still largely unexplored, however, it was demonstrated that toxin–antitoxin modules (TA) play an important role not only in biofilm formation, gene regulation, programmed cell death and regulation of mobile genetic elements, but also in persistence mechanism (Gerdes and Maisonneuve, 2012). TA modules, abundant in bacteria and archaea, are two-component systems formed by a toxin that inhibits cell growth and by an antitoxin that controls toxin activity. There are five known types of TA loci. Toxin modules are always of protein nature in types I and III TA, while antitoxins are small RNAs that block toxin at translational and post-translational levels. Antitoxins of types II, IV, and V TA are proteins (Wang et al., 2012). Type II TA is well-characterized and usually the two genes are organized in an operon. During normal conditions, toxin and antitoxin form a complex that leads to inactivation of the toxin. Stress conditions induce degradation of the antitoxin and the toxin module regulates different cellular functions (Gerdes and Maisonneuve, 2012). Sixteen type II TA modules are present in *B. cenocepacia* J2315, 12 of which are conserved among *B. cenocepacia* strains. They were found to be up-regulated during biofilm growth (Van Acker et al., 2014). After treatment with tobramycin or ciprofloxacin, overexpression of toxins belonging to these TA systems contributes to persistence (Van Acker et al., 2014). TA modules could be an interesting target to treat chronic infections, but their redundancy is a challenging obstacle (Van Acker et al., 2014).

NEW THERAPIES AND APPROACHES TO OVERCOME *B. cenocepacia* INFECTIONS

Until now, no standard treatment strategy has been described to eradicate *B. cenocepacia* chronic infections (Horsley and Jones, 2012; Regan and Bhatt, 2016). Several questions remain unanswered: the lack of correlation between *in vitro* and *in vivo* susceptibility data, the duration of therapy, the use of mono vs. combined antibiotic therapy (Gautam et al., 2015).

In general, the use of trimethoprim–sulfamethoxazole is recommended. If it cannot be administered, combinations containing first- and second-line agents, such as ceftazidime, meropenem, and penicillins (mainly piperacillin) can be considered according to the *in vitro* antimicrobial susceptibility patterns (Avgeri et al., 2009). As regarding penicillins (piperacillin–tazobactam and ticarcillin–clavulanate), different results were described by EUCAST and CLSI guidelines, so *in vitro* susceptibility assessment is required before administration.

Recommendations for infection prevention and control were reviewed by Saiman et al. (2014), while Tacconelli et al. (2014) described ESCMID guidelines for the management of infection control measures and reduce Gram negative transmission in hospitalized patients.

In this section we report a list of antibiotics in clinical use, a description of a new compound under clinical trial and several alternatives to standard antibiotics described in the literature during the last 5 years (**Figure 1**).

Antibiotics in Clinical Use

Aztreonam

Aztreonam is a monobactam antibiotic approved in 1986 for the treatment of infections caused by Gram negative bacteria. It targets penicillin binding protein 3 (PBP3), thus inhibiting bacterial cell wall synthesis.

The solution for inhalation (AZLI; Cayston®; Gilead Sciences, Inc.) showed *in vitro* activity against *Burkholderia* spp. (Bosso et al., 1991) and a double-blind, placebo-controlled, 24-week trial of treatment was undertaken in CF patients with chronic Bcc infection (Tullis et al., 2012). Despite the fact that prolonged inhalation was well-tolerated, no significant improvement in the lung function was observed. The authors hypothesized that this could be due to non-study antibiotic use that may have confounded any potential therapeutic effect (Tullis et al., 2014).

Doripenem

Doripenem is a carbapenem approved in 2007 by the FDA. It is very similar to meropenem, but it shows a greater *in vitro* activity against *P. aeruginosa* (Hilas et al., 2008).

Intermittent extended infusion of doripenem was successfully used in a patient with history of methicillin-sensitive *S. aureus*, *S. maltophilia*, *Pseudomonas stutzeri*, and *B. cenocepacia* infections and showing meropenem shortage (Zobell et al., 2014). The report demonstrated that it is possible to administer doripenem also in children and that its intermittent extended infusion is effective to improve pulmonary function.

Tobramycin

Tobramycin is an aminoglycoside which prevents the formation of the 70S ribosomal complex. Tobramycin inhalation powder (TOBI Podhaler, Novartis) has been approved by FDA in 2011 to manage CF patients infected with *P. aeruginosa*.

Kennedy et al. (2015) showed the efficacy of high-dose tobramycin in reducing *Burkholderia* biofilm thickness *in vitro*, suggesting a possible role as a suppressive therapy in CF. Subsequently, a pilot, open-label clinical trial of TOBI Podhaler administered twice daily for 28 days in adults and children with CF and chronic *B. cepacia* complex infection was carried out (Waters et al., 2017). A decreased pulmonary bacterial burden and inflammation was observed, and the majority of patients showed no or mild adverse events. However, lung function was not significantly improved, possibly indicating that randomized controlled trials of longer duration are necessary (Waters et al., 2017).

New Compounds in Clinical Trials

A search in the EU Clinical Trials Register[5] and ClinicalTrials.gov[6] using "cystic fibrosis and *Burkholderia*" keywords retrieved 12 and 8 results, respectively. Among the former, one was completed and the results are reported above in the "Antibiotics in clinical use" paragraph (Aztreonam), one had no results available and all the others were not pertaining to the search being not related to CF or to *Burkholderia* infections. Among the latter, one was about *Burkholderia dolosa* infections, two were completed and hence reported above in the "Antibiotics in clinical use" paragraph (Aztreonam and Tobramycin), for one the status had not been verified for more than 2 years, one was about *P. aeruginosa* infections, one was not related to infections but to microbiota of patients, and the last one had been withdrawn prior to enrollment.

For these reasons, the only trial worth reporting is the "Study of OligoG in cystic fibrosis subjects with *Burkholderia* spp. infection (SMR-2591)" which is ongoing in Germany. This is a randomized double-blind, placebo-controlled cross-over study of inhaled alginate oligosaccharide (OligoG) for the treatment of chronic *Burkholderia* infections in adults. Information about this trial is available at www.clinicaltrialsregister.eu/ctr-search/trial/2014-002125-35/DE. OligoG is a low-molecular-weight oligosaccharide enriched from sodium alginate polysaccharides. It is an oligomer composed of α-L-guluronic acid (>85%) and β-D-mannuronic acid (<15%). Alginate is produced by *P. aeruginosa* during its conversion to a mucoid phenotype in the formation of biofilms (Hentzer et al., 2001). Previous studies demonstrated an improved effect of antibiotics used in combination with OligoG due to perturbation of biofilm formation (Khan et al., 2012; Roberts et al., 2013). Patients (both males and females) with at least two sputum cultures positive for Bcc in the 12 months prior to the beginning of the study were

[5]https://www.clinicaltrialsregister.eu/ctr-search/search
[6]https://clinicaltrials.gov/ct2/home

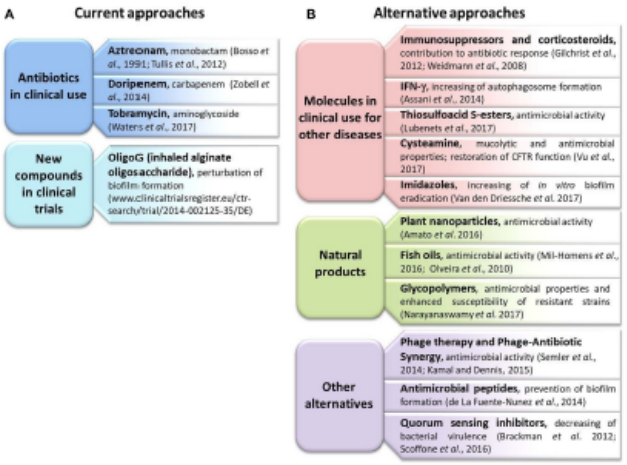

FIGURE 1 | Summary of the new therapies and approaches described in the review. **(A)** The current approaches utilized in clinical practice; **(B)** new alternatives.

recruited. Moreover, the eligibility criteria included the use of inhaled aztreonam. The main objective of this trial is to explore the efficacy of OligoG in reducing Bcc load in patients' sputum. The study will also evaluate the effect of inhaled OligoG on lung function, quality of life, rheology, safety and patient compliance with treatment.

Alternative Approaches

Molecules already in use to treat other clinical syndromes and products of natural origins are among the alternative approaches that can enhance current therapies or counteract the problem of resistance.

MOLECULES IN CLINICAL USE FOR OTHER DISEASES

Immunosuppressors and Corticosteroids

Immunomodulation has been suggested to contribute to the successful response to antibiotics in cases of cepacia syndrome (Gilchrist et al., 2012). This is probably due to the antagonist effect on the host response involved in the pathogenesis of cepacia syndrome. In one case, a patient was successfully treated with four intravenous antibiotics, oral corticosteroids, and cyclosporin (Gilchrist et al., 2012). In another case, four intravenous antibiotics, nebulised meropenem and tobramycin were used in combination with mycophenolate mofetil and tacrolimus (Weidmann et al., 2008). The role of immunosuppressant therapy in the management of *Burkholderia* infections is not completely understood, nevertheless it is worth considering this alternative approach for patients with poor prognosis.

IFN-γ

Cystic fibrosis macrophages show a suboptimal IFN-γ response during *B. cenocepacia* infection causing deficient autophagosome formation, therefore it has been suggested that IFN-γ administration may help clearance of these bacteria (Assani et al., 2014). So far, this has been demonstrated only in cell culture models and the efficacy *in vivo* has yet to be determined.

Thiosulfoacid *S*-Esters

Thiosulfoacid *S*-esters are organic compounds with established biological activity, already used as medicines, preservatives of vegetables, growth regulators, insecticides, and radioprotectors (Sotirova et al., 2012). Their chemical structure resembles that of natural compounds like garlic and onion derivatives (Block et al., 1996). In an attempt to find new molecules effective both against Gram-positive and negative microorganisms, Lubenets et al. (2017) synthesized *S*-esters of 4-acetyl, 4-trifluoroacetyl- and 4-(3-chloropropionylamino)-benzenethiosulfoacids and three of them showed MIC values within the micromolar concentration against *B. cepacia*. These molecules are examples of compounds worth further investigation to assess structure-activity relationships (SARs) and then prompt their practical application.

Cysteamine

Cysteamine has been investigated as a potential enhancer of antimicrobial therapy in CF patients due to its ability to disrupt disulfide bonds, thus achieving a mucolytic activity and improving biofilm penetration (Gahl et al., 1985). This

molecule is already approved for other diseases and its safety profile is known since 1994 (Gahl et al., 2007). Moreover, cysteamine showed antimicrobial activity against *P. aeruginosa*, so recently Fraser-Pitt et al. (2016) showed its effect in combination with antimicrobial agents used for the treatment of *Burkholderia*. In particular, they found that cysteamine was able to enhance the antimicrobial activity of tobramycin (even reversing resistance/insensitivity in 17 out of 36 strains), ciprofloxacin (reversing resistance/insensitivity in 10 strains), trimethoprim-sulfamethoxazole, but not ceftazidime. Furthermore, inhibition of bacterial biofilm was observed in presence of sub-inhibitory concentrations of cysteamine (Fraser-Pitt et al., 2016).

Interestingly, a very recent report showed that fatty acid cysteamine conjugates are able to promote transport of the misfolded CFTR to the surface of epithelial cells (Vu et al., 2017): in this way, besides having antimicrobial properties, cysteamine could be also useful to restore CFTR function in combination with potentiators and activators.

Imidazoles

In an attempt to identify compounds able to increase the susceptibility of *B. cenocepacia* biofilms to tobramycin, Van den Driessche et al. (2017) screened a repurposing library containing non-toxic compounds with already known metabolic properties. A total of 60 compounds were identified. Among them, four antifungal imidazoles (namely econazole, miconazole, oxiconazole, and ketoconazole) were able to significantly decrease the concentration of tobramycin necessary to completely eradicate Bcc biofilms. However, no potentiating effect could be observed in a 3D long epithelial cells model, nor in *Galleria mellonella* and mouse models of infection (Van den Driessche et al., 2017).

NATURAL PRODUCTS

Plant Nanoparticles

Essential oils (EOs) are complex extracts derived from aromatic plants comprising mixtures of aldehydes, terpenes, and phenols exhibiting broad spectrum antimicrobial activity (Bakkali et al., 2008). EO extracts containing carvacrol and thymol were shown to inhibit the growth of both clinical and environmental Bcc strains (Maida et al., 2014). Their mechanism of action relies on the partitioning of cytoplasmic membranes resulting in increased permeability, depletion of proton gradients, and disruption of ATP synthesis, ultimately leading to the death of bacterial cells.

First evidencies of antibacterial efficacy of thymol/carvacrol-loaded polymer nanoparticles against *B. cenocepacia* were reported by Amato et al. (2016) using polymer nanoparticles developed to overcome EO hydrophobicity, volatility and instability.

Fish Oils

Polyunsaturated fatty acids (PUFAs), such as essential omega-3 and omega-6 fatty acids, were shown to have antimicrobial activity, probably by disturbing cell membrane structures and associated functions like electron transport, proton gradient and enzymatic activities (Desbois and Smith, 2010). In particular, fish oils are a source of the omega-3 PUFA eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). Different papers described the use of omega-3 PUFAs to control *P. aeruginosa* infections *in vivo* (Tiesset et al., 2011; Caron et al., 2015) and *in vitro* (Tiesset et al., 2009), as well as *B. cenocepacia* infections (Mil-Homens et al., 2012). In particular, Mil-Homens et al. (2016) reported on their efficacy in treating *Burkholderia* infections and also as prophylactic therapy, using *G. mellonella* as infection model. Interestingly, as CF patients are deficient in fatty acids metabolism, administration of omega-3 PUFAs could be beneficial both to aid infection treatment and to improve respiratory, inflammatory, and nutritional parameters (Olveira et al., 2010).

Glycopolymers

Poly (acetyl, arginyl) glucosamine (PAAG) is a polycationic polysaccharide which represents a novel class of glycopolymers with antibacterial properties as well as synergy with antibiotics *in vitro* (Siedenbiedel and Tiller, 2012). Its mechanism of action relies on the interaction with the outer membrane of Gram negatives causing a depolarization which results in leakage of the intracellular contents and death. In particular, divalent cations located within the bacterial outer membrane electrostatically bind the LPS by the anionic phosphate groups (Rajyaguru and Muszynski, 1997). Recently, Narayanaswamy et al. (2017) showed that PAAG is effective to treat lung infections caused by Bcc in CF patients in combinations with Meropenem and Tobramycin as it enhances susceptibility of resistant strains.

OTHER ALTERNATIVES

Phage Therapy and Phage-Antibiotic Synergy

Phage therapy is the therapeutic application of bacterial viruses (bacteriophages) commercially developed in the 1930s to reduce or eliminate infection (Gravitz, 2012). This is considered a valuable alternative to chemotherapeutic agents due to the specificity of phages toward bacterial cells and their exponential replication highly enhancing the therapeutic potential (Alisky et al., 1998).

Semler et al. (2014) compared the activity of phages delivered as aerosol to that of phages delivered via an intraperitoneal (i.p.) route to treat *B. cenocepacia* infections in a murine model. Their results showed that infected mice receiving aerosolized phage treatments exhibited a significant decrease in bacterial loads within the lungs, which could not be observed in those receiving treatment via i.p. injection. In this way, they demonstrated that aerosolization provides more widespread and uniform particle deposition, while i.p. delivered phages reach only certain areas in the lungs and may be unable to co-localize with the bacteria in the lung lumen (Semler et al., 2014).

Phage-antibiotic synergy (PAS) is the effect that some antibiotics exert on the ability of phages to form larger plaque

under sub-lethal concentrations of the compound itself (Comeau et al., 2007). Ciprofloxacin, meropenem, and tetracycline exhibited PAS in combination with two *B. cenocepacia* phages enlarging plaque size (Kamal and Dennis, 2015). In particular, presence of ciprofloxacin and meropenem leads to the formation of elongated or filamented cells, and so phages may have increased access to phage receptors; moreover, cell clustering in presence of tetracycline allows phages to travel laterally across adjoined cell surfaces, again enhancing contact with phage receptors. PAS effect was not altered when treating antibiotic resistant cells, thus encouraging the use of this alternative method also with drug resistant strains (Kamal and Dennis, 2015).

Antimicrobial Peptides

Cationic antimicrobial peptides (CAMPs) have been isolated from very different organisms such as microorganisms, invertebrates, plants, and mammals. They are able to establish strong non-specific, hydrophobic and electrostatic interactions with bacterial cytoplasmic membranes (Godballe et al., 2011) and prevent cell adhesion via electrostatic bonds (Overhage et al., 2008).

Antimicrobial peptides able to block *B. cenocepacia* biofilm formation were described in de la Fuente-Núñez et al. (2014). These peptides are similar to CAMPs but have different SAR. They exert their activity by blocking the stringent response mediated through (p)ppGpp, two small signaling nucleotides (guanosine 5′-diphosphate 3′-diphosphate or ppGpp and guanosine 5′-triphosphate 3′-diphosphate or pppGpp) (Potrykus and Cashel, 2008). These antimicrobial peptides showed activity against both Gram negative and positives bacteria. The peptide described by de la Fuente-Núñez et al. (2014) directly interacts with (p)ppGpp and is able to cross bacterial membranes to reach the cytoplasm. It can prevent biofilm formation and promote biofilm dispersal, and also cell death in biofilms at concentrations sub-lethal for planktonic cells (de la Fuente-Núñez et al., 2014).

Quorum Sensing Inhibitors

In order to attenuate bacterial virulence, several quorum sensing inhibitors (QSI) have been developed in the last 15 years. Among them, analogs of the signal molecule Acyl-homoserine lactone (AHL) are obtained by modification of the acyl side chain, the lactone moiety (Ni et al., 2009) or the central amide moiety (Boukraa et al., 2011). Valuable AHL analogs, able to affect *B. cenocepacia* (as well as *P. aeruginosa*) QS and, in turn, inhibit and eradicate biofilm, have been described by Brackman et al. (2012).

More recently, another class of QSIs, namely diketopiperazines, was shown to inhibit the activity of the AHL synthase CepI of *B. cenocepacia* (Scoffone et al., 2016). These compounds interfered with the production of virulence factors, such as proteases and siderophores, as well as with biofilm formation, and showed good *in vivo* activity using a *Caenorhabditis elegans* infection model. Study results suggested that they could be considered for *in vivo* treatments combined with established or novel antimicrobials (Scoffone et al., 2016).

CONCLUSION

Even if the epidemiology of CF pathogens is continuously changing and *B. cenocepacia* is not one of the prevalent bacteria colonizing the lung of patients, it still remains a major threat due to its extreme resistance to antibiotics and its ability to cause a life-threatening CF complication known as cepacia syndrome. In the last years, several studies were aimed at deciphering the principal mechanisms of resistance in *Burkholderia*, however, many aspects have not yet been fully understood. This has important repercussions on the choice of antibacterial drugs to be used and on our knowledge of the physiology of a bacterium with a wide genome yet to be fully explored.

The lack of a standard therapy regimen makes the treatment of Bcc infections challenging and more studies are needed to improve survival and quality of life of CF patients. On the contrary, evidence-based eradication guidelines are available for *P. aeruginosa* allowing standardization of antimicrobial treatment and hence improving management of these infections (Mogayzel et al., 2014). The introduction of correctors and potentiators of the CFTR defects requires more studies of drug-drug interactions to predict treatment efficacy, however, this is not possible while a standard therapy for Bcc is not available. Since few patients are infected by *B. cenocepacia*, an increased effort to coordinate clinical trials and observational studies among different hospitals is needed, maximizing data necessary to standardize methods for evaluation of antibiotic susceptibility.

Since the development of new drugs is not trivial, it is necessary to improve the use of existing therapies while coordinating trials of new molecules which showed their potential *in vitro* and in animal models.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Investigating the Mechanism of Action of Diketopiperazines Inhibitors of the *Burkholderia cenocepacia* Quorum Sensing Synthase CepI: A Site-Directed Mutagenesis Study

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Quorum sensing (QS) is a bacterial intercellular communication process which controls the production of major virulence factors, such as proteases, siderophores, and toxins, as well as biofilm formation. Since the inhibition of this pathway reduces bacterial virulence, QS is considered a valuable candidate drug target, particularly for the treatment of opportunistic infections, such as those caused by *Burkholderia cenocepacia* in cystic fibrosis patients. Diketopiperazine inhibitors of the acyl homoserine lactone synthase CepI have been recently described. These compounds are able to impair the ability of *B. cenocepacia* to produce proteases, siderophores, and to form biofilm, being also active in a *Caenorhabditis elegans* infection model. However, the precise mechanism of action of the compounds, as well as their effect on the cell metabolism, fundamental for candidate drug optimization, are still not completely defined. Here, we performed a proteomic analysis of *B. cenocepacia* cells treated with one of these inhibitors, and compared it with a *cepI* deleted strain. Our results demonstrate that the effects of the compound are similar to the deletion of *cepI*, clearly confirming that these molecules function as inhibitors of the acyl homoserine lactone synthase. Moreover, to deepen our knowledge about the binding mechanisms of the compound to CepI, we exploited previously published *in silico* structural insights about this enzyme structure and validated different candidate binding pockets on the enzyme surface using site-directed mutagenesis and biochemical analyses. Our experiments identified a region near the predicted *S*-adenosylmethionine binding site critically involved in interactions with the inhibitor. These results could be useful for future structure-based optimization of these CepI inhibitors.

Keywords: quorum sensing, Acyl Homoserine Lactone synthase, synthase inhibitors, *Burkholderia cenocepacia*, homology model, site-directed mutagenesis

INTRODUCTION

Quorum sensing (QS) is a bacterial intercellular communication process, which relies on the synthesis and secretion of signal molecules (Sokol et al., 2007; Udine et al., 2013). The binding of these molecules to specific effectors mediates the regulation of the expression of major virulence factors such as proteases, siderophores, and toxins (Tomlin et al., 2005) and enhances the ability of bacteria to form biofilm. This last characteristic greatly impairs the diffusion of antibiotics enhancing resistance to antibacterial compounds. In this way, QS can be considered a good candidate drug target, as the interference with this pathway makes bacteria less virulent (Rasko and Sperandio, 2010).

This strategy could be particularly useful for the treatment of opportunistic pathogens, such as *Burkholderia cenocepacia*, a Gram negative bacterium which colonizes the lung of cystic fibrosis patients (Drevinek and Mahenthiralingam, 2010). These infections are particularly dangerous. This is due to the high rate of resistance to antibiotics caused by enzymatic inactivation, modification of target, poor cell wall permeability, and the presence of many efflux pumps (Scoffone et al., 2017). Thus, the identification of new compounds able to inhibit *B. cenocepacia* growth, as well as of new drug targets, is a prominent question.

B. cenocepacia J2315 possesses four QS systems composed by a synthase (I) and a receptor (R): CepIR, CciIR, the *Burkholderia* Diffusible Signal Factor (BDSF)-based system $RpfF_{BC}$, and the recently discovered non-ribosomal peptide synthetase-like cluster *ham* (Coenye, 2010; Spadaro et al., 2016; Jenul et al., 2018). The characterization of *B. cenocepacia* mutants lacking the synthases CepI and/or CciI and $RpfF_{BC}$ demonstrated an involvement of CepI in biofilm formation, protease production, and virulence, as well as an interplay among the Acyl Homoserine Lactone (AHL) systems CepIR and CciIR and the BDSF-based system (Udine et al., 2013).

We recently identified new diketopiperazine molecules, able to inhibit CepI *in vitro*, impairing the ability of *B. cenocepacia* to produce proteases, siderophores, and to form biofilm (Scoffone et al., 2016). These molecules did not possess any antimicrobial activity, nevertheless their administration significantly increased the survival of *Caenorhabditis elegans* nematodes infected with *B. cenocepacia*, suggesting that the virulence of the strain could be attenuated *in vivo*. All these data suggest the possibility of a combined treatment by using CepI inhibitors with antimicrobials, to improve the therapeutic strategies available against *B. cenocepacia* (Scoffone et al., 2016).

The current lack of molecular structure data on CepI prevents the possibility of 3D structure-assisted optimization studies of these new inhibitors. In our previous work, we generated a CepI homology model, and used it to perform *in silico* docking analyses of the diketopiperazine inhibitor 8b (**Figure 1**) ("Supplementary Materials and Methods"). Using this approach, we identified multiple candidate binding sites, localized far from the enzyme catalytic site, but in regions possibly still implicated in substrate recognition and catalysis (Scoffone et al., 2016).

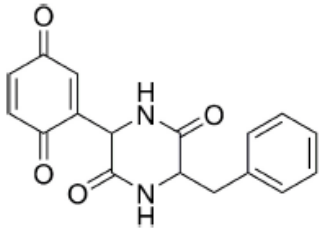

FIGURE 1 | Chemical structure of the (3S)-3-Benzyl-6-(3,6-dioxocyclohexa-1,4-dien-1-yl)piperazine-2,5-dione (8b) CepI Inhibitor.

Here, we confirmed that the cellular effects of 8b are indeed related to the inhibition of CepI, by analyzing the proteome of *B. cenocepacia* cells treated with the compound, and compared with that of the knock-out *cepI* strain. Moreover, we exploited a site-directed mutagenesis strategy to better define the crucial amino acid residues responsible for catalysis and recognition of the 8b inhibitor. Taken together, our results suggest a possible mechanism of CepI inhibition by the 8b compound through perturbations of a flexible loop involved in recognition and stabilization of the *S*-adenosylmethionine substrate, facilitating future drug discovery approaches based on the 8b chemical scaffold.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Plasmid pETSUMO-CepI (Scoffone et al., 2016) was used as template for each PCR mutagenesis experiment to generate amino acid substitutions using the primers listed in Supplementary Table S1. The site-directed mutagenesis was carried out as previously described in the PCR-based method (Bachman, 2013) using HotStar HiFidelity Polymerase (Qiagen) according to manufacturer's instructions.

Proteins Purification and Characterization

Wild type and mutant CepI were expressed in *Escherichia coli* BL21(DE3) cells and purified as previously described (Scoffone et al., 2016).

Far-UV circular dichroism (CD) measurements were performed with a Jasco-J700 spectropolarimeter (Jasco-Europe, Cremella, Italy) using a 1 mm path cell. Scans were conducted between 190 and 250 nm at a speed of 50 nm/min with a spectral band width of 2 nm and a sensitivity of 20 mdeg. CD spectrum measurements were performed at 25°C in 50 mM sodium phosphate pH 8.0, 50 mM KCl, and represent the average of 10 scans. The protein concentration was 4–5 μM. Spectra were analyzed using the DichroWeb online platform (Whitmore and Wallace, 2008).

CepI activity was determined according to Christensen et al. (2013). Reaction mixtures contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 0.005% Nonidet P-40, 0.13 mM 2,6-dichlorophenylindophenol (DCPIP), 70 μM Octanoyl-ACP (C8-ACP, prepared as reported previously) (Quadri et al., 1998; Cronan and Thomas, 2009), 40 μM *S*-adenosyl methionine (SAM), 4 μM CepI.

Steady-state kinetic parameters were determined by assaying the enzymes at eight different concentrations of substrates, using the Michaelis–Menten equation and Origin eight software.

IC_{50} was determined measuring the enzyme activity in presence of different concentrations of compound, and values determined with the following equation (Copeland, 2000), where $A_{[I]}$ is the enzyme activity at inhibitor concentration [I] and $A_{[0]}$ is the enzyme activity without inhibitor.

$$A_{[I]} = A_{[0]} \times \left(1 - \frac{[I]}{[I] + IC_{50}}\right)$$

Proteomics Analysis

Burkholderia cenocepacia Δ*cepI* (Udine et al., 2013) and J2315 (with or without 25 μM of 8b compound) were grown in 10 ml of LB medium until $OD_{600\ nm} > 2$. Cells were then harvested, resuspended in 0.2 ml of Tris-HCl 50 mM, pH 7.5, disrupted by sonication, and centrifuged at 12,000 rpm for 1 h at 4°C.

The amount of proteins present in the supernatant was quantified by bicinchoninic acid method (Smith et al., 1985), then 300 μg were precipitated with 10% (v/v) trichloroacetic acid. The obtained protein pellet was dissolved in 125 μL of rehydration buffer (8 M urea, 4% CHAPS (w/v), 65 mM DTE, 0.8% carrier ampholytes (v/v) and 0.5% bromophenol blue) and loaded onto 7 cm IPG strips, with nonlinear pH 3–10 gradient range (GE Healthcare), and strips rehydrated for 1 h at 20°C. The first-dimensional IEF was carried out at 15°C using an Ettan IPGphor system (GE Healthcare), by applying 30 V for 8 h, 120 V for 1 h, 500 V for 0.5 h, 1000 V for 0.5 h and 5000 V for 6 h, for a total of 29–30 kVh. The focused IPG strips were subjected to reduction/alkylation steps then loaded onto an 8 × 6 cm slabs, 12.5% SDS polyacrylamide gels. The 2-DE gels were stained with "Blue silver" (colloidal Coomassie G-250 staining), according to Candiano et al. (2004). Digital images of stained gels were acquired using VersaDoc Imaging Model 3000 (BioRad) and then subjected to quali/quantitative analysis using the PD Quest (BioRad) version 8.0.1 software. Scanned images were filtered and smoothed to remove background noise, vertical/horizontal streaking, gel artifacts and then normalized to eliminate the variability of each sample. The software then determined the amount of spots present and calculated their intensity by applying the following algorithm: peak value (ODs/image units) $*\sigma_x*\sigma_y$ (standard deviations in x and y).

For protein identification, the selected spots were carefully excised from the gel, washed twice with 100 mM ammonium bicarbonate buffer pH 7.8, 50% acetonitrile (ACN) and kept under stirring overnight, until complete destaining. After dehydration, gels were rehydrated by addition of 50 μL of 100 mM ammonium bicarbonate buffer pH 7.8, containing 20 ng/μL sequencing grade trypsin (Promega, Madison, WI, United States) and digestion was performed overnight at 37°C. The resulting peptides were extracted sequentially from gel matrix by treatment with 50 μL of 50% ACN in water, 5% trifluoroacetic acid and finally with 50 μL of 100% ACN. Each extraction involved 15 min of stirring followed by centrifugation and removal of the supernatant. The original supernatant and those obtained from sequential extractions were pooled, dried and stored at −80°C until mass spectrometric analysis. At the moment of use, the peptide mixture was solubilized in 100 μL of 0.1% formic acid (FA) for MS analyses.

The analyses were carried out on an LC-MS (Thermo Finnigan, San Jose, CA, United States) system consisting of a thermostated column oven Surveyor autosampler controlled at 25°C; a quaternary gradient Surveyor MS pump equipped with an UV/V is detector and an Ion Trap (LCQ Fleet™) mass spectrometer with electrospray ionization ion source controlled by Xcalibur software 2.0.7. Analytes were separated by RP-HPLC on a Jupiter (Phenomenex, Torrance, CA, United States) C_{18} column (150 × 2 mm, 4 μm, 90 Å particle size) using a linear gradient (2–60% solvent B in 60 min) in which solvent A consisted of 0.1% aqueous FA and solvent B of ACN containing 0.1% FA. Flow-rate was 0.2 mL/min. Mass spectra were generated in positive ion mode under constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, sheath gas flow 20 (arbitrary units), auxiliary gas flow 10 (arbitrary units), sweep gas flow 1 (arbitrary units), capillary temperature 210°C, tube lens voltage 105 V. MS/MS spectra, obtained by CID studies in the linear ion trap, were performed with an isolation width of 3 Da *m/z*, the activation amplitude was 35% of ejection RF amplitude that corresponds to 1.58 V.

Data processing was performed using Peaks Studio version 4.5 software. The mass list was searched against the SwissProt protein and *B. cenocepacia* databases, under continued mode (MS plus MS/MS) with the following parameters: trypsin specificity; five missed cleavages; peptide tolerance at 0.2 Da and MS/MS

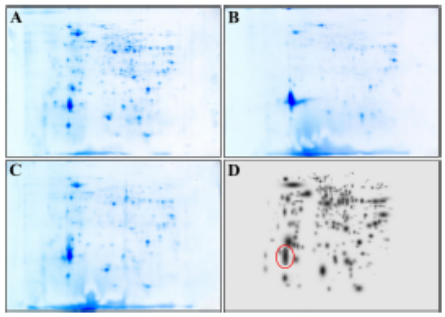

FIGURE 2 | Representative 2-DE map of *B. cenocepacia* J2315 untreated **(A)**, treated with 8b 25 μM **(B)** and of Δ*cepI* knock-out strain **(C)**. **(D)** represents the High Master Gel, a virtual image resulting from the correlation of the master gels of each sample group. Red circle highlight the spot corresponding to the giant cable pilus protein.

tolerance at 0.25 Da; peptide charge 1, 2, 3+ and experimental mass values: monoisotopic.

RESULTS

Diketopiperazine 8b Treatment and *cepI* Knock-Out Have the Same Phenotypic Effects

In a previous study, we identified a diketopiperazine molecule active against the QS synthase CepI, and impairing the production of virulence factors in *B. cenocepacia* J2315, as well as the ability of the bacterium to form biofilm (Scoffone et al., 2016).

To further confirm that the compound acts also intracellularly inhibiting the CepI synthase, we compared the proteomes of the wild type *B. cenocepacia* J2315 untreated and treated with 25 μM 8b, with that of the Δ*cepI* knock-out strain. To identify the bacterial proteins differentially expressed, parallel 2-DE analyses of protein extracts from each cell lysate were performed in triplicate. **Figures 2A–C** shows the representative maps of the three samples.

A match set was then created to compare the gels of each replicate and to match the spots present, and used to get a synthetic image (Master Gel) containing qualitative and

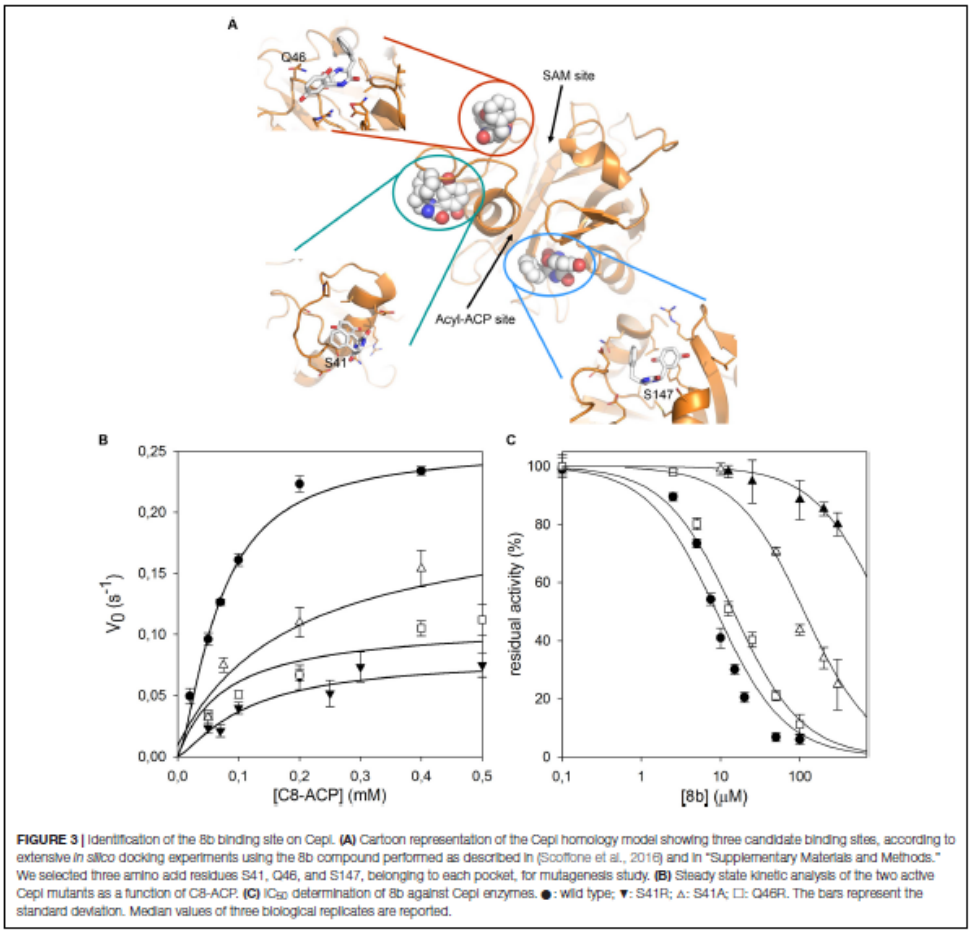

FIGURE 3 | Identification of the 8b binding site on CepI. **(A)** Cartoon representation of the CepI homology model showing three candidate binding sites, according to extensive *in silico* docking experiments using the 8b compound performed as described in (Scoffone et al., 2016) and in "Supplementary Materials and Methods." We selected three amino acid residues S41, Q46, and S147, belonging to each pocket, for mutagenesis study. **(B)** Steady state kinetic analysis of the two active CepI mutants as a function of C8-ACP. **(C)** IC_{50} determination of 8b against CepI enzymes. ●: wild type; ▼: S41R; △: S41A; □: Q46R. The bars represent the standard deviation. Median values of three biological replicates are reported.

quantitative data relative to all spots. The Master Gels from each group (*B. cenocepacia* J2315 untreated and treated, and Δ*cepI*) were matched to create a virtual image (High Master Gel, **Figure 2D**), containing all the common and uncommon spots. This higher match set allowed to determine the presence or absence of spots and the intensity values of common ones; typically, a mean of 240 protein spots were detected in each gel. The comparison of the 2-DE patterns revealed qualitative and quantitative differences between the three groups considered. In terms of presence/absence, the majority of spots were in common between *B. cenocepacia* J2315 cells treated with 8b and Δ*cepI* cells, while protein spots showed different density compared with the untreated J2315. Among them, one protein spot (marked in red in **Figure 2D**) showed higher density in 8b treated cells and Δ*cepI* profiles, and was excised from the gel for LC-MS/MS identification. This spot was found to correspond to the giant cable pilus protein CblA (Supplementary Table S2), which is known to contribute to *B. cenocepacia* virulence, being involved in persistence *in vivo* (Goldberg et al., 2011) and to adherence to respiratory epithelia (Sajjan et al., 2000). Moreover, the *cblA* gene is a useful marker for the *B. cenocepacia* ET12 lineage and has been linked with both pathogenicity and epidemic behavior (Mahenthiralingam et al., 2000). Thus, our data demonstrate that 8b mimics the effects of the deletion of *cepI*, confirming that the effects of the compound clearly rely on CepI inhibition.

Identification of the Diketopiperazine 8b Binding Site on CepI Enzyme

As no structural data on *B. cenocepacia* CepI are available, to improve the possibility of 3D structure-assisted optimization of the non-competitive inhibitor 8b, we previously generated a homology model of the enzyme and performed *in silico* docking studies, which enabled the identification of multiple candidate binding sites for the inhibitor (Scoffone et al., 2016 and "Supplementary Materials and Methods"). In particular, 8b could be docked with high score in at least three different pockets on the enzyme surface (**Figure 3A**). To understand if the inhibitor effectively binds to these sites, we therefore generated three different CepI mutants: S41R, Q46R, and S147R.

All mutants could be expressed and purified to homogeneity using the same procedure adopted for the wild type (Scoffone et al., 2016). Although these mutants were obtained with yields generally lower than wild type CepI, all proteins variants were found soluble. Moreover, CD spectra of the mutants did not show significant differences with that of the wild type, thus confirming that the introduced mutations did not affect the correct fold of the protein (Supplementary Figure S1).

Among these mutants, our biochemical experiments revealed that S147R was completely inactive (**Table 1**). To ascertain if the lack of activity was due to the introduction of a bulky and positively charged residue, we produced a new S147L mutant. However, this mutant was also inactive (**Table 1**), suggesting an essential role for the area around the amino acid 147 in the catalysis.

TABLE 1 | Enzymatic characterization of CepI mutants.

Enzyme	V_{max} (s^{-1})	K_m (mM)	compound 8b IC_{50} (mM)
Wild type	0.25 ± 0.009	0.068 ± 0.005	0.0072 ± 0.0002
R24Q	0.16 ± 0.020	0.074 ± 0.026	0.147 ± 0.017
E29Q	n. a.[a]	n. a.	n. a.
E40Q	0.09 ± 0.003	0.105 ± 0.001	0.053 ± 0.0026
S41A	0.16 ± 0.007	0.081 ± 0.006	0.113 ± 0.011
S41R	0.08 ± 0.010	0.113 ± 0.016	1.070 ± 0.075
Q46R	0.18 ± 0.024	0.078 ± 0.013	0.0192 ± 0.0035
S147L	n. a.	n. a.	n. a.
S147R	n. a.	n. a.	n. a.

[a]*n.a. not active.*

By contrast, the S41R and Q46R mutants did not show severe perturbations in their kinetic properties, showing K_m values unchanged respect to the wild type, and k_{cat} values two to three fold reduced (**Figure 3B** and **Table 1**). To understand whether any of these mutated residues could be involved in the binding of the inhibitor, we measured the IC_{50} of compound 8b toward each mutant enzyme. While Q46R was comparable to wild type CepI, S41R was practically insensitive to the compound (IC_{50} > 1 mM) (**Figure 3C** and **Table 1**), strongly suggesting an involvement of this residue in the mechanism of action of 8b. However, to ascertain if the high IC_{50} of the compound toward the S41R mutant was due to perturbations introduced by the bulky and positively charged arginine rather than to the lack of interaction with the serine, a more conservative mutant S41A was generated. This new mutant was still less sensitive to 8b, showing an IC_{50} 15-fold higher than that of the wild type (**Figure 3C** and **Table 1**), thus confirming a direct involvement of Ser 41 in inhibitor binding.

We therefore decided to expand our investigation to other amino acids belonging to the surface of this pocket region. This region is proximate to a loop that adopts a variety of conformations in homologous AHL synthases, with possible implications in SAM binding. In particular, we studied the role of R24, E29, and E40, predicted to constitute contact platform for the compound or for serine 41. Each of these residues was mutated into a glutamine (**Figure 4A**). Once again all these mutants did not show significant alteration of the K_m values; a small reduction was detected in their k_{cat}, with the exception of the E29Q which however displayed negligible enzymatic activity (**Figure 4B** and **Table 1**). Also in this case, the CD spectrum of E29Q did not show differences with respect to the wild type, thus excluding that the lack of catalytic activity is due to a misfolding of the mutant (Supplementary Figure S1).

By contrast, all the active mutants were significantly less inhibited by the 8b compound, showing IC_{50} values 10–15 fold higher respect to the wild type (**Figure 4C** and **Table 1**).

These results confirmed that CepI residues located in this region have a direct role in the binding and in the mechanism of inhibition of 8b against CepI, and experimentally support our previous hypotheses driven by *in silico* analyses.

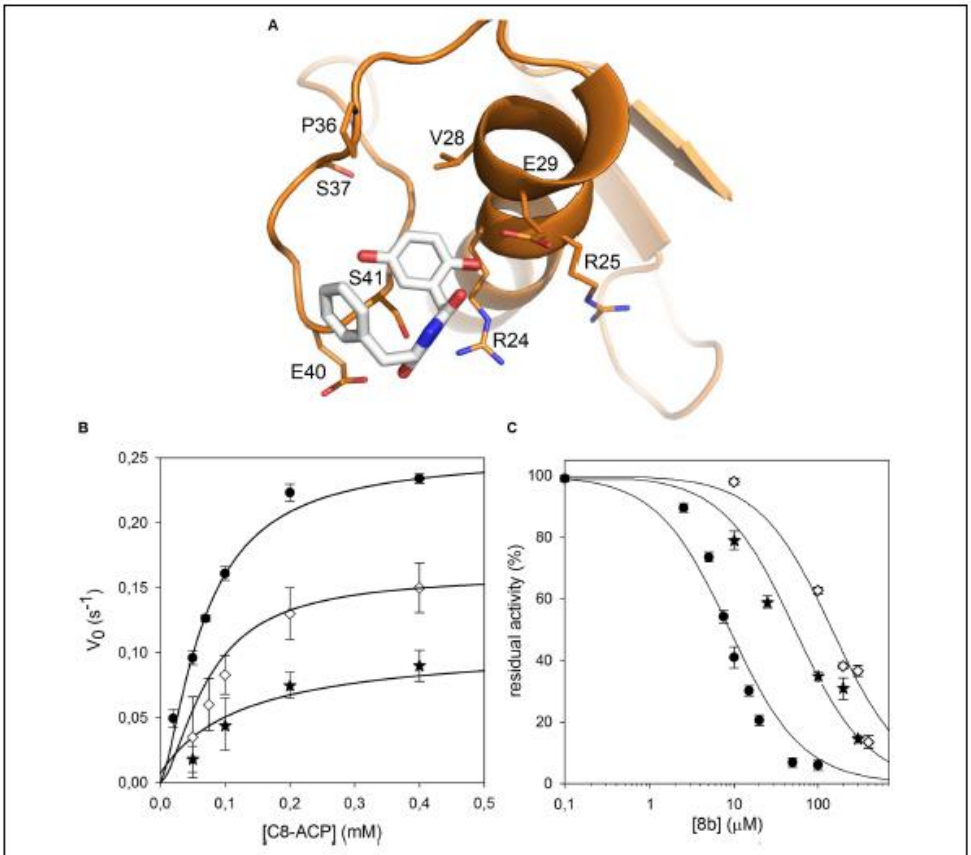

FIGURE 4 | Identification of the CepI amino acid residues involved in 8b binding. **(A)** Cartoon representation of the CepI homology model showing the possible interaction of 8b with the residues in proximity to the serine 41. The three amino acid R24, E29, and E40 were selected for mutagenesis. **(B)** Steady state kinetic analysis of the two active CepI mutants as a function of C8-ACP. **(C)** IC_{50} determination of 8b against CepI enzymes. ●: wild type; ◇: R24Q; ★: E40Q. The bars represent the standard deviation. Median values of three biological replicates are reported.

DISCUSSION

Quorum sensing inhibitors appear as very promising potentiators of the classical antibiotic therapy to treat dangerous infections such as those caused by *B. cenocepacia*, for which a lack of new solutions is massively reported (Horsley et al., 2011; Regan and Bhatt, 2016; Scoffone et al., 2017). Searching in PubMed for "quorum sensing inhibitors" retrieves 51 results in the first 5 months of 2018, which shows the increasing interest in alternative routes to counteract the emergence of resistance among bacteria such as *Proteus mirabilis* (Yu et al., 2018), *Salmonella* (de Almeida et al., 2018), *Pseudomonas aeruginosa* (Almohaywi et al., 2018), *Staphylococcus aureus* (Karathanasi et al., 2018).

We recently reported the discovery of diketopiperazines as inhibitors of the QS synthase CepI of *B. cenocepacia*. Beside the *in vitro* effect on the purified protein, these inhibitors showed interesting *in vivo* phenotypes, being able to decrease the production of proteases, siderophores, and lowering the ability of *B. cenocepacia* to form biofilm. Moreover, they increased the survival of infected *C. elegans* (Scoffone et al., 2016).

Here, we further confirmed that the compound acts also intracellularly by comparing the proteomes of the wild type *B. cenocepacia* J2315 untreated and treated with 25 μM 8b,

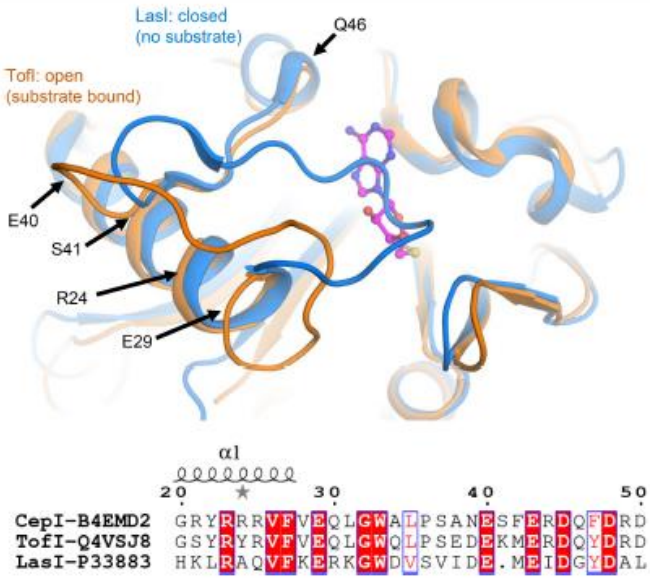

FIGURE 5 | Identification of CepI mutated residues based on structural similarity with the crystal structures of homologous AHL synthases TofI (Chung et al., 2011) (orange) and LasI (Gould et al., 2004) (blue). The position of the SAM substrate, derived from the crystal structure of TofI in complex with an analog, is shown with magenta ball-and-sticks. The position of residues 24, 29, 40, 41, and 46 are shown with black arrows. The amino acid composition of this region is partially conserved among AHL synthases, as highlighted in the bottom panel, in which the fully conserved residues are evidenced, while conservative changes are in red.

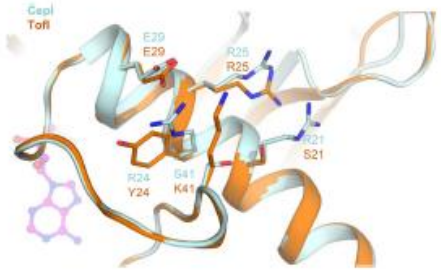

FIGURE 6 | Comparison of the amino acid network surrounding residue 41 in CepI and TofI. Shown is a superposition of the CepI homology model (light blue) with TofI crystal structure (Chung et al., 2011) (orange). Amino acid side chains are shown as sticks and labeled according to their numbering in both CepI and TofI. The position of the SAM substrate analog, as derived from TofI crystal structure, is shown as magenta ball-and-stick.

with that of the Δ*cepI* knock-out strain. Indeed, these last two cultures showed the same proteomic profile: in our experimental conditions, only the over production of the giant cable pilus protein CblA was detected. Cable pilus is a well known virulence factor, which together with the 22-kDa pilus-associated adhesin is mainly involved in binding of the host cells and, particularly, in the transmigration across the epithelium (Urban et al., 2005). However, no relationship between CepI and CblA has been reported so far. Thus, this result is quite unexpected and why the lack or inhibition of the QS synthase should enhance the expression of this virulence trait of *B. cenocepacia* is an intriguing open question which is worth investigating deeper.

In our previous work, we computationally generated an homology model of *B. cenocepacia* CepI and performed *in silico* docking procedures, which enabled the identification of three candidate binding sites for the most promising diketopiperazine compound 8b (Scoffone et al., 2016). To confirm the involvement of these pockets into 8b binding, we initially designed three different CepI mutants: S41R, Q46R, and S147R. As expected from homology model analysis, our biochemical experiments revealed that the area surrounding the amino acid 147 is likely involved in catalysis due to strong proximity of this residue to the main access cavity hosting the acyl-ACP substrates (**Figure 3A**). Intriguingly, mutant S41R was practically insensitive to the 8b compound, suggesting that the pocket around this residue may form the binding site of the compound. Thus, to confirm this, three other mutants were generated by modifying the

charged residues (R24, E29, and E40) around S41 into the polar, uncharged glutamine. Mutation E29Q abolished CepI enzymatic activity, suggesting roles for this residue in catalysis despite its distance from the enzyme's catalytic site. The other two mutants did not show significant alteration of the kinetic properties, nevertheless they were significantly less susceptible to inhibition by the 8b compound, confirming the mechanism of action and the involvement of the pocket in 8b binding.

Comparisons with homologous AHL synthases TofI (Chung et al., 2011) and LasI (Gould et al., 2004) structures indicate that CepI loop comprising neighboring residues 30–41 may be involved in conformational changes associated to interactions with the SAM substrate. These conformational changes may involve partial unfolding of the last helical turn comprising the fully conserved E29 residue. In this respect, isosteric alterations of the negatively charged character of this amino acid may result in increased structural instability and, possibly, interfere with the productive conformational changes necessary for SAM substrate binding (**Figure 5**).

Residue S41, identified as the most susceptible to compound 8b inhibition, is located at the C-terminal hinge of the flexible loop possibly subject to conformational changes upon SAM binding (**Figure 6**). Introduction of bulky, positively charged residues at this site (such as in the S41R mutant) may introduce strong repulsion due to interference with neighboring Arg21 and Arg25 residues and may possibly explain the dramatic variation observed in IC_{50} values (**Table 1**). Notably, homologous TofI possesses a Lys residue at this site, but at the same time its corresponding residue at position 21 is not an Arg, but a smaller Ser residue (**Figure 6**).

Together these results support our previous finding and confirm that compound 8b acts intracellularly by inhibiting CepI activity, thus interfering with the production of the QS signal molecule C8-AHL. Moreover, the identification of the pocket involved in inhibitory mechanism of 8b provides new information on the binding of the compound with CepI, which will be useful for a structure-based optimization of the diketopiperazine inhibitors.

AUTHOR CONTRIBUTIONS

SB, VS, and LC conceived and directed the project. SB, FF, and LC designed the experiments. VS, MF, VM, GT, and SB, carried out the experiments. SB, EDR, FF, GR, and LC conducted the data analysis and interpreted the results. SB, VS, FF, and LC wrote the manuscript. All the authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00836/full#supplementary-material

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Review

Quorum Sensing as Antivirulence Target in Cystic Fibrosis Pathogens

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Abstract: Cystic fibrosis (CF) is an autosomal recessive genetic disorder which leads to the secretion of a viscous mucus layer on the respiratory epithelium that facilitates colonization by various bacterial pathogens. The problem of drug resistance has been reported for all the species able to colonize the lung of CF patients, so alternative treatments are urgently needed. In this context, a valid approach is to investigate new natural and synthetic molecules for their ability to counteract alternative pathways, such as virulence regulating quorum sensing (QS). In this review we describe the pathogens most commonly associated with CF lung infections: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, species of the *Burkholderia cepacia* complex and the emerging pathogens *Stenotrophomonas maltophilia*, *Haemophilus influenzae* and non-tuberculous Mycobacteria. For each bacterium, the QS system(s) and the molecules targeting the different components of this pathway are described. The amount of investigations published in the last five years clearly indicate the interest and the expectations on antivirulence therapy as an alternative to classical antibiotics.

Keywords: quorum sensing; cystic fibrosis; bacterial infections

1. Introduction

Cystic fibrosis (CF) is a hereditary, autosomal recessive genetic disease associated with mutations in the gene encoding a membrane-bound chloride channel named cystic fibrosis transmembrane conductance regulator (CFTR) [1]. CFTR dysfunction has been correlated to the secretion of a viscous mucus layer on the respiratory epithelium that facilitates colonization by bacterial pathogens. In addition, a major hallmark of CF clinical phenotype is the dysregulation of innate immune functions, leading to chronic bacterial lung infections and inflammation [2]. Pulmonary disease is the primary cause of reduced life expectancy and death in CF patients [3]. The pathogens most commonly associated with CF lung infections include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, species of the *Burkholderia cepacia* complex as well as emerging pathogens, like *Stenotrophomonas maltophilia*, *Haemophilus influenzae* and non-tuberculous Mycobacteria [4].

Antibiotic therapies are implemented in order to eradicate these infections and slow down the deterioration of pulmonary function. However, by targeting essential bacterial physiological processes, antimicrobial compounds exert a strong selective pressure, facilitating the emergence and spread of resistant isolates [5]. New therapeutic strategies aimed at preventing pathogens from producing virulence factors, rather than killing them, represent an attracting alternative to the use of antimicrobial compounds. In particular, regulatory mechanisms controlling the expression of multiple virulence determinants constitute promising targets for antivirulence therapies [6,7].

Quorum sensing (QS) is a cell-to-cell communication process that allows bacteria to collectively modify their pattern of gene expression in response to changes in the cell density and species composition of the microbial community. Processes controlled by QS include the activation of bacterial defense mechanisms, such as the synchronized production of virulence factors (toxins, proteases, immune-evasion factors) and biofilm formation. These responses are activated in response to the extracellular concentration of small soluble autoinducer signal molecules that are produced and secreted by bacteria [8]. Autoinducer molecules comprise a diversity of molecular species such as oligopeptides, furanosyl borate diester (autoinducer-2, AI-2), acylated homoserine lactones (acyl-HSLs), the *Pseudomonas* quinolone signal molecule (PQS, 2-heptyl-3-hydroxy-4-quinolone) and integrated QS signal (IQS, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde) as well as the *Burkholderia cepacia* complex fatty acid molecule named diffusible signal factor (BDSF) [9–13]. Interestingly, bacteria usually do not rely on a single signal molecule but different QS-systems acting in parallel or in a hierarchical manner can be found within the same organism [8,14]. As autoinducers concentration increases with bacterial population density, changes in the concentration of autoinducers allow bacteria to monitor their cell numbers. Autoinducers are bound by specific receptors that reside either in the inner membrane or in the cytoplasm. Once a certain threshold of signal concentration is reached, a cascade of signaling events is triggered, leading to the modulation of the expression of hundreds of genes underlying various biological processes related to bacterial physiology, virulence, and biofilm formation [8].

QS is one of the most intensively studied targets for antivirulence therapy. As this process allows the concerted regulation of several virulence determinants without being essential for growth, targeting QS allows controlling bacterial pathogenesis while limiting selective survival pressure and emergence of antibiotic resistance [14].

Interference with QS systems therefore represents a promising strategy to address the emergence and spread of antibiotic resistance [7]. A great diversity of QS interfering agents has been described. These compounds can be either of natural or synthetic origin and can target different steps of the QS cell-to-cell communication process, by acting as inhibitors or agonists of signal molecule biosynthesis, signal molecule detection, or signal transduction.

Plant-derived compounds have been known since ancient times as having beneficial properties, including antimicrobial activity. Plant-derived secondary metabolites have been widely explored for their ability to inhibit QS. To test the inhibitory activity of natural compounds, different methods have been developed. The ability of phytochemicals to inhibit violacein production in the sensor strain *Chromobacterium violaceum* (CV12472) is a common assay used to evaluate anti-QS activity [15,16]. In *C. violaceum*, synthesis of the violet pigment violacein is regulated by QS in response to the concentrations of the autoinducers C6-AHL and C4-AHL [17]. Since this QS-regulated trait is easily observable by disc diffusion assay, *C. violaceum* is widely used as biosensor strain for screening anti-QS molecules. More specific and targeted screening methods for anti-QS activity include biofilm formation and eradication assays by crystal violet staining [18–20], quantification of QS-regulated virulence traits (e.g., pyocyanin production in *P. aeruginosa*, alpha-hemolysin secretion by *S. aureus*, protease production) as well as gene expression analysis of known QS-targets [21,22].

In this review, we will describe the QS systems of the main pathogens associated with CF lung infections and we will provide an overview of the main, so-far developed antivirulence compounds targeting these cell communication and regulatory processes, focusing our attention on papers published in the last five years.

2. *Staphylococcus aureus*

Staphylococcus aureus is a ubiquitous non-motile Gram-positive coccus, which can be found in the anterior nares and skin of humans. It is an aerobe and a facultative anaerobe bacterium, able to form biofilms, which can cause skin, soft tissue, and respiratory infections, osteomyelitis, endocarditis, and can colonize medical device implants. It can cause bacteraemia in 30–50% of healthy people with chronic nasal carriage [23]. Within two years of the introduction of methicillin in

clinical practice, *S. aureus* strains developed resistance through the acquisition of the *mecA* gene, thus being defined as Methicillin Resistant (MRSA) [24]. Treatment of Methicillin Sensitive strains (MSSA) includes the use of fusidic acid in combination with oxacillin or dicloxacillin (or rifampicin in case of penicillin allergy) given for 14 days [25]. Among the currently used drugs to treat MRSA we can find fusidic acid, trimethoprim-sulfamethoxazole, tetracyclines, linezolid, clindamycin, levofloxacin, glycopeptides, rifampin, aminoglycosides, and tigecycline [26]. Newer medications for MRSA include quinopristin/dalfopristin, daptomycin (for skin infections and bacteriaemia, but not for pneumonia), and fosfomycin in combination with tobramycin [26].

2.1. *S. aureus* Infections in cystic fibrosis

In CF patients *S. aureus* is implicated in early lung damage [27], but it is also associated with lower respiratory tract inflammation [28]. Chronic infections include high bacterial density, frequent exacerbations, and inflammation. Co-infection with *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* appear to be particular risk markers for more severe lung disease [29], while in the absence of other infections the prognosis is more favorable [30]. MRSA represent a great concern leading to an increased rate of decline in lung function and a high risk of death [31,32]. At the same time, the emergence of small colony variants (SCVs) is associated with worse lung function [33].

S. aureus is often the first pathogen isolated in CF children and the most prevalent one during childhood [34]. From adolescence to adulthood the prevalence of *S. aureus* decreases gradually, but a significant percentage of adults harbor the pathogen [34]. *S. aureus* infections prevalence seems to vary from country to country and over time: in the USA it changed from 30% in 1990 to 60% in 2016 [34]. On the contrary, the UK CF Registry shows a reducing proportion of children infected by *S. aureus*, with 16% of 0–3 year-olds and 23.7% of 4–7 year-olds [35], while in 1994 up to 60% of babies included in a randomized trial were positive [36]. Also, the type of strains is different in the USA respect to Europe: in particular, a three-fold greater annual prevalence of MSSA and an eight-fold greater annual prevalence of MRSA was reported in the USA compared to the UK [26].

Although the high prevalence of infections caused by *S. aureus* no international guidelines for the treatment in CF patients exist [37]. As an example, in UK an anti-staphylococcal prophylactic therapy in younger children is used [38], while in other countries the infection is treated only if symptoms occur or if specimens from airway are cultured positive [39].

2.2. Quorum Sensing Systems of *S. aureus*

Two main QS systems have been described so far in *S. aureus*: the Accessory gene regulator (Agr, Figure 1) and the LuxS systems [40].

2.2.1. The Agr System

The *agr* locus consists of two divergent transcriptional units, RNAII and RNAIII, which are under the control of P2 and P3 promoters, respectively [41]. The RNAII locus contains the genes *agrB*, *agrD*, *agrC* and *agrA* which are transcribed on the complementary strand [42].

The *agrD* gene codes for a peptide which is a precursor of the extracellular AutoInducing Peptide (AIP), the QS signal molecule produced by the Agr system. The AIP harbors a thiolactone ring and an exocyclic N-terminus tail [9].

AgrB is a transmembrane endopeptidase that modifies the thiolactone, cleaves the C-terminal, and exports the AIP [43].

The *agrC* gene encodes a receptor histidine kinase that autophosphorylates and, subsequently, transfers a phosphoryl group to the response regulator AgrA [44]. Then, AgrA activates the P2 promoter region for RNAII, thus leading to an autoinduction, the P3 promoter region for RNAIII [45], but also the transcription of the phenol-soluble modulin psmα and psmβ, a family of staphylococcal peptide toxins [46].

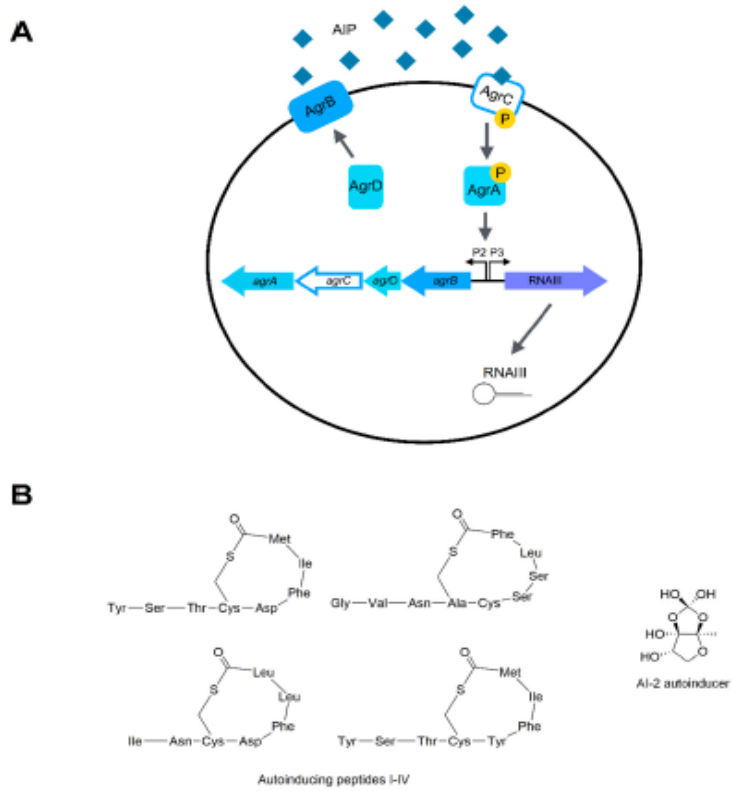

Figure 1. The Agr QS system of *S. aureus*. (**A**) The autoinducing peptide (AIP) is produced by AgrD and exported by AgrB. The two component system AgrC/AgrA is activated by AIP. AgrA binds the P2 and P3 promoter regions activating the *agr* quorum sensing feedback mechanism and RNAIII expression. (**B**) Chemical structures of autoinducing peptides I–IV and autoinducer-2 (AI-2).

RNAIII contains the gene which encodes the δ-toxin (an exoprotein that lyses eukaryotic host cells), and regulates other genes required for exotoxin secretion, degradative exoenzyme production, and biofilm disassembly [47].

There are four *S. aureus* Agr allelic variants (I to IV) that produce four AIPs which differ in a few amino acid residues (Figure 1B). AIPs function as QS activator in the *S. aureus* cells that produce them, while they generally inhibit QS in *S. aureus* strains that produce different AIPs [47]. Agr groups have been also correlated to specific biotypes: for example, most toxic shock syndrome strains belong to Agr group III, while vancomycin susceptible strains belong to group II and host exfoliation, producing strains belonging to group IV [48].

It has been shown that Agr is inactive during biofilm formation, mainly related to the increased expression of adhesins. In contrast, Agr is active in the detachment process, facilitating bacterial dissemination to other sites [49]. On the other hand, the upregulation of Agr enhances the production of virulence factors important for the progression of many staphylococcal diseases, including pneumonia [50], endocarditis [51], septic arthritis, and osteomyelitis [52,53], and skin and soft tissue infections [54].

2.2.2. The LuxS System

The LuxS system employs the AI-2 autoinducer, a furanosyl borate diester [55]. It regulates the capsule synthesis, biofilm formation (through the *icaR* locus), antibiotic susceptibility, and virulence [56,57]. However, the role of the LuxS system in *S. aureus* QS is not completely clear, as it is involved in metabolism, nor have AI-2 receptors been described so far [58].

2.3. Molecules Targeting QS in S. aureus

Molecules targeting QS in *S. aureus* can be classified into natural and synthetic products. Among natural molecules, Agr, generic QS, biofilm, δ toxin, and PT13 inhibitors have been described. Among synthetic products we can enumerate Agr, generic QS, biofilm and SarA inhibitors (Figure 2).

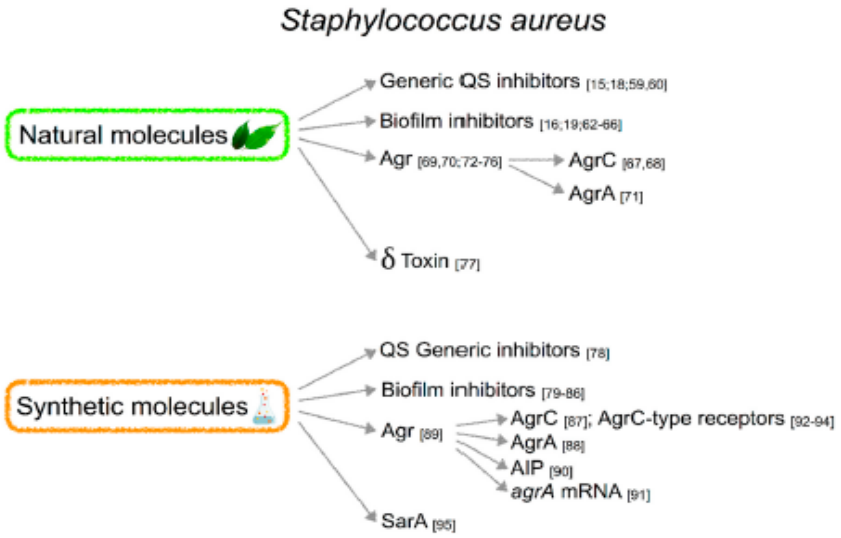

Figure 2. Targets of natural and synthetic molecules active against *S. aureus* quorum sensing.

2.3.1. Natural Molecules

The chemical structure of natural molecules inhibiting QS of *S. aureus* is reported in Figure 3A.

Generic QS Inhibitors

Since plants are considered the greatest source for obtaining new antimicrobials, Monte and collaborators explored the antimicrobial activity of four phytochemicals, finding that 7-hydroxycoumarin and indole-3-carbinol affected the motility and QS activity of *S. aureus* [15].

Among generic QS inhibitors of *S. aureus*, an Agr-like peptide from *Clostridium difficile* [59] and myricetin [60] were described. The first one was shown to affect the gene and protein expression profiles of different *S. aureus* strains and inhibited the production of Hla and LukS-PV toxins which are particularly important in *S. aureus* pathogenesis, suggesting its potential use as 'antipathogenic' therapy for *S. aureus* infections [59]. Myricetin is a flavonoid contained in fruits, vegetables, tea, berries, and red wine with proven beneficial pharmacological properties [61]. It has been shown to affect both surface and secreted proteins, decreasing the production of several *S. aureus* virulence factors, including adhesion, biofilm formation, hemolysis, and staphyloxanthin production, without interfering with growth, and thus being an alternative multi-target antivirulence candidate [60].

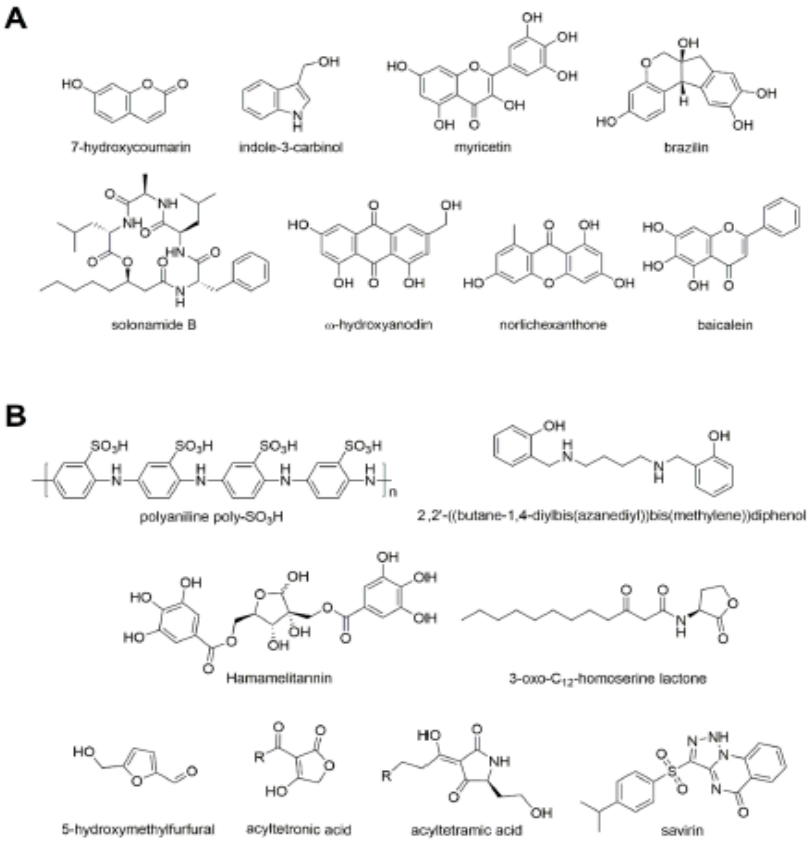

Figure 3. Chemical structures of natural (**A**) and synthetic (**B**) molecules active against *S. aureus* QS systems.

Finally, the alanine rich protein PT13 from *Populus trichocarpa* has been shown to suppress the expression of various QS dependent virulence factors in *S. aureus*, including biofilm-related genes, cell adhesion, and bacterial attachment [18].

Biofilm Inhibitors

Mare colostrum, the mare's first milk, has been shown as a promising source for isolating next-generation antibacterials [16]. It exhibited inhibitory activities against virulence factors produced by *S. aureus*, such as spreading ability, hemolysis, protease, and lipase activities. Moreover, mare colostrum showed a strong inhibitory activity against biofilm formation and eradication [16].

Brazilin is a principal active component of the herbal medicine *Caesalpinia sappan* L [62]. Different studies demonstrated its multiple biological properties, including immune system modulatory, antioxidant, anti-inflammatory, antiplatelet, antihepatotoxicity, and antitumor activities [62], as well as its antimicrobial activity [63]. In 2018, Peng and collaborators used a biofilm model of *S. aureus* to establish in vitro inhibitory effects of brazilin on biofilm formation [19]. Indeed, this molecule was able to inhibit and destroy *S. aureus* biofilm, to reduce the production of the extracellular polymeric

matrix and to inhibit the QS system, thus supporting its use as a novel drug and treatment strategy for *S. aureus* biofilm-associated infections [19].

Among natural agents able to inhibit biofilm formation in *S. aureus*, the action of essential oils has been investigated. *Eucalyptus globulus* essential oil, and its main component 1,8-cineole, has been reported to be effective against MRSA [64], while Sharifi et al. [65] investigated the effects of *Thymus daenensis* and *Satureja hortensis* essential oils on some *S. aureus* isolates showing a significant inhibitory effect on biofilm formation.

S. aureus expresses several phenol-soluble modulins which are produced in the late growth phase in an *agr* QS dependent manner. They show persister reducing activity which has been associated with lytic activity against bacterial membranes [66]. This way, it has been proposed that these toxins increase the ability of antibiotics to kill persister cells present in the biofilm [66].

Agr Inhibitors

Solonamide B is a cyclodepsipeptide isolated from the marine bacterium *Photobacterium halotolerans* that strongly reduces expression of RNAIII, interfering with the binding of *S. aureus* AIPs to sensor histidine kinase AgrC and is the first described natural compound with these characteristics [67]. In 2018, Hansen and collaborators synthesized an array of 27 analogues identifying an analogue resembling solonamide B in amino acid sequence with more potent AgrC inhibitory activity [68].

Manuka honey is another Agr system inhibitor described between 2014 and 2018 [69]. It has been reported as a broad-spectrum antimicrobial agent [70] which is used to treat topical wounds. In order to better understand its mode of action, Jenkins and collaborators performed proteomic and genomic analysis to investigate its effects on MRSA strains [69]. The *agr* gene was found among the genes with decreased expression, thus the authors concluded that a decreased expression of virulence genes will impact MRSA pathogenicity.

ω-hydroxyanodin from *Penicillium restrictum* is a polyhydroxyanthraquinone. It has been shown to prevent *agr* signaling by all four *S. aureus agr* alleles [71]. In particular, it inhibited QS by direct binding to AgrA, thus preventing its interaction with the *agr* P2 promoter. Its efficacy has been demonstrated in a mouse model of *S. aureus* infection where it decreased dermonecrosis in association with enhanced bacterial clearance and reductions in inflammatory cytokine transcription and expression at the site of infection [71].

Hamigera ingelheimensis, a metabolically prolific Eurotiales, was observed to be producing an unknown congener, designated as avellanin C [72]. Its chemical structure was determined and its ability to inhibit *S. aureus* QS was reported in 2015 by measuring the decrease in luminescence intensity from a *S. aureus* transformant that carried a plasmid encoding luciferase gene under *agr* P3 promoter upon a treatment with 0.5–200 μM of compound [72].

Also, the QS inhibitory activity of chestnut leaf extracts, which are rich in oleanene and ursene derivatives, has been assessed against all *S. aureus agr* alleles, suggesting a role for non-biocide inhibitors of virulence in future antibiotic therapies [73].

Norlichexanthone is a small non-reduced tricyclic polyketide produced by fungi and lichens. It is able to reduce expression of α-hemolysin and RNAIII thus lowering *S. aureus* toxicity towards human neutrophils and reducing its ability to form biofilms [74].

In 2016 Chen and collaborators showed that baicalein treatment reduced staphylococcal enterotoxin A and α-hemolysin levels downregulating the QS regulators *agrA*, RNAIII, and *sarA*, and gene expression of *ica* [75]. Moreover, it was able to inhibit *S. aureus* biofilm formation, to destroy biofilms, and to increase the permeability of vancomycin, supporting its use as a novel drug candidate [75].

Finally, ajoene is a sulfur-rich molecule from garlic which has been shown to reduce expression of key QS regulated virulence factors in *S. aureus* lowering RNAIII expression and, in turn, of hemolysins and proteases [76].

δ-Toxin Production Inhibitors

Recently, Khan and collaborators selected nine plants from the Sudhnoti district of Pakistan used in the ethnopharmacological tradition for the treatment of infectious and inflammatory diseases to check their activity against *S. aureus* and other bacteria [77]. Some of the extracts exhibited significant QS inhibition in a reporter strain for *S. aureus agr* I and one of them also for *agr* I–III with a significant drop in δ-toxin production.

2.3.2. Synthetic Molecules

The chemical structure of synthetic molecules inhibiting QS of *S. aureus* is reported in Figure 3B.

Generic QS Inhibitors

Three biaryl hydroxyketone compounds showed efficacy in MRSA-infected animal models and combination therapy with cephalothin or nafcillin revealed survival benefits [78]. These data suggested a possible employment of obsolete antibiotic therapies in combination with these novel quorum-quenching agents.

Biofilm Inhibitors

Sub-inhibitory concentrations of azithromycin have been shown to decrease the biofilm formation in MRSA in a dose-dependent manner [79].

In 2015, Gizdavic and collaborators showed that functionalized polyanilines significantly disrupted and killed bacterial cells present in pre-established forty-eight-hour static biofilms of *S. aureus* [80].

An acyclic diamine, (2,20-((butane-1,4-diylbis(azanediyl)bis(methylene))diphenol) showed good antimicrobial and antibiofilm activity, being capable of reducing the virulence factors expression. Moreover, confocal laser scanning microscope analysis showed biofilm reduction as well as bacterial killing, suggesting its role as lead compound for further studies in alternative therapeutic approaches [81].

Many studies have been performed also on Hamamelitannin, shown to increase *S. aureus* biofilm susceptibility towards vancomycin through the TraP receptor by affecting cell wall synthesis and extracellular DNA release [82]. Vermote and collaborators synthesized many derivatives [83,84] which resulted in the identification of an analogue that increases the susceptibility of *S. aureus* towards antibiotics in vitro, in *Caenorhabditis elegans*, and in a mouse mammary gland infection model, without showing cytotoxicity [85].

Also, 5-hydroxymethylfurfural has been shown to reduce the ability of *S. aureus* to form a biofilm up to 82% [86], while silver and ruthenium nanoparticles caused a significant reduction in biofilm formation (46%) of a clinical MRSA isolate. Indeed, RNA sequencing demonstrated down-regulation of many biofilm-associated genes and of genes related to virulence [86].

Agr Inhibitors

Among synthetic inhibitors of the *S. aureus* Agr system, two kind of small molecules were synthesized: a series of 3-oxo-C12-HSL, tetramic acid, and tetronic acid analogues [87] and Savirin [88]. The former are noncompetitive inhibitors of the AIP activated AgrC receptor and in vivo reduced nasal cell colonization and arthritis [87]. Savirin (*S. aureus* virulence inhibitor) hits the transcriptional regulator AgrA, preventing virulence gene upregulation. Also, savirin showed efficacy in murine skin infection models, abating tissue injury and promoting clearance [88].

In 2014 O'Rourke and collaborators identified a set of peptides displayed on virus-like-particles that bound with high specificity to AP4-24H11. Immunization with a subset of these particles protected against pathogenicity in a mouse model of *S. aureus*, paving the way for the development of a mimotope vaccine [89].

Serum lipoproteins are dual purpose molecules that contribute to both cholesterol homeostasis and host innate defense. The apolipoprotein B100 (apoB100) prevents *agr* activation by binding and sequestering AIP. ApoB48, the N-terminal 2152 amino acids of apoB100, has been shown to antagonize *S. aureus* QS. Since they are produced by enterocytes in the form of chylomicrons, these data suggested a previously unrecognized role for chylomicrons and enterocytes in the host innate defense against *S. aureus* QS-mediated pathogenesis [90].

Peptide-conjugated locked nucleic acids targeting *agrA* mRNA were developed to inhibit *agr* activity and arrest the pathogenicity of MRSA strains. They were shown to inhibit the expression of virulence genes that are upregulated by Agr and showed high levels of protection in a mouse skin infection model [91].

Analogues of a native AIP-III signal able to inhibit AgrC-type QS receptors and attenuate virulence phenotypes in *S. aureus* [92], as well as AIP-II peptidomimetics with a conserved hydrophobic motif [93] or linear peptide-like molecules [94] suggested that the AIP scaffold is amenable to structural mimicry for the development of synthetic QS inhibitors.

SarA Inhibitors

The quorum regulator SarA of *S. aureus* up-regulates the expression of many virulence factors, including biofilm formation. Through an in silico approach, Balamurugan and collaborators synthesized 2-[(Methylamino)methyl]phenol, which showed antibiofilm and antivirulence activity against clinical *S. aureus* strains [95].

3. *Pseudomonas aeruginosa*

P. aeruginosa is a social, ubiquitous, opportunistic Gram-negative pathogen able to cause infections in many different niches of the human body, such as respiratory and urinary tracts [96]. It is highly invasive, toxigenic and adaptable to different surfaces and tissues. Infections occur frequently in immunocompromised individuals and in particular in cystic fibrosis (CF) patients. In 2017 carbapenem-resistant *P. aeruginosa* has been listed in the highest category of "critical" pathogens with urgent need for new treatments by the World Health Organization (WHO) [97]. *P. aeruginosa* is a member of the large group of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) and it could be considered a "superbug" due to its pathogenesis and transmission. The extensive use of antibiotics increases the development of multidrug-resistant *P. aeruginosa* strains that leads to the failure of the therapies against this bacterium [98]. In this scenario, the identification of new and alternative strategies for prevention and treatment of infection is essential.

3.1. *P. aeruginosa* Infections in cystic fibrosis

Chronic *P. aeruginosa* colonizations in CF patients are recalcitrant to antibiotic treatments and they are associated with loss of lung function, morbidity and mortality [99]. The majority of the CF patients become positive for *P. aeruginosa* infections during the lifespan and it is still one of the major causes of death associated with this genetic disorder [100]. Initially, patients are infected by the nonmucoid strains of *P. aeruginosa*, but during the time mutations could occur in *mucA*, encoding an anti-sigma factor, leading to a switch to the mucoid phenotype characterized by the overproduction of polysaccharide alginate [101].

Nowadays, several treatments have been applied to handle early *P. aeruginosa* infection, such as inhaled antibiotics like colistin and tobramycin [102,103], oral ciprofloxacin [104], or an intravenous combination of an aminoglycoside with a beta-lactam [105]. Nevertheless, there is insufficient information to determine the antibiotic strategy that should be used for early *P. aeruginosa* infections eradication in CF patients [106].

3.2. Quorum Sensing Systems of P. aeruginosa

P. aeruginosa is one of the model organisms in QS study and its complex QS systems play a key role in virulence. Four QS systems were described in *P. aeruginosa*: LasI/LasR, RhlI/RhlR, Pqs and Iqs (Figure 4A). Their specific signal molecules are *N*-oxododecanoyl-L-homoserine lactone (OdDHL or $3OC_{12}$-HSL), *N*-butanoyl-L-homoserine lactone (BHL or C_4-HSL), the *Pseudomonas* quinolone signal (PQS), and the integrated quorum sensing signal (IQS), respectively (Figure 4B). These systems are deeply intertwined and LasI/LasR is at the top of the hierarchical organization. LasI/LasR and RhlI/RhlR are *N*–acylhomoserine lactone (AHL) circuits homolog of LuxI/LuxR and are activated by an increased cell density. Both these systems are represented by a Lux-type synthase and a LuxR-type receptor. The LasI synthase produces the signal molecule OdDHL which is detected by the cytoplasmic receptor LasR. In the second system, the signal molecule BHL is produced by the synthase RhlI and sensed by the receptor RhlR. The two receptors LasR and RhlR are the transcriptional regulators that control the expression of nearly 10% of the *P. aeruginosa* genome (approximately 300 genes) [107]. The third system is the *P. aeruginosa* quinolone signal (PQS) system in which the signal molecule PQS is produced by PqsABCDE and PqsH and detected by the receptor PqsR [108]. The fourth QS system, activated by phosphate and iron starvation, is still under investigation. Its signal molecule is the IQS synthesized by AmbBCDE [109].

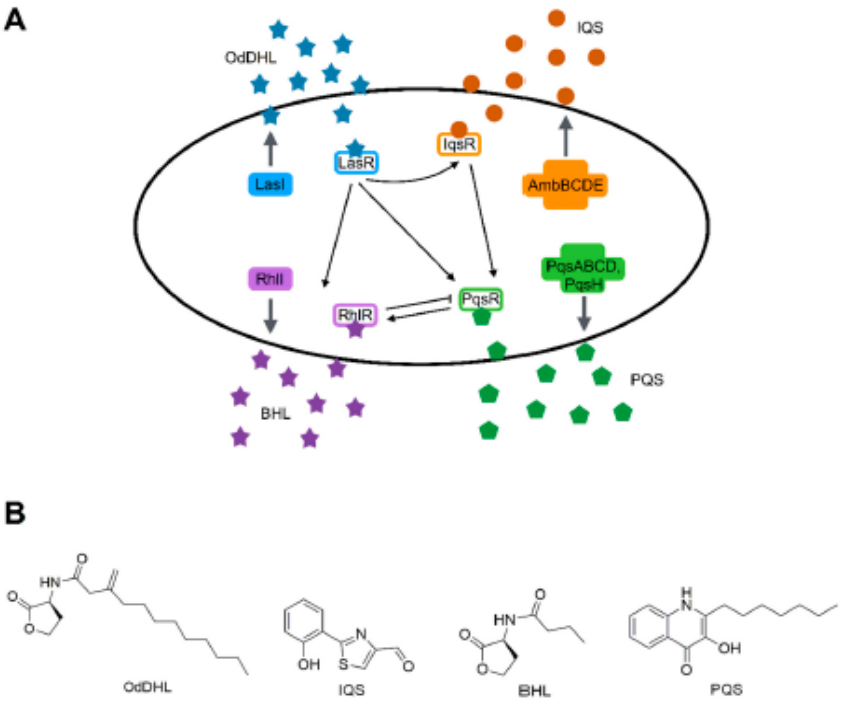

Figure 4. The QS systems of *P. aeruginosa*, Las, Iqs, Rhl and Pqs and their interactions. (**A**) OdDHL, *N*-(3-oxododecanoyl) homoserine lactone; IQS, integrating quorum sensing signal; BHL, *N*-butyryl-L-homoserine lactone; PQS, *Pseudomonas* quinolone signal. Arrows indicate positive regulation, T-bars negative regulation. (**B**) Chemical structures of QS signal molecules.

Additionally, *P. aeruginosa* produces other 50 AHQs, discovered by LC/MS of the culture supernatant, the majority of which is still uncharacterized [110].

The *las* system is at the top of the signalling hierarchy and, when activated by its molecule OdDHL, induces the transcription of *rhlI/rhlR*, *lasI* and of the other virulence genes [111]. On the other hand, when RhlR is activated by the BHL signal it induces the expression of *rhlI* and of its own regulon. At the same time, the operon *pqsABCDE* is induced by the *las* system and repressed by rhl and it is modulated by the ratio between OdDHL and BHL [112]. The PQS system induces the transcription of *rhlI* and, in turn, of the RhlI/RhlR system [111,113]. The IQS system is controlled by LasI/LasR during growth in rich medium [109]. PQS signal is produced mainly during the late phase of growth, suggesting its prominent role under stressful conditions [113,114].

Among the virulence factors modulated by QS signals there is the LasB elastase controlled by OdDHL and the BHL signals and involved in the degradation of the proteins of the matrix; the pyocyanin, necessary for immune evasion and controlled by all the three QS systems (OdDHL, BHL and PQS); the protease LasA, for the disruption of the epithelial barrier and the alkaline protease, degrading the proteins of the host defence, both controlled by OdDHL. Also, rhamnolipids, inducing necrosis of immune cells, are controlled by BHL and factors that enhance colonization, such as the LecA lectin regulated by PQS system [11]. QS systems control the production of virulence factors necessary to survive during the host invasion in the early and late stages of infection and in *P. aeruginosa* are required for complete virulence in different hosts: nematodes, fruit flies, zebrafish and mice [115]. *P. aeruginosa* strains deficient in QS systems are significantly less virulent and cytotoxic and induce lower level of tissue damage during colonization [116]. Notably, *P. aeruginosa* clinical isolates from chronic infections showed mutations in the gene *lasR* and some of these mutants still have a functional LasR while others have uncoupled the LasI/LasR system from the RhlI/RhlR [117]. Moreover, stress conditions during infection of the host such as phosphate and iron limitation induce virulence factor production through RhlR and IQS activation [109,118]. It has been demonstrated that there is a correlation between the concentration of some QS signal molecules and the level of pulmonary exacerbation [119]. Hence, some studies were focused on the correlation between the QS and the mucoid phenotype, showing that strains with this phenotype have a reduction of 3-oxo-C12-HSL, C4-HSL and AQ-dependent QS systems during the early stationary phase, while in the late stationary phase expression levels were comparable with the wild type strain [120].

All these results showed how QS systems have a key role during *P. aeruginosa* adaptation to the host and environmental changes.

3.3. Molecules Targeting QS in P. aeruginosa

Also, in this case, both natural and synthetic molecules targeting QS in *P. aeruginosa* have been described (Figure 5).

3.3.1. Natural Products

There is a huge amount of literature describing natural compounds active against *P. aeruginosa* QS (Figure 6A). Among these natural products with anti-biofilm activity there is the cranberry extract rich in proanthocyanidins (cerPAC) that acts by decreasing the concentration of virulence factors and protecting *Drosophila melanogaster* from *P. aeruginosa* PA14 fatal infection. *lasIR* and *rhlIR* genes were downregulated and molecular docking studies proposed that CerPAC binds to QS transcriptional regulators [121].

A natural plant phenolic compound, Coumarin, has been described as a QS inhibitor with a strong anti-virulence activity. Coumarin is effective against protease and pyocyanin production and blocks biofilm formation. Furthermore, transcriptome analysis highlighted that several genes involved in *las*, *rhl*, *pqs*, and also IQS systems were downregulated in *P. aeruginosa* PAO1 biofilm treated with Coumarin [21].

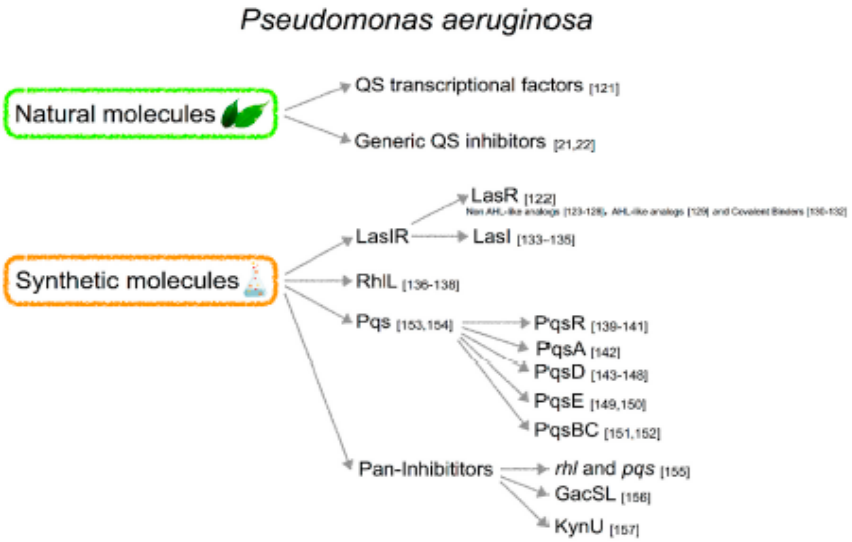

Figure 5. Targets of natural and synthetic molecules active against *P. aeruginosa* quorum sensing.

The natural compound Baicalin extracted from *Scutellaria baicalensis* inhibits *P. aeruginosa* biofilm formation at sub-MICs concentrations and enhances the activity of bactericidal compounds in vitro. Moreover, it decreases the expression levels of QS-regulatory genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsR* and *pqsA*) and the treatment of *C. elegans* reduces the pathogenesis of *P. aeruginosa* infection and increases the activation of Th1-induced immune response to induce bacterial clearance [22].

3.3.2. Synthetic Molecules

The chemical structure of synthetic molecules inhibiting QS of *P. aeruginosa* is reported in Figure 6B.

Inhibitor of LasIR QS System

The LasIR system is an attractive target to interfere with QS and a recent work demonstrates that blocking the LasR receptor prevents the binding of LasR to the target DNA [122]. LasR inhibitors can be classified into three groups: non-AHL-like antagonists, AHL-like antagonists, and covalent binders.

In order to block LasR activity, a possibility is to modify the chemical and enzymatic stability of the molecule. Among the non-AHL-like antagonists there is an indole derivative characterized by low levels of inhibition (65% at 250 μM). More recently, a compound with a glycine ethyl ester branch has been described and tested in the *P. aeruginosa* reporter strain MH602. It blocks the activation of LasR and slightly decreases the pyocyanin production [123]. The same group identified a glyxoamide-based macrocycle able to inhibit the biofilm formation in the reporter strain *P. aeruginosa* MH64 at 250 μM [124].

Among 25 nonsymmetrical azines, another study identified two compounds that inhibit the LasR receptor-based QS system in a plasB-gfpASV-based bioassay and decrease biofilm formation in *P. aeruginosa* [125].

Another hybrid compound was identified using a structure-based scaffold hopping approach combining a triphenyl derivative (already known to agonize LasR) with LasR antagonists producing a more stable molecule. This new compound shows an IC_{50} of 4.8 μM in the *E. coli lasR* reporter strain [126]. Two different patents described two molecules, an *N*-thioacyl-homoserine lactone and

a pyrrolidin-2-ol derivative: the first is active against Las, Pqs and Rhl QS system at sub-inhibitory concentration [127], while the second blocks both LasR and RhlR at high concentration (400 μM) [128].

In order to block LasR activity, a family of compounds was designed with an aliphatic tail and a pyrone headgroup; one of these showed the strongest activity at a ligand concentration of 100 μM in the biofilm assay. The interaction between this molecule and LasR was predicted using in silico modeling [129].

In order to interfere with LasR function, many studies developed LasR covalent inhibitors based on the core structure of the ligand. In particular, a previously studied isothiocyanate-based inhibitor, ITC-12, showing covalent non-competitive inhibition at low micromolar concentration, but also able to activate LasR [130], has been modified adding an electronegative halogen. The new compound successfully protects *C. elegans* from *P. aeruginosa* infection [131]. Finally, O'Brien and colleagues synthesized a group of irreversible binders of LasR among which the lead inhibitor is able to decrease *P. aeruginosa* pyocyanine and biofilm production [132].

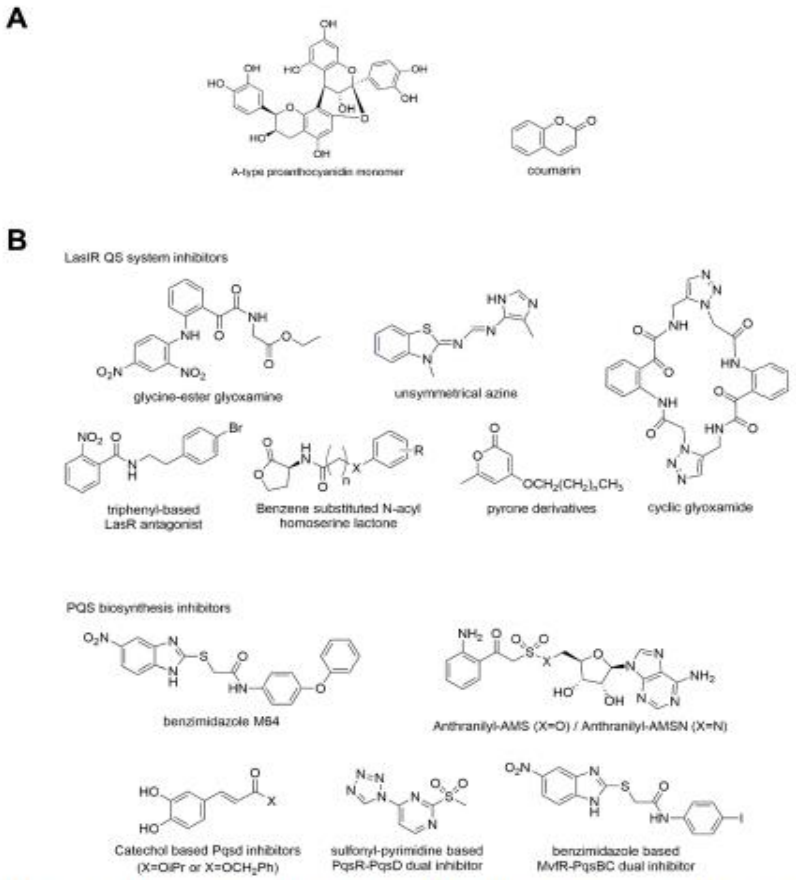

Figure 6. Chemical structures of natural (**A**) and synthetic (**B**) molecules active against *P. aeruginosa* QS systems.

LasI is another interesting drug target and several studies identified molecules blocking its activity. Unfortunately, there are few examples of LasI inhibitors and the majority described only a putative interaction predicted by docking analysis. Among a group of synthetic and natural compounds, the trans-cinnamaldehyde was identified. This is a strong inhibitor of AHL synthases, decreasing *P. aeruginosa* PAO1 pyocyanin production [133]. Using molecular docking analysis, it has been demonstrated that trans-cinnamaldehyde binds to LasI, interacting with the substrate binding site [133].

The (z)-5-octylidenethiazolidine-2, 4-dione (TZD-C8) is a potent inhibitor of biofilm, swarming motility and QS signal production, and in silico docking studies predicted the affinity of this compound for LasI pocket [134].

In another study on marine *Streptomyces* extracts, it has been shown that some fatty acid lead molecules have synergistic or individual anti-biofilm activity and the most active compounds were successfully docked against the protein LasI [135].

3.3.3. Inhibitors of rhl Quorum Sensing System

A group of synthetic molecules was tested for inhibition of the *Pseudomonas* QS receptor RhlR: among these the most effective compound is an analog of a native autoinducer, the meta-bromo-thiolactone (mBTL), that prevents virulence factor production such as pyocyanin, biofilm formation and protects *C. elegans* and human lung epithelial cells from *P. aeruginosa* infections [136]. The in vivo target of this compound is RhlR and mBTL functions as an agonist of RhlR blocking pyocyanin production down-regulating the Pqs circuit [137]. The same group characterized a family of strong agonists of RhlR that repress the Pqs signal cascade, revealing the Rhl-Pqs cross-talk as a new QS target [138].

3.3.4. Inhibition of PqsR

PqsR could be considered an important target in the development of QSIs. One of the PqsR inhibitors described is an HHQ derivative that once in the cell is converted in a strong PqsR agonist by the synthase PqsH. The molecule was further optimized by introducing a $CONH_2$ group which reduced mortality caused by *P. aeruginosa* in an animal model [139].

Another group of PqsR inhibitors was identified using a whole-cell high throughput screen and structure-activity relationship (SAR) analysis: these new molecules block pro-persistence and pro-acute PqsR-dependent signals [140]. These compounds contain the structural backbone of benzamide and a benzimidazole moiety with a thioether bond: they are highly potent with IC_{50} values of 200–350 nM for HHQ, PQS and pyocyanin [140]. The optimization of these molecules resulted in a robust inhibitor called M64 with a significant therapeutic efficacy against acute and persistent infections in mice, also in combination with antibiotic therapy [140]. Moreover, M64 interferes with biofilm formation and potentiates the antibiofilm activity of currently used antibiotics [141].

Inhibitors of PQS Biosynthesis

One of the key enzymes of the PQS biosynthesis is the anthranilyl-CoA synthase PqsA, for this reason it is an attractive target for the development of QS inhibitors. Sulfonyladeonsine-based substrate analogs were synthetized: anthranilyl-AMS and anthranilyl-AMSN that decreased HHQ and PQS levels but not pyocyanin production in *P. aeruginosa* [142].

PqsD is involved in the production of the 2-aminobenzoylacetate-CoA, the second step of HHQ biosynthesis. The first PqsD antagonists derived from FabH inhibitors (a homolog of PqsD) and their optimization produced two molecules that compete better for the substrate binding pocket [143]. In another study, urea-based PqsD inhibitors were described and improved, producing compounds with high inhibitory activity (IC_{50} of 0.14 and 0.36 μM) [143]. Unfortunately, their intracellular activity was not evaluable, probably because they were subjected to efflux [144].

PqsD shares some features (size of the active site, catalytic residues) with chalcone synthase (CHS2) of *Medicago sativa*. Starting from these affinities, Allegretta and co-workers evaluated the

inhibitory activity of some selected substrates of CHS2 on PqsD. The new inhibitors were characterized by a catechol structure, a saturated linker with at least two carbons and an ester moiety [144]. One of these compounds showed a promising inhibitory activity (IC_{50} of 7.9 μM) and was able to reduce the HHQ production. Surface Plasmon Resonance revealed that this molecule does not bind to the protein active site, but it is near the entrance of the substrate channel [145].

The 2-sulfonylpyrimidines were reported as dual inhibitors targeting the PQS receptor PqsR and the synthase PqsD. Bioisosteric replacement was used to improve their functionality and allowed to obtain a new dual inhibitor with enhanced efficacy [146]. This molecule reduced biofilm formation, pyocyanin and pyoverdine release and restored ciprofloxacin activity. Moreover, it protected the larvae of *Galleria mellonella* from *P. aeruginosa* infections [146].

Two groups of benzamidobenzoic acids were described as RNAP and PqsD inhibitors. Studying the structural modification needed to increase their selectivity against PqsD, Hinsberger and co-workers identified a new molecule which strongly blocks PqsD activity (IC_{50} of 6.2 μM), but without activity against RNAP [147].

Recently, a new strategy to screen PqsD inhibitors in a *E. coli* cellular model system has been described [148]. Through this technique, a covalent inhibitor derived from the anthranilic acid core of the native substrates was identified, which caused a global inhibition of quinolone biosynthesis in *P. aeruginosa* [148].

Another enzyme that plays a central role in the HHQ biosynthesis is the thioesterase PqsE: its functions are not completely elucidated, but it is involved in the regulation of numerous genes coding for biofilm production and virulence determinants [149]. PqsE contributes to the regulation of bacterial virulence producing an alternative ligand that activates RhlR QS receptor in the absence of the 4-HSL. During the elucidation of PqsE function, molecules that bind at the PqsE catalytic site and that inhibit its thioesterase activity were identified [149]. Further investigations are needed because these ligands failed to alter the levels of the PqsE-regulated virulence factor pyocyanin and to influence the interaction with RhlR [150].

The final step of HH biosynthesis involves the heterodimer PqsBC. Taking advantage of a benzamide-benzamidazole (BB) core structure (described as PqsR inhibitor) the first class of inhibitors targeting simultaneously MvfR and PqsBC has been identified [151]. The authors showed that PqsBC inhibition blocks acute virulence behaviours, interfering with the conversion of 2-ABA into the signal molecule HHQ [151]. The dual inhibition allows to decrease acute and chronic virulence factors. Moreover, these molecules have a more potent efficacy against antibiotic tolerance [151].

In another study, the effect of already described Pqs inhibitors was evaluated on PqsBC activity, underlining that these compounds are less effective in the reduction of HHQ levels. Indeed, these compounds affect the distribution of QS molecules and not their production [152].

There are also other compounds described as Pqs inhibitors with unspecific targets. The 4-aminoquinoline derivatives are effective QS and biofilm inhibitors in *P. aeruginosa*, characterized also by a weak bactericidal activity. Among these molecules, compounds interfering with PQS signalling, able to decrease pyocyanin production and biofilm formation were identified [153].

In order to characterize new PqsR antagonists, in silico docking analysis, together with screening with *P. aeruginosa* mCTX::PpqsA-lux chromosomal promoter fusion were performed. The resulting hits blocked alkylquinolone and pyocyanin production in both *P. aeruginosa* PAO1-L and PA14. Among these, one compound, stable in the plasma, reduced biofilm formation and increased the efficacy of tobramycin [154].

3.3.5. "PAN-INHIBITORS" of QS

Previous studies highlighted the efficacy of ajoene against QS in *P. aeruginosa*, hence a screening of in-house compound library identified a sulfuric compound (resembling ajoene) able to block QS. The optimization of this molecule was carried out using SAR and a benzothiazole derivative was the

most potent [155]. These derivatives reduced the production of virulence factors (elastase, rhamnolipids and pyocyanin) and decreased *P. aeruginosa* infection in the animal model [155].

Another group identified benzothiazole-based HK inhibitors that alter multiple virulence factors, in particular the compounds called Rilu-4 and Rilu-12 decreased significantly the production of PQS signal molecule, toxins and altered the motility of the bacteria, acting on the functionality of the two-component system GacS/GacA [156].

The products of *Petiveria alliacea*, *S*-phenyl-L-cysteine sulfoxide, antagonized QS pathways and biofilm formation and *P. aeruginosa* showed a down-regulation of many QS-dependent virulence operons and a misregulation of genes involved in metabolic pathways such as the one of PQS biosynthesis. Indeed, *S*-phenyl-L-cysteine sulfoxide is able to inhibit the KynU enzyme (kynureninase), reducing the PQS production in vivo [157].

4. *Burkholderia cepacia*

4.1. *B. cepacia* Infections in cystic fibrosis

Burkholderia cepacia complex (Bcc) is a group of 22 closely related Gram-negative bacterial species isolated from soil, water, plants, industrial settings, hospitals and from infected patients [158–161]. These bacteria have peculiar characteristics, as the ability to degrade toxic xenobiotics and the capacity of promoting the growth of crops, that make them interesting for biotechnological applications in agriculture and industry [162].

Unfortunately, Bcc bacteria are better known from the early 1980s as opportunistic human pathogens which cause persistent and severe infections in CF patient airways, as well as in chronic granulomatous disease or in immunocompromised individuals. The acquisition of these infections, which can occur both from the environment and from other patients, is very dangerous, because they are inherently highly resistant to the majority of the antibiotics used in current clinical treatments [163,164], making the eradication extremely challenging. This wide antibiotic resistance is shared by clinical and environmental *Burkholderia* strains, because of the conservation of the genes coding for the resistance mechanisms [165]. Moreover, in up to 20% of the cases the infection leads to the cepacia syndrome, a lethal necrotizing pneumonia associated with bacteremia [166]. Although all the Bcc species are able to cause an infection in CF patients, *Burkholderia cenocepacia* and *Burkholderia multivorans* are the most common isolates from these patients [4]. *B. cenocepacia* includes the epidemic strains ET-12 and Czeck strain, which spread in the 1990s within people with CF in Canada and Europe, besides the PHDC and the Midwest clone, dominant in United States [167]. These strains are particularly virulent and transmissible and is demonstrated that they possess several genes coding for virulence factors [168]. The expression of these genes is mainly regulated by QS.

4.2. *Quorum Sensing Systems of B. cepacia*

Bcc uses multiple QS systems for the cell-to-cell communication, and while some are common to each species, others are species or strain-specific (Figure 7A). QS in Gram-negative bacteria usually involves the production and sensing of *N*-acyl-homoserine lactone (AHL) molecules synthesized by a LuxI homolog and bound by a transcriptional regulator of the LuxR family (Figure 7B) [169]. This system is represented in all Bcc species by CepIR, which is composed of the synthase CepI that synthesizes *N*-octanoyl-homoserine-lactone (C8-HSL) and, to a minor extent, *N*-hexanoyl-homoserine-lactone (C6-HSL), and CepR that can act both as positive and negative transcriptional regulator [170].

Some epidemic strains of *B. cenocepacia* possess also a pathogenicity island named cenocepacia island (cci) which contains the genes coding for a second AHL-based QS system known as CciIR. Conversely to CepI, CciI produces mainly C6-HSL and a lower amount of C8-HSL, activating the cognate receptor CciR [171].

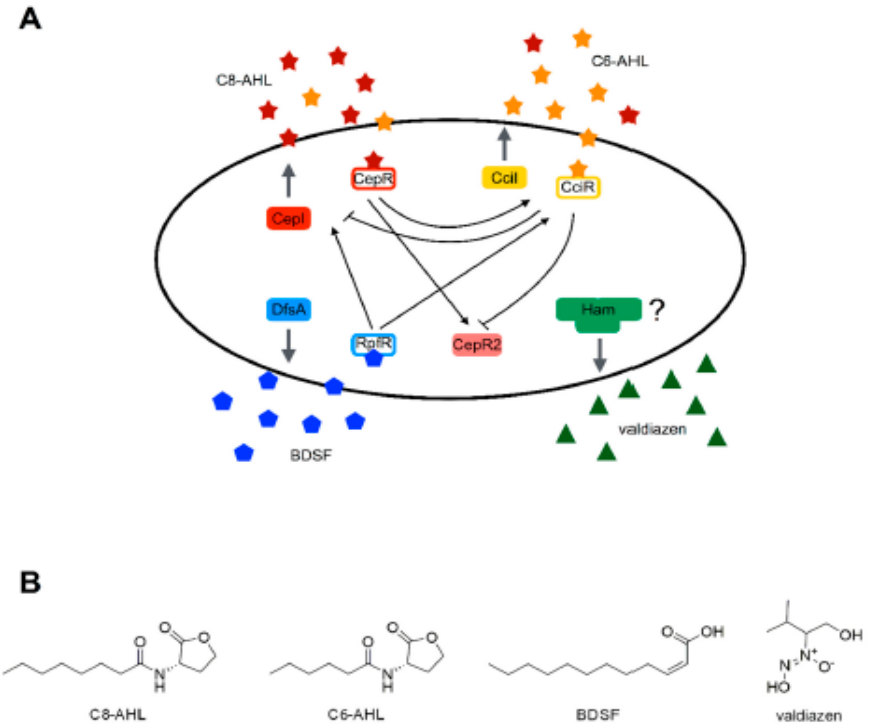

Figure 7. The QS systems of *B. cenocepacia*, Cep, Cci, Rpf and Ham and their interactions. (**A**) The interactions of the Ham system are not completely elucidated. C8-HSL, *N*-octanoyl-homoserine-lactone; C6-HSL, *N*-hexanoyl-homoserine-lactone; BDSF, Burkholderia diffusible signal factor and valdiazen. Arrows indicate positive regulation, T-bars negative regulation. (**B**) Chemical structures of QS signal molecules.

B. cenocepacia has also an orphan LuxR homolog, CepR2, not coupled with any synthase in the genome, indeed its activation is independent from AHLs, even though recently, it has been discovered that its activity is antagonized by C8-HSL [172,173].

Among the Bcc, another AHL-mediated QS system has been characterized only in other two species, *Burkholderia vietnamiensis*, which has the BviIR system that synthesizes C10-HSL [174], and *Burkholderia ambifaria*, which has the CepI2R2 system that mainly produces the hydroxylated AHLs, 3OHC10-HSL and 3OHC12-HSL [175].

The other leading signal molecule used by all Bcc species for cellular communication, is the *Burkholderia* diffusible signal factor (BDSF), a fatty acid molecule similar to the diffusible signal factor originally described in *Xanthomonas* [12,176]. In *B. cenocepacia* BDSF or cis-2-dodecenoic acid is synthesized by the bifunctional crotonase DfsA or $RpfF_{Bc}$, the counterpart of RpfF of *Xanthomonas*, [177] and sensed by the soluble receptor RpfR [13,178]. This protein, activated in the presence of BDSF, degrades the c-di-GMP through its phosphodiesterase activity and allows the activation of the global transcriptional regulator GtrR which works in complex with RpfR itself [179].

Furthermore, another sensor protein has been identified, BCAM0227, which controls only a subgroup of genes regulated by BDSF (Figure 7B) [180]. *B. cenocepacia* is able to produce also a

2-heptyl-4(1H)-quinolone (HHQ) molecule, a known signaling factor in *Burkholderia pseudomallei*, but not yet characterized in this species [181]. Besides a modified HHQ, the 4-hydroxy-3-methyl-2-heptenylquinoline (HMAQ-C7:2′) is produced by the other Bcc species *B. ambifaria* [182].

Finally, the last signal molecule discovered in the Bcc has been a diazeniumdiolate compound named valdiazen, which controls the expression of more than 100 genes in *B. cenocepacia* H111 [183]. This molecule is probably the first member of a new wider class of signal compounds in *Burkholderia*. These QS systems communicate with each other through a very complex and regulated network of interactions that is just partially characterized so far, considering that new regulators are discovered periodically and some interactions are not fully understood as yet.

The AHL based QS systems directly interact, and indeed CepR positively regulates the expression of *cepI* by a positive feedback regulation, but also the *cciIR* operon and, at least in *B. cenocepacia* H111, the gene *cepR2*. However, it is also a repressor of its own expression. The *B. cenocepacia* QS regulator, CciR, is instead a transcriptional repressor, negatively regulating the expression of *cepI* by a negative feedback regulation, *cepR2* and its own expression [184]. The orphan regulator CepR2 does not need the presence of AHL to be active and to repress its own expression, as it inhibits the activator CepS, an AraC-type transcription factor, and blocks the transcription in an unusual way [173]. Unlike the other two regulators, CepR2 does not interfere with CepIR and CciIR systems [172].

Until a few years ago, there was no evidence about cross interactions between the two main QS systems of *Burkholderia*, CepIR and BDSF, but today it is assessed that they are part of a bigger regulon. Indeed, it has been demonstrated that BDSF controls the AHL production, activating the RpfR-GtrR complex and directly promoting the expression of the AHL synthase gene cepI [185,186]. Moreover, in the ET-12 strain of *B. cenocepacia* the synthase gene *cciI* is regulated in the same manner [185].

Several other regulators are known to take part in the QS regulation, such as CepS, ShvR, YciR, SuhB, YciL, BCAM1871, AtsR, BCAM1869, BCAM0258, BceR, RqpR, contributing to the complexity of the network [173,187–193].

QS systems are known to control the expression of next to hundred genes in *Burkholderia*, and several of these are regulated together by more than one system [184,194,195]. In human infections, it has been proved that the bacterium needs active QS systems to infect the lungs of CF patients successfully, because they control also the expression of the genes coding for virulence factors [196]. The virulence factors directly associated with QS are the extracellular zinc metalloprotease ZmpA and ZmpB [12,194], the siderophores ornibactin and pyochelin [184,197], the flagellar motility [180,184], the type III and type VI secretion systems [180,184], the biofilm formation [13,198], the LysR regulator ShvR [199], the protein BCAM1871 not characterized as yet [187], and the nematocidal protein AidA [172,184]. Therefore, in order to decrease the virulence of these infections and to overcome antibiotic resistance, molecules able to hit the QS regulation have been screened and characterized.

4.3. Molecules Targeting QS in B. cenocepacia

The combination of quorum sensing inhibitors (QSI) with antibiotics is a useful strategy to control the infections caused by Bcc bacteria. Although promising results have been obtained in the last years, only few active molecules in Bcc have been studied, principally because of the low number of studies performed on these bacteria. The molecules characterized as active QSI are both natural compounds and synthetic molecules [20,200–202] which are able to interfere with the biofilm formation and in turn to increase the efficacy of the antibiotic treatment (Figure 8A). Moreover, it has been proved that some of these QSI can decrease the virulence in infection models [200,202]. Within them, there are both AHL analogues, that act as agonist or antagonist of the natural AHL [201], and molecules with completely different structures and so different mechanisms of action [202,203]. Recently, a new set of synthetic diketopiperazines has been tested in our laboratory for their ability to inhibit QS, and among them we discovered two molecules with a very interesting activity against CepI of *B. cenocepacia* J2315 [202]. Besides these, the only other molecule further studied in the last years has been the

baicalin hydrate [204], a flavonoid initially characterized as QSI for *Pseudomonas aeruginosa* in 2008 by Zeng et al. [205].

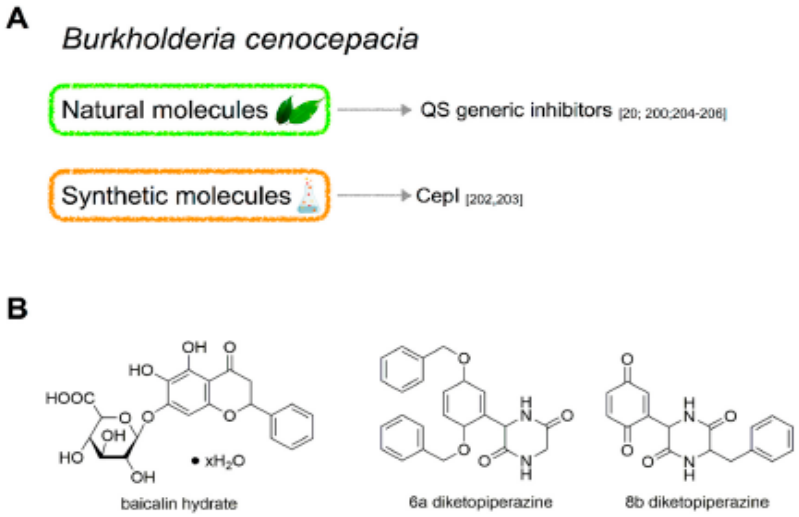

Figure 8. (**A**) Targets of natural and synthetic molecules active against *B. cenocepacia* quorum sensing. (**B**) Chemical structures of natural and synthetic molecules active against *B. cenocepacia* QS systems.

4.3.1. Natural Molecules

Baicalin hydrate (BH, Figure 8B) is a polyphenolic molecule belonging to the class of the flavonoids, isolated from the roots of *Scutellaria baicalensis*, and firstly characterized as QSI in a work consisting in the screening of compounds with antibacterial activity of the Traditional Chinese Medicine [205]. Polyphenols are already known to be biofilm and swarming motility inhibitors in *B. cenocepacia* H111 [206]. BH has been tested as QSI on some microorganisms including Bcc bacteria, obtaining very promising results. These studies uncovered its ability to impair the biofilm formation in *B. cenocepacia* and *B. multivorans* [20], and the ability to increase the effect of tobramycin against sessile cells both in vitro and in vivo [200]. The combination of BH and tobramycin results in a significantly reduced mortality after *Burkholderia* infections in *C. elegans* and *G. mellonella* models and in a strongly reduced pulmonary bacterial load in infected mice, compared to the antibiotic treatment alone [200]. Although the potential of this molecule is fully recognized, the mechanism of action of BH remains unclear. BH enhances the bactericidal activity of tobramycin and other aminoglycosides, even though the effect is strain-specific, increasing the formation of reactive oxygen species, by acting on several pathways such as cellular respiration, glucarate metabolism and biosynthesis of putrescine [204]. On the contrary, the QSI action of BH in this case may not be the main mechanism that leads to this synergistic effect as hypothesized by Slachmuylders et al. [204], leaving a question open for further studies.

4.3.2. Synthetic Molecules

2,5-Diketopiperazines (DKPs, Figure 8B) are a class of cyclic dipeptides, isolated mainly from Gram-negative bacteria, but also from Gram-positive bacteria, Archaea and Fungi, which were characterized as agonists of LuxR-type proteins and accordingly a new class of QS molecules and interspecies signals [207,208]. However, this theory is still debated, because of a study which proved that DKPs did not interact directly with TraR, LasR and LuxR [209]. However, being DKPs an

established QSI, in order to find CepI inhibitors of *B. cenocepacia* J2315, we have tested ten newly synthesized molecules, designed adding a redox moiety to the DKPs scaffold, against the enzymatic activity of the recombinant purified synthase CepI. We have found two active compounds, named 6a and 8b, classified as non-competitive inhibitor of the synthase. Even though the two molecules did not show any synergistic activity in combination with the currently most used antibiotics against planktonic cells, they were able to significantly reduce the production of siderophores and proteases, and besides to interfere with the biofilm formation [202]. These results have been confirmed also in a *C. elegans* infection model, showing that the two DKPs could protect the nematodes from the infection of *B. cenocepacia* J2315. Moreover, it has been verified that 6a and 8b have a very low toxicity on HeLa cells, making the compounds good candidates for future experimentations in humans [202]. A further study has been performed on the molecule 8b, showing that the effect of the compound on *B. cenocepacia* J2315 is comparable to the deletion of *cepI*, and also that its binding pocket is likely localized close to the predicted *S*-adenosylmethionine binding site of CepI [203], adding information that may be considered in the future to improve its biological activity by chemical modifications.

5. Emerging CF Pathogens

5.1. Stenotrophomonas maltophilia

5.1.1. *S. maltophilia* Infections in cystic fibrosis

Stenotrophomonas maltophilia is a Gram-negative rod which represents an important emerging nosocomial pathogen, responsible for infectious diseases and death, particularly in immunosuppressed or immunocompromised patients or in subjects carrying medical implants [210]. Among the emerging CF pathogens, *S. maltophilia* infection is considerably variable from center to center, ranging from 3–30%, with an increasing in prevalence in this population [211]. In CF patients, infection with *S. maltophilia* has been associated to poor outcomes, decreased lung function, and increased risk of transplantation or death [212].

Nevertheless, the effect of *S. maltophilia* on lung function decline is not clear, and there is no consensus about the management of patients with this infection. For instance, in some cases it is considered a colonizer, and thus no specific treatments are carried out, while in other cases it is treated with specific antibiotics, but with no consensus on the optimal regimen [213].

Anyway, the treatment is very difficult, as *S. maltophilia* is intrinsically resistant to several antimicrobials, and able to acquire new resistances by horizontal gene transfer [214]. Indeed, *S. maltophilia* is usually present in environmental water reservoirs, a highly competitive niche that favors not only the acquisition of resistance genes, but also the establishment of communication networks with the neighboring microorganisms [215].

5.1.2. Quorum Sensing Systems of *S. maltophilia*

The principal QS system of *S. maltophilia* relies on the Diffusible Signal Factor (DSF) cis-11-methyl-2-dodecenoic acid, which regulates bacterial motility, biofilm formation, antibiotic resistance, and virulence [216,217]. Differing from other DSF producing bacteria, such as *B. cenocepacia* and *P. aeruginosa*, in *S. maltophilia*, the genes encoding the QS proteins co-localize in the regulation of pathogenicity factors (Rpf) cluster, and are organized in two adjacent operons convergently transcribed [217]. The first operon encodes the fatty acid ligase RpfB and the synthase RpfF, whereas the second operon contains the genes coding for the sensor kinase RpfC and the cytoplasmic regulator RpfG [216]. The peculiarity of the *S. maltophilia* DSF system is the presence of two variants of the rpf cluster, rpf-1 and rpf-2, which are associated with two RpfC-1 and RpfC-2 variants. Interestingly, the association of these variants is fixed, whereby the strains harboring RpfF-1 necessarily carry the RpfC-1 variants and *vice versa*. The RpfF variants differ in first 108 amino acids, while the RpfC variants display a different number of trans-membrane regions at the N-terminal portion, 10 for RpfC-1 and 5 for RpfC2. To date, the *rpf-1*

variant has been detected in the 55.5% of the isolates, while the *rpf-2* in the 44.5% [216]. These two variant strains show differences in DSF synthesis, perception, and in regulation of biological processes. Differently from the *rpf-1* strains, which under standard growth conditions produce DSF, the *rpf-2* needs extra copies of the *rpfF-2* gene or the absence of RpfC-2 to produce DSF [216]. Nonetheless, RpfF-1 and RpfF-2 enzymes have both acyl-ACP dehydratase and thioesterase activity, and catalyze the conversion of (R)-3-hydroxy-11-methyl-dodecanoyl-ACP into DSF [217]. This thioesterase activity is not specific, and the enzymes are able to cleave different medium and long chain acyl-ACP, producing free fatty acids that are released in extracellular environment [217]. Among these fatty acids, the mostly produced in *S. maltophilia* is the 13-methyltetradecanoic acid (iso-15:0). This molecule is synthetized by the biosynthetic pathway of the DSF, suggesting a connection between DSF and membrane synthesis. Indeed, iso-15:0 modulates the DSF in *rpf-1* strains, being sensed by RpfC-1, which thus releases RpfF-1 that can start DSF synthesis [217]. By contrast, in *rpf-2* strains the 5-transmembrane sensor RpfC-2 does not have promiscuous perception, which leads to the repression of RpfF-2 also in the presence of iso-15:0 or other fatty acids. Indeed, the RpfF-2 RpfC-2 complex can dissociate only upon sensing the DSF itself. Thus, in *rpf-2* strains the DSF production is triggered by the presence of exogenous DSF [217].

Interspecies communication through DSF signal molecules is a quite common phenomenon also for *S. maltophilia*. For instance, DSF produced by *S. maltophilia* was found to influence *P. aeruginosa*, particularly regarding biofilm formation, antibiotic resistance, virulence and persistence in lungs of CF patients [218]. Moreover, although *S. maltophilia* has been shown not to produce AHLs, it has been found that it responds to AHL signal molecules produced by *P. aeruginosa* [219]. Indeed, in the *S. maltophilia* genome there are 15 putative LuxR that lack the cognate LuxI, and are widely spread throughout bacteria [220]. Among them, the SmoR, containing the typical N-terminal AHL-binding domain and the C-terminal helix-turn-helix DNA-binding domain, was demonstrated to bind in vitro oxo-C8- homoserine lactone. Moreover, *S. maltophilia* swarming motility was found to be strongly stimulated in the presence of a *P. aeruginosa* supernatant containing high levels of AHLs, indicating that SmoR senses the AHL signals of neighboring bacteria [219]. By contrast, the strain of *S. maltophilia* BJ01 was found to produce a compound (cis-9-octadecenoic acid) which possesses quorum quenching activity and which is able to inhibit biofilm formation of *P. aeruginosa* [221].

5.1.3. Molecules Targeting QS in *S. maltophilia*

Despite the increasing incidence of multi-resistant *S. maltophilia* clinical isolates, and the potential of quenching DSF communication as a promising therapeutic approach, no active compounds, nor QS molecules degrading enzymes, have been reported until now.

5.2. *Haemophilus Influenzae*

5.2.1. *H. influenzae* Infections in cystic fibrosis

Nontypeable *H. influenzae* (NTHi) is a common commensal of the upper airways, which can cause different infections, such as otitis media, bronchitis, sinusitis, and pneumonia. Moreover, chronic infection can occur in patients with diseases of lower respiratory tracts, including chronic obstructive pulmonary disease, bronchiectasis and CF [222]. In particular, NTHi is the most common colonizer of the airways in infants with CF [4]. NTHi is indeed recovered from about 20% of children under the age of 2 years and has a peak of prevalence at 30% in children 2 to 5 years old, decreasing to less than 10% in adults [34]. Furthermore, about 80% of children with *P. aeruginosa* had previous infections with *S. aureus* or NTHi. NTHi has been found involved in chronic infections and exacerbations of CF lung disease, and associated with lower lung function impairment in infants [223].

NTHi is able to produce biofilm, also in the lower airway of CF patients [224]. Similarly, to several pathogens, within biofilm it is intrinsically resistant to antibiotics, and has been demonstrated to be

able to persist during infection in multicellular biofilm communities [225]. Moreover, it has been found that low concentrations of different antibiotics stimulate biofilm formation [226].

5.2.2. Quorum Sensing Systems of *H. influenzae*

Biofilm regulation in NTHi is mainly mediated by the autoinducer-2 (AI-2) QS signal that relies on the LuxS/RbsB system [227] and is widely distributed among different bacteria [169]. Interestingly, AI-2 signaling can have different roles among different species, promoting the formation and maturation of the biofilm, or the biofilm dispersal and cells release [228].

In NTHi, AI-2 promotes biofilm formation and prevents its dispersal during the maturation process. Indeed, it has been shown that if the expression of *luxS* is interrupted, thus decreasing the production of AI-2, biofilm dispersal occurs, suggesting a role of AI-2 also in the regulation of the lipooligosaccharides that are known to mediate adherence [229]. In this context, the recent discovery that the levels of AI-2 regulate the expression of the glycosyltransferase GstA [229] paves the way to new investigations to better define how the bacteria form and persist into biofilms, and to find novel possible antivirulence targets. For instance, the AI-2 receptor RbsB has been suggested as a promising target, since mutations in its gene are associated with alterations of biofilm formation and maturation in an in vivo model of otitis media [227,229]. In addition to the LuxS/RbsB system, biofilm in NTHi is regulated by the two-component histidine kinase QseB/QseC system [230]. To date, little is still known about this system in NTHi that, differently from the well characterized QseBC of *E. coli* and *Salmonella enterica*, does not respond to epinephrine or norepinephrine, but is activated only by ferrous iron or zinc and has been designated as a ferrous-iron-responsive system (FirS) [231].

5.2.3. Molecules Targeting QS in *H. influenzae*

A novel approach against NTHi, based on vaccine targeting bacterial adhesive proteins and biofilm mediators, was recently demonstrated to prevent otitis media on a polymicrobial infection model [232]. In particular, it was demonstrated that antisera against the NTHi type IV pili PilA were able to in vitro disrupt and prevent the dual species biofilm formed by NTHi and *Moraxella catarrhalis*. Moreover, the bacteria released from biofilm were significantly more sensitive to different antibiotics [233]. Since NTHi and *M. catarrhalis* have been found together also in the lungs of children with CF [234], this strategy of immunization in combination with antibiotics can provide a novel approach for the treatment of these biofilm-associated infections [233] (Figure 9A).

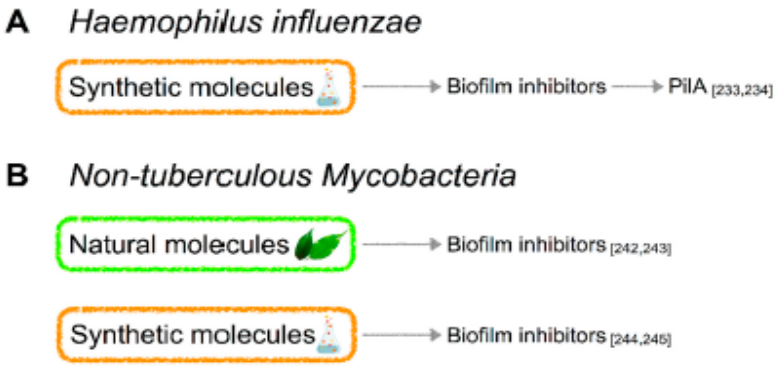

Figure 9. Targets of natural and synthetic molecules active against *Haemophilus influenzae* (**A**) and Non-tuberculous Mycobacteria (**B**) quorum sensing.

5.3. Non-Tuberculous Mycobacteria (NTM)

5.3.1. NTM Infections in cystic fibrosis

Non-tuberculous mycobacteria (NTM) are microorganisms usually isolated from environmental sources, such as soil and water. NTM can be divided in rapid growers (i.e., *Mycobacterium abscessus* complex, *Mycobacterium fortuitum*), that take less than 7 days to grow, or slow growers (i.e., *Mycobacterium avium* complex, *Mycobacterium kansasii*). Among NTM, some species are associated with human disease, particularly the *M. avium* complex (MAB: including *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium chimaera*) and the *Mycobacterium abscessus* complex (MABSC: that comprises *Mycobacterium abscessus*, *Mycobacterium massiliense*, and *Mycobacterium bolletii*). NTM are considered opportunistic pathogens organisms, particularly prevalent in patients with CF, non-CF bronchiectasis, and in chronic obstructive pulmonary disease [235]. The incidence and prevalence of NTM in CF patients is increasing and highly variable, with a prevalence ranging between 2% and 28% [236]. NTM infections in CF patients, particularly of *M. abscessus*, are associated with an increased morbidity and mortality, and a rapid lung function decline [237]. Moreover, the treatment is particularly challenging due to an intrinsic resistance to several antibiotics, including those commonly used in CF infections [238].

5.3.2. Quorum Sensing Systems of NTM

Most mycobacteria, including NTM such as *M. abscessus* and *M. avium*, are known to form biofilm, suggesting the possibility that these organisms may have QS systems, but experimental validation is still lacking [239]. Nevertheless, bioinformatics analysis demonstrated the presence of homologs of LuxR in *Mycobacterium tuberculosis*, which have been found also in different other mycobacteria [240]. Moreover, the existence of QS in mycobacteria is also indirectly suggested by the fact that their signal transduction phospho-relay cascade uses different di-cyclic or modified nucleotides as second messenger, including c-di-GMP. Indeed, c-di-GMP is ubiquitous in several bacteria, and known to be involved in virulence and biofilm formation [241].

5.3.3. Molecules Targeting QS in NTM

Due to the increasing incidence of NTM infections, together with their intrinsic resistance to several antibiotics, particularly when in biofilms, the research of new compounds with biofilm dispersal activity is fundamental (Figure 9B).

Among the natural products, essential oils, which are secondary plant metabolites, have been suggested as potential antimicrobial and antivirulence compounds [242]. For instance, the essential oil of *Cymbopogon flexuosus* (a plant generally known as lemongrass) was recently demonstrated to have antimicrobial activity against rapidly growing NTM, being also able to efficiently disperse biofilm, as well as to inhibit its formation [243].

On the other hand, Flores and co-workers evaluated the antibiofilm activity of different antibiotics used in the treatment of mycobacterial infections (amikacin, ciprofloxacin, clarithromycin, doxycycline, imipenem and sulfamethoxazole), but none of them was able to significantly prevent biofilm formation or to promote biofilm dispersal [244]. Nevertheless, it was recently found that sulfamethoxazole when in complex with metal ions, particularly in complex with Au, showed a markedly enhanced antibiofilm activity [245]. Moreover, showing these compounds a good safety profile and antibacterial activity, they have been suggested as potential therapeutic agents [245].

Thus, although NTM QS is still very poorly known, these effective antibiofilm compounds demonstrate the potential of this target to develop novel therapeutic molecules.

6. Conclusions

In this review we enumerated the principal CF pathogens which cause lung infections: *S. aureus*, *P. aeruginosa*, *B. cepacia* complex bacteria, and the emerging pathogens *S. maltophilia*, *H. influenzae* and

non-tuberculous Mycobacteria. As their common peculiarity is the high degree of drug resistance, in the last years a lot of work has been done to find an alternative therapeutic solution: the research on QS inhibitors, able to attenuate the virulence of these strains, has merged as a promising strategy. In this way, both synthetic and natural products were evaluated for their ability to interfere with various components of the QS machinery, although sometimes their target has not been found yet.

In particular, for *S. aureus* we described generic QS inhibitors, both of natural and synthetic origin (Figures 2 and 3). Moreover, among natural molecules, some are able to inhibit biofilm formation, the Agr system at different levels, and the δ toxin; while synthetic compounds hit biofilm formation, the Agr system as well as the QS regulator SarA (Figure 2).

Among *P. aeruginosa* QS inhibitors, we described natural generic and transcriptional factors inhibitors (Figures 5 and 6A). The synthetic molecules reported target all the QS systems of this bacterium: LasIR, RhlIL, and Pqs. Also, Pan-inhibitors have been described (Figures 5 and 6B).

As regarding *B. cepacia* complex, natural generic QS inhibitors have been characterized, while among the synthetic molecules, only CepI inhibitors have been described so far (Figure 8).

Conversely, despite the incidence of multi-resistant *S. maltophilia* clinical isolates, and the potential of quenching DSF communication, no active compounds have been reported until now. The unique report about *H. influenzae* biofilm disruption regards synthetic antisera against the PilA, which render the cells released from biofilm significantly more sensitive to different antibiotics (Figure 9A). Finally, natural essential oils and synthetic sulfamethoxazole in complex with metal ions showed good activity against Non-tuberculous Mycobacteria biofilm (Figure 9B).

The huge amount of literature published in the last five years clearly demonstrates that QS is a good candidate to find alternative therapeutic approaches to face the increasing problem of antibiotic resistance. The compounds described have a great potential as antivirulence molecules, but can also be proposed for combined therapies to improve the activity of existing drugs, paving the way for alternative treatments.

On the other hand, potential challenges for the use of these compounds have been described. These include the possible incurrence of drug resistance [246–248], and the limitations of the current animal infection models for QS [246]. Moreover, it seems that, in order to develop truly effective QSI, a better understanding of the virulence and of the behavior of pathogens during infections is needed [249]. Finally, despite QSI having been published in the literature since the 1990's, none of them is in clinical trial yet, leading to the conclusion that further efforts are needed in order to exploit these new approaches for the treatment of bacterial infections; yet a lot of alternatives are being explored.

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Abbreviations

AHL	Acyl Homoserine Lactone
CF	Cystic Fibrosis
DSF	Diffusible Signal Factor
NTM	Non-tuberculous Mycobacteria
QS	Quorum sensing
QSI	Quorum sensing inhibitor

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Research paper

The cell division protein FtsZ as a cellular target to hit cystic fibrosis pathogens

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ABSTRACT

Cystic fibrosis is a rare genetic disease characterized by the production of dehydrated mucus in the lung able to trap bacteria and rendering their proliferation particularly dangerous, thus leading to chronic infections. Among these bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa* play a major role while, within emerging pathogens, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex species, as well as non-tuberculous mycobacteria are listed. Since a common feature of these bacteria is the high level of drug resistance, cell division, and in particular FtsZ, has been explored as a novel therapeutic target for the design of new molecules with antibacterial properties. This review summarizes and provides insight into recent advances in the discovery of compounds targeting FtsZ: the majority of them exhibit anti-staphylococcal activity, while a few were directed against the cystic fibrosis Gram negative pathogens.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by more than 2,000 mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) encoding gene [1]. These are responsible for the malfunctioning of the ion channel, leading to the depletion of the airway surface liquid [2], as well as to its depth reduction due to liquid desiccation in the periciliary layer [3]. The resulting mucus stasis and dehydration strongly favour the lack of pathogens clearance, especially in the lung. In addition, bacteria trapped in the mucus are particularly difficult to eradicate, resulting in chronic respiratory infections which account for most CF deaths [4].

Among the bacteria which colonize the lung of CF patients, *Staphylococcus aureus* represents the most frequently isolated bacterium [5]. Of note, *S. aureus* is often the first pathogen to colonize CF children and the most prevalent during childhood. From adolescence to adulthood it decreases gradually, but a significant percentage of adults harbours this pathogen. In CF patients, *S. aureus* is implicated in early lung damage but it is also associated with lower respiratory tract inflammation [5]. Chronic infections include high bacterial density, frequent exacerbations, and inflammation.

Another bacterium frequently isolated from CF patients' sputum is *Pseudomonas aeruginosa*. It is particularly dangerous since it can adapt to the lung environment through genetic and phenotypic modifications [6], including the acquisition of antibiotic resistance, suppression of motility and the transition from a non-mucoid to a mucoid phenotype [7].

On the other hand, an increasing number of emerging and challenging pathogens, which include the Gram-negatives *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *Burkholderia cepacia* complex species plus the Gram positives non-tuberculous mycobacteria, has been reported within the last years [8].

In order to fight all these microorganisms, antibiotic therapy must be optimized depending on the species and on the susceptibility: for instance, in the presence of *S. aureus* infections, patients usually are administered with monotherapy (β-lactams), while aminoglycosides are used to treat *P. aeruginosa*. More frequently, a combination of drugs must be used, including polymyxins, β-lactams and fluoroquinolones [9].

Indeed, a common feature of these pathogens is the high level of resistance to antibiotics. The selection of bacteria resistant to the

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most commonly used antibacterial drugs is a forthcoming danger to human health. Until now, the antibiotic targets are enumerated among components of the cell wall biosynthesis or of the nucleic acid and protein synthesis. In this scenario, the identification of new compounds targeting alternative pathways essential for microorganism survival is fundamental.

In this mini-review, we describe the cell division machinery, and in particular its key protein FtsZ, as a novel therapeutic target against which many molecules have been synthesized and tested: many of them showed anti-staphylococcal activity, while a few were directed against the CF Gram negative pathogens.

2. Cell division and FtsZ as a target to fight cystic fibrosis pathogens

One of the most studied bacterial pathways is cell division machinery that is considered a potential reservoir of new cellular targets. The first step of cell division is the construction of the FtsZ polymers scaffold bound to the membrane by the "early" proteins: FtsA, ZipA and the Zaps proteins (ZapA, ZapB, ZapC and ZapD). This structure determines the site of the future cell division and its assembly is necessary for the recruitment of the "late" proteins, involved in the real synthesis of the two new cell poles. Many of the cell division machinery components are conserved in bacteria and they are called "Fts" (filamentation temperature sensitive) because of the conditional filamentation phenotype of mutants lacking them, while proteins involved in the regulation of this process are less conserved. Cell division is orchestrated together with genome duplication and chromosome segregation [10].

Many different antibacterial compounds have been studied to hit both cell division and genome maintenance machineries. Most of the work has been focused on design anti-FtsZ molecules, even if also the other proteins of the cell division machinery are considered interesting putative drug targets.

Among the potential cell division inhibitors, a peculiar class not further discussed in this review, but that is worth to mention, are the antimicrobial peptides. These compounds are widely considered good candidates for the development of new therapies, being active against both Gram negative and Gram positive bacteria and rarely inducing resistance [11,12]. It has been demonstrated that some of them could target the divisome [13–16] even if, in most cases, only as a supportive mechanism of action, exerting their antimicrobial activity mainly through the impairment of the membrane structure.

2.1. Cell division proteins as potential drug-target

The interaction among FtsZ, FtsA and ZipA is essential for the cell division process, for this reason small compounds and mimetic peptides that block these interactions could be interesting [17]. The second step of cell division involves different complexes of proteins, including FtsEX, structurally similar to an ABC transporter. The ATP-binding function of FtsE is a key step for the machinery assembly. New antibiotics could be focused on the inhibition of this ATPase activity or of the formation of this protein complex. Another "late" protein is FtsW, an integral membrane protein which interacts with PBP3 for the synthesis of septal peptidoglycan [18]. For its key role, FtsW could be considered a suitable target for antibiotics, considering that the *in vitro* activity assay is available [19]. Moreover, the synthesis of lipid II competitors could be of interest to block the FtsW function. Another protein complex is the one formed by FtsQ, FtsL and FtsB, a group of membrane proteins with the largest domain in the periplasm, which form the structural core of late division proteins. Many data coming from *in vitro* reconstitution [20], small-angle neutron and X-ray scattering [21], modelling, cross-linking [22] and crystallography [23] helped to obtain data on the surface interaction of these proteins. Novel competitive inhibitors could be identified using this information. At the same time, it has been hypothesized that FtsL stability is a control point in the divisome formation and for this reason it could represent a new opportunity to design inhibitors.

2.2. FtsZ as drug target

Most of the cell division inhibitors described in literature target FtsZ.

FtsZ is the primary cytoskeletal protein and the best characterized component of bacterial cell division, it shares 40–50% sequence identity among most bacterial and archaeal species, while it is an ancestral homolog of the tubulin (<20% identity). It forms the Z-ring at the cell division site, polymerizing with a head to tail interaction using GTP molecules to couple the FtsZ monomers [24]. The polymerization depends on the hydrolysis of the GTP to GDP. The binding site of GTP is composed of two FtsZ monomers. The larger part of the cofactor is bound by the T2, T3 and T4 loops at the N-terminal domain of the FtsZ monomer, while the T7 loop of the other FtsZ molecule binds the γ-phosphate and the magnesium ion. The interface between the two monomers constitutes the active site of the GTP hydrolysis, that is the result of the nucleophilic attack on the γ-phosphate; the GDP-bound FtsZ induces the disassembling of the polymers. Many crystal structures of FtsZ are available from ten different bacterial species [25–29].

The N- and C-terminal domains are separated by an interdomain branched along the H7-core helix. The absence of this interdomain in the tubulin structure paves the way to design small selective molecules binding this region. On the other hand, the C-terminal part of FtsZ is characterized by a globular domain, followed by a disordered region (called linker region), whose crystal structure is still unresolved, and by a conserved C-terminal peptide. The linker regions vary in length in different species, but all of them have a key role in tethering the membrane anchor proteins, FtsA and ZipA, using the conserved C-terminal peptide and in the interaction with the other divisome components [30,31]. The FtsZ monomers are added to one end of the polymer and removed from the other opposite site, generating a directional movement through the treadmilling, which coordinates the synthesis of the cell wall (Fig. 1) [32,33].

FtsZ function could be targeted in three different sites: the

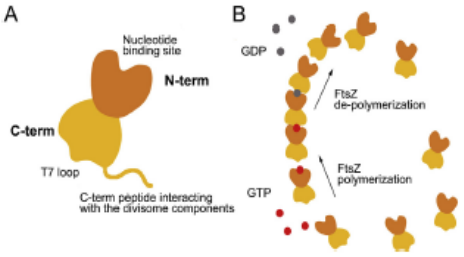

Fig. 1. Schematic representation of FtsZ structural features and of FtsZ polymerization process. (A) The monomer of FtsZ in which the domains and structural elements are indicated. (B) Polymerization/de-polymerization process. FtsZ monomers bind one GTP molecule (red dot) and form the polymer by a head to tail interaction. Upon the hydrolysis of GTP to GDP (grey dot), the polymer is disassembled from the opposite site, generating the treadmilling movement. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

nucleotide binding domain [25], the synergistic T7 loop [34], and the interdomain which connects the N and C terminal parts [35].

3. Bacterial cell division inhibitors

3.1. Compounds effective against Staphylococcus aureus

Staphylococcus aureus is an ubiquitous Gram positive bacterium, which can cause important skin and respiratory infections, as well as osteomyelitis and endocarditis. In CF people, *S. aureus* is often associated with early lung damage and lower respiratory tract inflammation [36], and in particular co-infection with *P. aeruginosa* and *S. maltophilia* is associated with severe lung disease [37]. A great problem in the treatment of CF patients occurred with the appearance of the *S. aureus* strains resistant to methicillin, soon after its introduction in clinical practice [5]. Indeed, Methicillin Resistant *S. aureus* (MRSA) infection leads to an increased rate of lung function decline, and is associated with a higher risk of death [38]. *S. aureus* methicillin sensitive infections are usually treated for 14 days with fusidic acid in combination with oxacillin/dicloxacillin or rifampicin, while for MRSA the therapy can include other drugs, such as trimethoprim-sulfamethoxazole, aminoglycosides, levofloxacin, linezolid, glycopeptides, tetracyclines, and tigecycline [39]. However, despite the increase in prevalence of *S. aureus* infections, currently there are no international guidelines for CF patients treatment [40].

As already said, due to its great potential as a novel therapeutic drug target of novel antimicrobial agents, divisome, and in particular FtsZ, have been extensively investigated searching for new therapies. Currently, different series of FtsZ inhibitors with anti-staphylococcal activity have been identified through both high throughput screening and rational drug design and optimization [41]. These inhibitors can be classified based on the origin, as natural or synthetic, or on the mechanism of action on FtsZ, for instance by inhibition of the polymerization, rather than of the GTPase activity.

3.1.1. Benzamides

Among the first described FtsZ inhibitors, 3-Methoxybenzamide (**1**), a small molecule which inhibited cell division in *Bacillus subtilis*, showed a weak antibacterial activity [42]. Nevertheless, this compound represented the starting point for a fragment-based approach which led to the discovery of the 2,6-difluorinated derivative PC190723 (**2**) [35]. This compound, with potent anti-staphylococcal activity, also in an *in-vivo* mouse model, is characterized by the presence of a 6-chloro-2-methylthiazols[5,4-b]pyridine group instead of the methoxyl group [35] (Fig. 2). However, (**2**) was rather unsuitable for clinical development, as it displayed poor pharmacokinetic properties. To overcome this issue, an N-Mannich base derivative of (**2**) was designed. This compound, TXY436 (**3**) (Fig. 2), a prodrug that at physiological conditions rapidly converts to (**2**), showed significantly improved pharmaceutical properties showing both intravenous and oral *in vivo* efficacy against MRSA (Table 1) [43].

Thus, starting from the scaffold of (**2**), several derivatives have been investigated in order to improve the pharmacokinetic properties. However, being the amide function essential for the biological activity of the compound, the efforts have been mainly directed to the ether-linked substituent at position 3. For instance, Chiodini and colleagues in 2015 [44] introduced a 1,4-benzodioxan-2-ylmethyl residue. The introduction of the heteroalicycle benzodioxane gave interesting possibility of optimization, particularly the enantiomer switch. The best compound (**4**), bearing a 7-chloro-1,4-benzodioxanyl (Fig. 2), was highly active against *S. aureus*, but with low toxicity on mammalian cells, with the S enantiomer 5-fold more potent than the R [44]. Further SAR studies demonstrated the importance of the O at 1-position of the benzodioxane nucleus for the activity of the compound [45]. Moreover, they confirmed that hydrophobic 7-substitution of the benzodioxane core is favorable, while hydrophilic substituents lead to loss of activity [45,46].

Moreover, different other substituents in 3-position of (**1**) have been explored. Bi and colleagues, for instance, explored different 3-O-arylalkyl-2,6-difluorobenzamide, obtaining some derivatives with interesting activity against MRSA, such as the 3-bromoalkoxy (**5**) and 3-alkyloxy derivatives (**6**) (Fig. 2) (Table 1) [47]. Moreover, the same research group designed a series of isoxazol-3-yl- and isoxazol-5-yl-containing benzamide derivatives [48], considering the wide spectrum of activities and therapeutic potential of the isoxazole scaffold [49,50]. Indeed, through computational docking and structure-based optimization, they achieved the compound (**7**), characterized by a (tert-butyl)phenyl)isoxazol-3-yl- group (Fig. 2), which displayed good activity against MRSA (Table 1).

3.1.2. Quinolines and quinazolines

Using a high throughput screen of more than 18,000 compounds against the purified FtsZ protein, Margalit et al. identified five different compounds that inhibited the GTPase activity. These compounds, named zantrins, showed two different effects on FtsZ polymerization, with compounds Z1, Z2 and Z3 that destabilized the polymers assembly, while compounds Z4 and Z5 caused a hyperstability of the FtsZ filaments [51]. Among them, Zantrin Z3 compound (**8**) (Fig. 3) showed a broad spectrum antibacterial activity, with a modest activity also against MRSA (Table 1). Despite its rather low antibacterial activity, this scaffold was subjected to different SAR studies. For instance, Nepomuceno and colleagues demonstrated that the benzo[g]quinazoline can be replaced by a smaller quinazoline moiety (**9**) (Fig. 3), without a loss of activity of the compound [52]. Similarly, a quinoline derivative (**10**) active against *Mycobacterium tuberculosis*, was demonstrated to inhibit FtsZ [53], and more recently different quinolinium derivatives (**11**–**13**) were found to show more efficient inhibition on different drug resistant bacteria, including MRSA strains (Table 1) [54–58]. By *in silico* docking studies, it was suggested that quinolinium derivatives interact with the C-terminal interdomain cleft of FtsZ, interfering with the GTPase activity, thus inhibiting the polymerization [55].

3.1.3. Quinuclide-based and tri-substituted pyrimidine GTPase activity inhibitors

Through a structure-based virtual screening against the GTP binding site of *Methanococcus jannaschii* FtsZ, Chan and colleagues identified a weak quinuclide-based inhibitor (**14**) (Fig. 4), with modest antibacterial activity [59]. This structure was then re-docked against a *S. aureus* FtsZ model, affording a series of optimized structures. The most active inhibitors (**15**), (**16**) and (**17**) were characterized by the presence of a pyridine and a pentanyl substituents on the pyrimidine ring, instead of the methyl and thiophene groups, respectively (Fig. 4). The pyridine, in particular, was predicted to interact with the guanine binding site of the protein through a network of hydrogen bonds, while the pentanyl moiety was allocated in a hydrophobic groove. The oxopentanoic acid moiety was substituted by an uncharged substituent, with an increase in lipophilicity, that probably increases the permeation across the bacterial membrane. In compound (**15**), a furfuryl alcohol was introduced, which can maintain the interaction with the binding site of the triphosphate group of the GTP through a series of hydrogen bonds. Nevertheless, this interaction seems to be not essential for FtsZ inhibition, as the best compounds of these three derivatives (**17**) had an amine in this position [59]. As reported also for other FtsZ inhibitors, these compounds showed a

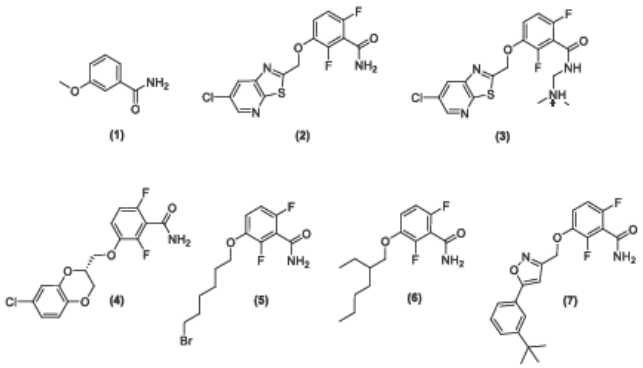

Fig. 2. Structure and development of the different benzamide-derived FtsZ inhibitors.

Table 1
Biological characterization of FtsZ inhibitors against *S. aureus* strains.

Compound	MIC MSSA (μg/ml)	MIC MRSA (μg/ml)	IC_{50} FtsZ (μM)	Ref.
(1)	n.d	n.d	n.d.	[42]
(2)	1	1	55 (ng/ml)	[35]
(3)	0.5–1	0.5	n.d.	[43]
(4)	0.25–1	n.d.	n.d.	[44]
(5)	1	2	n.d.	[47]
(6)	1	8	n.d.	[47]
(7)	2	2	n.d.	[48]
(8)	5	10	n.d.	[51]
(9)	n.d.	n.d.	24	[52]
(10)	n.d	n.d	n.d.	[53]
(11)	2	2	n.d.	[54]
(12)	2	2	n.d.	[55]
(13)	2	2	n.d.	[57]
(14)	897 (μM)		317	[59]
(15)	50.5 (μM)		77.2	[59]
(16)	26.9 (μM)		55.7	[59]
(17)	24.6 (μM)		37.5	[59]
(18)	3	6	20% inhibition at 50 μM	[61]
(19)	2	2	n.d.	[62]
(20)	n.d.	n.d.	10.0	[69]
(21)	2	2	37.8	[69]
(22)	16	64	n.d	[72]
(23)	>64	>64	n.d.	[72]
(24)	2	2–4	N.D.	[72]
(25)	n.d.	n.d.	3	[73]
(26)	0.06	0.25	n.d.	[74]
(27)	5	n.d.	n.d.	[75]
(28)	5	n.d.	n.d.	[75]
(29)	5	n.d.	n.d.	[75]
(30)	2	2–4	n.d.	[76]

broad antibacterial activity, and significant synergistic effects when in combination with β-lactams [60].

However, as the complexity of the chiral quinuclidine limited the development of this scaffold, the same authors explored the possibility to simplify this compound introducing simple amines [61]. To this purpose, they performed a docking study starting from (**15**), maintaining the pyrimidine moiety in the guanine binding site and replacing the quinuclidine with different substituted amines such as homopiperazines, piperazines or linear amines. Moreover, the importance of the 4-pyridyl group in position 2 of the pyrimidine was also explored. All the compounds that displayed improved efficacy compared with (**15**) were characterized by the presence of the 4- pyridyl, confirming the essentiality of this moiety, and of a benzyl substituted homopiperazine ring [61]. These compounds were active against several MRSA clinical isolates (Table 1), and the most effective compound (**18**) was demonstrated to be active in an *in vivo G. mellonella* infection model, with low toxic effects [61]. A further optimization of this scaffold was achieved re-introducing the thiophene group at position 6 of the pyrimidine [62]. Indeed, for compound (**19**), the best from this series active against several MRSA clinical isolates, without toxicity against human erythrocytes, no resistant mutants arose in a resistance generation experiment using *S. aureus* [62].

3.1.4. Natural compounds: sanguinarine and berberine derivatives

Several natural compounds with antibacterial activity have been

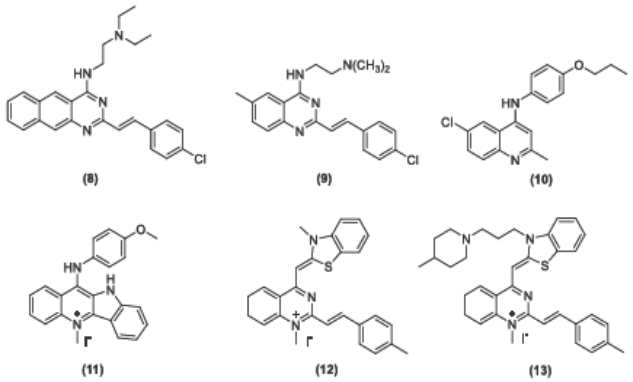

Fig. 3. Structure of quinoline and quinazoline-based FtsZ inhibitors.

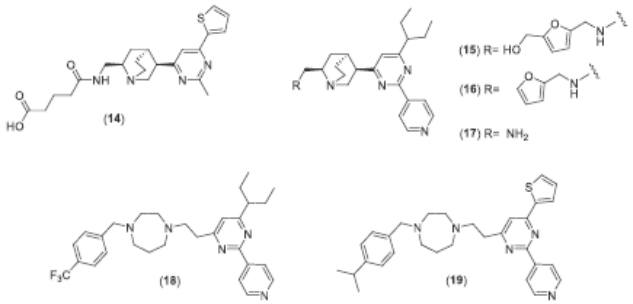

Fig. 4. From quinuclide to tri-substituted pyrimidine GTPase inhibitors.

identified as FtsZ inhibitors, including curcumins [63], coumarins [64], totarol [65], cinnamaldehyde derivatives [66], madurahydroxylactones isolated from *Nonomuraea* [67], as well as new antimicrobial molecules produced by endophytic *Penicillium janthinellum* [68]. However, the most promising compounds against MRSA appear to be polycyclic alkaloid compounds, characterized by a quaternary pyridinium core, such as berberine (**20**) (Fig. 5), that is derived from *Berberis* spp. [34].

In particular, *in silico* studies of (**20**) suggested that the binding of the compound with FtsZ could perturb the geometry of the GTPase site of the protein [34]. Indeed, it was demonstrated that berberine binds FtsZ with very high affinity, but the *in vivo* efficacy against *S. aureus* was quite modest [34].

Nevertheless, through a combination of structure-assisted drug design and *in vitro* assays, Sun and co-workers synthesized a series of phenoxy alkyl derivatives of (**20**), in which the introduction of a hydrophobic function in position 9 of the isoquinoline significantly improved the properties, with the best compound (**21**) showing low micromolar Minimal Inhibitory Concentration (MIC) against MRSA strains (Table 1) [69].

Starting from an in-house isoquinoline alkaloid library, Song and colleagues identified 13-n-octylberberine derivatives as well as cycloberberines with promising antimicrobial activities [70,71]. In particular, the two 13-phenyl derivative (**22**) and the cycloberberine (**23**) were exploited to design and synthesize a novel series of 13-substituted cycloberberines. Indeed, the introduction of the benzyl group in position 13 of (**23**) significantly improved the antimicrobial activity, and SAR studies demonstrated that electron-donating group at this position was beneficial for the potency. The most effective compound from this series (**24**) showed significant activity against several MRSA strains (Table 1), with high stabilities in blood and good *in vivo* safety profiles, demonstrating the value of this class of compounds as novel anti-MRSA drugs [72].

Sanguinarine (**25**) (Fig. 5), derived from *Sanguinaria canadensis*, is another quaternary pyridinium FtsZ inhibitor with modest antibacterial activity, but active also against mammalian tubulin, thus potentially toxic [73]. Nevertheless, starting from this scaffold, Liu and co-workers developed a series of improved methylphenanthridium derivatives, such as compound (**26**) showing interesting activity against several MRSA strains (Table 1) [74].

3.2. Compounds effective against Gram negative pathogens

Gram negative bacteria infections, which include principally *P. aeruginosa*, *B. cepacia complex* and *S. maltophilia*, are the main cause of death in CF patients [77]. Among these pathogens, *P. aeruginosa* is the most common, with the majority of patients that become positive during the lifespan. Although currently there are

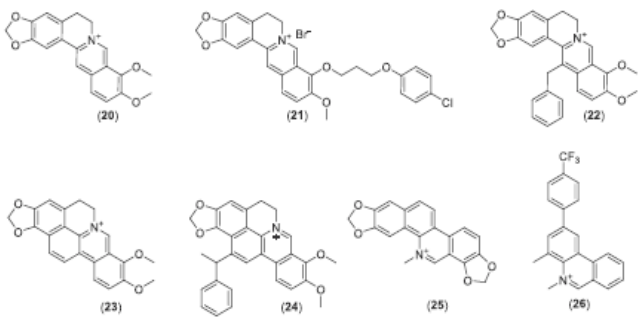

Fig. 5. Structures of the berberine and sanguinarine derivatives.

several treatments to handle early infections, carbapenem-resistant *P. aeruginosa* are emerging, and are considered among the "critical" pathogens with urgent need for novel treatments.

Burkholderia cepacia complex is a group of 22 Gram negative related bacteria, that can infect CF patients, leading to a fatal systemic infection known as cepacia syndrome. These bacteria, including *B. cenocepacia*, are commonly present in the environment and are naturally resistant to several antibiotics, thus the treatment of these infections is particularly challenging [78].

S. maltophilia infection is very variable, from 3 to 30%, with increasing prevalence in CF patient population, and is associated to decreased lung function with poor outcomes and increased risk of death. The treatment is very difficult, being *S. maltophilia* intrinsically resistant to different antibiotics, moreover, there is no currently consensus about the management of the patients [79].

For all these reasons, there is an urgent need of novel therapeutic approaches against CF infections [80].

Despite the essentiality of FtsZ, the majority of the inhibitors showed only weak or moderate activity against Gram negative bacteria, and very few drugs were significantly active against the principal CF pathogens [48,54–56].

Sangeeta and co-workers, starting from the benzimidazole scaffold known to inhibit FtsZ, performed *in silico* studies that led to novel benzimidazolium compounds that showed a broad significant activity against both Gram positive and negative bacteria, including *P. aeruginosa* [75]. In particular, a library of benzimidazoles, benzylated benzimidazoles and dibenzyl benzimidazolium salts was docked in the four cavities that are present in the *S. aureus* FtsZ structure. From this analysis, benzyl benzimidazolium compounds showed the best properties, and were selected to be synthetized and assayed. Among these compounds, the substituted dibenzyl-2-methyl-benzoimidazolium (**27**–**29**) (Fig. 6) displayed significant activity against a panel of different bacteria, including *S. aureus*, *Macrococcus caseolyticus*, *E. coli* and *P. aeruginosa*, with MICs ranging from 5 to 10 μg/ml [75].

More recently, FtsZ was recognized as the target of a benzothiadiazole compound (**30**) (Fig. 6) active against *B. cenocepacia*, with an MIC of 8 μg/ml [81]. The compound was previously identified through a phenotypic screening of an in-house library, but at that time it was not possible to identify the target [82]. Compound (**30**) was subsequently demonstrated to be very effective not only against *B. cenocepacia*, including several clinical isolates, but also against other members of the *Burkholderia cepacia* complex and against different Gram positive bacteria, including MRSA (Table 1) [81]. Moreover, being (**30**) poorly soluble, nanocrystal-embedded

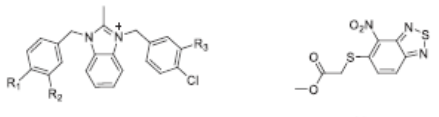

Fig. 6. Structure of the FtsZ inhibitors showing broad specificity against both Gram negative and Gram positive bacteria.

dry powders for inhalation have been developed [76]. This formulation was also demonstrated to diffuse through artificial CF mucus, and showed encouraging effects, being active against *B. cenocepacia* also in a biofilm model, and showing efficacy in combination with piperacillin in an *in vivo G. mellonella* infection model [76].

In Fig. 7 the main class of FtsZ inhibitors here described, along with their proposed binding site and mechanism of action are summarized.

4. Conclusions

Chronic infections represent a main cause of death among cystic fibrosis patients. Although the most prevalent species are represented by *S. aureus* and *P. aeruginosa* [5], other pathogens are particularly dangerous. An example is *B. cenocepacia*, a Gram negative bacterium affecting a small percentage of CF patients, but extremely difficult to eradicate and preventing the admission of patients to transplant list [84]. Among the other relevant CF pathogens, *S. maltophilia*, *A. xylosoxidans*, and non-tuberculous mycobacteria play a major role in lung infections.

Unfortunately, a common feature of these bacteria is the high level of drug resistance, which can be ascribed to mutations in genes of the antibiotics cellular target, enzymatic inactivation of the drug, reduction of cell wall permeability, or an increased activity of efflux pumps. Although usually combinatorial therapies are used to face multiple or single infections [9], they still represent the leading cause of death for CF patients. In this context, the development of novel compounds to fight chronic infections is fundamental to improve and prolong CF people life.

The divisome, i.e. the complex of proteins involved in bacterial cell division, seems particularly suitable to find new targets for the

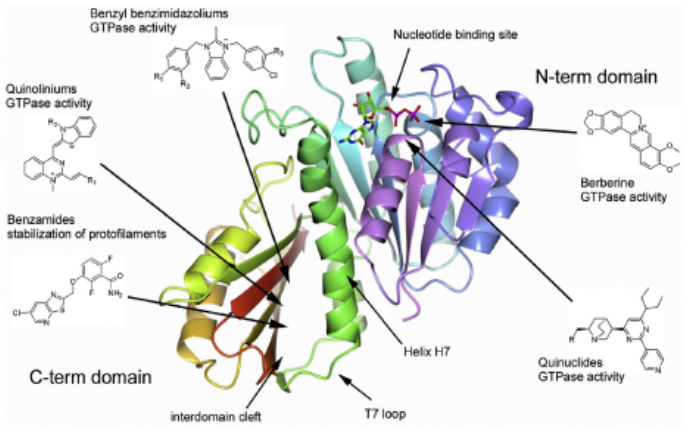

Fig. 7. Representation of the main classes of antimicrobial compounds inhibiting FtsZ, along with their proposed mechanism of action and binding site in FtsZ structure (PDB: 3VBO [83]).

discovery of new antibacterials: indeed, this pathway is essential for bacterial growth and quite different from its eukaryotic counterpart. In particular, the master regulator of cell division, FtsZ, is highly conserved and well characterized from the biochemical and structural point of view and this is a good starting point for the research on new antibiotics.

As described in the review, most compounds targeting FtsZ described so far are effective against *S. aureus*: indeed, benzamides, quinolines, quinazolines, pyrimidines, indole derivatives and natural compounds show key features which lead to their optimization as lead compounds. Currently, the most promising compounds appear to be the benzamide based prodrugs of PC190723 (i.e. compound (**3**)), which showed not only *in vivo* oral efficacy, but also good safety and pharmacokinetic properties.

By contrast, for the treatment of Gram negatives, only benzimidazolium and benzothiadiazole compounds are reported as antibacterial FtsZ inhibitor. However, the lack of efficacy of several FtsZ inhibitors against Gram negatives bacterial is conceivably due to permeability or efflux issue, rather than a failure to inhibit the target.

Unfortunately, none of these compounds reached the clinical trial phase until now: indeed, even if these compounds showed good activity against the protein activity, they were not so effective *in vivo*.

This is not uncommon when a target-based approach is used to develop new drugs and it is corroborated by the fact that the currently used therapies were all discovered through a cell-based screening method [85].

On the other hand, being cell division an ideal drug target, it is worth to continue studying the proteins which compose the divisome, and in particular FtsZ, and to find a way to improve the chemical properties of molecules targeting them.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chemical, Metabolic, and Cellular Characterization of a FtsZ Inhibitor Effective Against *Burkholderia cenocepacia*

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There is an urgent need for new antimicrobials to treat the opportunistic Gram-negative *Burkholderia cenocepacia*, which represents a problematic challenge for cystic fibrosis patients. Recently, a benzothiadiazole derivative, C109, was shown to be effective against the infections caused by *B. cenocepacia* and other Gram-negative and-positive bacteria. C109 has a promising cellular target, the cell division protein FtsZ, and a recently developed PEGylated formulation make it an attractive molecule to counteract *Burkholderia* infections. However, the ability of efflux pumps to extrude it out of the cell represents a limitation for its use. Here, more than 50 derivatives of C109 were synthesized and tested against Gram-negative species and the Gram-positive *Staphylococcus aureus*. In addition, their activity was evaluated on the purified FtsZ protein. The chemical, metabolic and cellular stability of C109 has been assayed using different biological systems, including quantitative single-cell imaging. However, no further improvement on C109 was achieved, and the role of efflux in resistance was further confirmed. Also, a novel nitroreductase that can inactivate the compound was characterized, but it does not appear to play a role in natural resistance. All these data allowed a deep characterization of the compound, which will contribute to a further improvement of its properties.

Keywords: *Burkholderia cenocepacia*, new antimicrobials, drug resistance, cell division, cystic fibrosis

INTRODUCTION

Cystic fibrosis (CF) patients are continuously subjected to antibiotic therapies due to lung colonization both by Gram-positive and –negative bacteria. Among the latter, *Burkholderia cenocepacia* represents a main concern being responsible for the so-called "*cepacia* syndrome," a necrotizing pneumonia which can lead to patient's death (Salsgiver et al., 2016). Indeed, this opportunistic pathogen is among the bacterial species CF patients should be worried about (Jones, 2019). Moreover, infections caused by *B. cenocepacia* are still a major contraindication to lung transplantation, although certain centers do admit infected patients in the list (Dupont, 2017). An

important characteristic, which renders these bacteria particularly dangerous, is their resistance toward most antibiotics used in clinical practice (Scoffone et al., 2017). This limits the therapeutic options available to treat the infections, and there are no standardized set of antibiotics for treatment. In this context, new antibacterials are highly necessary, although their development may be limited by poor commercial interest in *Burkholderia* infections because they are rare, with approximately 2.6% of CF patients infected with these bacteria (https://www.cff.org/Research/Researcher-Resources/Patient-Registry/).

During the last few years, we focused our attention on this topic, proposing new targets, such as the glutamate racemase (Israyilova et al., 2016), new strategies, such as the inhibition of quorum sensing (Scoffone et al., 2016; Buroni et al., 2018) and new drugs (Scoffone et al., 2014), to fight *B. cenocepacia* infections. We found that the benzothiadiazole derivative C109 is highly effective against *B. cenocepacia* (Scoffone et al., 2015) and other Gram-negative and-positive bacteria, including *Mycobacterium abscessus* (Hogan et al., 2018). We were also able to identify the cellular target of C109 as the highly conserved cell division protein FtsZ (Hogan et al., 2018). This protein has recently emerged as a new promising target for the development of pharmacological agents against CF pathogens (Buroni et al., 2020). This not only justifies the broad-spectrum activity of C109, but also validates it as a robust molecule that hits an essential pathway, which is evolutionarily distant from its eukaryotic counterpart.

Due to the poor solubility of C109, we recently described the development of PEGylated nanocrystals in which the compound was stabilized with D-α-tocopheryl polyethylene glycol 1000 succinate embedded in hydroxypropyl-β-cyclodextrin (Costabile et al., 2020). This powder formulation allows its re-dispersion in water for *in vitro* aerosolization. The ability of these C109 nanocrystals to diffuse through artificial mucus and possess low toxicity toward human bronchial epithelial cells was also demonstrated (Costabile et al., 2020). The great potentiality of this formulation has been shown also by its activity against both planktonic and sessile *B. cenocepacia* strains, and by its efficacy in combination with piperacillin (Costabile et al., 2020).

Despite its promising activity and low toxicity, we previously showed that C109 can be extruded out of the cell by an RND efflux pump (Scoffone et al., 2015). For this reason, in the present work we synthesized and characterized more than 50 C109 derivatives, by performing a deep structure-activity relationship (SAR) analysis to screen for compounds less prone to efflux. The Minimal Inhibitory Concentration (MIC) of all the compounds and their activity against the purified FtsZ protein were assessed. The C109 resistance mechanism, chemical, metabolic and cellular stability were also studied at the single-cell level.

MATERIALS AND METHODS

Chemical Synthesis of C109 Derivatives

The chemical synthesis of C109 derivatives is described in **Supplementary Data**.

Bacterial Strains and Growth Conditions

Burkholderia cenocepacia strains, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PAO1, and *Staphylococcus aureus* ATCC 25923 were grown in Luria-Bertani (LB) medium (Difco), if not differently specified, with shaking at 200 rpm, or on LB agar, at 37°C.

MIC Determination and Checkerboard Assays

The effectiveness of C109 compound and of its derivatives against *B. cenocepacia* J2315, FCF19, and FCF22, *E. coli* ATCC 25922, *P. aeruginosa* PAO1, and *S. aureus* ATCC 25923 was assessed determining MICs in LB medium by the 2-fold microdilution method in U-bottom 96-well microtiter plates, and inoculating about 10^5 CFU. The microtiter plates were incubated at 37°C for 48 (for *B. cenocepacia*) or 24 h (for all the other strains) and growth was determined by the resazurin method (Martin et al., 2006). A solution of resazurin sodium salt (Sigma Aldrich) was prepared at 0.01% in distilled water and filter-sterilized. Thirty microliter of resazurin solution were added to each well after 1 or 2 days of incubation at 37°C, and the microtiters were re-incubated at the same temperature for 4 h. The MIC was defined as the lowest concentration of the drug that prevented a change in color from blue to pink, which indicates bacterial growth.

Checkerboard assays of C109 or its derivatives with concentrations ranging from 0 to 128 μg/ml in combination with the efflux pump inhibitor PaβN MC-207.110 (0–128 μg/ml) were set up, as previously described (Saiman et al., 1996) and the results determined using the resazurin MIC method.

Three independent biological replicates were used for each MIC determination.

Quantitative Reverse Transcription PCR (qRT-PCR) of RND-9 Efflux Pump and Nitroreductase Genes

Total RNA was extracted from *B. cenocepacia* J2315, FCF19, and FCF22 clinical isolates (1×10^9 CFU) using the RiboPure Bacteria Kit (Ambion), following the manufacturer's instructions. A 30 min incubation of each sample with DNaseI (Ambion) was performed, following the manufacturer's protocol. One-microgram of total RNA was used for cDNA generation using the QuantiTect reverse Transcription kit (Qiagen) according to the manufacturer's instructions, but diluting the cDNA 1:2 before qRT-PCR.

qRT-PCRs were performed on *BCAM1946* gene using the primers BCAM1946Rtfor (5′- TGCTCGTCGTGATCCTGTTT-3′) and BCAM1946Rtrev (5′- CGAACAGCGTGAGCGTATTG-3′) and on *BCAL0539* using primers NitroIntFor (5′-GTGTCGCCGTATATCGA-3′) and NitroIntRev (5′-TTCTCGTCGCGGCCGAT-3′). All reactions were performed on a Rotor-Gene-6000 cycler (Corbett), using the Quanti-Tect SYBR Green PCR Kit (Qiagen), according to manufacturer's instructions. Cycling conditions were: 95°C for 15 min (1 cycle), 94°C for 15 s followed by 54°C for 30 s (for *BCAM1946*) or 40°C for 30 s (for *BCAL0539*) and 72°C for 30 s (40 cycles). A melting curve analysis was included at the end of each run.

Each sample was spotted in triplicate and control samples without cDNA were included in each experiment. *BCAM0918* (*rpoD*) gene was used as reference gene with the primers 0918F (5′-GCCAACCTGCGTCTCGT-3′) and 0918R (5′-AACTTGTCCACCGCCTT-3′), using an annealing temperature of 50°C. The fold difference in gene expression between the FCF19 and FCF22 and J2315 strains was assessed using the comparative Ct-method (Livak and Schmittgen, 2001). The results are the average of three independent replicates. Mann–Whitney test was used to determine if differences in expression were significant ($P < 0.05$).

FtsZ Proteins Expression and Purification and Enzymatic Assay

The gene *ftsZ* (*BCAL3457*) was amplified from *B. cenocepacia* FCF19 using the primers pet28presFtsZfor (5′-ATGGGTCGC GGATCCCTGGAAGTTCTGTTCCAGGGGCCCATGGAA TTCGAAATGCTGGA-3′) and pet28ftsZrev (5′-TGCGGC CGCAAGCTTTCAGTCAGCCTGCTTGCGCA-3′). The gene *ftsZ* of *P. aeruginosa* was amplified from the genomic DNA using the primers ftsZPAOfor28a (5′-ATGGGTCGCGGA TCCCTGGAAGTTCTGTTCCAGGGGCCCTTTGAACTG GTCGATAACAT 3′) and ftsZPAOrev28a (5′-TGCGGCCGC AAGCTTTCACAGGCCGGTGGCGACTAC-3′). The PCR products were cloned into the pET-28a vector (Novagen) using the In-Fusion HD Cloning kit (TaKaRa), according to the manufacturer's instructions.

All the recombinant FtsZ proteins were expressed at 20°C overnight in *E. coli* BL21(DE3) cells, upon induction with 0.5 mM IPTG, and purified using the same protocol assessed for the wild-type *B. cenocepacia* FtsZ (Hogan et al., 2018).

GTPase enzyme activity was determined using a pyruvate kinase/L-lactic dehydrogenase (PK/LDH) spectrophotometric coupled assay (Ingerman and Nunnari, 2005). The assays were performed at 30°C, in a reaction mixture containing 50 μM Hepes pH 7.5, 5 mM $MgCl_2$, 5 mM KCl, 10 U PK/LDH, 0.25 mM NADH, 0.25 mM PEP, and 5–10 μM FtsZ, and initiated by the addition of 0.5 mM GTP. Alternatively, GTPase activity was determined by measuring the release of phosphate using the malachite green assay (Baykov et al., 1988).

Steady-state kinetic analysis was performed by assaying the activity at different GTP concentrations and parameters determined by fitting the data to the Michaelis-Menten equation using Origin 8 software. All experiments were performed in triplicate.

Initial Inhibition assays were performed at 100 μM of each compound (stock solution 20 mM in DMSO) and, for compounds significantly active, the inhibitory concentration (IC_{50}) was determined. To this purpose, GTPase activity of FtsZ was evaluated in the presence of different concentrations of compounds, ranging from 0.5 μM to 100 μM, and IC_{50} value was determined by the Equation 1 using Origin 8 software:

$$A_{[I]} = A_{[0]} \times \left(1 - \frac{[I]}{[I] + IC_{50}}\right) \quad (1)$$

where $A_{[I]}$ is the enzyme activity at inhibitor concentration [I] and $A_{[0]}$ is the enzyme activity without inhibitor.

Metabolic Transformation of C109 in *B. cenocepacia* Cultures

To identify the products of C109 transformation in the cellular environment, 200 ml *B. cenocepacia* J2315 culture were grown overnight at 37°C in LB medium, then 20 mg C109 were added. After 1.5 h, a chloroform extraction (100 ml × 3) was performed. Organic phase obtained from the extraction was evaporated, residues were resuspended in hexane/ethyl acetate 9:1 and subjected to flash column chromatography (Merck SiO2 60, 230–400 mesh). Visualization of metabolites was achieved under UV light at a wavelength of 254 nm.

The isolated metabolites were analyzed in UPLC/MS. The chromatographic analysis was performed with a JASCO X-LC (Lecco, Italy) system, coupled with a Thermo Fisher Scientific (Milan, Italy) LTQ XL HESI-MS/MS system. Chromatography was performed on a Waters Acquity column, 3 um particle size, 0.3 ml/min, gradient 10 min from 90:10 H_2O/MeCN to 100% MeCN, then 4 min in 100% MeCN. The analyses were performed in full-scan from 150 and 2,000 u.m.a. in positive mode, and base peaks were analyzed with dependent scan method with @CID = 32 eV. Run were also monitored by recording the absorbance at 220 nm.

Nitroreductase BCAL0539 Expression, Purification, and Characterization

In order to obtain the *B. cenocepacia* putative nitroreductase (BcNR), the *BCAL0539* gene was amplified using the primers pet28BC0539for (5′-ATGGGTCGCGGATC*CCTGGAAGT TCTGTT*CCAGGGGCCCATGTCCGTCCCCACTGCTT-3′) and pet28BC0539rev (5′-TGCGGCCGCAAGCTTTCAAGC GAAAAAGCGCGCG-3′), and PCR products cloned into the pET-28a vector by the "In-Fusion® HD Cloning." To facilitate the purification process, the forward primer was designed to insert the sequence encoding the cleavage site of the PreScission Protease (GE Healthcare) downstream the N-terminal His-tag sequence (italicized in the primer sequence). Best protein expression was achieved in *E. coli BL21*(DE3) cells, by induction with 0.5 mM IPTG overnight at 25°C. Cells were frozen at −20°C until use.

Frozen cells were resuspended in 50 mM potassium phosphate pH 8.0, 500 mM KCl (buffer A) containing 1 mM phenylmethylsulfonylfluoride, lysed by sonication, and clarified by centrifugation at 50,000 × *g* for 1 h. Cell free extract was then applied on a HisTrapFF Crude (1 ml, GE Healthcare) column, washed with 50 mM imidazole in buffer A, then BcNR was eluted with 250 mM imidazole in the same buffer. Proteins were dialyzed against 50 mM potassium phosphate pH 8.0, 150 mM KCl (buffer B), containing 1 mM DTT, treated with PreScission Protease to remove the N-terminal histidine tag, then further purified by size exclusion chromatography on a HiLoad 16/60 Superdex-75 column (GE Healthcare) equilibrated in buffer B. Protein was concentrated to 10 mg/ml and stored at −80°C until use. As BcNR was produced as a flavoprotein, we explored

whether the prostetic group is covalently bound to the enzyme. To this purpose, 0.5 µl of the protein (2 mg/ml) were incubated at 100°C for 10 min, and centrifuged for 20 min at 12,000 × *g*. The denaturated protein was resuspended in the same initial volume of 2% SDS, and the spectra of both supernatant and resuspended pellet were recorded.

Enzyme activity assay was determined at 37°C using 4-nitrobenzoic acid or C109 as a substrate and NADPH as cofactor, by measuring the decrease in absorbance at 340 nm of NADPH ($\varepsilon = 6{,}220\ M^{-1}\ cm^{-1}$). The reaction mixture typically contained 50 mM potassium phosphate pH 8.0, 50 mM KCl, 5–10 µM BcNR, and reactions were started by adding the substrate. Steady-state kinetics parameters were determined by assaying the enzyme at variable concentrations of substrate (10–500 µM). The experiments were performed in triplicate, and the kinetic constants K_m and k_{cat} determined by fitting the data to the Michaelis-Menten equation using Origin 8 software.

To determine the BcNR metabolite of C109, 5 mg of compound were incubated with 10 mg of BcNR in 50 mM potassium phosphate pH 8.0, 200 µM NADPH, at 37°C, in a final volume of 35 ml for 1 h under agitation. The reaction mixture was then partitioned between water and dichloromethane (DCM), the aqueous layer was extracted with DCM, and combined organic layer washed with brine and dried over Na_2SO_4. Solvent was removed under reduced pressure and residue purified by flash column chromatography with hexane-ethyl acetate 8:2 as eluent. The isolated metabolites were analyzed in UPLC/MS, as described above. Analysis were performed in full-scan from 90 and 1,000 u.m.a. and base peaks were analyzed with dependent scan method with @CID = 28 eV.

Time-Lapse Microscopy

B. cenocepacia J2315 was cultured over-night in Middlebrook 7H9 broth, supplemented with 10% (v/v) DS enrichment (20 g dextrose, 8.5 g sodium chloride in 1 L water) and 5% (v/v) of a casamino acids solution (10 g casamino acids in 100 ml water). Cells were diluted 10-fold in the same fresh 7H9 medium, and 5 µl of the diluted cell suspension were inoculated into a custom-made microfluidic *hexa*-device, as previously described (Manina et al., 2019). Silicone tubing (0.76 mm ID) was connected to each inlet and outlet ports of the *hexa*-device to enable medium circulation. Two 50 ml syringes were connected to the inlet tubing, and prewarmed 7H9 medium was pumped through the microfluidic device at 15 µl/min. The outlet tubing were inserted into a waste receptacle containing bleach. The syringes were switched to 7H9 medium containing C109 (100 µg/ml) when appropriate. Bacteria were imaged in phase contrast, using a UPLFLN100XO2/PH3/1.30 objective (Olympus) and a high-speed sCMOS camera, with the help of an automated epifluorescence inverted microscope (DeltaVision Elite, GE Healthcare). The microscope was equipped with an environmental chamber maintained at 37°C. Images were recorded at 20-min intervals and up to 46 h. Exposure conditions were as follows: phase 50% T, 150 ms. In each experiment, 190 XY fields were imaged. The same experimental set-up was carried out twice.

Single-Cell Image Analysis

Manual segmentation of individual cells and analysis of image stacks were performed using the ImageJ 1.52a software. The Selection Brush Tools was used to draw polygons corresponding to the shape of individual cells and to extract the cell planar area (μm^2). Measurements of the cell area were repeated throughout the generation time of individual cells, to calculate the growth rate by fitting an exponential curve. Division and lysis events were scored from the total number of cells constituting a microcolony before and during both the first and second exposure to C109, and used to calculate both division and lysis rates, as previously described (Manina et al., 2015).

RESULTS

Chemistry and Structure-Activity Relationship of C109 Derivatives

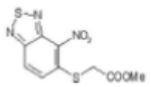
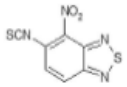
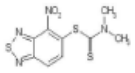
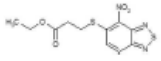
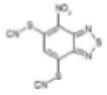
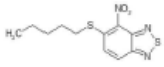
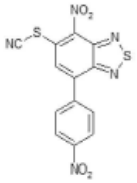
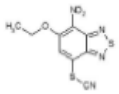
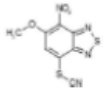
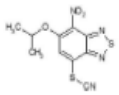
During our research in the field of the discovery of new anti-tuberculosis and anti-leprosy agents, we synthesized broad spectrum ortho-nitrodithiocarbomoyl heterocycles and analogs (Makarov et al., 2006). As a continuation of this research, we investigated the activity of some of these compounds on *B. cenocepacia* and surprisingly only compound C109 showed significant activity in whole cell screening (Scoffone et al., 2015). So, the primary aim of our current study was to improve the chemical properties of C109 in order to find new derivatives with lower MIC values toward *B. cenocepacia* and/or other bacteria, lower toxicity and, possibly, not extruded out of the cell by efflux mechanisms. To study structure activity and to design new active derivatives of C109, we used known medicinal chemistry methods to synthesize a broad spectrum of compounds and to probe their activity.

After screening 51 C109 derivatives (**Supplementary Table 1**), 23 compounds that showed inhibitory activity were examined to understand structure activity relationship.

The primary compound, C109, is a methyl [(4-nitro-2,1,3-benzothiadiazol-5-yl)thio]acetate (Scoffone et al., 2015). As a first step, we investigated the activity of several heterocyclic compounds having methylthioacetate moiety in ortho position to nitro group and observed that 2,1,3-benzothiadiazol scaffold is essential to preserve antibacterial activity. Also, our efforts to change methylthioacetate moiety to other sulfur containing nucleophilic substituents, such as dithiocarbamates, rhodano, imidothiocarbamate did not lead to any compounds with significant activity. Therefore, all other modifications have been limited with efforts to modify the nitro group and methylthioacetate side chain. The nitro group was explored to understand the essentiality of this reactive moiety. All the compounds lacking this group failed to be active both against the FtsZ activity and all the bacteria tested, confirming its essentiality.

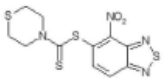
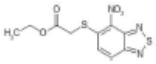
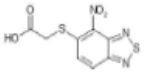
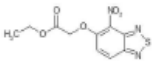
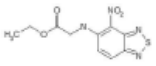
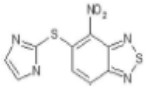
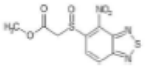
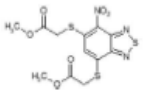
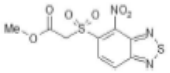
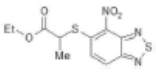
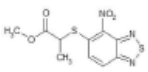
Another way to modify the C109 compound was achieved through the synthesis of derivatives close to the original molecule, by modifying the size or hydrophobicity of the side chain, and introducing different moieties or atoms. This part of the molecule

TABLE 1 | Chemical structure, MICs and IC_{50} of C109 and of selected derivatives.

Molecule	Chemical structure	MIC (μg/ml)				Inhibition of Bc FtsZ*	
		B. cenocepacia J2315	*E. coli 25922*	*S. aureus 25923*	*P. aeruginosa PAO1*	At 100 μM	IC_{50} (μM)
C109		8	8	4	128	Yes	8
10026149		32	16	2	16	Yes	20
10126130		>128	512	32	>128	Yes	10
10226047		>512	>512	>512	>128	No	-
10626056		>64	>512	8	>128	Yes	52
10726015		>64	>512	512	>128	No	-
11026176		>512	>512	4	>128	Yes	17
11026177		>512	>512	4	64	Yes	19
11126009		>128	>512	8	>128	Yes	4
11126010		>128	>512	4	>128	Yes	15

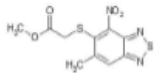
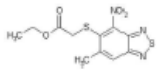
(Continued)

TABLE 1 | Continued

Molecule	Chemical structure	MIC (μg/ml)				Inhibition of Bc FtsZ*	
		B. cenocepacia J2315	*E. coli* 25922	*S. aureus* 25923	*P. aeruginosa* PAO1	At 100 μM	IC_{50} (μM)
11126015		>128	>512	32	>128	Yes	36
11426142		32	8	4	32	Yes	21
11426177		>128	>512	256	ND	No	–
11626109		512	>512	>512	ND	No	–
11726041		>256	>512	>256	ND	No	–
11726042		>256	>512	>256	ND	No	–
11726256		128	128	8	>256	No	–
11726257		16	8	8	128	Yes	3
11726258		256	>256	256	>256	Yes	15
11826109		256	128	16	>256	Yes	30
11826110		16	32	32	>256	No	–
11826383		16	16	64	256	Yes	30

(Continued)

TABLE 1 | Continued

Molecule	Chemical structure	MIC (μg/ml)				Inhibition of Bc FtsZ*	
		B. cenocepacia J2315	*E. coli* 25922	*S. aureus* 25923	*P. aeruginosa* PAO1	At 100 μM	IC_{50} (μM)
11926141		>256	>256	32	>256	Yes	100
11926142		>256	>256	32	>256	Yes	100

*ND, Values Not Detected because of the resistance detected in the other bacteria. * Burkholderia cenocepacia purified FtsZ protein.*

was explored as it can influence not only the binding of the compound to the molecular target FtsZ, but also the permeability across cell wall, as well as the affinity for efflux systems. For instance, the corresponding ethyl acetate derivative (11426142) partially lost activity against both bacteria and the purified enzyme (**Table 1**). The same was observed in the case of exchange of one proton inside chain on methyl group (11826110). We also observed completely loss of activity in the case of exchange of sulfur atom to oxygen (11726041), or nitrogen (11726042), or side chain length extension (10226047) (**Table 1**).

Then, we explored typical metabolic processes such as hydrolysis, oxidation and reduction during which C109 can be transformed into its active metabolites. Hydrolysis followed by free acid formation led to the isolation of compound 11426177 (**Table 1**). Although this compound can be easily transferred to water soluble salt, unfortunately it is not active against *B. cenocepacia*. During oxidation process, we isolated two compounds with different level of sulfur oxidation. The result of mono oxidation, the sulfinyl derivative 11726257, showed an activity on *B. cenocepacia* similar to C109 (**Table 1**). A deeper oxidation of sulfur, which resulted in the sulfonyl derivative 11826109 showed loss of activity. We also studied the reduction process of nitro group of xenobiotics: several chemical methods of reduction were explored but, surprisingly, in all cases we isolated only products of the reduction followed by cyclisation of the thiozine ring with formation of the interesting tricyclic compound 7*H*-[1,2,5]thiadiazolo[3,4-*f*][1,4]benzothiazin-8(9*H*)-one 11626109. This considerable modification of C109 structure caused loss of activity (**Table 1**).

Collectively these data show that the C109 molecule is very sensitive to any change, and that its structure is therefore most likely "terminal" for this class of compounds and for the activity against *B. cenocepacia*.

C109 Derivatives Exhibit a Broad Spectrum Efficacy

Overall, none of the derivatives showed better MIC values against *B. cenocepacia* than C109 (**Table 1** and **Supplementary Table 1**). To check whether the compounds were effective against other Gram-negatives, the MICs against *E. coli* were evaluated, as described in Material and Methods, showing results very similar to those achieved for *B. cenocepacia* (**Table 1**). The activity of the identified compounds was assessed also against *P. aeruginosa* and the Gram-positive *S. aureus*. Seven of them (10626056, 11026176, 11026177, 11126009, 11126010, 11126015, and 11726256) showed activity only against *S. aureus*, indicating that probably these molecules cannot enter the Gram-negative cells, due to the different composition of the cell wall and the presence of the outer membrane. However, compounds 10026149 and 11426142 had better MIC values than C109 against *P. aeruginosa* (16 and 32 μg/ml, respectively vs. 128 μg/ml).

To extend the basic antimicrobial inhibitory activity screening, we also determined the ability of all the C109 derivatives, showing activity against at least one of the microorganisms, to inhibit their cellular target, i.e., the cell division protein FtsZ. The IC_{50} of the selected compounds was assayed on the *B. cenocepacia* FtsZ recombinant protein, as previously described (Hogan et al., 2018). As expected, all the compounds showed inhibitory activity, having an IC_{50} ranging from 3 μM (11726257) to 100 μM (11926141 and 11926142, **Table 1**), thus confirming that the resistance identified is probably due to either limited cell penetration or increased efflux.

Being that 11426142 was effective against all the microorganisms tested, including *P. aeruginosa*, and showing an IC_{50} against FtsZ of 21 μM (**Table 1**), we also determined its toxicity against a CF bronchial epithelial (CFBE41o-) cell line using the MTT assay, as previously described (Hogan et al., 2018). The toxic concentration at 50% (TC_{50}) of C109 on these cell lines is about 75 μM (Hogan et al., 2018; Costabile et al., 2020), while 11426142 showed a TC_{50} of 50 μM (data not shown), confirming once more that C109 remains the best compound in our hands.

Analysis of C109 Resistant Strains

As we previously described that a mechanism of resistance to C109 relies on efflux mediated by the RND-9 transporter (Scoffone et al., 2015), we decided to investigate the resistance of *P. aeruginosa*, being the MIC of C109 equal to 128 μg/ml. A checkerboard assay, using increasing concentrations of C109 (from 0 to 128 μg/ml) in combination with the efflux pump inhibitor PaβN MC-207.110, was set up as described in Materials

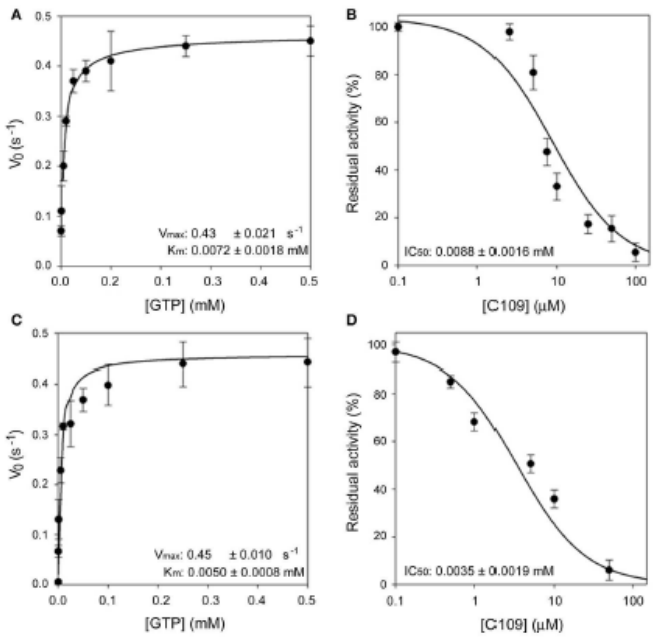


FIGURE 1 | Enzymatic characterization of FCF19-FtsZ and PAO1-FtsZ. **(A)** Steady state kinetic analysis of FCF19-FtsZ mutant as a function of GTP. **(B)** IC_{50} determination of C109 toward FCF19-FtsZ mutant. **(C)** Steady state kinetic analysis of PAO1-FtsZ mutant as a function of GTP. **(D)** IC_{50} determination of C109 toward PAO1-FtsZ.

and Methods. The use of the efflux pump inhibitor PaβN MC-207.110 (Pagès et al., 2005) in combination with the C109 molecule showed a synergistic effect. In fact, in the presence of 16–128 μg/ml of MC-207.110, the C109 MIC decreased to 1 μg/ml (data not shown). These data clearly indicate that an efflux mechanism is responsible for the resistance of *P. aeruginosa* to C109.

Moreover, the *ftsZ* gene from *P. aeruginosa* PAO1 was cloned and expressed in *E. coli* using the same protocol followed for FtsZ from *B. cenocepacia* (see Materials and Methods). The purified PAO1-FtsZ showed kinetic parameters comparable to the ones of *B. cenocepacia* enzyme and the protein was sensitive to the effect of C109, being the IC_{50} even lower than toward *B. cenocepacia* FtsZ (3.5 ± 1.9 μM vs. 8.2 ± 1.3 μM), showing that the compound is able to inhibit the activity of this protein (**Figure 1**). As the two proteins share a 53% amino acid sequence identity, this result was not unexpected but further confirmed that the conserved FtsZ protein is the cellular target of C109.

Next, we decided to investigate the origin of resistance to C109 in two clinical isolates of *B. cenocepacia*, namely FCF19 and FCF22 (Papaleo et al., 2010) for which C109 MIC is 128 and 256 μg/ml, respectively. Initially, we checked whether the gene encoding the C109 target, *ftsZ*, was mutated in these clinical isolates. Indeed, three mutations were found in the *ftsZ* gene of FCF19 strain, which lead to the following amino acidic changes: Pro347Gln; Ala348Pro; Gln349His. In contrast, no mutations were found in the *ftsZ* gene of FCF22 strain compared to the sequence of the reference strain J2315. Consequently, FCF19-FtsZ was expressed and purified in the same conditions of the wild-type FtsZ and showed to have kinetic parameters identical to those of the wild-type FtsZ (**Figure 1**). Moreover, the IC_{50} of C109 on FCF19-FtsZ resulted to be almost identical to the one for the wild-type protein (8.8 ± 1.6 μM), thus demonstrating that the mutated residues in FCF19-FtsZ are not responsible for the resistance phenotype.

Based on these findings, we sought whether the RND-9 efflux pump was implicated in the resistance phenotype of the two clinical isolates. We performed qRT-PCR analyses to check the level of expression of the RND-9 encoding gene. We found that the *BCAM1946* gene, encoding the RND-9 transporter, was overexpressed in both FCF19 and FCF22 clinical isolates of 12 and 20 times, respectively, compared to the wild-type J2315

TABLE 2 | MICs and RND-9 qRT-PCR results of *B. cenocepacia* FCF19 and FCF22 strains.

Strain	C109 MIC (μg/ml)	C109 MIC in the presence of MC-207.110 (μg/ml)	10026149 MIC (μg/ml)	11426142 MIC (μg/ml)	RND-9 overexpression ± S.D.
FCF19	128	8	64	32	12 ± 1.2
FCF22	256	16	256	32	20 ± 3.5

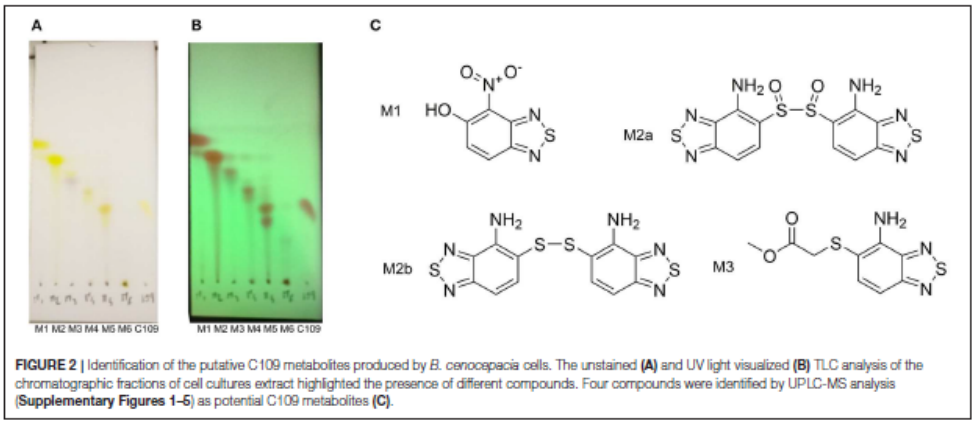


FIGURE 2 | Identification of the putative C109 metabolites produced by *B. cenocepacia* cells. The unstained **(A)** and UV light visualized **(B)** TLC analysis of the chromatographic fractions of cell cultures extract highlighted the presence of different compounds. Four compounds were identified by UPLC-MS analysis (**Supplementary Figures 1–5**) as potential C109 metabolites **(C)**.

strain (**Table 2**). As we previously isolated C109 resistant strains that carried mutations in the RND-9 regulator encoding gene (*BCAM1948*) or in the intergenic region, in between the first gene encoding RND-9 (*BCAM1947*) and the regulator (Scoffone et al., 2015), we sequenced the same region also in the two clinical isolates FCF19 and FCF22. However, no mutations were found in either the *BCAM1948* coding sequence or the intergenic region.

In order to determine whether the inhibition of the activity of efflux could affect the MIC of C109 in these strains, a checkerboard assay was set up using the compound C109 and the efflux pump inhibitor PaβN MC-207.110, as described above for *P. aeruginosa*. The MIC value of the C109 molecule in the presence of 128 μg/ml of the efflux inhibitor (**Table 2**) was reduced in both clinical isolates (from 128 μg/ml to 8 μg/ml for the strain FCF19 and from 256 to 16 μg/ml for FCF22), and comparable to the wild-type strain J2315. These data clearly indicate that an unidentified efflux mechanism is responsible for the resistance of these two clinical isolates.

Finally, the MIC of the C109 derivatives, having a lower MIC against *P. aeruginosa* (namely, 10026149 and 11426142), was determined also against the clinical isolates of *B. cenocepacia*, naturally resistant to C109, aiming to check whether they may be more effective and not subject to efflux. Both FCF19 and FCF22 clinical isolates showed resistance to the compound 10026149, whereas the MIC of the compound 11426142 was lower compared to the MIC of the compound C109 (32 μg/ml for both strains, **Table 2**). This value, which is identical to the MIC against *B. cenocepacia* J2315, did not change in the presence of different concentrations (0–128 μg/ml) of the MC-207.110 efflux inhibitor. In contrast, the MIC of 11426142 decreased from 32 to 2 μg/ml in the presence of MC-207.110 when the checkerboard assay was carried out using *P. aeruginosa*.

Metabolic Transformation of C109 Upon Contact With *B. cenocepacia* Cells

The TLC of the chromatographic fractions of C109 metabolites (**Figure 2**) showed that at least 6 different compounds could be isolated. All these fractions were subjected to UPLC-MS analysis to characterize the compounds, allowing us to identify four putative C109 metabolites, present in fractions 1 (M1), 2 (M2a and M2b) and 3 (M3) (**Figure 2** and **Supplementary Figures 1–4**). Moreover, fraction M5 partly contained untransformed C109 (**Supplementary Figure 5**), whereas it was not possible to identify the compounds present in fractions 4 and 6 (data not shown).

The identified metabolites should derive from hydrolysis of C109 between the sulfur atom and the benzothiazole or the methylacetate moieties, and from reductive reactions particularly on the nitro group of the compound. To verify if these conversions lead to the inactivation of the compound, the MIC of these metabolites was determined using the resazurin method. None of the metabolites retained an antimicrobial activity, being all the MIC values >256 μg/ml (data not shown).

B. cenocepacia Nitroreductase BCAL0539 Has the Potential to Inactivate C109

Since in three out of four identified metabolites of C109 the nitro group resulted to be reduced to amino, leading to loss of activity, we investigated whether an enzyme could be responsible for such reaction. Searching in the *Burkholderia* Genome

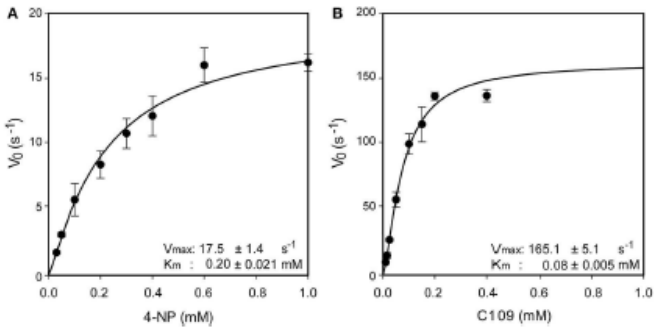


FIGURE 3 | Steady state kinetic analysis of BcNR, using 4-nitrobenzoic acid **(A)** and C109 **(B)** as substrate.

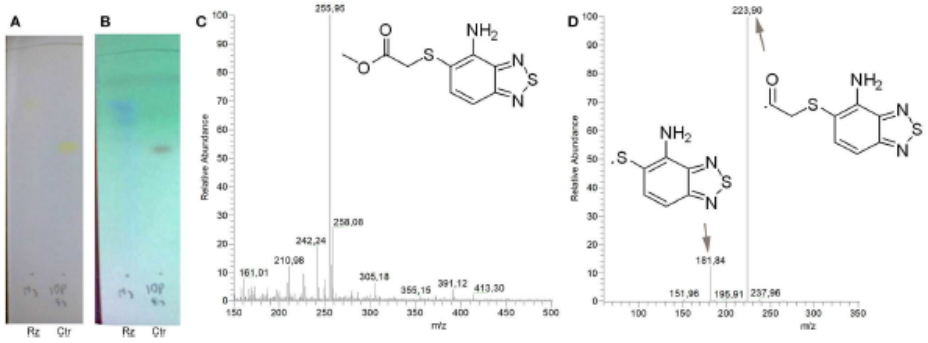


FIGURE 4 | Identification of BcNR C109 metabolites. Unstained **(A)** and UV light visualized **(B)** TLC analyses of the BcNR reaction using C109 as substrate (Rz), compared with the control performed with the same reaction mixture but without NADPH, demonstrated that the enzyme is able to transform C109 compound. The MS analysis [**(C)** full mass, and **(D)** fragmentation pattern] of the purified reaction products demonstrated that BcNR catalyzes the reduction of the *nitro* group of C109.

Database (www.burkholderia.com) we found the *BCAL0539* gene, which encodes a putative nitroreductase/4-nitrobenzoic reductase, belonging to the FMN—NAD(P)H-dependent nitroreductase family. To probe the role of this enzyme in the metabolism of C109, we produced the recombinant *B. cenocepacia* nitroreductase BCAL0539 (BcNR) protein in *E. coli*, and purified to homogeneity with a good yield (about 2 mg of protein per g of cells). The UV-vis spectrum of BcNR showed two peaks at 370 and 450 nm, demonstrating that the enzyme is expressed as a flavoprotein (**Supplementary Figure 6**). We found that the cofactor was not covalently linked to the enzyme because, after heat denaturation, the resuspended enzyme lost the two typical peaks of the flavin, which were instead present in the spectrum of the supernatant (**Supplementary Figure 7**). Since the enzyme is predicted to be a NAD(P)H dependent nitroreductase that uses 4-nitrobenzoic acid, we carried out a spectrophotometric activity assay, which follows the decrease in absorbance at 340 nm of the cofactor. Moreover, the enzyme activity was also assayed by using C109 as substrate. As shown in **Supplementary Figure 8**, BcNR is strictly NADPH-dependent, and is able to reduce both 4-nitrobenzoic acid and C109. Furthermore, the kinetic analysis showed that the enzyme is able to use C109 very efficiently, showing a specificity constant of 2063.7 ± 20.1 s^{-1} mM^{-1}, about 20-fold higher than that for 4-nitrobenzoic acid, due to a 10-fold higher V_{max} and about a 2-fold lower K_m values (**Figure 3** and **Supplementary Table 2**).

To identify and characterize the C109 reaction product of BcNR, 5 mg of the compound were incubated for 1 h with the enzyme, in the presence of NADPH. The reaction mixture was then extracted with DCM, products isolated by silica gel chromatography and analyzed by mass spectrometry (**Figure 4**). Only a major metabolite was found, corresponding to the analogous amino metabolite of C109 M3, isolated in cell cultures (**Figure 2**),

demonstrating that BcNR catalyzes the nitroreduction of the C109 compound.

To assess whether the resulting BcNR reduction product could inhibit *B. cenocepacia* growth, we carried out a resazurin-based assay. We set up three reactions: (i) a complete reaction in the presence of 1 mM C109; (ii) a reaction without the cofactor NADPH; (iii) a reaction without C109. All these reactions were assayed along with the C109 *nitro* derivative 11626109 (**Table 1**), revealing that the metabolite resulting from the complete reduction is not active against *B. cenocepacia* growth (as 11626109), while in the absence of NADPH the BcNR does not work, C109 is not reduced and, consequently, still active. All these data confirm a role of BcNR in the inactivation of the compound, thus suggesting a possible involvement of the enzyme in the resistance mechanism to C109.

For this reason, the *BCAL0539* gene expression was analyzed in the wild type J2315 and in the clinical isolates FCF19 and FCF22, which are resistant to C109 (see above). However, the *BCAL0539* gene was not expressed, either in the wild type or in the resistant strains, implying that the BcNR is not implicated in C109 inactivation and resistance mechanism.

Time-Lapse Microscopy of *B. cenocepacia* Treated With C109 Reveals a Fraction of Possible Persistent Cells

The killing dynamics of *B. cenocepacia* with the compound C109 at the bulk-population level were shown to be concentration dependent and biphasic (Scoffone et al., 2015). Biphasic killing represents the typical pattern of drug persistence, whereby the initial linear mortality of the population is followed by a second phase where a subpopulation of cells is able to endure inhibitory drug concentrations in the absence of genetic mutations (Van den Bergh et al., 2017). In contrast to drug-resistant cells, which continue to replicate in the presence of the drug, drug-persistent cells either derive from pre-existing non-growing cells or slow down their growth during drug exposure, and their progeny is killed with kinetics similar to that of the initial population (Van den Bergh et al., 2017; Balaban et al., 2019). Possible

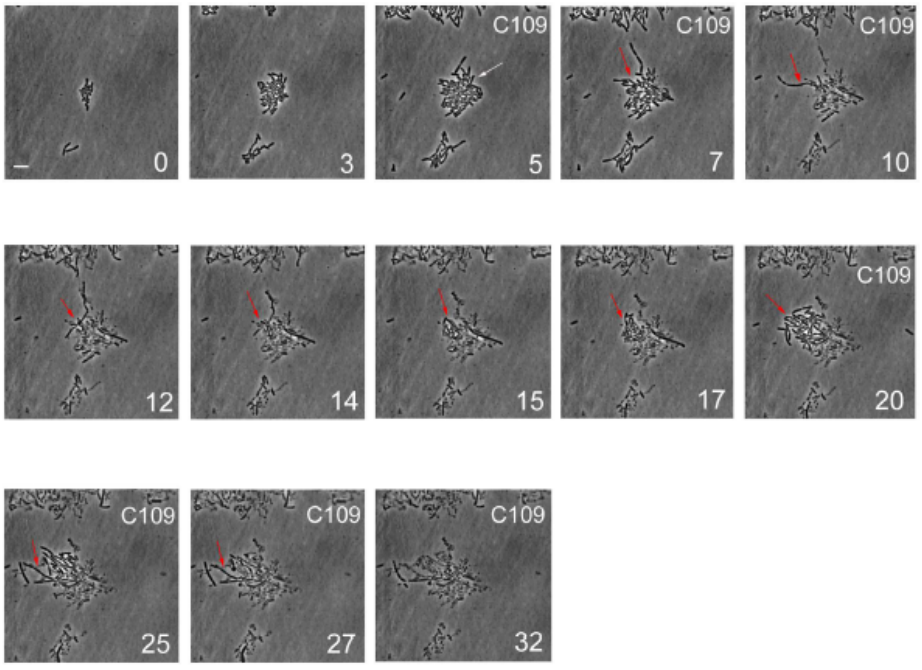


FIGURE 5 | Single-cell imaging of *B. cenocepacia* cells treated with C109. Contrast-phase image stacks of *B. cenocepacia* J2315 during the time-lapse microscopy in the microfluidic chamber. The white arrow indicates a cell that after the C109 treatment died, while a filamentous cell is indicated by the red arrow. Time is indicated in h at the bottom-right corner of each picture. C109 treatment (12.5X-MIC) is indicated on the top-right corner. Scale bar in the first picture is 5 μm.

mechanisms that can bring about heterogeneous responses to drugs, such as drug persistence, include uneven expression and partitioning of efflux systems among single bacterial cells (Pu et al., 2016; Bergmiller et al., 2017). Importantly, the mechanism of resistance to the C109 compound is mediated by the RND-9 efflux transporter (Scoffone et al., 2015). To examine the C109 killing dynamics of *B. cenocepacia* at the single-cell level, we carried out a time-lapse microscopy experiment, divided into several steps. Wild type *B. cenocepacia* J2315 cells were first seeded into a microfluidic device (Manina et al., 2019), and perfused with prewarmed 7H9 medium for 3 h, to allow the cells to adapt to the microfluidic environment. After a few events of cell division, the cells were exposed to 100 μg/ml of C109 (12.5X-MIC for the wild-type strain used in this work) for 4 h, equivalent to about 4 replication cycles. Cells were then perfused with C109-free 7H9 medium for 12 h, enabling the recovery and expansion of surviving cells. Finally, to understand whether the survivors had acquired genetic resistance, cells were exposed a second time to C109 (12.5X-MIC) for 24 h, equivalent to about 24 replication cycles. During the time-lapse imaging, cells were monitored every 20 min for a total of 46 h. The total number of cells, the single-cell area, and the number of division and lysis events were measured during each step of the experiment.

During the first 3 h phase, *B. cenocepacia* cells were able to adapt to the microfluidic device and replicate every 60 min (**Figures 5**, **6**). After the first treatment with C109 (12.5X-MIC), 70% of the cells died from lysis(**Figures 5**, **7A**) and the growth rate and the number of cell divisions decreased (**Figures 6**, **7B**), until cells stopped dividing (**Figure 7B**). During the recovery phase in fresh 7H9 medium, 3.5 % of the remaining intact cells (30%) were able to resume growth and the cell number and the division rate increased again (**Figures 5**, **7A,B**). After, the cells that had survived and resumed growth were treated for the second time with C109 (12.5X-MIC), we found that the compound caused again a decline in cell population by 92%, and finally cells stopped dividing (**Figure 7A**). Importantly, during the second treatment with C109 the population derived from the subpopulation of survivors showed a lysis rate comparable to the lysis rate of the initial population subject to the first treatment with C109, implying the absence of stable genetic resistance in the survivors, and reminiscent of a phenotype conceivably ascribable to persistent cells (**Figure 7B**).

From the single-cell point of view, the *B. cenocepacia* cell population exhibited two different phenotypes in the presence of C109 compound. During the first treatment with C109, 70% of the cells slowly decreased their rate of growth and division (**Figures 5**, **6B**, **7B**), increasing their area of 2-fold, and then died by lysis (**Figures 5**, **7C**). These results were expected and consistent with previous observations upon inhibition of an essential component of the cell divisome by treatment with C109, and in case of other FtsZ inhibitors (Scoffone et al., 2015; Hogan et al., 2018; Tripathy and Sahu, 2019). The lytic phenotype could be explained because FtsZ is an essential component of the cell divisome and controls the cell wall biosynthesis at the septum site (Yang et al., 2017). Interestingly, 3.5% of cells decreased their growth rates, but were still able to grow and underwent considerable filamentation, increasing the cell area up to 5-fold

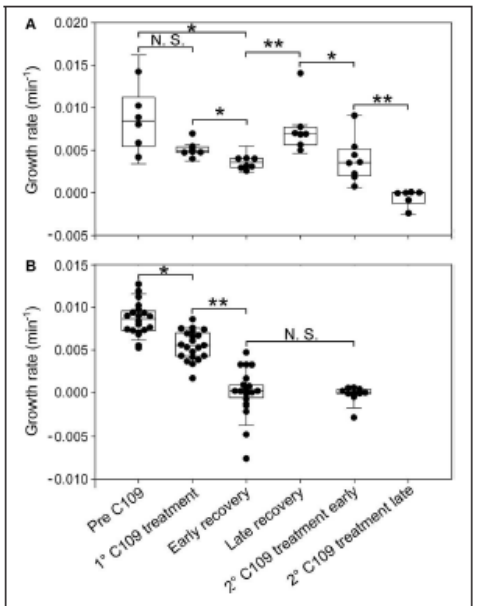


FIGURE 6 | Single-cell analysis of growth rate for different cell categories. Single-cell growth rate of survivor cells **(A)** and cell dead by lysis **(B)** prior to and following C109 exposure ($n = 30$). Early recovery was defined between 6 and 10 h and late recovery was defined between 10 and 18 h, from the beginning of the experiment. 2nd C109 treatment early is defined between 19 and 22 h and 2nd C109 treatment late is defined between 22 and 46 h from the beginning of the experiment. The data shown are from 2 independent experiments. Black lines indicate mean ± SD. Asterisks denote significance by Paired samples *T*-test: N.S., not significant; $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

(**Figures 5**, **6A**, **7C** and **Supplementary Movie 1**). During C109 washout with the fresh medium, 30% of these filamentous cells started again to divide from both poles, decreasing the cell area to 1.5 μm^2, which is comparable to the size of healthy wt cells (**Figures 5**, **6A**, **7B,C** and **Supplementary Movie 1**), while the other cells remained frozen to the initial state. To determine whether the cell population that expanded from the cells that survived the first treatment with C109 had become genetically resistant, we treated the cells a second time with the C109 compound for 24 h. The population showed again both phenotypes; namely, 94% of the cells stopped to divide during the treatment (**Figures 5**, **6A**, **7A,B** and **Supplementary Movie 1**), while a smaller population (about the 6% of the total cell number) showed again the elongated phenotype (**Figures 5**, **7C** and **Supplementary Movie 1**). These cells were unable to divide but increased their area up to 10-fold (8–10 μm^2) the normal size of the cell (**Figures 5**, **7C** and **Supplementary Movie 1**). Then, these elongated cells stopped to increase their area and died during the exposure to C109 (**Figures 5**, **7C** and **Supplementary Movie 1**).

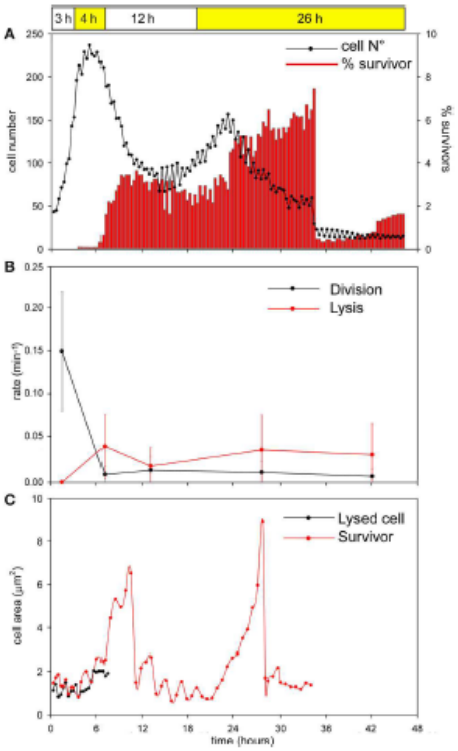


FIGURE 7 | Single-cell analysis of *B. cenocepacia* cells treated with C109. **(A)** Total cell number (black line) and percentage of filamentous cells (red bars) for each time point during the time-lapse experiment. Data points are mean values of 8 different frames taken during two independent experiments. **(B)** Rates of cell division and lysis (mean ± SEM). Data points are mean values of different frames ($n = 10$) before, during and after C109 treatment, taken during two independent experiments. **(C)** Cell area measurements (μm^2) of two-cells: cell dead by lysis (black line) and survivor filamentous cell (red line). The two cells are representative of 50 cells analyzed in two independent experiments.

These results indicate that *B. cenocepacia* cells exposed to C109 produce a subpopulation with a phenotype ascribable to possible persistent cells. In particular, these survivor cells remain elongated in presence of the compound but restore their normal size and life cycle once the C109 is removed, giving rise to a new non-filamentous population. The latter population, once treated again with C109, shows again both phenotypes, i.e., a fraction of cells (94%) that is again sensitive to C109 and a second fraction of elongated cells (6%) that is still sensitive to C109 extended exposure, and does not show any sign of resistance. Among a representative population of 3,000 elongated cells that formed during the first exposure to C109, 8 cells (0.27%) resumed normal growth during drug washout and, being sensitive to the second exposure to C109, were ascribable to possible persistent cells.

DISCUSSION

Burkholderia spp infections represent a problematic threat for CF patients. However, due to the low infection rate, the development of novel antimicrobials has been limited and remains an urgent priority for these patients (Regan and Bhatt, 2019). For this reason, in the past few years we identified and characterized the FtsZ inhibitor C109, which showed strong activity against *B. cenocepacia*, including several clinical isolates (Scoffone et al., 2015; Hogan et al., 2018). Moreover, this compound proved to be active against both planktonic and sessile *B. cenocepacia* strains, and against different other Gram-positive and -negative bacteria (Hogan et al., 2018; Costabile et al., 2020).

However, despite its great potential, this compound displays two main drawbacks. First, we showed that C109 is a substrate of the RND-9 efflux pump (Scoffone et al., 2015), and efflux pumps are known to be among the most common mechanisms of resistance to antibiotics in *B. cenocepacia* (Perrin et al., 2010). Second, the compound is characterized by the presence of a nitro group, which is considered a structural alert in a potential drug, due to its possible association with mutagenicity and genotoxicity (Nepali et al., 2019). In addition, this nitro group could be the substrate of modifying nitroreductase enzymes, which can alter the efficacy of the compound (Nepali et al., 2019).

In particular, we explored the effects of the typical metabolic reactions of hydrolysis, oxidation and reduction on the activity of C109. However, none of the synthesized derivatives showed improved potency. Similarly, the modification introduced to synthetize close derivatives, such as acetylation, methylation of the benzothiadiazole group, substitution of the sulfur atom with oxygen or nitrogen atom, or the extension of the side chain, gave no improvement.

It is noteworthy that the majority of the derivatives, showing worsened potency in *B. cenocepacia* and in *E. coli*, maintained a similar or improved activity in *S. aureus*, further confirming that the main drawback of these compounds is the difficulty to access the cell, or to be maintained into Gram-negative cells. Indeed, the main issue of these compounds is that they are good substrate of efflux pumps, as we demonstrated in both *B. cenocepacia* clinical isolates and *P. aeruginosa*.

The other drawback of our benzothiadiazole compound is the presence of an essential nitro group, that could be subjected to different modifications. It is noteworthy that modifications occurring to nitro group could lead to the inactivation of the drug (Nepali et al., 2019), whereas in the case of pro-drugs these modifications can cause their activation (Mori et al., 2017). Thus, to better characterize the behavior of C109, we investigated how it can be metabolized by *B. cenocepacia* cells. As expected, the main transformations taking place are reductive reactions on the nitro moiety, and hydrolytic reactions occurring between the sulfur atom linking the benzothiazole with the methylacetate. We were able to isolate and characterize certain C109 metabolites, but

all of them were shown to be inactive, demonstrating that the compound is not subjected to any activation process. However, having found that C109 is intracellularly inactivated through nitroreduction, we looked for possible enzymes responsible for this reaction. We found that *B. cenocepacia* possesses a gene encoding the putative nitroreductase BcNR. However, we exclude that BcNR confers resistance to the compound, being its basal level of expression negligible. At this stage, we cannot exclude that there are other possible endogenous nitroreductases that could be responsible for the inactivation of C109 in *B. cenocepacia*, although none has been reported in the literature so far.

The C109 compound is also particularly susceptible to the action of the RND-9 efflux pump, which is over-expressed in two clinical isolates we tested (Papaleo et al., 2010; Scoffone et al., 2015). Remarkably, stochastic expression of efflux pumps can induce a fraction of the bacterial population to become persistent to drugs (Pu et al., 2016; Bergmiller et al., 2017). Furthermore, it has been recently proposed that recurrent drug-persistent infections, such as those caused by *B. cenocepacia* in CF patients, may promote the onset of genetic drug resistance (Balaban et al., 2019). Here we decided to assess the single-cell behavior of a clonal population of *B. cenocepacia* J2315 when confronted with the compound C109, by using quantitative time-lapse imaging. Although the majority of the bacterial population stopped dividing and died during treatment, consistent with the essentiality of FtsZ and with the mislocalization of FtsZ-GFP in *E. coli* cells treated with C109 (Hogan et al., 2018), a small fraction of *B. cenocepacia* cells was still viable and exhibited a considerable filamentous phenotype, which reflects the ability of a subpopulation to tolerate dysfunctional cytokinesis. During the recovery in C109-free medium, these surviving cells gave rise to a new population with normal cell size that, following a second treatment, was still sensitive to C109 and did not develop stable resistance to the compound. These results imply that these transient filamentous cells could be considered cells with a phenotype ascribable to possible persisters, which will be subjected to further investigations. Interestingly, we found that cells that survived the C109 compound originated from metabolically active cells, which had a growth rate comparable to drug-sensitive cells, as previously described in different bacterial species (Van den Bergh et al., 2017). Although non-growing populations are usually enriched with drug-persistent cells, the phenomenon of persistence is not exclusively linked to the absence of growth ahead of drug exposure, but is multifactorial and can also be associated with the environmental conditions; species-specific factors; pre-existing cell-to-cell phenotypic variation; the drug class and the dynamics of drug exposure (Van den Bergh et al., 2017; Balaban et al., 2019; Goormaghtigh and Van Melderen, 2019; Manina et al., 2019). Here we report that the single-cell growth rate prior to C109 exposure is not predictive of survival toward C109 compound in *B. cenocepacia*. At present, we cannot discriminate whether C109-survivors might result from the exposure to the compound, or might derive from a subpopulation having a distinct phenotype before exposure to the compound. In the future, it will be interesting to explore whether stochastic over-expression of RND-9 or of another efflux pump may be responsible for the onset of persistence in a subpopulation of *B. cenocepacia*. We think that a better understanding of this phenomenon may help to conceive a drug combination that makes C109 more effective against *B. cenocepacia*.

In conclusion, within this work, we thoroughly characterized the potential of the C109 scaffold for the development of new antimicrobial compounds against microbial species that are life-threatening for CF patients. Despite most of the derivatives being effective against the cellular target FtsZ *in vitro*, none showed improved activity against Gram negative bacteria. However, five of these derivatives (10026149, 11026176, 11026177, 11126010, 11426142) showed promising activity against the CF and nosocomial pathogen *S. aureus*, suggesting another possible application for this class of compounds.

DATA AVAILABILITY STATEMENT

All necessary datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

SB, VM, and GR contributed conception and design of the study. LC expressed and purified the proteins and performed enzymatic and metabolic assays. VS performed cloning, enzymatic assays, and time lapse experiments. GT expressed and purified the proteins and performed qRT-PCR. GB analyzed data. OR and NM synthesized and analyzed all tested compounds. AP performed MS assays and analysis. GM performed time lapse experiments. SB performed microbiological experiments. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00562/full#supplementary-material

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Molecular Characterization of the *Burkholderia cenocepacia dcw* Operon and FtsZ Interactors as New Targets for Novel Antimicrobial Design

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Abstract: The worldwide spread of antimicrobial resistance highlights the need of new druggable cellular targets. The increasing knowledge of bacterial cell division suggested the potentiality of this pathway as a pool of alternative drug targets, mainly based on the essentiality of these proteins, as well as on the divergence from their eukaryotic counterparts. People suffering from cystic fibrosis are particularly challenged by the lack of antibiotic alternatives. Among the opportunistic pathogens that colonize the lungs of these patients, *Burkholderia cenocepacia* is a well-known multi-drug resistant bacterium, particularly difficult to treat. Here we describe the organization of its division cell wall (*dcw*) cluster: we found that 15 genes of the *dcw* operon can be transcribed as a polycistronic mRNA from *mraZ* to *ftsZ* and that its transcription is under the control of a strong promoter regulated by MraZ. *B. cenocepacia* J2315 FtsZ was also shown to interact with the other components of the divisome machinery, with a few differences respect to other bacteria, such as the direct interaction with FtsQ. Using an in vitro sedimentation assay, we validated the role of SulA as FtsZ inhibitor, and the roles of FtsA and ZipA as tethers of FtsZ polymers. Together our results pave the way for future antimicrobial design based on the divisome as pool of antibiotic cellular targets.

Keywords: cell division; FtsZ; *Burkholderia cenocepacia*; drug resistance; new drug targets

1. Introduction

Bacterial division is a very complex and spatiotemporally regulated process in which several proteins cooperate for the formation of the divisome. The process integrates DNA replication, chromosome segregation and septum formation making its full characterization very challenging [1,2]. Nevertheless, within the past 30 years many components of the divisome have been discovered and functionally characterized, in particular in the model organisms *Caulobacter crescentus* [3], *Escherichia coli* [4] and *Bacillus subtilis* [5]. In *E. coli*, division machinery formation is composed of two temporally distinct phases: the early and the late. The assembly of the essential part of the divisome is primed by the polymerization of the tubulin-like protein FtsZ at the mid cell, stabilized and anchored to the inner membrane by the interactions with FtsA and ZipA [6]. The proteins ZapA, ZapC and ZapD cross-link FtsZ polymers increasing their stability [7–9]. The localization of the protein complex FtsEX (the main regulator of the peptidoglycan hydrolysis at the septum for the modeling of the new cell poles) to the forming Z ring, via FtsE-FtsZ interaction, concludes the early phase [10]. Then, the late phase occurs, after a physiological programmed delay, recruiting concurrently: FtsK,

a DNA translocase and a linker of the downstream proteins to the ring [11,12]; FtsQ, FtsL, FtsB, known to form a complex acting as a protein scaffold but also having regulatory activity [13]; FtsW, recently emerged as a peptidoglycan glycosyltransferase [14]; FtsI, the ring peptidoglycan transpeptidase [15]; and FtsN, triggering the activation of the machinery and starting the septation [16].

The key component of this structure is definitely FtsZ, the prokaryotic homolog of the tubulin, that shares with its eukaryotic counterpart the GTPase activity and the related ability to form dynamic polymers through head-to-tail interactions, even though the assembly occurs with an opposite polarity [17]. During the divisome assembly, FtsZ directly interacts with most of the proteins involved becoming, after the polymerization, their molecular scaffold [18]. Moreover, the FtsZ interactome includes negative modulators that prevent the Z-ring formation in wrong places, such as: MinCD, that inhibits its formation at the cell poles [19]; SlmA, that prevents its assembly over the nucleoid [20]; SulA, that blocks the polymerization of FtsZ in case of SOS response induction [21]. The role of the FtsZ polymers is not limited to the structural function, indeed they are thought to generate the force that lead to mid cell constriction [22] and guide the synthesis of septal wall through a treadmilling behavior [23,24].

In *E. coli*, the gene *ftsZ*, together with *ftsL*, *I*, *W*, *Q*, *A*, is localized within the division cell wall (*dcw*) operon, a gene cluster involved in bacterial division and cell wall synthesis [25]. This cluster is extremely conserved across bacteria, even if gene number and order change following the cell shape [26]. For this reason, the above mentioned genes are thought to be the only ones absolutely required for a successful division, since all the others, encoded outside the *dcw* region, are demonstrated to be not essential in certain conditions [4]. The importance of this cluster lies also in its complex transcriptional control, probably contributing to the spatiotemporal coordination of the division. The complexity is given by many promoters and regulatory factors within the *dcw* sequence, required to modulate the expression of different transcriptional units based on growth phase and condition [25,27]. This regulation is not fully characterized yet, but in general it follows different pattern in different organisms [28–32].

In several bacteria the *dcw* operon includes, often at the 5′ of the cluster, the gene encoding the transcriptional regulator MraZ, classified within the AbrB-like superfamily of DNA-binding proteins [33,34]. Although its DNA-binding ability had already been hypothesized, only recently Eraso et al. [35] demonstrated its role in the transcriptional control of the *dcw* operon in *E. coli*: by binding a repeated sequence upstream of the first gene of the operon, MraZ represses its transcription starting from the promoter P_{mra} (*mraZ1p*). This regulation represses the expression of the first 11 genes, including its own, from *mraZ* to *murC* [35]. Furthermore, MraZ was demonstrated to affect about 23% of *E. coli* gene expression during the early logarithmic phase [35]. Despite its almost ubiquitous presence in prokaryotes, MraZ has a different role in phylogenetically distant organisms, since in mycoplasmas it was described as a transcriptional activator [36].

This increasing knowledge of the division mechanisms has risen the awareness on the potentiality of this pathway as a pool of new drug targets, given the essentiality of most proteins involved. Indeed, blocking a single component of this machinery often leads to the impairment of the entire process. In a worldwide context of antimicrobial resistance spreading, the research of novel potential cellular targets is necessary. The decreasing of the antibiotic efficacy represents a threat principally for patients suffering from recurrent infections, as people with cystic fibrosis (CF). Indeed, respiratory failure caused by chronic lung infections represents the leading cause of death for these patients [37]. Many opportunistic pathogens colonize the lungs of these people but *Burkholderia cenocepacia*, a member of the *Burkholderia cepacia* complex [38], is notorious as one of the deadliest. Upon infection, it is associated in most cases to a fast decline of pulmonary function and, in up to 20% of the infected patients, it causes a fulminant necrotizing pneumonia [39]. Moreover, it can spread from patient-to-patient, as demonstrated from the devastating outbreaks in Europe, Canada, and USA [40]. The presence of several virulence factors in its uncommonly large genome [41], and the acquisition of pathogenicity islands [42] explain the high levels of virulence of the infections. Besides, the *B. cenocepacia* natural resistance to almost every available antibiotic makes its eradication extremely challenging [43–45].

In recent years, we found a newly synthesized benzothiadiazole derivative, C109, showing bactericidal activity at low doses against *B. cenocepacia* [46]. This compound resulted to be a potent FtsZ GTPase inhibitor, able to impair the division of a broad-spectrum of Gram-positive and -negative pathogens, given the high conservation of the molecular target across bacteria [47]. Further in-depth characterizations [48] and the development of a PEGylated nanoparticles formulation for aerosol administration [49] validated the C109 as a promising novel antibacterial compound. Moreover, the reported activity of FtsZ inhibitors against *Staphylococcus aureus* and CF Gram-negative pathogens corroborates the potential of this protein as molecular target for new antimicrobials [50]. However, also FtsZ interactors, especially the proteins encoded in the *dcw* cluster, are considered excellent candidates, since the inhibition of their activities, besides the perturbation of their interactions with FtsZ, can critically impair bacterial division, leading to bacterial death [50].

In this study, we aimed at performing the transcriptional analysis of the *dcw* operon and the characterization of its regulator MraZ in *B. cenocepacia*, using molecular techniques. We also wanted to identify the FtsZ interactors using a bacterial adenylate cyclase two-hybrid system, as well as co-sedimentation assays. The achieved results provide new insights into *B. cenocepacia* division mechanisms, focusing the attention on the essential divisome proteins transcription and on their molecular interactions, giving an overview of the key elements of this pathway.

2. Results

2.1. Characterization of the dcw Operon in B. cenocepacia J2315

2.1.1. The *dcw* Cluster of *B. cenocepacia* J2315 Has the Conserved Gene Organization Shared by Rod-Shaped Bacteria

To determine the localization and the gene organization of the *dcw* operon in *Burkholderia cenocepacia* J2315, the *Burkholderia* genome database (https://www.burkholderia.com) [51] was used. The cluster encompasses a DNA region of 19.158 bp (3803412–3784254) and is composed of 17 genes (Figure 1), localized on the negative strand with the same transcriptional polarity. It is located on chromosome 1 [41] and, despite the remarkable gene redundancy in the *B. cenocepacia* huge genome, it is present in a single copy. Regarding gene organization, it shows the same arrangement seen in *Escherichia coli*, containing the same closely packed genes from *mraZ* to *ftsZ*, with the only remarkable difference found in the insertion of the gene BCAL3456, a thioredoxin reductase, between *ftsZ* and *lpxC* (*envA*) (Figure 1).

Figure 1. Representation of the 17 genes, and their transcriptional orientation, composing the *dcw* cluster on chromosome 1 of *B. cenocepacia* J2315. The main promoter P_{mra}Bc is represented by the black arrow at the 5′ of the operon, whereas the only transcription terminator is localized at the 3′, downstream of *lpxC*.

2.1.2. The dcw Operon is Transcribed as a Polycistronic mRNA from mraZ to ftsZ

In order to determine whether the *dcw* operon is subdivided into transcriptional units, its entire DNA sequence was analyzed using the bacterial rho-independent transcription terminator prediction software TransTermHP (http://transterm.ccb.jhu.edu/query.php) [52]. A transcription terminator site (3784225–3784210) was found downstream of the gene *lpxC*, but no other putative terminators were found within the operon sequence. For this reason, it has been speculated that the cluster could be transcribed as a single polycistronic mRNA. To confirm this theory, a RT-PCR approach was used. Primers were designed to test the co-transcription of gene pairs, analyzing the whole sequence from *mraZ* to *lpxC*. The RNA was extracted from cells during mid-log phase in LB medium, and subsequently

retrotranscribed using the reverse primers listed in Table S1, each one designed to anneal at the 5′-end of a different *dcw* operon gene. The resulting cDNAs were used as templates in PCRs performed with the same reverse primers used for retrotranscription coupled with forward primers complementary to the 3′-end of the upstream gene. The primer pairs and the corresponding fragment lengths are reported in Table S1. The amplicons of all the gene pairs from *mraZ* to *ftsZ* were obtained, confirming that each gene is transcribed together with the flanking neighbor genes, demonstrating that in *B. cenocepacia* J2315 they can be transcribed as a single polycistronic mRNA of about 17 kb. Conversely, no fragments were amplified when cDNAs of the gene pairs *ftsZ-BCAL3456* and *BCAL3456-lpxC* were used as PCR templates, suggesting that the two genes are transcribed as single mRNAs. However, as mentioned above, no transcription terminators have been found downstream of the genes *ftsZ* and *BCAL3456* using the TransTermHP software, probably because a different mechanism, such as rho-dependent transcription terminators, is involved in blocking the transcription.

Subsequently, the presence of a promoter able to activate the transcription of the first 15 genes of the operon, was investigated. In *E. coli*, the transcription of the first 9 genes of the *dcw* operon starts 38 bp upstream of the *mraZ* gene and is under the direct control of the P_{mra} promoter [53]. A bioinformatic analysis of the non-coding region upstream of the *B. cenocepacia mraZ* gene (3803413–3803798) was carried out using the BDGP Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html), employing the default parameters for predictions of promoters in prokaryotes. The search resulted in the identification of a 50 bp putative promoter sequence starting 141 bp upstream of the first translated codon of *mraZ* (Figure 2).

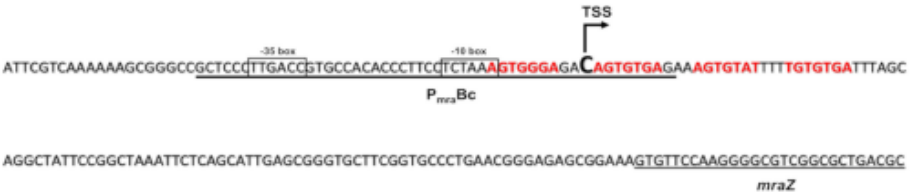

Figure 2. Organization of the P_{mra}Bc promoter sequence. The promoter sequence (P_{mra}Bc) is underlined in bold, with the −10 and −35 boxes, the cytosine representing the transcription start site (TSS) of the *dcw* operon, and the repeats of the putative MraZ binding site highlighted in red.

In order to experimentally confirm its promoter activity, two overlapping fragments of 289 bp and 161 bp, respectively, were amplified using the primers dcwPROMOfor1_2 and dcwPROMOrev2, and dcwPROMOfor2_2 and dcwPROMOrev2 (Table S2). These were cloned into the pSU11 vector, in which the MCS is upstream of a promoterless *lacZ* gene, obtaining pSU11-161 and pSU11-289 (Table S3). The two plasmids were transferred from *E. coli* to *B. cenocepacia* K56-2 by conjugation and the expression of LacZ was assessed by β-galactosidase activity assay, culturing the cells in LB medium and harvesting them in early stationary phase. The tested fragments had comparable activities (empty pSU11: 2.2 ± 0.92; pSU11-161: 101.8 ± 4.7 MU; pSU11-289: 87.7 ± 5.6 MU) (Figure 3A), demonstrating that the putative sequence has indeed promoter activity and that the 161 bp fragment contains the minimal transcription promoter P_{mra}Bc (Figure 2) of the *dcw* operon in *B. cenocepacia*.

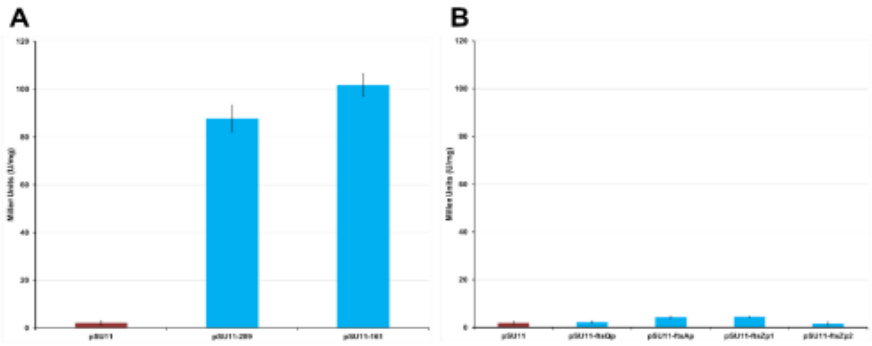

Figure 3. Comparison of the promoter activities of the tested fragments expressed in Miller units. (**A**) Both fragments, 289 bp (pSU11-289) and 161 bp (pSU11-161), amplified from the intergenic region upstream of *mraZ*, have a strong promoter activity, containing the promoter of the first 15 genes of the *dcw* operon P_{mra}Bc. (**B**) The four fragments covering the *ddl-ftsA* region of the *dcw* cluster show a promoter activity comparable to the negative control (empty pSU11 vector, red bars), demonstrating the absence of additional promoters. Results are expressed as mean of 3 experiments and the error bars represent the standard deviation.

As the expression of *ftsZ* is known to be strictly regulated in many microorganisms [29,31,54], the presence of additional promoters in the *ddl-ftsA* region of *B. cenocepacia* J2315 was investigated. To this purpose BDGP Neural Network Promoter Prediction software was used, as previously described. From this analysis, no putative promoters were predicted, but nevertheless the sequence was experimentally tested using *lacZ* fusions. Thus, four sequential fragments were amplified: ftsQp, using murCPROMOfor1 and ddlPROMOrev1; ftsAp, using ftsQPROMOfor1 and ftsQPROMOrev1; ftsZp1, using ftsAPROMOfor1 and ftsAPROMOrev1; ftsZp2, using ftsAPROMOfor2 and ftsAPROMOrev2. In this way, the whole *ddl-ftsA* region was tested (Table S2, Supplementary Materials Figure S1). These fragments were cloned upstream of the promoterless *lacZ* gene into the pSU11 vector, creating the pSU11-ftsQp, pSU11-ftsAp, pSU11-ftsZp1, pSU11-ftsZp2 (Table S3). The promoter activity of the fragments was tested in *B. cenocepacia* K56-2 by β-galactosidase activity assay. For this experiment, the cultures were harvested when entering the stationary phase, having an OD_{600} around 2. Indeed, in *E. coli* several promoters of this region increase their activity during the stationary phase, once the growth rate decreases [54]. Despite this, all the tested sequences showed no activity (empty pSU11: 1.93 ± 0.55 MU; pSU11-ftsQp: 2.20 ± 0.38 MU; pSU11-ftsAp: 4.22 ± 0.49 MU; pSU11-ftsZp1: 4.38 ± 0.21 MU; pSU11-ftsZp2: 1.63 ± 0.71 MU) (Figure 3B), confirming that under standard laboratory culture conditions transcription starts only from the P_{mra}Bc, with no intermediate promoters able to further regulate *ftsZ* expression.

Finally, the transcription start site was identified as described in Materials and Methods as a cytosine located 100 bp upstream of the first translated codon of *mraZ*, at the 3′-end of the P_{mra}Bc promoter sequence (Figure 2).

2.1.3. The Protein Mraz Is the Transcriptional Regulator of the dcw Operon

In order to identify the DNA binding site of *B. cenocepacia* MraZ, an electrophoretic mobility shift assay (EMSA) was performed. First, MraZ was expressed and purified as described in Materials and Methods. Then, two fragments were amplified using the 6-carboxyfluorescein (6-Fam) labeled primers. The two probes overlapped for 137 bp and encompassed the whole intergenic DNA region upstream of the gene *mraZ*. In particular, fragment BS1-6-Fam, containing the 5′ half of the region from −386 bp to −136 bp upstream of *mraZ* gene, was obtained using the primers mraZBS1for and mraZBS1rev

(Table S2); instead, fragment BS2-6-Fam, containing the 3′ half of the region from −250 bp to −1 bp upstream of gene *mraZ*, was amplified with mraZBS2for and mraZBS2rev (Table S2). Both probes were 250 bp long and were tested in the presence of different MraZ concentrations in the assay, demonstrating that the protein is able to bind BS2-6-Fam (Figure 4A and Figure S2), but not BS1-6-Fam. The specificity of the binding was confirmed by adding a 20× excess of non-labeled BS2 fragment or a 20× excess of a non-specific 250 bp competitor in the binding reaction (Figure 4B and Figure S2). Subsequently, the BS2 fragment sequence was analyzed to find a putative MraZ DNA binding site, searching for a repeated GTG motif, known to be conserved in the binding sites of evolutionary distant bacteria, such as *B. subtilis*, *E. coli* and mycoplasmas [36]. The sequence was identified as series of four repeats of seven nucleotides separated by a 3-nucleotide spacer region (Figure 2). In particular, it is located downstream of the putative P_{mra}Bc −10 box, as in *E. coli* [35], and it overlaps with the *dcw* operon transcription start site (Figure 2).

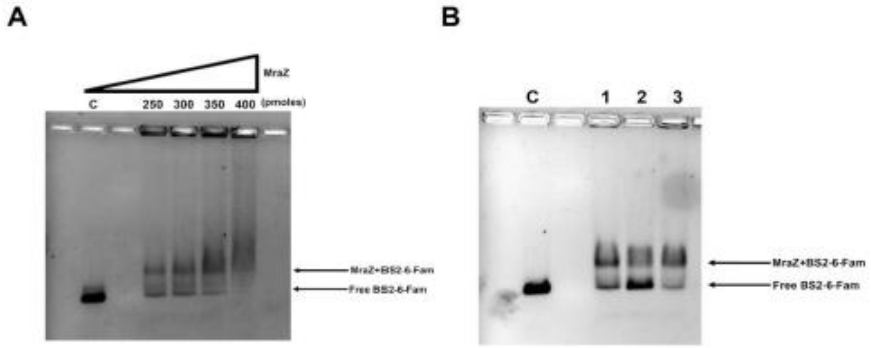

Figure 4. Electrophoretic mobility shift assay of the MraZ protein. (**A**) The gel represents the band shift using a constant BS2-6-Fam DNA amount (C, 0.34 pmoles) and increasing quantities of MraZ. (**B**) Demonstration of the specificity of the binding of MraZ to the fragment BS2, using a fixed amount of protein (300 pmoles) and BS2-6-Fam (0.34 pmoles) (lane 1), a 20× excess of non-labeled BS2 fragment which competes for the binding of MraZ (lane 2), or a 20× concentration of non-specific, non-labeled competitor (a 250 bp *cepI* fragment) (lane 3).

2.2. *Study of the FtsZ Interactors in B. cenocepacia In Vivo and In Vitro*

2.2.1. BACTH Analysis of *B. cenocepacia* Cell Division Proteins

To explore the divisome of *B. cenocepacia*, proteins homologous to the well characterized *E. coli* cell division machinery were selected. First, the designed proteins (FtsA, SulA, ZipA, ZapA, FtsE, FtsQ, FtsI, FtsN) were tested for pairwise interactions with FtsZ using the Bacterial Adenilate Cyclase Two Hybrid (BACTH) assay. DNA fragments encoding these proteins were systematically cloned into the four vectors of the BACTH system (pUT18, pUT18C, pKT25 and pKNT25) to obtain recombinant plasmids expressing the proteins of interest fused at the C- or N-terminus of either the T18 or the T25 fragment of the *Bordetella pertussis* adenylate cyclase. To test the putative interactions between FtsZ and the other proteins, an *E. coli cya* deficient strain (BTH101) was used and co-transformed with pairs of recombinant vectors expressing the T18 and T25 protein hybrids. The functional complementation efficiencies between the hybrids were evaluated using β-galactosidase activity (in solid and in liquid media). Only the pairs of vectors that showed a significant β-galactosidase activity on solid media underwent further investigations (quantification of the β-galactosidase in liquid medium and in vitro protein-protein interaction studies).

The overexpression of the selected proteins did not affect the growth of the *E. coli* host cells (data not shown). As negative control, the *E. coli* BTH101 strain transformed with the two empty vectors pUT18 and pKT25 was used. For each protein, the four different constructs (Table S3) were used to test interaction with FtsZ by qualitative evaluation of β-galactosidase activity on solid medium. Levels of β-galactosidase activity at least four-fold higher than that measured for BTH101(pKT25/pUT18) cells were considered indicative of an interaction. As shown in Figure 5, the BTH101 strain co-transformed with the vectors both expressing FtsZ has a β-galactosidase activity five-fold higher than the control strain. This result indicates that FtsZ monomers interact each other with a head to tail interaction, as previously reported in *E. coli* [55]. Indeed, this kind of interaction is necessary for the GTPase activity and protein polymerization of FtsZ.

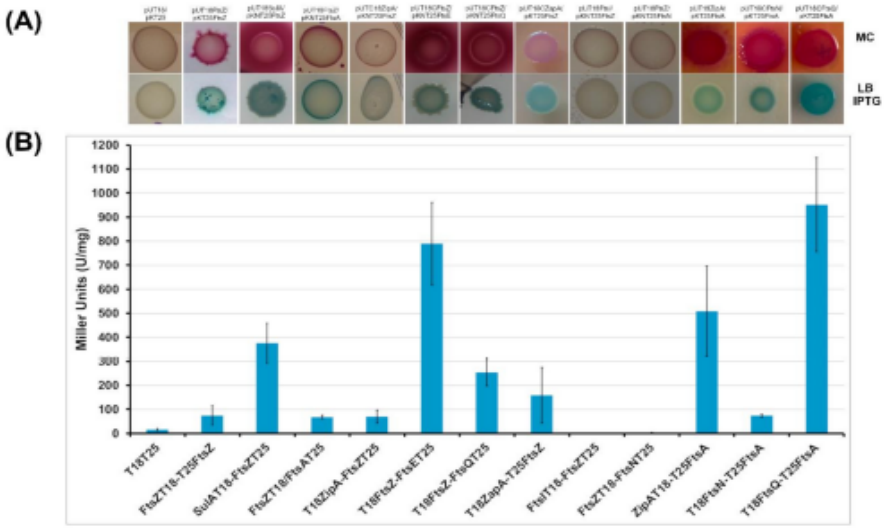

Figure 5. BACTH analysis of interactions between Fts proteins. (**A**) *Escherichia coli* strain BTH101 was co-transformed with two-hybrid vector plasmids (Table S3) expressing different hybrid proteins, as indicated. Transformants were spotted onto nutrient agar plates containing IPTG (LB IPTG) and X-Gal, or on MacConkey agar plates (MC) and incubated at 30 °C for 40 h. Blue coloration on LB IPTG or pink coloration on MC indicates a positive interaction. (**B**) The efficiencies of functional complementation between the indicated hybrid proteins were quantified by measuring β-galactosidase (expressed in Miller Units) activities in suspensions of *E. coli* BTH101 cells harboring the corresponding plasmids, as described in Materials and Methods. Each bar represents the mean value from results of at least three independent cultures. The *E. coli* BTH101 harboring the empty vectors was used as control.

Among proteins involved in cell division control, in *E. coli* and in *Pseudomonas aeruginosa* SulA has been described to block FtsZ activity in case of DNA damage [56]. The putative SulA of *B. cenocepacia* is codified by the gene pBCA006. The BACTH assay showed that the putative SulA interacts using its N-terminal part with FtsZ (Figure 5A,B), with a β-galactosidase activity twenty-five-fold higher than the control. These results confirmed the involvement of the protein in the mechanism of cell division.

Another key factor of bacterial division machinary, togheter with FtsZ, is FtsA. In this case the two-hybrid assay showed that the co-transformed strain containing *B. cenocepacia* FtsZ and FtsA has a β-galactosidase activity five-fold higher than the control strain (Figure 5A,B). This result indicates that a physical interaction occurs between the N-terminal domains of FtsA and FtsZ.

Among the early proteins involved in first steps of cell division, ZipA has been described in other bacteria to help the theatering of FtsZ to the membrane [57]. In this case, results indicate that *B. cenocepacia* FtsZ interacts with the C-terminus of ZipA, since the β-galactosidase activity value obtained from the two hybrid assay is 4.5 fold higher than the control (Figure 5A,B).

The last FtsZ-associated protein in *E. coli* that was analyzed is ZapA. The two-hybrid assay showed that the BTH101 strain carrying the vectors with *B. cenocepacia* FtsZ and ZapA has a β-galactosidase activity ten-fold higher than the control strain (Figure 5A,B), demonstrating the interaction of ZapA with the C-terminus of FtsZ.

Regarding the late proteins of cell division, the interaction between FtsZ and FtsE, FtsQ, FtsI and FtsN was evaluated. Results showed that the BTH101 clone expressing FtsZ and FtsE has a β-galactosidase activity fifty-fold higher than the control. This result demonstrates a physical interaction between FtsZ and the N-terminal region of FtsE in vivo. Moreover, the BACTH assay showed that the BTH101 strain transformed with both the vectors carrying FtsZ and FtsQ had a β-galactosidase activity sixteen-fold higher than the control strain. This result demonstrates a newly identified interaction between the C-terminal part of FtsZ and the N-terminal part of FtsQ, a key-player of the divisome reported to connect the early and the late proteins of cell division in *E. coli* [58]. In the search for other FtsZ interactors among the late components of cell division, two-hybrid assays were performed on clones expressing FtsZ together with FtsI or FtsN. In these cases, clones did not show any significative β-galactosidase activity (Figure 5A,B).

After the discovery that *B. cenocepacia* FtsZ interacts with both FtsA and ZipA, the physical interaction between FtsA and ZipA, previously described in *E. coli* [59] was investigated using the BACTH assay. The experiment revealed that the clone expressing FtsA and ZipA had a β-galactosidase activity thirty two-fold higher than the control strain. In *E. coli* FtsA interacts also with FtsN to connect early and late proteins [60]. In this work, we show that *B. cenocepacia* clones with FtsA and FtsN have a β-galactosidase activity five fold-higher than the control (Figure 5A,B). These results revealed that FtsA is able to interact with the N-terminal part of ZipA and that FtsA and FtsN interact each other with their C-termini. The interaction between FtsA and FtsQ was also tested, revealing that the early division protein FtsA of *B. cenocepacia* interacts also with the late protein FtsQ (Figure 5A,B), as previously described in *E. coli* [58].

2.2.2. SulA Blocks FtsZ Polymerization In Vitro

The BACTH assay highlighted the in vivo interaction of FtsZ and the putative SulA protein of *B. cenocepacia*. To confirm this physical interaction and its role in cell division, we expressed and purified *B. cenocepacia* SulA and carried out a co-sedimentation assay to check its effect on FtsZ polymerization. The co-sedimentation assay was performed in the absence and in the presence of SulA. In the presence of its co-factor GTP, FtsZ alone is able to polymerize, allowing the recovery of a good amount of protein in the pellet fraction (Figure 6A,B).

When SulA was added to the polymerization assay, in an equimolar concentration of FtsZ and in the presence of GTP to start the reaction, FtsZ was recovered only in the supernatant fraction, indicating that it was not able to polymerize. As a control, FtsZ in the presence of GDP (that could not polymerize) was recovered only in the supernatant (Figure 6A,B). These results demonstrated that the putative SulA of *B. cenocepacia* blocks FtsZ polymerization in vitro. Hence, it is possible to ascribe to this protein the role of regulator of FtsZ activity, as previously described in *E. coli*, where it blocks FtsZ activity in case of DNA damage during cell division [61].

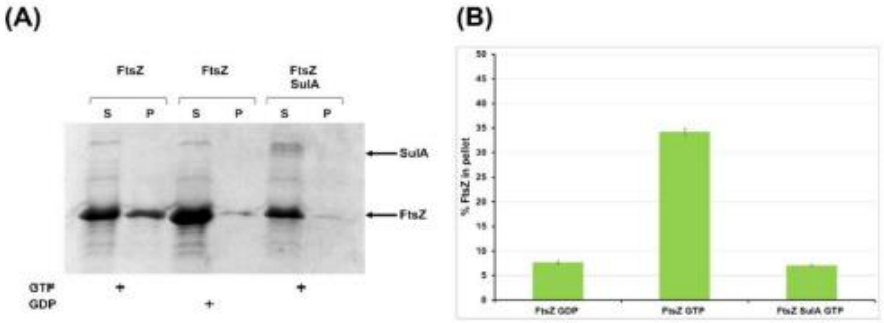

Figure 6. Effect of *B. cenocepacia* SulA on FtsZ in vitro polymerization. FtsZ was induced to polymerize in the presence or absence of SulA. (**A**) The polymeric FtsZ was collected by sedimentation and the amount of FtsZ in the pellets (P) was estimated by coomassie-blue staining of the SDS-PAGE. S, supernatant. (**B**) The bars indicate the relative quantification of FtsZ percentage in the pellet obtained by densitometry analysis. Data are the average ± SD of three independent experiments.

2.2.3. FtsZ and FtsA In Vitro Interaction

FtsA and FtsZ interact in vivo as shown by the BACTH assay. To characterize this interaction in vitro, the *B. cenocepacia* FtsA was expressed, purified and used for co-precipitation assays in the presence of FtsZ and of vesicles that allow FtsA to interact with the membranes via its C-terminal amphipathic helix [62]. The liposomes co-sedimentation showed that the presence of lipids did not affect FtsZ polymerization, being about the 50% of the protein in the pellet fraction (Figure 7).

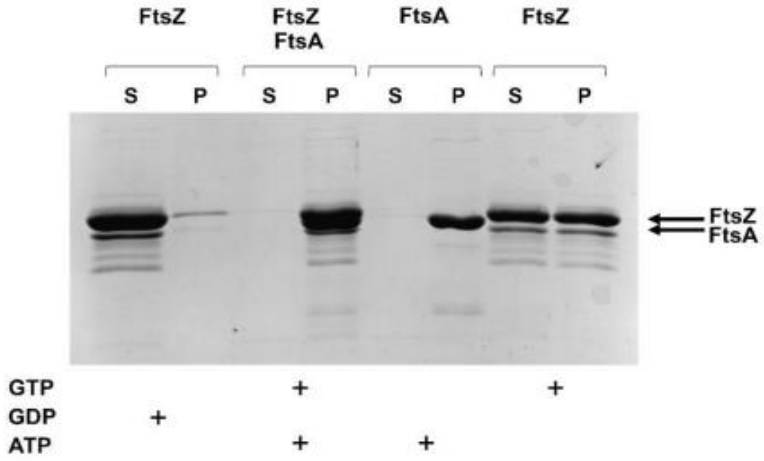

Figure 7. In vitro reconstitution of the FtsZ/FtsA interaction. FtsZ was induced to polymerize in the presence or absence of FtsA and GTP or GDP. The co-sedimentation was carried out in the presence of vesicles. The polymeric FtsZ and FtsA were collected by sedimentation and the amount of proteins in the pellet (P) was estimated by coomassie-blue staining of SDS-PAGE. S, supernatant.

FtsA polymerized completely in the presence of ATP and vescicles (Figure 7), while in absence of ATP the protein was recovered only in the supernatant (data not shown). In the presence of both FtsZ and FtsA and their cofactors (GTP and ATP), all proteins were recovered in the pellet fraction (Figure 7). This result indicated that FtsA interaction with FtsZ increased the sedimentation of FtsZ in the pellet.

2.2.4. FtsZ Interacts with ZipA In Vitro

To confirm the physical interaction between *B. cenocepacia* FtsZ and ZipA, a co-sedimentation assay was set up (Figure 8A–C).

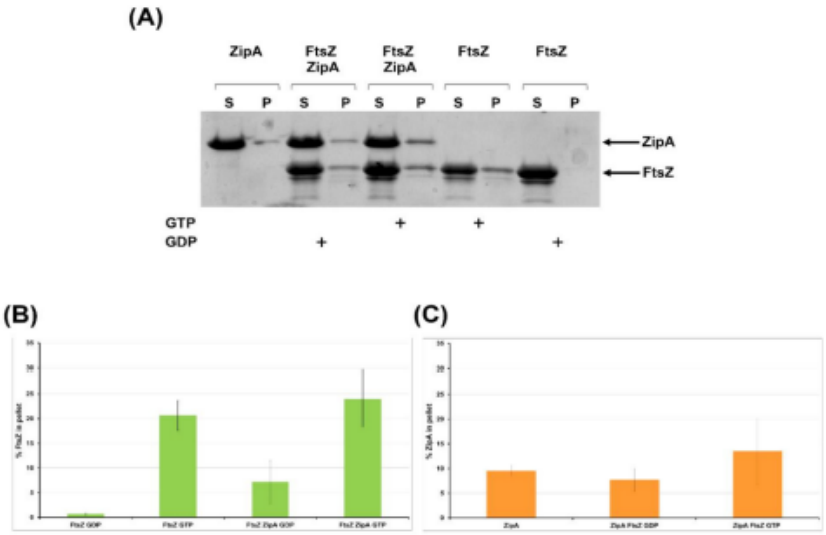

Figure 8. In vitro reconstitution of the FtsZ/ZipA interaction. FtsZ was polymerized in the presence or in the absence of ZipA with GTP or GDP. (**A**) The polymeric FtsZ was collected by sedimentation and the amount of FtsZ in the pellets (P) was estimated by coomassie-blue staining of the SDS-PAGE. S, supernatant. (**B**) and (**C**) The bars indicate the relative quantification of FtsZ (**B**) and ZipA (**C**) percentage in the pellet obtained by densitometry analysis. Data are the average ±SD of three independent experiments.

As reported in Figure 8, in the presence of ZipA and GTP the quantity of the polymerized FtsZ increased. Moreover, in the presence of ZipA and GDP, when the polymerization is not induced, FtsZ is present in the polymerized fraction together with ZipA (Figure 8A–C). In the presence of FtsZ and GTP, the quantity of ZipA in the polymerized fraction increased (Figure 8A–C). These results confirmed that *B. cenocepacia* ZipA interacts with FtsZ in its monomeric and filamentous forms.

3. Discussion

Cystic Fibrosis (CF) patients suffer from persistent lung infections throughout life and, among them, those caused by *B. cenocepacia* are the most dangerous. The accelerated deterioration of lung functions [39] and the significant increase of the mortality rate after lung transplantation, which remains the last option for patients with end-stage CF [63], make this pathogen a serious threat. For this reason, the characterization of new molecules active against this microorganism, as the C109 compound [46], is fundamental to expand the therapeutic options available, given its resistance to nearly every antibiotic currently used in clinics. However, the importance of the discovery of the C109 is not exclusively due to

its potent antimicrobial activity, but also to the identification of its molecular target, the division protein FtsZ [47]. Indeed, this demonstrated the potential of the essential division proteins as druggable targets for a target-based drug design approach against *B. cenocepacia*, paving the way to the present in-depth study of the genetics and the protein interactions regulating the division process of this poorly characterized but very complex bacterium.

Starting from the identification of the *dcw* operon within the multireplicon genome of *B. cenocepacia*, we localized it on the chromosome 1, encompassing 17 tightly packed genes that reflect the conserved genetic arrangement of this cluster found in even very phylogenetically distant bacilli [26]. This gene order conservation across genera is known to be very rare [64]. However, in both Gram-positive and Gram-negative rod-shaped bacteria, the *dcw* gene arrangement is essential for the maintenance of the shape [26,65]. Indeed, this trait clearly undergoes to a constant positive selection absent for the other known shapes, as cocci, that probably have arisen after a consistent *dcw* gene order rearrangement [66]. In bacilli, the ordered clustering of *fts* and *mur* genes, involved in septation and peptidoglycan synthesis, gives an advantage to the synthesis of long transcriptional units. According to the genomic channeling theory proposed by Mingorance et al. [67], this can facilitate the compartmentalization of their translation and so promote the ordered assembly of protein complexes specifically required at the division site. The transcription of the *dcw* operon in *E. coli* and *B. subtilis*, the best characterized bacilli, is indeed organized in many long polycistronic mRNAs [29,68]. The presence of a transcript containing all the 16 genes was postulated in *E. coli*, observing that the transcription of the distal genes was greatly influenced by the activity of the promoters localized at the 5′ of the cluster [27,69]. Here we demonstrated the presence of an operon-wide transcript in *B. cenocepacia*, showing that the transcription can proceed from *mraZ* to *ftsZ*, giving rise to a long mRNA including 15 of the 17 *dcw* genes.

The transcription was controlled only by the active promoter here characterized, P_{mra}Bc, localized in the non-coding region upstream of the first gene of the *dcw* operon, like its analogue P_{mra} in *E. coli* [53]. Accordingly, the transcription start site was experimentally identified at the 3′ of the P_{mra}Bc sequence. Instead, the only terminator site identified was localized downstream of the last gene *lpxC*, as already reported in *E. coli* [70].

The presence of shorter transcription units was considered unlikely because of the lack of characterized or predicted promoters and terminator sites within the entire operon sequence. It is widely known that the timing of expression and the cytoplasmic concentration of FtsZ must be accurately controlled because it strongly influences the cell septation [65]. This regulatory function is usually carried out by promoters and regulatory sequences localized upstream of the *ftsA-ftsZ* region, as described in *E. coli* [54], *B. subtilis* [29], and *Bacillus mycoides* [31]. Intriguingly, in *B. cenocepacia* the distal region of the operon is characterized by the absence of active promoters, suggesting the presence of post-transcriptional control mechanisms involved in the modulation of the expression of *ftsZ*. Regarding the presence of a regulator of the transcription starting from P_{mra}Bc, we demonstrated that in *B. cenocepacia* the protein MraZ is involved in this process, as already shown in *E. coli*, *Mycoplasma gallisepticum*, and *Corynebacterium glutamicum* [35,36,71]. In our experiments, the protein showed a strong binding affinity for a specific DNA fragment containing a series of four repeats of seven nucleotides. Each repeated sequence is probably recognized by MraZ as an octameric toroidal complex, as demonstrated in Mollicutes by Fisunov et al. [36], forming high molecular complexes with DNA.

After an accurate analysis of the *dcw* operon of *B. cenocepacia*, we focused our attention on the characterization of protein-protein interactions of the cell division machinery components. The cell division proteins of *B. cenocepacia* were selected based on the sequence homology with the best characterized cell division interactomes of *E. coli*. Proteins putatively involved in cell division have an intermediate level of conservation with the proteins of *E. coli*. By using the BACTH system, the interactions between the *B. cenocepacia* FtsZ and each protein selected was evaluated. The BACTH assay revealed that FtsZ monomers interact with each other with a head to tail interaction. This result

confirmed what previously reported in other bacteria: FtsZ needs this interaction to complete the GTPase active site that allows protein polymerization [55].

Regarding the proteins that control cell division, SulA, described in *E. coli* and in other Gram-negative bacteria [56,61,72] blocks FtsZ activity in case of DNA damage. The results obtained using the BACTH system showed that the interaction between FtsZ and the putative SulA homologous (pBCA006) of *B. cenocepacia* occurs between their N-terminal parts. Moreover, using a co-sedimentation assay, we demonstrated that the putative SulA blocks FtsZ polymerization in vitro, confirming its role as FtsZ regulator also in *B. cenocepacia*.

Another key-player of the first steps of cell division is FtsA, for which the interaction with FtsZ has been experimentally described through many different approaches in *E. coli* [73]. As reported in the results, using the BACTH system we first reported that *B. cenocepacia* FtsA interacts with its N-terminal part with FtsZ in vivo. To confirm this interaction, an in vitro-sedimentation assay was carried out in the presence of vesicles. The results showed that *B. cenocepacia* FtsA is able to polymerize in the presence of ATP and vesicles and, when FtsZ and GTP were added to the reaction, FtsA increased the quantity of FtsZ recovered in the pellet fraction. These data suggest that FtsA polymers tether FtsZ to the membrane with a higher affinity compared to the previously described FtsA of *E. coli*, where FtsZ is not completely recovered in the pellet in the presence of FtsA [62,74]. Further mutational studies and pull-down experiments will clarify the FtsA behavior in *B. cenocepacia*.

In *E. coli* the early protein ZipA is one of the first protein interacting with FtsZ. Results obtained from the BACTH assay showed that ZipA of *B. cenocepacia* interacts with the C-terminal part of FtsZ, similarly to what previously obtained in *E. coli* [75]. Furthermore, the interaction between FtsZ and ZipA was evaluated through the in vitro sedimentation assay, showing that ZipA tethers FtsZ in the pellet fraction also when the polymerization is not induced. These results confirmed the in vitro interaction between FtsZ and ZipA of *B. cenocepacia*, as previously reported in *E. coli* [57].

Looking at the late proteins involved in cell division, FtsE, a member of the ABC transporter superfamily, has an important role in the connection of the Z ring with the late division proteins and in the initiation of the bacterial cell constriction [76,77]. Here, a physical in vivo interaction between the N-terminal region of FtsE and the C-terminal region of FtsZ of *B. cenocepacia* was validated. This type of interaction was previously described in *E. coli* using an in vitro pull-down experiment [78] and the BACTH system [10].

In *E. coli* FtsQ acts as a sensing mechanism that promotes the passage from early to late phases of cell division [58]. Using the bacterial two hybrid assay, we demonstrate that FtsQ interacts directly with FtsZ in *B. cenocepacia*, while in *E. coli* FtsQ interacts with different components of cell division, such as FtsI, FtsN, FtsW and FtsK [79].

On the other hand, the BACTH assay revealed no other interactions between FtsZ and the components of the late phase of cell division FtsI and FtsN as in *E. coli*.

Although it has been demonstrated that there is an order of the division protein recruitment, there are evidences in *E. coli* that these proteins interact each other outside of the division process [80]. Using two-hybrid experiments, we demonstrated that in *B. cenocepacia* the scenario is similar: indeed, FtsA is able to interact using its C-terminal part with both ZipA and FtsN, two proteins involved in different phases of cell division.

The cell division machinery is a well conserved biological system in bacteria, but there are some peculiarities for each bacterial species, as previously described in *Neisseria gonorrhoeae* in which, for example, ZipA does not interact with the other components of the divisome and FtsA interacts with FtsW [81]. For instance, in this work we described for the first time the interaction between FtsZ and FtsQ. All these results pave the way to the identification of new drug target candidates in the high multi-drug resistant *B. cenocepacia*, which will be used to search for novel compounds able to inhibit bacterial growth.

4. Materials and Methods

4.1. Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this work are listed in Table S4. *E. coli* and *B. cenocepacia* cultures were growth in Luria-Bertani (LB) broth (Difco, BD, Sparks, MD, USA) with shaking at 200 rpm at 37 °C or in LB agar plate at 37 °C. *E. coli* culture for BACTH complementation assay was grown in LB with shaking at 200 rpm at 30 °C. The *E. coli* XL1-Blue strain was used in all of the cloning steps of the BACTH system. BACTH complementation assays were carried out with the *E. coli cya* strain BTH101 (Table S4).

For plasmid selection and maintenance, the following antibiotics were used: Kanamycin (Applichem, Panreac, Darmstadt, Germany) 50 μg/mL (*E. coli*); Gentamicin (Sigma, St. Louis, MO, USA) 20 μg/mL (*E. coli*), 500 μg/mL (*B. cenocepacia*); Ampicillin (Applichem, Panreac, Darmstadt, Germany) 100 μg/mL (*E. coli*), Chloramphenicol (Sigma) 34 μg/mL (*E. coli*), Streptomycin (Sigma) 100 μg/mL (*E. coli*). During the BACTH complementation assays, media were supplemented with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 40 μg/mL 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

4.2. B. cenocepacia J2315 RNA Extraction and RT-PCR

The total RNA of *B. cenocepacia* J2315 was extracted following the protocol of the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) but adding a preliminary step in order to improve the cell lysis efficiency: bacterial cells, resuspended in TRI Reagent® (Zymo Research) and mixed with zirconia beads (Ambion, Austin, TX, USA) in a 2 mL bead beating tube, were subjected to 2 cycles of 4 min at the maximum speed of bead beating using the Minilys personal homogenizer (Montigny-le-Bretonneux, France). The obtained RNA was treated twice with the DNA-free™ DNA Removal Kit (Invitrogen, Carlsbad, CA, USA) to completely remove DNA contaminations. After that, the RNA was retrotranscribed following the ProtoScript® II Reverse Transcriptase (NEB, Ipswich, MA, USA) protocol. cDNAs were then used as templates to amplify by PCR specific fragments using primers annealing on two consecutive *dcw* cluster genes. All the primers used for reverse transcription PCR and cDNA PCR are listed in Table S1. The amplicons obtained were purified and sequenced and the specificity of the results was validated using PCR negative controls (using RNA as template for the cDNA amplification) and positive controls (amplification of the genomic DNA using the cDNA primers).

4.3. Promoter Activity Assessment by β-Galactosidase Activity Assay

The *dcw* promoter activity of *B. cenocepacia* J2315 were measured by β-galactosidase activity assay taking advantage of the pSU11 vector [82] containing transcriptional *lacZ* fusions. First, two overlapping fragments, containing the putative *dcw* promoter, were amplified by PCR from the intergenic region upstream of the gene *mraZ*, using dcwPROMOrev2 coupled with dcwPROMOfor1_2 or dcwPROMOfor2_2 primers (Table S2). Additional four fragments, covering the entire *ddl-ftsA* region, were amplified by PCR to check the presence of active promoters in the distal part of the operon by using the primers murCPROMOfor1-ddlPROMOrev1, ftsQPROMOfor1-ftsQPROMOrev1, ftsAPROMOfor1-ftsAPROMOrev1, ftsAPROMOfor2-ftsAPROMOrev2 (Table S2). The purified fragments were cloned upstream of the promoterless *lacZ* reporter gene into the pSU11 vector, previously digested with *Hind*III and dephosphorylated using alkaline phosphatase (Roche, Basel, Switzerland), following the In-fusion HD Cloning Kit protocol (Takara, Shiga, Japan). The resulting plasmids (Table S3) were transferred from *E. coli* to *B. cenocepacia* K56-2 by triparental mating. The β-galactosidase assay was carried out as described before [83] with minor changes. Bacterial cultures were grown overnight in liquid LB, diluted 1/50 in fresh LB and recultured for six hours. Then, the precise OD_{600} were measured and cells from 1 mL of culture were harvested by centrifugation, resuspended in the same volume of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 pH = 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM DTT) and permeabilized with 25 μL of chloroform and 25 μL of 0.1% SDS. The bacterial suspensions were vortexed for 10 s and 500 μL of each were diluted 1/2 in Z-buffer and incubated at 28 °C for 10 min. 200 μL

of o-nitrophenyl-β-D-galactoside (ONPG) solution (4 mg/mL in Z-buffer) were added to each sample to start the reaction; then the samples were vortexed, and further incubated at 28 °C. When the samples turned yellow, the reactions were stopped adding 500 µL of 1 M Na_2CO_3. Before the spectrophotometric quantification, samples were centrifuged at 15,000× *g* for 10 min at room temperature and finally 1 mL of cell debris-free supernatant was used to measure the absorbance at 420 nm and 550 nm, allowing the calculation of the promoter activity (Miller Units).

4.4. 5′-Rapid Amplification of cDNA Ends (5′-RACE)

For the identification of the *dcw* operon transcription start site in *B. cenocepacia* J2315, 5′ rapid amplification of cDNA ends (5′-RACE) was performed. The RNA extraction, DNase treatment and reverse transcription were carried out as mentioned above, using the primer mraZRACErev1 (Table S2) for the RT-PCR. Subsequently, to degrade the RNA of the resulting cDNA-RNA heteroduplex and improve the further step yield, the sample was treated with ribonuclease H (Promega, Madison, WI, USA) adding 2 U of enzyme directly in 20 uL of RT-PCR mix after the reverse transcription reaction and incubating at 37 °C for 30 min. Then, the cDNA was purified and a tail of polyadenosine was added to the 3′ end by terminal deoxynucleotidyl transferase (Promega) reaction, according to the manufacturer's instructions. The cDNA was amplified by PCR using the polyT universal forward primer RA1 (Table S2) coupled with the reverse primer mraZRACErev2 (Table S2). The reaction product of the first PCR was used as template for the nested PCR performed with the primers RA2 and mraZRACErev3 (Table S2), designed to amplify a fragment within the first product sequence. The resulting amplified fragment was sequenced to identify the *dcw* cluster transcription start site.

4.5. Cloning, Expression, and Purification of B. cenocepacia MraZ

The gene *mraZ* (*BCAL3471*) of *B. cenocepacia* J2315 was amplified from the genomic DNA by PCR using the primers mraZpET28aFOR and mraZpET28aREV (Table S2). The two primers were designed following the In-fusion HD Cloning Kit protocol instructions (Takara) and the PreScission protease (GE Healthcare, Chicago, IL, USA) cleavage site was added to the forward primer to remove the 6-histidine tag from the protein. The purified fragment was cloned into the linearized pET28a (Merck Millipore, Burlington, MA, USA) vector, digested with *Bam*HI/*Hind*III restriction enzymes, by recombination according to the In-fusion HD Cloning Kit protocol, obtaining the pET28a-MraZ (Table S3). To express the protein MraZ, *E. coli* BL21(DE3) strain was used. 3L of LB supplemented with Kanamycin were inoculated 1/50 with the overnight grown starter culture and incubated at 37 °C with shaking until OD_{600} = 0.6 was reached. At this point, the protein expression was induced with 0.5 mM of IPTG and the culture was incubated overnight at 18 °C. The cells were harvested by centrifugation, resuspended in buffer A (25 mM Tris-HCl pH = 7.5, 300 mM NaCl, 5 mM $MgCl_2$, glycerol 5%), supplemented with 1 mM of the non-specific protease inhibitor Phenylmethanesulfonyl fluoride (PMSF) and lysed by sonication. The lysate was centrifuged at 50,000× *g* for 30 min and the protein was purified from the cell-free extract using immobilized metal affinity chromatography (IMAC) on a HiTrap TALON crude column (1 mL, GE Healthcare). The purified protein was dialyzed against the buffer B (50 mM Tris-HCl pH = 7.5, 300 mM NaCl, 5 mM $MgCl_2$, glycerol 5%, 1 mM Dithiothreitol (DTT)) and digested with PreScission protease (GE Healthcare) to remove the 6-histidine tag. Finally, the protein MraZ was further purified in the same buffer on a nickel nitrilotriacetic acid resin (Ni-NTA, Qiagen, Hilden, Germany) packed in a column, quantified, and stored at −80 °C.

4.6. Electrophoretic Mobility Shift Assay (EMSA)

To characterize the binding site of the transcriptional regulator MraZ, electrophoretic mobility shift assay (EMSA) was used. The non-coding region upstream of *mraZ*, containing the putative binding site, was amplified by PCR using mraZBS1for, mraZBS1rev and mraZBS2for, mraZBS2rev primers (Table S2). These primers were labeled with 6-carboxyfluorescein to be easily detectable on the gel using the ChemiDoc XRS+ System (Bio-Rad, Milano, Italia) and were designed to amplify the

two DNA probes that consisted in two overlapping fragments of 250 bp covering the whole intergenic region. The fragments were named BS1-6-Fam and BS2-6-Fam and their sequences were confirmed by sequencing. 0.34 pmoles of the labeled DNA probes were incubated with increasing quantities of MraZ (250, 300, 350, 400 pmoles) in a reaction volume of 25 µL in binding buffer (20 mM Tris-HCl pH = 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT, 150 ng/µL BSA, 50 ng/µL deoxyribonucleic acid from herring sperm) for 30 min at 37 °C and loaded on an agarose gel 1.5%. The electrophoretic run was performed in TBE 0.5× for 1 h at 100 V at 4 °C in the dark, after an initial equilibration of the gel for 30 min at 60 V in TBE 0.5×. The gel was then visualized using the ChemiDoc XRS+ System (Bio-Rad). The specificity of the binding was verified repeating the same experiment adding a 20× excess of specific (non-labeled BS2 fragment) or non-specific (non-labeled 250 bp fragment amplified by PCR from the gene *cepI* of *B. cenocepacia* J2315 with mraZBSNSfor and mraZBSNSrev (Table S2) primers), DNA probe competitors.

4.7. Construction of the Recombinant Plasmid of the BACTH Complementation Assays

The genes coding for FtsZ, FtsA, FtsE, FtsQ, FtsI, FtsN, ZipA, ZapA and SulA were amplified by using the appropriate primers listed in Table S5 and the genomic DNA of *B. cenocepacia* J2315 as template. The PCR products were inserted by recombination, following the In-fusion HD Cloning Kit protocol instruction (Takara), into the plasmids of the BACTH system (Euromedex, Souffelweyersheim, France) pUT18, pUT18C, pKT25 and pKNT25, previously digested with the corresponding restriction enzymes, obtaining the vectors listed in Table S3. The resulting recombinant plasmids expressed hybrid proteins in which the polypeptides of interest were fused to the C- or N-termini of the T25 and T18 fragments of adenylate cyclase, respectively.

4.8. BACTH Complementation Assays

For BACTH complementation assays, recombinant pUT18, pUT18C, pKT25 and pKNT25 carrying the selected genes were used in various combinations to cotransform BTH101 cells. The transformants were plated onto LB-IPTG-XGal medium containing Kanamycin and Ampicillin and incubated at 30 °C for 48 h. Efficiencies of interactions between different hybrid proteins were evaluated in solid media (LB and MacConkey) and in liquid culture quantifying the β-galactosidase activity. The β-galactosidase assays were performed as described previously, on strains grown in LB broth in the presence of 0.5 mM IPTG, Ampicillin and Kanamycin at 30 °C for 18 h. A level of β-galactosidase activity at least four-fold higher than that measured for BTH101 (pUT18/pKT25) cells was considered to indicate an interaction.

4.9. Cloning, Expression, and Purification of pBCA006, the Putative B. cenocepacia SulA

The protein pBCA006 of *B. cenocepacia* J2315, the putative SulA, was expressed using the following protocol. The gene *pBCA006* was amplified from genomic DNA by PCR using the primers sulApBADM41for and sulApBADM41rev (Table S2). The PCR product was inserted by recombination, following the In-fusion HD Cloning Kit protocol instruction (Takara), into the pBADM-41 plasmid, previously digested with *Nco*I/*Eco*RI, obtaining the pBADM-41-SulA (Table S3). This plasmid was co-transformed by electroporation with the pG-Tf2 vector (Chaperone plasmid set, Takara), expressing the *E. coli* chaperones GroEL-GroES and tig upon induction, into *E. coli* TOP10 competent cells. The consequent inducible overexpression of the GroEL-ES complex in this strain assisted the correct folding of MBP-SulA, increasing the quantity of soluble protein produced, which otherwise was completely accumulated within inclusion bodies given the intrinsic instability of SulA. The cells were grown in 3 L of LB supplemented with Ampicillin and Chloramphenicol, and 5 ng/mL of Tetracycline to induce the chaperones expression, until OD_{600} = 0.6 was reached, then the protein expression was induced with 0.2% of L-arabinose overnight at 18 °C. The cells were collected by centrifugation, resuspended in buffer C (50 mM Tris-HCl pH = 7, 5 mM $MgCl_2$, glycerol 5%) supplemented with 1 mM of PMSF and lysed by sonication. Subsequently, the lysate was centrifuged at 50,000× *g* for 30 min and a solution of ATP pH = 7 was added to the supernatant obtaining a final concentration of

5 mM, in order to facilitate the detachment of GroEL from MBP-SulA. After 15 min of incubation at 4 °C, the extract was loaded on a MBPTrap HP (5 mL, GE Healthcare), eluting MBP-SulA complexed with GroEL with 10 mM of maltose in buffer C. In order to eliminate the chaperone contamination, the eluate was further purified using cation exchange chromatography. A column of SP Sepharose Fast Flow resin (GE Healthcare) was used and the proteins were eluted in buffer C with a (0–1 M) KCl gradient. The fractions containing exclusively MBP-SulA were merged, desalted using a HiPrep 26/10 Desalting column (GE Healthcare), and eluted in buffer D (20 mM Tris-HCl pH = 7.9, 50 mM KCl, 1 mM EDTA, glycerol 10%). Finally, the protein was quantified and stored at −80 °C.

4.10. Cloning, Expression, and Purification of B. cenocepacia FtsA

To express the protein FtsA of *B. cenocepacia* J2315, the gene *ftsA* (*BCAL3458*) was amplified by PCR, using the primers ftsASUMOfor and ftsASUMOrev (Table S2), with genomic DNA as template. The fragment obtained was purified and inserted into the pETSUMO (Invitrogen) plasmid, using the In-fusion HD Cloning Kit protocol (Takara), and then the pETSUMO-FtsA (Table S3) was transformed into *E. coli* BL21(DE3) competent cells by electroporation. The culture was grown in LB supplemented with Kanamycin and the protein production was induced with 0.5 mM of IPTG overnight at 20 °C. The cells were harvested by centrifugation, resuspended in buffer E (50 mM Tris-HCl pH = 7.5, 300 mM NaCl, 5 mM $MgCl_2$, glycerol 5%) containing 1 mM of PMSF and lysed by sonication. The lysate was clarified by centrifugation at 50,000× *g* for 30 min and the supernatant was applied on a HisTrap HP nickel column (1 mL, GE Healthcare), eluting the protein with 250 mM imidazole in buffer E. Then, the purified protein was dialyzed overnight against buffer B and the SUMO protease was used to remove the SUMO protein. A further purification step was carried out by size exclusion chromatography, using a HiLoad 16/60 Superdex-75 column (GE Healthcare) in buffer D, obtaining the purified FtsA that was concentrated to 5 mg/mL and stored at −80 °C.

4.11. Cloning, Expression, and Purification of B. cenocepacia ZipA

To express the soluble fragment of the protein ZipA of *B. cenocepacia* J2315, the gene *zipA* (*BCAL2097*) was amplified by PCR, using the primers ZipASUMOFor and ZipASUMOrev (Table S2), with genomic DNA as template. The fragment obtained was purified and inserted into the pETSUMO (Invitrogen) plasmid, using the In-fusion HD Cloning Kit protocol (Takara), and then the pETSUMO-ZipA (Table S3) was transformed into *E. coli* BL21(DE3) competent cells by electroporation. The culture was grown in LB supplemented with Kanamycin and the protein production was induced with 0.5 mM of IPTG overnight at 18 °C. The cells were harvested by centrifugation, resuspended in buffer E containing 1 mM of PMSF and lysed by sonication. The lysate was clarified by centrifugation at 50,000× *g* for 30 min and the supernatant was applied on a HisTrap HP nickel column (1 mL, GE Healthcare), eluting the protein with 250 mM imidazole in buffer E. Then, the purified protein was dialyzed overnight against buffer B and the SUMO protease was used to remove the SUMO protein. A further purification step was carried out by size exclusion chromatography, using a HiLoad 16/60 Superdex-75 column (GE Healthcare) in buffer D, obtaining the purified ZipA that was concentrated to 10 mg/mL and stored at −80 °C.

4.12. Co-Sedimentation Assays of FtsZ in the Presence of ZipA or SulA

The reaction mixtures containing 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 12 µM FtsZ, 12 µM ZipA or SulA and 2 mM GTP or GDP were incubated for 10 min at 30 °C and 300 rpm to allow the polymerization to occur. Samples were then ultracentrifuged at 350,000× *g* for 10 min at 25 °C, and the supernatant was immediately separated from the pellet, which contains the protein polymers. The samples were analyzed by SDS-PAGE on 12% polyacrylamide gels. The quantification of FtsZ polymerization was performed as previously described [84], using densitometry by calculating the percentage of the relative intensity of the bands.

4.13. Co-Sedimentation Assays of FtsZ in the Presence of FtsA and Vesicles

Vesicles were prepared using *E. coli* total lipid extract (Avanti Polar Lipids , Alabaster, AL, USA) in 25 mM PIPES (pH 6.8), 300 mM KCl and 10 mM $MgCl_2$ using sonication. The reaction mixtures contained 2 mg/mL of vesicles with pre-spun proteins at a final concentration of 12 µM, 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, and 2 mM GTP, GDP or ATP. The reactions were incubated for 10 min at 30 °C and 300 rpm to allow the polymerization to occur, then samples were centrifuged at 20,000× *g* at 20 °C for 25 min. The supernatants were immediately removed for analysis, the pellets resuspended with 25 mM PIPES (pH 6.8), 300 mM KCl and 10 mM $MgCl_2$ (same volume as the supernatants) and solubilized with SDS gel loading buffer. Samples were analysed by SDS–PAGE on 12% polyacrylamide gels.

5. Conclusions

The importance of cell division as a new source of drug targets deserved a careful description in the multi-drug resistant bacterium *Burkholderia cenocepacia*, for which a lack of knowledge was evident. In this work we described the organization of the division cell wall (*dcw*) cluster, in which 15 genes can be transcribed as a polycistronic mRNA from *mraZ* to *ftsZ*. We also highlighted that the transcription is under the control of a strong promoter regulated by MraZ. Moreover, the interaction of FtsZ with the other components of the divisome machinery was shown. Importantly, the direct interaction with FtsQ has never been reported before. We validated the role of SulA as FtsZ inhibitor, and the roles of FtsA and ZipA as tethers of FtsZ polymers. Our results lead to a deep knowledge of cell division in *B. cenocepacia*, paving the way for future antimicrobial design based on the divisome.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/9/12/841/s1, Figure S1: Representation of the distal genes of the *dcw* cluster. Table S1: List of primers used for the transcription analysis of the *dcw* operon, and expected length of the PCR fragments. Table S2: List of primers used for gene and promoter sequences cloning into plasmids, EMSA and 5′-RACE. Table S3: List of plasmids used in this work. Table S4: List of strains used in this work. Table S5: List of primers used for cloning the divisome genes into the BACTH plasmids.

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