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Social interactions in the *Burkholderia cepacia* complex : biofilm formation and quorum sensing

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Burkholderia cepacia complex bacteria are opportunistic pathogens that cause respiratory tract infections in susceptible patients, mainly in people with cystic fibrosis (CF). There is convincing evidence that *B. cepacia* complex bacteria can form biofilms, not only on abiotic surfaces (e.g. glass and plastics), but also on biotic surfaces like epithelial cells, leading to the suggestion that biofilm formation plays a key role in the persistent infection of CF lungs. In this review I present an overview of (i) the molecular mechanisms involved in *B. cepacia* complex biofilm formation, (ii) the increased resistance of sessile *B. cepacia* complex cells and (iii) the role of quorum sensing in *B. cepacia* complex biofilm formation.

Executive summary

Molecular basis of *B. cepacia* complex biofilm formation

- QS systems are required for formation of mature biofilms
- Adhesive structures (pili, flagella) are involved in initial stages of biofilm formation
- Five different exopolysaccharides (including cepacian) are produced by *B. cepacia* complex species ; these are likely involved in persistence and invasiveness
- Cepacian enhances the formation of thick, mature biofilms

Antimicrobial resistance in *B. cepacia* complex biofilms

- *B. cepacia* complex bacteria are higly resistant against a wide range of antimicrobial agents
- Single antibiotics or (double/triple) antibiotic combinations with good *in vitro* activity often fail to clear bacteria from the lung
- Comparing antimicrobial susceptibility between planktonic and sessile cells requires similar experimental conditions
- For most antibiotics, the concentrations required to inhibit growth of exponential-phase planktonic cultures and freshly-adhered biofilms is similar ; likewise, the concentrations required to kill planktonic cells in stationary-phase cultures and sessile cells in mature biofilms are also similar.
- Sessile *B. cepacia* complex cells tend to be less susceptible to tobramycin than their planktonic counterparts
- Marked differences in susceptibility to disinfectants can be observed between planktonic and sessile *B. cepacia* complex cells, with the latter being considerably more resistant
- In contrast to *Pseudomonas aeruginosa*, agents interfering with iron-dependent cellular processes (e.g. gallium) have little effect on *B. cepacia* complex biofilms

Role of QS in *B. cepacia* complex biofilms

- *N*-acyl homoserine lactones are used for cell-cell communication in *B. cepacia* complex bacteria
- In some *B. cepacia* complex species the use of 4-hydroxy-2-alkylquinolines and/or cis-2-dodecenoic acid signalling molecules has been described
- AHL-based QS plays an important role in biofilm formation in all *B. cepacia* complex species, but most likely not in all conditions
- Inhibitors of the QS system also affect biofilm formation in *B. cepacia* complex bacteria

Mixed species biofilms

- *B. cepacia* complex bacteria can form mixed species biofilms with *P. aeruginosa*

The *Burkholderia cepacia* complex is a group of closely-related Gram-negative bacteria, belonging to the *β-Proteobacteria*. At present, the *B. cepacia* complex contains 17 species : *Burkholderia cepacia, Burkholderia multivorans, Burkholderia cenocepacia, Burkholderia stabilis, Burkholderia vietnamiensis, Burkholderia dolosa, Burkholderia ambifaria, Burkholderia anthina, Burkholderia pyrrocinia, Burkholderia ubonensis, Burkholderia latens, Burkholderia diffusa, Burkholderia arboris, Burkholderia seminalis, Burkholderia metallica, Burkholderia contaminans* and *Burkholderia lata* [1-4]. *B. cepacia* complex species are notorious for causing respiratory tract infections in susceptible individuals, most notably people with cystic fibrosis (CF) or chronic granulomatous disease (CGD) [5,6]. The problematic treatment of *B. cepacia* complex infected patients and the capacity of these bacteria to cause severe inflammation, progressive decline of the lung function and invasive infections, have led to intensive research on a broad variety of potential virulence factors. These virulence traits include the resistance against antimicrobial agents, the formation of biofilms and the synthesis of exopolysaccharides (EPS), the adherence to mucin and epithelial cells, the ability to invade epithelial cells and macrophages, the production of catalases, superoxide dismutases and other reactive oxygen species (ROS)- degrading/scavenging proteins, the secretion of lipases, proteases, haemolysins and siderophores and the use of quorum sensing (QS) signals that modulate the expression of genes involved in virulence [7]. Over the past decade, biofilm formation by various *B. cepacia* complex bacteria has been described (see for example references [8-14]). Interestingly, *B. cepacia* complex bacteria were not only found to be capable of colonizing various abiotic surfaces (e.g. glass and plastics), but were also found to form biofilms on biotic surfaces like epithelial cells [9]. This has led to the suggestion that biofilm formation plays a key role in the persistent infection of CF lungs [15,16].

Molecular basis of *B. cepacia* complex biofilm formation

Several studies have focussed on the factors required for initial surface adhesion and subsequent biofilm formation and maturation [17-20]. Adhesion to surfaces is often mediated through specialized surface structures like fimbriae or pili and in many organisms also requires flagellar motility [21]. *B. cepacia* complex bacteria are equipped with mesh, filamentous, spine, spike and/or cable pili [22]. Although cable pili are well known for their role in adhesion to epithelial cells, genes encoding these pili are not widely distributed in the *B. cepacia* complex and not all *cblA*-positive strains produce the pili [23]. Hence, their exact role (and the role of other pili) in *B. cepacia* complex biofilm formation is at present unclear [16]. A screening of 5000 random insertion mutants of *B. cenocepacia* H111 to determine their ability to form biofilms, led to the identification of 13 mutants defective in the formation of a mature biofilm [18]. The genes involved either encoded

surface proteins or regulatory factors or they were required for the biogenesis and maintenance of the outer membrane. In addition, some of the identified mutants were defective in QS, confirming previous research on the necessity of the CepIR QS system for the formation of mature biofilms in *B. cenocepacia* H111 [17] (see below for more details). Confocal laser scanning microscopy images revealed markedly different ultrastructures for the mutant biofilms compared to the wild type biofilms [18]. The same study demonstrated that swarming motility is not required for the formation of mature *B. cenocepacia* biofilms; yet, mutant strains lacking functional flagella were severely impaired in their initial adhesion. These results were confirmed by Saldias et al. [19], who demonstrated that RpoN (a sigma factor that regulates flagellar motility) is required for biofilm formation. A putative sensor kinase response regulator AtsR has also been identified as an important regulator of biofilm formation in *B. cenocepacia*, however none of the genes regulated by AtsR have been identified thus far [20].

Alginate, a high molecular weight acetylated polymer composed of nonrepetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids, is an important component of the *Pseudomonas aeruginosa* biofilm matrix, but other EPS (including Pel and Psl) are also involved [24]. The ability of *B. cepacia* complex bacteria to produce EPS has been studied extensively and five different EPS, including PS-I, PS-II (cepacian) and levan, have been identified [25]. Cepacian, a heteropolysaccharide with a heptasaccharide basic unit, is the most frequently identified EPS in *B. cepacia* complex bacteria and it has been isolated from mucoid as well as from non-mucoid strains [25-30]. Although not systematically studied so far, it appears that some *B. cepacia* complex strains exclusively produce cepacian, while others also co-produce small(er) amounts of other extracellular polysaccharides [8,29].

The genes in the *bceABCDEFGHIJKL* gene cluster encode the enzymes required for the formation of the sugar precursors, the glycosyltransferases and the proteins involved in polymerization and export of cepacian [31]. Interestingly, the *B. cenocepacia* type strain, *B. cenocepacia* J2315, does not produce cepacian, as a frameshift mutation is present in *bceB* (BCAM0856, coding for a putative glycosyltransferase) [31-33]. Cepacian is often acetylated [29,31,34,35], and although the role of cepacian acetylation remains unknown, it may protect the bacteria from opsonisation and phagocytosis, as is the case with alginate in *P. aeruginosa* [35,36].

B. cepacia complex EPS also scavenges ROS and it interferes with neutrophil chemotaxis [37]; the latter characteristics might explain why production of EPS enhances persistence in a mouse model system [10,38]. In *P. aeruginosa* infected CF patients, mucoid alginate-producing isolates are associated with a progressive deterioration of the lung function and with an increased resistance against antimicrobial agents and the immune system. In addition, excessive alginate production is found to be important for the formation of thick *P. aeruginosa* biofilms in the CF lung [39]. Cunha

et al. [10] reported that cepacian is not required for biofilm formation, although it may enhance the formation of thick, mature biofilms. Contrary to the non-mucoid to mucoid phenotype switch in *P. aeruginosa*, the transition from a mucoid tot non-mucoid phenotype has been observed in *B. cepacia* complex isolates from chronically infected CF patients [40]. This observation suggests that EPS production is involved in the persistence of the infections, whereas the loss of EPS production might increase the invasiveness of these strains [39].

Further research is required to elucidate the role of biofilm formation and/or EPS production in the persistence and virulence of *B. cepacia* complex bacteria [16].

Antimicrobial resistance in *B. cepacia* complex biofilms

Mechanisms of resistance in planktonic *B. cepacia* complex cells

B. cepacia complex bacteria are highly resistant against multiple antibiotics, disinfectants as well as preservatives [41]. This panresistance is caused by various mechanisms, including limited permeability of the outer membrane, changes in lipopolysaccharide (LPS) structure and the presence of several multidrug efflux pumps, inducible chromosomal β-lactamases and altered penicillin-binding proteins [7,41]. *B. cepacia* complex bacteria are intrinsically resistant to cationic antimicrobial agents due the unique structure of their LPS. In addition, porins can severely limit the permeability of the outer membrane [41] and this decreased porin permeability has been associated with resistance against chloramphenicol, trimethoprim, ciprofloxacin and β-lactam antibiotics [42,43]. Multiple Resistance-Nodulation-Division (RND) efflux pumps have been identified in the genome of *B. cenocepacia* J2315 [44]. One of these efflux pumps is highly upregulated in the presence of chloramphenicol, whereas another efflux pump appears to be involved in the resistance to fluoroquinolones and streptomycin [44,45]. Furthermore, Nair et al. [46] reported that efflux of chloramphenicol, trimethoprim and ciprofloxacin is mediated by a *P. aeruginosa* MexAB-OprM homolog in *B. cenocepacia*. The role of all other putative RND efflux pumps for which genes were identified in the *B. cenocepacia* J2315 genome is at present unclear [44]. Inducible β-lactamases impair the antibiotic activity of β-lactamase sensitive penicillins and various genes encoding these inducible β-lactamases (two class A, one class C and one class D β-lactamase) are present within the *B. cenocepacia* J2315 genome [32]. Thus far no aminoglycoside inactivating enzymes have been confirmed experimentally but genes encoding a putative aminoglycoside O-phosphotransferase and a putative aminoglycoside O-adenyltransferase have been identified in the *B. cenocepacia* J2315 genome sequence [32,41]. In addition, although co-trimoxazole is still used in patients chronically infected with *B. cepacia* complex bacteria, resistance to trimethoprim (due to

184 the production of a altered trimethoprim dihydrofolate reductase) has frequently been reported [47-49].

186

Resistance mechanisms in biofilms

188 Several mechanisms are thought to be involved in biofilm antimicrobial resistance, including slow penetration of the antimicrobial agent in the biofilm, biofilm heterogeneity (including the presence 190 of zones of slow or no growth), presence of a small population of extremely resistant "persister" cells and biofilm-specific adaptive stress response [50-52]. Apart from this last mechanism (see 192 next sections) most of these mechanisms have not been investigated in *B. cepacia* complex biofilms.

194 The rate of transport of antimicrobial agents into biofilms is important, unlike for planktonic cells where mixing a suspension of the latter with an antimicrobial agent rapidly exposes all cells to the 196 full dose. However, if the rate of antibiotic penetration through a biofilm is decreased relative to the rate of transport through a liquid then the bacteria may be exposed to a gradually increasing dose of 198 the antibiotic and may have time to mount a defensive response to the agent [53]. Penetration of antimicrobial agents into microbial biofilms has been the subject of debate for almost 30 years 200 [54,55], resulting in literature reports in favour as well as against the presence of a diffusion barrier in biofilms (reviewed in [55]). While physicochemical calculations indicate that for most 202 compounds these limitations are not expected to contribute to a substantial decrease in penetration [55,56], experimental data suggest that this retardation is often compound- and/or biofilm-specific. 204 This is clearly illustrated by the penetration of antimicrobial agents into the alginate-containing *P. aeruginosa* biofilm. The penetration of aminoglycoside antibiotics such as gentamicin and 206 tobramycin is considerably slower than that of β-lactam antibiotics, which can be explained by the observation that, unlike β-lactams, aminoglycosides bind very well to alginate [57-59]. In addition, 208 the presence of inactivating compounds can greatly influence the effective penetration : e.g. extracellular β-lactamase activity can have a dramatic effect on the penetration of penicillins in 210 *P.aeruginosa* biofilms [60,61].

Gradients of nutrients, oxygen, signalling molecules and metabolic end products occur in biofilms 212 due to differences in metabolic activities and/or differences in transport of molecules. As a result of these gradients, considerable structural, chemical and biological heterogeneity can be found within 214 a biofilm [62]. Cells at the surface of a biofilm usually do not experience shortage of oxygen and/or nutrients, but in deeper areas of the biofilm depletion of these essential compounds may lead to the 216 presence of metabolically inactive cells that are less suceptible to the action of antimicrobial compounds and are difficult to eradicate [62].

The presence of "persister cells" is also a possible mechanism responsible for the increased resistance in sessile microbial populations. These persisters can tolerate the presence of certain antimicrobial agents (i.e. they are not killed) and can be considered as specialised survivor cells [63-65]. Persisters are not mutants, but phenotypic variants of the wild type (WT) which, after removal of the selective pressure (i.e. the antimicrobial treatment) give rise to a WT culture which again contains only a small fraction of persisters [66]. Persister cells have already been observed in several microorganisms, including the bacteria *P. aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* and the dimorphic fungus *Candida albicans* [63,66-70]. While the phenomenon of persisters is not limited to biofilm populations, it has been observed that the fraction of persisters is often higher in sessile populations than in planktonic populations, although there can be marked species- and antibiotic-dependent differences [69,71,72]. Recently it was shown that in planktonic *P. aeruginosa* cultures, the fraction of persisters increases in response to the quorum sensing molecule 3-oxo-C12 homoserine lactone and the secreted phenazine pyocyanin, linking antimicrobial resistance due to persister formation with QS [73].

Biofilm-specific adaptive stress responses will be discussed in the next sections.

Effect of antibiotics and disinfectants on *B. cepacia* complex biofilms

Caraher et al. [74] showed that for *B. multivorans*, *B. cenocepacia* and *B. dolosa* strains (2 strains each), the minimal concentrations required to inhibit biofilms (minimal biofilm inhibitory concentration, MBIC) were considerably higher than the minimum inhibitory concentrations (MIC) for planktonic cells, when the β-lactams meropenem and piperacillin-tazobactam were tested. However, ceftazidime (also a β-lactam), ciprofloxacin, azithromycine, and the aminoglycosides tobramycine and amikacin, were effective at inhibiting *B. cepacia* complex biofilms, although high concentrations were often required. In a larger study, Peeters et al. [75] investigated the *in vitro* activity of six antibiotics (ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycine and trimethoprim/sulfametoxazole) against 38 *B. cepacia* complex isolates (2-4 strains per species). The growth inhibitory concentrations for exponentially growing planktonic cells and freshly adhered (4 h) sessile cells were similar. This somewhat surprising finding was in contrast with results from other studies, and this is likely due to differences in methodology. In order to allow a correct comparison between MIC and BIC, experimental conditions should be identical ; this is illustrated by the observation that resistance to ciprofloxacine and ceftazidime changes dramatically during exponential growth of both planktonic and sessile cultures [76]. When bactericidal activity against stationary phase planktonic cultures and mature (24 h old) biofilms was evaluated, major differences were also not observed, although a marked decrease in the susceptibility of sessile cells towards tobramycine was observed [68]. Combining multiple antibiotics to increase the *in vitro*

effect against biofilm-grown *B. cepacia* complex isolates is not straightforward. Dales et al. [77] showed that 59% of 47 *B. cepacia* complex isolates recovered from CF patients (the majority belonging to *B. cenocepacia*) were resistant to all 27 double antibiotic combinations tested, while 18% of all isolates were resistant to all 67 triple antibiotic combinations. The most effective double antibiotic combination was meropenem combined with high-dose (200 µg/ml) tobramycin (35% of all isolates were inhibited), while the most effective triple antibiotic combination contained meropenem, piperacillin-tazobactam and high-dose tobramycin (53% of all isolates were inhibited). With regard to resistance to disinfectants, marked differences in susceptibilities between planktonic and sessile *B. cenocepacia* cultures were observed [78]. While treatments with acetic acid (1.25%, 15-60 min), dettol (5%, 5-30 min), ethanol (70%, 2-10 min), hot water (70°C, 15-60 min), hydrogen peroxide (0.5-3%, 30 min), cetrimide (0.15%, 15 min) and NaOCl (0.05% - 0.3%, 5 min) resulted in reductions of at least 99.999% in planktonic cultures, sessile populations were more resistant. Especially treatments with acetic acid, chlorhexidine, hydrogen peroxide and NaOCl did not result in the eradication of all sessile *B. cenocepacia* cells. The reduced susceptibility to oxidising disinfectants not only has implications for infection control practices but, as these oxidative agents are being produced by neutrophils as part of the endogenous defence against microorganisms [79], may also have implications for pathogenesis. Reduced activity of a cetylpyridinium chloride (CPC) nanoemulsion against sessile *B. cepacia* complex strains has also been reported and the median increase in MBIC and MBEC compared to the respective MICs and MBCs for the six *B. cepacia* complex strains tested was 12-fold and 11-fold, respectively [80].

Effect of agents that interfere with iron-dependent cellular processes

In 2005, Banin et al. showed that iron is essential for the development of mature, structured, *P. aeruginosa* biofilms, as mutations in the high-affinity pyoverdine iron acquisition system result in thin, unstructured, biofilms [81]. Subsequently, it was shown that physiological concentrations of the iron-binding glycoprotein lactoferrin inhibited the growth of planktonic and sessile *B. cepacia* complex cells, and that *B. cepacia* complex biofilms formed in the presence of lactoferrin contained significantly less biomass than untreated biofilms after 24 h. However, several *B. cepacia* complex strains were capable of overcoming this antibiofilm effect and at 48 h treated biofilms were very similar to untreated ones. Addition of lactoferrin also decreased the MBIC for rifampicin (2-4 fold) [82].

Kaneko et al. [83] very convincingly showed that gallium (a transition metal capable of disrupting iron-dependent cellular processes) inhibits *P. aeruginosa* biofilm formation and kills sessile cells *in vitro*. In addition, gallium was shown to be effective in several *in vivo P. aeruginosa* infection models. However, using a similar approach it was recently shown that the effect of gallium against

B. cepacia complex biofilms is rather limited [84]. In the presence of physiological iron concentrations, biofilms formed by the *B. cenocepacia* strains investigated appeared to be insensitive to gallium (concentrations up to 64 mg/l), while only a moderate effect was observed for *B. multivorans* and *B. dolosa*. These experiments were carried out with gallium nitrate ; subsequent experiments with gallium maltolate revealed that the latter was even less effective (own unpublished data).

Molecular response of sessile *B. cenocepacia* cells to antibiotics and disinfectants

As described above, sessile *B. cenocepacia* cells are relatively insenstive to oxidising disinfectants like H_2O_2 and NaOCl [78]. When the transcriptional response of treated and untreated *B. cenocepacia* biofilms was compared, it was observed that exposure to these agents resulted in an upregulation of 315 (4.4%) and 386 (5.4%) genes, respectively, while transcription of 185 (2.6%) and 331 (4.6%) genes was decreased in response to H_2O_2 or NaOCl treatments, respectively [85]. Not surprisingly, many of the upregulated genes in the treated biofilms are involved in (oxidative) stress responses, emphasizing the importance of the efficient neutralization and scavenging of ROS. In addition, multiple upregulated genes encode proteins that are necessary to repair ROS-induced cellular damage. In agreement with what was observed for *P. aeruginosa* [86], *ahpC* and *ahpF* were highly upregulated, while *katB* was only modestly upregulated [85]. Treatments with H_2O_2 or NaOCl also resulted in the increased transcription of several organic hydroperoxide resistance (*ohr*) genes, including BCAS0085.

To my knowledge the molecular response of sessile *B. cepacia* complex cells to antibiotics has not been investigated. However, the changes in gene expression of planktonic *B. cenocepacia* J2315 cells in response to sub-inhibitory concentrations of meropenem, trimethoprim/sulfamethoxazole and amikacin were studied [87]. Only a small number of genes appeared up- or downregulated, and the upregulated genes included genes encoding β-lactamases (meropenem), acetyltransferase (amikacin) and *aidA* (trimethoprim). The latter gene encodes a nematocidal protein [88] that is positively regulated by the QS regulator CepR and negatively by another QS regulator, CciR [89,90] (see below for details). Many of the genes upregulated in the presence of antibiotics were also upregulated in planktonic stationary phase cultures, suggesting that they may also be relevant in the biofilm mode of life [87].

Role of QS in *B. cepacia* complex biofilms

N-acyl homoserine lactone based QS

QS systems control the expression of various virulence factors and these systems are considered to be important regulators of pathogenicity [91]. In *B. cepacia* complex bacteria (like in many other Gram-negative bacteria), the cell-density dependent coordination of gene expression involves the use of *N*-acyl homoserine lactone (AHL) signalling molecules [16]. The AHL-based QS system was first discovered and studied in detail in the bioluminescent *Vibrio* species *Vibrio harveyi* and *Vibrio fischeri* [92,93]. AHLs are produced intracellularly by an AHL synthase (LuxI homologue) and will accumulate extracellularly. Once a certain threshold concentration is reached, AHLs will bind to a response regulator (LuxR homologue) and the resulting complex will bind to a promotor, activating the transcription of QS regulated genes. Synthesis of AHLs was found to be widespread among *B. cepacia* complex species, although strain- and species-dependent differences were observed [8,94,95]. The CepIR QS system is found in all *B. cepacia* complex species [16]. In this system, the AHL synthase CepI predominantly synthesizes *N*-octanoyl-homoserine lactone (C8-HSL) as well as smaller amounts of *N*-hexanoyl-homoserine lactone (C6-HSL). When the population density is sufficiently high, these AHLs will bind to CepR. This interaction will cause a conformational change in the latter regulatory protein and will result in the induction or repression of the transcription of the CepR target genes. Functions regulated by CepIR include the production of extracellular proteases (ZmpA and ZmpB), chitinases and the nematocidal protein AidA, swarming motility, biofilm maturation and ornibactin synthesis [90,96] (TABLE 1). In *B. cepacia* ATCC 25416, CepR also influences the expression of RpoS, an alterative sigma factor that controls the gene expression in stationary phase cultures and in cultures exposed to certain environmental stresses [97]. A recent screening of a random promoter library in *B. cenocepacia* K56-2 identified 89 genes to be regulated directly or indirectly by the CepIR QS system [98]. A comparison of the protein-expression pattern in a *B. cenocepacia* H111 wild type and H111 *cepI* mutant showed that 5% of the proteome was downregulated in the mutant strain, whereas 1% was upregulated [99]. Using known CepR-regulated genes, a consensus *cep* box (i.e. CepR binding site) motif was created and subsequently used to identify promotor regions that may be regulated by CepR [100] (FIGURE 1). Using this approach, 65 genes potentially regulated by CepR were identified, belonging to several functional classes : cell surface or membrane (9.2%), metabolism (33.9%), phages (3.1%), regulatory genes (21.5%), transport (4.6%), and secretion (4.6%). 12.3% of the *cep* boxes were located in promotor regions of genes encoding hypothetical proteins, 10.8% were located in genes encoding proteins of unknown function [100].

In addition to the transcription regulation of the above mentioned target genes, a positive feedback control of *cepI* by CepR, allowing a rapid AHL-signal amplification, has been described [101]. The discovery of three higher-level CepR regulators (YciR, SuhB and YciL) has added another layer of complexity to the already complex QS network [18]. In addition to the CepIR QS system, other QS

systems have been described within the *B. cepacia* complex. These include the CciIR, the CepR2 and the BviIR QS systems [16]. The CciIR system is only present in epidemic *B. cenocepacia* strains containing the pathogenicity island called "cenocepacia island (*cci*)" and its function depends on the production of C6-HSL by CciI [98]. CepR2 is an orphan LuxR homolog in *B. cenocepacia* that influences the expression of several CepR and CciR regulated genes, but does not require an AHL for activation [96]. Besides the CepIR system, *B. vietnamiensis* strains are also equipped with a second QS system BviIR that relies on the production of *N*-decanoyl homoserine lactone (C10-HSL) [16]. An overview of the various systems in *B. cenocepacia* K56-2 and their interactions is shown in FIGURE 1. A selection of phenotypes and/or proteins regulated by the various systems in in *B. cenocepacia* K56-2 is shown in TABLE 1.

Other QS systems in the *B. cepacia* complex

Besides the AHL-based QS system, other QS systems employing 4-hydroxy-2-alkylquinolines (HAQ) [102] or *cis*-2-dodecenoic acid ("*Burkholderia* diffusible signal factor", BDSF) [103] can be found in some *B. cepacia* complex strains.

In *P. aeruginosa*, the *Pseudomonas* quinolone signal (PQS) is synthesised by enzymes encoded in the *pqsABCDE* operon : these enzymes generate 2-heptyl-4-quinolone (HHQ) from anthranilic acid, which is then converted to PQS by the FAD-dependent monoxygenase PqsH. PQS regulates the RhlI/R QS system but at the same time the *las* and *rhl* systems act antagonistically on *pqsR*, the regulator of the *pqsABCDE* operon [102]. *B. cenocepacia* J415 was shown to produce HHQ, but not PQS [104]. In another member of the *B. cepacia* complex, *B. ambifaria*, a novel type of HAQs was identified, i.e. HAQs containg a methyl group and designated 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) [105]. The genes involved in the biosynthesis of these HMAQs are located in the *hmqABCDEFG* operon ; mutations in *hmqA* or *hmqG* (encoding a putative methyltransferase) increase the production of AHLs in *B. ambifaria*, suggesting an important role for HMAQs in regulating QS-controlled phenotypes [105].

BDSF is structurally similar to the diffusible signal factor in *Xanthomonas campestris* and its synthesis requires the presence of the *X. campestris rpfF* homologue [103]. This gene (BCAM0581 in *B. cenocepacia* J2315) is also present in other sequenced *B. cepacia* complex genomes. Mutations in BCAM0581 are associated with growth defects, reduced virulence gene expression and attenuated virulence in a zebrafish infection model [106]. BDSF also inhibits germ tube formation in the human-pathogenic dimorphic fungus *Candida albicans*, potentially preventing infection with this fungus [103]. Interestingly, several AHL-regulated virulence genes in *B. cenocepacia* (including *zmpA* and *orbI*) are also under control of BDSF [106].

QS and biofilm formation in the *B. cepacia* complex

The link between QS and biofilm formation has extensively been studied in *P. aeruginosa*. Davies et al. [107] first showed that a functional QS system is required for the formation of differentiated biofilms with a marked threedimensional structure ("mushrooms"). In contrast, in other studies no [108] or only minor [109] differences were observed between a *lasI* deletion mutant and the wild type *P. aeruginosa* strain. However, QS inhibitors turned out to affect *P. aeruginosa* biofilm structure [110] and biofilms of mutants in which both the Las and the Rhl system were mutationally inactivated were more sensitive to tobramycin, hydrogen peroxide and leukocytes [111], suggesting a key role for QS in biofilm formation and resistance. Huber et al. [17] showed that QS also plays an important role in the development of *B. cenocepacia* H111 biofilms development. While biofilms formed by *cep* mutants are initially indistinguishable from biofilms formed by the wild type strain, the mutant biofilms did not develop further into mature biofilms. The QS – regulated genes required for this biofilm maturation have not been identified yet, but it has been speculated that FimA (the major subunit of type 1 fimbriae and produced under QS control) could be involved [16]. Futher analysis of a set of QS mutants of *B. cenocepacia* K56-2 [112] confirmed and extended the initial observations of Huber et al. [17]. *cepI* and *cepR* mutants formed biofilms with less biomass than the wild type biofilms, but neither the *cciI* nor the double *cciI cepI* mutant was deficient in biofilm formation. In the *cciI* mutant, C6- and C8-HSL are still being produced by the functional copy of *cepI* but for the double *cciI cepI* double mutant this phenotype is surprising, as very little biofilm formation would be expected in the absence of a functional AHL signal [112].

The *cciR* mutant and the *cepR cciIR* mutant were both defective in biofilm formation. In the *cciR* mutant C6-HSL is still being produced by CepI and CciI, but this molecule may be less efficient in binding to and/or activation of CepR than C8-HSL, resulting in (partial) inhibition of Cep-system regulated genes and biofilm formation [112]. In terms of susceptibility to antimicrobial agents, the individual *cepIR* and *cciR* mutants were not different from the wild type, but the *cepI cciI* mutant formed biofilms that were more sensitive to ciprofloxacin. Finally, biofilms formed by *cciI* and *cepI* mutants were more sensitive to removal by sodium dodecyl sulfate (TABLE 2). The observation that QS plays an important role in biofilm development in *B. cenocepacia* was subsequently extended to several other *B. cepacia* complex species [113] and it seems reasonable to assume that QS plays an important role in biofilm formation in all *B. cepacia* complex species. Nevertheless, it should be noted that results from one of the earliest studies investigating the link between QS and biofilm indicated that QS is likely not involved in regulating *B. cepacia* complex biofilm formation under all growth conditions [8].

In *P. aeruginosa*, PQS is also involved in biofilm formation, and addition of PQS enhances attachment of cells, possibly by inducing the production of the LecA lectin and/or the release of

extracellular DNA [102]. It is at present unclear whether HHQs, HMAQs and/or other molecules similar to HAQs are involved in biofilm formation in the *B. cepacia* complex.

Inhibition of QS as a novel approach to treat *B. cepacia* complex biofilm-related infections

Considering the major role of QS in *B. cepacia* complex biofilm formation, QS inhibitors have been proposed as potential novel antibiofilm agents [114,115]. In contrast to several QS systems in various other species, the AHL-based QS system of *B. cenocepacia* is not inhibited by furanone compounds [115]. Riedel et al. developed a set of QS blockers based on the structure of 3-oxo-C12-HSL. These included N-(2-thienylcarbonyl)-4-bromo-1,5-dimethyl-1H-pyrazole-3-carbohydrazide (compound 1) and N-(6-tert-butyl-2,3-dihydro-2-methylpyridazin-4-yl)-5-chlorothiophene-2-carbohydrazide (compound 3) [116]. Besides drastically reducing the production of several virulence factors, compound 3 also turned out to be a potent inhibitor of biofilm formation in *B. cenocepacia* H111 (reduction of biofilm biomass with appr. 60%). Similar results were obtained for this compound when other *B. cenocepacia* and *B. multivorans* strains were tested [14]. In addition, compounds that were previously described as QS inhibitors also affected biofilm formation in *B. cepacia* complex bacteria [14]. More detailed investigation into the mode of action of several of these compounds revealed that at least some of them (including cinnamaldehyde and compound 3) promote detachment at later stages of the biofilm development, rather than interfering with the early phases of biofilm development. This is in agreement with the observations that QS mutants are not affected in early biofilm formation, but do show defects at later stages of the process [17].

Mixed species biofilms

It has been shown that *B. cepacia* complex bacteria can form mixed species biofilms with *P. aeruginosa*, with sessile cells of both species being in close association [11,15,117]. In biofilms formed in various mixed-species biofilm model systems (including flow chambers and alginate beads in mouse lung tissue), both organisms produce AHL QS molecules, but interspecies signalling appeared to be unidirectional : while *B. cenocepacia* H111 reacts to signals produced by *P. aeruginosa* PAO1, the opposite is not the case [15]. It is likely that during chronic infections of the CF lung, both pathogens form mixed biofilms as well and as such the interactions between both bacteria merit further study.

Conclusion

Biofilm formation and QS are important and wide-spread processes, that significantly impact the management and treatment of *B. cepacia* complex infections. Factors involved in biofilm formation are the QS system(s), adhesive structures and various exopolysaccharides. When tested under comparable conditions, the MIC and MBIC, and the MBC and MBEC, respectively, were comparable for most antibiotics, although marked differences could be observed for tobramycine and various disinfectants. Various QS systems can be found in *B. cepacia* complex systems and it has been shown that the AHL-based system plays an important role in regulating biofilm formation.

Future perspectives

Although considerable progress has been made, a more comprehensive evaluation of the genes involved in biofilm formation is required in order to get a better understanding of the entire process. Besides identifying which genes are involved, a better insight into the dynamics of their expression will shed light on the role of these different genes under various conditions. At the same time, more research is needed to obtain a clearer picture of what factors are involved in the resistance of sessile *B. cepacia* complex cells. This may ultimately lead to the development of novel drugs with increased activity against *B. cepacia* complex bacteria. As it was clearly shown that QS plays an important role in biofilm development, QS inhibitors (alone or in combination with conventional antimicrobial agents) may hold great promise to treat *B. cepacia* complex infections.

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Figure 1. (A) Organisation of the different quorum sensing systems in *B. cenocepacia* K56-2. Based on data previously reported in [16,90]. Regular arrows indicate flow from DNA to protein to AHL molecule (and subsequently to AHL-receptor complex, designated with *). Bold arrows indicate positive regulation. Dashed lines indicate negative regulation. CepR*, CciR* and CepR2* regulate the expression of a wide range of target genes, see [90] and Table 1 for details. **(B) Graphical representation of the *cep* box motif.** The relative sizes of the letters indicate their likelihood of occurring at a particular position. Based on sequences reported in [100] and created with MEME Suite (http://meme.sdsc.edu/).

Table 1. Reciprocal regulation of gene expression *B. cenocepacia* K56-2 by CepIR, CciR and CepR2.

	CepIR	CciR	CepR2
Motility	+	-	
Zinc metalloproteases	+	+, -	-
AidA (nematocidal protein)	+	-	-
Type VI secretion	+	-	
Heat shock proteins	+	-	+
Efflux pumps	+, -	+, -	+, -
Flp type pilus	+	-	
FimA (fimbrial protein)	+	-	
Lectins	+	-	-
Ferric ornibactin transport	-	+	

Data from [90].

Table 2. Phenotype of various *B. cenocepacia* K56-2 QS mutants.

	Biofilm biomass compared to WT	MBEC (µg/ml) of ciprofloxacin	Sensitive to removal by SDS (24 h)
K56-2 (WT)	NA	512	No
Mutation in :			
cepI	Reduced	512	Yes
cepR	Reduced	1024	Yes
cciI	Not affected	1024	ND
cciR	Reduced	512	ND
cepI cciI	Not affected	64	ND
cepR cciIR	Reduced	ND	ND

Data are from [112]. MBEC, minimum biofilm eradicating concentration. WT, wild type. NA, not applicable. ND, not determined