Two quorum sensing systems control biofilm formation and virulence in members of the *Burkholderia cepacia* complex

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The *Burkholderia cepacia* complex (Bcc) consists of 17 closely related species that are problematic opportunistic bacterial pathogens for cystic fibrosis patients and immunocompromised individuals. These bacteria are capable of utilizing two different chemical languages: *N*-acyl homoserine lactones (AHLs) and *cis*-2-unsaturated fatty acids. Here we summarize the current knowledge of the underlying molecular architectures of these communication systems, showing how they are interlinked and discussing how they regulate overlapping as well as specific sets of genes. A particular focus is laid on the role of these signaling systems in the formation of biofilms, which are believed to be highly important for chronic infections. We review genes that have been implicated in the sessile lifestyle of this group of bacteria. The new emerging role of the intracellular second messenger cyclic dimeric guanosine monophosphate (c-di-GMP) as a downstream regulator of the fatty acid signaling cascade and as a key factor in biofilm formation is also discussed.

Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of closely related species with large genomes comprising multiple replicons. Bcc species are notable for their ability to metabolize a wide range of organic compounds and to thrive in many different environments.[1-4] The Bcc currently contains 17 defined species, which include opportunistic pathogens that are best known for infecting immunocompromised patients or individuals suffering from cystic fibrosis (CF), where they can cause a fatal pneumonia known as "cepacia syndrome".[5] One of the major problems associated with Bcc infection is their intrinsic resistance to antibiotic therapy. Several strains of the Bcc species *B. multivorans*, *B. cenocepacia*, *B. cepacia*, and *B. dolosa* have been shown to be highly transmissible between patients, with *B. cenocepacia* and

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B. multivorans accounting for the majority of CF infections.[6-8] Biofilm formation is a common trait of Bcc strains and has been associated with the persistence of Bcc infections and the increased resistance to antibiotics relative to planktonic cells.[9-13] It has been shown that in many Bcc strains the formation of biofilms, as well as the expression of virulence factors and secondary metabolites, is under quorum sensing (QS) control.[14-17] QS is a cell density-dependent regulatory mechanism used by bacteria to coordinate gene expression by the aid of diffusible self-produced signal molecules. The purpose of this review is to summarize the current knowledge of QS in members of the Bcc and discuss its role in biofilm formation and pathogenicity.

AHL-Based QS Systems in the Bcc

All Bcc members encode at least one QS system that consists of homologs of the LuxR and LuxI proteins of *Vibrio fischeri*, where LuxI synthesizes an AHL signal and LuxR is an AHL receptor protein that activates or represses gene expression by binding to a consensus sequence (the so-called *lux* box) in the promoter regions of target genes.[18] AHL signal molecules can differ in the length and substitution of their acyl side chains. In many cases transcription of *luxI* is activated by the LuxR/AHL complex, providing a signal amplification mechanism via positive feed-back regulation.[19]

AHL production in the Bcc is strain-dependent with respect to both the quantity and the type of AHL molecules. Within the Bcc the CepIR QS system is fully conserved.[20-23] CepI directs the synthesis of *N*-octanoyl-homoserine lactone (C8-HSL) and minor amounts of *N*-hexanoyl-homoserine lactone (C6-HSL; **Fig. 1**).[14,22] Epidemic strains of *B. cenocepacia* belonging to the ET12 lineage carry the *B. cenocepacia* genomic island (*cci*), which encodes an additional QS system named CciIR.[24] The AHL synthase CciI produces C6-HSL and minor amounts of C8-HSL, which are bound by the cognate receptor CciR. The CepIR and CciIR systems interact, with CepR positively regulating the expression of the *cciIR* operon and CciR negatively regulating *cepI* expression.[15] While CepR is mainly a positive regulator, CciR

acts as a negative regulator of gene expression. In *B. vietnamiensis* strains, CepR is required for expression of yet another QS system (BviIR), which utilizes *N*-decanoyl-homoserine lactone (C10-HSL).[25] Phenotypic assays as well as global transcript and protein analyses using *cepIR* and *cciIR* mutant strains have shown that AHL-mediated QS controls various functions, including swarming motility, biofilm formation and the production of virulence factors, such as proteases (e.g., the metalloproteases ZmpA and ZmpB), siderophores, toxins, and antifungal agents.[14-16,26,27]

AHL-mediated QS is fine-tuned by additional regulators. The AHL synthase-encoding genes and their cognate receptor genes are normally located in close proximity to each other in the genome. However, whole genome sequencing identified LuxR homologs in significant excess relative to the number of AHL synthases. Although these unpaired, "orphan" or "solo" LuxR proteins are not associated with an AHL synthase, most of them require AHLs in order to function.[28-30] An orphan LuxR homolog, CepR2, has been identified in all *B. cenocepacia* strains sequenced so far but not in other Bcc species. While CepR2 is not involved in the regulation of *cepIR* or *cciIR*, CepR is required for CepR2-mediated activation of pyochelin production in *B. cenocepacia* strain H111.[31] In contrast, the CepR2 in *B. cenocepacia* strain K56-2 acts as a repressor; moreover, in this strain expression of CepR2 is repressed by CciR and its activity is antagonized by AHLs.[32] Recently, CepR2 of strain K56-2 has been shown to function as an anti-activator of CepS, an AraC-type regulator-encoding gene located adjacent to *cepR2*. CepS was shown to act downstream of CepR2 by activating gene expression in the absence of CepR2.[32]

Additional regulators involved in fine-tuning AHL-mediated QS have been identified in *B. cenocepacia*. A hypothetical conserved protein encoded by *BCAM1871*, which is downstream and co-transcribed with *cepI*, was shown to induce AHL activity and to positively regulate *cepIR* and *cciIR* expression.[33] In addition, BCAM1871 activates expression of the LysR-type regulator ShvR, which is highly conserved among members of the Bcc and is located in the vicinity of the **a**ntifungal **c**luster (*afc*) on plasmid pC3 (formerly chromosome 3).[33,34] ShvR has been shown to negatively control *cepIR* and *cciIR* expression while positively affecting biofilm formation.[35,36] Another negative regulator of *cepI* and *cciI* expression and their target genes is the recently identified membrane hybrid sensor kinase AtsR (**A**dhesion and **t**ype **s**ix secretion system **r**egulator) which is lacking a DNA binding domain, suggesting control through a signal transduction cascade.[37] AtsR has been shown to be a global regulator, which controls expression of virulence factors also in the absence of the CepIR QS system. The exact mode of action of AtsR as well as of the other regulators affecting the QS circuitry in Bcc strains remain to be elucidated.

Fatty Acid Signal-Based QS in the Bcc

In 2008, Boon et al. reported the identification of a novel fatty acid signal molecule that is produced by several *B. cenocepacia* strains.[38] The structure of the molecule synthesized by *B. cenocepacia* J2315 was identified as *cis*-2-dodecenoic acid, referred to
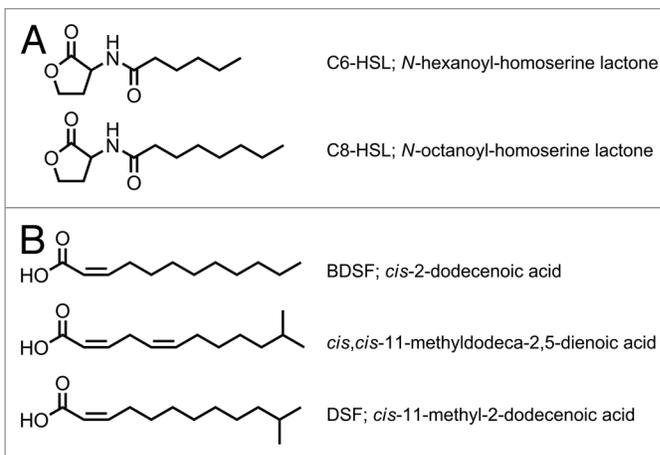
Figure 1. The different QS molecules produced by Bcc species. (**A**) Signaling molecules in AHL-based QS. (**B**) Signaling molecules in DSF family-based QS.

as BDSF (**B**urkholderia **d**iffusible **s**ignal **f**actor). BDSF is structurally related to DSF (**d**iffusible **s**ignal **f**actor, *cis*-11-methyl-2-dodecenoic acid), which was first isolated from supernatants of *Xanthomonas campestris* pv *campestris*.[39,40] In this plant pathogen DSF is activating the RpfCG two component system, leading to a lowered cellular c-di-GMP level and concomitantly to the expression of target genes involved in biofilm dispersal and virulence.[41,42] DSF is synthesized by the gene products of *rpfF* and *rpfB* encoding a putative enoyl-CoA hydratase and a putative long-chain fatty acyl CoA ligase, respectively.[42,43] In *B. cenocepacia* BDSF is synthesized by an RpfF homolog (BCAM0581 in *B. cenocepacia* J2315), named RpfF$_{Bc}$.[38,44] RpfF$_{Bc}$ is a bifunctional crotonase having both dehydratase and thioesterase activities, which enables the enzyme to directly convert the acyl carrier protein (ACP) thioester of 3-hydroxydodecanoic acid into *cis*-2-dodecenoic acid.[44] BDSF accumulates in a cell density-dependent manner with maximum levels observed in the late stationary phase.[45] However, in contrast to the AHL-dependent CepIR system, the expression of the BDSF synthase is not subject to positive feedback regulation.[14,45] The biological activity of BDSF is dependent on the *cis* configuration of the fatty acid, as neither the *trans* isomer of BDSF nor lauric acid were found to be active.[46] Moreover, supplementation of the medium with DSF rescued the phenotypes of an *rpfF$_{Bc}$* mutant, providing further evidence that the configuration of the double bond is a critical structural feature of the signal molecule. This result also suggests that *cis*-2-unsaturated fatty acids may be used for interspecies communication. Not only can DSF substitute for BDSF in *B. cenocepacia* but it has also been shown that BDSF is able to activate DSF-dependent responses in *X. campestris*, suggesting that cross-species signaling between these organisms is possible.[38] In this context it is worth noting that the production of DSF-family signal molecules is widespread among bacteria and therefore cross-talk via *cis*-2-unsaturated fatty acids may be a common phenomenon.[47,48] A recent study showed that DSF-family molecules, including BDSF, are present in sputum taken from CF patients and it was suggested that interspecies DSF-mediated

bacterial interactions occur in the CF lung and may influence the efficacy of antibiotic treatment.[49] Finally, BDSF has been shown to inhibit germ tube formation of *Candida albicans*, indicating that BDSF also serves as an interkingdom signal.[38,50]

The BDSF-regulated QS system is involved in the control of several functions. Mutation of $rpfF_{Bc}$ resulted in decreased motility, reduced adherence to porcine mucin, diminished exopolysaccharides (EPS) production and lowered protease activity.[46,51,52] In addition, the BDSF mutant strains were found to be more susceptible to antimicrobials and their ability to form biofilms was shown to be strongly reduced.[53,54]

BDSF perception. Recent work has shown that the gene adjacent to $rpfF_{Bc}$ encodes the BDSF receptor protein RpfR (BCAM0580 in *B. cenocepacia* J2315).[46] RpfR contains a GGDEF, an EAL, and a PAS domain. PAS domains are able to bind a chemically diverse range of small-molecules, including hemes, flavins, di- and tricarboxylic acids, divalent metal cations, amino acids and coumaric acid.[55] RpfR and RV1364c, a σ^F regulatory protein of *Mycobacterium tuberculosis*, are the only proteins known to bind directly a fatty acid through their PAS domains.[46,56] GGDEF and EAL are highly conserved domains involved in c-di-GMP turnover with diguanylate cyclase and phosphodiesterase activities, respectively.[57] Binding of BDSF to the PAS domain causes an allosteric conformational change of RpfR and thereby stimulates the c-di-GMP phosphodiesterase activity of the protein.[46] Consequently, in the presence of BDSF RpfR lowers the intracellular c-di-GMP level. Noteworthy, RpfR is the first example of a c-di-GMP metabolic enzyme that is directly activated by a QS signal molecule.[46]

McCarthy et al. identified an additional putative BDSF sensor, BCAM0227, in *B. cenocepacia* J2315.[53] This protein shares 35.6% identity with RpfC of *X. campestris*. BCAM0227 contains a histidine kinase phosphoacceptor domain (HisKA), a CheY-like receiver domain, and a C-terminal histidine phosphotransfer (HPt) domain. However, at least two lines of evidence indicate that the BCAM0227 signaling system may only play a subordinate role in responding to accumulated BDSF signals. First, disruption of *BCAM0227* only affected the expression of a subset of genes belonging to the BDSF regulon. Second, while mutation of *BCAM0227* and $rpfF_{Bc}$ has a similar effect on biofilm architecture, the two mutants differed drastically in motility and in adherence to porcine mucin. In contrast, a *rpfR* mutation leads to identical phenotypes as $rpfF_{Bc}$ mutations, including reduced motility, reduced biofilm formation, lowered proteolytic activity and attenuated virulence.[27,46]

The BDSF and AHL stimulons overlap. In order to identify BDSF-regulated genes, transposon mutagenesis, global transcript and protein profiling analyses were performed.[27,51,53] In *B. cenocepacia* J2315, $rpfF_{Bc}$ was found to regulate 372 genes with functions including motility, attachment, stress tolerance, virulence, transport, signal transduction, multidrug resistance, and detoxification.[53] Among these $rpfF_{Bc}$-dependent genes, 65 were found to be also regulated by the putative BDSF sensor BCAM0227. The mapping of the *B. cenocepacia* H111 BDSF stimulon by RNA-Seq and shotgun proteomics confirmed BDSF-dependent regulation of genes known to be involved in biofilm formation and protease activity.[27] Among the genes positively regulated by BDSF, many are known to be controlled by the CepIR QS system, suggesting that both signal molecules are required for full expression of some functions, including the large surface protein BapA and the EPS cepacian.[27,58] However, the contribution of each of the systems in the regulation of target genes was found to be variable.[27] While some genes are mainly regulated by either BDSF (e.g., *bclACB*) or AHLs (e.g., *aidA* or *cepI*), maximum transcription of *bapA* requires the presence of both signal molecules. On the basis of these results we proposed a model in which the BDSF and AHL QS systems operate in parallel to regulate specific as well as overlapping sets of genes (**Fig. 2**).[27] BDSF-dependent signaling results in a reduction of the intracellular c-di-GMP levels, which in turn leads to differential expression of target genes.[46] Hence, it is very likely that an as-yet unidentified regulator (or regulatory cascade) responds to changes in the intracellular c-di-GMP level. Whether this regulator is independent of the CepIR system or the two QS regulatory cascades converge in one regulator remains to be elucidated.

Evidence for overlapping QS operons was also obtained from phenotypic investigations. For example, biofilm formation of a *cepI* $rpfF_{Bc}$ double mutant in strain H111 could only be restored to the level of the wild type when the medium was supplemented with both signal molecules.[27] This is fully congruent with the finding that optimal expression of BapA, a large secreted protein required for biofilm formation, is dependent on both QS systems.[59] Likewise, maximal proteolytic activity was dependent on both signal molecules although BDSF mutants of strains H111 and J2315 were at least partially complemented in the presence of AHLs.[45] This effect has been attributed to the decreased amounts of AHLs produced by the BDSF mutant as a consequence of lowered *cepI* transcription.[27] In addition to *cepI*, *cciI* was also found to be BDSF-regulated.[54] Taken together, the data suggest that the BDSF- and AHL-dependent QS circuitries interact with each other although the molecular mechanism is currently not understood.

The BDSF-dependent QS system is widespread. Like the CepIR system, the RpfFR system is highly conserved within the Bcc.[27,52] Furthermore, by using a BDSF biosensor, the production of BDSF (or other cross-reacting *cis*-2-unsaturated fatty acids) was detectable in all currently described Bcc species (our unpublished data). These results confirm and extend a previous survey that demonstrated the synthesis of BDSF in nine Bcc strains (*B. ambifaria, B. anthina, B. cenocepacia, B. dolosa, B. lata, B. multivorans, B. pyrrocinia, B. stabilis*, and *B. vietnamiensis*) by using high-performance liquid chromatography (HPLC) and mass spectrometry.[52] This study also showed that *B. anthina, B. stabilis*, and *B. pyrrocinia* synthesize, in addition to BDSF, the DSF-family compound *cis,cis*-11-methyldodeca-2,5-dienoic acid. *B. multivorans* was found to produce not only these two molecules but also DSF.[52] It has been suggested that the synthesis of different DSF family molecules is due to the genetic background of the bacterial strain rather than to variations in the BDSF synthase genes.[44,52] These data clearly show that cell-to-cell communication by the aid of *cis*-2-unsaturated fatty acids is a widespread phenomenon within the Bcc. It is also worth noting that $RpfF_{Bc}$ homologs
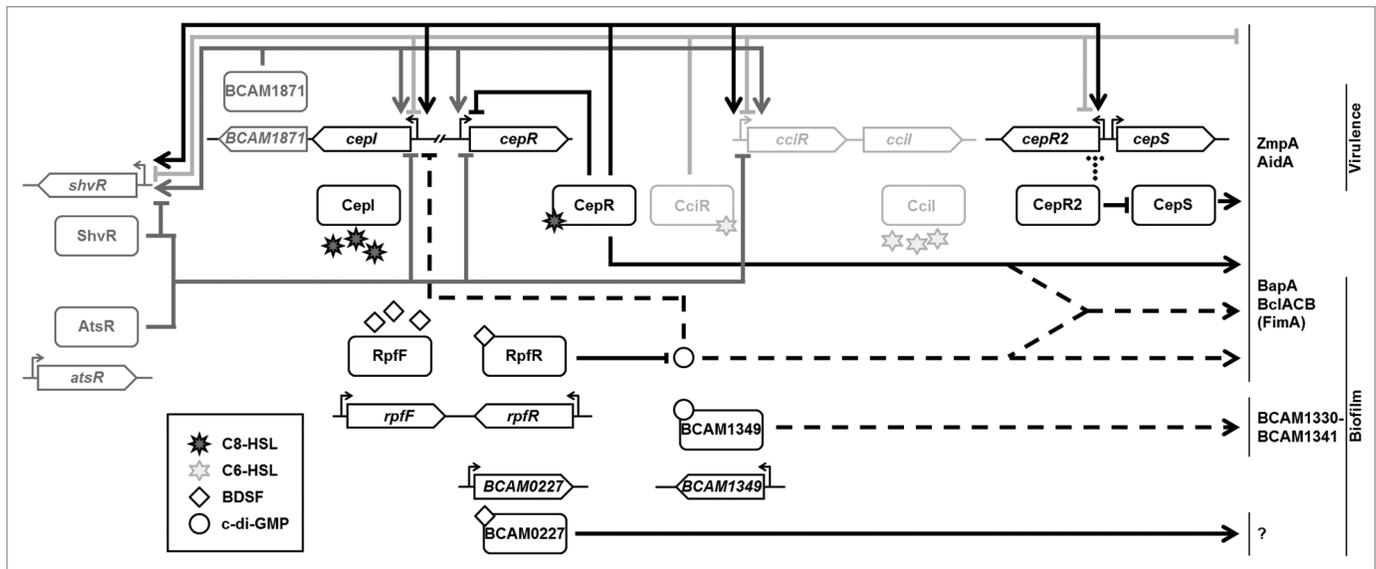
Figure 2. Model of the QS network in *B. cenocepacia*. Depicted in black is the "core" QS circuitry of *B. cenocepacia* that is present in all strains and that consists of the CepIR and the RpfFR systems, as well as the downstream regulators CepR2, CepS, BCAM1349 and BCAM0227. The regulators ShvR, AtsR and BCAM1871, which fine-tune AHL-dependent QS, are shown in dark gray. The second AHL-dependent QS system, CciIR, which is only present in strains of the ET-12 lineage, is shown in light gray. The dashed lines indicate regulation of target genes through the second messenger *c*-di-GMP. The dotted line indicates contradictory experimental data regarding the autoregulation of *cepR2*.[31,32]

have also been identified in *Burkholderia* species not belonging to the Bcc, and in other genera including *Achromobacter*, *Yersinia*, *Serratia*, *Enterobacter*, *Pantoea*, *Cronobacter*, *Rahnella*, *Erwinia*, and *Yokenella*.[46] This supports the idea that DSF family signal molecules may also serve as interspecies signals.[47,49,53]

The Role of QS in Pathogenicity of Members of the Bcc

The first Bcc strain was isolated from macerated onion tissue.[60] This organism, subsequently named *B. cepacia,* was found to be the causative agent of soft rot of onions. Other Bcc species have also been described as phytopathogens of banana (*B. cenocepacia*) and apricot (*B. seminalis*).[61-63] However, their pathogenic potential is not restricted to plants. Bcc species are important opportunistic pathogens and are particularly problematic for individuals suffering from CF, chronic granulomatous disease or in immunocompromised patients. The clinical manifestation of a Bcc infection in CF patients ranges from asymptomatic carriage to a rapidly progressing fatal pneumonia, the so-called "cepacia syndrome".[2] Importantly, all but two Bcc species (*B. latens* and *B. metallica*) have been isolated from both environmental and clinical sources and thus it is not possible to distinguish between environmental and clinical strains.[2,4,64] For example, from 381 clinical isolates examined in a study by Baldwin et al., more than 20% were indistinguishable by multilocus sequence typing (MLST) from environmental source isolates.[65] Using PFGE fingerprinting along with other typing methods, LiPuma et al. showed that the epidemic CF strain prevalent in the mid-Atlantic region of the United States and Europe is identical to isolates recovered from agricultural soil.[66] Furthermore, a recent study

showed that Bcc species isolated from patient sputum and from the waterbody of a freshwater lake in China showed similar virulence properties in different pathogenicity models.[67] Hence, in the absence of patient-to-patient transmission, the natural environment is the most likely source of Bcc infections in humans.[6]

Over the past decade, substantial progress has been made in identifying and characterizing the virulence determinants and infection mechanisms of Bcc strains (for a review see ref. 68). Since Bcc strains can use a wide range of plants and animals as infection hosts, diverse disease models have been developed. Plant models include alfalfa (*Medicago sativa*) seedlings, onion (*Allium cepa*), and lettuce (*Lactuca sativa* L. var *longifolia*); invertebrate models employ nematodes (*Caenorhabditis elegans*), the larvae of the greater wax moth (*Galleria mellonella*) and fruit flies (*Drosophila melanogaster*); vertebrate infection models make use of zebrafish (*Danio rerio*) embryos, rats, and mice.[67-70] The role of QS in virulence has been investigated for several Bcc strains in various infection models. The results of these investigations have been recently summarized in an excellent review by Subramoni and Sokol.[70] The bottom line is that both AHL-dependent QS systems (CepIR and CciIR) contribute to virulence in different vertebrate infection models whereas only the CepIR system is required for virulence in alfalfa (in the case of strain H111 but not strain K56-2) and *C. elegans*. Interestingly, the AHL-dependent QS systems seem to have no effect on virulence of *B. cenocepacia* in the *G. mellonella* and *D. melanogaster* infection models. Although it has been reported that a *cepI* mutant of H111 is less virulent in *G. mellonella*,[69] this turned out to be due to a second-site mutation present in this strain (our unpublished results). By contrast, a *cepI* mutant of the plant growth-promoting *B. ambifaria* strain showed reduced survival in the *D. melanogaster* host

model.[71] Mutants defective in BDSF signaling have so far only been constructed in *B. cenocepacia* strains J2315 and H111. An *rpfF$_{Bc}$* mutant of J2315 showed decreased mortality in both a mouse agar bead and a zebrafish infection model as well as reduced killing of *G. mellonella*.[45,51,53] Inactivation of either *rpfF$_{Bc}$* or *rpfR* attenuated the virulence of the strain H111 in *C. elegans*.[46] It will be of great interest to investigate whether BDSF-deficient mutants are also attenuated in other infection models and to examine the importance of DSF family-based signaling systems for virulence of other Bcc species.

The use of the above mentioned infection models provided convincing evidence that QS is involved in the pathogenicity of Bcc species. Rather than controlling one universal pathogenicity factor, QS regulates the expression of a variety of virulence factors, which vary in their importance for infections in different host organisms.[69] For example, the zinc metalloproteases ZmpA and ZmpB, which are regulated by both AHL and BDSF, were found to be important for the pathogenicity in mammalian hosts and *D. melanogaster*, whereas they do not contribute to pathogenicity in *C. elegans*, *G. mellonella*, or alfalfa.[27,69,72] The stringently AHL-regulated protein AidA, is essential for killing of *C. elegans*, but is not required for virulence in any other infection model.[69] A comprehensive list of QS-regulated virulence factors can be found in the review of Subramoni and Sokol.[70]

A few clinical studies have provided evidence that an intact QS system is important for the pathogenicity of *Burkholderia* species during infection of CF patients. Analysis of sputum samples revealed that AHLs can be detected in the majority of the samples originating from patients infected with *B. cepacia* or *Pseudomonas aeruginosa*, but not *Staphylococcus aureus*.[73] This is in accordance with another study, where AHLs could be extracted from mucopurulent respiratory secretions from 13 cystic fibrosis patients infected with *P. aeruginosa* and/or Bcc strains.[74] Furthermore, McKeon et al. showed that a functional QS system is maintained in *B. cenocepacia* and *B. multivorans* during the course of infection in CF patients. Forty-five sequential isolates of these two species isolated over a time course of 2 to 16 y from individuals suffering from CF were analyzed for their ability to produce AHLs. Interestingly, all but one isolate produced AHLs. Further analyses revealed that there is a selective advantage for strains with a functional CepIR system and that mutations in these genes are therefore very rare.[75] Additional evidence for an important role of AHL-mediated QS was obtained by analyzing the transcriptome of two clonal variants of *B. cenocepacia* isolated during long-term colonization of a CF patient.[76] In this study, more than 1000 genes were found to be differentially expressed, including *cciR* and 94 genes previously known to be regulated by CciR. Likewise, DSF signals have been shown to be present in CF sputa and correlated well with patient colonization by *Stenotrophomonas maltophilia* and/or *B. cenocepacia* (see above).[49]

The Role of QS in Biofilm Formation of Members of the Bcc

Evidence that has accumulated over the past decade suggests that QS plays an important role in the development of bacterial biofilms. Cells within the biofilm are embedded in a self-produced extracellular matrix and are in close contact to each other, thus representing a high cell density community. The biofilm matrix may also act as a diffusion barrier for signal molecules, creating an ideal environment for the induction of QS.

The influence of AHL-mediated QS on biofilm formation has been investigated using a quorum-quenching approach, i.e., the enzymatic degradation of AHL signal molecules.[77] This survey revealed that the CepIR system controls biofilm formation in the great majority of the Bcc strains tested. A role of AHL signal molecules in biofilm formation was first reported for *B. cenocepacia* H111. In this study it was shown that inactivation of either *cepI* or *cepR* impaired biofilm maturation and resulted in thinner biofilms when compared with the one formed by the wild type.[16] Subsequent work showed that the influence of AHL-based QS on biofilm formation of *B. cenocepacia* K56-2 (harboring two QS systems, *cepIR* and *cciIR*), is more complex: while mutations in either *cepI*, *cepR* or *cciR* led to reduced biofilms, mutations in *cciI* or in *cepI* and *cciI* did not affect biofilm formation.[26] It was not until 2012 that the QS-regulated factors that link QS and biofilm formation were identified.[58] To this end, it was necessary to first unravel the underlying molecular mechanism of biofilm development in the Bcc.

QS-regulated functions affecting biofilm formation in members of the Bcc. The initial step in biofilm development is the adhesion of cells to a surface. This event is often dependent on specialized surface appendages such as fimbriae or flagella, which can act as adhesins.[78] By the use of transmission electron microscopy (TEM), five types of pili were identified in Bcc strains: mesh (Msh), filamentous (Fil), spine (Spn), spike (Spk), and cable pili (Cbl).[79] Although the cable pilus Cbl has an important role in the adhesion to epithelial cells (and is therefore thought to be of clinical relevance), only a few strains from the Bcc harbor the *cblA* gene coding for this pilus and not all *cblA*-positive strains produce the pilus.[80] In *B. cenocepacia* H111, a gene encoding a homolog of the *E. coli* FimA type I pilus was identified and shown to be controlled by the CepIR system.[81] This gene (*fimA, BCAL1677*) is part of an operon that also contains genes coding for a chaperone–usher secretion apparatus (*BCAL1678–1681*).[58,82] Type I fimbriae are important in some organisms for the adhesion to surfaces;[83] however, a *fimA* mutant of *B. cenocepacia* H111 was neither defective in biofilm biomass nor in biofilm architecture on abiotic surfaces.[58,84] Since additional genes coding for pili are present in the H111 genome, including a cluster encoding a type IV Flp-type pilus, it is plausible that these may compensate for the absence of FimA, at least under the conditions tested. In *B. pseudomallei* the *pilA* gene, which codes for a type IV pilus, was demonstrated to be essential for microcolony development but was not required for adherence to human cells in culture.[85] In conclusion, the exact role of pili in biofilm development of *Burkholderia* sp remains to be elucidated.

To identify the genes involved in the late stages of biofilm development, a collection of 5000 random transposon insertion mutants in *B. cenocepacia* H111 was screened using a microtiter dish-based assay.[59] Thirteen mutants that exhibited defects in biofilm formation without being impaired in growth were isolated.

Inspection of the biofilms formed by these mutants using confocal laser-scanning microscopy (CLSM) revealed dramatic differences in their morphologies when compared with the one of the parent strain. The genes responsible for the different biofilm architectures encoded several functions, including surface proteins, proteins implicated in the biogenesis and maintenance of the outer membrane and, importantly, regulators and proteins involved in QS.[59]

One of the genes identified in this screen was *bapA*, which codes for the **b**iofilm **a**ssociated **p**rotein **A** (BapA). BapA belongs to a family of large surface proteins that are usually secreted via a type I secretion system and are believed to remain loosely associated with the cell surface.[86,87] Several members of this family of proteins have been shown to have a role in biofilm formation in different bacterial species. Examples of these large proteins are LapA and LapF from *P. putida* KT2440,[88] Bap of *S. aureus*,[89] Esp of *Enterococcus faecalis*,[90] LapA from *P. fluorescens*,[86] and BapA of *Salmonella enterica*.[87] Importantly, *bapA* expression was found to be controlled by the CepIR system in a combined transcriptome and proteome analysis of *B. cenocepacia* H111 and is to date the only member of the family of large surface proteins whose expression is QS-regulated.[58] BapA has been shown to be of crucial importance for biofilm formation on abiotic surfaces, influencing both the architecture and the biomass of the biofilm. The *bapA* gene is co-transcribed with three genes encoding a type I export machinery. Mutants in this exporter were indistinguishable from a *bapA* mutant, suggesting that it is involved in the secretion of BapA.[58]

In the study of Inhülsen et al., a third operon, *bclACB* (*BCAM0184-186* in *B. cenocepacia* J2315), was identified among the AHL-regulated genes that influenced biofilm morphogenesis.[58] This operon encodes for three lectins, which share a PA-IIL-like C-terminal domain. BclB and BclC have additional N-terminal domains. BclA forms homodimers with a strict specificity for oligomannose-type oligosaccharides, present on human glycoproteins.[91,92] BclC forms hexamers and has an N-terminal domain displaying a TNF-α-like fold with fucose-binding properties. Since BclC contains two different lectin domains, it is considered to be a super-lectin, with dual carbohydrate specificity.[93,94] The third lectin produced by *B. cenocepacia*, BclB, is the least characterized one but it was recently shown that it may be associated with the bacterial cell surface.[58] The biofilms formed by a *bclACB* mutant strain were found to have an altered architecture, with hollow microcolonies that are not observed in wild-type biofilms. Interestingly, this defect could only be rescued after complementation with an intact *bclACB* operon, suggesting that the three lectins are not redundant and that all three lectins are needed for biofilm structural development.[58]

The role of EPS in biofilm formation of Bcc strains. In addition to protein components, EPS is an important constituent of the biofilm matrix, affecting cell attachment and the mechanical stability of the biofilm. EPS is produced by the majority of Bcc species and cepacian, a polysaccharide with a branched heptasaccharide repeating unit, was shown to be particularly widespread among Bcc species.[95,96] Cepacian plays a role in biofilm maturation but was not required for the initial steps of biofilm development.[10]

Two gene clusters, *bce-I* and *bce-II,* encode the enzymes necessary for cepacian biosynthesis.[96,97] Interestingly, no clear correlation could be established between the ability of 108 Bcc strains to produce EPS or to form biofilms in vitro and the clinical outcome of the infections they caused in different patients.[10] In another study an inverse correlation between EPS production and decline of CF lung function by Bcc bacteria has been reported.[98] This suggests that non-mucoid isolates are associated with increased disease severity while the mucoid phenotype may be associated with bacterial persistence. In support of this hypothesis it has been shown that an EPS-producing clinical *B. cenocepacia* isolate was able to inhibit chemotaxis and production of reactive oxygen species (ROS) of neutrophils in vitro, both essential components of innate neutrophil-mediated host defenses.[99] Although it has been demonstrated that production of EPS is controlled by an AHL-dependent QS system in plant-associated *Burkholderia* species,[100] there is no evidence that this is also the case in members of the Bcc. A possible mechanism involved in the regulation of cepacian biosynthesis is mediated by the RNA chaperone Hfq, a protein known to regulate target mRNAs by small regulatory non-coding RNAs. In the clinical isolate *B. cepacia* IST408, the deletion of the *hfq* gene strongly reduces cepacian production, which could be explained by either a small RNA molecule or by pleiotropic effects caused by the lack of Hfq.[101]

The role of BDSF and c-di-GMP in biofilm formation of Bcc strains. The widespread bacterial second messenger c-di-GMP has been identified as a key player in the transition of bacteria from the planktonic to the sessile lifestyle. It is generally accepted that high levels of intracellular c-di-GMP promote biofilm formation.[57]

Fazli et al. demonstrated that overproduction of c-di-GMP by expressing the GGDEF domain protein YedQ from *E. coli* in *B. cenocepacia* H111 resulted in the formation of a pellicle at the air-liquid interface in a static liquid culture as well as in wrinkled colony morphology on a solid medium.[102] The latter phenotype was exploited to identify the c-di-GMP effector BCAM1349, which was shown to be not only required for these two phenotypes but is also important for virulence in a *G. mellonella* wax moth larvae infection model.[102] In a subsequent study the authors showed that BCAM1349 controls expression of a 12-gene cluster, *BCAM1330-BCAM1341*, which encodes an EPS of unknown structure.[84] This polysaccharide has been shown to provide structural stability to flow-cell grown *B. cenocepacia* H111 biofilms. In the presence of c-di-GMP, BCAM1349 binds to the promoter region of *BCAM1330* and stimulates transcription of the gene cluster.

Recent work has demonstrated that biofilm formation of *B. cenocepacia* is also controlled by the RpfFR QS system.[46,51] Mutants in either *rpfF_Bc* or *rpfR* were found to be severely impaired in biofilm formation.[46] In the presence of BDSF, RpfR decreases the cellular level of c-di-GMP, thereby affecting several cellular behaviors including biofilm formation (see above). Among the RpfR-regulated genes we identified *bapA,* explaining at least in part the effect of the BDSF-dependent QS system on biofilm formation of *B. cenocepacia*.[27] Hence, expression of BapA is subject to control by both QS systems operating in this

organism, underpinning the central importance of this large surface protein for biofilm formation by *B. cenocepacia*.

In a recent study a biofilm model was designed that enables long-term selection for daily adherence to and dispersal from a plastic bead in a test tube.[12] In this model, cells must form a biofilm on a plastic bead, which is then used to inoculate another tube containing a fresh bead, so that bacteria must remain adherent during transfer and then disperse to colonize the new bead.[12] Adaptive mutations that occurred over a time period of approximately 1050 generations were identified by sequencing DNA from mixed communities (metagenomes), the complete genomes of representative clones, and alleles of 60 alternative clones from multiple time points. Most interestingly, mutations in the *rpfR* gene were found to be critical for the early evolution of the biofilm community. These mutations occurred either in conserved residues of the GGDEF domain, in the PAS sensor domain, or deleted the entire *rpfR* gene together with 94 other genes. These data suggest that the BDSF signaling system may play a key role for the transition from the planktonic to the biofilm lifestyle and vice versa.

QS affects resistance of Bcc biofilms. It is well established that Bcc strains growing as a biofilm exhibit a markedly increased resistance to both antibiotics and disinfectants, limiting not only the treatment options for CF patients but also complicating the implementation of effective infection control measures in hospitals.[103-106] QS appears to be a highly valuable novel target for treatment of biofilm-related Bcc infections,[23] as QS inhibitors were shown to interfere effectively with biofilm formation and detachment of *B. multivorans* and *B. cenocepacia*.[107-110] Most intriguingly, some QS inhibitors, including baicalin hydrate and cinnamaldehyde, have been shown to improve the success of antibiotic treatment in vitro and in vivo by increasing the susceptibility of bacterial biofilms and reducing virulence during infection, demonstrating the potential of QS blockers as antibacterial and antibiofilm agents.[111]

Outlook

Members of the Bcc are multilingual talents that master at least two chemical languages. The AHL-dependent CepIR system is fully conserved within the Bcc and more than ten years of research have shown that this regulatory circuit controls biofilm formation, proteolytic activity, motility, antifungal activity, and pathogenicity in the large majority of Bcc strains. More recent work has identified several additional regulators, including other QS systems relying on AHLs, LuxR solos, and transcriptional factors, that finetune CepR-dependent gene expression. The BDSF-dependent cell-to-cell communication system is also involved in the regulation of biofilm formation and virulence. Although recent work has shown that the two QS regulons partly overlap it is presently not known whether the two QS systems operate independently of each other or converge and control the expression or the activity status of a yet to be discovered common regulator, which in turn regulates expression of target genes.[27] It will therefore be of great importance to identify the c-di-GMP effector that links BDSF perception through RpfR and target gene expression.

The intracellular second messenger c-di-GMP is believed to play a key role for the lifestyle of bacteria, with high levels being typical for sessile cells during chronic infections and low levels for planktonic cells and acute infections.[57,112] Interestingly, *B. cenocepacia* H111 appears to be an exception to this rule, as inactivation of the BDSF QS system leads to an elevated cellular c-di-GMP level and a concomitant reduction in the strain's ability to form surface-associated biofilms.[27,46] On the other hand, artificially elevated intracellular levels of c-di-GMP were shown to promote a wrinkly colony phenotype, pellicle and biofilm formation.[102] The transcriptional regulator BCAM1349 was shown to bind c-di-GMP, thereby activating the production of several biofilm matrix components, including polysaccharides and fimbriae. Under which natural conditions the intracellular c-di-GMP levels are elevated and thus promote the formation of a pellicle rather than a submerged biofilm remains to be elucidated.

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