High intracellular c-di-GMP levels antagonize quorum sensing and virulence gene expression in *Burkholderia cenocepacia* H111

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**Abstract**

The opportunistic human pathogen *Burkholderia cenocepacia* H111 uses two chemically distinct signal molecules for controlling gene expression in a cell density-dependent manner: N-acyl-homoserine lactones (AHLs) and *cis*-2-dodecenolic acid (BDSF). Binding of BDSF to its cognate receptor RpfR lowers the intracellular c-di-GMP level, which in turn leads to differential expression of target genes. In this study we analysed the transcriptional profile of *B. cenocepacia* H111 upon artificially altering the cellular c-di-GMP level. One hundred and eleven genes were shown to be differentially expressed, 96 of which were downregulated at a high c-di-GMP concentration. Our analysis revealed that the BDSF, AHL and c-di-GMP regulons overlap for the regulation of 24 genes and that a high c-di-GMP level suppresses expression of AHL-regulated genes. Phenotypic analyses confirmed changes in the expression of virulence factors, the production of AHL signal molecules and the biosynthesis of different biofilm matrix components upon altered c-di-GMP levels. We also demonstrate that the intracellular c-di-GMP level determines the virulence of *B. cenocepacia* to *Caenorhabditis elegans* and *Galleria mellonella*.

**INTRODUCTION**

The soluble molecule bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is an important intracellular second messenger in bacteria [1]. c-di-GMP is a key factor controlling the transition between motile and sessile lifestyles, with high c-di-GMP levels being indicative of the biofilm mode of growth [2, 3]. In addition to its role in biofilm formation, c-di-GMP is also involved in the regulation of many cellular functions, including the biosynthesis of exopolysaccharides (EPSs), motility, antibiotic production and virulence [4–7]. The intracellular c-di-GMP concentration is modulated by the opposing activities of diguanylate cyclase (DGC) and cyclic nucleotide phosphodiesterase (PDE), which are in turn regulated by extra- or intracellular factors [8, 9]. DGCs produce c-di-GMP from two molecules of GTP and PDEs hydrolyse c-di-GMP to give linear pGpG or two GMP molecules. The GGDEF motif is essential for the enzymatic activity of DGCs, while the PDE activity is associated with EAL and HD-GYP domains [10]. The number of genes encoding GGDEF, EAL and HD-GYP proteins varies greatly among microorganisms [11]. Having numerous c-di-GMP-controlling proteins allows the organism to fine-tune its regulation of target genes, and hence its adaptation to the prevailing environment. Such complex systems, with components that work in parallel, can only function specifically if GGDEF and EAL domain proteins are normally sequestered. Such a sequestration could be temporal or spatial and is likely to involve specific protein–protein interactions [3, 12]. c-di-GMP-dependent regulatory systems are often part of more complex signalling networks, for example in phosphorylation or quorum sensing (QS) cascades [13, 14]. QS is a mechanism that regulates gene expression in a cell density-dependent manner and is based on the synthesis, release and detection of small signal molecules [15–17].
Burkholderia cenocepacia is a Gram-negative opportunistic pathogen that belongs to the Burkholderia cepacia complex (Bcc), a group of 20 closely related bacterial species [18, 19]. Bcc strains are able to inhabit diverse niches and can also cause life-threatening disease in immunocompromised patients [20–24]. Previous work has shown that the production of several virulence factors, including extracellular hydrolytic enzymes, secondary metabolites and biofilm, is QS-regulated in members of the Bcc [25–28]. B. cenocepacia H111 has two types of QS systems, one relying on N-acyl-homoserine lactone (AHL) and the other on cis-2-dodecanoyl (Burkholderia diffusible signal factor, BDSF) as signalling molecules [25, 28, 29]. The BDSF-based system consists of the BDSF synthase RpfIIC and its receptor RpfR [30]. Upon binding of BDSF to RpfR, which contains PAS-GGDEF-EAL domains, the PDE activity of RpfR is stimulated and the cellular c-di-GMP level is reduced [30]. A range of target genes, including the large surface protein bapA and the lectin operon bcLA, are at least partly regulated by BDSF [28, 31].

In this study, phenotypic changes of B. cenocepacia H111 in response to artificially modified intracellular c-di-GMP levels were analysed and alterations in the global transcriptional profile were determined by RNA-Seq. One hundred and eleven genes were differentially expressed with the large majority being downregulated at high intracellular c-di-GMP concentration. The c-di-GMP regulon overlaps substantially with both QS regulons. Our results show that c-di-GMP is involved in the regulation of various functions affecting the physiology and virulence of B. cenocepacia H111 and demonstrate that c-di-GMP is a negative regulator of QS in this organism.

METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains, plasmids and oligonucleotides used are listed in Tables S1 and S2 (available in the online Supplementary Material).

Unless otherwise stated, strains were grown aerobically at 37°C in LB Lennox broth (Difco) at 225 r.p.m. or on LB Lennox plates (1.5% agar). Antibiotics were used at the concentrations (in µg ml⁻¹) indicated in parentheses: for Escherichia coli, ampicillin (100), kanamycin (25), gentamicin (10) and chloramphenicol (30); and for B. cenocepacia, kanamycin (100), gentamicin (20) and chloramphenicol (60). Plasmid pBBR-rpfRAAL [30] was digested with XhoI and HindIII and the rpfRAAL was ligated into plasmid pBRR1MCs-5 cut with the same enzymes. Plasmids were delivered to B. cenocepacia by triparental mating, as described previously [25].

RNA-Seq analysis

Total RNA of cultures grown to late exponential growth phase (OD₆₀₀ 2.0) was extracted and processed by using a hot acid phenol protocol [32]. Briefly, a cell pellet was resuspended in 1.5 ml ice cold buffer A (20 mM sodium acetate pH 5.5, 1 mM EDTA) and added to a mixture of 160 µl 10% SDS, 2 ml buffer A and 3.5 ml acid phenol. The suspension was vigorously mixed for 30 s and incubated for 7 min at 65°C with an additional vortexing step of 1 min in between. The aqueous phase was separated and extracted with 3 ml phenol/chloroform/isoamyl alcohol and then with 2.5 ml chloroform. Total RNA was precipitated at −80°C overnight and quality control was performed by using RNA Nano Chips (Agilent 2100 Bioanalyzer; RNA integrity number, RIN >8). To remove the 5S rRNA which would interfere with the subsequent mRNA enrichment procedure, 50 µg of total RNA in a volume of 100 µl was run through RNeasy Mini kit columns (Qiagen). DNA depletion was achieved by a treatment with RQI RNase-Free DNase I (Promega) and SUPERaseIn RNase Inhibitor (Ambion) and verified by PCR using primers rhlA_F and rhlA_R (target: rhlA). Depletion of mRNA was performed by using the MICROBExpress kit (Ambion) with two times 5 µg RNA per strain, as described previously [33]. If subsequent bioanalyser results suggested incomplete mRNA removal, this step was repeated on the pooled samples. For the synthesis of cDNA and library generation, the Ovation Prokaryotic RNA-Seq System (Nugen) and the Ovation Ultralow Library Systems (Nugen) were used. The libraries (10 nM of each) were pooled and sequenced in one sequencing lane. The sequencing was performed on an Illumina HiSeq 2000, with 100 bp single-end reads. The sequencing reads were mapped to the B. cenocepacia H111 genome using CLC Genomics Workbench v4.9 (CLCbio) allowing up to two mismatches per read. Orthologue mapping was performed as described previously [28]. To obtain a list with differentially expressed genes in response to changes in c-di-GMP levels, the unique read counts of strains H111/pPA5295, H111/pBBR5 and H111/pRpfRAAL were analysed with the R-package DESeq2 [34]. Data are based on two independent replicates. Genes with an adjusted P-value <0.05 were considered. The RNA-Seq raw data files are accessible through the GEO Series accession number GSE92442.

Extraction and quantification of c-di-GMP

c-di-GMP was quantified on the bacterial cultures used for RNA-Seq analysis by harvesting 5 ml. Nucleotide extraction was performed as described by Spangler et al. [35] with slight modifications: cXMP was omitted and evaporation to dryness was performed using a vacuum concentrator. Quantification by LC-MS/MS was performed as described in [36].

Phenotypic assays

Proteolytic activity was determined on skimmed milk plates. To this end, bacterial overnight culture (5 µl) was spotted on a skimmed milk plate (LB Lennox, 2% skimmed milk, 1% agar) and incubated for 24 h at 37°C before photographic documentation. The assay was repeated at least three times. Biofilm formation was quantified in a microtitre dish assay as described previously [25]. Briefly, overnight cultures were diluted to an OD₆₀₀ of 0.01 in minimal medium supplemented with 10 mM citrate and 100 µl of this suspension was added per well to a 96-well plate. After

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48 h of static incubation at 30 °C, the planktonic cells were removed and 100 μl of a 1% (w/v) aqueous solution of crystal violet was added. Following 20 min incubation at room temperature, the wells were washed with distilled water, the dye was solubilized with 120 μl DMSO and the absorbance was determined at 570 nm. AHL and BDSF levels were quantified by the aid of biosensors as described previously [28]. Siderophore production was analysed by the chromo azuro S (CAS) assay [37] with the following modification: LB PIPES [7.65 g PIPES (Sigma), 2.5 g Bacto tryptone, 1.25 g Bacto yeast extract, 1 g NaCl in 225 ml H₂O] was used instead of MM9 salts and casamino acids. Five microlitres of bacterial overnight cultures were spotted on plates prior to incubation for 24–48 h at 37 °C. Pathogenicity assays with Caenorhabditis elegans and with Galleria mellonella were performed as described by Agnoli and coworkers [38]. Data shown are from three independent replicates. The pathogenicity score was calculated as described previously [38].

RESULTS

**Altered c-di-GMP levels affect multiple phenotypes in B. cenocepacia H111**

To study the effect of different intracellular c-di-GMP levels on the physiology of B. cenocepacia H111 we employed plasmids expressing RpfR variants with mutated catalytic residues in either the EAL or the GGDEF domain. As shown in Fig. 1, expression of rpfR<sub>AAAL</sub>, in which the glutamic acid residue of the RpfR EAL motif was substituted by alanine, rendering the protein incapable of c-di-GMP degradation, resulted in a dramatic increase of the c-di-GMP level from approximately 20 ng ml<sup>−1</sup> to more than 2000 ng ml<sup>−1</sup>. Conversely, expression of rpfR<sub>GGAAF</sub>, in which the GGDEF domain required for c-di-GMP biosynthesis being inactivated, did not significantly reduce the c-di-GMP level (data not shown). As a control, we used plasmid pPA5295, which constitutively expresses the PDE gene PA5295 of Pseudomonas aeruginosa from the lac promoter of pBBR1MCS-5. Expression of PA5295 from a different plasmid in Caulobacter crescentus reduces the intracellular c-di-GMP level to below the detection limit [39]. Similarly, introduction of pPA5295 into B. cenocepacia H111 reduced the intracellular c-di-GMP level below the detection limit (Fig. 1). Characterization of these strains revealed that the c-di-GMP level did not affect growth rates but had a great influence on various phenotypes (Fig. 2; data not shown). Strain B. cenocepacia H111/pRpfR<sub>AAAL</sub> lacked proteolytic and swarming activities, and was deficient in swimming motility, siderophore production and biofilm formation in microtiter trays, whereas pellicle formation was not affected. Interestingly, the low c-di-GMP strain B. cenocepacia H111/pPA5295 formed no pellicle at all. The B. cenocepacia H111/pPA5295 strain exhibited increased swimming and swarming motility but all other phenotypes tested were not affected.

The effect of altered c-di-GMP levels on virulence was assessed in the two infection hosts C. elegans and G. mellonella. While the wild-type carrying the empty plasmid pBBR1MCS-5 and the low c-di-GMP strain B. cenocepacia H111/pPA5295 were virulent to C. elegans (pathogenicity score 3), the nematodes showed no sign of sickness and propagated well when fed on the high c-di-GMP strain H111/pRpfR<sub>AAAL</sub> (pathogenicity score 0, Fig. 3a, Table S3). The high c-di-GMP strain was also highly attenuated in the G. mellonella infection model. While more than half of the larvae had died by 48 h post infection with B. cenocepacia H111/pBBR5 or the low c-di-GMP strain H111/pPA5295, most B. cenocepacia H111/pRpfR<sub>AAAL</sub>-infected larvae remained alive. The difference in virulence between the strains was even more pronounced 72 h post infection (Fig. 3b).

**c-di-GMP downregulates AHL-mediated QS**

As previous work had demonstrated that virulence among other phenotypes affected by c-di-GMP are regulated by QS in B. cenocepacia H111 [25, 30], we tested the influence of altered c-di-GMP levels on the production of signal molecules. We found that the high c-di-GMP strain B. cenocepacia H111/pRpfR<sub>AAAL</sub> produced greatly reduced amounts of AHJs relative to B. cenocepacia H111/pBBR5, while the AHL level of B. cenocepacia H111/pPA5295 was virtually indistinguishable from the control (Fig. 4a). The BDSF level was sixfold reduced in strain H111/pRpfR<sub>AAAL</sub> but was unaltered in H111/pPA5295 relative to the control strain (Fig. S1). AidA, which is a major virulence factor in the C. elegans infection model, is one of the most stringently AHL-regulated proteins in B. cenocepacia H111 [27, 28, 40].
Western blot analysis of strains with different c-di-GMP levels demonstrated that expression of AidA is downregulated at a high intracellular c-di-GMP level, consistent with the reduced AHL production of the strain (Fig. 4b). Supplementation of the growth medium with 200 nM AHL partially restored AidA expression in *B. cenocepacia* H111/pRpfR AAL. These results indicate that downregulation of AidA is a direct consequence of lowered AHL production at a high intracellular c-di-GMP level.

**c-di-GMP regulon overlaps with both QS regulons in *B. cenocepacia* H111**

To determine how altered c-di-GMP levels affect global transcription patterns in *B. cenocepacia* H111, we performed RNA-Seq of the wild-type harbouring the empty plasmid (H111/pBBR5), the high c-di-GMP strain (H111/pRpfRAAL) and the low c-di-GMP strain (H111/pPA5295). The percentage of uniquely mapped reads varied between 19 and 32%. Using a ≥3-fold change cut-off, we found a total of 111 genes to be significantly differentially expressed upon alterations of the c-di GMP level (adjusted *P*-value <0.05), i.e. wild-type versus high or low c-di-GMP level or low versus high c-di-GMP level (Tables 1 and S4). Ninety-six of these genes were downregulated in H111/pRpfRAAL, suggesting that c-di-GMP is predominantly a negative regulator in *B. cenocepacia* H111. Only 15 genes were upregulated in the high c-di-GMP strain.

In agreement with our phenotypic characterization, many of the differentially regulated genes were previously shown to be QS-controlled (Table S4, Fig. 5). Expression of 24 genes was regulated by c-di-GMP and both QS systems. These genes include *aidA*, the zinc metalloprotease *zmpB*, the lectins *bclACB*, the large surface protein *bapA*, the adjacent type I secretion system (I35_6022–24 in H111 and BCAM2140-42 in J2315), and a large cluster coding for a...
non-ribosomal peptide synthetase (I35_4188–95 in H111 and BCAM0190-96 in J2315). In the high c-di-GMP strain, expression of cepI was 10-fold downregulated relative to the wild-type, in agreement with the reduced AHL production by this strain (Fig. 4a). This also explains the substantial overlap (42 genes) of the CepR and c-di-GMP regulons (Table S4). We also found rpfF<sub>Bc</sub> and rpfR were upregulated under high c-di-GMP conditions. However this is an artefact, as in the high c-di-GMP strain, rpfR<sub>AAL</sub> as well as part of rpfF<sub>Bc</sub> is expressed from the introduced plasmid. When comparing H111/pBBR5 with H111/pPA5295, neither rpfF<sub>Bc</sub> nor rpfR were significantly regulated.

Comparison of the low c-di-GMP strain with the high c-di-GMP strain identified five and 12 genes involved in cepacian (bceA, bceC, bceE, bceF, gtaB) and pyochelin (I35_6113–26 in H111; BCAM2221-35 in J2315) biosynthesis, respectively (Tables 1 and Table S4). These genes are weakly induced at low and slightly repressed under high c-di-GMP conditions. We have previously shown that all the cepacian and eight of the 12 pyochelin biosynthesis genes are positively regulated by BDSF [28], in agreement with the PDE activity of RpfR. We observed that strain B. cenocepacia H111/pRpfR<sub>AAL</sub> formed a smaller halo relative to the wild-type on CAS plates (Fig. 2a). The halo had also lost its pinkish colour, indicative of the absence of pyochelin. We observed that B. cenocepacia H111/pRpfR<sub>AAL</sub> produced lower amounts of EPS on mannitol agar plates, suggesting reduced cepacian production (data not shown).

**Genes regulated by c-di-GMP but unaffected by QS**

Our RNA-Seq analysis also identified 51 genes that are regulated by c-di-GMP, but are affected by neither RpfF<sub>Bc</sub> nor CepR (Table S4). Many of these genes are located within three clusters that code for the phenylacetate catabolic pathway (Fig. 6) [41]. All of these genes, with the exception of paaF and paaR, were more than twofold downregulated in the high, compared to the low, c-di-GMP strain. Interestingly, we also identified four genes (I35_5889, bscD, bscC, bcsV) of the type III secretion system cluster (I35_5881–93...
Forty-five genes were identified that showed differential expression upon altered c-di-GMP level (DESeq analysis: fold change ≥5 and adjusted P-value <0.05). WT: H111/pBBR5, high: H111/pPBr

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<td>BCAM2224</td>
<td>TonB-dependent outer membrane receptor for ferric-pyochelin</td>
<td>fptA</td>
<td>1.32</td>
<td>-1.60</td>
<td>6.57</td>
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<td>I35_6117</td>
<td>BCAM2225</td>
<td>Eflux pump, fused inner membrane and ATPase subunits in pyochelin gene cluster</td>
<td>pchI</td>
<td>1.41</td>
<td>-1.50</td>
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<td>Pyochelin biosynthetic protein PchG, oxidoreductase (NAD-binding)</td>
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<td>I35_6120</td>
<td>BCAM2230</td>
<td>Pyochelin synthetase, non-ribosomal peptide synthetase module</td>
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<td>1.66</td>
<td>-1.41</td>
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<td>Dihydroaeruginosato synthetase, non-ribosomal peptide synthetase modules</td>
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<td>BCAM2232</td>
<td>2,3-Dihydrobenzoate-AMP ligase (pyochelin) siderophore</td>
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<td>BCAM2233</td>
<td>Pyochelin biosynthetic protein, putative thioesterase</td>
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<td>Isochorismate pyruvate-lyase (pyochelin) siderophore</td>
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<td>-1.49</td>
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<td>BCAS0292</td>
<td>virulence factor in C. elegans model</td>
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<td>4.78</td>
<td>-1.28</td>
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in H111 and BCAM2045-57 in J2315 among the exclusively c-di-GMP-regulated genes (Tables 1 and S4).

To analyse whether the gene expression changes induced by high c-di-GMP levels in B. cenocepacia H111 are specific for the cyclase activity of RpFR_{AA}, we also performed an RNA-Seq analysis of B. cenocepacia H111 carrying plasmid pYedQ. This plasmid expresses the GGDEF-domain protein YedQ of E. coli and was previously shown to increase the intracellular c-di-GMP level in B. cenocepacia [42]. In B. cenocepacia H111/pYedQ we found 61 genes with altered expression (≥2-fold change cut-off, P-value <0.05) compared to B. cenocepacia H111/pBBR5 (Table S4). Of the genes differentially regulated in B. cenocepacia H111/pRpFR_{AA}, 18 were differentially regulated in B. cenocepacia H111/pYedQ, including bapA, cepI, the protease zmpA and the paa gene clusters (Tables 1 and S4). Most intriguingly, 26 and 37 genes respectively were exclusively regulated by expression of YedQ and RpFR_{AA,} (≥3-fold change, P-value <0.05). Given that in both cases the intracellular c-di-GMP levels were massively increased relative to the wild-type, our data suggest that the differential regulation of these genes is linked to specific interactions of the c-di-GMP-synthesizing enzymes with other regulatory proteins.

**DISCUSSION**

To investigate the influence of c-di-GMP on global gene expression in B. cenocepacia H111, we profiled the transcriptomes of strains with altered intracellular c-di-GMP levels. Our results indicate that the second messenger molecule c-di-GMP is a predominantly negative regulator of B. cenocepacia H111 gene expression. The most striking effect of a high intracellular c-di-GMP level was the strong downregulation of cepI transcription and concomitantly of AHL-mediated gene expression. This explains many of the phenotypic changes observed in response to altered c-di-GMP levels. We found that a high intracellular c-di-GMP concentration abolished proteolytic activity, reduced siderophore production and diminished biofilm formation in microtitre trays. High c-di-GMP levels also attenuated virulence of B. cenocepacia H111 to C. elegans and G. mellonella.

Global RNA-Seq analyses revealed a substantial overlap of the c-di-GMP regulon with the AHL regulon, which confirmed the results of the phenotypic characterization. Given that the BDSF-based QS system affects the cellular c-di-GMP level [30] an overlap of the c-di-GMP and the BDSF-dependent transcriptomes was expected. Interestingly, not all of the BDSF-regulated genes were found among the c-di-GMP-regulated genes and vice versa. There are several reasons for this observation: (i) we applied stringent criteria to filter for differentially expressed genes in our transcriptome analyses and some of the jointly regulated genes may not have passed the filter criteria in one of the experiments; (ii) in addition to RpFR an additional, subordinate, BDSF receptor has been characterized in B. cenocepacia strain J2315 (the histidine kinase BCAM0227), which triggers a c-di-GMP-independent signal transduction pathway [43]; (iii) B. cenocepacia H111 encodes 23 proteins that contain an EAL and/or GGDEF domain and an unknown number of c-di-GMP binding proteins (Table S5). Artificial modification of c-di-GMP levels may lead to differential expression of genes whose expression is, under certain environmental conditions, stimulated by other c-di-GMP-dependent regulatory cascades.

Among the c-di-GMP- but not QS-regulated genes is the phenylacetate degradation pathway, which was downregulated in the high c-di-GMP strains. The phenylacetate catabolic pathway is a central route through which different aromatic compounds, such as styrene, phenylethylamine and phenylalanine, are aerobically degraded and funneled into the TCA cycle [44, 45]. In B. cenocepacia, the
phenylacetate degradation pathway is encoded by genes arranged in three clusters, which are repressed by glucose and succinate and induced in synthetic cystic fibrosis sputum medium [41, 46–48]. This pathway has been associated with pathogenicity of \textit{B. cenocepacia} in several studies [41, 49, 50]. The attenuated virulence was attributed to transcriptional inhibition of the \textit{cepIR} QS system by phenylacetate that is released by the mutants [51]. Our results are in agreement with a role of the AHL-dependent QS system in the regulation of the phenylacetate pathway and show that high c-di-GMP levels repress signal molecule production.

Previous work has shown that plasmid pC3 is essential for pathogenicity of \textit{B. cenocepacia} H111 to both \textit{C. elegans} and \textit{G. mellonella} [38]. In this study we identified \textit{zmpA} and \textit{aidA} as known pathogenicity factors in mice and \textit{C. elegans}, respectively, among the c-di-GMP-regulated genes on pC3. Both genes, however, do not play a role in virulence in the \textit{G. mellonella} model [52]. Moreover, many other genes known to contribute to pathogenicity of \textit{B. cenocepacia} against wax moth larvae are not among the c-di-GMP-regulated genes, including \textit{obrA}, \textit{pvdA} [52], the flagellum genes \textit{flIC} and \textit{flIj}, the LPS core polysaccharide genes \textit{hldA} and \textit{waaC} [53], I35_4218 (BCAM0227 in J2315) [43] and I35_4271 (BCAM0224 in J2315) [54]. Interestingly, the tyrosine kinase \textit{bceF}, one of the c-di-GMP-regulated genes identified in our study, was recently shown to contribute to virulence of \textit{B. cepacia} IST408 in the \textit{G. mellonella} infection model and to biofilm formation in \textit{B. cenocepacia} K56-2 [55, 56]. Given that \textit{bceF} is encoded by the cepacian biosynthesis cluster I, it will be of interest to investigate the role of cepacian in pathogenicity to \textit{G. mellonella}.

In several pathogens, the expression of secretion systems is c-di-GMP-regulated (discussed in [57]). In this study, we found four genes encoding components of the type III secretion system (T3SS) to be downregulated under high c-di-GMP conditions in \textit{B. cenocepacia}. T3SSs, which have evolved to deliver effector proteins from the bacterial cytoplasm into the host cell cytosol, are found in plant as well as in animal pathogens [58]. In \textit{B. cenocepacia} the T3SS is only poorly characterized. A mutant in \textit{bscN}, which encodes a component of the T3SS of \textit{B. cenocepacia} J2315, was attenuated in a mouse infection model, with significantly lower bacterial recovery from the lungs and spleens and less pronounced histopathological changes of lung tissue when compared to the wild-type [59]. Furthermore, a \textit{bscN} mutant showed reduced killing of \textit{C. elegans} and was less competitive than the \textit{B. cenocepacia} K56-2 wild-type in the \textit{Drosophila melanogaster} infection model [52, 60]. Interestingly, while some genes of the T3SS were reported to be BDSF-regulated in \textit{B. cenocepacia} J2315, they were not among the BDSF-regulated genes in strain H111 [28, 43].

A link between QS and c-di-GMP has been reported for several bacteria. In \textit{Vibrio cholerae} QS triggers a signalling cascade that controls expression of HapR, which represses both biofilm formation and virulence factor production [61, 62]. HapR represses biofilm formation directly (through controlling \textit{vpsT}) as well as indirectly by reducing the levels of c-di-GMP. In the plant pathogen \textit{Xanthomonas campestris}, the diffusible signalling factor (DSF), a member of the family of cis-2-unsaturated fatty acid signal molecules like BDSF, is required for plant pathogenicity [63]. In this bacterium DSF activates the RpfCG two-component system, which in response lowers the cellular c-di-GMP level and thereby induces expression of target genes involved in biofilm dispersal and virulence [64, 65]. In all these examples the QS systems regulate the intracellular c-di-GMP levels in response to the bacterial population density. This is fully in line with the BDSF-dependent RpfQR QS system in \textit{B. cenocepacia}, which was shown to reduce the intracellular c-di-
GMP concentration [30]. However, here we report for the first time that, we believe, the c-di-GMP level controls production of a QS signal by demonstrating that expression of the cefI AHL synthase is massively downregulated in high c-di-GMP cells. Consequently, not only is expression of virulence factors repressed but also the ability to form biofilms in microtitre plates (Fig. 2f). This is likely to be due to the observed downregulation of the expression of the large surface protein BapA, which has been demonstrated to be essential for biofilm formation [25, 27]. This finding is in disagreement with the commonly accepted view that high c-di-GMP levels are indicative of the biofilm mode of growth. Moreover, previous work has shown that artificial elevation of the c-di-GMP level by introducing plasmid pYedQ into B. cenocepacia H111 leads to a wrinkly macrocolony phenotype, promotes pellicle formation and stimulates biofilm formation in flow-cells [42]. It has been shown that these effects are caused by increased expression of an EPS encoded by the BCAM1330-BCAM1341 gene cluster [42] that is transcriptionally activated by the c-di-GMP-binding regulator BCAM1349 [66]. Our global transcription analysis of B. cenocepacia strain H111/pYedQ identified 61 differentially expressed genes when compared to the wild-type strain (Table 1). However, neither BCAM1349 nor the BCAM1330-BCAM1341 gene cluster was regulated under the conditions used for the RNA-Seq analysis. Our data suggest that these genes are not expressed in LB medium and in agreement with this we observed pellicle formation in NYG medium but not in LB medium (Fig. 2, data not shown). Collectively, these results suggest that high c-di-GMP conditions can promote the formation of certain biofilm types through increased polysaccharide production under particular nutritional conditions while QS-dependent formation of submerged biofilms in microtitre plates is suppressed. Additional work will be required to unravel how QS and c-di-GMP fine-tune the expression of biofilm matrix material to determine which type of biofilm is formed in a particular environment.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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