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RESEARCH ARTICLE

Pellicle Biofilm Formation in *Burkholderia cenocepacia* J2315 is Epigenetically Regulated through WspH, a Hybrid Two-Component System Kinase-Response Regulator

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ABSTRACT The chemosensory signal transduction system Wsp regulates biofilm formation and related phenotypes by influencing cyclic-di-GMP (c-di-GMP) levels in bacterial cells. This is typically achieved by activation of the diguanylate cyclase WspR, through phosphorylation of its phosphoreceiver domain. The Wsp system of *Burkholderia cenocepacia* J2315 is in one operon with the hybrid response regulator/histidine kinase *wspH,* but lacks the diguanylate cyclase *wspR* which is located in a different operon. The expression of *wspH*, the first gene in the *B. cenocepacia* Wsp operon as well as pellicle biofilm formation are epigenetically regulated in *B. cenocepacia* J2315. To investigate whether WspH regulates pellicle biofilm formation, several mutants with altered expression of *wspH* were constructed. Mutants with increased expression of *wspH* showed accelerated pellicle biofilm formation, reduced swimming motility and increased c-di-GMP levels. This was independent of WspR phosphorylation, showing that WspR is not the cognate response receiver for histidine kinase WspH.

IMPORTANCE Biofilms are surface-attached or suspended aggregates of cells, that are problematic in the context of bacterial infections, as they provide protection from antibiotic treatment. *Burkholderia cenocepacia* can colonize the lung of immunocompromised patients and forms biofilms that increase its recalcitrance to antibiotic treatment. Pellicles are biofilms which form at an air-liquid interface to take advantage of the higher oxygen concentrations in this environment. How quickly pellicles are formed is crucial for the fitness of obligate aerobic bacteria such as *B. cenocepacia*. Cyclic-di-GMP (c-di-GMP) levels determine the transition between planktonic and biofilm lifestyle, and WspH controls c-di-GMP production. WspH is therefore important for the fitness of *B. cenocepacia* in environments with gradients in oxygen concentration, such as the human lung.

KEYWORDS *Burkholderia*, Wsp, biofilm, c-di-GMP

Burkholderia cenocepacia is an obligate aerobic Gram-negative betaproteobacterium which can mainly be found in the rhizosphere, but can also act as opportunistic pathogen, particularly in individuals with cystic fibrosis (1, 2). *B. cenocepacia* possesses a high innate resistance to antibiotics which makes infections difficult to treat (3), and the ability to form biofilms, which adds to its recalcitrance to antibiotic treatment (4, 5).

Recently, the role of DNA methylation in *B. cenocepacia* J2315 was investigated (6, 7) and it was observed that a mutant in which a gene encoding a methyltransferase (BCAL3494) was deleted displayed increased biofilm and pellicle formation, and reduced motility. Analyzing promoter sites upstream of annotated genes methylated by BCAL3494 revealed a methylated motif 26–30 bases upstream of the transcription start site of gene BCAM0820, overlapping the putative -30 box (6). Expression of gene BCAM0820 was increased in the methyltransferase deletion mutant compared to wild type, indicating that methylation in

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the promoter region of this gene has an impact on its expression level. BCAM0820 is a hybrid response regulator/histidine kinase which is in an operon with genes homologous to the *wsp* genes of *Pseudomonas* spp., and hence the name *wspHRR* was proposed for this gene (8), recently shortened to *wspH* (9).

The Wsp (wrinkly spreader phenotype) system in *Pseudomonas aeruginosa* constitutes a chemosensory signal transduction system similar to chemotaxis and motility regulating sensory systems (10). It influences biofilm formation and motility via an increase in intracellular concentration of cyclic-di-GMP (c-di-GMP) due to activation of WspR, which contains a diguanylate cyclase (DGC) domain (10). However, the homologous *B. cenocepacia wsp* operon does not include *wspR*, nor any other gene with a DGC domain. Nonetheless, this *wsp* operon has been implicated in biofilm formation in *B. cenocepacia* (8, 11, 12).

To explore whether an increased expression of *wspH*, as observed in the DNA methyltransferase mutant, has an influence on *B. cenocepacia* biofilm formation and related phenotypes, we constructed several mutants with altered *wspH* and *wspA-F1* gene expression. When *wspH* was induced, pellicle biofilm formation was accelerated, motility was reduced and c-di-GMP levels were elevated. This confirms that expression of *wspH* can be linked to the phenotypes observed in the DNA methyltransferase deletion mutant.

RESULTS AND DISCUSSION

***wspH* operon organization in *Burkholderia* spp. and its expression in mutants.** The *wsp* cluster in *B. cenocepacia* and other *Burkholderia cepacia* complex bacteria has a similar operon organization as in *Pseudomonas* spp., in that *wspA* is followed by *wspB* to *wspF1*, however, the last gene of the *Pseudomonas wsp* cluster, *wspR*, is not encoded in the *Burkholderia wsp* operon (Fig. 1A). *wspH* is located upstream of the *wsp* genes in all *Burkholderia* spp. (9). The whole cluster is preceded by one transcription start site (13), suggesting *wspH* and *wspA-F1* form a single operon in *B. cenocepacia* J2315 (Fig. 1A), which we designated as *wspH* operon for the sake of clarity.

A *wspR* homolog (BCAM1161) is encoded on replicon two of the *B. cenocepacia* J2315 genome, 350 kb distal to the *wspH* operon (Fig. 1A). This large distance to the *wspH* operon locus might be the reason that this gene has previously not been investigated in the context of *wsp* genes (9). *wspR* consists of a DGC domain and a phosphoreceiver domain (Fig. 1B) that is homologous to the one in *wspH* (63% similarity on the amino acid level). It is in a cluster with *wspF2*, which has 66% similarity to *wspF1*, suggesting that the *wsp* cluster genes were separated and partially duplicated by genome rearrangement in a *Burkholderia* spp. ancestor.

Genome analysis revealed that the organization of *wsp* genes into two clusters is present in all *Burkholderia* spp., except for members of the pseudomallei group, which lack *wspR*. Other bacteria of the family Burkholderiaceae, such as *Paraburkholderia* and *Caballeronia* spp., harbor the *wsp* cluster in its canonical form, with genes *wspA-R* in one operon, and without *wspH*.

We modulated the activity of the *wspH* operon (i) by replacing the native promoter upstream of *wspH* with a rhamnose-inducible promoter (P_{rhaB}) (14) yielding conditional mutant cWsp-1, and (ii) by introducing P_{rhaB} upstream of *wspA* (BCAM0821) yielding conditional mutant cWsp-2 (Fig. 1A, Table 1). To distinguish the effect of increased expression of the whole *wsp* cluster from the effect of overexpressing *wspH* alone, mutant WspH-OV was created in which only *wspH* is expressed in *trans* from a replicative plasmid, under the control of P_{rhaB}. To test whether any resulting phenotype depends on *wspR* activation, the conserved phosphorylation site in the *wspR* receiver domain, which is required for activity (15, 16) was altered (Fig. 1B). The resulting mutant, *wspR72*, was then transformed with the same plasmids as described above, yielding mutants *wspR72*-1, *wspR72*-2, and *wspR72*-OV (Table 1).

Expression levels of *wsp* genes in wild type (WT) and mutant strains were determined in shaking planktonic cultures after 60 min pulse expression from P_{rhaB} (Fig. 1C).

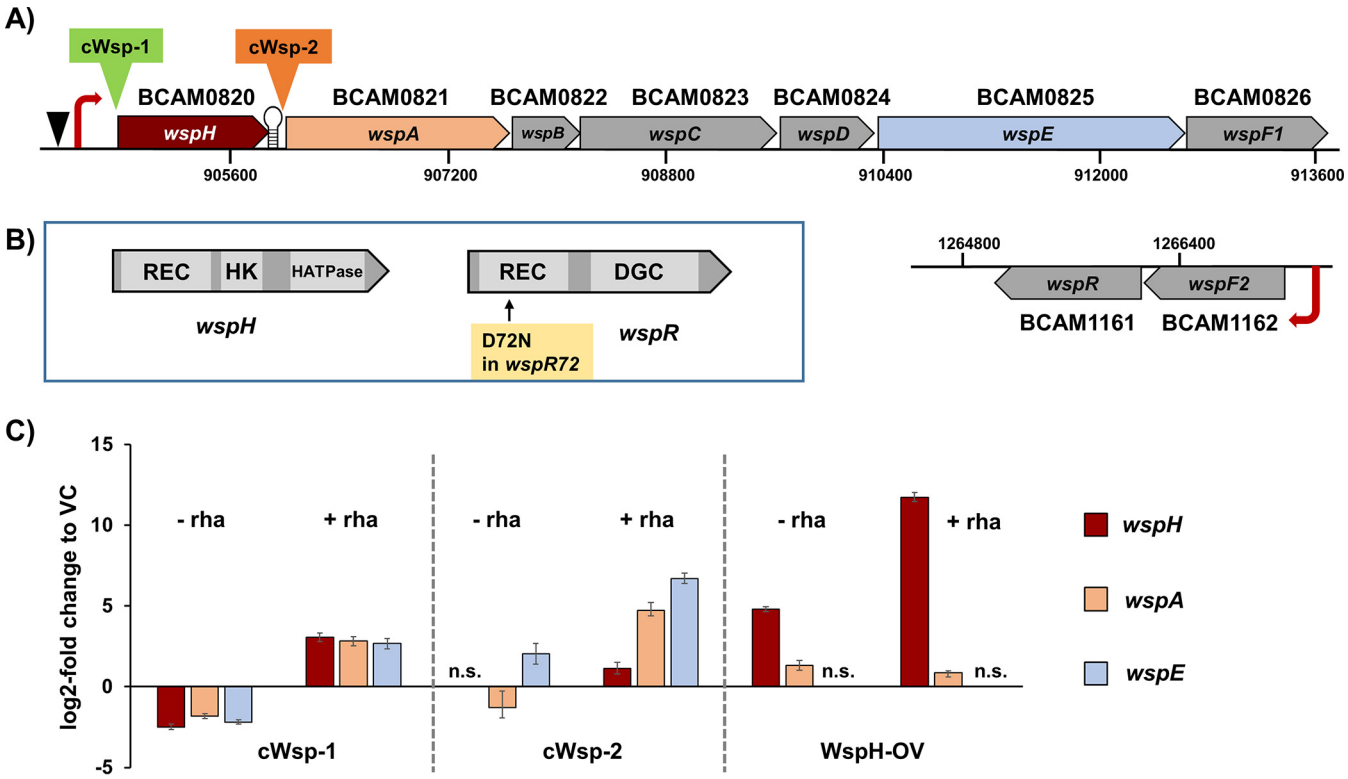

FIG 1 Schematic of *wsp* cluster genes in the *B. cenocepacia* J2315 genome and description of mutants generated in the course of this study. A) *wsp* genes are located in two separate clusters on replicon two, both have an upstream transcription start site (TSS, red arrows, positions 904562 and 1267311, (13)). A rho-independent terminator structure is predicted upstream of *wspA*, but this intergenic region has no TSS. The black triangle depicts the methylated motif 26–30 nt upstream of the TSS. Green and orange triangles indicate the location of P_{rhaB} in mutants cWsp-1 and cWsp-2. B) WspH contains three domains, a response receiver (REC), a histidine kinase (HK) and a histidine kinase-like ATPase (HATPase) domain. WspR contains a diguanylate cyclase output (DGC) and a receiver (REC) domain, where D72 is the phosphorylation site. Mutant *wspR72* was constructed by changing nucleotide 214 from G to A, replacing aspartate at position 72 with asparagine. C) Expression change of *wsp* cluster genes in mutants cWsp-1, cWsp-2 and WspH-OV, compared to vector control (VC), determined by qPCR. Only significant values with $P \geq 0.05$ (three biological replicates) are shown, error bars represent standard deviation. RNA was extracted from planktonic cultures, rhamnnose (rha) was added 1 h before harvest. n.s.: not significant.

This setup was chosen to enable monitoring the immediate effect of gene induction, with minimal interference from a reduced growth rate or from aggregation. Without rhamnose, planktonic growth of mutants was indistinguishable from vector control (VC), while adding rhamnose attenuated growth in mutants cWsp-1, WspH-OV and *wspR72*-OV (Fig. S1 in the supplemental material).

In mutant cWsp-1, *wspH* as well as *wspA* and *wspE* were simultaneously induced in the presence of rhamnose, showing that *wspH* and the downstream *wsp* genes form a single operon, despite the presence of a terminator structure downstream of *wspH*. Expression of *wspH* in mutant cWsp-2 was only slightly induced by the addition of rhamnose, while expression of *wspA* and *wspE* increased to a larger extent.

In mutant WspH-OV, expression of *wspH* was increased even in the absence of rhamnose (Fig. 1C), showing that leaky expression from P_{rhaB} was sufficient to increase overall *wspH* transcript abundance if *wspH* was present on a replicative plasmid (copy number 30–40, (17)). In the presence of rhamnose, WspH-OV showed a growth defect, identifiable as elongated cells (Fig. S2 in the supplemental material), indicating that extensive overexpression of *wspH* might be toxic for the cells. The phenotype of mutant WspH-OV was therefore mainly assessed in the absence of rhamnose.

Pellicle biofilm formation is accelerated by overexpressing *wspH*. Pellicle biofilm formation has obvious advantages for an obligate aerobic bacterium like *B. cenocepacia*. In static culture, oxygen is available at high concentration only at the air-liquid interface (ALI) and being able to colonize this interface enhances growth (18).

When grown statically, *B. cenocepacia* J2315 forms pellicle biofilms at the ALI, reaching a maximum extent after approximately 48 h (6). Mature pellicles in glass tubes

TABLE 1 Strains and plasmids

Strain or plasmid	Description	Source/reference
B. cenocepacia		
J2315	CF sputum isolate (LMG16656)	(34)
VC	vector control, J2315 with plasmid pSC200_M1327	This study
cWsp-1	J2315 wt with plasmid pSC200_M0820	(6)
cWsp-2	J2315 wt with plasmid pSC200_M0821	This study
WspH-OV	J2315 with plasmid pM0820_OV	This study
wspR72	J2315 with mutation D72N in *wspR*	This study
wspR72-VC	*wspR*-D72N with plasmid pSC200_M1327	This study
wspR72-1	*wspR*-D72N with plasmid pSC200_M0820	This study
wspR72-2	*wspR*-D72N with plasmid pSC200_M0821	This study
wspR72-OV	*wspR*-D72N with plasmid pM0820_OV	This study
E. coli		
DH5α λ *pir*	Propagation of suicide plasmid pSC200 and derivatives	Lab collection
DH5α	Propagation of helper plasmid pRK2013 and pSCrhaB2 and derivative	Lab collection
Plasmids		
pSC200	pGpΩTp derivative, ori_{R6K}, *rhaR, rhaS,* P_{rhaB}, *dhfr*, suicide plasmid with a rhamnose-inducible promoter	(35)
pRK2013	Helper plasmid; ori_{colE1}, RK2 derivative, Kan^r, *mob*$^+$, *tra*$^+$	(36)
pSC200_M1327	pSC200 carrying fragment of BCAM1327, modified to include a stop codon	This study
pSC200_M0820	pSC200 carrying fragment of BCAM0820	This study
pSC200_M0821	pSC200 carrying fragment of BCAM0821	This study
pSCrhaB2	ori_{pBBR1}, *rhaR*, *rhaS*, P_{rhaB}, *dhfr*, replicative plasmid with a rhamnose-inducible promoter	(37)
pM0820_OV	pSCrhaB2 carrying entire gene BCAM0820	This study
pGPI-SceI-XCm	*I-SceI* restriction site, ori_{R6K} *dhfr*, Cm^r	(38)
pDAI-SceI-SacB	*I-SceI* nuclease, Tet^r, counter selectable marker SacB, ori_{pBBR1}	(38)

were visually inspected, and pellicle formation over time was tracked in microtiter plates, using an optotracer which fluoresces after binding to biofilm components. Only pellicle biofilm material emitted fluorescence, which was confirmed by epifluorescence microscopy (Fig. S3 in the supplemental material).

Overexpressing *wspH* accelerated pellicle formation, as seen by the increase in optotracer fluorescence after approximately 20 h of incubation in mutants WspH-OV and cWsp-1 (Fig. 2A). At this time point, all mutants had reached early stationary phase (Fig. S5 in the supplemental material) and the sudden increase in fluorescence is therefore independent of cell density. Pellicles formed in glass tubes were thin and disintegrated easily. When overexpressing *wspH*, visible growth was confined to the ALI and no planktonic growth was visible in the lower liquid phase (Fig. 2B). Overexpressing the *wsp* genes in mutant cWsp-2 had only a small effect on timing of pellicle formation (Fig. 2A) and on residual planktonic growth (Fig. 2B). This mild phenotype could be caused by the limited rhamnose-induced upregulation of *wspH* in cWsp-2 (Fig. 1C).

Mutants with increased pellicle biofilm formation also displayed increased formation of biofilm attached to the wells of microtiter plates (Fig. 3A), which is probably due to increased pellicle formation at point of measurement (24 h). Crystal violet staining was mostly associated with a zone near the ALI, and less with the submerged sections of the microtiter wells.

Swimming motility in soft agar was also reduced when *wspH* was overexpressed (Fig. 3B). Interestingly, mutant cWsp-1 showed a stronger reduction in swimming diameter than WspH-OV, although *wspH* is induced to a larger extent in mutant WspH-OV. Impaired swimming motility of mutant cWsp-1 could be responsible for the lack of visible planktonic growth in static culture observed for this mutant (Fig. 2B). Cells which do not establish themselves at the ALI probably sink to the bottom of the tube where they do not grow due to lack of oxygen.

Increased expression of *wspH* coincides with elevated intracellular c-di-GMP levels and induction of c-di-GMP-regulated genes. Pellicle biofilm formation and swimming motility are known to be regulated by intracellular c-di-GMP levels (19).

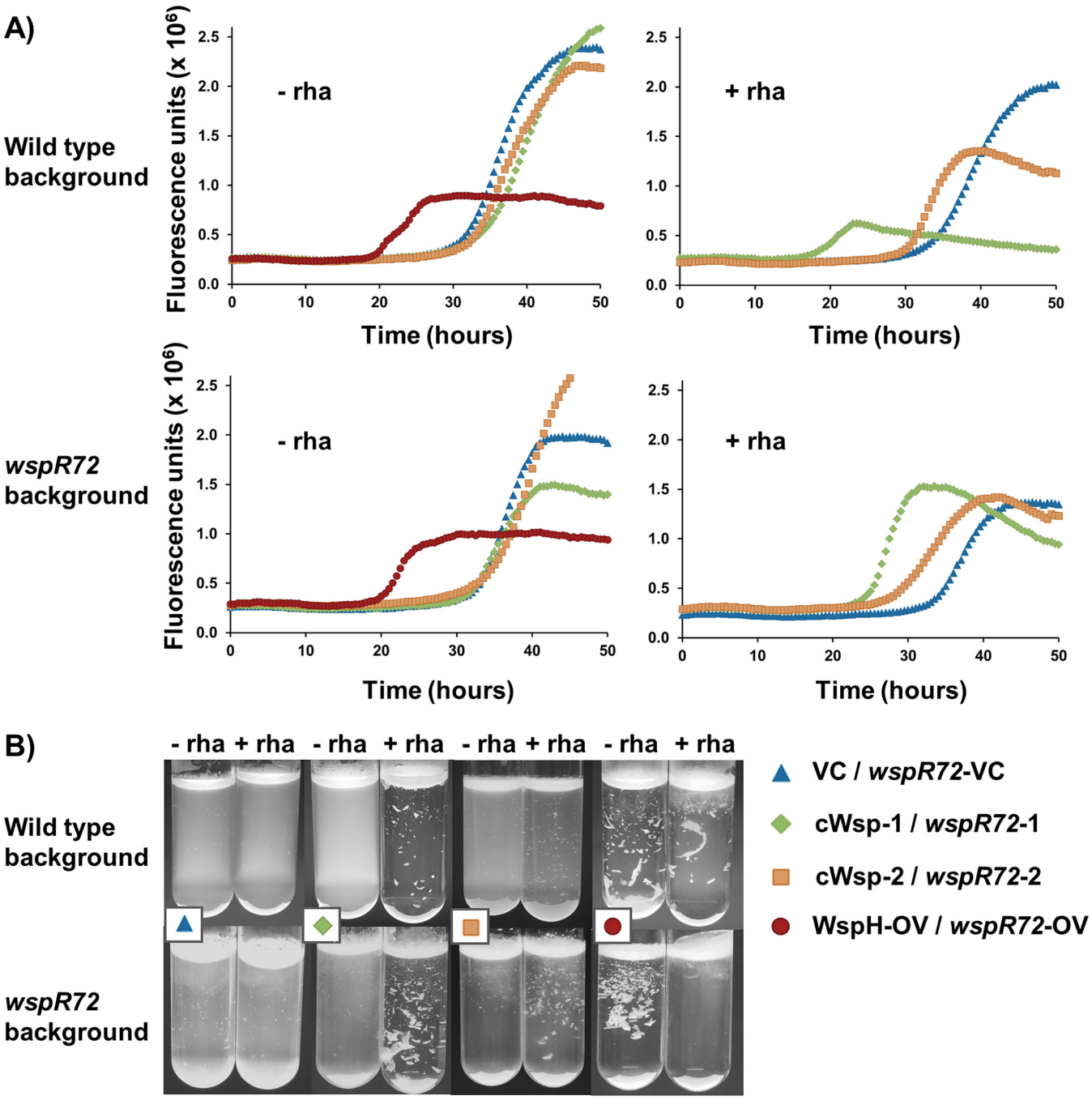

FIG 2 Effect of *wspH* and *wspH* cluster induction on pellicle biofilm formation in *B. cenocepacia*. A) Pellicle biofilm formation was tracked in microtiter plates, using an optotracer which fluoresces upon binding to biofilm components. Pellicle biofilms are formed earlier in mutant WspH-OV in absence and in cWsp-1 in the presence of rhamnose (rha), conditions in which *wspH* is overexpressed. Data shown are from a representative experiment; all three biological replicates are presented in Fig. S4 in the supplemental material. B) Pellicle biofilms formed by *B. cenocepacia* J2315 are fragile and disintegrate easily. All cell material is located in the pellicle biofilm in mutant WspH-OV, and in cWsp-1 in the presence of rhamnose; aggregates are larger, and no planktonic growth is visible. In cWsp-2 in the presence of inducer, this effect is less pronounced. VC: vector control.

Correspondingly, overexpressing *wspH* lead to an increase in c-di-GMP concentrations in shaking planktonic cultures as well as in cells grown statically as pellicle biofilm (Fig. 4). Static cultures contained considerably more c-di-GMP than planktonic cultures, in accordance with the paradigm that sessile or biofilm cells have an increased level of c-di-GMP (19).

Expression of several genes known to respond to increased c-di-GMP concentration were analyzed by qPCR. Expression changes in genes regulated by c-di-GMP were

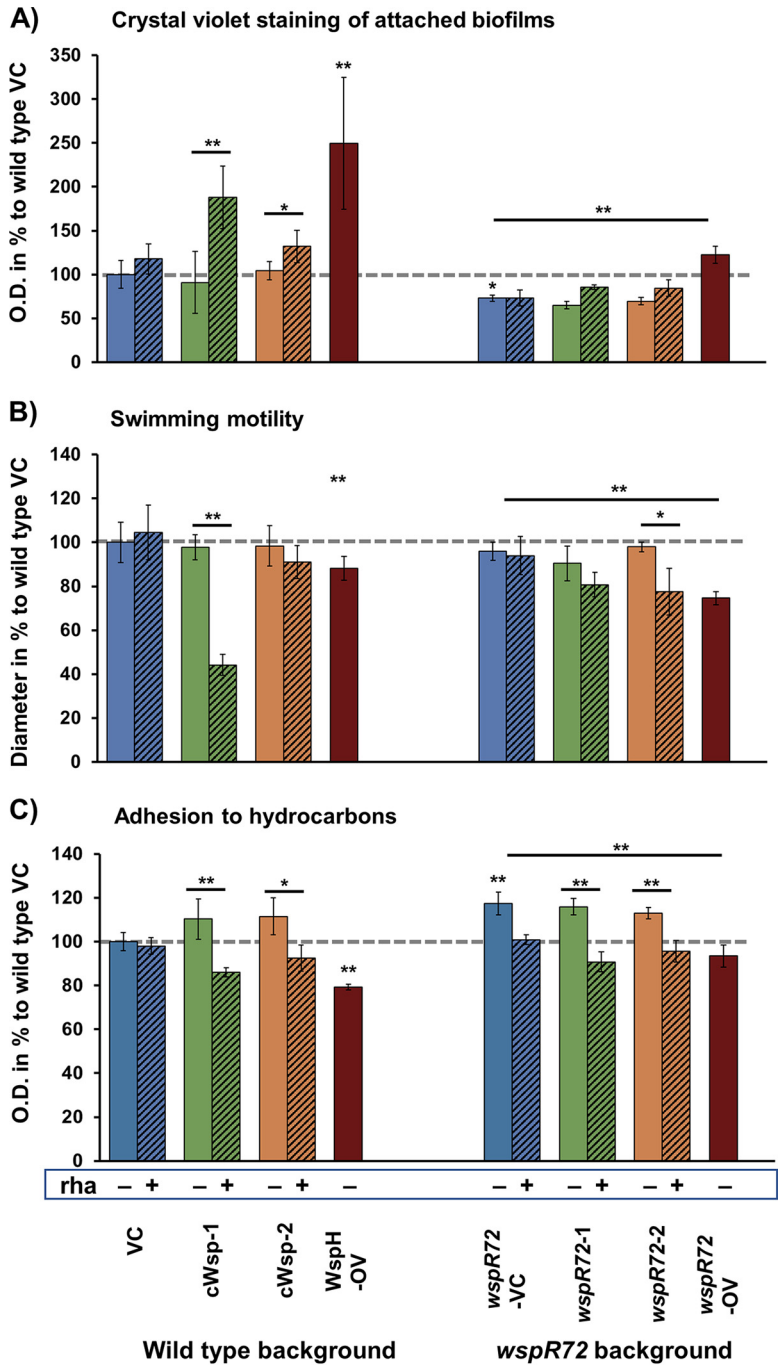

FIG 3 Effect of *wsp* gene overexpression on biofilm formation, swimming motility and pellicle hydrophobicity. A) Biofilm attached to the walls of 96-well styrene microtiter plates was quantified with crystal violet staining. Biofilms were grown for 24 h. B) Swimming motility in soft agar, measured after 48 h. C) Cell surface hydrophobicity was measured as adhesion to hydrocarbons. A reduced O.D. of the aqueous phase corresponds to increased affinity for the hydrocarbon phase. All values are represented as % compared to wild type vector control (VC) grown without rhamnose (rha). * $P \leq 0.05$, ** $P \leq 0.01$ (compared to wild type VC without rhamnose, if not otherwise indicated by a black line). Error bars represent standard deviation. All experiments were performed with at least three biological replicates.

more pronounced in pellicles than in planktonic cultures (Table 2), corresponding to the overall higher c-di-GMP levels.

Pellicle formation depends on production of exopolysaccharides, but the exact nature of these exopolysaccharides varies between *Burkholderia* species and strains. In *B. cenocepacia*

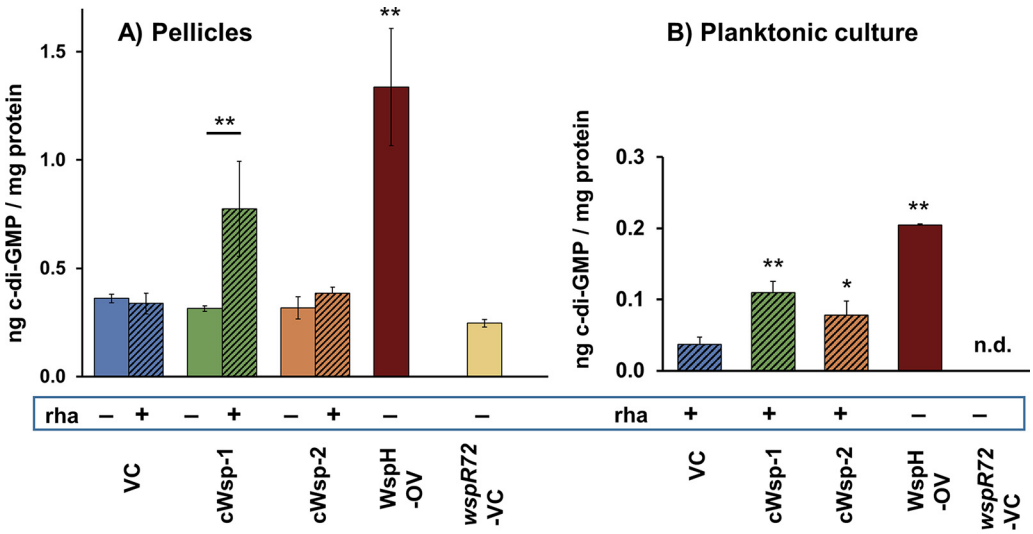

FIG 4 Cyclic di-GMP content in static (A) and shaking planktonic (B) cultures. Note that background c-di-GMP levels for wild type VC were approximately 10-fold lower in planktonic culture than in static culture. * $P \leq 0.05$, ** $P \leq 0.01$ (compared to wild type VC without rhamnose, if not otherwise indicated by a black line). Error bars represent standard deviation. Experiments were performed with at least three biological replicates for pellicles, and two replicates for planktonic cultures. n.d.: not detected, rha: rhamnose.

strain H111, a cluster of c-di-GMP-regulated genes involved in production of an exopolysaccharide essential for pellicle biofilm formation was identified (5, 20). The cluster, named *bep*, consists of 12 genes and is regulated by the c-di-GMP-binding regulator BerA (21). Representatives of this gene cluster, *bepB* (BCAM1331) and *manC* (BCAM1340), as well as *berA* (BCAM1349), were upregulated in pellicle biofilms when *wspH* was overexpressed (Table 2).

In *B. glumae*, cellulose, produced by the *bcs* gene cluster, is a major essential component of pellicle biofilms (22). The same genes are not necessary for pellicle formation in *B. cenocepacia* H111 (20), but they are regulated by BerA (5). In the present study, the first gene of the *bcs* cluster (BCAL1389) was downregulated during induction of *wspH*.

Expression of flagellar genes *fliC* (BCAL0114) and *motA* (BCAL0126) was strongly repressed in cWsp-1 pellicles in the presence of rhamnose, whereas only *fliC* was substantially repressed

TABLE 2 Expression change of genes involved in pellicle formation or responsive to increased c-di-GMP concentrations in *B. cenocepacia* J2315 and its mutants as determined by qPCR[a]

		Pellicle growth					Planktonic growth					
Gene	Rhamnose	cWsp-1 +	cWsp-1 −	cWsp-2 +	WspH-OV −	*wspR72*-VC −	cWsp-1 +	cWsp-1 −	cWsp-2 +	cWsp-2 −	WspH-OV +	WspH-OV −
wspH	BCAM0820	3.2	−2.6	1.7	5.3	(−0.1)	3.1	−2.5	1.1	(0.8)	11.7	4.8
wspA	BCAM0821	3.5	(−2.0)	6.3	(1.1)	(0.1)	2.8	−1.8	4.7	−1.3	(0.9)	1.3
wspE	BCAM0825	4.0	−1.7	4.6	(0.4)	(−0.1)	2.7	−2.2	6.7	2.1	1.1	(0.9)
wspF2	BCAM1162	(−0.6)	(−0.3)	(−0.3)	(−0.3)	(−1.0)	(−0.6)	(0.1)	(0.5)	(0.3)	(−0.7)	(0.6)
wspR	BCAM1161	(−0.7)	(0.3)	(−0.9)	(-1.0)	(0.8)	(0.3)	(0.2)	(0.5)	(0.4)	(0.0)	(0.9)
berA	BCAM1349	2.3	(−0.2)	2.1	4.1	(0.0)	(0.3)	(−0.4)	ND	ND	(0.2)	(0.9)
bepB	BCAM1331	1.5	(−0.2)	(0.9)	3.1	(0.1)	−0.5	(−0.3)	ND	ND	(0.2)	(0.3)
manC	BCAM1340	2.0	(−0.3)	2.4	4.3	(0.1)	(0.0)	(0.2)	ND	ND	(0.3)	0.5
bcsB	BCAL1389	−2.1	(−0.2)	2.0	(0.2)	(−0.3)	(0.1)	(0.0)	ND	ND	−2.8	−1.3
fliC	BCAL0114	−8.4	(0.4)	(−2.4)	−9.2	(−0.2)	(0.0)	(0.4)	ND	ND	−1.7	−0.9
motA	BCAL0126	−4.8	(0.1)	(1.8)	(−1.9)	(0.4)	(−0.3)	(0.0)	ND	ND	−3.1	−1.3
fimA	BCAL1677	3.4	(−1.1)	3.4	4.5	(-0.3)	(-0.4)	−1.1	ND	ND	4.9	4.0
cepI	BCAM1870	(−0.1)	(−0.1)	(−0.5)	(−1.2)	(−0.1)	(0.5)	−1.0	ND	ND	(1.4)	(−0.2)

[a]Values are presented as Log_2 fold changes compared to VC grown in the same condition. qPCR was performed on three biological replicates, Statistical testing was performed on Log_2 fold changes compared to average VC, values $p \leq 0.05$ were reported as significant. Values in brackets: not significant. ND: not determined. The position of mutations for cWsp-1, cWsp-2 and *wspR72* is depicted in Fig. 1.

in WspH-OV pellicles. This could explain why cWsp-1 showed a stronger reduction in swimming motility than WspH-OV, although WspH-OV has higher *wspH* mRNA and c-di-GMP levels. c-di-GMP levels alone are not responsible for this phenotype, and overexpression of the whole *wspH* operon could affect flagellar rotation.

Fimbriae are upregulated in *B. cenocepacia* H111 by c-di-GMP, but not necessary for pellicle formation in this strain (20). In the present study, *fimA* is induced by *wspH* overexpression, confirming its regulation by c-di-GMP.

cepI (BCAM1870), a N-acylhomoserine lactone synthase gene, previously reported to be c-di-GMP regulated in *B. cenocepacia* H111 (23), did not change expression significantly upon rhamnose addition (Table 2). It is therefore unlikely that increased pellicle formation is caused by activated quorum sensing in *B. cenocepacia* J2315.

Overexpressing *wspH* increases cell surface hydrophobicity. The product of the *bep* gene cluster in *B. cenocepacia* H111 was recently identified as a water insoluble exopolysaccharide, named Bep (*Burkholderia cepacia* exopolysaccharide), and consisting of repeated tetrasaccharide units containing glucose, galactose and mannose ([3)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-α-D-Man*p*-(1→]$_n$) (24). Pellicle biofilms need to float on the ALI (18) and it is therefore logic to hypothesize that inducing *wspH* would coincide with increased formation of Bep.

Cell surface hydrophobicity of pellicle biofilms formed in glass tubes was determined via a microbial adhesion to hydrocarbons test (Fig. 3C). Overexpression of *wspH*, but also of *wsp* genes, lead to a modest but significant increase in hydrophobicity, as measured by a decrease in residual optical density in the aqueous phase after shaking. However, no Bep was detected in WspH-OV mutant pellicles by NMR spectroscopy (Fig. S6 in the supplemental material). Considering the increased cell surface hydrophobicity, it appears probable that while Bep production might be slightly increased when *wspH* is overexpressed, the overall amount of Bep in *B. cenocepacia* J2315 pellicles is too low to be detected by the approach used. Strain H111 forms stronger and more voluminous pellicles (24) than strain J2315 and likely produces more exopolysaccharides, making it easier to detect them.

The effect of overexpressing *wspH* is independent of an intact phosphorylation site in *wspR*. Mutants in *wspR72* background were subjected to the same experiments as mutants in WT background. *wspR72*-VC still forms pellicles, albeit with a decreased pellicle biofilm hydrophobicity compared to wild type VC (Fig. 2B and Fig. 3C). Therefore, pellicle formation in *B. cenocepacia* J2315 does not depend on phosphorylation of WspR. Overexpressing *wspH* or the *wsp* operon in the *wspR72* background had a similar effect on pellicle formation as in WT background. Pellicle formation was accelerated (Fig. 2A) and pellicle hydrophobicity was enhanced in *wspR72*-1 and *wspR72*-OV (Fig. 3C). However, all mutants in the *wspR72* background formed less attached biofilm in microtiter plates compared to the corresponding mutants in the WT background, and only *wspR72*-OV showed enhanced biofilm formation compared to the control (Fig. 3A). In contrast to mutant cWsp-1, swimming motility and planktonic growth of *wspR72*-1 was not much affected when grown in medium with rhamnose (Fig. 3B, Fig. S1 in the supplemental material). Expression of *wspR* did not change in any *wspR72* mutant compared to wild type VC (data not shown), and expression of c-di-GMP regulated genes was not significantly different from wild type VC (Table 2).

c-di-GMP was not detected in *wspR72*-VC planktonic cultures, in contrast to wild type VC. This suggests that inactivation of the phosphorylation site in WspR might lower the base c-di-GMP levels in planktonic culture but has no effect on the elevated levels of c-di-GMP in sessile cells.

Putative role of WspR in *B. cenocepacia* J2315. *wspR* is one of 25 genes encoded in the *B. cenocepacia* J2315 genome with a putative function in c-di-GMP metabolism (25), 12 of which have a DGC domain. The overall c-di-GMP levels in a bacterial cell are the result of a coordinated action of several c-di-GMP metabolizing enzymes. The function of *wspR* (BCAM1161) was investigated in *B. cenocepacia* strain H111 by phenotyping a markerless deletion mutant (25), and in strain K56-2 by inserting a suicide plasmid into the receiver domain of *wspR* (26), leaving the DGC domain intact. Motility was used as

an indicator for a change in overall c-di-GMP levels in the above mutants. Deletion of *wspR* had no impact (25), but insertion of a plasmid into the receiver domain of *wspR* attenuated motility (26). The plasmid insertion could have increased the activity of the DGC domain, resulting in elevated c-di-GMP levels. These results indicate that WspR is not active under standard laboratory conditions but can be activated by changes to its receiver domain; and they confirm that the DGC domain of WspR is functional in *B. cenocepacia*.

wspR and *wspF2* did not change expression in the conditions and mutants tested in the present study (Table 2), their expression is therefore not affected by overexpressing or silencing *wspH* or the main *wsp* operon. This is not surprising since WspR DGE activity is regulated by phosphorylation of the receiver domain and by product feedback-inhibition (16); regulation at the transcriptional level likely plays little role in determining WspR activity.

The results obtained in the present study show that WspR is probably not the primary cognate receptor of WspH, but it is possible that WspR is phosphorylated by WspE, and that this has an additive effect on certain phenotypes when both *wspH* and *wspA-E* are overexpressed simultaneously. For example, coordinated overexpression of the whole *wspH* operon had a larger effect on motility than overexpressing *wspH* alone, and this was not observed in mutant *wspR72*. On the one hand, c-di-GMP levels in the cell could be higher when overexpressing the entire *wspH* operon. On the other hand, activating WspR via WspE could have a direct effect on a key player of flagellar movement via physical protein-protein interactions, as c-di-GMP signaling can be highly specific via local interactions (27).

Putative role of WspH in *B. cenocepacia*. Adapting to changing environments by receiving external signals and translating these into gene expression changes is crucial for bacteria, to enable survival and growth in dynamic habitats. Bacterial signal transduction systems are needed for transferring external signals to a transcriptional regulator. These signal transduction systems often have multiple components, including membrane-bound signal receptors, kinases, phosphatases and phosphotransferases, forming a phosphorelay system and thereby physically separating input and output domains. Phosphorelay systems with many components enable integrating multiple input stimuli into one output signal, and such systems can be regulated at more than one point, making fine-tuning of the response possible (28).

A *wsp* operon that is preceded by *wspH* has so far only been found in *Burkholderia* spp; the possible advantage of such a setup is the integration of multiple signals to amplify the output signal, which in this case is an increased c-di-GMP level combined with increased Ber production. The two-component system histidine kinase BCAL1445 has been proposed as another member of this phosphorelay system (12), as a potential further signal receiver relaying to the output signal. Moreover, the regulator RpfR, which has a DGC and a phosphodiesterase domain and which responds to the quorum sensing signal cis-2-dodecenoic acid, is involved in regulating Ber production via c-di-GMP (29). Therefore, the Wsp system and quorum sensing can contribute to the same output signal.

Another advantage of the Wsp system setup in *B. cenocepacia* could be integration of an endogenous signal to an exogenous signal that is received by WspA. Expression of WspH is affected by DNA methylation, linking its expression to cell division and replication status. Combining this with the Wsp system can enable a response to the (yet unknown) exogenous signal(s) only if the cell is in a certain physiological state, thus increasing the c-di-GMP levels over a certain threshold only if the cell is dividing.

The complexity of the Wsp phosphorelay system in *B. cenocepacia* indicates that it is advantageous for this bacterium to establish a biofilm at a variety of surfaces using the same response, while fine-tuning this response to enable niche differentiation within a biofilm (29).

Summary. Increased expression of epigenetically regulated *wspH* resulted in the same phenotype as observed for a methyltransferase deletion mutant in *B. cenocepacia* J2315. *wspH* and downstream genes *wspA-E* form a single operon. Inducing *wspH*

increased c-di-GMP content of cells, accelerated pellicle formation, reduced flagellar motility, and increased biofilm formation and cell surface hydrophobicity. The phosphoreceiver function of WspR is not necessary for the effect of *wspH* overexpression, hence the cognate phosphoryl group acceptor and DGC of WspH are still unknown. Increased expression of *wspH* alone can enhance biofilm formation, independently of increased expression of *wspA-E*.

MATERIALS AND METHODS

Strains and media. All strains and plasmids used in this study are listed in Table S1 in the supplemental material. *B. cenocepacia* was routinely cultivated in Luria-Bertani broth (Lennox formulation with 5 g/L NaCl). Experiments were performed in a phosphate buffered mineral medium (2 g/L NH_4Cl, 4.25 g/L $K_2HPO_4 \times 3\ H_2O$ (ChemLab), 1 g/L $NaH_2PO_4 \times H_2O$, 0.1 g/L nitrilotriacetic acid, 0.003 g/L $MnSO_4 \times H_2O$, 0.003 g/L $ZnSO_4 \times 7\ H_2O$, 0.001 g/L $CoSO_4 \times 7\ H_2O$, 0.2 g/L $MgSO_4 \times 7\ H_2O$, and 0.012 g/L $FeSO_4 \times 7\ H_2O$; Sigma-Aldrich). Organic components were 5 g/L glycerol (Scharlab), 5 g/L yeast extract (Lab M), 2 g/L Bacto peptone (BD Biosciences) for a medium high in carbohydrates and amino acids which promotes pellicle formation, or 5 g/L glucose, 0.5 g/L yeast extract and 0.5 g/L Bacto peptone for a medium with low background fluorescence for experiments with optotracers.

Agar (Neogen) was added at a concentration of 0.3 or 1.2% as required. Organic and inorganic components of the medium were autoclaved separately.

For cultivation of *B. cenocepacia* mutants the medium was supplemented with 800 μg/mL trimethoprim (TP), added as stock solution (80 mg/mL TP in DMSO) after autoclaving. For biofilm and pellicle formation experiments, TP was added as powder, the medium stirred for 1 h and then filter sterilised. For induction of genes from P_{rhaB}, the medium was supplemented with 0.2% rhamnose (Sigma-Aldrich). *E. coli* strains were cultivated in Luria Bertani medium (Lab M) supplemented with 50 μg/mL trimethoprim or kanamycin or 20 μg/mL tetracycline (Sigma). Incubation temperature was 37°C for all cultures and experiments.

Mutant construction. Inserts for promoter replacement mutants were amplified using PrimeSTAR GXL DNA polymerase (TaKaRa), and double-digested with NdeI and XbaI (Promega). As vector control, plasmid pSC200 was integrated into hypothetical gene BCAM1327, using an insert with a substituted base to create an in-frame stop codon.

Purified DNA fragments (PCR cleaning kit, Macherey-Nagel) were ligated into the restricted plasmids using T4 DNA ligase (Promega) and ligation reactions were used for heat transformation of *E. coli* DH5α λ*pir*. The sequence of inserts was confirmed by Sanger sequencing.

Plasmids were transformed into *B. cenocepacia* J2315 by triparental mating (30), using a selective medium with 800 μg/mL TP and 50 μg/mL gentamicin (Sigma-Aldrich). Ex-transformants were screened for correct insertion of suicide plasmids by PCR using primers annealing in the rhamnose-inducible promoter of pSC200 and downstream of the DNA fragment in the genome. Primers are listed in Table S1 in the supplemental material.

For introducing a point mutation in *wspR*, a 1009 nt long DNA fragment of *wspR* was synthesized by GenScript (https://www.genscript.com). The fragment contained the substituted base and was flanked by XbaI restriction sites. The DNA fragment was digested with XbaI, gel-cleaned, cloned into XbaI-digested suicide plasmid pGPI-SceI-XCm, and transformed into *B. cenocepacia* J2315 by an adapted allelic exchange method (6). Successful mutants were screened for by Sanger sequencing.

Biofilm quantification. Biofilms were grown in U-bottom microtiter plates as described previously (6), using 100 μL medium per well, for 24 h. Planktonic cells were removed, the wells washed once with physiological saline, 130 μL/well of a 0.1% crystal violet solution (Sigma-Aldrich) added, and left at room temperature for 15 min. The crystal violet solution was removed, the plate washed once with water and left to dry for 30 min, 150 μL/well 95% ethanol (Chem-Lab) was added, and the plate shaken at 600 rpm for 5 min. The absorbance was measured at 590 nm in a plate reader (Envision, Perkin Elmer).

Swimming motility. 20 mL of hot medium containing 0.3% agar was measured into Petri plates which were left open to solidify and dry for 1 h. A hole was formed into the middle of the agar using a sterile 1 mL pipette tip, and 2 μL liquid culture with 10^8 CFU/mL was pipetted into it. The plate was left to dry for another 30 min, to prevent swarming motility across the agar surface, and incubated for 48 h.

Pellicle formation. Pellicles were grown in borosilicate glass tubes with 15 mm diameter. 5 mL medium were inoculated with 10^7 CFU/mL and incubated stationary for 48 h. Photos were taken with an Olympus E-PL3 digital camera using uplighting from a light box. Microscopy was performed with an Olympus BX41 microscope using phase contrast or epifluorescence with a 530–550 nm bandwidth excitation filter, and a long pass 590 nm emission filter.

Pellicle biofilm formation was tracked over time using optotracer molecules that become fluorescent when binding to biofilm extracellular components (EbbaBiolight 680, Ebba Biotech, Sweden). Cells were grown in clear U-bottom microtiter plates in a glucose-rich medium, using 10^7 CFU/mL inoculum, incubated in an Envision plate reader (Perkin Elmer). The optotracer was added to the medium at begin of the experiment. Fluorescence was measured every 30 min at 555 nm excitation and 685 nm emission, optical density (O.D.) was measured in parallel at 590 nm.

Cell surface hydrophobicity measurement. Pellicle biofilms formed by *B. cenocepacia* J2315 are very fragile and rapidly disintegrate upon moving or shaking, so the entire content of the glass tubes was used for analysis of cell surface hydrophobicity.

Cell surface hydrophobicity was determined via microbial adhesion to hydrocarbons, using a modified method described by Rosenberg (31). Contents of 5 tubes were pooled, pelleted by centrifugation

and homogenized in PUM buffer (16.9 g/L K_2HPO_4, 7.3 g/L KH_2PO_4, 1.8 g/L urea, 0.2 g/L $MgSO_4 \times 7\ H_2O$, pH 7.1) by pipetting up and down. 5 mL of cell suspension normalized to optical density (O.D.) 1.0 in PUM buffer was pipetted into a borosilicate glass tube with 15 mm diameter and overlaid with 0.4 mL *n*-hexadecane (Sigma). The tubes were fixed onto a vortex with tube adapter (VV3, VWR), vortexed for 2 min at 2,500 rpm, and left to stand for 5 min for phase separation. Then the residual O.D. of the lower phase was measured at 590 nm in a photometer (Milton Roy).

Exopolysaccharide analysis by NMR spectroscopy. Exopolysaccharide purification was performed as described in Bellich et al. (24). In brief, pellicle material, collected as described for adherence to hydrocarbons measurement, was freeze-dried and polysaccharides extracted by stirring in 0.3 M NaOH for 3 h at 12°C. The extract was centrifuged and an aliquot of 0.6 mL was lyophilized. For comparison, purified Bep polysaccharide (0.8 mg) was dissolved in 0.3 M NaOH and lyophilized. The samples were lyophilized, exchanged twice with D_2O and $^{1H\ NMR}$ spectra were recorded with a 500 MHz Varian spectrometer.

Growth curves, RNA extraction and qPCR. Growth of the mutants created for this study was monitored with a Cell Growth Quantifier (Aquila Biolabs, Germany) in 250 mL flask in a shaking incubator, at 100 rpm. The same conditions were used for generating samples for RNA extraction from planktonic cultures. Cells were grown without rhamnose to an O.D. of 0.5, then rhamnose was added for induction of P_{rhaB} and cells harvested 60 min later. Pellicles were grown statically for 36 h in glass tubes, rhamnose was added at the beginning of incubation. They were harvested by vortexing and pooling the content of five glass tubes. Cells were snap cooled, pelleted by centrifugation and stored at −80°C for max. 1 week. RNA extraction, cDNA generation and qPCR was performed as described previously (32). All primer sequences are listed in Table S1 in the supplemental material.

c-di-GMP extraction and quantification. c-di-GMP was extracted from planktonic cultures and from pellicles, which were grown as described above for RNA extraction, except planktonic cultures were harvested 3 h after adding rhamnose. Cultures were centrifuged at 2,500 × *g* and 4°C for 20 min. Pellets were resuspended in 500 μL extraction buffer (acetonitrile/methanol/water 2:2:1, vol/vol/vol), stored at −20°C for 1 h, heated to 95°C for 10 min, cooled on ice and centrifuged at 20,800 × *g*. The supernatant was transferred into a new tube and stored at −20°C over night. The residual cell pellet was used for protein quantification with the Pierce 660 nm Protein Assay (Thermo Scientific). Centrifugation and transfer of supernatant was repeated, and the extraction buffer evaporated in a Speed Vac (Eppendorf) at 30°C.

c-di-GMP was quantified from dried extracts using the General Cyclic Diguanylate ELISA kit (MyBioSource) according to manufacturer's instructions. The extracts were re-dissolved in the sample diluent provided with the kit.

Computational analysis and statistics. Homologous genes were screened for using BLASTp (33); genes were defined as homologous at >95% query coverage and >60% amino acid similarity.

For statistical analysis, One-way ANOVA with a Tukey Post-hoc test using SPSS (v. 28) was performed to determine statistical significance. If variances were not equal, a non-parametric Kruskal-Wallis test was applied. O.D. values from analysis of cell hydrophobicity, biofilms and diameters from swimming motility assays were normalized to their corresponding vector control, in percent, before statistical analysis.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.

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REFERENCES

1. Drevinek P, Mahenthiralingam E. 2010. *Burkholderia cenocepacia* in cystic fibrosis: Epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect 16:821–830. https://doi.org/10.1111/j.1469-0691.2010.03237.x.
2. LiPuma JJ. 2010. The changing microbial epidemiology in cystic fibrosis. Clin Microbiol Rev 23:299–323. https://doi.org/10.1128/CMR.00068-09.
3. Mahenthiralingam E, Baldwin A, Dowson CG. 2008. *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. J Appl Microbiol 104:1539–1551. https://doi.org/10.1111/j.1365-2672.2007.03706.x.
4. Coenye T. 2010. Social interactions in the *Burkholderia cepacia* complex: biofilms and quorum sensing. Future Microbiol 5:1087–1099. https://doi.org/10.2217/fmb.10.68.
5. Fazli M, O'Connell A, Nilsson M, Niehaus K, Dow JM, Givskov M, Ryan RP, Tolker-Nielsen T. 2011. The CRP/FNR family protein Bcam1349 is a c-di-GMP effector that regulates biofilm formation in the respiratory pathogen *Burkholderia cenocepacia*. Mol Microbiol 82:327–341. https://doi.org/10.1111/j.1365-2958.2011.07814.x.
6. Vandenbussche I, Sass A, Pinto-Carbó M, Mannweiler O, Eberl L, Coenye T. 2020. DNA methylation epigenetically regulates gene expression in *Burkholderia cenocepacia* and controls biofilm formation, cell aggregation, and motility. mSphere 5:e00455-20. https://doi.org/10.1128/mSphere.00455-20.
7. Vandenbussche I, Sass A, Van Nieuwerburgh F, Pinto-Carbó M, Mannweiler O, Eberl L, Coenye T. 2021. Detection of cytosine methylation in *Burkholderia cenocepacia* by single-molecule real-time sequencing and whole-genome bisulfite sequencing. Microbiology 167:e001027. https://doi.org/10.1099/mic.0.001027.
8. Cooper VS, Staples RK, Traverse CC, Ellis CN. 2014. Parallel evolution of small colony variants in *Burkholderia cenocepacia* biofilms. Genomics 104:447–452. https://doi.org/10.1016/j.ygeno.2014.09.007.
9. Kessler C, Mhatre E, Cooper V, Kim W. 2021. Evolutionary divergence of the Wsp signal transduction systems in Beta- and Gammaproteobacteria. Appl Environ Microbiol 87:e01306-21. https://doi.org/10.1128/AEM.01306-21.

10. Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102:14422–14427. https://doi.org/10.1073/pnas.0507170102.
11. Traverse CC, Mayo-Smith LM, Poltak SR, Cooper VS. 2013. Tangled bank of experimentally evolved *Burkholderia* biofilms reflects selection during chronic infections. Proc Natl Acad Sci U S A 110:E250–E259. https://doi.org/10.1073/pnas.1207025110.
12. O'Rourke D, FitzGerald CE, Traverse CC, Cooper VS. 2015. There and back again: consequences of biofilm specialization under selection for dispersal. Front Genet 6:18. https://doi.org/10.3389/fgene.2015.00018.
13. Sass AM, Van Acker H, Förstner KU, Van Nieuwerburgh F, Deforce D, Vogel J, Coenye T. 2015. Genome-wide transcription start site profiling in biofilm-grown *Burkholderia cenocepacia* J2315. BMC Genomics 16:775. https://doi.org/10.1186/s12864-015-1993-3.
14. Cardona ST, Mueller CL, Valvano MA. 2006. Identification of essential operons with a rhamnose-inducible promoter in *Burkholderia cenocepacia*. Appl Environ Microbiol 72:2547–2555. https://doi.org/10.1128/AEM.72.4.2547-2555.2006.
15. Goymer P, Kahn SG, Malone JG, Gehrig SM, Spiers AJ, Rainey PB. 2006. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*: II. Role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. Genetics 173:515–526. https://doi.org/10.1534/genetics.106.055863.
16. De N, Pirruccello M, Krasteva PV, Bae N, Raghavan RV, Sondermann H. 2008. Phosphorylation-independent regulation of the diguanylate cyclase WspR. PLoS Biol 6:e67. https://doi.org/10.1371/journal.pbio.0060067.
17. Lynch MD, Gill RT. 2006. Broad host range vectors for stable genomic library construction. Biotechnol Bioeng 94:151–158. https://doi.org/10.1002/bit.20836.
18. Armitano J, Méjean V, Jourlin-Castelli C. 2014. Gram-negative bacteria can also form pellicles. Environ Microbiol Rep 6:534–544. https://doi.org/10.1111/1758-2229.12171.
19. Römling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol Mol Biol Rev 77:1–52. https://doi.org/10.1128/MMBR.00043-12.
20. Fazli M, McCarthy Y, Givskov M, Ryan RP, Tolker-Nielsen T. 2013. The exopolysaccharide gene cluster Bcam1330–Bcam1341 is involved in *Burkholderia cenocepacia* biofilm formation, and its expression is regulated by c-di-GMP and Bcam1349. Microbiologyopen 2:105–122. https://doi.org/10.1002/mbo3.61.
21. Fazli M, Rybtke M, Steiner E, Weidel E, Berthelsen J, Groizeleau J, Bin W, Zhi BZ, Yaming Z, Kaever V, Givskov M, Hartmann RW, Eberl L, Tolker-Nielsen T. 2017. Regulation of *Burkholderia cenocepacia* biofilm formation by RpoN and the c-di-GMP effector. BerB Microbiology Open 6:e00480. https://doi.org/10.1002/mbo3.480.
22. Kwak G-Y, Choi O, Goo E, Kang Y, Kim J, Hwang I. 2019. Quorum sensing-independent cellulase-sensitive pellicle formation is critical for colonization of *Burkholderia glumae* in rice plants. Front Microbiol 10:3090. https://doi.org/10.3389/fmicb.2019.03090.
23. Schmid N, Suppiger A, Steiner E, Pessi G, Kaever V, Fazli M, Tolker-Nielsen T, Jenal U, Eberl L. 2017. High intracellular c-di-GMP levels antagonize quorum sensing and virulence gene expression in *Burkholderia cenocepacia* H111. Microbiology (Reading) 163:754–764. https://doi.org/10.1099/mic.0.000452.
24. Bellich B, Jou IA, Caterino M, Rizzo R, Ravenscroft N, Fazli M, Tolker-Nielsen T, Brady JW, Cescutti P. 2020. *Burkholderia cenocepacia* H111 produces a water-insoluble exopolysaccharide in biofilm: structural determination and molecular modelling. Int J Mol Sci 21:1702. https://doi.org/10.3390/ijms21051702.
25. Richter AM, Fazli M, Schmid N, Shilling R, Suppiger A, Givskov M, Eberl L, Tolker-Nielsen T. 2018. Key players and individualists of cyclic-di-GMP signaling in *Burkholderia cenocepacia*. Front Microbiol 9:3286. https://doi.org/10.3389/fmicb.2018.03286.
26. Kumar B, Sorensen JL, Cardona ST. 2018. A c-di-GMP-modulating protein regulates swimming motility of *Burkholderia cenocepacia* in response to arginine and glutamate. Front Cell Infect Microbiol 8:56. https://doi.org/10.3389/fcimb.2018.00056.
27. Hengge R. 2021. High-specificity local and global c-di-GMP signaling. Trends Microbiol 29:993–1003. https://doi.org/10.1016/j.tim.2021.02.003.
28. Cashin P, Goldsack L, Hall D, O'Toole R. 2006. Contrasting signal transduction mechanisms in bacterial and eukaryotic gene transcription. FEMS Microbiol Lett 261:155–164. https://doi.org/10.1111/j.1574-6968.2006.00295.x.
29. Mhatre E, Snyder DJ, Sileo E, Turner CB, Buskirk SW, Fernandez NL, Neiditch MB, Waters CM, Cooper VS. 2020. One gene, multiple ecological strategies: A biofilm regulator is a capacitor for sustainable diversity. Proc Natl Acad Sci U S A 117:21647–21657. https://doi.org/10.1073/pnas.2008540117.
30. Craig FF, Coote JG, Parton R, Freer JH, Gilmour NJL. 1989. A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. J Gen Microbiol 135:2885–2890. https://doi.org/10.1099/00221287-135-11-2885.
31. Rosenberg M. 2006. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. FEMS Microbiol Lett 262:129–134. https://doi.org/10.1111/j.1574-6968.2006.00291.x.
32. Sass AM, Coenye T. 2020. Low iron-induced small RNA BrrF regulates central metabolism and oxidative stress responses in *Burkholderia cenocepacia*. PLoS One 15:e0236405. https://doi.org/10.1371/journal.pone.0236405.
33. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. https://doi.org/10.1093/nar/25.17.3389.
34. Mahenthiralingam E, Coenye T, Chung JW, Speert DP, Govan JRW, Taylor P, Vandamme P. 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. J Clin Microbiol 38:910–913. https://doi.org/10.1128/JCM.38.2.910-913.2000.
35. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, Campopiano DJ, Govan JRW, Valvano MA. 2007. A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. J Bacteriol 189:3639–3644. https://doi.org/10.1128/JB.00153-07.
36. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76:1648–1652. https://doi.org/10.1073/pnas.76.4.1648.
37. Cardona ST, Valvano MA. 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. Plasmid 54:219–228. https://doi.org/10.1016/j.plasmid.2005.03.004.
38. Hamad MA, Skeldon AM, Valvano MA. 2010. Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. Appl Environ Microbiol 76:3170–3176. https://doi.org/10.1128/AEM.03024-09.