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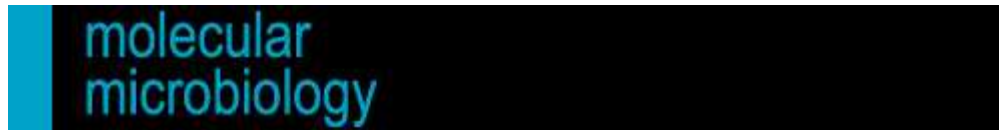
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A sensor kinase recognizing the cell-cell signal BDSF (cis-2-dodecenoic acid) regulates virulence in Burkholderia cenocepacia



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1 **A sensor kinase recognizing the cell-cell signal BDSF (*cis*-2-dodecenoic acid)**
2 **regulates virulence in *Burkholderia cenocepacia***

3
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27 **RUNNING TITLE:** Perception of BDSF in *Burkholderia cenocepacia*

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SUMMARY

Burkholderia cenocepacia is an opportunistic human pathogen that uses *cis*-2-dodecenoic acid (BDSF) as a quorum-sensing signal to control expression of virulence factors. BDSF is a signal molecule of the diffusible signal factor (DSF) family that was first described in the plant pathogen *Xanthomonas campestris*. The mechanism of perception of this signal and the range of functions regulated in *B. cenocepacia* are however unknown. A screen for transposon mutants unable to respond to exogenous signal identified BCAM0227 as a potential BDSF sensor. BCAM0227 is a histidine sensor kinase with an input domain unrelated to that of RpfC, the DSF sensor found in xanthomonads. Transcriptome profiling established the scope of the BDSF regulon and demonstrated that the sensor controls expression of a subset of these genes. A chimaeric sensor kinase in which the input domain of BCAM0227 replaced the input domain of RpfC was active in BDSF signal perception when expressed in *X. campestris*. Mutation of BCAM0227 gave rise to reduced cytotoxicity to CHO cells and reduced virulence to Wax moth larvae and in the agar-bead mouse model of pulmonary infection. The findings identify BCAM0227 as a novel BDSF sensor and a potential target for interference in virulence-related signalling in *B. cenocepacia*.

1 INTRODUCTION

2 Intercellular signalling is known to regulate functions that contribute to the
3 persistence and virulence of a number of human bacterial pathogens. These bacteria
4 use structurally diverse signal molecules to monitor aspects of their environment such
5 as cell density (quorum sensing) or confinement to niches and to modulate their
6 behaviour accordingly (Ng and Bassler, 2009; Ryan and Dow, 2008; Shank and
7 Kolter, 2009; Waters and Bassler, 2005; Williams and Camara, 2009)). Interference
8 with such signalling affords a rational approach to aid the treatment of infections, but
9 requires an understanding of both the signalling mechanisms and their role in bacterial
10 behaviour. The identification of sensors for bacterial signal molecules is an important
11 element of such a strategy as these proteins can present particularly attractive targets
12 for interference (Rasko and Sperandio, 2009; 2010).

13
14 Bacteria of the *Burkholderia cepacia* complex (Bcc) are opportunistic pathogens that
15 colonise the lungs of cystic fibrosis (CF) sufferers. One of the most problematic of
16 these CF pathogens is *Burkholderia cenocepacia*, which accounts for between 50-
17 90% of all Bcc infections diagnosed in CF patients. This species is characterized by
18 low responsiveness to antibiotic therapy and the ability to cause significant reductions
19 in lung function and includes strains exhibiting a high virulence potential as well as
20 strains with a well-documented history of patient-to-patient transmission (Govan *et*
21 *al.*, 2007; Mahenthiralingam *et al.*, 2005; Saldias and Valvano, 2009).

22
23 In common with many Gram-negative bacteria, *B. cenocepacia* uses *N*-acyl
24 homoserine lactones (*N*-AHLs) as signals. These signal molecules are synthesized by
25 proteins of the LuxI family and bind to transcriptional regulators of the LuxR family
26 to regulate gene expression. *B. cenocepacia* has two pairs of LuxIR systems; the
27 CepIR system is present in all species of the Bcc whereas the CciIR system is only
28 present in isolates of *B. cenocepacia* containing the *cenocepacia* island (*cci*) found in
29 highly transmissible *B. cenocepacia* strains such as the notorious ET12 lineage
30 (Malott *et al.*, 2005; O'Grady *et al.*, 2009; Sokol *et al.*, 2007). CepI is responsible for
31 the synthesis of primarily oxanoyl-homoserine lactone (C8-HSL) with lesser amounts
32 of hexanoyl-HSL (C6-HSL). CciI primarily synthesizes C6-HSL with lesser amounts
33 of C8-HSL produced. Diggle and colleagues (Diggle *et al.*, 2006) reported that in
34 addition to *N*-AHLs, *B. cenocepacia* produces a diverse set of 2-alkyl-4(1*H*)-

1 quinolones (AHQs), including 2-heptyl-4(1*H*)-quinolone (HHQ), but does not
2 synthesize 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), which is a signal molecule in
3 *Pseudomonas aeruginosa*. The proposed role of HHQ in signalling in *B. cenocepacia*
4 has yet to be confirmed.

5
6 Until recently, these molecules (*N*-AHLs and AHQs) were the only cell-cell signals
7 defined in *B. cenocepacia*. However, work by Boon and colleagues demonstrated that
8 *B. cenocepacia* J2315 also produces *cis*-2-dodecenoic acid, a molecule structurally
9 related to the diffusible signal factor (DSF; 11-methyl-*cis*-2-dodecenoic acid) found in
10 xanthomonads. Synthesis of *cis*-2-dodecenoic acid by *B. cenocepacia* requires
11 BCAM0581, which is related to RpfF, the DSF synthase of *Xanthomonas campestris*
12 (Barber *et al.*, 1997; Wang *et al.*, 2004). The *Burkholderia* signal molecule (called
13 BDSF) is able to activate DSF-dependent responses in *X. campestris* and to inhibit
14 germ tube formation in *Candida albicans*, suggesting a role in interspecies and
15 interkingdom communication. Furthermore, BDSF also plays a role as an intraspecies
16 signal in *B. cenocepacia* J2315 (Deng *et al.*, 2009; Ryan *et al.*, 2009). Mutation of
17 BCAM0581 gives rise to multiple phenotypic effects including reduction in motility,
18 altered adherence to mucin, reduced biofilm formation and attenuated virulence in the
19 *Galleria mellonella* larva and zebrafish infection models. For most of the phenotypes
20 that were tested, addition of exogenous BDSF restored the wild-type phenotype to the
21 BCAM0581 mutant (Deng *et al.*, 2009; Ryan *et al.*, 2009).

22
23 Although BDSF and DSF are structurally similar molecules, bioinformatic analysis
24 suggests that the signal perception mechanisms associated with BDSF signalling in *B.*
25 *cenocepacia* are different from those established for DSF in the xanthomonads. In
26 these latter bacteria, the sensor kinase RpfC is implicated in DSF perception leading
27 to alterations in bacterial physiology and gene expression. The genome of *B.*
28 *cenocepacia* J2315 however does not encode a histidine kinase with a sensory input
29 domain related to that of RpfC, suggesting the existence of an alternative sensor for
30 the BDSF signal. Here we have addressed the mechanism of BDSF perception by *B.*
31 *cenocepacia* J2315 by screening a transposon mutant library in a BCAM0581
32 background for mutants unable to respond to exogenous BDSF. This approach
33 identified that the histidine sensor kinase BCAM0227 had a possible role in signal
34 perception. Comparative transcriptome profiling established that the sensor is required

for expression of a subset of genes in the BDSF regulon. A chimaeric sensor kinase in which the input domain of BCAM0227 replaced the input domain of RpfC was active in BDSF signal perception when expressed in *X. campestris*. Notably, homologs of this sensor are found in other *Burkholderia* species, which include the Bcc. Taken together the findings indicate that BCAM0227 is a novel sensor involved in BDSF perception in a signalling system that regulates virulence.

RESULTS

Identification of transposon mutants that have altered expression of BDSF regulated genes

We have previously shown that mutation of *BCAM0581* in *B. cenocepacia* J2315 leads to reduced expression of the cable pilus gene *cblD* and that addition of BDSF restores expression to wild-type (Ryan *et al.*, 2009). This observation formed the basis of a screen for genes involved in BDSF perception. A panel of transposon mutants was created in the *BCAM0581* background using mariner transposon mutagenesis and was assessed for a loss of responsiveness to exogenous BDSF using a *cblD::lux* reporter (see Experimental procedures). Approximately 3,000 mutants were screened for altered bioluminescence in the presence of 50 μ M BDSF. A total of 74 mutants that demonstrated altered levels or kinetics of expression of the reporter fusion was isolated.

The sites of transposon insertion for non-responsive mutants were mapped to the genome of *B. cenocepacia* J2315 by inverse PCR and sequencing of amplicons (see Experimental procedures). Southern blot analysis confirmed that only a single copy of the transposon was inserted into the chromosome in these non-responsive mutants (data not shown). Insertion sites, which were all different, were mapped to 20 genetic loci with 9 genes found more than once (Table 1). None of the mutants showed impaired growth in complex or minimal media, suggesting that the altered response to BDSF could not be attributed to differences in growth.

One insertion was mapped to *BCAM2758* (*cblS*), a known positive regulatory gene of *cblD* (Tomich *et al.*, 2003; Tomich and Mohr, 2003). In addition, mutations in several genes encoding enzymes involved in metabolic processes (such as *BCAM1685*, *BCAL2745* and *BCAL2731*), in genes encoding hypothetical proteins (*BCAL3149*,

1 *BCAL1791*, *BCAM2836*, *BCAM1242*) and in genes encoding regulatory proteins
2 including a putative diguanylate cyclase (*BCAM2836*), a putative two-component
3 sensor kinase (*BCAM0227*), methyl-accepting chemotaxis protein (*BCAM1572*) were
4 identified. Since a histidine kinase (RpfC) is implicated in the perception of BDSF-
5 like molecules in *X. campestris*, subsequent work focused on the potential role of the
6 putative sensor kinase *BCAM0227* in BDSF perception.

7 8 **BDSF regulates the expression of genes associated with a range of cellular** 9 **functions**

10 The findings outlined above together with our previous work indicated that both
11 BDSF and *BCAM0227* regulate expression of the *cblD* gene. To examine the extent
12 of the overlap between those genes regulated by BDSF and those regulated by
13 *BCAM0227*, we compared the global gene expression patterns of specific *B.*
14 *cenocepacia* J2315 mutants using a custom microarray (Drevinek *et al.*, 2008). In the
15 first set of experiments, the transcriptomes of the wild-type *B. cenocepacia* J2315 and
16 a *BCAM0581* deletion mutant (constructed as described in the Experimental
17 procedures) were compared.

18
19 Comparison of wild-type and the *BCAM0581* deletion mutant (BDSF-minus
20 strain) established that expression of 372 genes was significantly influenced (> 2 fold
21 change) by mutation of *BCAM0581* (Fig. 1; Table S1). The *BCAM0581*-regulated
22 genes could be grouped into 10 functional categories; selected genes in each category
23 are shown in Table 2. The analysis revealed that *BCAM0581* controls genes
24 associated with a range of biological functions that include bacterial motility and
25 attachment, stress tolerance, virulence, regulation, transport, multidrug resistance,
26 detoxification and signal transduction. Genes associated with virulence that are
27 regulated by *BCAM0581* included *rpoN* (Saldias and Valvano, 2009), *hfq* (Sousa *et*
28 *al.*, 2010), *shvR* (Bernier *et al.*, 2008) and those encoding the components of the Bcs
29 type III secretion system (Tomich *et al.*, 2003) (Fig. 1B).

30
31 Changes in expression of genes involved in synthesis and/or action of cable pilus and
32 virulence genes are consistent with previous findings that bacterial motility, virulence
33 and attachment are regulated by the BDSF signal (Boon *et al.*, 2008; Ryan *et al.*,
34 2009). The effects of mutation of *BCAM0581* on expression of *fliJ*, *cblS* (*BCAM2758*)

1 and *clbD* (*BCAM2759*) as revealed by the microarray experiments confirmed our
2 previous findings on *BCAM0581* regulation of these genes, which were based on
3 quantitative Real Time PCR (qRT-PCR) studies (Ryan *et al.*, 2009).

4
5 For other selected genes, qRT-PCR was used to confirm the alteration in expression
6 as revealed by the microarray. Fifteen genes with altered expression and one control
7 gene with unaltered expression were chosen (Fig. S1A). These genes were selected to
8 be representative of: (i) a range of observed changes in expression (from 2- to 50-
9 fold), and (ii) different functional classes of genes with altered expression. For all
10 genes tested, the alteration in expression measured by qRT-PCR mirrored that seen by
11 microarray (Table 3; Fig. S1A).

12
13 *In trans* expression of the wild-type *BCAM0581* gene in the *BCAM0581* deletion
14 mutant restored the transcript level of all fifteen genes to that of the wild-type, as
15 tested by qRT-PCR (Fig. S1A). Importantly, the transcript level for 11 of the genes
16 was also restored to wild-type by exogenous addition of 5 μ M BDSF (Fig. S1A).
17 However for *BCAM0495*, *BCAS0297*, *BCAS0353* and *BCAS0387*, addition of BDSF
18 up to a concentration of 50 μ M was unable to restore wild-type levels of transcript
19 (Table 3; Fig. S1A). The findings may suggest that *BCAM0581* has other roles in the
20 bacterial cell in addition to BDSF biosynthesis that influences expression of certain
21 genes. Deng and colleagues (Deng *et al.*, 2009) have proposed for example that
22 *BCAM0581* has an additional metabolic function.

23 **BCAM0227 regulates the expression of a sub-set of genes in the BDSF regulon**

24 Comparative transcriptome profiling of the *BCAM0227* deletion mutant (sensor-
25 minus) and the wild-type parent established that expression of 78 genes was
26 significantly influenced (> 2 fold change) by the loss of the sensor (Table 2; Table
27 S2). Again for selected genes, qRT-PCR was used to confirm the alteration in
28 expression as revealed by the microarray (Table 3; Fig. S1B).

29
30
31 Comparisons of transcriptome profiles revealed that the expression of 65 genes was
32 influenced by mutation of both *BCAM0581* and *BCAM0227* (Fig. 1). Furthermore, the
33 expression of all 65 genes was altered in the same direction compared to wild-type in
34 the two mutants. Also *BCAM0227* did not regulate expression of *BCAM0495*,

1 *BCAS0297*, *BCAS0353* and *BCAS0387* whose transcription was not responsive to
2 addition of exogenous BDSF (see above). These findings are consistent with the
3 hypothesis that *BCAM0227* is involved in BDSF perception leading to expression of
4 this subset of genes, although they do not exclude the scenario that BDSF and
5 *BCAM0227* have independent actions on gene expression.

6 7 **The sensor kinase protein *BCAM0227* influences a sub-set of BDSF-regulated** 8 **phenotypes**

9 We have previously shown that mutation of *BCAM0581* and associated loss of BDSF
10 synthesis has a pleiotropic phenotypic influence in *B. cenocepacia* J2315, affecting
11 biofilm formation, motility and adhesion. To further examine the possible role of
12 *BCAM0227* in BDSF signalling, we assessed the effect of deletion of *BCAM0227* on
13 the same phenotypes. Although mutation of the *BCAM0581* leads to a reduction in
14 motility and adherence to mucin, mutation of *BCAM0227* did not significantly alter
15 these traits (Fig. 3).

16
17 In contrast, mutations in both genes influenced biofilm formation by bacteria growing
18 in flow cells in minimal medium (Fig. 3; see Experimental procedures). Under these
19 conditions, the wild-type and mutant strains formed characteristic microcolonies after
20 initial surface attachment. With the wild-type, biofilms rapidly developed and covered
21 most of the available surface space within a period of 4 days (Fig. 3A). At this time,
22 the biofilms showed substantial staining with propidium iodide. However, biofilm
23 formation by the mutants never appeared to pass the microcolony stage, bacteria did
24 not colonize the entire surface during the course of the experiment and little staining
25 by propidium iodide was evident (Fig. 3B, E). The introduction of cloned *BCAM0227*
26 or *BCAM0581* genes into the appropriate mutant strains restored biofilm formation
27 and architecture to that seen of the wild-type in each case (Fig. 3, C, F). Addition of
28 exogenous BDSF at 100 nM to cultures of the *BCAM0581* mutant also restored the
29 formation of biofilm structures similar to those of the wild-type (Fig. 3D). In contrast,
30 biofilm architecture and formation by the *BCAM0227* mutant was not altered by
31 addition of exogenous BDSF (data not shown). Staining with propidium iodide will
32 reflect not only the occurrence of dead bacteria but also the presence of extracellular
33 DNA, a component that is an important part of the biofilm matrix for most bacteria,

and which may be produced via pathways controlled by cell-cell signalling (Allesen-Holm *et al.*, 2006; Whitchurch *et al.*, 2002).

The different effects of mutation of *BCAM0581* and *BCAM0227* on motility, and adherence to mucin but similar effects on biofilm formation suggest that the sensor kinase *BCAM0227* is involved in regulation of a subset of BDSF-regulated phenotypes. This conclusion was further supported by measurement of the susceptibility of *BCAM0581* and *BCAM0227* mutant strains to different antibiotics. *B. cenocepacia* is known to exhibit high levels of intrinsic resistance to many antimicrobials (Nzula *et al.*, 2002). Mutation of *BCAM0581* gene led to an increased susceptibility to aminoglycosides, erythromycin, chloramphenicol and ampicillin but had no effect on susceptibility to other antibiotics tested (Table S3). In contrast, mutation of *BCAM0227* did not alter resistance to any of the antibiotics tested. These findings correlate with the observation that mutation of *BCAM0581* but not *BCAM0227*, led to down-regulation of a number of genes involved in multidrug efflux, resistance and detoxification (Fig. 1B). Whether these particular genes play a functional role in antibiotic resistance remains to be determined.

Assessment of the role of *BCAM0227* in BDSF perception by domain swapping

Using the Simple Modular Architecture Research Tool (SMART), *BCAM0227* was predicted to be a hybrid two-component sensor of 1011 amino acids with a histidine kinase phosphoacceptor (HisKA) domain (residues 529–594), histidine kinase domain (residues 641–757), CheY-like receiver domain (residues 781–892) and a C-terminal Histidine phosphotransfer (HPT) domain (residues 920–996) (Fig. 4A). The sensory input domain of *BCAM0227* has predicted to have two transmembrane helices and a large loop located in the periplasm (Fig. 4B). This organization differs from that of RpfC, the DSF sensor from *X. campestris*, which has five predicted transmembrane helices with very limited periplasmic loops (Fig. 4A, B).

We hypothesized that the sensory input of *BCAM0227* was responsible for the perception of BDSF. In order to test this proposed function, domain-swapping experiments were performed in which the portion of RpfC comprising the sensory input domain was replaced with the sensory input domain of *BCAM0227*. A hybrid construct was made (see Experimental procedures) and the functional response to

BDSF addition was tested. Constructs expressing either full-length RpfC or the chimaeric protein BCAM0227 (residues 1-367)-RpfC (residues 172–726) were introduced into an *rpfFrpfc* double mutant of *X.campestris*. This mutant strain is defective in the production of extracellular endoglucanase and is insensitive to addition of BDSF or DSF (Ryan *et al.*, 2008). Expression of RpfC in the *rpfFrpfc* mutant conferred sensitivity to added DSF at concentrations as low as 100 nM and added BDSF at concentrations as low as 10 μ M as measured by the induction of extracellular endoglucanase activity. Importantly, expression of the chimeric BCAM0227-RpfC protein also allowed endoglucanase production in the presence of 10 μ M BDSF or 10 μ M DSF, and activation of the system was seen below these concentrations (Fig. 4C). Similar effects are also seen after expression of a chimeric sensor protein in which the input domain and HisKA domain of BCAM0227 replaces the input domain and HisKA domain of RpfC generating chimeric sensor protein BCAM0227 (residues 1-594)- RpfC (residues 253–726) (Fig. S2).

This induction of endoglucanase was specific to BDSF or DSF. A range of compounds of related structure, including *trans*-11-methyl dodecenoic acid, *cis*-2-decenoic acid, 11-methyl dodecanoic acid and farnesoic acid showed little or no activity when tested at concentrations of 50 μ M, although some activity was seen at 1 mM (Fig. 4C). Importantly we have shown previously that chimaeric proteins in which the input domain of unrelated sensors are fused to RpfC have no activity in sensing either DSF (Ryan *et al.*, 2008) or BDSF (data not shown). Taken together, these observations support the contention that BCAM0227 has a direct role in BDSF perception.

BDSF signalling contributes to cytotoxicity and virulence in *B. cenocepacia*

We have previously reported that mutation of *BCAM0581* led to a reduction of virulence of *B. cenocepacia* in the wax moth (*Galleria mellonella*) larvae model, indicating a role for BDSF signalling in virulence. Transcriptome analysis discussed above identified that genes encoding the Bcs type III secretion system and a number of other virulence-associated factors were down regulated in both the *BCAM0581* and *BCAM0227* mutant strains. These observations prompted us to examine the role of the BCAM0227 sensor in both virulence in the insect model and cytotoxicity to mammalian cells.

The *BCAM0227* mutant showed reduced ability to cause disease in *Galleria mellonella*. The minimum inoculum of the wild-type required to kill all of 10 larvae challenged was 10 ± 5 CFU ($n = 10$). With the *BCAM0227* mutant, a higher level of bacteria ($100,000 \pm 1000$ CFU) was required to kill all 10 larvae, indicating a reduction in virulence. A similar alteration in virulence is seen with the *BCAM0581* mutant. Complementation of the *BCAM0581* and *BCAM0227* mutant strains with the cognate wild-type gene restored virulence to wild-type levels of virulence; the minimum inoculum level to kill all larvae was between 10 and 15 CFU. (All values are the means of measurements on quadruplicate sets of ten larvae; the whole experiment was repeated twice with essentially the same result). A reduction in the rate of killing of larvae by *BCAM0581* and *BCAM0227* mutants was also seen when comparisons were made using an inoculum of 10^6 CFU of each strain (Fig. 5A). Complementation of the *BCAM0581* and *BCAM0227* mutant strains with the cognate wild-type gene restored wild-type levels of virulence (Fig. 5A). Importantly, addition of BDSF to the inoculum at 100 nM restored virulence to the *BCAM0581* mutants, but had little effect on the *BCAM0227* strain (Fig. 5A).

Previous studies have established a correlation between the ability of strains of *B. cenocepacia* and *B. cepacia* to induce cytotoxicity in cultured mammalian cells and the virulence of these strains to mice (Bylund *et al.*, 2006; Bylund *et al.*, 2005; Cieri *et al.*, 2002; Tomich *et al.*, 2003). As a first step towards testing the role of BDSF signalling in mammalian virulence, we examined the effects of wild-type, *BCAM0227* and *BCAM0581* mutant strains on cytotoxicity to CHO cells (see Experimental procedures). Cytotoxicity was measured by quantifying the release of lactate dehydrogenase (LDH) into the supernatants of cultured CHO cells after a short co-cultivation period (6 h) with bacteria. At a multiplicity of infection (MOI) of 50:1, the wild-type *B. cenocepacia* J2315 strain induced a stronger cytotoxic effect than either the *BCAM0227* or *BCAM0581* mutant (Fig. 5B). The reduction in cytotoxic phenotype in these mutants could be restored to the level of the wild-type strain by complementation (Fig. 5B). Addition of 100 nM BDSF had a significant effect on increasing the cytotoxicity of the *BCAM0581* strain although no significant effect on cytotoxicity of the *BCAM0227* mutant strain was evident (Fig. 5B).

On the basis on these findings, we extended these studies to utilise the agar bead mouse model of pulmonary infection. C57Bl/6 mice were infected intra-tracheally with *B. cenocepacia* wild-type, *BCAM0227* and *BCAM0581* mutant strains embedded in agar beads and tested for virulence. Mice infected with the wild-type strain displayed 50% mortality after 8 days post-infection. In contrast, none of mice infected with the mutants appeared to decline during the experiment, and all survived to 8 days post-infection (Table 4). The lethality wild-type was restored to the mutant strains by complementation *in trans* with a wild-type copy of the appropriate gene (Table 4). The effects of mutation of *BCAM0581* and *BCAM0227* on virulence were associated with a substantial reduction in the number of bacteria that could be isolated from the lungs and spleen of the sacrificed mice (Table 4).

These findings from all three infection models indicate that both the BDSF-synthase *BCAM0581* and sensor kinase *BCAM0227* significantly contribute to virulence of *B. cenocepacia* J2315. Addition of BDSF to the *BCAM0581* mutant restores the cytotoxicity and virulence to *G. mellonella*. In contrast, addition of BDSF to the *BCAM0227* mutant gave no alteration in phenotype, suggesting that the regulatory effect of BDSF on virulence is exerted through the sensor kinase.

DISCUSSION

Previous work has established that BDSF acts as an intraspecies or quorum sensing signal in *B. cenocepacia* and has identified *BCAM0581* as the BDSF synthase (Boon *et al.*, 2008; Deng *et al.*, 2009; Ryan *et al.*, 2009). The mechanism of signal perception and the full range of genes controlled by signalling have however remained obscure. Here we have utilized a transposon mutagenesis screen, transcriptome profiling and domain swapping experiments to identify *BCAM0227* as a sensor for BDSF and to establish the extent of the BDSF regulation in *B. cenocepacia*. Our findings indicate that *BCAM0227* acts in regulation of a subset of genes of the BDSF regulon and has a role in virulence of *B. cenocepacia* in both insect and mammalian models.

BCAM0227 was one of a number of genes in which transposon insertion led to a loss of responsiveness to exogenous BDSF, as measured by *cbiD* expression. The role of

these other gene products in regulation of *cbiD* is currently unclear; we do not know if they are elements within the BDSF signalling cascade or whether their regulatory action is independent of BDSF signalling. Intriguingly expression of some of these genes including *BCAM1009*, *BCAL3149*, *BCAM2758* and *BCAS0021* was influenced by BDSF (Table S1).

The extent of the BDSF regulon

Comparative transcriptome profiling identified that 390 genes were under the control of BCAM0581 under the conditions tested (Table S1). Genes under BCAM0581 regulation include those involved in bacterial motility and attachment, stress tolerance, regulation, transport, multidrug resistance, detoxification and signal transduction. Our qRT-PCR analysis of 23 selected genes indicated that the expression of 19 of these genes could be restored to wild-type by addition of exogenous BDSF. Complementation with *BCAM0581* *in trans* however restored expression of all of these genes to wild-type. The findings suggest that the regulatory role of BCAM0581 in *B. cenocepacia* is not restricted to the synthesis of BDSF. Deng and colleagues (Deng *et al.*, 2009) have proposed that BCAM0581, which is a member of the crotonase/enoyl CoA hydratase superfamily of proteins, may have a metabolic function. Another possibility is that accumulation of a precursor of BDSF within the cell of the *BCAM0581* mutant may have a regulatory impact that cannot be reversed by addition of exogenous BDSF. Currently nothing is known about the substrate(s) or the enzymatic action of BCAM0581. Nevertheless, addition of exogenous BDSF to the *BCAM0581* mutant restores expression of the majority of genes and all of the phenotypes that we have tested (including virulence) to wild-type levels. Overall these findings indicate that BDSF signalling has a broad regulatory influence in *B. cenocepacia*, which reflects the regulatory impact of DSF in *X. campestris* (He *et al.*, 2006).

A number of laboratories have proposed criteria to define signalling molecules involved in intraspecies communication (Monds and O'Toole, 2008; Winzer *et al.*, 2002). Such signals should be produced during specific stages of growth, under certain physiological conditions, or in response to changes in the environment; the signal should accumulate extracellularly and be recognised by a specific receptor; accumulation of the signal should generate a concerted response, once a critical

threshold concentration has been reached; most importantly, the cellular response to the signal should extend beyond physiological changes required to metabolize or detoxify it. The work shown here and previously (Deng *et al.*, 2009; Ryan *et al.*, 2009) indicates that BDSF satisfies all of these criteria and thus can be considered a true cell-cell signal molecule.

It is established that *B. cenocepacia* also utilises *N*-AHLs as intracellular signals to regulate gene expression through the two LuxR family proteins CepR and CciR (O'Grady *et al.*, 2009). This raises the issue as to the extent to which the regulatory influences of the two classes of signal overlap. Comparison of the BDSF regulon described here with those genes whose expression is regulated by CepR and CciR (O'Grady *et al.*, 2009) indicates only a partial regulatory overlap (Fig. S3). Furthermore, we have previously shown that mutation of *BCAM0581* does not influence the levels of *N*-AHLs produced by *B. cenocepacia* J2315 (Ryan *et al.*, 2009) and the transcriptome profiling of O'Grady and colleagues (O'Grady *et al.*, 2009), which was done in the K56-2 strain, does not indicate any influence of CepR and CciR on expression of *BCAM0581*.

BCAM0227 is a novel sensor for DSF-family signals

The findings from the domain-swapping experiments provide direct evidence for a role for *BCAM0227* in the perception of BDSF. This contention is supported by the observation that *BCAM0227* is required for the induction of *cbiD* gene expression in response to exogenously added BDSF. Furthermore *BCAM0227* regulates a sub-set of genes under BDSF control but does not regulate expression of *BCAM0581* and shows no alteration in BDSF levels (data not shown), consistent with a role in signal perception rather than signal generation. The sensory domain of *BCAM0227* is however quite different from that of RpfC, the DSF sensor in xanthomonads. Bioinformatics analysis predicts that RpfC has 5 transmembrane helices with periplasmic and cytoplasmic loops of less than 20 amino acids. In contrast, *BCAM0227* is predicted to have 2 transmembrane helices and a large periplasmic loop of 300 amino acids (Fig. 4). The precise mechanism by which either sensor recognises BDSF or DSF remains to be determined.

Transduction of the DSF signal in xanthomonads involves modulation of levels of the second messenger cyclic di-GMP through the action of RpfG, an HD-GYP domain regulator with cyclic di-GMP phosphodiesterase activity (Ryan *et al.*, 2006; Ryan *et al.*, 2010). In the genome of *X. campestris*, *rpfG* is part of the *rpfGHC* operon that is convergently transcribed with *rpfF*, which encodes the DSF synthase. Although nothing is known of the BDSF signal transduction mechanism beyond BCAM0227, we speculate that this may involve BCAM0228, which encodes an OmpR-like two component response regulator with a DNA-binding domain, rather than an RpfG-like regulator.

Notably, mutation of *BCAM0227* affects expression of only a subset of the genes within the BDSF regulon. Furthermore *BCAM0227* does not influence all phenotypes that are regulated by *BCAM0581*. For example, although *BCAM0227* is involved in biofilm formation and virulence, it does not affect motility or adherence to mucin. This partial overlap of regulatory influence may suggest the existence of further sensing mechanisms for BDSF in *B. cenocepacia*. Although bioinformatic studies of *B. cenocepacia* identified no homolog of RpfC, a sensor kinase related to *BCAM0227* (*BCAM0218*) was identified. However RT-PCR experiments showed that the putative *BCAM0218* sensor did not regulate any of a number of genes under BDSF regulation and domain-swapping experiments using the input domain of *BCAM0218* provided no evidence for a role in BDSF perception (data not shown). Hence the nature of any additional sensory mechanisms for BDSF remains obscure. Furthermore we cannot rule out that BDSF may have biosurfactant properties that play a biological role. For example other quorum sensing molecules from Gram-negative organisms have been shown to influence surface movement and attachment by their surfactant properties (Daniels *et al.*, 2004; 2006).

The broader relevance of findings

Bioinformatic studies suggest that BDSF signalling is widespread in the genus *Burkholderia*. Homologues of *BCAM0227* occur widely within bacteria of the Bcc, including *B. pseudomallei* and *B. ambifaria*, as well as in other species that are plant associated. Homologs of *BCAM0581*, which encodes the BDSF synthase, are also found in a number of *Burkholderia* spp. but not in *B. pseudomallei*. The occurrence of the sensor in the absence of a signal synthase in *B. pseudomallei* suggests that this

organism may be able to sense a signal that it does not make. Such “eavesdropping” on bacterial signalling has been described for a number of bacteria and a range of signals. A pertinent example is the sensing of DSF by *Pseudomonas aeruginosa*, an organism that has no close homolog of RpfF (the DSF synthase of xanthomonads) or BCAM0581. DSF perception in *P. aeruginosa* is mediated by PA1396, a sensor kinase with an input domain of related amino acid sequence to the sensory input domain of RpfC. Signal perception leads to alteration of expression of genes involved in increased tolerance to cationic antimicrobial peptides (Ryan *et al.*, 2008). *P. aeruginosa* can also recognize BDSF through the action of PA1396 suggesting a possible role for BDSF in interspecies signalling with *B. cenocepacia* that counterbalances the role for *N*-AHLs in (unidirectional) signalling from *P. aeruginosa* to *B. cenocepacia*. Intriguingly, Davies and Marques, (2009) have recently shown that *P. aeruginosa* does make a signal of the DSF family (cis-2-decenoic acid) but this molecule is not recognised by BCAM0227 or PA1396 (unpublished data) and the synthase for cis-2-decenoic acid has not yet been identified.

In conclusion, we have shown that BCAM0227 is a sensor kinase that is an element of the BDSF mediated cell-cell signalling pathway of *B. cenocepacia* that contributes to the virulence of this organism. In the context of the global rise of anti-microbial resistance and the slower development of novel antibiotics, cell-cell signalling pathways are being evaluated as potential drug targets. Our findings make a contribution towards the greater understanding of the mechanisms of signal generation and perception that will be important for the development of novel therapeutic strategies to combat bacterial infections.

EXPERIMENTAL PROCEDURES

Bacterial strains, growth conditions and media.

The bacterial strains used in this study are listed in Supporting Table S4. Cultures were routinely grown at 37°C, in Luria broth (LB) with shaking or on 1.5% LB agar plates. For promoter::lux assays, strains were grown in 0.25% trypticase soy broth with 5% Bacto-Peptone (Difco™). When appropriate, the following concentrations of antibiotics were used: 50 µg/ml of gentamicin (Gm) and 100 µg/ml of tetracycline (Tc). Antibiotics were purchased from Sigma-Aldrich. Methods used for assay of virulence factors, adherence to mucin and motility are detailed in (Ryan *et al.*, 2009).

DNA manipulations.

Molecular biological methods such as isolation of plasmid and chromosomal DNA, PCR, plasmid transformation as well as restriction digestion were carried out using standard protocols (Sambrook *et al.*, 1989). PCR products were cleaned using the PCR purification kit (Qiagen™) and DNA fragments were recovered from agarose gels using Qiaquick minielute gel purification kit (Qiagen™). Oligonucleotide primers were purchased from Sigma-Genosys™.

Construction of chromosomal reporter fusions.

The protocol used for the construction of the *lux* reporter fusion for *cbiD* was a modification of the RED recombination protocols previously described by (Gerlach *et al.*, 2007; Lesic and Rahme, 2008). The *cbiD* gene was amplified by PCR using forward and reverse primers cbiD-F (5'GGATCCAAGCGCCAGCAGGACGTGCTGATG-3') and cbiD-R (5'GGATCCGAGACTGGTGGACGACGGC-3') to include the region 250 bp upstream of the gene and was cloned into pGLuc. The *cbiD::lux* fusion removed by restriction digest using *HindIII* and *XbaI* and cloned into pME6032-RedS cut with the same enzymes. This construct was electroporated into *B. cenocepacia* wild-type or *BCAM0581* mutant. Transconjugants were grown in LB to an optical density at 600 nm of 0.4 at 37°C and recombination was induced by addition of 0.2% L-arabinose for 1.5 h. The correct integration of the reporter cassette was confirmed by colony PCR using a reverse primer specific to the reporter and a forward primer specific to the upstream region of the target gene.

Transposon mutagenesis and screening.

A library of mutants in the *B. cenocepacia* *BCAM0581* strain carrying the *cbiD::lux* fusion was constructed using Mariner transposon vector pBT20 as previously described (Kulasekara *et al.*, 2005). Briefly, 100 ng of transposon-containing plasmid was electroporated into newly prepared electrocompetent *B. cenocepacia* *BCAM0581* mutant cells. Strains carrying the transposon insertion were identified by selection on Gm. The library of mutants was screened for loss of responsiveness of *cbiD::lux* expression to exogenous BDSF. Strains were inoculated into microtiter wells containing LB medium with 50nmol BDSF (Allesen-Holm *et al.*, 2006a) by using a

Qpix robot (Genetix™). The luciferase activity lysates of bacteria from each well were quantified as described previously (Deiwick *et al.*, 1999). Briefly, equal amounts of bacterial cells as adjusted by determination of optical density at 600 nm (OD600) of the cultures were harvested by centrifugation, and lysed. Luciferase activity in bacterial cell lysates was assayed using Luciferase Assay Kit (Promega™) and quantified in black microtiter plates by a Wallac Victor 2 1420 Multilabel Counter plate reader (Perkin Elmer™).

Identification of transposon insertion sites.

Genomic DNA was extracted from transposon mutants of interest as previously described (Sambrook *et al.*, 1989). The nucleotide sequence flanking the transposon insertion site was amplified by PCR as previously described (Friedman and Kolter, 2004) using the primers: Rnd1-TnM (5'-GTGAGCGGATAACAATTTTCACACAG-3'), and Rnd2-TNm (5'-ACAGGAAACAGGACTCTAGAGG-3'). Amplicons were sequenced using the TnM specific primer: TnMseq (5'-CACCCAGCTTTCTTGACAC-3'). Sequence data generated was examined with BLASTn, BLASTx (www.ncbi.nlm.nih.gov/BLAST/), and VGE BLAST (www.vge.ac.uk/BLAST/) software. Bioinformatic analysis of the BCAM0227 sensor kinase was performed using SMART (<http://smart.embl-heidelberg.de/>).

Cloning and expression of input-BCAM0227-RpfC chimeric protein.

For the construction of BCAM0227-RpfC chimeric sensor protein, the coding sequences for the input domain of BCAM0227 modules (amino acid residues 1 -383) and N-terminal of RpfC (residues 249 - 726) were amplified using primers InBCAM0227-F (5'-GGATCCATGGGGGCACCCTGGCCGACCGC-3') and BCAM0227-R (5'-AAGGCTTGCGCACCAGCAGCCACCACAC-3'); RpfCCTerm-F (5'-AAGGCTTGCGATGACCCGTGCAGTGCGC-3') and RpfCCterm-R (5'-GGATCCCTATTCGTTGCTACGGGGGGA-3') respectively. These primers contain restriction sites for *Hind*III and *Bam*HI (underlined). These amplified products were diluted, mixed and subjected to further PCR amplification using the primers F (5'-GGATCCATGGGGGCACCCTGGCCGACCGC-3') and R (5'-AAGCTTCTATTCGTTGCTACGGGGGGA-3'). The final amplicon was cloned into TOPO (Invitrogen™) and verified by sequencing. The product was excised from TOPO using *Hind*III and *Bam*HI and ligated into pLAFR3 that had been cut with the

1 same enzymes. This construct was conjugated into *X. campestris* strains 8004 (wild-
2 type), the *rpfC* mutant and *rpfC**CrpfF* double mutant using triparental mating as
3 described previously (Ryan *et al.*, 2008).

5 **Construction of complementing clone.**

6 To complement the *B. cenocepacia* BCAM0227 mutant, the wild-type gene
7 BCAM0227 was amplified by PCR from genomic DNA of *B. cenocepacia* with the
8 primers (see Supporting Table S4) with the following thermal cycling conditions:
9 95°C for 5 min followed by 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 2
10 min, with a final extension at 72°C for 10 min. The resulting amplicon was digested
11 with the restriction enzymes *Hind*III and *Bam*HI and ligated into pBBR1MCS cut
12 with the same enzymes. The resulting plasmid, pBCAM0227, was introduced into the
13 *B. cenocepacia* BCAM0227 mutant by conjugation.

15 **RNA manipulations.**

16 *B. cenocepacia* strains was subcultured to obtain an initial optical density at 600 nm
17 of 0.8 in LB and grown without selection. Total RNA was isolated using a RiboPure
18 bacterial RNA isolation kit (Ambion™) and subjected to DNase treatment. Samples
19 were confirmed to be free of DNA prior to cDNA synthesis by PCR using *Taq*
20 polymerase (Invitrogen™).

22 **Microarray sample preparation and hybridization.**

23 Three independent RNA samples from *B. cenocepacia* strains grown for 24 h were
24 used in microarray experiments. Gene expression profiles were generated using
25 custom *B. cenocepacia* J2315 microarrays (Agilent™) (Drevinek *et al.*, 2008). A
26 reference pool of J2315 cDNA was fluorescently labelled with Cy3 while the test
27 cDNA samples (WT J2315, BCAM0581 and BCAM0227) were fluorescently labelled
28 with Cy5. cDNA generation and labelling was performed using the CyScribe Post-
29 Labelling kit (GE Healthcare™) according to the manufacturer's protocol. Spike-in
30 controls (Agilent™) were included into the labelling procedure for quality control
31 purposes. cDNA purification was performed by ethanol precipitation and the labelled
32 cDNA was purified using a CYSCRIBE GFX purification kit and eluted with water.

34 Hybridization and washing of arrays were performed according to the two-colour

microarray based gene expression analysis protocol (Agilent™) with minor modifications where the 25× fragmentation buffer was omitted and the cDNA mix and 10× blocking agent were heat-denatured for 3 min at 98°C and cooled to room temperature before adding the hybridization buffer. Washing of microarrays was performed including acetonitrile as well as stabilization and drying solution (Agilent™). A G2565 BA microarray scanner and the scan control software (Agilent™) were used. Scanning resolution was set to 5 µm and the scan region was adjusted to 61 × 21.6 mm. The extended dynamic range function was switched on with 100% and 10% photomultiplier gain settings. Images were analysed with the feature extraction software (Agilent™).

Microarray data analysis.

Microarray data analysis was performed using GeneSpring GX 11.1 software (Agilent™). The enhanced Agilent FE import method was used to preprocess the initial data before per-spot and per-chip normalizations were performed for all arrays. After filtering, genes were selected on the basis of changes, for which a 2-fold cutoff was used for comparison. The entire microarray data set has been deposited in the ArrayExpress database <http://www.ebi.ac.uk/arrayexpress> under accession number (waiting accession number).

Quantitative RT-PCR.

RT-PCR was performed using a cDNA synthesis kit (Invitrogen™). For quantitative RT-PCR, quantification and melting curve analyses were performed with a LightCycler ® (Roche™) and QuantiFast SYBR Green (Qiagen™) according to manufacturers' instructions. RT-PCR reactions were performed in triplicate, and the data shown are from at least two independent experiments. Expression levels were normalized to levels of the 16S RNA gene transcript in each experiment.

Antimicrobial susceptibility testing.

Susceptibility experiments were carried out as previously described by (Hassett *et al.*, 1999) with the following modifications. Selected bacterial strains were grown shaking aerobically over night at 37°C. For sensitivity experiments for most compounds were carried out by adding a 0.1 ml suspension of stationary phase bacteria spread over the entire surface of LB agar plates. After 17 h of growth at 37°C, the stationary phase

organisms were removed from the agar surface with 10 ml of 0.9% saline and a sterile glass rod. Filter paper disks impregnated with various concentrations of selected compounds were placed on the agar surface. For H₂O₂ and NaOCl sensitivity experiments, suspensions (0.1 ml) were diluted in 3 ml of molten soft agar (0.6%), poured onto the surface of L agar plates, and the agar was allowed to solidify. Filter paper disks (7 mm, Whatmann) saturated with 10 ml of 30% H₂O₂ were placed on the agar overlay. Sensitivity to each compound was then recorded as the mean \pm SE ($n = 9$) of the diameter of growth inhibition after 24 h of growth at 37°C. The results represent the average of three independent replicas.

Cultivation and imaging of biofilms in flow chambers.

Biofilms were grown in flow chambers with individual channel dimensions of 1 \times 4 \times 40 mm. The flow system was assembled and prepared as described by (Sternberg and Tolker-Nielsen, 2006). The flow chambers were inoculated by injecting 350 μ l of overnight culture diluted to an OD₆₀₀ of 0.01 into each flow channel with a syringe. After inoculation, flow channels were left without flow for 1 h, after which medium flow at a linear rate of 0.2 mm/s was started using a Watson Marlow 205S peristaltic pump. All microscopic observations and image acquisitions were performed on an LSM510 confocal laser scanning microscope (CLSM) (Carl ZeissTM) equipped with detectors and filter sets for monitoring Syto9 fluorescence. Simulated three-dimensional images, shadow projections and sections were generated using the ImarisTM software package.

Cytotoxicity assay.

Cytotoxicity was assessed by measuring release of lactate dehydrogenase (LDH) from CHO (Chinese hamster ovary) cells. The CHO cells were routinely grown in F-12 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. Prior to infection, confluent CHO cells were washed and incubated with F-12 containing 1% FBS and 2 mM glutamine. *B. cenocepacia* was grown overnight in LB, subcultured into fresh LB, and grown to mid-log phase. 2.5×10^5 CHO cells per well were infected with *B. cenocepacia* at an initial multiplicity of infection (MOI) of 50:1 in triplicate experiments. Culture supernatants were collected after 6 hours of infection and centrifuged for 10 min at 3,220 \times g to sediment bacteria and CHO cells. LDH in the supernatant was measured with a Roche LDH kit in accordance with the

manufacturer's instructions. Percentage LDH release was calculated relative to that of the uninfected control, which was set at 0% LDH release, and that of cells lysed by Triton X-100, which was set at 100% LDH release.

***G. mellonella* killing assays.**

Larvae were stored in wood chips at 4°C. A 10-µl Hamilton syringe was used to inject 5-µl aliquots of bacterial suspensions into *G. mellonella* via the hindmost left proleg. 10-fold serial dilutions of bacteria starting at 2×10^8 CFU per ml were made in PBS supplemented with 100 µg/ml of ampicillin. 5 µl of each dilution was injected into each of ten larvae. Control larvae were injected with 5 µl of PBS plus 100 µg/ml of ampicillin. Following injection, larvae were placed in a static incubator in the dark at 30°C, the optimum temperature for insect growth and development. Larvae were scored as dead or alive at 48 and 72 h post-infection. The minimum inoculum required to kill all 10 larvae was determined for each strain.

Mouse infection model.

C57Bl 6 mice were infected with *B. cenocepacia* strains by using the agar bead model of lung infection (Bylund *et al.*, 2006; Bylund *et al.*, 2005; Cieri *et al.*, 2002). Bacteria were incorporated into agar beads, and 1×10^9 CFU were inoculated into each mouse intra-tracheally. The animals were sacrificed after 8 days post-infection, and the lungs and spleens were harvested, homogenized, and cultured on LB agar to assess the severity of infection.

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12 **FIGURES AND TABLES LEDGENDS**

13 Table 1. Location of mariner transposon insertions in the *Burkholderia cenocepacia*
14 J2315 genome leading to lack of responsiveness to exogenous BDSF.

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16 Table 2. Mutation of *BCAM0581* and *BCAM0227* in *Burkholderia cenocepacia*
17 affects expression of genes in a number of functional groups, as revealed by
18 microarray analysis. The sensor kinase *BCAM0227* regulates a sub-set of the genes
19 regulated by the BDSF synthase *BCAM0581*.

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21 Table 3. Comparison of effects of mutation of *BCAM0581* and *BCAM0227* on
22 expression of selected genes in *Burkholderia cenocepacia* as measured by microarray
23 and quantitative RT-PCR.

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25 Table 4. BDSF signalling is associated with lethality in an agar bead mouse model of
26 infection. Mice were infected intra-tracheally using agar beads with 1×10^9 CFU of *B.*
27 *cenocepacia* J2315 wild-type or strains with mutations in *BCAM0581* or *BCAM0227*.
28 Survival of mice was monitored over a period of 8 days. Animals were sacrificed 8
29 days post-infection, and the bacterial loads in the lungs and spleen were determined.

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31 Fig. 1. Genes differentially expressed in *BCAM0581* or *BCAM0227* mutants
32 compared to the wild-type *B. cenocepacia* J2315 as revealed by microarray analysis.
33 (A) Venn diagrams showing the overlap of genes whose expression is influenced by
34 both *BCAM0227* and *BCAM0581*: (i) up-regulated; (ii) down-regulated gene subsets.

(B) Detailed analysis of expression of (i) genes involved in the regulation of stress response and detoxification and (ii) genes involved in the regulation of virulence and pathogenesis from microarray analysis representing the effects of mutation of *BCAM0227* and *BCAM0581* on global gene expression with decreased (green), increased (red) or no significant change (grey) in expression in each mutant compared to J2315 according to microarray analysis. Only changes in transcript level >2 fold were deemed significant.

Fig. 2. Mutation of genes encoding *BCAM0581* and *BCAM0227* does not have the same effect on motility and adherence to porcine mucin. (A) Although the *BCAM0581* mutant showed reduced motility in 0.6% Eiken agar, no alteration was seen in the *BCAM0227* mutant. Motility of the *BCAM0581* mutant was restored to wild-type level by complementation or addition of 50 μ M BDSF to the plates. Each experiment was performed at least three times in triplicate. (B). The *BCAM0581* mutant showed a reduction in adherence to porcine mucin while mutation of *BCAM0227* mutant had no effect. Adherence of the *BCAM0581* mutant was restored to wild-type level by complementation. Each experiment was performed at least three times in triplicate. Error bars represent the standard errors of the mean.

Fig. 3. Mutation of genes encoding *BCAM0581* and *BCAM0227* has similar effects on biofilm architecture within biofilms of *B. cenocepacia* J2135. Images are of 4-day-old biofilms in flow cells that were stained with both Syto9 (green) to stain all bacteria and propidium iodide (red) to stain dead cells and extracellular DNA. Separate images for each fluorescent dye were merged. The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. Scale bars, 20 μ m. The *B. cenocepacia* J2135 strains were (A) wild-type; (B) *BCAM0581* mutant; (C) *BCAM0581* complemented mutant; (D) *BCAM0581* mutant plus 100 nM BDSF. (E) *BCAM0227* mutant; (F) *BCAM0227* complemented mutant.

Fig. 4. *BCAM0227* is a two-component sensor with a role in BDSF perception. (A) Domain architecture of *BCAM0227* and of RpfC, the DSF/BDSF sensor of xanthomonads as revealed by the SMART algorithm. Key: Bars: transmembrane helices; HisKA: histidine kinase phosphoacceptor domain; HATPase_c: histidine

kinase domain; REC: two component receiver domain; HPt: histidine phosphotransfer domain. The HPt domain in BCAM0227 is not identified by SMART, but as a Pfam domain. The black arrows indicate the region of the input of BCAM0227 that was used to construct the chimaeric protein BCAM0227 (residues 1-367)-RpfC (residues 172–726). (B) Predicted topology of RpfC, which has 5 predicted transmembrane helices, and BCAM0227, which has two transmembrane helices in the bacterial inner membrane (IM). (C) Domain swapping experiments between BCAM0227 and RpfC indicate that BCAM0227 is a sensor of BDSF. These experiments were conducted by expression of chimeric sensors in the *rpfF**rpfC* double mutant of *X. campestris* and measurement of the effects of addition of exogenous DSF/BDSF on the DSF-regulated phenotype of endoglucanase production. Expression of RpfC in this mutant background has no effect on endoglucanase activity (which is very low), but endoglucanase activity was increased upon addition of exogenous DSF or BDSF at 50 μ M. Similar effects are also seen after expression of a chimeric sensor protein in which the input domain of BCAM0227 replaces the input domain of RpfC; the *rpfF**rpfC* strain expressing this chimeric protein can recognize synthetic BDSF and DSF at 50 μ M to induce endoglucanase activity. In contrast, addition of *trans*-11-methyl-2-dodecenoic acid and *cis*-2-decenoic acid (*cis*-DA) at 50 μ M did not induce endoglucanase production in this strain, although some effects were seen at the higher concentration of 1 mM.

Fig. 5. BCAM0227 and BCAM0581 contribute to cytotoxicity and virulence by *Burkholderia cenocepacia* J2135. (A) The *BCAM0227* and *BCAM0581* mutant strains showed reduced virulence in the *Galleria mellonella* larva infection model. Larvae were injected with 10^4 CFU of bacteria and monitored for survival 24 h post-infection. Addition of BDSF (100nM) restored virulence of the *BCAM0581* mutant to wild-type but had no effect of the virulence of the *BCAM0227* mutant strain. Each bar is representative of the results for five independent trials ($n = 10$), and standard deviations are shown. (B) Mutation of *BCAM0227* and *BCAM0581* leads to reduced cytotoxicity to CHO cells as measured by release of LDH. Cytotoxicity was expressed as a percentage of the total amount of LDH released from cells treated with 1% Triton X-100 (TX-100). Cytotoxicity of the *BCAM0581* strain was restored to the wild-type level by complementation with *BCAM0581* or inclusion of BDSF (100nM) in the

assay. Addition of 100 nM BDSF had no significant effect on cytotoxicity of the *BCAM0227* mutant strain, although complementation with *BCAM0227* restored cytotoxicity to wild-type levels. Error bars represent the SDs for triplicate experiments.

SUPPORTING INFORMATION

Table S1. A complete list of genes with altered expression in *BCAM0581* mutant compared to wild-type J2315.

Table S2. A complete list of genes with altered expression in *BCAM0227* mutant compared to wild-type J2315. The genes that are also regulated by *BCAM0581* (BDSF synthase) are indicated.

Table S3. Antimicrobial susceptibilities of *B. cenocepacia* J2315, *BCAM0227* and *BCAM0581* mutants

Table S4. Bacterial strains, plasmids and primers used in this study.

Fig. S1. (A) Real-time RT-PCR analysis of the effects of mutation of *BCAM0581* grown in LB (OD₆₀₀ 0.8) on transcript levels for *BCAM0495*, *BCAM1265*, *BCAM1619*, *BCAM2039*, *BCAM2041*, *BCAM2045*, *BCAM2051*, *BCAM2759*, *BCAL0114*, *BCAL0813*, *BCAS0297*, *BCAS0299*, *BCAS0353*, *BCAS0387* and *BCAS0230*. Data were normalized to 16S RNA and each is presented as the fold change with respect to the wild-type *B. cenocepacia* J2315 for each gene. Data (means \pm SD) are representative of four independent biological experiments. White columns: effects of mutation of *BCAM0581*; Grey columns: effects of complementation of *BCAM0581* mutation; Black columns: effects of addition of 100 nM exogenous BDSF. (B) Real-time RT-PCR analysis of the effects of mutation of *BCAM0227* grown in LB (OD₆₀₀ 0.8) on transcript levels for *BCAM0495*, *BCAM1265*, *BCAM1619*, *BCAM2039*, *BCAM2041*, *BCAM2045*, *BCAM2051*, *BCAM2759*, *BCAL0114*, *BCAL0813*, *BCAS0297*, *BCAS0299*, *BCAS0353*, *BCAS0387* and *BCAS0230*. Data were normalized to 16S RNA and each is presented as the fold change with respect to the wild-type *B. cenocepacia* J2315 for each gene. Data (means \pm SD) are representative of four independent biological experiments. White

columns: effects of mutation of *BCAM0227*; Grey columns: effects of complementation of *BCAM0227* mutation; Black columns: effects of addition of 100 nM exogenous BDSF.

Fig. S2. Domain swapping experiments between *BCAM0227* and *RpfC* indicate that *BCAM0227* is a sensor of BDSF. These experiments were conducted by expression of chimeric sensors in the *rpfFrpfC* double mutant of *Xcc* and measurement of the effects of addition of exogenous BDSF on the DSF-regulated phenotype of endoglucanase production. Expression of *RpfC* in this mutant background has no effect on endoglucanase activity (which is very low), but endoglucanase activity was increased upon addition of exogenous BDSF at 50 μ M. Similar effects are also seen after expression of a chimeric sensor protein in which the input domain and HisKA domain of *BCAM0227* replaces the input domain and HisKA domain of *RpfC* generating chimeric sensor protein *BCAM0227* (residues 1-594)- *RpfC* (residues 253–726); the *rpfFrpfC* strain expressing this chimeric protein can recognize synthetic BDSF at 50 μ M to induce endoglucanase activity. In contrast, addition of *trans*-11-methyl-2-dodecenoic acid and *cis*-2-decenoic acid (*cis*-DA) at 50 μ M did not induce endoglucanase production in this strain, although some effects were seen at the higher concentration of 1 mM.

Fig. S3. Regulatory overlap of BDSF signalling and the quorum sensing networks of *CepIR* and *CciIR* in *B. cenocepacia*. Microarray data from expression profiles of *BCAM0581*, *BCAM0227* mutants (as reported here) and *cepR* or *cciR* mutants as reported by O'Grady *et al.*, (2009) are compared.

Table 1. Location of transposon insertions found in the *Burkholderia cenocepacia* J2315 genome leading to lack of responsiveness to exogenous BDSF.

Disrupted gene ^a	Polar effect possible? ^b	Number of different transposon mutants ^c	Gene product
<i>BCAL0223</i>	N	1	50S ribosomal protein L1
<i>BCAL1543</i>	N	2	major facilitator superfamily protein
<i>BCAL1740</i>	N	2	major facilitator superfamily protein
<i>BCAL1791</i>	N	1	hypothetical protein
<i>BCAL1957</i>	Y	1	putative sugar transferase
<i>BCAL2276</i>	N	1	putative FAD dependent oxidoreductase
<i>BCAL2731</i>	N	3	ATP-dependent Clp protease adaptor protein
<i>BCAL2745</i>	N	1	putative CoA transferase family protein
<i>BCAL3149</i>	N	1	hypothetical protein
<i>BCAM0227</i>	N	2	hybrid two-component system kinase-response regulator protein
<i>BCAM0580</i>	Y	2	RNase II stability modulator
<i>BCAM0700</i>	N	2	TetR family regulatory protein
<i>BCAM1009</i>	N	2	putative acyltransferase
<i>BCAM1242</i>	N	1	hypothetical protein
<i>BCAM1572</i>	N	1	methyl-accepting chemotaxis protein
<i>BCAM1685</i>	N	1	<i>nirB</i> ; putative nitrite reductase

<i>BCAM2758</i>	N	1	<i>cblS</i> ; two-component regulatory system, sensor kinase protein
<i>BCAM2836</i>	N	5	putative diguanylate cyclase
<i>BCAS0021</i>	Y	1	putative CoA-transferase
<i>BCAS0212</i>	Y	2	hypothetical protein

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- a. None of these gene disruptions affect bacterial growth in complex media and minimal media.
b. Possibility of Tn5 insertion affecting expression of other (downstream) genes. Y: yes; N: no.
c. Number of different Tn5 insertion mutations mapped to the same gene.

Table 2. Classification of a selection of BDSF-regulated genes by functional groups.

Class	Gene name or ID ^a	Fold changes ^b	
		BCAM0581 vs WT	BCAM0227 vs WT
Flagellar synthesis, attachment, motility and chemotaxis	<i>fliC</i> (BCAL0114)	5.5	5.4
	<i>motA</i> (BCAL0126)	2.2	NC ^c
	<i>motB</i> (BCAL0127)	2.0	NC
	<i>cheD</i> (BCAL0133)	2.1	2.3
	BCAM2758	-2.1	-2.1
	BCAM2759	-2.0	-2.0
	BCAL1525	-3.0	NC
	BCAL1828	-5.4	NC
	<i>pilA</i> (BCAS0299)	-2.1	NC
	<i>flp</i> (BCAS0308)	-2.8	NC
Membrane components and transporters	BCAS0066	2.1	NC
	<i>aqpZ</i> (BCAL0121)	-2.6	NC
	BCAL1546	-11.1	-11.5
	<i>ompA</i> (BCAL2645)	-2.1	NC
	BCAM1697	-3.0	-2.3
	BCAM1931	-2.0	NC
	BCAM2142	-2.4	NC
	BCAM2188	-2.2	NC
	BCAM2334	-3.5	-4.0
	BCAS0014	-2.1	NC
Stress	<i>ctc</i> (BCAL0799)	2.1	NC
	BCAM0291 (<i>uspA</i>)	2.3	NC
	BCAM0292 (<i>uspB</i>)	2.1	NC
	<i>cspD</i> (BCAL0368)	-3.6	-2.9
	BCAL2054	-2.4	NC
	BCAM1619	-3.1	-2.3
	BCAM2803	-2.5	NC
Multidrug resistance and detoxification	<i>mfs</i> (BCAL1740)	2.4	NC
	<i>mip</i> (BCAM0163)	2.6	NC
	<i>czcB</i> (BCAM0712)	2.2	NC
	<i>mrtP</i> (BCAM1202)	4.6	NC
	<i>norM</i> (BCAL2907)	-2.1	NC
	BCAM0783	-2.2	NC
	BCAM1132	-2.1	NC
	BCAM2545	-2.1	NC
	<i>oprM</i> (BCAL2820)	-125.3	NC

	<i>BCAL2821</i>	-10.3	NC
	<i>tetR (BCAL2812)</i>	-16	NC
	<i>BCAS0014</i>	-2.1	NC
	<i>bpeA (BCAS0766)</i>	-3	NC
	<i>BCAS0592</i>	NC	-2.1
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Ion transport and metabolism	<i>glpK (BCAL0925)</i>	3.9	NC
	<i>BCAL1989</i>	2.6	NC
	<i>BCAL1830</i>	2.4	NC
	<i>BCAM0296</i>	2.2	NC
	<i>BCAM0855</i>	3.4	NC
	<i>BCAM0858</i>	2	NC
	<i>BCAM2833</i>	3.2	NC
	<i>catC (BCAM0803)</i>	-2.1	NC
	<i>pqqC (BCAM2362)</i>	-2.1	NC
	<i>BCAM0080</i>	-4.6	-4.1
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Virulence and pathogenesis	<i>BCAM2041 (bcscR)</i>	-2.2	NC
	<i>BCAM2045 (bcscU)</i>	-2.8	-2.2
	<i>BCAM2050 (bcscK)</i>	-3.8	NC
	<i>BCAM2051 (bcscJ)</i>	-2.5	-2.2
	<i>afcA (BCAS0222)</i>	-2.7	NC
	<i>BCAS0225 (shvR)</i>	-2.3	NC
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Energy production and conversion	<i>BCAM0284</i>	4.0	NC
	<i>BCAS0488</i>	2.2	NC
	<i>BCAL0063</i>	-2.6	NC
	<i>BCAM1473</i>	-2.0	NC
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Transcription regulators	<i>asnC (BCAL0628)</i>	2.3	NC
	<i>BCAM0595</i>	2.7	NC
	<i>BCAM2190</i>	2.4	NC
	<i>BCAM2039</i>	-42.5	-33.2
	<i>fis (BCAM2365)</i>	-2.5	NC
	<i>BCAL1230</i>	-3.3	NC
	<i>BCAL0197</i>	-2.1	NC
	<i>BCAL3169</i>	-2.1	NC
	<i>BCAS0767</i>	-2.7	NC
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Signal transduction	<i>BCAL3497</i>	2.7	NC
	<i>BCAS0069</i>	2.6	NC
	<i>BCAS0264</i>	2.3	NC
	<i>BCAM2426</i>	-2.5	-2.2
	<i>BCAS0589</i>	-5.2	NC
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Regulatory	<i>BCAL3368</i>	3.2	NC
	<i>flhD (BCAL0124)</i>	2.1	NC
	<i>asnC (BCAL0628)</i>	2.2	NC
	<i>BCAS0134</i>	2.1	NC
	<i>BCAL0812</i>	-4.0	NC
	<i>rpoN (BCAL0813)</i>	-2.5	NC
	<i>hfq (BCAL1879)</i>	-2	NC

	<i>gntR</i> (BCAM2329)	-2.4	NC
	<i>BCAM2025</i>	-2.2	NC
	<i>BCAM0238</i>	-3.2	NC
	<i>tetR</i> (BCAL2823)	-2.1	NC
Hypothetical proteins and others	<i>BCAL0112</i>	2.7	2.4
	<i>BCAM0634</i>	NC	3
	<i>BCAM2024</i>	3.4	2.4
	<i>BCAM2377</i>	4.1	2.7
	<i>BCAL0108</i>	-4	-3.5
	<i>BCAS0269</i>	-2	-2.1
	<i>BCAS0297</i>	NC	-2

- 1 a. Gene numbers are from http://www.sanger.ac.uk/Projects/B_cenocepacia. Annotations and
2 functional assignments are from *B. cenocepacia* J2315 (Holden *et al.*, 2009).
3 b. A complete outline of expression changes in both *BCAM0581* and *BCAM0227* mutant backgrounds
4 is detailed in Table S1 and S2.
5 c. NC: No significant (< 2 fold) change in gene expression compared to wild-type.

1 **Table 3.** Summary of microarray and quantitative RT-PCR analysis of selected genes.

Gene name ^a	Gene product and function ^a	Microarray gene expression relative to wild-type ^b		RT-qPCR gene expression analysis relative to wild- type ^c		
		<i>BCAM0581</i>	<i>BCAM0227</i>	<i>BCAM0581</i>	<i>BCAM0227</i>	<i>BCAM0581</i> (+BDSF)
<i>BCAM0495</i>	putative DNA-binding protein	-12.1	-5	-9.5 (1.5)	-6.1 (0.4)	ND ^d
<i>BCAM1265</i>	putative amino acid permease	-2.5	NC ^d	-2.0 (0.3)	ND	-3.1 (0.4)
<i>BCAM1619</i>	putative DNA-binding cold-shock protein	-3.1	-2.5	-2.9 (0.9)	-3.1 (0.3)	ND
<i>BCAM2039</i>	putative transcriptional regulator	-42.5	-33.5	-7.3 (1.1)	-5.2 (0.4)	ND
<i>BCAM2041</i>	type III secretion system protein (BcscR)	-2.2	NC	-2.2 (0.4)	-2.0 (0.5)	ND
<i>BCAM2045</i>	type III secretion system protein (BcscU)	-2.8	-2.3	-2.5 (0.9)	-2.1 (0.7)	ND
<i>BCAM2051</i>	type III secretion system protein (BcscU)	-2.7	-2.1	-2.6 (0.8)	-2.1 (0.9)	ND
<i>BCAM2758</i>	cable pilus (CblS)	-2.1	-2.1	-2.2 (0.5)	-2.1 (0.3)	ND
<i>BCAM2759</i>	cable pilus (CblD)	-2	-2	-2.5 (0.6)	-2.4 (0.9)	ND
<i>BCAL0114</i>	flagellin (FliC)	5.5	5.4	6.3 (1.2)	6.9 (1.8)	ND
<i>BCAL0132</i>	chemotaxis protein methyltransferase (CheR)	2.7	2.2	3.1 (0.9)	2.2 (1.2)	ND
<i>BCAL0813</i>	RNA polymerase factor sigma-54, (RpoN)	-2.5	NC	-2.9 (0.4)	ND	ND
<i>BCAL2820</i>	efflux system outer membrane protein (OprM)	-125	NC	-15.2 (1.6)	-2 (1.1)	ND
<i>BCAS0297</i>	hypothetical protein	-5.3	-2.1	-2.0 (1.2)	-2.3 (0.9)	-3.8 (0.4)
<i>BCAS0299</i>	flp type pilus subunit (PilA)	-2.1	NC	-2.5	ND	ND
<i>BCAS0353</i>	putative aldose 1-epimerase	-2.6	-3	-4.0 (0.2)	-3.5 (0.3)	-2.2 (0.5)
<i>BCAS0387</i>	putative haloacid dehalogenase-like hydrolase	-4.3	NC	-3.1 (0.2)	ND	-2.5 (1.1)

2 a. Gene numbers are from http://www.sanger.ac.uk/Projects/B_cenocepacia. Annotations and functional assignments are from *B. cenocepacia* J2315.

3 b. Change (fold) in *BCAM0581* or *BCAM0227* mutant compared to wild-type determined by microarray analysis.

1 c. Real-time RT-PCR analysis of gene expression after: (i) mutation of *BCAM0581* (ii) mutation of *BCAM0227* and exogenous addition of BDSF to *BCAM0581* mutant.
2 Gene expression is presented as the *x*-fold change relative to expression in *B. cenocepacia* J2315 wild type and was normalized for cDNA content using 16S RNA. Data
3 (means \pm SD) are representative of three independent biological experiments.
4 d. NC: No significant change (< 2 fold) in gene expression compared to wild-type in microarray; ND: no differences seen by qRT-PCR.
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For Peer Review

1 **Table 4.** BDSF signalling is associated with lethality in an agar bead mouse model of
2 infection. Mice were infected intra-tracheally using agar beads with 1×10^9 CFU of *B.*
3 *cenoepectia J2315* wild-type or strains with mutations in *BCAM0581* or *BCAM0227*.
4 Survival of mice was monitored over a period of 8 days. Animals were sacrificed 8
5 days post-infection, and bacterial loads in the lungs and spleen were determined.
6

Strain	No. of mice per group	No. of surviving mice 8 days post-infection	Log CFU per (mean +/- S.D.)	
			Lung	Spleen
J2315	6	3	5.3 (+/- 0.01)	3.3 (+/- 0.05)
<i>BCAM0581</i>	6	6	0.8 (+/- 0.01)	0.2 (+/- 0.01)
<i>BCAM0227</i>	6	6	1.1(+/- 0.01)	0.7 (+/- 0.01)
<i>BCAM0581</i> (pBAM0581)	6	3	6.3 (+/- 0.01)	2.3 (+/- 0.05)
<i>BCAM0227</i> (pBCAM0227)	6	3	5.9 (+/- 0.01)	2.9 (+/- 0.05)

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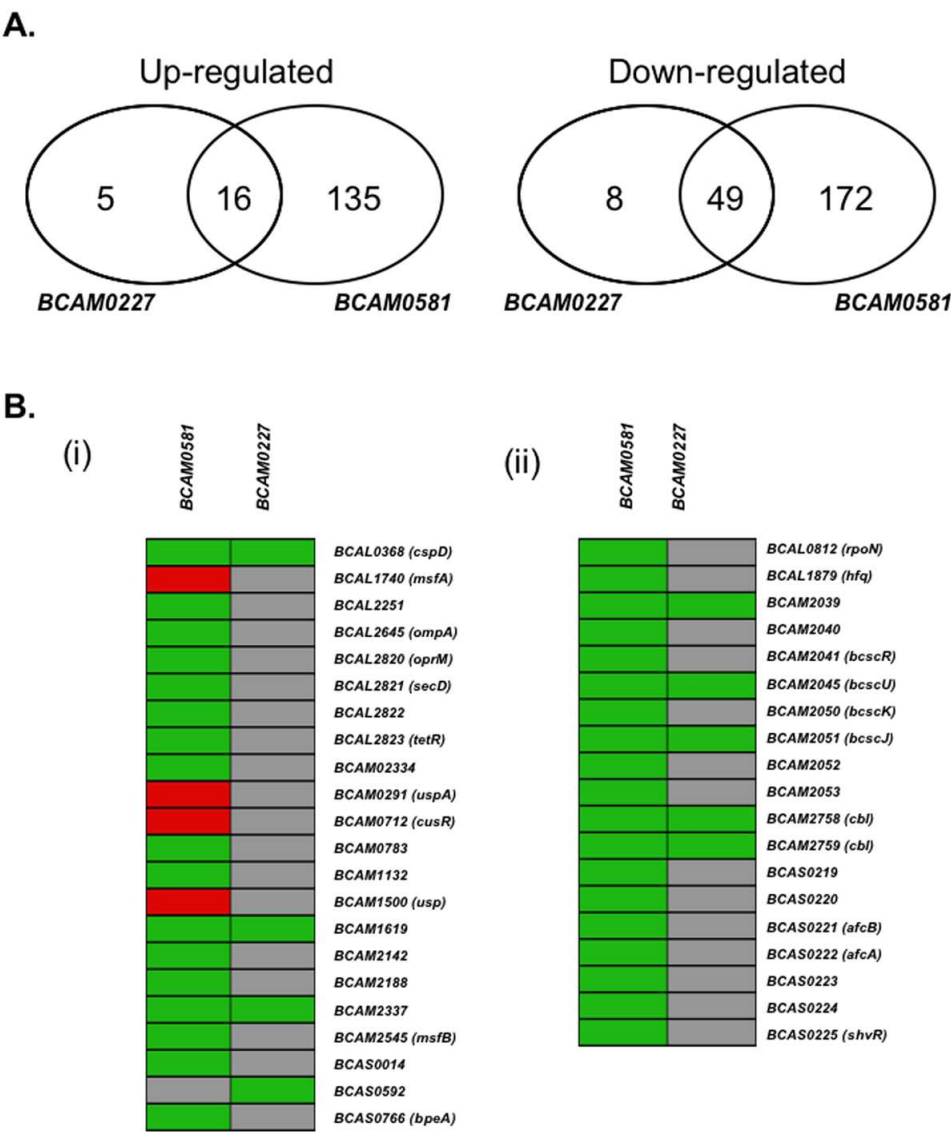


Fig. 1. Genes differentially expressed in BCAM0581 or BCAM0227 mutants compared to the wild-type *B. cenocepacia* J2315 as revealed by microarray analysis.
105x127mm (300 x 300 DPI)

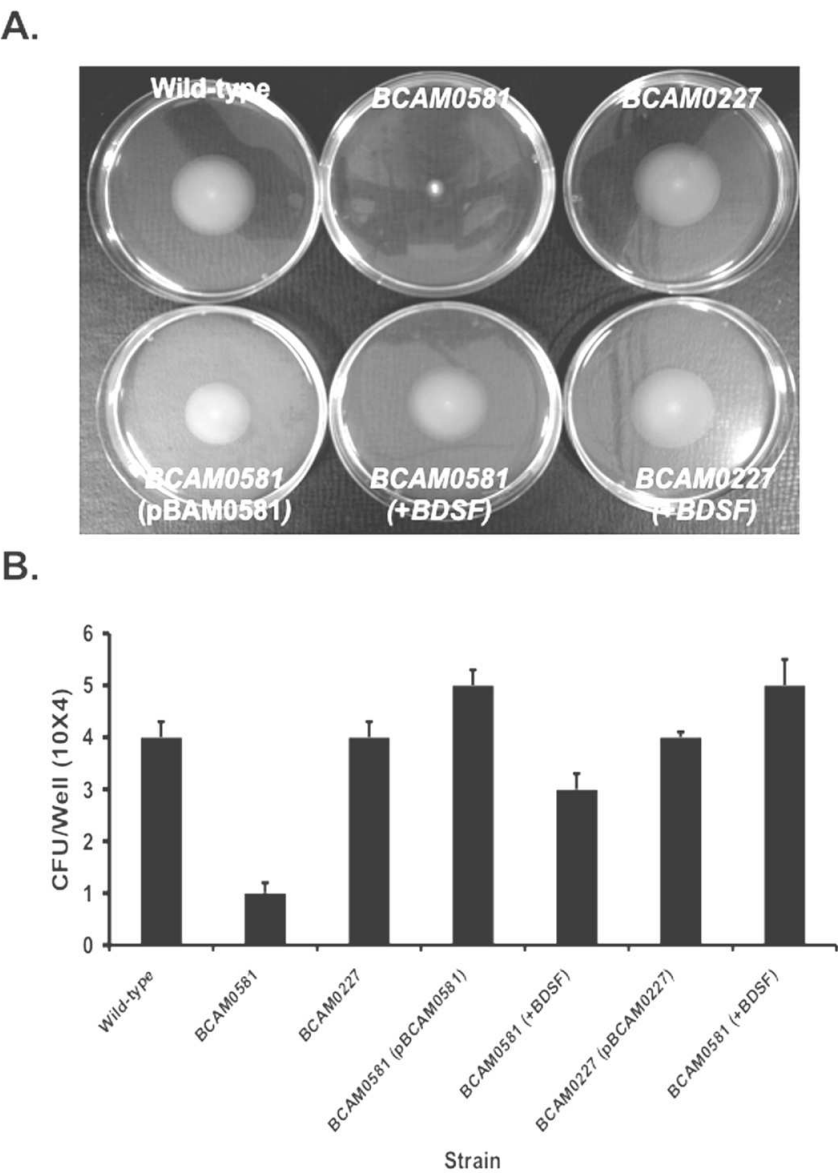


Fig. 2. Mutation of genes encoding BCAM0581 and BCAM0227 does not have the same effect on motility and adherence to porcine mucin.
95x127mm (300 x 300 DPI)

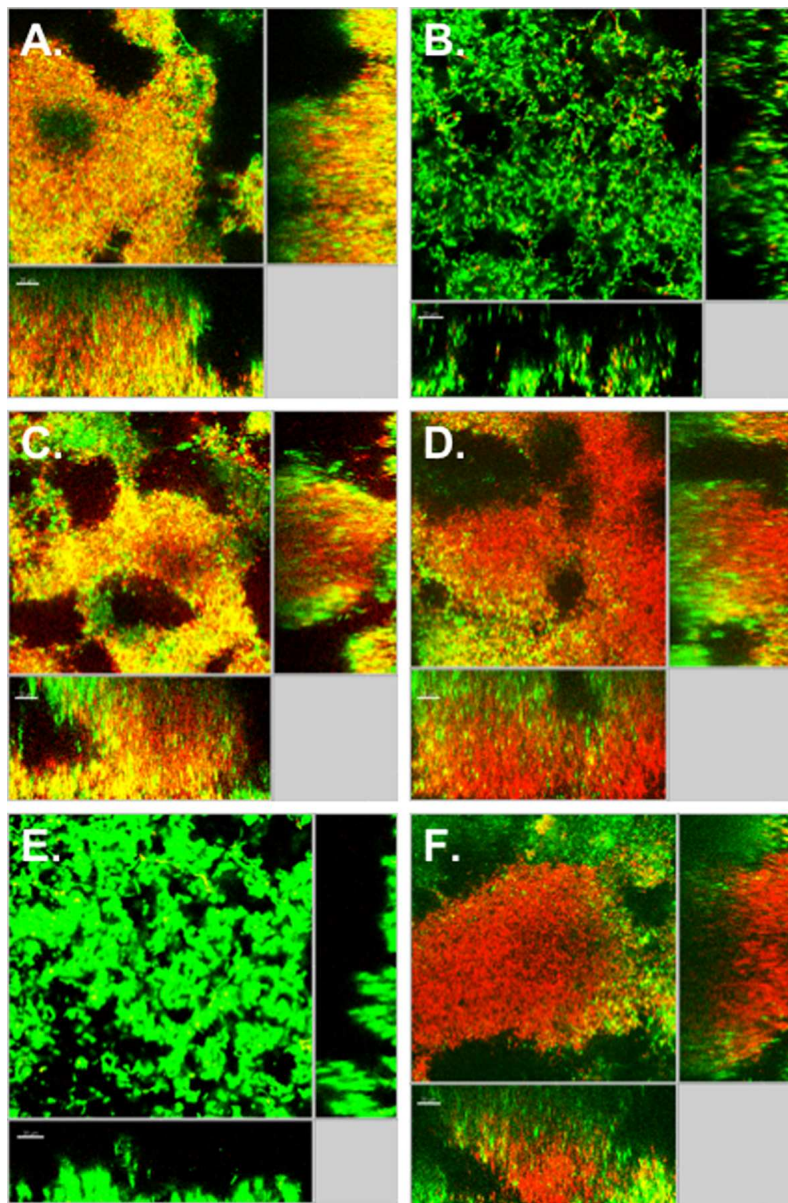
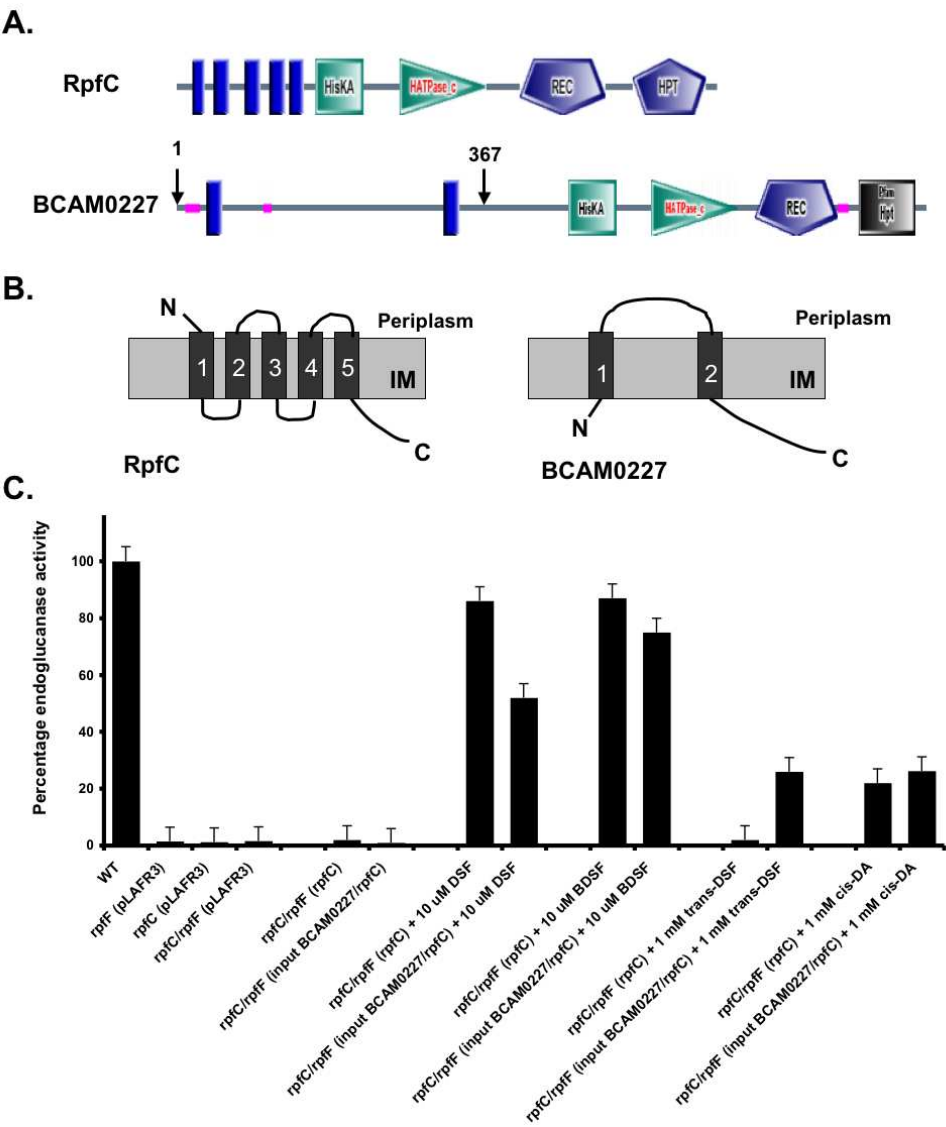


Fig. 3. Mutation of genes encoding BCAM0581 and BCAM0227 has similar effects on biofilm architecture within biofilms of *B. cenocepacia* J2135.
101x152mm (300 x 300 DPI)



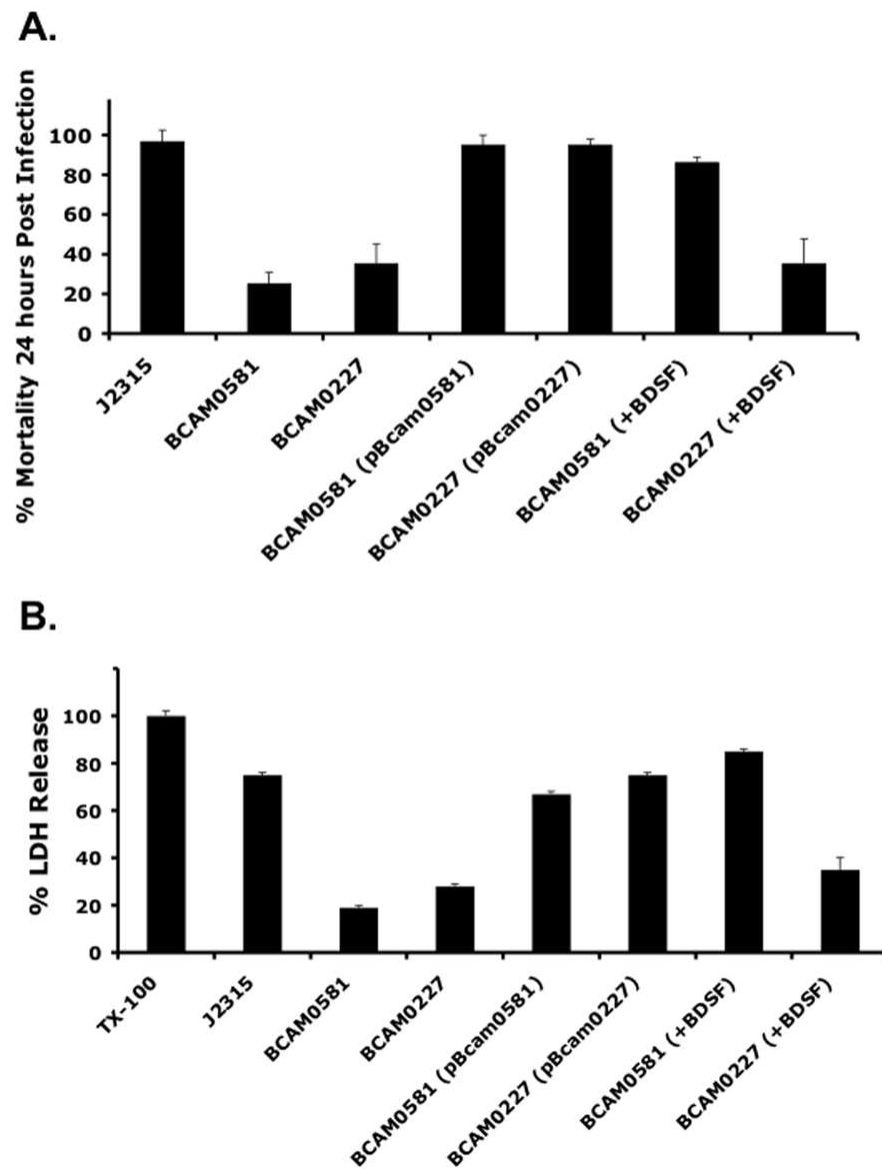


Fig. 5. BCAM0227 and BCAM0581 contribute to cytotoxicity and virulence by *Burkholderia cenocepacia* J2315
76x101mm (300 x 300 DPI)