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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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Complete List of Authors:	<p>McCarthy, Yvonne; National University of Ireland, Department of Microbiology          Yang, Liang; Technical University of Denmark, DTU-Biosys          Twomey, Kate; National University of Ireland, Department of Microbiology          Sass, Andrea; Cardiff School of Biosciences, Microbiology          Tolker-Nielsen, Tim; University of Copenhagen, Department of International Health, Immunology and Microbiology.          Mahenthiralingam, Eshwar; Cardiff School of Biosciences, Microbiology          Dow, J. Maxwell; National University of Ireland, Department of Microbiology          Ryan, Robert; National University of Ireland, Department of Microbiology</p>
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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

4 Yvonne McCarthy<sup>1</sup>, Liang Yang<sup>2</sup>, Kate B. Twomey<sup>1</sup>, Andrea Sass<sup>3</sup>, Tim Tolker-  
5 Nielsen<sup>4</sup>, Eshwar Mahenthiralingam<sup>3</sup>, J. Maxwell Dow<sup>1</sup> and Robert P. Ryan<sup>1\*</sup>

6

7 <sup>1</sup>BIOMERIT Research Centre, Department of Microbiology, Biosciences Institute,  
8 University College Cork, Cork, Ireland.

9

10 <sup>2</sup>Centre for Biomedical Microbiology, BioCentrum-DTU, Technical University of  
11 Denmark, DK-2800 Lyngby, Denmark.

12

13 <sup>3</sup>Cardiff School of Biosciences, Cardiff University, Cardiff.

14

15 <sup>4</sup>Department of International Health, Immunology and Microbiology. Faculty of  
16 Health Sciences. University of Copenhagen, Copenhagen, Denmark.

17

18

19

20 **\*Corresponding author:** R.P.Ryan, BIOMERIT Research Centre, Department of  
21 Microbiology, Biosciences Institute, University College Cork, Cork, Ireland

22 **Tel. No:** +353-21-4901316; **Fax. No:** +353-21-4275934; **E-mail:** r.ryan@ucc.ie

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

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1 **SUMMARY**

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

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## 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; Mahenthalingam *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

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

## 8 RESULTS

### 9 Identification of transposon mutants that have altered expression of BDSF 10 regulated genes

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

21  
22 The sites of transposon insertion for non-responsive mutants were mapped to the  
23 genome of *B. cenocepacia* J2315 by inverse PCR and sequencing of amplicons (see  
24 Experimental procedures). Southern blot analysis confirmed that only a single copy of  
25 the transposon was inserted into the chromosome in these non-responsive mutants  
26 (data not shown). Insertion sites, which were all different, were mapped to 20 genetic  
27 loci with 9 genes found more than once (Table 1). None of the mutants showed  
28 impaired growth in complex or minimal media, suggesting that the altered response to  
29 BDSF could not be attributed to differences in growth.

30  
31 One insertion was mapped to *BCAM2758* (*cbiS*), a known positive regulatory gene of  
32 *cbiD* (Tomich *et al.*, 2003; Tomich and Mohr, 2003). In addition, mutations in several  
33 genes encoding enzymes involved in metabolic processes (such as *BCAM1685*,  
34 *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 *cbiD* 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 *cenoepectia* J2315 mutants using a custom microarray (Drevinek *et al.*, 2008). In the  
15 first set of experiments, the transcriptomes of the wild-type *B. cenoepectia* 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*, *cbiS* (*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  $\mu$ M BDSF (Fig. S1A).  
17 However for *BCAM0495*, *BCAS0297*, *BCAS0353* and *BCAS0387*, addition of BDSF  
18 up to a concentration of 50  $\mu$ 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,

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

3

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

18

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

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

29

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

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

15  
16 This induction of endoglucanase was specific to BDSF or DSF. A range of  
17 compounds of related structure, including *trans*-11-methyl dodecenoic acid, *cis*-2-  
18 decenoic acid, 11-methyl dodecanoic acid and farnesoic acid showed little or no  
19 activity when tested at concentrations of 50  $\mu$ M, although some activity was seen at 1  
20 mM (Fig. 4C). Importantly we have shown previously that chimaeric proteins in  
21 which the input domain of unrelated sensors are fused to RpfC have no activity in  
22 sensing either DSF (Ryan *et al.*, 2008) or BDSF (data not shown). Taken together,  
23 these observations support the contention that BCAM0227 has a direct role in BDSF  
24 perception.

25

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

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

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

17

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

33

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

12

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

19

20

## 21 **DISCUSSION**

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

32

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

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

6

### 7 ***The extent of the BDSF regulon***

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

28

29 A number of laboratories have proposed criteria to define signalling molecules  
30 involved in intraspecies communication (Monds and O'Toole, 2008; Winzer *et al.*,  
31 2002). Such signals should be produced during specific stages of growth, under  
32 certain physiological conditions, or in response to changes in the environment; the  
33 signal should accumulate extracellularly and be recognised by a specific receptor;  
34 accumulation of the signal should generate a concerted response, once a critical

1 threshold concentration has been reached; most importantly, the cellular response to  
2 the signal should extend beyond physiological changes required to metabolize or  
3 detoxify it. The work shown here and previously (Deng *et al.*, 2009; Ryan *et al.*,  
4 2009) indicates that BDSF satisfies all of these criteria and thus can be considered a  
5 true cell-cell signal molecule.

6

7 It is established that *B. cenocepacia* also utilises *N*-AHLs as intracellular signals to  
8 regulate gene expression through the two LuxR family proteins CepR and CciR  
9 (O'Grady *et al.*, 2009). This raises the issue as to the extent to which the regulatory  
10 influences of the two classes of signal overlap. Comparison of the BDSF regulon  
11 described here with those genes whose expression is regulated by CepR and CciR  
12 (O'Grady *et al.*, 2009) indicates only a partial regulatory overlap (Fig. S3).

13 Furthermore, we have previously shown that mutation of *BCAM0581* does not  
14 influence the levels of *N*-AHLs produced by *B. cenocepacia* J2315 (Ryan *et al.*, 2009)  
15 and the transcriptome profiling of O'Grady and colleagues (O'Grady *et al.*, 2009),  
16 which was done in the K56-2 strain, does not indicate any influence of CepR and  
17 CciR on expression of *BCAM0581*.

18

### 19 ***BCAM0227 is a novel sensor for DSF-family signals***

20 The findings from the domain-swapping experiments provide direct evidence for a  
21 role for *BCAM0227* in the perception of BDSF. This contention is supported by the  
22 observation that *BCAM0227* is required for the induction of *cbiD* gene expression in  
23 response to exogenously added BDSF. Furthermore *BCAM0227* regulates a sub-set  
24 of genes under BDSF control but does not regulate expression of *BCAM0581* and  
25 shows no alteration in BDSF levels (data not shown), consistent with a role in signal  
26 perception rather than signal generation. The sensory domain of *BCAM0227* is  
27 however quite different from that of RpfC, the DSF sensor in xanthomonads.

28 Bioinformatics analysis predicts that RpfC has 5 transmembrane helices with  
29 periplasmic and cytoplasmic loops of less than 20 amino acids. In contrast,  
30 *BCAM0227* is predicted to have 2 transmembrane helices and a large periplasmic  
31 loop of 300 amino acids (Fig. 4). The precise mechanism by which either sensor  
32 recognises BDSF or DSF remains to be determined.

33

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

10

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

27

### 28 ***The broader relevance of findings***

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

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

16

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

25

## 26 **EXPERIMENTAL PROCEDURES**

### 27 **Bacterial strains, growth conditions and media.**

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

1

**2 DNA manipulations.**

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

9

**10 Construction of chromosomal reporter fusions.**

11 The protocol used for the construction of the *lux* reporter fusion for *cbID* was a  
12 modification of the RED recombination protocols previously described by (Gerlach *et*  
13 *al.*, 2007; Lesic and Rahme, 2008). The *cbID* gene was amplified by PCR using  
14 forward and reverse primers *cbID*-F  
15 (5'GGATCCAAGCGCCAGCAGGACGTGCTGATG-3') and *cbID*-R (5'GGATCC  
16 GAGACTGGTGGACGACGGC-3') to include the region 250 bp upstream of the  
17 gene and was cloned into pGLuc. The *cbID::lux* fusion removed by restriction digest  
18 using *Hind*III and *Xba*I and cloned into pME6032-RedS cut with the same enzymes.  
19 This construct was electroporated into *B. cenocepacia* wild-type or *BCAM0581*  
20 mutant. Transconjugants were grown in LB to an optical density at 600 nm of 0.4 at  
21 37°C and recombination was induced by addition of 0.2% L-arabinose for 1.5 h. The  
22 correct integration of the reporter cassette was confirmed by colony PCR using a  
23 reverse primer specific to the reporter and a forward primer specific to the upstream  
24 region of the target gene.

25

**26 Transposon mutagenesis and screening.**

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

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

8

### 9 **Identification of transposon insertion sites.**

10 Genomic DNA was extracted from transposon mutants of interest as previously  
11 described (Sambrook *et al.*, 1989). The nucleotide sequence flanking the transposon  
12 insertion site was amplified by PCR as previously described (Friedman and Kolter,  
13 2004) using the primers: Rnd1-TnM (5'-GTGAGCGGATAACAATTTTCACACAG-  
14 3'), and Rnd2-TNm (5'-ACAGGAAACAGGACTCTAGAGG-3'). Amplicons were  
15 sequenced using the TnM specific primer: TnMseq (5'-  
16 CACCCAGCTTTCTTGACAC-3'). Sequence data generated was examined with  
17 BLASTn, BLASTx ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)), and VGE BLAST  
18 ([www.vge.ac.uk/BLAST/](http://www.vge.ac.uk/BLAST/)) software. Bioinformatic analysis of the BCAM0227 sensor  
19 kinase was performed using SMART (<http://smart.embl-heidelberg.de/>).

20

### 21 **Cloning and expression of input-BCAM0227-RpfC chimeric protein.**

22 For the construction of BCAM0227-RpfC chimeric sensor protein, the coding  
23 sequences for the input domain of BCAM0227 modules (amino acid residues 1 -383)  
24 and N-terminal of RpfC (residues 249 - 726) were amplified using primers  
25 InBCAM0227-F (5'-GGATCCATGGGGGCACCCTGGCCGACCGC-3') and  
26 BCAM0227-R (5'-AAGGCTTGCGCACCAGCAGCCACCACAC-3'); RpfCCTerm-  
27 F (5'-AAGGCTTGCGATGACCCGTGCAGTGCGC-3') and RpfCCTerm-R (5'-  
28 GGATCCCTATTCGTTGCTACGGGGGGA-3') respectively. These primers contain  
29 restriction sites for *Hind*III and *Bam*HI (underlined). These amplified products were  
30 diluted, mixed and subjected to further PCR amplification using the primers F (5'-  
31 GGATCCATGGGGGCACCCTGGCCGACCGC-3') and R (5'-  
32 AAGCTTCTATTCGTTGCTACGGGGGGA-3'). The final amplicon was cloned into  
33 TOPO (Invitrogen™) and verified by sequencing. The product was excised from  
34 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**rpfF* 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

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

11

### 12 **Microarray data analysis.**

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

20

### 21 **Quantitative RT-PCR.**

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

28

### 29 **Antimicrobial susceptibility testing.**

30 Susceptibility experiments were carried out as previously described by (Hassett *et al.*,  
31 1999) with the following modifications. Selected bacterial strains were grown shaking  
32 aerobically over night at 37°C. For sensitivity experiments for most compounds were  
33 carried out by adding a 0.1 ml suspension of stationary phase bacteria spread over the  
34 entire surface of LB agar plates. After 17 h of growth at 37°C, the stationary phase

1 organisms were removed from the agar surface with 10 ml of 0.9% saline and a sterile  
2 glass rod. Filter paper disks impregnated with various concentrations of selected  
3 compounds were placed on the agar surface. For H<sub>2</sub>O<sub>2</sub> and NaOCl sensitivity  
4 experiments, suspensions (0.1 ml) were diluted in 3 ml of molten soft agar (0.6%),  
5 poured onto the surface of L agar plates, and the agar was allowed to solidify. Filter  
6 paper disks (7 mm, Whatmann) saturated with 10 ml of 30% H<sub>2</sub>O<sub>2</sub> were placed on the  
7 agar overlay. Sensitivity to each compound was then recorded as the mean  $\pm$  SE  
8 ( $n = 9$ ) of the diameter of growth inhibition after 24 h of growth at 37°C. The results  
9 represent the average of three independent replicas.

10

### 11 **Cultivation and imaging of biofilms in flow chambers.**

12 Biofilms were grown in flow chambers with individual channel dimensions of  
13 1  $\times$  4  $\times$  40 mm. The flow system was assembled and prepared as described by  
14 (Sternberg and Tolker-Nielsen, 2006). The flow chambers were inoculated by  
15 injecting 350  $\mu$ l of overnight culture diluted to an OD<sub>600</sub> of 0.01 into each flow  
16 channel with a syringe. After inoculation, flow channels were left without flow for  
17 1 h, after which medium flow at a linear rate of 0.2 mm/s was started using a Watson  
18 Marlow 205S peristaltic pump. All microscopic observations and image acquisitions  
19 were performed on an LSM510 confocal laser scanning microscope (CLSM) (Carl  
20 Zeiss™) equipped with detectors and filter sets for monitoring Syto9 fluorescence.  
21 Simulated three-dimensional images, shadow projections and sections were generated  
22 using the Imaris™ software package.

23

### 24 **Cytotoxicity assay.**

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

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

#### 4 5 ***G. mellonella* killing assays.**

6 Larvae were stored in wood chips at 4°C. A 10- $\mu$ l Hamilton syringe was used to inject  
7 5- $\mu$ l aliquots of bacterial suspensions into *G. mellonella* via the hindmost left proleg.  
8 10-fold serial dilutions of bacteria starting at  $2 \times 10^8$  CFU per ml were made in PBS  
9 supplemented with 100  $\mu$ g/ml of ampicillin. 5  $\mu$ l of each dilution was injected into  
10 each of ten larvae. Control larvae were injected with 5  $\mu$ l of PBS plus 100  $\mu$ g/ml of  
11 ampicillin. Following injection, larvae were placed in a static incubator in the dark at  
12 30°C, the optimum temperature for insect growth and development. Larvae were  
13 scored as dead or alive at 48 and 72 h post-infection. The minimum inoculum  
14 required to kill all 10 larvae was determined for each strain.

#### 15 16 **Mouse infection model.**

17 C57Bl 6 mice were infected with *B. cenocepacia* strains by using the agar bead model  
18 of lung infection (Bylund *et al.*, 2006; Bylund *et al.*, 2005; Cieri *et al.*, 2002). Bacteria  
19 were incorporated into agar beads, and  $1 \times 10^9$  CFU were inoculated into each mouse  
20 intra-tracheally. The animals were sacrificed after 8 days post-infection, and the lungs  
21 and spleens were harvested, homogenized, and cultured on LB agar to assess the  
22 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.

15

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*.

20

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.

24

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  $1 \times 10^9$  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.

30

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.

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

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

19

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

30

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

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

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

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

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## 6 **SUPPORTING INFORMATION**

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

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

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

16

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

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19 Fig. S1. (A) Real-time RT-PCR analysis of the effects of mutation of *BCAM0581*  
20 grown in LB (OD<sub>600</sub> 0.8) on transcript levels for *BCAM0495*, *BCAM1265*,  
21 *BCAM1619*, *BCAM2039*, *BCAM2041*, *BCAM2045*, *BCAM2051*, *BCAM2759*,  
22 *BCAL0114*, *BCAL0813*, *BCAS0297*, *BCAS0299*, *BCAS0353*, *BCAS0387* and  
23 *BCAS0230*. Data were normalized to 16S RNA and each is presented as the fold  
24 change with respect to the wild-type *B. cenocepacia* J2315 for each gene. Data  
25 (means  $\pm$  SD) are representative of four independent biological experiments. White  
26 columns: effects of mutation of *BCAM0581*; Grey columns: effects of  
27 complementation of *BCAM0581* mutation; Black columns: effects of addition of  
28 100 nM exogenous BDSF. (B) Real-time RT-PCR analysis of the effects of mutation  
29 of *BCAM0227* grown in LB (OD<sub>600</sub> 0.8) on transcript levels for *BCAM0495*,  
30 *BCAM1265*, *BCAM1619*, *BCAM2039*, *BCAM2041*, *BCAM2045*, *BCAM2051*,  
31 *BCAM2759*, *BCAL0114*, *BCAL0813*, *BCAS0297*, *BCAS0299*, *BCAS0353*, *BCAS0387*  
32 and *BCAS0230*. Data were normalized to 16S RNA and each is presented as the fold  
33 change with respect to the wild-type *B. cenocepacia* J2315 for each gene. Data  
34 (means  $\pm$  SD) are representative of four independent biological experiments. White

1 columns: effects of mutation of *BCAM0227*; Grey columns: effects of  
2 complementation of *BCAM0227* mutation; Black columns: effects of addition of  
3 100 nM exogenous BDSF.

4  
5 Fig. S2. Domain swapping experiments between *BCAM0227* and RpfC indicate that  
6 *BCAM0227* is a sensor of BDSF. These experiments were conducted by expression  
7 of chimeric sensors in the *rpfFrpfC* double mutant of *Xcc* and measurement of the  
8 effects of addition of exogenous BDSF on the DSF-regulated phenotype of  
9 endoglucanase production. Expression of RpfC in this mutant background has no  
10 effect on endoglucanase activity (which is very low), but endoglucanase activity was  
11 increased upon addition of exogenous BDSF at 50  $\mu$ M. Similar effects are also seen  
12 after expression of a chimeric sensor protein in which the input domain and HisKA  
13 domain of *BCAM0227* replaces the input domain and HisKA domain of RpfC  
14 generating chimeric sensor protein *BCAM0227* (residues 1-594)- RpfC (residues  
15 253–726); the *rpfFrpfC* strain expressing this chimeric protein can recognize  
16 synthetic BDSF at 50  $\mu$ M to induce endoglucanase activity. In contrast, addition of  
17 *trans*-11-methyl-2-dodecenoic acid and *cis*-2-decenoic acid (*cis*-DA) at 50  $\mu$ M did  
18 not induce endoglucanase production in this strain, although some effects were seen at  
19 the higher concentration of 1 mM.

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

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2 **Table 1.** Location of transposon insertions found in the *Burkholderia cenocepacia*

3 J2315 genome leading to lack of responsiveness to exogenous BDSF.

Disrupted gene <sup>a</sup>	Polar effect possible? <sup>b</sup>	Number of different transposon mutants <sup>c</sup>	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.

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b. Possibility of Tn5 insertion affecting expression of other (downstream) genes. Y: yes; N: no.

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c. Number of different Tn5 insertion mutations mapped to the same gene.

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

Class	Gene name or ID <sup>a</sup>	Fold changes <sup>b</sup>	
		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 <sup>c</sup>
	<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
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
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
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
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
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
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
	BCAM2025	-2.2	NC
	BCAM0238	-3.2	NC
	<i>tetR</i> (BCAL2823)	-2.1	NC
<hr/>			
Hypothetical proteins and others	BCAL0112	2.7	2.4
	BCAM0634	NC	3
	BCAM2024	3.4	2.4
	BCAM2377	4.1	2.7
	BCAL0108	-4	-3.5
	BCAS0269	-2	-2.1
	BCAS0297	NC	-2

- 1 a. Gene numbers are from [http://www.sanger.ac.uk/Projects/B\\_cenocepacia](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.

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

Gene name <sup>a</sup>	Gene product and function <sup>a</sup>	Microarray gene expression relative to wild-type <sup>b</sup>		RT-qPCR gene expression analysis relative to wild-type <sup>c</sup>		
		<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 <sup>d</sup>
<i>BCAM1265</i>	putative amino acid permease	-2.5	NC <sup>d</sup>	-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 (CbIS)	-2.1	-2.1	-2.2 (0.5)	-2.1 (0.3)	ND
<i>BCAM2759</i>	cable pilus (CbID)	-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](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  $1 \times 10^9$  CFU of *B.*  
 3 *cenozoepacia 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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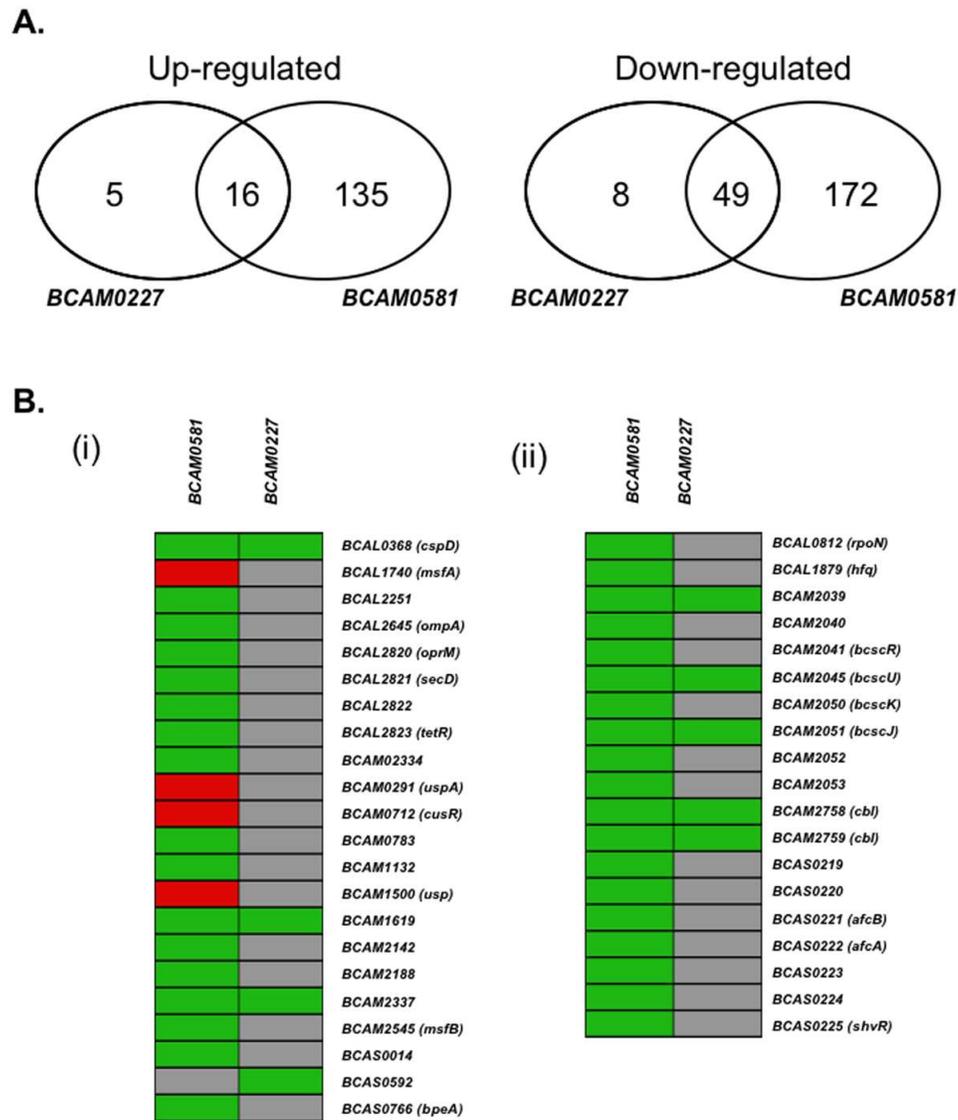
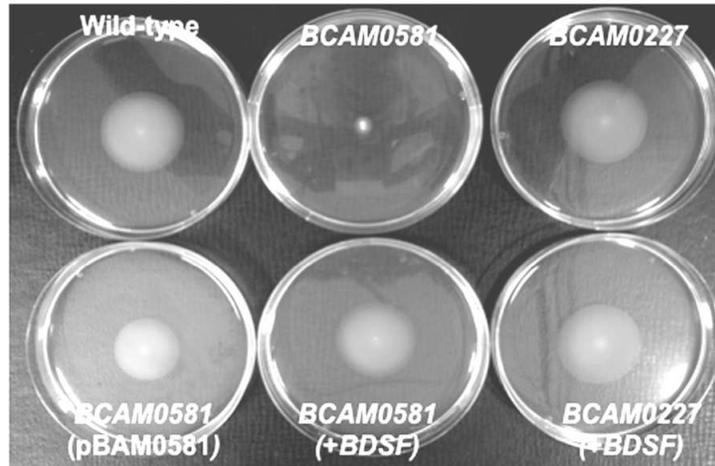


Fig. 1. Genes differentially expressed in BCAM0581 or BCAM0227 mutants compared to the wild-type *B. cenocepacia* J2315 as revealed by microarray analysis.  
105x127mm (300 x 300 DPI)

A.



B.

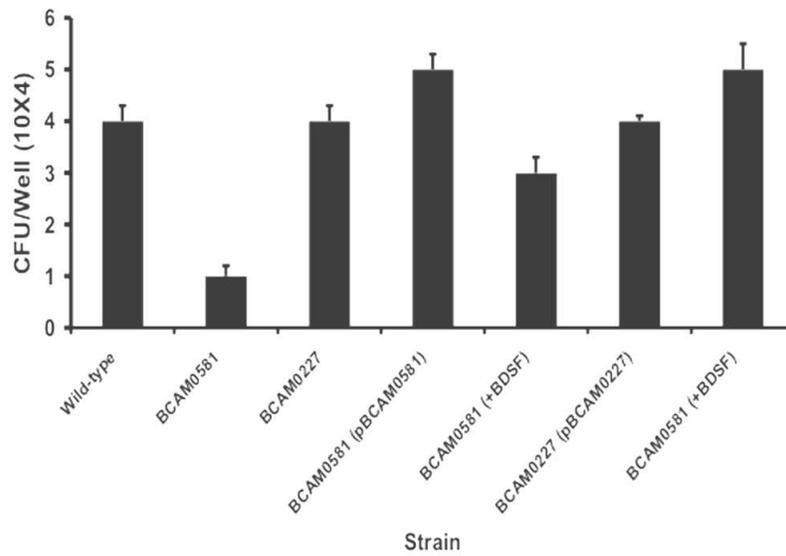


Fig. 2. Mutation of genes encoding BCAM0581 and BCAM0227 does not have the same effect on motility and adherence to porcine mucin.  
95x127mm (300 x 300 DPI)

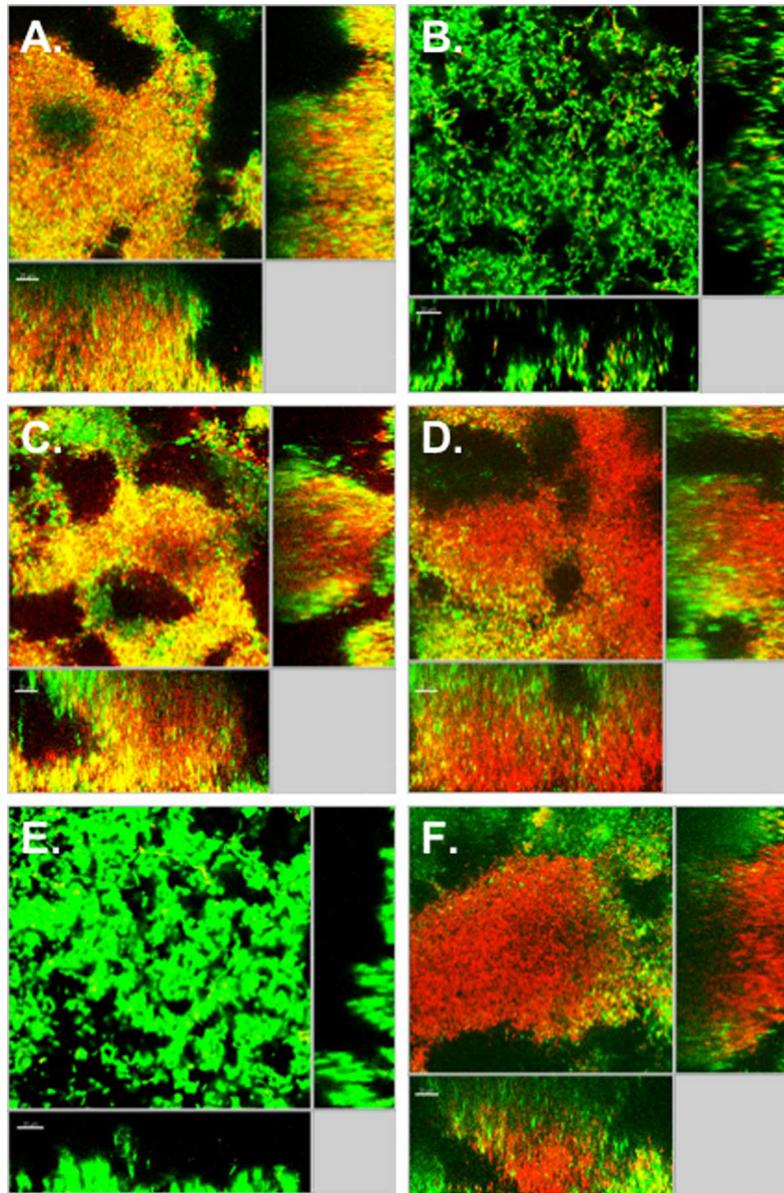
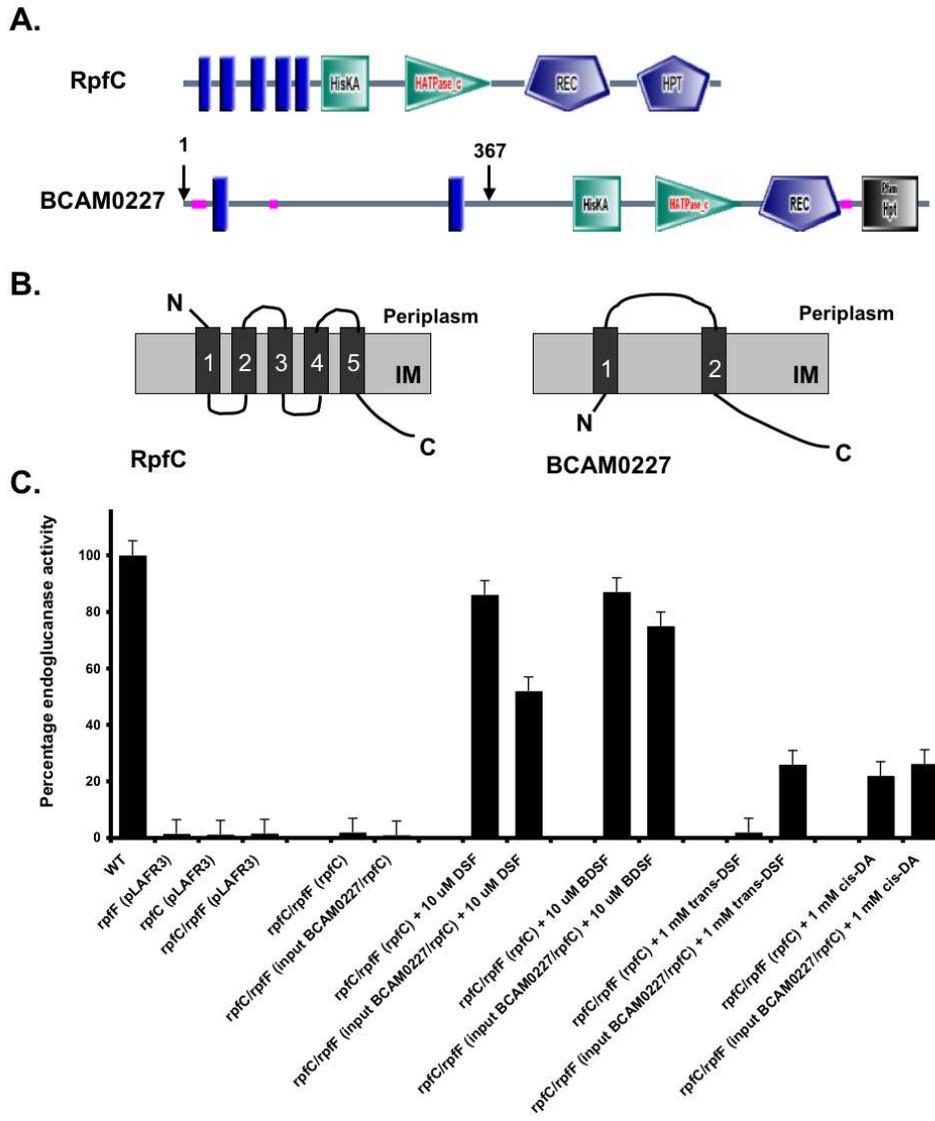


Fig. 3. Mutation of genes encoding BCAM0581 and BCAM0227 has similar effects on biofilm architecture within biofilms of *B. cenocepacia* J2135.  
101x152mm (300 x 300 DPI)



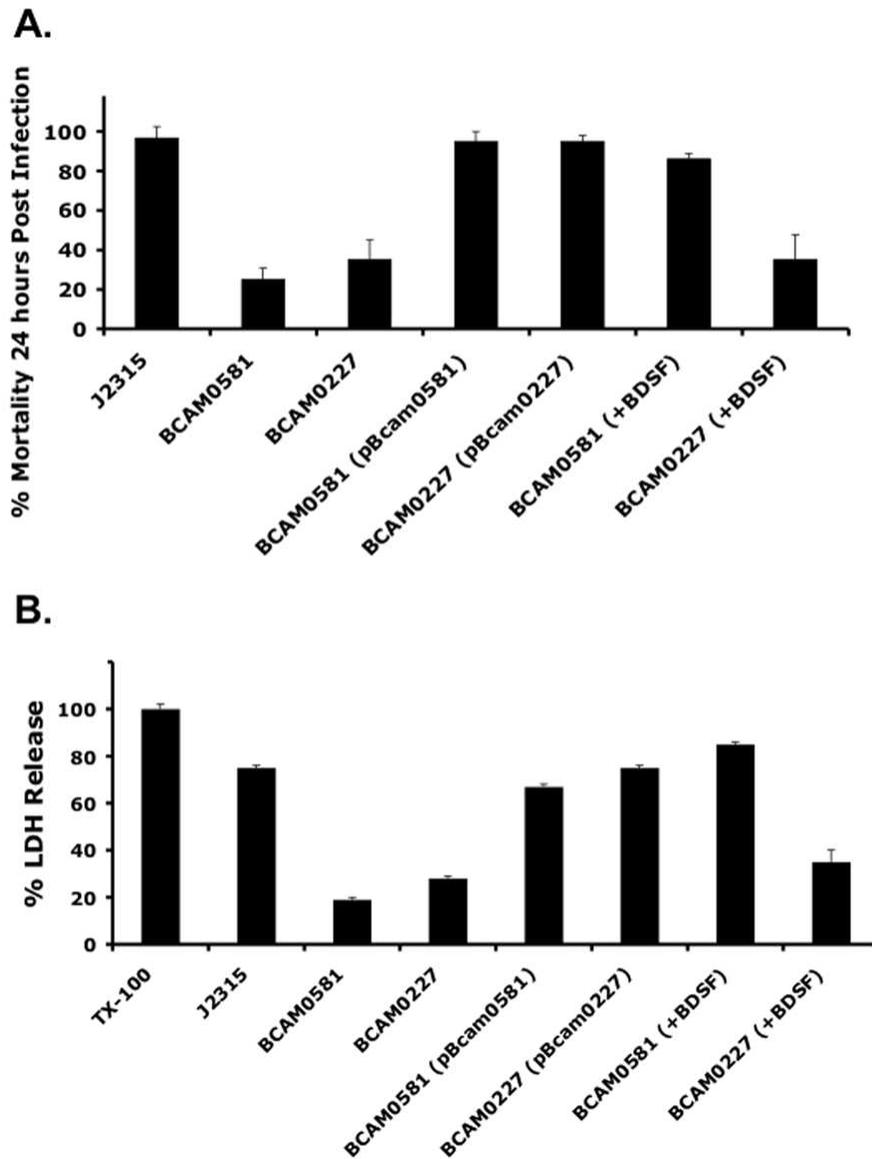


Fig. 5. BCAM0227 and BCAM0581 contribute to cytotoxicity and virulence by *Burkholderia cenocepacia* J2135  
76x101mm (300 x 300 DPI)