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Evidence for the widespread production of DSF family signal molecules by members of the genus *Burkholderia* by the aid of novel biosensors

Angela Suppiger, Claudio Aguilar and Leo Eberl*
Department of Microbiology, Institute of Plant Biology, University of Zurich, Zurich, Switzerland

Summary

Many bacteria employ *cis*-2-unsaturated fatty acids, referred to as DSF (diffusible signal factor) family signals, to communicate with each other. Such systems have been shown to control biofilm formation, motility, production of hydrolytic enzymes and expression of virulence factors. We report the construction of novel biosensors on the basis of components of the *Burkholderia*-DSF (BDSF) dependent circuitry of *Burkholderia cenocepacia* H111 and evaluated their utility for detecting the production of DSF family signal molecules. We show that a *luxAB*-based biosensor responds to nM levels of synthetic BDSF and is suitable to detect a wide range of *cis*-2 fatty acid molecules. Using this biosensor we show that the production of DSF family molecules is widespread among members of the *B. cepacia* complex and demonstrate for the first time that DSF-based molecules are also produced by plant-associated *Burkholderia* species.

Introduction

Evidence has accumulated that many social behaviours of bacteria, including biofilm formation and the production of public goods, are regulated by quorum sensing (QS) (Fuqua *et al*., 1994; Waters and Bassler, 2005; Galloway *et al*., 2011). Quorum sensing is a regulatory mechanism that allows bacteria to coordinate gene expression in a cell density-dependent manner. Such systems rely on the constitutive release of small diffusible signal molecules that accumulate in the environment. When a critical threshold concentration of the signal molecule is reached it interacts with a cognate receptor protein, resulting in the activation or repression of target genes (Whitehead *et al*., 2001; Fuqua and Greenberg, 2002; Zhang and Dong, 2004). The most widespread signal molecules produced by Gram-negative bacteria belong to the family of *N*-acylhomoserine lactones (AHLs) (Whitehead *et al*., 2001). However, many other structurally unrelated molecules have been identified that can serve as QS signals (Ryan and Dow, 2008; Deng *et al*., 2011; Galloway *et al*., 2011). Among these molecules are the *cis*-2-unsaturated fatty acids, often referred to as DSF (diffusible signal factor) family signals (Deng *et al*., 2011). The first molecule of the DSF family, *cis*-11-methyl-2-dodecenoic acid, was identified in supernatants of the phytopathogen *Xanthomonas campestris* pv. *campestris* (Xcc) (Barber *et al*., 1997). Subsequently, fatty acid-based QS systems were also identified and characterized in members of the genera *Xylella*, *Burkholderia, Stenotrophomonas* and *Pseudomonas* (Fouhy *et al*., 2007; Boon *et al*., 2008; Davies and Marques, 2009; Beaulieu *et al*., 2013). These DSF-dependent QS systems have been shown to control the production of virulence factors and are thus essential for full pathogenicity of these organisms (Deng *et al*., 2011).

Many strains belonging to the genus *Burkholderia* produce both AHL and DSF family molecules, which operate in parallel to regulate specific as well as overlapping sets of genes (Schmid *et al*., 2012). Initial work showed that *Burkholderia cenocepacia* produces *cis*-2-dodecenoic acid, which was named BDSF (*Burkholderia* diffusible signal factor) (Boon *et al*., 2008). *Burkholderia* diffusible signal factor is synthesized by the enoyl-CoA hydratase $RpfF_{Bc}$ (Bi *et al*., 2012) and is sensed by the receptor protein RpfR, which contains Per/Arnt/Sim (PAS)-GGDEF-EAL domains (Deng *et al*., 2012). Upon binding of BDSF to the PAS domain, the c-di-GMP phosphodiesterase activity of RpfR is stimulated, which in turn lowers the intracellular c-di-GMP level. Global analyses of the *B. cenocepacia* H111 $RpfF_{Bc}$ regulon identified several BDSF-regulated genes, including the lectin operon *bclACB* and the large surface protein *bapA*. However, many of the identified genes were found to be maximally expressed only when both BDSF- and AHL-dependent QS systems are active (Deng *et al*., 2012; Schmid *et al*., 2012). Homologs of $RpfF_{Bc}$ and RpfR could be identified not only in many *Burkholderia* species, but also in strains belonging to the genera *Achromobacter*,

Received 13 July, 2015; revised 8 October, 2015; accepted 17 October, 2015. *For correspondence. E-mail leberl@botinst.uzh.ch; Tel. +41-44-634-8220; Fax +41-44-634-8204.

Yersinia, *Serratia*, *Enterobacter* and *Cronobacter*, suggesting that fatty acid-based signalling as described for *B. cenocepacia* may be even more widespread than anticipated (Deng *et al*., 2012). In order to detect DSF family molecules, bioassays have been developed that are based on the restoration of endoglucanase production of a *rpfF* mutant of Xcc (strain 8523) or the induction of an *engXCA*::*gfp* or an *engXCA*::*gusA* gene fusion (Barber *et al*., 1997; Slater *et al*., 2000; Newman *et al*., 2004; Wang *et al*., 2004). The latter system was shown to be capable of detecting 0.5 μM DSF or 60 μM BDSF (Wang *et al*., 2004).

Here, we describe the construction and characterization of novel *luxAB*- and (green fluorescent protein) *gfp*-based biosensors that respond to various *cis*-2-unsaturated fatty acids. The *luxAB* bioluminescent biosensor was found to be highly sensitive, detecting concentrations of BDSF as low as 10 nM. We used this novel tool to screen various *Burkholderia* strains for the production of *cis*-2 fatty acid signal molecules and show that its biosynthesis is particularly widespread among members of the *B. cepacia* complex (Bcc). We also show for the first time that DSF-based molecules are also produced by plant-associated *Burkholderia* species.

Results

Construction of a luxAB*-based biosensor for the detection of DSF family signal molecules*

In a recent study we have demonstrated that the *bclACB* lectin operon (l35_4184-82) is strongly BDSF-regulated, while the AHL-dependent CepIR system only marginally affects expression of this operon (Schmid *et al*., 2012). Using the promoter region of the *bclACB* operon we engineered the sensor plasmid pAN-L15, on which the BDSF-dependent promoter drives the expression of the *luxAB* genes (Material and Methods S1). The sensor plasmid has a broad host range and can easily be transferred to different bacteria via conjugation (Kovach *et al*., 1995). To test the suitability of the sensor plasmids, we introduced it into two different *B. cenocepacia* H111 genetic backgrounds: the *rpfF*$_{Bc}$ mutant H111-rpfF$_{Bc}$ and the *cepI rpfF*$_{Bc}$ double mutant H111ΔcepI rpfF$_{Bc}$. After addition of BDSF to the growth medium, a significantly stronger induction response was observed when the sensor plasmid was in the H111-rpfF$_{Bc}$ background relative to the double mutant (Fig. 1). Expectedly, no activation of the sensor was observed in an *rpfR* mutant background (Fig. S1). This is in agreement with our previous finding that for maximum expression of *bclACB*, both the AHL- and the BDSF-dependent QS system have to be intact (Schmid *et al*., 2012). We therefore used the H111-rpfF$_{Bc}$ genetic background for subsequent experiments.

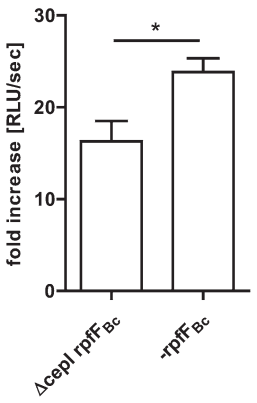

Fig. 1. The genetic background influences sensitivity of the sensor plasmid pAN-L15. Plasmid pAN-L15 was introduced into the QS double mutant *B. cenocepacia* H111ΔcepI rpfF$_{Bc}$ or the *rpfF*$_{Bc}$ mutant *B. cenocepacia* H111-rpfF$_{Bc}$. Strains were grown in Luria–Bertani (LB) medium (kanamycin 100 μg ml^{-1}) to an OD600 = 1.8–2.0, then diluted 1:1 in LB and supplemented with 10 μM BDSF (Sigma). Samples were incubated for 20 h at 30°C and then assayed for bioluminescence as described in Material and Methods S1. Fold increase is relative to the bioluminescence of the control without BDSF. Error bars indicate standard error of the mean, $n = 3$. * $P < 0.05$ (ANOVA, one-way, Bonferroni post-test with 95% confidence interval).

Previous work has identified BCAM0227 as an additional BDSF receptor in *B. cenocepacia* J2315 (McCarthy *et al*., 2010). We therefore tested whether activity of the *bclACB* promoter on pAN-L15 is influenced by BCAM0227. However, in line with the previous finding that BCAM0227 is only involved in the regulation of a subset of the BDSF-regulated genes and does not control expression of the *bclACB* lectin operon in *B. cenocepacia* H111 (McCarthy *et al*., 2010; Deng *et al*., 2012), we did not observe any effect of this regulator on the functionality of the biosensor (Fig. S1).

The luxAB*-based BDSF biosensor is sensitive to nM levels of BDSF*

To test the sensitivity of the biosensor, various concentrations of synthetic BDSF were added to bacterial cultures of the reporter strain H111-rpfF$_{Bc}$ / pAN-L15 and incubated for 20 h at 30°C. Induction of luminescence was measured after addition of decanal, the luciferase substrate. Our results show that the biosensor responded to concentrations as low as 10 nM of synthetic BDSF, reaching a maximum level of bioluminescence with 100 nM of BDSF (Fig. 2).

The H111-rpfF$_{Bc}$ */ pAN-L15 biosensor responds to fatty acids with cis-2 configuration*

To characterize the specificity of the H111-rpfF$_{Bc}$ / pAN-L15 biosensor, we measured the induction of lumines-

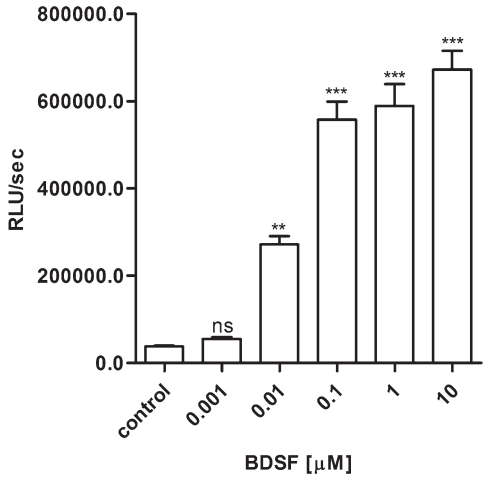

Fig. 2. The *luxAB*-based biosensor H111-rpfF$_{Bc}$ / pAN-L15 is highly sensitive for BDSF. The sensor strain was grown (as described in the figure legend of Fig. 1) in the presence of BDSF with concentrations ranging from 1 nM to 10 μM. Error bars indicate standard error of the mean, $n = 3$. ns, not significant; ** $P < 0.0001$ (ANOVA, one-way, Dunnett's post-test with 95% confidence interval).

cence after the addition of molecules structurally related to BDSF as well as lauric acid and C8-HSL. These experiments showed that both DSF and *cis*-2-decenoic acid activated the biosensor at 1 μM, while no signal above the background was observed when other fatty acids or C8-HSL were tested at biological relevant concentrations of 10 μM and 200 nM, respectively (Fig. 3A; Fig. S2). All active compounds share a *cis*-2 configuration. By contrast, *trans*-2-decenoic acid did not induce the biosensor (Fig. 3B). This is in line with the previous finding that the *cis*-2 configuration appears to be essential for the activity as a signal and for binding to the receptor protein RpfR (Wang *et al*., 2004; Deng *et al*., 2012).

Production of DSF family signals is widespread among members of the Bcc

We next evaluated the suitability of the biosensor to detect the production of *cis*-2 fatty acids by bacteria in simple cross-streak assays. We cross-streaked the wild type *B. cenocepacia* H111, the *cepI* mutant H111ΔcepI, the *rpfF$_{Bc}$* mutant H111-rpfF$_{Bc}$ and the double mutant H111ΔcepI rpfF$_{Bc}$ against the biosensor. Signals could only be detected for the wild type and the *cepI* mutant, suggesting that the biosensor does not cross-react with the AHL-dependent QS system (Fig. 4A). We also investigated whether the biosensor could be used for the detection of *cis*-2 fatty acid signal molecules in culture supernatants. To this end, cell free culture supernatants were added to cultures of the biosensor and bioluminescence was measured in a plate reader (Synergy HT; Bio-Tek, Germany) after 20 h incubation. In agreement with the results obtained from the cross-streak experiments, the biosensor

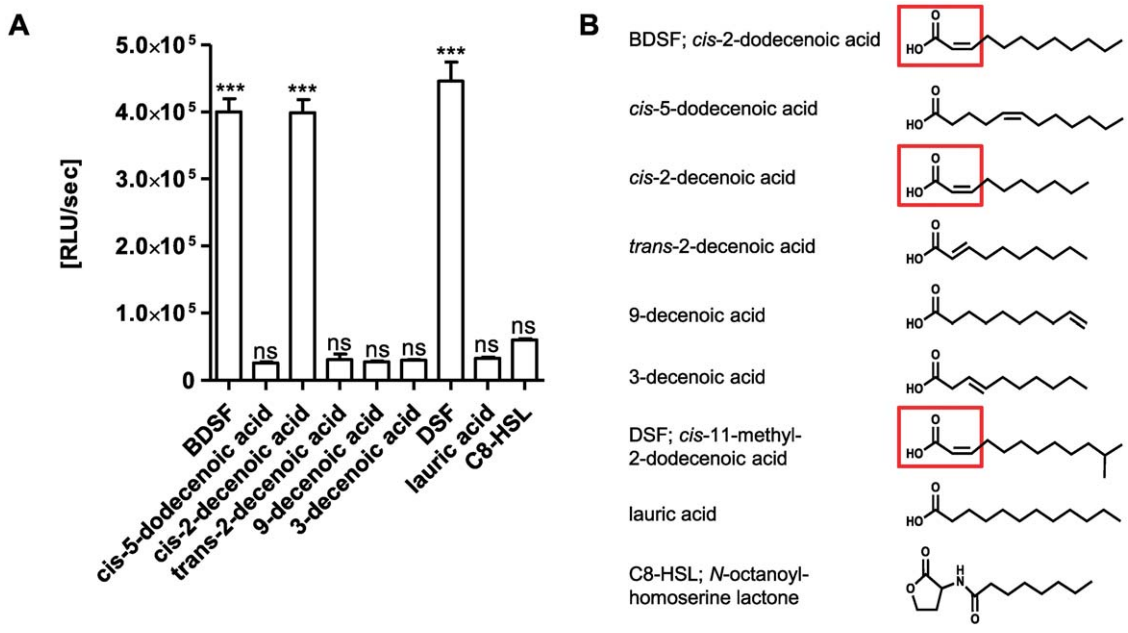

Fig. 3. The sensor plasmid pAN-L15 is responsive to various fatty acids with a *cis*-2 configuration.
A. The sensor strain *B. cenocepacia* H111-rpfF$_{Bc}$ / pAN-L15 was grown in Luria–Bertani medium supplemented with different *cis*-2 fatty acids (10 μM, Sigma), lauric acid (10 μM, Sigma) or C8-HSL (200 nM, Sigma). Error bars indicate standard error of the mean, $n = 3$; ns, not significant; *** $P < 0.0001$ (ANOVA, one-way, Bonferroni post-test with 95% confidence interval).
B. Chemical structures of the tested compounds; the *cis*-2 configuration of respective fatty acids is depicted in a red box.

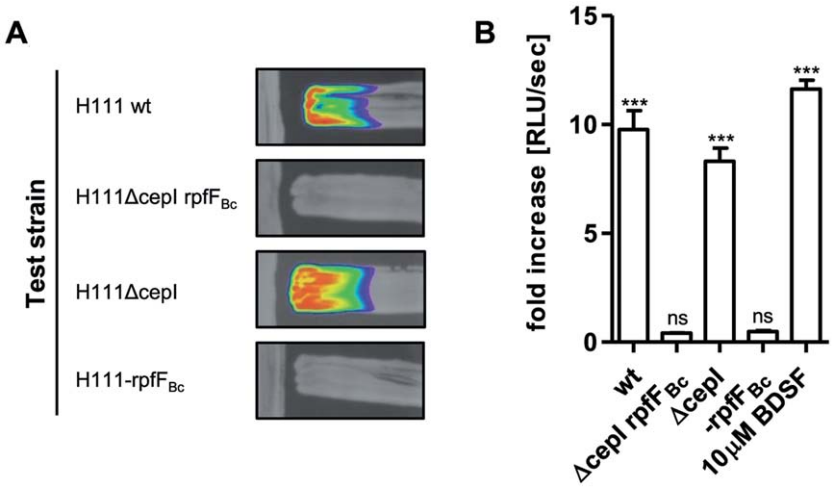

Fig. 4. Activation of the *B. cenocepacia* H111-rpfF$_{Bc}$ / pAN-L15 biosensor.
A. The biosensor strain (horizontal) and test strains (vertical) were cross-streaked on Luria–Bertani (LB) agar plates as described in Material and Methods S1. Only strains producing BDSF (*B. cenocepacia* H111 and H111ΔcepI) induced reporter gene expression.
B. The sensor strain *B. cenocepacia* H111-rpfF$_{Bc}$ / pAN-L15 was grown for 20 h at 30°C in LB medium supplemented 1:1 with supernatants of the *B. cenocepacia* H111 wild type, the *cepI rpfF$_{Bc}$* double mutant, the *cepI* mutant H111 ΔcepI and the *rpfF$_{Bc}$* mutant H111-rpfF$_{Bc}$ or BDSF (10 μM). Samples were assayed for bioluminescence as described in Material and Methods S1. Fold increase is relative to reporter gene induction in medium only. Error bars indicate standard error of the mean, $n = 4$. ns, not significant; *** $P < 0.0001$ (ANOVA, one-way, Dunnett's post-test with 95% confidence interval).

was significantly activated in the presence of supernatants of strains synthesizing BDSF but not by the supernatant of H111-rpfF$_{Bc}$ or H111ΔcepI rpfF$_{Bc}$ (Fig. 4B).

A Blast search identified RpfF$_{Bc}$ and RpfR homologs in all available *Burkholderia* genome sequences (Table S1) (Winsor *et al*., 2008), suggesting that the production of DSF family signals may be widespread within this genus. Cross-streak and liquid culture assays were performed to test various strains for the production of DSF family signal molecules. Under the conditions tested, we found that production of DSF family signals is common among all members of the Bcc (the novel species *Burkholderia pseudomultivorans* was not tested) (Table S1). *Burkholderia sordidicola* (LMG22029), a strain outside of the Bcc, showed production of a DSF family signalling molecule in both liquid and cross-streak experiments. *Burkholderia caledonica* (LMG19076), *Burkholderia graminis* (LMG18924), *Burkholderia phenoliruptrix* (LMG22037), *Burkholderia terricola* (LMG20594) and *Burkholderia plantarii* (LMG9035) induced the biosensor in cross-streak experiments but not significantly in liquid culture, suggesting condition-dependent production of DSF-based signalling molecules (Fig. S3, Table S1).

Construction of a GFP-based BDSF biosensor for single cell, real time investigations

Previous work has shown that the BDSF-dependent QS system of *B. cenocepacia* controls biofilm formation (Deng *et al*., 2012; Schmid *et al*., 2012). To visualize the production of BDSF within biofilms, we constructed a transcriptional fusion of the *bclACB* promoter with an unstable variant of the green fluorescent protein (GFPmut3*), yielding plasmid pAN-G3. The functionality of this sensor plasmid was confirmed by cross-streak experiments against different *B. cenocepacia* strains (Fig. S4). Next, we transferred the sensor plasmid into the wild-type *B. cenocepacia* H111 and used this transgenic strain to monitor the spatial and temporal production of BDSF during biofilm development in a flow-through chamber system. In this setup, fluorescent cells were first observed when microcolonies were formed (approximately 24 h after inoculation) and at this time point only very few fluorescent cells were found outside of microcolonies (Fig. 5). In 48 h old biofilms, virtually all cells within the microcolonies were fluorescent and many induced cells were also observed outside the microcolonies. These results indicate that a critical population density is required to trigger the QS response, an expression pattern that is compatible with an expected BDSF-dependent cross-induction of cells within microcolonies. In order to exclude the possibility that activation of the biosensor occurs by routes other than the BDSF-dependent QS system in biofilms, we also transferred the biosensor into the *rpfF$_{Bc}$* mutant as well as into the *cepI rpfF$_{Bc}$* double mutant and monitored fluorescence during biofilm development. In agreement with previous work (Deng *et al*., 2012; Schmid *et al*., 2012), we observed that these strains formed less and structurally different biofilms. More importantly, fluorescence was greatly

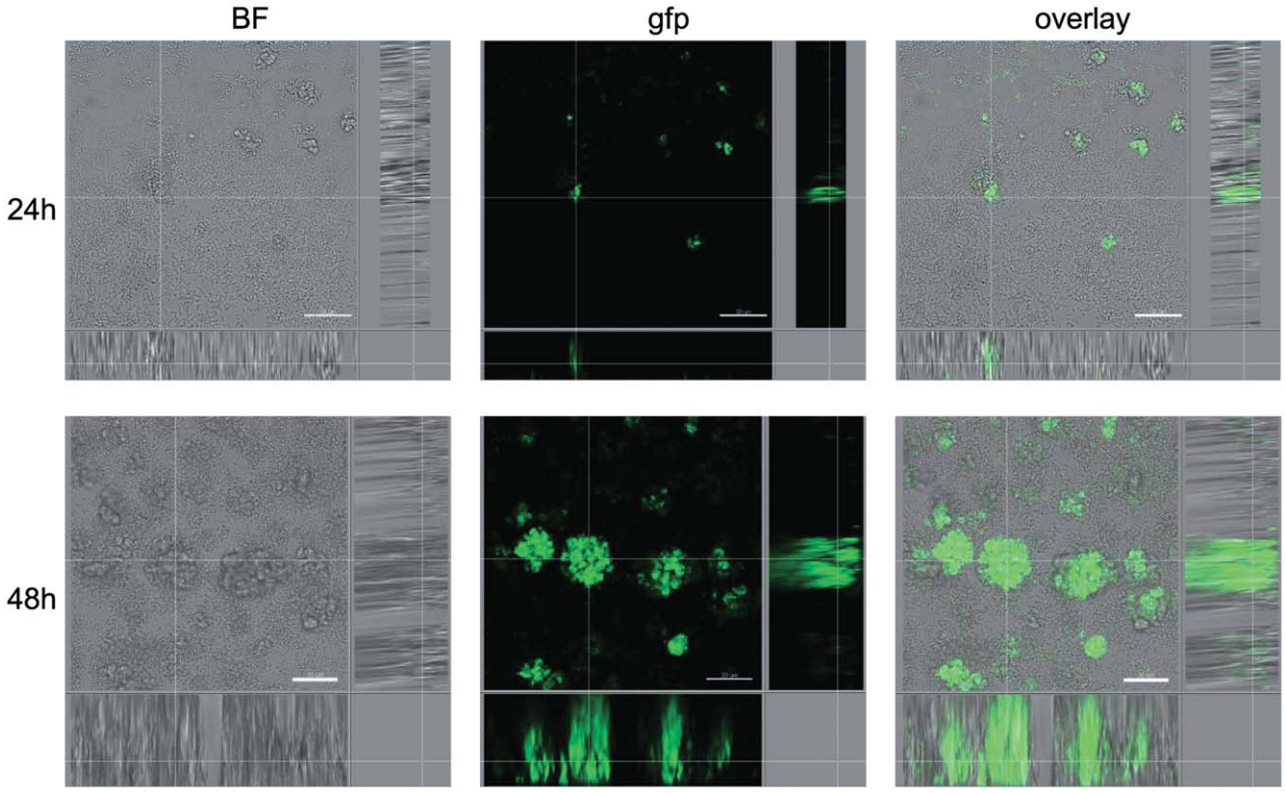

Fig. 5. Visualization of BDSF production in a *B. cenocepacia* biofilm. Biofilms of *B. cenocepacia* H111 harbouring the *gfp*-based sensor plasmid pAN-G3 were cultivated in a flow chamber system as described previously (Inhulsen *et al*., 2012). After 24 and 48 h the biofilms were inspected with a confocal laser scanning microscope (Leica, DM 5500 Q, Wetzlar, Germany) with a 40 × 1.15 oil objective. Data were analysed with Leica Application Suite (Mannheim, Germany) and the Imaris 7.7.2 software package (Bitplane, Zurich, Switzerland). Green fluorescence is indicative of BDSF production. BF, bright field; scale bar: 30 μM).

reduced in the biofilm of the *rpfF*$_{Bc}$ mutant and was found to be further reduced in the biofilm of the *cepI rpfF*$_{Bc}$ double mutant (Fig. S5). Exposure of a 48 h old biofilm of H111ΔcepI rpfF$_{Bc}$ to 10 μM BDSF for 3 h markedly increased fluorescence. In conclusion, these experiments strongly support a model in which the RpfFR system is the major regulator of *bclACB* expression while the CepIR system only plays a subordinate role.

Discussion

Evidence has accumulated over the past years that many Gram-negative bacteria are able to synthesize and respond to fatty acid signal molecules (Boon *et al*., 2008; Deng *et al*., 2011). This class of signals has been shown to play important roles in intraspecies, interspecies and even interkingdom communication (Boon *et al*., 2008; Deng *et al*., 2010; 2013; Alavi *et al*., 2013; Ryan *et al*., 2015). At present, it is not entirely clear how widespread the production of these signals is, but a bioinformatic analysis revealed that RpfF homologs are present in many bacterial genomes (Deng *et al*., 2012). The biosensors developed in this study will facilitate the identification of the respective signal molecules and provide a tool for investigating the underlying signalling pathways. We show that the *luxAB*-based biosensor is very sensitive to BDSF (as low as 10 nM), but is also activated by other fatty acids containing a *cis*-2 configuration, which was previously shown to be critical for biological activity (Wang *et al*., 2004). Although the GFP version of the biosensor is less sensitive, we show that it is a highly valuable tool for single cell, real time studies. It is worth noting that both sensor plasmids have an origin of transfer as well as a broad host origin of replication and therefore can be transferred to a wide range of bacteria (Kovach *et al*., 1995).

In this study we have employed the biosensor to survey various *Burkholderia* species for the production of DSF family signal molecules. In agreement with the available bioinformatic information we found that all tested strains of the Bcc were capable of activating the biosensor. The Bcc is a group of 18 closely related species, which are notable for their ability to metabolize a wide range of organic compounds and to thrive in many different environments (Peeters *et al*., 2013). However, they are also known as opportunistic pathogens that are capable of infecting

immunocompromised patients or individuals suffering from cystic fibrosis. This analysis extends previous work that showed, by using high-performance liquid chromatography and mass spectrometry, that BDSF is not only produced by *B. cenocepacia* but also by 8 additional species of the Bcc, namely *B. ambifaria*, *B. anthina*, *B. dolosa*, *B. lata*, *B. multivorans*, *B. pyrrocinia*, *B. stabilis* and *B. vietnamiensis,* with some of these species synthesizing additional molecules, such as *cis*,*cis*-11-methyldodeca-2,5-dienoic acid or DSF (Deng *et al*., 2010). Here we show for the first time the production of DSF family signals by *Burkholderia* strains that belong to a cluster of approximately 30 species that appear to be exclusively associated with plants and are beneficial for their hosts (Compant *et al*., 2008; Gyaneshwar *et al*., 2011; Suarez-Moreno *et al*., 2012). Previous work has demonstrated that the RpfFR system in *B. cenocepacia* is essential for virulence in different infection hosts (Deng *et al*., 2012). Given that members of the beneficial *Burkholderia* clade are not pathogenic, it will be of great interest to unravel the functions regulated by DSF signals in these bacteria.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The BDSF sensor RpfR but not BCAM0227 is required for *bclACB* promoter activity. The sensor plasmid pAN-L15 was introduced into the *rpfF*Bc, the *rpfR* and the *BCAM0227* mutants and the recombinant strains were tested for biosensor activity in the presence (+) or absence (-) of 10 μM BDSF. White, H111-rpfF_{Bc} / pAN-L15; Grey, H111-rpfR / pAN-L15; Black, H111–bcam0227 / pAN-L15. Error bars indicate SEM, n>2; * P<0.05; ns, not significant (ANOVA, one-way, Bonferroni post-test with 95% CI). RLU, relative light units.
Fig. S2. Specificity and sensitivity of the H111-rpfF_{Bc} / pAN-L15 biosensor. Induction of bioluminescence was measured after the addition of molecules structurally related to BDSF in concentrations ranging from 0.001 μM to 100 μM. Fold increase is relative to the bioluminescence of the control without BDSF.
Fig. S3. *Burkholderia* strains tested for the production of DSF family signal molecules in liquid culture bioassays. Supernatants of overnight cultures of various *Burkholderia* strains (names are according to Table S1) were tested for the induction of the biosensor *B. cenocepacia* H111-rpfF_{Bc} / pAN-L15. Error bars indicate SEM, n=2.
Fig. S4. The *gfp*-based biosensor is induced by BDSF-synthesizing strains. The sensor strain *B. cenocepacia* H111-rpfF_{Bc} / pAN-G3 (horizontal) was tested for induction by test strains (vertical) in cross-streak experiments. Only BDSF producers (*B. cenocepacia* H111 wt and H111ΔcepI) induce reporter gene expression. Background level was reduced to the autofluorescence level of the strains (1000 cts).
Fig. S5. The activity of the biosensor is strongly reduced in H111–rpfF_{Bc} and H111ΔcepI rpfF_{Bc} biofilms when compared to biofilms formed by the wild type strain. Biofilms were grown in flow-through cells as described previously (Inhulsen *et al*., 2012). A 48 h old biofilm of H111ΔcepI rpfF_{Bc} was supplemented with 10 μM BDSF for 3 h. Biofilms were analysed after 72 h of incubation using a confocal laser scanning microscope (Leica, DM 5500 Q, Wetzlar, Germany). Green fluorescence (upper panel) is indicative of BDSF production. BF, bright field (lower panel); scale bar: 30 μM.
Table S1. *Burkholderia* strains tested in cross-streak experiments for induction of the *luxAB*-based biosensor H111-rpfF_{Bc} / pAN-L15 and presence of RpfR and RpfF_{Bc} orthologs in sequenced *Burkholderia* species.
Table S2. Bacterial strains and plasmids used in this study.
Material and Methods S1. Materials and Methods used in this study.