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ORIGINAL ARTICLE

A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition

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In addition to producing lethal antibiotics, microorganisms may also use a new form of antagonistic mechanism in which signal molecules are exported to influence the gene expression and hence the ecological competence of their competitors. We report here the isolation and characterization of a novel signaling molecule, *cis*-2-dodecenoic acid (BDSF), from *Burkholderia cenocepacia*. BDSF is structurally similar to the diffusible signal factor (DSF) that is produced by the RpfF enzyme of *Xanthomonas campestris*. Deletion analysis demonstrated that *Bcam0581*, which encodes an RpfF homologue, was essential for BDSF production. The gene is highly conserved and widespread in the *Burkholderia cepacia* complex. Exogenous addition of BDSF restored the biofilm and extracellular polysaccharide production phenotypes of *Xanthomonas campestris* pv. *campestris* DSF-deficient mutants, highlighting its potential role in inter-species signaling. Further analyses showed that *Candida albicans* germ tube formation was strongly inhibited by either coculture with *B. cenocepacia* or by exogenous addition of physiological relevant levels of BDSF, whereas deletion of *Bcam0581* abrogated the inhibitory ability of the bacterial pathogen. As *B. cenocepacia* and *C. albicans* are frequently encountered human pathogens, identification of the BDSF signal and its activity thus provides a new insight into the molecular grounds of their antagonistic interactions whose importance to microbial ecology and pathogenesis is now becoming evident.

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Introduction

Microbe–microbe interactions are ubiquitous in various natural ecosystems. Not surprisingly, various forms of symbiosis and antagonism have surfaced. It has been known for a long time that microorganisms may produce antibiotics to inhibit or stop the growth of their competitors to gain competitive advantages. Recently, evidence is accumulating for a new form of microbial antagonism, which was tentatively designated as signal interference (Zhang and Dong, 2004). This type of antagonism acts not by killing, but instead by influencing the signal-mediated gene expression of the competitors and thus tips the balance of interaction (Dong *et al.*, 2004; Hogan *et al.*, 2004). For keeping up their competitive advantages, many microorganisms appear to produce, release and respond collectively to species-specific small signal molecules to coordinate a range of important activities, such as virulence factor production, antibiotics biosynthesis and biofilm formation. This cell–cell communication mechanism is commonly known as quorum sensing (Whitehead *et al.*, 2001; Fuqua and Greenberg, 2002). Logically, microorganisms might also boost their competitive strength in ecosystems by interfering with the quorum-sensing signaling of their competitors (Zhang and Dong, 2004).

Candida albicans causes various forms of candidiasis ranging from mucosal infections to serious systemic infections. Mutants defective in morphological transitions during infection are avirulent (Lo *et al.*, 1997; Braun *et al.*, 2000; Saville *et al.*, 2003; Zheng *et al.*, 2003). Therefore, this ability to switch between yeast and hyphal-form is an important aspect of its pathogenesis. Intriguingly, the chance of infection by *C. albicans* seems to be influenced by the presence of certain bacterial pathogens. While the fungal pathogen can be found in various groups of patients who have undergone

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treatments with broad-spectrum antibiotics, it is rarely found in individuals suffering from cystic fibrosis that are chronically infected with *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Kerr, 1994). This putative antagonism seems to be at least partially explained by the recent finding that the long-chain quorum-sensing signal 3-oxo-C12HSL produced by *P. aeruginosa* at physiological relevant level was sufficient to inhibit *C. albicans* yeast-to-hyphae transition (Hogan *et al.*, 2004). However, C8-HSL, the major quorum-sensing signal produced by *B. cepacia* (Lewenza *et al.*, 1999; Riedel *et al.*, 2001), was not able to suppress the fungal filamentation (Hogan *et al.*, 2004).

Burkholderia cepacia complex (*Bcc*) has emerged as a major opportunist pathogen for immunocompromised individuals in particular the patients with cystic fibrosis and chronic granulomatous disease (Isles *et al.*, 1984; Goldmann and Klinger, 1986; Mahenthiralingam *et al.*, 2005). The Bcc consist of at least nine species that are phenotypically similar but genetically distinct, and all the species are capable of causing infections (Coenye *et al.*, 2001; Mahenthiralingam *et al.*, 2005). Among them, *Burkholderia cenocepacia* constitutes the majority of the transmissible and epidemic strains and is highly virulent (Mahenthiralingam *et al.*, 2001, 2002). In this study, we report the identification and characterization of a novel signaling molecule designated as BDSF from *B. cenocepacia*. BDSF is a structural homologue of diffusible signal factor (DSF), which is the quorum-sensing signal produced by the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*). In addition, we demonstrate that the ORF *Bcam0581* is essential for BDSF production and can genetically substitute for the DSF synthase gene *rpfF* in *Xcc*. Furthermore, we show that the dimorphic transition of *C. albicans* was inhibited by either exogenous addition of BDSF or coculturing with *B. cenocepacia*.

Materials and methods

Bacteria strains and growth conditions

B. cenocepacia J2315, a cystic fibrosis clinical isolate, was obtained from the American Type Culture Collection, Manassas, VA, USA. It is the representative strain of the highly transmissible ET12 clone (Govan *et al.*, 1993). *X. campestris* pv. *campestris* strain 8004 and its *rpfF* deletion mutant 8004dF were described previously (Wang *et al.*, 2004; He *et al.*, 2006b). *Xcc* strains were maintained at 30 °C in YEB medium (Zhang *et al.*, 2002), while *Escherichia coli* and *B. cenocepacia* strains were grown in Luria-Bertani (LB) broth at 37 °C. The following antibiotics were supplemented when necessary: rifampicin, 50 μg ml^{-1}; tetracycline, 10 μg ml^{-1} (*Xcc* and *E. coli*) or 400 μg ml^{-1} (*B. cenocepacia*); gentamycin, 100 μg ml^{-1}; and trimethoprim, 300 μg ml^{-1}. *C. albicans* SC5314 were grown either in GMM medium consisting of 6.7 g of Bacto yeast nitrogen base (Difco, Sparks, MD, USA) and 0.2% glucose or in minimum medium (pH 7.2). The latter consists of K_2PO_4, 60 mM; KH_2PO_4, 30 mM; citrate, 20 mM; $(NH_4)_2SO_4$, 15 mM; $MgSO_4 \cdot 7H_2O$, 0.8 mM; $CaCl_2$, 90 μM; $FeSO_4$, 30 μM; $MnCl_2$, 15 μM; and Casamino acids, 0.5%. DSF was added to the medium in a final concentration of 5 μM unless otherwise indicated.

Bioassay of BDSF

The assay was performed as described previously using the biosensor strain FE58 (Wang *et al.*, 2004). Briefly, 4-mm diameter wells were introduced on prepared bioassay plates and 20 μl concentrated culture was added to each well. Alternatively, single colonies were spotted on bioassay plates. The plates were incubated at 30 °C overnight. DSF activity is indicated by the presence of a blue halo around the well or colony.

BDSF purification

B. cenocepacia J2315 was grown in LB overnight with agitation at 37 °C. Seventy liters of culture supernatant was collected by centrifugation and extracted with equal volume of ethyl acetate. The crude extract (organic phase) was dried using a rotary evaporator and dissolved with methanol. The mixture was subjected to flash column chromatography using a silica gel column (12 × 150 mm, Biotage Flash 12 M cartridge) and eluted with ethyl acetate–hexane (20:80 v/v). The active fractions were detected using the DSF bioassay and pooled. These were then concentrated, subjected to flash column chromatography again and eluted with ethyl acetate–hexane (10:90 v/v). The purity of the collected active components was analyzed by high-performance liquid chromatography using a C18 reverse-phase column (4.8 × 250 mm, Waters, Milford, MA, USA), eluted with methanol–water (80:20 v/v) at a flow rate of 1 ml min^{-1}.

Structure analysis and synthesis of BDSF

^{1}H, ^{13}C, ^{1}H-^{1}H COSY, distortionless enhancement by polarization transfer, heteronuclear multiple bond coherence and heteronuclear multiple quantum coherence (HMQC) nuclear magnetic resonance (NMR) spectra in $CDCl_3$ solution were obtained using a Bruker DRX400 spectrometer operating at 400 MHz for ^{1}H or 100.5 MHz for ^{13}C. High-resolution electrospray ionization mass spectrometry was performed on a Finnigan/MAT MAT 95XL-T mass spectrometer. Conditions used were as stated before (Wang *et al.*, 2004). BDSF was synthesized by Favorsky rearrangement of the corresponding 1,3-dibromo-2-dodecanone as described previously (Wang *et al.*, 2004).

Construction of Bcam0581 in-frame deletion mutants
B. cenocepacia J2315, a cystic fibrosis clinical isolate, was used as the parental strain to generate the *Bcam0581* in-frame deletion mutants. The upstream and downstream regions flanking *Bcam0581* were isolated using two PCR primer pairs, that is BCAM0581KO_LF (5′-ggatccctcgagatgcttgtcgaa), BCAM0581KO_LR (5′-aagcttggtatgtcctcgtgagatgtg); and BCAM0581KO_RF (5′-aagcttcgcacggtgtaatgcgac), BCA M0581KO_RR (5′-tctagaggatcc acgtatcgcgtgttctcgctg), respectively. This resulted in removal of 852 bp of the 864 bp *Bcam0581* coding sequence. To facilitate construction, *Bam*HI and *Hin*dIII sites were included in the upstream fragment, whereas *Hin*dIII and *Xba*I sites were tagged to the downstream fragment. The PCR products were cleaved with respective enzymes and ligated to the suicide vector pEX18Tc (Hoang *et al.*, 1998). The construct, verified by DNA sequencing, was introduced into *B. cenocepacia* J2315 by tri-parental mating with pRK2013 (Figurski and Helinski, 1979) as the mobilizing plasmid. The *B. cenocepacia* transconjugants were selected on LB agar plates containing tetracycline and gentamycin. Colonies harboring second crossover events were selected on LB agar containing 10% sucrose. *Bcam0581* deletion mutants (Δ*Bcam0581*) were identified by colony PCR using the primer pair BCAM0581KO_LF and BCAM0581KO_RR described above.

Complementation of strains 8004dF and ΔBcam0581
The coding region of *Bcam0581* was amplified via PCR using primers pair BCAM0581-F (5′-ggatccatgcaactccaatcccatcc) and BCAM0581-R (5′-aagctttacaccgtgcgcagctt). The product was digested with *Bam*HI and *Hin*dIII and ligated separately to plasmid vectors pMSL7 (Lefebre and Valvano, 2002) and pLAFR3 at the same enzyme sites. The resultant construct was conjugated into Δ*Bcam0581* and 8004dF, respectively, by tri-parental mating. The transconjugants of 8004dF and Δ*Bcam0581* were selected on YEB agar plates containing rifampicin and tetracycline and on LB agar plates supplemented with gentamycin and trimethoprim, respectively.

Extracellular polysaccharide and biofilm analysis
For quantification of extracellular polysaccharide (EPS) production, 10 ml of overnight culture ($OD_{600} \sim 2.5$) were centrifuged at 12 000 r.p.m. for 20 min. The collected supernatants were mixed with 2.5 volumes of absolute ethanol and the mixture was incubated at 4 °C for 30 min. The precipitated EPS was isolated by centrifugation and dried overnight at 55 °C before determination of dry weights.

For analysis of biofilm formation, a single colony of each strain was inoculated and grown overnight at 37 °C with agitation in 5 ml of YEB medium. As a positive control, 5 μM of DSF was added to *Xcc 8004dF* and methanol was added to the wild-type strain as solvent control. Multiple samples were obtained after overnight incubation and visualized with a phase contrast microscope (Olympus BX50). Imaging was performed using an Olympus DP70 digital camera.

Microscopic analysis and quantification of germ tube formation in C. albicans
For testing the effect on *C. albicans* germ tube formation, BDSF and other compounds were diluted to appropriate concentrations in methanol. The overnight culture of *C. albicans* strain SC5314 grown in GMM medium were diluted 10-fold in fresh GMM medium containing the 3 kDa fraction of fetal calf serum at a final concentration of 20%. The 3 kDa fraction was prepared by filtration of fetal calf serum through a membrane (Millipore, Billerica, MA, USA) with a 3 kDa pore size and collection of the filtrates. This preparation was necessary as it drastically reduced the crude serum-induced aggregation but retained the potent germ tube induction capability, thereby allowing accurate quantification. The testing signal molecules were added separately to appropriate final concentrations as indicated and the cells were induced for 3 h at 37 °C. For growth experiments, overnight cultures ($OD_{600} \approx 1.0$) were diluted to $OD_{600} \approx 0.06$ in GMM medium and cultured at 30 °C with agitation (200 r.p.m.). Coculture experiments were performed in minimum medium using three *B. cenocepacia* strains, which displayed a similar growth rate. Fresh bacilli and yeast-form *C. albicans* were cultured together in the ratio of 20:1 with *E. coli* DH5α as a negative control. The mix cultures were grown for 15 h at 30 °C and then 3 h at 37 °C for induction of germ tube formation. Quantification of germ tube formation was performed using a phase contrast microscope (Olympus BX50) by counting about 400 *C. albicans* cells per sample. Imaging was achieved using a Leica DMR Fluorescence microscope with ×100 objective and a Hamamatsu digital camera interphased with METAMORPH software (Universal Imaging, Downingtown, PA, USA).

Results

Detection of DSF-like activity in B. cenocepacia
In our preliminary screening, several environmental isolates, belonging to *Burkholderia* spp. based on 16S rDNA analysis, were found capable of producing DSF-like signals when assayed using the DSF biosensor *Xcc* strain FE58 (Wang *et al.*, 2004). For further characterization, strain *B. cenocepacia* J2315, which is a clinical isolate with genome sequence available (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_cenocepacia), was obtained from ATCC. A single colony of strain J2315 was spotted on the bioassay plate containing the biosensor. A blue halo, which indicates the presence of DSF activity,

was detected after incubation at 30 °C overnight (Figure 1a), suggesting that *B. cenocepacia* J2315 may secrete a DSF-like molecule, which was tentatively designated as BDSF.

Bcam0581 is the rpfF homologue of B. cenocepacia *J2315 essential for production of BDSF*

In *Xcc*, a putative enoyl-CoA hydratase encoded by *rpfF* is a key enzyme for DSF biosynthesis. Mutation of *rpfF* abolishes DSF production and reduces the DSF-mediated virulence gene expression (Barber *et al.*, 1997; Wang *et al.*, 2004; He *et al.*, 2006b). To identify the gene responsible for BDSF biosynthesis, the RpfF homologue was searched in the genome of *B. cenocepacia* J2315 by using the BLAST program (Altschul *et al.*, 1990). The top hit is a peptide encoded by *Bcam0581* showing a 37.2% identity with RpfF with an e-value of 1.8×10^{-44}. The gene is located on chromosome 2 and encodes a protein of 32 kDa. Interrogation of the *B. cenocepacia* J2315 genome sequence with the protein sequence of Bcam0581 did not reveal the presence of paralogues. Domain analysis using the pfam database version 22.0 (Finn *et al.*, 2006) showed that Bcam0581 contained an enoyl-CoA hydratase domain similar to the RpfF enzyme of *Xcc* (Figure 2a).

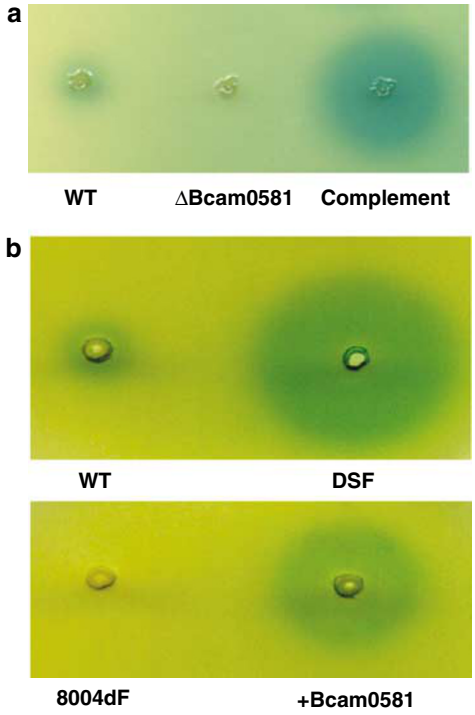

Figure 1 *B. cenocepacia* produced diffusible signal factor (DSF)-like signals. (**a**) DSF bioassay with *B. cenocepacia* J2315 and derivatives. WT, wild-type *B. cenocepacia* J2315; complement, Δ*Bcam0581*(pMLS7-*Bcam0581*). (**b**) Expression of *Bcam0581* in the DSF-deficient mutant *8004dF* of *X. campestris* pv. *campestris* (*Xcc*) restored DSF production. WT, wild-type *Xcc* 8004; +*Bcam0581*, *8004dF*(pLAF3-*Bcam0581*); DSF, 5 μl of DSF (5 μM). The presence of a blue halo around the site of inoculation indicates the presence of DSF-like activity.

The *rpfF* of *Xcc* is located within the same locus as *rpfC* and *rpfG*, which encode a DSF sensor and cognate response regulator, respectively. In contrast, no *rpfC* or *rpfG* homologue was found in the vicinity of *Bcam0581* (Figure 2b), suggesting a different origin of evolution of the BDSF system in *B. cenocepacia*. The *Bcam0581* gene appears to be a single transcriptional unit and is flanked by *Bcam0582* and *Bcam0580*. The former encodes a 73 kDa hypothetical protein and the latter a 73.3 kDa protein with PAS, diguanylate cyclase (GGDEF) and a phosphodiesterase (EAL) domains. This domain structure is identical to that of PdeA from *Xcc* (*Xc2324*), which had been shown to regulate the synthesis of extracellular enzymes under oxygen-limited conditions (Ryan *et al.*, 2007). In addition, protein blast analysis found that the peptide encoded by *Bcam0580* was about 39% identical to PdeA. Taken together, these observations indicate that Bcam0580 is a putative homologue of PdeA.

To verify the role of *Bcam0581* in BDSF biosynthesis, the gene was deleted via homologous recombination. This resulted in a complete loss of BDSF production, and expression of the wild-type *Bcam0581* gene in the deletion mutant resulted in overproduction of the signal (Figure 1a).

Bcam0581 is a functional homologue of rpfF

It was curious whether *Bcam0581*, which shows a moderate similarity to *rpfF*, could functionally replace the latter. For this purpose, the coding region of *Bcam0581* was PCR-amplified and cloned under the control of the *tac* promoter in the expression vector pLAFR3. The resultant construct was conjugated into the *rpfF* deletion mutant 8004dF (He *et al.*, 2006b). As shown in Figure 1b, expression of *Bcam0581* in the mutant restored DSF production. Furthermore, biofilm dispersal and EPS production were restored to wild-type levels in the strain 8004dF expressing *Bcam0581* (Figure 3). Given that *Xcc* is rather stringent in recognition and response to DSF-type signal molecules (Wang *et al.*, 2004), these data suggest that *Bcam0581* may produce the same DSF signal or a closely related structural analogue.

BDSF purification and structural analysis

The active component was collected from 70 l of *B. cenocepacia* J2315 culture supernatants by ethyl acetate extraction and purified by flash column chromatography. About 7 mg of BDSF was obtained from the combined active fractions after evaporation of the solvent. This was estimated at approximately 98% purity, based on the analysis by high-performance liquid chromatography.

The ^{13}C NMR spectrum showed that there were 12 peaks, which represent 12 carbons as stated below (Figure 4b). The nine ^{13}C peaks in the range of 14–32 p.p.m. were most likely from acyclic hydrocarbons. The ^{1}H-^{13}C correlated HMQC data

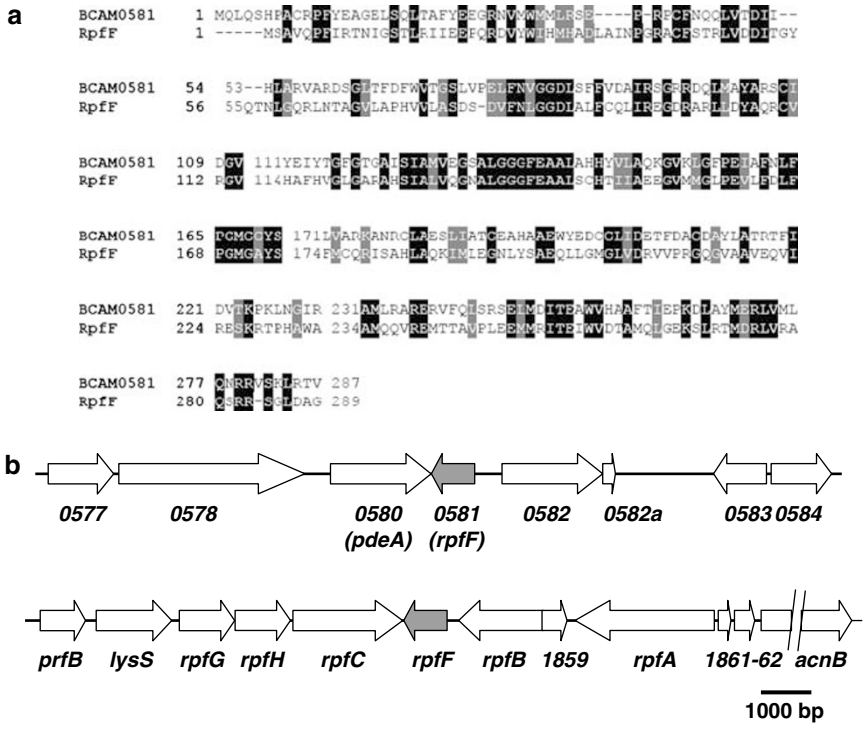

Figure 2 Comparison of the peptide sequence and genomic organization of *rpfF* and its homologue from *B. cenocepacia*. (**a**) Alignment of protein sequences of *Bcam0581* with RpfF from Xcc. The black and gray shading indicates the identical and similar residues, respectively. (**b**) Genomic organization of the *Bcam0581* region in *B. cenocepacia* J2315 (top) and that of *rpfF* region in *Xcc* (bottom). The diffusible signal factor synthesis and response cluster (*rpfA–rpfG*) is flanked by peptide chain release factor (*prfB*); lysyl-tRNA synthase (*lysS*); aconitate hydratase (*acnB*) and two genes encoding hypothetical proteins (*1861* and *1862*). The region around the *rpfF* homologue *Bcam0581* of *B. cenocepacia* consists of the genes encoding two transporters of the major facilitator superfamily (*Bcam0577* and *Bcam0584*), a putative 5-oxoprolinase (*Bcam0578*), a PdeA homologue (*Bcam0580*), two hypothetical proteins (*Bcam0582* and *Bcam0582a*) and an AraC-type transcription regulator (*Bcam0583*).

indicated that the peak at 14.08 p.p.m. was correlated with the three aliphatic protons at 0.88 p.p.m. (Figure 4a), probably representing a terminal CH_3 group. The remaining eight ^{13}C peaks in the range of 14–32 p.p.m. represented eight CH_2 groups based on HMQC and distortionless enhancement by polarization transfer spectra, suggesting the presence of an aliphatic chain. Among three carbon signals over 100 p.p.m., two at 118.91 and 153.43 p.p.m. were correlated with the two olefinic protons at 5.78 and 6.34 p.p.m., respectively, and were assigned to a double bond by HMQC spectrum. These two olefinic protons coupled to each other with a coupling constant of 11.5 Hz, thus establishing the *cis* configuration of the double bond. A quaternary carbon peak at 171.34 p.p.m. should be assigned to a carbonyl, which conjugated to the double bond based on the ^{1}H-^{13}C correlated heteronuclear multiple bond coherence spectrum. Furthermore, high-resolution electrospray ionization mass spectrometry analysis of the purified BDSF revealed a molecular ion $(M\text{-}H)^-$ with an m/z of 197.1532, suggesting a molecular formula of $C_{12}H_{21}O_2$ (197.1542). Taken together, these data indicate that BDSF is *cis*-2-dodecenoic acid (Figure 4c), a closely related structural analogue of DSF. In addition, we synthesized *cis*-2-dodecenoic acid and found that its ^{1}H and ^{13}C NMR spectra and biological activity were virtually indistinguishable from those of natural BDSF (data not shown).

BDSF inhibited the germ tube formation by C. albicans

BDSF is structurally related to farnesol and highly similar to DSF. Both were shown to inhibit germ tube formation by *C. albicans* (Hogan *et al.*, 2004; Wang *et al.*, 2004). For determination of the potential inhibitory activity of BDSF against *C. albicans*, the signal was added to the fresh fungal yeast cells (Figure 5a). Farnesol and methanol were used as a positive and solvent control, respectively. After incubation at 37 °C for 3 h, more than 90% of *C. albicans* cells in solvent control formed germ tubes (Figure 5b), and farnesol at a final concentration of 5 μM slightly reduced the length of germ tubes but seemed to have no effect on germination (Figure 5c). In contrast, BDSF at a final concentration of 5 μM caused a marked reduction of germ tube germination and elongation (Figure 5d).

The above data suggest that BDSF is a highly potent inhibitor. Its activity was thus further compared quantitatively with several similar molecules, including DSF, 3OC12HSL and farnesol (Figures 5e and f), using the same medium and

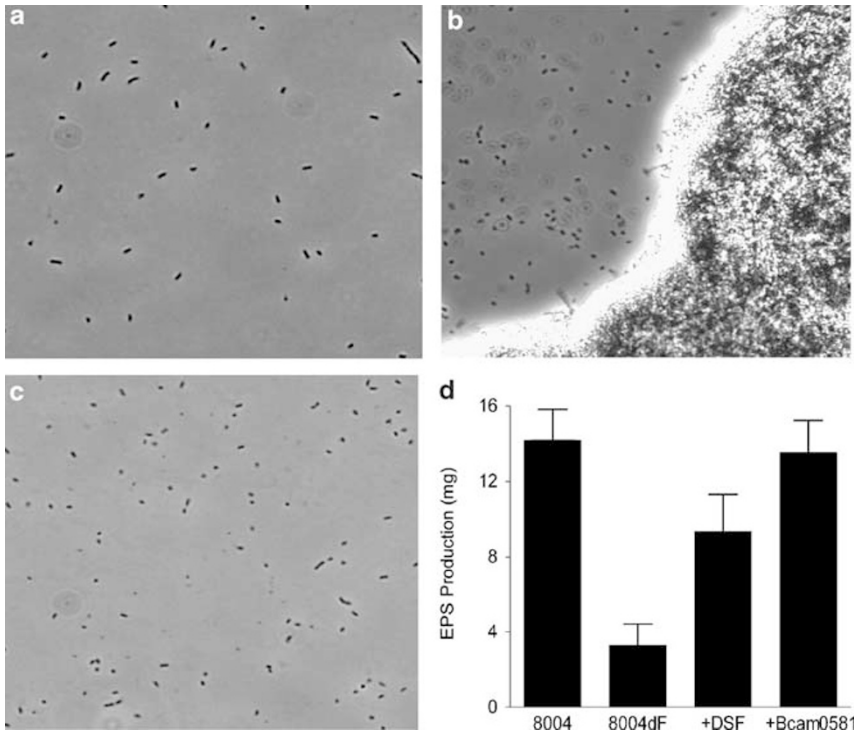

Figure 3 Complementation of the *Xcc* diffusible signal factor (DSF)-deficient mutant 8004dF by expression of the *B. cenocepacia* gene *Bcam0581*. (**a**) The cells of wild-type *Xcc* strain 8004 grew in planktonic (free-floating) form. (**b**) The *rpfF* deletion mutant 8004dF derived from strain 8004 grew in biofilm (cell aggregates at the right-hand side of the photo) form. (**c**) Expression of *Bcam0581* in *trans* in the mutant 8004dF dispersed the bacterial biofilm. (**d**) Extracellular polysaccharide (EPS) production levels. +DSF, exogenous addition of 5 μM of DSF; +*Bcam0581*, mutant 8004dF expressing *Bcam0581* in *trans*. Cells were observed under ×200 magnification and the error bars show the standard deviations.

growth condition as described previously (Hogan *et al*., 2004). At a final concentration of 25 μM, none of them were able to inhibit yeast growth, but showed varied inhibitory effects on *C. albicans* germ tube formation with BDSF being the most effective, followed by DSF, farnesol and 3OC12HSL (Figure 5f). Consistent with the previous observation (Hogan *et al*., 2004), farnesol at a final concentration of 100 μM caused about 45% reduction of germ tube formation (Figure 5f). It is also interesting to note that farnesol, 3OC12HSL and DSF at 100 μM did no affect the yeast cell growth, but strikingly, BDSF at the same concentration showed a detrimental effect on yeast cell growth (Figure 5e). Further titration to a final concentration of 5 μM, BDSF and DSF resulted in about 60% and 12% reduction in germ tube formation, respectively, whereas farnesol and 3OC12HSL had no obvious effect (Figure 5f). Interestingly, even at 0.5 μM, BDSF outperformed its close structural analogue DSF at 5 μM by reducing about 15% germ tube formation (Figure 5f).

ΔBcam0581 displays reduced ability to inhibit germ tube formation when cocultured with C. albicans

To determine the ecological significance of BDSF, we grew *C. albicans* together with *B. cenocepacia* or its BDSF-deficient mutants as a mixed culture under hyphae-inducing conditions. Observation under the microscope showed that the presence of the wild-type *B. cenocepacia* J2315 significantly decreased the fungal morphological transition as a large percentage of *C. albicans* cells grew in yeast-form (Figure 6a). In contrast, majority of the fungal cells appeared as filaments when grown together with the BDSF-deficient mutant Δ*Bcam0581* (Figure 6b). Interestingly, the deletion mutant Δ*Bcam0581* formed heavy cell aggregates together with *C. albicans* hyphal cells (Figure 6b), whereas overexpression of *Bcam0581* in the mutant resumed the planktonic phenotype and abolished the yeast-to-hyphal transition of *C. albicans* (Figure 6c). Quantitative analysis found that approximately 50% of the *C. albicans* cells counted were grown in hyphae-form when cocultured with Δ*Bcam0581*, which was close to the *E. coli* control mix culture where approximately 65% fungal cells were of hyphae-form. However, coculture of *C. albicans* with the wild-type *B. cenocepacia* J2315 decreased the hyphae cell level to about 20% (Figure 6d). The fungal hyphae cell level was further reduced down to only about 5% by the complementary strain that overproduced BDSF (Figures 1a and 6d), highlighting a BDSF dosage-dependent effect.

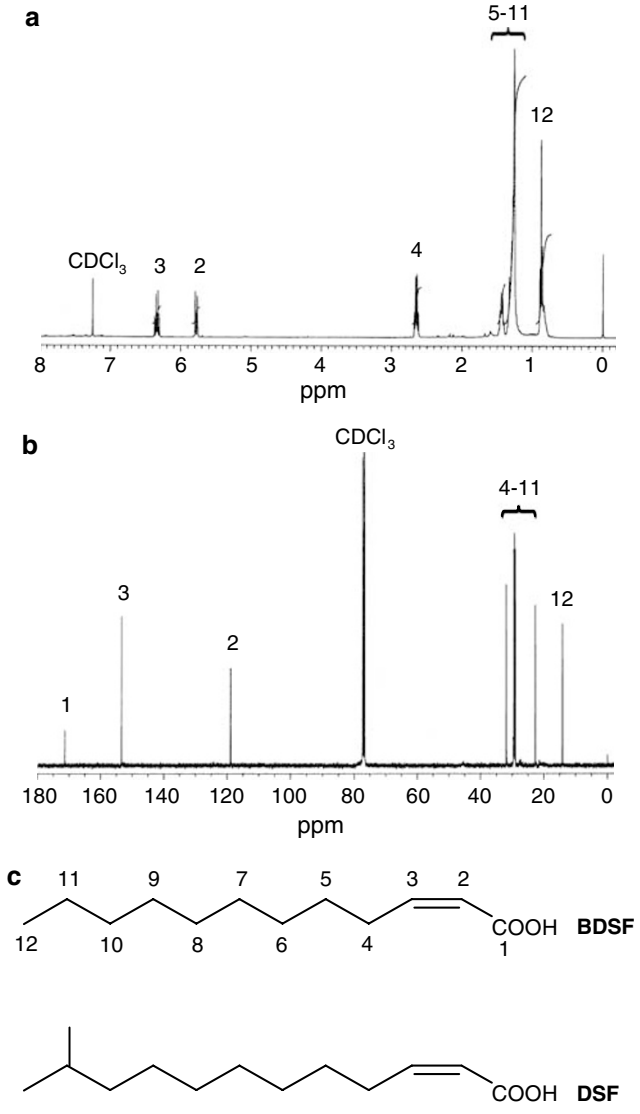

Figure 4 Purification and structural characterization of BDSF. (**a**) ^{1}H NMR spectral of BDSF. (**b**) ^{13}C NMR spectra of BDSF. (**c**) The predicted chemical structure of BDSF compared to diffusible signal factor. NMR, nuclear magnetic resonance.

Discussion

The results of this study show that the human opportunistic bacterial pathogen *B. cenocepacia* produces a potential cell–cell communication signal, which was structurally characterized as *cis*-2-decenoic acid by mass spectrometry and NMR analysis (Figures 1 and 4). The structure of BDSF is similar to but not identical with the DSF (*cis*-11-methyl-2-decenoic acid) signal produced by the plant bacterial pathogen *Xcc* (Wang *et al.*, 2004). The only difference between the two molecules is at the C-11 position where BDSF lacks a methyl group. DSF is a well-characterized quorum-sensing signal that regulates a few hundreds genes encoding diverse biological functions through its signaling network comprising RpfC/RpfG two component system and a few transcriptional regulators (Ryan *et al.*, 2006; He *et al.*, 2006a, b, 2007). *Xcc* belongs to the γ-subdivision of proteobacteria, whereas *B. cenocepacia* is a member of the distantly related β-subdivision. Identification of BDSF from *B. cenocepacia* has strengthened the notion that DSF may represent a new class of conserved signals for bacterial cell–cell communications (Wang *et al.*, 2004).

The BDSF biosynthesis by *B. cenocepacia* is encoded by the gene *Bcam0581.* This is supported by several lines of evidence. First, the peptide encoded by *Bcam0581* shares about 37% identity and a conserved enoyl-CoA hydratase domain with RpfF (Figure 2), the key enzyme known for DSF biosynthesis in *Xcc* (Barber *et al.*, 1997; Wang *et al.*, 2004; He *et al.*, 2006b). Second, expression of *Bcam0581* in the *rpfF* deletion mutant of *Xcc* restored the biofilm dispersal and EPS production to the wild-type level (Figure 3). Third, deletion of *Bcam0581* in *B. cenocepacia* abolished the BDSF biosynthesis (Figure 1). Blast searches revealed the *Bcam0581* homologues in the other five *Bcc* genomovars with greater than 95% identity. They are *B. cepacia sp 383* (genomovar I), *B. multivorans* (genomovar II), *B. vietnamiensis* (genomovar V), *B. dolsa* (genomovar VI) and *B. ambifaria* (genomovar VII). The presence of the *Bcam0581* homologue in the remaining three *Bcc* genomovars is not clear as their genome sequences are not yet available. Thus, like *CepIR*, which is found in all strains of *Bcc* (Lutter *et al.*, 2001), the BDSF signaling system may also be widely conserved in *Bcc*.

Among the several reported bacterial and fungal signals, including the 3OC12HSL from *P. aeruginosa*, DSF produced by *X. campestris* and farnesol produced by *C. albicans* itself, BDSF showed the highest potency on inhibition of filament formation by *C. albicans* (Figure 5). The previous assays performed in different laboratories showed that DSF is more effective than 3OC12HSL for inhibition of germ tube formation (Hogan *et al.*, 2004; Wang *et al.*, 2004). This is consistent with our data that treatment of the fungal cells with about 25 μM DSF or four times more 3OC12HSL resulted in about 45% reduction in germ tube formation (Figure 5f). Highly significantly, BDSF was able to inhibit germ tube formation in approximately 70% of the cells at 5 μM. As DSF and BDSF differ only in the methyl group substitution at C-11 position, revealing such a structure–activity relationship is useful for further drug design and development. Furthermore, our data showed that at a high concentration (100 μM), BDSF caused a complete growth inhibition of *C. albicans*, whereas DSF and other signals had no effect (Figure 5e). While the corresponding molecular mechanisms remain to be further investigated, these apparent dosage-dependent dual functions of BDSF on the fungal morphology and growth may present an exciting prospect for treatment of *C. albicans* infections.

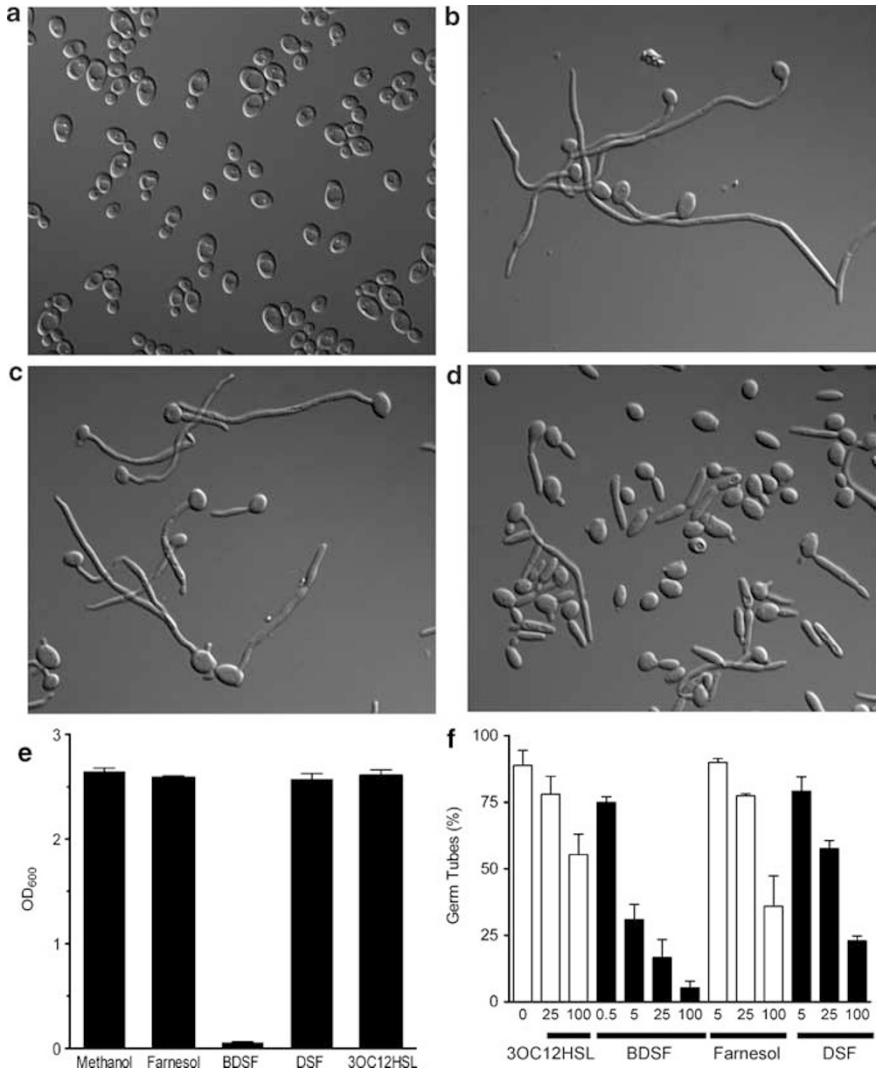

Figure 5 The effect of BDSF on *C. albicans* growth and germ tube formation. *C. albicans* cells were grown under non-induction conditions (30 °C) (**a**), or under induction conditions (serum extract, 37 °C) (**b**). In (**c** and **d**), the cells were grown under the same condition as in (**b**) but treated with 5 μM of farnesol and BDSF, respectively. The photos were taken 3 h after induction. (**e**) The effect of signal molecules on *C. albicans* yeast cell growth. The OD_{600} was determined 24 h after growth at 30 °C with agitation. (**f**) Comparison of the inhibitory activity of BDSF and related signals on germ tube formation of *C. albicans* 3 h after induction. The experiment was performed twice, and each time at least 400 cells were counted per treatment. The error bars show the standard deviations.

The yield of 7 mg of pure BDSF isolated from 70 l of cultures translates to a minimum of 0.5 μM present in overnight cultures of *B. cenocepacia* without taking into account the expected losses during the purification process. Significantly, even at this concentration, BDSF reduced the hyphal growth of *C. albicans* by about 15% (Figure 5f), which suggests that the signal might play a role in cross-kingdom microbial competition in ecosystems. This speculation was demonstrated by the findings that deletion of the *Bcam0581* gene significantly compromised the inhibitory effect of *B. cenocepacia* on the hyphal growth of *C. albicans*; and the mutant phenotype was rescued by complementation with the same gene (Figure 6). The ability to maintain an infection at a susceptible host site that is likely populated by several microbial species could be a combination of several factors. These may include the evolved survival mechanisms that are unique to ecological niche as well as signal interference and communication systems that can result in a competitive edge. There is increasing evidence that inter-genus and cross-kingdom communication is a widespread phenomenon (Zhang and Dong, 2004; Bassler and Losick, 2006). The ability of BDSF to phenotypically influence two organisms of different evolutionary lineage underscores its potential as a cross-kingdom and inter-genus signal, which may have a significant impact on the ability of Bcc to establish and maintain an infection in the host.

This work has uncovered a new DSF-like signal from bacterial pathogen *B. cenocepacia* and

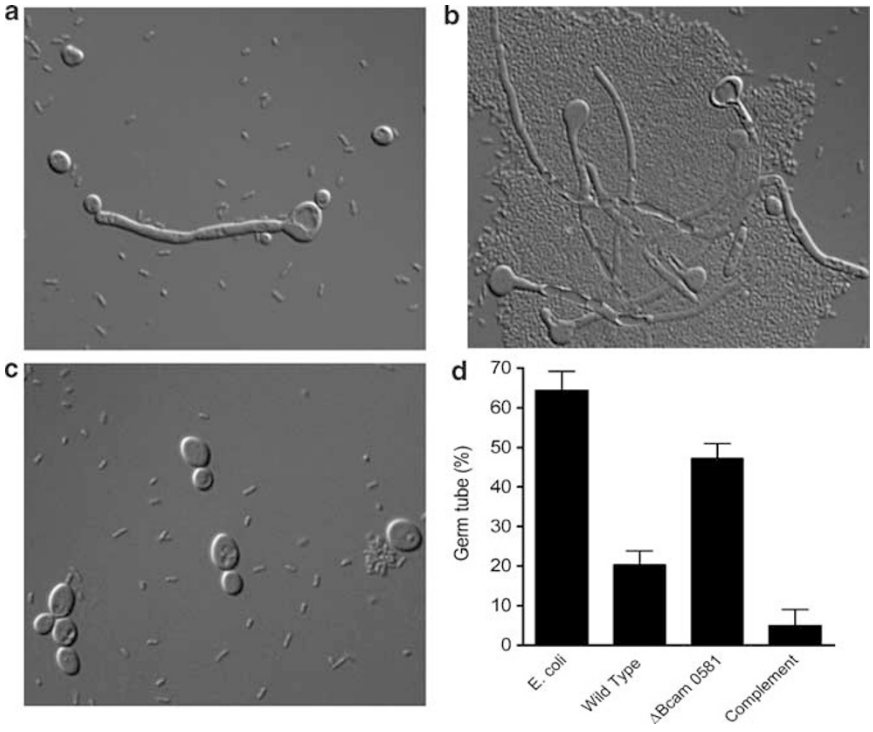

Figure 6 The inhibitory effect of *B. cenocepacia* on *C. albicans* germ tube formation was dependent on the BDSF synthase gene *Bcam0581*. *C. albicans* cells cocultured with (**a**) wild-type *B. cenocepacia* J2315, (**b**) BDSF-deficient mutant Δ*Bcam0581* and (**c**) the complemented strain Δ*Bcam0581*(pMLS7-*Bcam0581*). (**d**) The percentage of germ tube formation of *C. albicans* in the presence of different bacterial strains. *E. coli* strain DH5α was included in the coculture experiment as a negative control. Experiment was repeated three times and each time at least 400 cells were counted per sample. The error bars show the standard deviations.

demonstrated its role in microbe–microbe interaction under *in vitro* conditions. Elucidation of the chemical structure and the gene encoding for synthesis of BDSF, which is highly conserved in *Bcc* complex, provides a new platform to explore potential genetic and signaling mechanisms that may modulate the physiology and virulence of these important bacterial pathogens. Furthermore, the finding that BDSF is a highly potent inhibitor on *C. albicans* hyphal growth raises intriguing questions on the molecular mechanism of signal interference and the potential role of this signal in competition between *B. cenocepacia* and *C. albicans* under *in vivo* conditions. Particular noteworthy is that the *Bcc* and *C. albicans* are frequent inhabitants of human and animals (Kerr, 1994; Hermann *et al.*, 1999), identification of BDSF from *B. cenocepacia* thus underpins the potential ecological significance of DSF-like signals in bacteria–fungi interactions and competitions.

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