THE BDSF-DEPENDENT CELL-CELL COMMUNICATION IN *BURKHOLDERIA CENOCEPACIA*

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SUMMARY

The *Burkholderia cepacia* complex (*Bcc*) has emerged as a major opportunist pathogen for the immunocompromised individuals in particular the patients with cystic fibrosis and chronic granulomatous disease. *Bcc* consist of at least 9 species that are phenotypically similar but genetically distinct, and all the species are capable of causing infections. Among them, *B. cenocepacia* constitutes the majority of the transmissible and epidemic strains and is highly virulent. In this study, a novel signaling molecule designated as BDSF from *B. cenocepacia* has been identified and characterized. BDSF is a structural homologue of DSF, which is the quorum sensing signal produced by the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*). In addition, the genetic analysis has demonstrated that the ORF *Bcam0581* is essential for BDSF production and can genetically substitute for the DSF synthase gene *rpfF* in *Xcc*. Furthermore, the dimorphic transition of *Candida albicans* was shown to be inhibited by exogenous addition of BDSF or co-culturing with *B. cenocepacia*. These data indicate that 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. As *B. cenocepatia* and *C. albicans* are frequently encountered human pathogens, identification of BDSF signal and its activity thus provide a new insight into the molecular grounds of their antagonistic interactions, whose importance to microbial ecology and pathogenesis is now becoming evident.

The role of BDSF in the *B. cenocepacia* intraspecies cell-cell communication was subsequently characterized. The results show that production of BDSF is under the stringent transcriptional control and the molecule accumulates in a cell density-dependent manner, typically found with quorum sensing signals. A *B. cenocepacia* J2315 mutant with a deleted *Bcam0581* gene, which encodes an enzyme essential for BDSF production, exhibited a growth defect in minimal but not in rich medium, decreased virulence gene expression and attenuated virulence in a zebrafish infection model. Exogenous addition of BDSF to the mutant rescues virulence gene expression but fails to restore its growth defect in minimal medium. Further analysis shows that Bcam0581, but not BDSF, is associated with the *B. cenocepacia* ATP biogenesis. The genetic and biochemical analyses showed that some of the BDSF-regulated genes are also controlled by the AHL-dependent QS system and are thus co-regulated by two cell-cell signaling systems. These data demonstrate that in addition to the role in the interspecies signal interference, BDSF and its synthase are also important for virulence and physiology of *B. cenocepacia*.

Pseudomonas aeruginosa usually share the same niche as *B. cenocepacia* in cystic fibrosis patients. As a human pathogen, this bacterium is a major source of opportunistic infections in both immunocompromised individuals and cystic fibrosis patients. Besides to be a QS signal in *B. cenocepacia*, BDSF was revealed to interfere with *P. aeruginosa* through inhibiting on its QS systems and type III secretion system (T3SS). The bioassay results showed that exogenous addition of BDSF decreased the transcriptional expression of *lusR*, *rhlI*, *rhlR* and *pqsR*, which was consistent with the reduction of the production of BHL, PQS and virulence factors. Moreover, treatment with BDSF inhibited the gene

expression of master regulators and effectors of T3SS of *P. aeruginosa*, finally causing the attenuation of T3SS-meidated cytotoxicity of *P. aeruginosa* to HeLa cell model.

Given the important roles of BDSF in the intraspecies and interspecies cell-cell communication, it becomes intriguing to determine whether BDSF is conserved in other members of the *Burkholderia cepacia* complex. By using a combination of high performance liquid chromatography, spectrometry and biological activity analysis, the results showed that five out of the nine genomovars of the *B. cepacia* complex produce BDSF as a sole DSF-family signal, whereas the other four genomovars produce not only BDSF but also a new DSF-family signal. This new signal was characterized as *cis*-11-methyldodeca-2, 5-dienoic acid (CDSF). Interestingly, it was found that DSF, which was originally identified in *Xanthomonas campestris*, is produced by one member of the *B. cepacia* complex, i.e., *B. multivorans*. Biological activity analysis showed similar to DSF and BDSF, the newly identified CDSF is a potent signal in regulation of biofilm formation and virulence factor production of *Xcc* and *B. cenocepacia*, and in modulation of morphological transition of *Candida albicans*. These results present further evidence that the DSF-like molecules are widely conserved signals with roles not only in the bacterial quorum sensing but also in microbial ecology through interspecies cell-cell communication.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Quorum sensing (QS) in Bacteria

1.1.1 AHL-mediated QS in Gram-negative bacteria

Over the last twenty years, it has become increasingly clear that many microorganisms can coordinate their communal behavior in a process known as "quorum sensing" (QS) (Fuqua *et al*., 1994; Fuqua *et al*., 2001; Miller and Bassler, 2001). This cell-to-cell communication mechanism is employed to play a central role in mediating the bacterial cooperative behavior. In Gram-negative bacteria, most of the quorum-sensing signals are identified as *N*-acyl homoserine lactones (AHL), which are synthesized by the *N*-acylhomoserine lactone synthases known as the LuxI homologs. After diffusing outside the bacterial cells, AHL molecules accumulate in the culture supernatants at a rate proportional to the increase in cell density. Once the AHL concentration exceeds a certain threshold value, these signals return back and bind to the AHL response receptors known as the LuxR homologs. These intracellular receptors are transcriptional regulators, whose activity alters upon binding the AHL ligand, thereby eliciting a change in target gene transcription (Fig. 1-1A).

The first QS system was described in *Vibrio fischeri* (Neaslon *et al*., 1970; Eberhard, 1972), a symbiotic species that provides its marine eukaryotic hosts with light. Light emission depends on the transcription of the luciferase operon, which occurs when the concentration of the autoinducer reaches a threshold level. The working model of QS in *V.*

fischeri is that LuxI constitutively produces *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which passively diffuses across the membrane. Hence, along with the increase of bacterial cell density, the OHHL signal molecules accumulate accordingly in the culture supernatants. At the same time, LuxR is expressed and intracellularly binds to OHHL. Upon binding to the lignad OHHL, LuxR undergoes a conformational change, which in turn promotes the multimerized protein to bind to the operator region called the *lux* box, which is localized at upstream of *luxICDABEG*, thereby stimulating the binding of RNA polymerase to the intervening promoter region (Urbanowski *et al*., 2004). In 1995, the second AHL molecule was identified in *V. fischeri* as *N*-octanoyl-(L)-homoserine lactone (OHL) (Gilson and Dunlap, 1995). Intriguingly, the synthesis of this molecule was found to be catalyzed by AinS, which has no similarity to LuxI. AinS employs the same basic substrates for OHL synthesis (SAM and octanoyl-ACP) as LuxI (Hanzelka *et al*., 1999). This finding suggests that the QS signalling systems have evolved at least twice during evolutionary history in *V. fischeri*. The AinS-derived OHL is produced at relatively low cell densities when the bacteria do not need to luminescence. At higher cell densities, the induction of *luxR* expression activates the expression of *luxICDABEG*. Thus, the QS *in V. fischeri* is a finely-tuned, sequential process that ensures the proper timing of the gene expression during bacterial growth.

After discovery of the AHL-mediated QS systems in *V. fischeri*, subsequently, similar AHL quorum sensing signals were identified in some other bacterial species, including *Agrobacterium tumefaciens* (Zhang *et al*., 1991; Zhang *et al*., 1993; Piper *et al*., 1993), *Erwinia carotovora* (Bainton *et al*., 1992; Jones *et al*., 1993) and *Pseudomonas*

aeruginosa (Passador *et al*., 1993). These discoveries indicate that a common gene regulation mechanism similar to the *V. fischeri* bioluminescence autoinduction system indeed exists in diverse bacterial species. After that, many more bacterial species have been known to produce the AHL-family quorum sensing signals (Pearson *et al*., 1994; Throup *et al*., 1995; Bassler *et al*., 1997; Cha *et al*., 1998; Costa and Loper, 1997; Swift *et al*., 1997; Gotschlich *et al*., 2001). These bacteria utilize the AHL-dependent quorum sensing systems to regulate different biological functions, which include bioluminescence, plasmid transfer, biofilm formation, virulence, antibiotics production and swarming motility. Taken together, the accumulated lines of evidences have indicated that the AHL-dependent system is a global gene regulation mechanism in Gram-negative bacteria

1.1.2 Oligopeptide-mediated QS in Gram-positive bacteria

Similar to the AHL-dependent QS in Gram-negative bacteria, there are also several processes in Gram-positive bacteria which are regulated in a cell-density-dependent manner. These quorum sensing systems promote the genetic competence of *Bacillus subtilis* and *Streptococcus pneumoniae*, as well as the virulence response in *Streptococcus aureus* and the production of antimicrobial peptides (AMPs) by several other Gram-positive species. Different from the AHL-type QS in Gram-negative bacteria which use fatty acid derivatives as signals, Gram-positive bacteria utilize amino acids and short peptides usually existing as oligopeptides which are about 5-17 residues in length (Lazazzera and Grossman, 1998).

The regulation model of the oligopeptide-mediated QS in Gram-positive bacteria is distinct from the LuxRI type in Gram-negative bacteria. They usually utilize a two-component regulatory system based bacterial QS circuit. In this regulatory process, the secreted peptide signals are detected by the input domain of a membrane-bound sensor histidine kinase, which is a typical sensor component of the two-component signal transduction system consisting of a sensor and response-regulator protein. This sensing will use phosphorylation to transfer information to the cognate response regulator, which then regulates the target gene expression directly or indirectly (for reviews see Stock *et al*., 2000; Parkinson and Kofoid, 1992) (Fig. 1-1B). Another common feature in many of these quorum-sensing modes is the involvement of a dedicated ATP-binding cassette (ABC) exporter in the secretion of the peptide signal. Furthermore, in the AIP two-component QS system, before being secreted out, the precursor peptide is modified to generate the final AIP signals, which then are exported by the corresponding ABC transporter to start the QS regulation process (Fig. 1-1B).

1.1.3 Other QS signals

AI-2 was originally recognized as a quorum-sensing signal in *Vibrio harveyi* by Bassler *et al*. (1993). Since then, this type of signaling system has been discovered in many gram-negative bacteria. The AI-2-type signaling is involved in the regulation of the bioluminescence in *V. harveyi* (Bassler *et al*., 1994), the type III secretion in *Escherichia coli* O157:H7 (Sperandio *et al*., 1999), and the virulence factor VirB in *Shigella flexneri* (Day and Maurelli, 2001). Over 400 genes could be influenced by AI-2 as indicated by the microarray analysis conducted with *E. coli* strains (DeLisa *et al*., 2001;

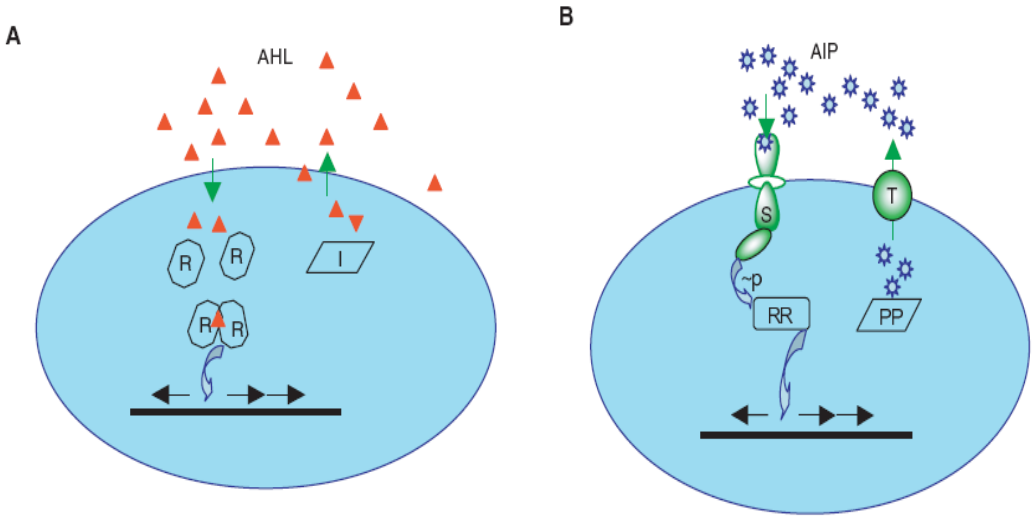


Fig. 1-1. Quorum sensing models in bacteria (copied from Zhang and Dong, 2004). (A) The LuxIR type QS system in Gram-negative, the LuxI protein (I) catalyses the synthesis of AHL signals. As bacterial cells proliferate, the accumulated AHL signals initiate QS signalling by binding to LuxR-type transcription factors (R). The LuxR-AHL complex induces the expression of target genes. (B) The AIP two-component system QS in Gram-positive QS system, precursor peptide (PP) are modified and the resulting QS signals (AIP) exported by an ABC transporter (T). The AIP signals are detected by sensor-histidine kinases (S), and the sensory information is transferred to the cognate response regulator (RR) by phosphorylation relay (P), which induces the target gene expression.

Sperandio *et al*., 2001). Furthermore, it has been previously shown that AI-2 signals from *Salmonella enterica* cross communicate and activate the *V. harveyi* indicator strain (Surette *et al*., 1998). This describes AI-2 as a signal not only for intracellular regulation but also for interspecies communication.

A new class of autoinducers was recently identified in strains of *Pseudomonas* based on their ability to activate the AHL biosensors. Structural analysis indicated that these new signal molecules were the diketopiperazines (DKPs) cyclo(L-Ala-L-Val) and cyclo(L-Pro-L-Tyr), respectively (Holden *et al*., 1999). Additional DKPs have also been identified in *Proteus mirabilis*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Pseudomonas fluorescens*, and *Pseudomonas alkaligenes* (Degrassi *et al*., 2002; Holden *et al*., 1999). However, the DKPs need a much higher concentration to activate AHL biosensors (Holden *et al*., 1999), which indicates that these compounds may not play a significant role in the natural environment. Another report of cyclic dipeptides from *Pseudomonas putida* suggests that the DKPs are copurified with AHLs and are capable of activating various bacterial AHL biosensors (Degrassi *et al*., 2002). Interestingly, DKPs act as AHL antagonists in some strains and as agonists in others. This ability of DKPs from one bacterial strain to cross-communicate with the quorum-sensing networks of unrelated bacteria adds complexity and diversity to the quorum-sensing languages.

Another cell density factor was proposed to be involved in the population density-dependent regulation of the *nod* genes in *Bradyrhizobium japonicum*, which is a gram-negative, nitrogen-fixing symbiont of many leguminous plants. The *nod* genes are

repressed at high population densities, and this is mediated by small diffusible signal molecules present in conditioned media of wild-type bacterial cultures (Loh *et al*., 2002b). Structural characterization elucidated that the cell density factor is 2-{4-[[4-(3-aminooxetan-2-yl) henyl] (imino) methyl] phenyl} oxetan-3-yl amine, which has been named as bradyoxetin. This structure of bradyoxetin is similar to that of a siderophore, mugeneic acid. In accordance with this similarity, bradyoxetin appears to be regulated by iron concentrations in the medium. It is maximally produced under iron-deficient conditions and repressed when Fe^{3+} is provided in excess (Loh *et al*., 2002a). A preliminary analysis suggested that bradyoxetin-like signal molecules were present in the extracts of various *Rhizobium* strains as well as other species of the α-proteobacterial group (Loh *et al*., 2002a).

Additionally, besides the signals mentioned above, a few more QS signals have been identified in bacteria, including the AI-3 autoinducer in *E. coli* (Sperandio *et al*., 2003), and the diffusible signal factor (DSF) in *Xanthomonas campestris* (Barber *et al*., 1997; Wang *et al*., 2004). The structure of DSF was recently identified as *cis*-11-methyl-2-dodecenoic acid (Wang *et al*., 2004). A detailed literature review of the DSF signals will be described in the later section of this chapter.

1.2 Quorum Sensing in *Burkholderia cenocepacia*

1.2.1 The *Burkholderia cepacia* complex

Burkholderia cepacia is an opportunistic pathogen that infects the immunocompromised patients with cystic fibrosis and chronic granulomatous disease (McDowell *et al*., 2004;

Mohr *et al*., 2001). It was first discovered in 1949 by Walter Burkholder at the Cornell University in rotting onions and described as a human pathogen in 1950 (Burkholder, 1950). The first "epidemic" strain of *Burkholderia cepacia* was isolated in the UK in 1986 (Govan *et al*., 1993). Infected with *B. cepacia*, cystic fibrosis patients will suffer from cepacia syndrome, a necrotizing peneumonia with fever and occasionally bacteremia, which causes a rapid and pulmonary decline (Isles *et al*., 1984). Although the clinical outcomes are different for individuals, infections in some patients cause serious outcomes and even fatal disease (Mahenthiralingam *et al*., 2001; Mahenthiralingam *et al*., 2002). Moreover, isolates of the *Burkholderia cepacia* complex (Bcc) can spread between CF patients through contact, which mostly occurs inside hospital. Sequence variation in the 16S rRNA gene is generally useful for differentiating bacterial species. However, among the Bcc species there is limited sequence diversity in this gene, which share a high similarity of more than 97.7%. Nevertheless, *recA* shows a high level of variation and becomes one of the main methods to distinguish members of the Bcc complex (Coenye *et al*., 2001). A taxonomic approach based on multiple tests known as polyphasic taxonomy was used to define the species diversity of Bcc, which includes tests such as biochemical profile analysis, whole-cell protein profile and fatty acid analysis, 16S rRNA and *recA* gene sequencing, as well as DNA-DNA hybridization (Coenye *et al*., 2001; Vandamme *et al*., 1997). These taxonomic methods divided the *Burkholderia cepacia* complex into at least nine distinct genomic species or genomovars. They are *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolsa*, *Burkholderia ambifaria*, *Burkholderia anthina*, and *Burkholderia pyrrocinia* (Lipuma 2005). Within

this group, the most transmissible and epidemic strains are *B. cenocepacia* and *B. multivorans*, which are highly virulent, causing significant mortality among CF patients (Mahenthiralingam *et al*., 2001; Mahenthiralingam *et al*., 2002; Rebecca *et al*., 2005).

1.2.2 *Burkholderia cenocepacia* and its pathogenicity

Burkholderia cenocepacia belongs to the genomovars III of the *B. cepacia* complex. It comprises about 83% of all the *B. cepacia* complex strains isolated from the CF patients in Canada (Speert *et al*., 2002), and 50% of the isolates in the United States (Lipuma *et al*., 2001). Unlike most other pathogens in CF, which typically remain confined to the endobronchial spaces, *B. cenocepacia* can traverse airway epithelium to cause bacteremia and sepsis.

Besides humans, *B. cenocepacia* can also infect a wide range host such as rodents (Bernier *et al*., 2003; Speert *et al*., 1999), nematodes (Kothe *et al*., 2003), unicellular organisms (Fehlner-Gardier *et al*., 2002), and plants (Bernier *et al*., 2003), suggesting that *B. cenocepacia* is likely to produce multiple, potentially novel virulence factors, which consist of lipase, protease, chitinase and siderophores (Gotschlich *et al*., 2001). Several putative genomic DNA fragments enriched for the virulence genes of *B. cenocepacia* have been identified; and sequences analysis demonstrated that some of these putative genomic DNA fragments are unique for this genomovars genus.

1.2.3 QS systems in *Burkholderia cenocepacia*

Studies showed that *B. cenocepacia* utilizes the *N*-acyl homoserine lactones (AHL)-dependent quorum-sensing system to regulate particular genes in a cell density-dependent mechanism. It has the LuxI/R homologs, CepIR, which is widely distributed among the *B. cepacia* complex strains (Lewenza *et al*., 1999; Gotschlich *et al*., 2001; Lutter *et al*., 2001). This regulatory system consists of the AHL synthase CepI and the transcriptional regulator CepR (Gotschlich *et al*., 2001; Lewenza *et al*., 1999). CepI directs the synthesis of *N*-octanoylhomoserine lactones (C8-HSL, OHL) and minor amount of *N*-hexanoylhomoserine lactones (C6-HSL, HHL). At a low density, the cells produce basal levels of AHLs by the CepI synthase, which then diffuse to the environment across the cell membrane. As the cell density increases, the diffusible AHL signals molecules accumulate in the growth medium; when it reaches the threshold level, these signals interact with the cognate receptor CepR; and the CepR-AHL complex then in turn increase or repress the expression of the target genes. An interesting feature of the CepIR system is that CepR negatively regulates its own expression but positively regulates the *cepI* expression at the transcriptional level (Lewenza and Sokol, 2001). Additionally, this QS system positive controls expression of extracellular proteases, chitinases, swarming motility, and biofilm formation (Lewenza *et al*., 1999; Huber *et al*., 2001).

B. cenocepacia strains contain a genomic island designated as the **c**eno**c**epacia **i**sland (cci). This pathogenicity island is a horizontally acquired genomic region. It has an atypical GC content; and it is the first pathogenicity island that actually encodes classical LuxRI homologs (Baldwin *et al*., 2004). The cci island contains an *N*-acylhomoserine lactones synthase gene named *cciI*, and a response regulator gene, *cciR*. Phylogenetic

analysis demonstrated that the CepIR and CciIR systems are distinct from each other. AHL production analysis indicated that CciI catalyzes the synthesis of *N*-hexanoyl-L-homoserine (C6-HSL, HHL) and minor amounts of *N*-octanoyl-L-homoserine lactones (C8-HSL, OHL) (Malott *et al*., 2005), which is in direct contrast to the CepIR system. Research shows that the CciIR QS system is involved in regulation of some virulence factors production and the bacterial swarming motility (Hacker and Kaper, 2000). It is also required for persistence and inflammation in rat lung infection model (Mahenthiralingam *et al*., 2005).

In addition, *B. cenocepacia* as well as other *Burkholderia* species have been reported to produce 2-heptyl-4-quinolone (HHQ), the precursor of the *Pseudomonas aeruginosa* quinolone signal molecule (PQS); and it has been suggested that HHQ may also be used for cell-cell communication (Diggle *et al*., 2006a; Diggle *et al*., 2006b). In the case of the human pathogen *Burkholderia pseudomallei*, loss of HHQ production was shown to affect colony morphology and increase the elastase production (Diggle *et al*., 2006a; Diggle *et al*., 2006b).

1.3 DSF cell-cell communication system

1.3.1 The wide distribution of the DSF-family signals in bacteria

Recently, the diffusible signal factor (DSF) family signals have been implicated as a novel family of cell-cell communication signals in many bacteria species (Table 1-1) (He and Zhang, 2008). DSF was originally recognized in *Xanthomonas campestris* pv. *campestris* (*Xcc*) as a novel regulatory system required for pathogenicity (Barber *et al*.,

1997). Subsequently, the DSF-like activity or similar signals were identified in *Xanthomonas axonopodis* pv *citri* (Andrade *et al*., 2006), *Stenotrophomonas maltophilia* (Fouhy *et al*., 2007), *Xanthomonas oryzae* pv. *oryzae* (Tang *et al*., 1996; Chatterjee and Sonti, 2002) and *Xylella fastidiosa* (Newman *et al*., 2004). Wang *et al*. (2004) tested 31 bacterial strains from 13 bacterial species, and found that 25 strains from 10 species produce the DSF-like activity, including *Xcc*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas albilineans*, *Pseudomonas aeruginosa*, *Mycobacterium avium*, *Mycobacterium chelonae*, *Mycobacterium smegmatis*, *Mycobacterium intracellulare* and *Mycobacterium kansasii*. These evidences support the notion that the DSF-family signals are a new family of QS "language" which appears to be widely distributed in bacteria.

1.3.2 Regulatory mechanisms of DSF signaling systems

1.3.2.1 DSF signaling pathway in *X. campestris*

Similar to the QS system in Gram-positive bacteria, the DSF-dependent cell-cell communication system in *Xcc* employs a two-component system in the signal detection and transduction. In *Xcc,* RpfF is responsible for DSF signal production, while RpfC and RpfG constitute a two-component system for DSF perception and signal transduction (Barber *et al*., 1997; Slater *et al*., 2000; He *et al*., 2006a). In the RpfC/RpfG two-component system, RpfC is a hybrid sensor kinase consisting of five transmembrane domains (TM), a histidine kinase (HK) domain, a receiver (REC) domain, and a histidine phosphotransfer (HPT) domain. RpfG contains two major domains: a receiver domain

Table 1-1. The DSF family signals are widely conserved and modulate various biological functions in the Gram-negative bacterial pathogens.

Bacterium	DSF-like Signal	RpfF similarity	DSF-mediated Biological functions	Reference
Xanthomonas campestris pv. *campestris*	*cis*- Δ 2-11-methyl-dodecenoic acid	100%	Virulence and biofilm dispersal	Wang *et al*., 2004
Xanthomonas campestris pv. *vesicatoria*	ND	97%	ND	Thieme *et al*., 2005
Xanthomonas axonopodis pv. *glycines*	ND	97%	ND	Thomthampitak *et al*., 2008
Xanthomonas oryzae pv. *oryzicola*	ND	96%	ND	ND
Xanthomonas oryzae pv. *oryzae*	ND	95%	Virulence, tetracycline resistance and iron uptake	Chatterjee & Sonti, 2002
Stenotrophomonas maltophilia	*cis*- Δ 2-11-methyl-dodecenoic acid and seven other similar signals	80%	Virulence, antibiotic resistance and ferric citrate uptake	Huang & Wang, 2007b
Xylella fastidiosa	12-methyl-tetradecanoic acid	67%	Virulence, Biofilm formation and vector transmission	Colnaghi Simionato *et al*., 2007
Xanthomonas axonopodis pv. *citri*	ND	54%	Virulence	Andrade *et al*., 2006
Methylobacillus flagellatus	ND	40%	ND	Chistoserdova *et al*., 2007
Thiobacillus denitrificans	ND	40%	ND	Beller *et al*., 2006
Leptospirillum sp.	ND	39%	ND	Lo *et al*., 2007
Mariprofundus ferrooxydans	ND	37%	ND	ND
Sulfurovum sp.	ND	37%	ND	Nakagawa *et al*., 2007

and an HD-GYP domain, with conserved HD and GYP motifs (Ponting *et al*., 1999). The signaling mechanism by which RpfC/RpfG employs to transmit the DSF signal has been characterized. Substitution of the conserved residues associated with phosphorelay in the HK-, REC-, HPT-domain in RpfC or the receiver domain in RpfG abolishes the DSF-dependent functions, such as modulation of virulence factor production and biofilm dispersal (He *et al*., 2006a). Deletion of the signal receiver domain in RpfC and RpfG caused the same results. The addition of DSF to the *rpfG* or *rpfC* mutant can not restore the production of EPS and extracellular enzymes to the levels of wild-type strain (He *et al*., 2006a). Based on these results, it was concluded that the RpfC/RpfG system uses the conserved phosphorelay mechanisms to transduce the DSF signal in modulation of virulence factors production (He *et al*., 2006a). The conclusion was further strengthened by the reconstruction of the RpfC/RpfG mediated DSF signaling in *Pseudomonas aeruginosa* (Ryan *et al*., 2006).

Besides functioning as the DSF sensor, RpfC negatively controls the DSF production through a novel posttranslational mechanism involving protein-protein interaction (He *et al*., 2006a). It has been shown that RpfC interacts with RpfF tightly and specifically (He *et al*., 2006a; Andrade *et al*., 2006). This coupled with the fact that the *rpfC* mutants produce higher level of DSF than the wild type suggesting a novel model of autoregulation of QS signal biosynthesis. At a lower cell density, the extracellular concentration of DSF is below a threshold and RpfC is locked in an unphosphorylated state. To keep the basal level of DSF synthesis, RpfC tightly binds to RpfF to limit its DSF synthase activity. At high cell density, the accumulated DSF triggers the

autophosphorylation of RpfC and release of RpfF, leading to the enhance production of DSF signals.

Several lines of evidences support the notion that RpfG acts as a downstream phosphodiesterase to degrade the second messenger cyclic-di-GMP through HD-GYP domain. Purified HD-GYP domain can degrade cyclic-di-GMP to generate GMP, whereas it does not have any effect on other nucleotide analogues, including ATP, GTP, GMP, cGMP, and cAMP (Ryan *et al*., 2006). Point mutation of the conserved residues H and D of the HD-GYP domain abrogates both the enzymatic activity against cyclic-di-GMP and the regulatory activity on the virulence factor production. Combined with the finding that the HD-GYP domain alone can substitute RpfG in regulation of EPS and extracellular enzymes production (He *et al*., 2006b; Ryan *et al*., 2006), it becomes clear that the response regulator RpfG performs its regulatory activity by enzymatic degradation of cyclic-di-GMP to coordinate the expression of the downstream genes in the DSF signaling pathway.

The conserved global regulator Clp, which shows a strong homology to the cAMP nucleotide receptor protein Crp of *Escherichia coli*, is essential for the DSF-dependent regulation of virulence factor production (He *et al*., 2007). The expression of *clp* is slightly upregulated by DSF through the RpfC/RpfG two-component system, although its expression in the *rpfF* mutant remains relatively high (He *et al*., 2007). Clp is a c-di-GMP effector. It specifically binds to c-di-GMP with high affinity and induces the allosteric

conformational changes, which finally abolishs the interaction between Clp and its target gene promoter (Tao *et al*., 2009).

1.3.2.2 DSF signaling pathway in *X. fastidiosa*

Cell–cell signaling in *Xylella fastidiosa* is mediated by a different fatty acid, 12-methyl-tetradecanoic acid, which is a homologue of DSF of *Xcc* (Colnaghi Simionato *et al*., 2007). Similar to *Xcc*, RpfF and RpfC are found to be employed for the production and perception of putative DSF signal (Newman *et al*., 2004; Chatterjee *et al*., 2008). However, two distinct pathways for DSF perception in *X. fastidiosa* have been indicated by the divergent expression patterns of biofilm and virulence related genes (Chatterjee *et al*., 2008). It was proposed that RpfF/RpfC share the same downstream pathway for the negative regulation of virulence genes, but use different pathways for the activation or inhibition of genes required for biofilm formation. The next challenge will be to identify the downstream regulators specifically responsible for the different pathways in biofilm and virulence. It also remains unclear that whether Clp is involved in the DSF signaling pathways in *X. fastidiosa*. The results from *Xcc* and *X. fastidiosa* seem to suggest that the DSF-signaling systems may share a general role in the modulation of virulence but they may differ in the regulatory mechanisms among different bacterial pathogens.

1.3.2.3 DSF signaling pathway in *S. maltophilia*

The pathogen *Stenotrophomonas maltophilia* has also evolved a DSF system for coordinating the gene expression. Besides producing the DSF of *Xcc*, *S. maltophilia* WR-C employ RpfF and RpfB to produce seven other structural derivatives that are not shared

by *Xcc* (Ryan *et al*., 2008). In addition, a cyclic AMP (cAMP) receptor protein (CRP) acts to positively regulate the *rpfF* expression, possible by binding the upstream regions of the *rpfF* promoter (Huang and Wong, 2007). Through this transcription regulation, DSF positively regulates the expression of FecA, a ferric citrate receptor important for acquisition of iron from the environment. Whether the RpfC/RpfG two-component system is involved in the regulation of *fec* by DSF signal is unknown. In addition, the direct binding of the *rpfF* promoter by Crp remains to be investigated.

1.3.3 Biological functions of the DSF signaling systems

Numorous studies of the *Xcc* DSF-deficient mutants have established the importance of the DSF signaling system in regulation of the production of virulence factors and of the bacterial virulence. Reduced production of EPS and extracellular enzymes, such as proteases and cellulases, were observed in the mutant *rpfF*::Tn*5lac* of *Xcc* strain 8004 and the *rpfF* deletion mutant of XC1 (Tang *et al*., 1991; Barber *et al*., 1997; He *et al*., 2006b). Null mutation of *rpfF* in *Xcc* 8004 or XC1 results in the substantial decrease in the production of EPS and extracellular enzymes and notable reduction of virulence to plant hosts (Barber *et al*., 1997; He *et al*., 2007), suggesting that DSF positively controls the production of virulence factors and regulates the bacterial virulence. Another important DSF-dependent biological function is the biofilm dispersal. Mutation of the *rpfF* gene in both *Xcc* strains 8004 and XC1 causes the formation of cell aggregates (biofilm) (Dow *et al*., 2003; He *et al*., 2006b). Moreover, addition of DSF to the *rpfF* mutant abolishes biofilm formation and the mutant grows in planktonic form, suggesting a critical role of DSF in the modulation of *Xcc* switching between the planktonic grow

mode and the biofilm growth form. Although the mechanism of DSF in the regulation of biofilm dispersal remains unclear, genome scale analysis of DSF regulon appears to provide rich information on the potential genetic pathways and the associated biological functions (He *et al*., 2006b). In addition to the role of virulence and biofilm dispersal, DSF is also involved in regulation of other biological functions of importance such as flagellum synthesis, resistance to toxin and oxidative stress, iron uptake and aerobic respiration (He *et al*., 2006b).

In *X. oryzae* pv. o*ryzae*, a pathogen of rice, the DSF signaling system positively regulates the pathogenesis. However, different from *Xcc*, insertion mutation of *rpfF* of *Xoo* leads to proficient production of EPS and extracellular enzymes, and the mutant exhibits an unusual tetracycline susceptibility phenotype and growth deficiency under low iron conditions (Chatterjee and Sonti, 2002). Mutation of *rpfC* in *Xoo* has no effect on the production of extracellular enzymes, but reduces the level of the EPS biosynthesis and virulence (Tang *et al*., 1996).

The DSF signaling system in *S. maltophilia* plays a role in regulation of various biological functions. Mutation of *rpfF* in *S. maltophilia* gives rise to reduced motility, decreased production of extracellular protease, decreased tolerance to a range of antibiotics and heavy metals, altered LPS structure and formation of cell aggregates, which are functionally connected with the virulence of *S. maltophilia* (Fouhy *et al*., 2007). The study from other group indicates that inactivation of *rpfF* or *rpfB* in *S. maltophilia* strain WR-C decreases the transcription of *fecA*, which encodes a ferric citrate receptor

that transports exogenous siderophore-ferric citrate from the environment into the bacterial periplasm, and addition of synthetic DSF restores the *fecA* expression (Huang and Wong, 2007), suggesting a link between the *rpf*/DSF system and ferric citrate uptake.

In *X. fastidiosa*, RpfF is sufficient for biosynthesis of a DSF-like signal, which appears to be different from the DSF of *Xcc* in the chemical structure. It was tentatively identified as 12-methyl-tetradecanoic acid (Colnaghi Simionato *et al*., 2007). Mutation of *rpfF* in *X. fastidiosa* gives rise to enhanced virulence, impaired insect transmission, and inability of biofilm formation in the insect foregut but not in plant (Newman *et al*., 2004). In contrast to the *rpfF* mutants, the *rpfC* mutants are deficient in virulence, migration along xylem vessels, insect transmission and have a hyper-attachment phenotype (Chatterjee *et al*., 2008). Consistent with their differences in virulence, the expression patterns of many downstream genes are not always the same in the *rpfF* and the *rpfC* mutants. RpfF and RpfC both positive regulate the virulence related genes such as *tolC* , *pglA*, *PD0279*; however, they show the opposite effects on the expression of the biofilm related genes such as *fimA*, *hxfA-B*, *gumJ*, indicating the gene regulation may be occurred by two different pathways involving the DSF signaling system in *X. fastidiosa*.

1.3.4 The role of the DSF-family signals in microbial ecology

DSF signals not only control a range of biological functions through intraspecies signaling; they also play an important role in the microbial ecology (Wang *et al*., 2004; Ryan *et al*., 2008). Among different *Xanthomonads*, inter-species scommunication easily occurs in may be due to the same or similar chemical structure of DSF signals produced

by these closely related bacterial pathogens (Wang *et al*., 2004). Actually, DSF-dependent inter-species communication is not restricted to *Xanthomonads*. Ryan *et al.* (2008) reported that DSF produced by *S. maltophilia* influenced the biofilm formation of *P. aeruginosa* through *PA1396*, which encodes a sensor kinase with a similar input domain as that of RpfC in *X. campestris*. This evidence implicates DSF as the "language" in the cross-talk between *S. maltophilia* and *P. aeruginosa*. Moreover, besides affecting the structure of biofilm, exogenous addition of DSF leads to up-regulation of a number of proteins involved in stress tolerance; and the enhanced tolerance of *P. aeruginosa* to polymyxins B and E (Ryan *et al*., 2008).

Besides involving in the inter-species cross-talk, DSF was shown to inhibit the morphological transistion of *Candida albicans* through signal interference (Wang *et al*., 2004). DSF is structurally similar to farnesoic acid, which is an autoregulatory substance in *C. albicans*. Farnesoic acid inhibits *C. albicans* germ tube formation and plays a key role on the regulation of morphological transition in *C. albicans* (Oh *et al*., 2001). Studies showed that DSF is not only structurally similar to farnesoic acid; it can also substitute farnesoic acid to inhibit the germ tube formation of *C. albicans* (Wang *et al*., 2004). These studies support that DSF has dual functions on the genetic regulation at the intraspecies level and the cross-talk through inter-species and inter-kingdom communications.

1.4 Aims and scope of this study

Previous studies indicated that quorum-sensing mechanisms are widely conserved in bacteria, and employed to coordinate the communal behaviors such as biofilm formation, swarming motility, plasmid transfer, antibiotic resistance and virulence factors production (Pearson, *et al*., 1994; Whiteley, *et al*., 1999; de Kievit and Iglewski, 2000; Williams *et al*., 2000). Recently, diffusible signal factors (DSF) have emerged as a novel family of cell-cell communication signals in a range of bacterial species (He and Zhang, 2008). Different from the AHL-dependent QS, the DSF signaling system usually utilizes a two-component transduction system to perceive and transmit signal to the downstream steps (Slater *et al*., 2000; Newman *et al*., 2004; He *et al*., 2006a; Chatterjee *et al*., 2008). Although the functions and the molecular mechanisms of DSF signaling systems have been partially elucidated in *Xcc* and some other Gram-negative bacterial pathogens, there are still many unanswered questions that need to be resolved. Additionally, there is no any evidence for the interactions between AHL-dependent QS system and DSF signaling system; and the knowledge of DSF to be used as a "language" in inter-species and inter-kingdom communication is also limited.

In this study, our main aim is to characterize a novel DSF signaling system in *Burkholderia cenocepacia*; and to explore its interaction with the AHL-mediated QS system. In addition, its role on the microbial ecology will also be investigated. This study will enrich our understanding of this novel DSF signaling pathway, and the roles of the DSF-familiy signals in the intraspecies and interspecies cell-cell communications. Especially, it may also shed light on the potential intriguing relationship between the AHL-mediated QS system and the DSF-dependent signaling system in *B. cenocepacia*.

This thesis is divided into 6 chapters.

Chapter 1 introduces and discusses the literatures related to this study.

Chapter 2 describes the characterization of BDSF signal and its interference with *Candida albicans* morphological transition.

Chapter 3 reports the identification of the biological roles of the QS signal BDSF and its synthase.

Chapter 4 states the structural and functional characterization of the DSF-family signals produced by the *B. cepacia* complex.

Chapter 5 describes the attenuation of virulence of *Pseudomonas aeruginosa* by BDSF through downregulation of the QS and type III secretion systems.

Chapter 6 is the general discussions and further study.

CHAPTER 2

A NOVEL DSF-LIKE SIGNAL FROM *BURKHOLDERIA CENOCEPACIA* INTERFERES WITH *CANDIDA ALBICANS* MORPHOLOGICAL TRANSITION

2.1 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 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-tohyphae 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).

The 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*.

2.2 Methods and Materials

2.2.1 Bacterial 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^{o}C in YEB medium (Zhang *et al*., 2002), while *Escherichia coli* and *B. cenocepacia* strains were grown in Luria-Bertani (LB) broth at 37^{o}C. The following antibiotics were supplemented when necessary: rifampicin, 50 μgml^{-1}; tetracycline, 10 μgml^{-1} (*Xcc* and *E. coli*) or 400 μgml^{-1} (*B. cenocepacia*); gentamycin, 100 μgml^{-1}; and trimethoprim, 300 μgml^{-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, 60mM; KH_2PO_4, 30mM; 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.

2.2.2 Bioassay of BDSF signal

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.

2.2.3 Purification of BDSF signal

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 × 150mm, Biotage Flash 12M cartridge) and eluted with ethyl acetate–hexane (20:80 v/v). The active fractions were detected using the DSF bioassay and pooled; and then were 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 × 250mm, Waters, Milford, MA, USA), eluted with methanol–water (80:20 v/v) at a flow rate of 1ml min^{-1}.

2.2.4 Structural 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 400MHz for ^{1}H or 100.5MHz 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).

2.2.5 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 (50-ggatccctcgagatgcttgtcgaa), BCAM0581KO_LR (50-aagcttggtatgtcctcgtgagatgtg); and BCAM0581KO_RF (50-aagcttcgcacggtgtaatgcgac), BCAM0581KO_RR (50-tctagaggatccacgtatcgcgtgttctcgctg), respectively. This resulted in removal of 852 bp of the 864 bp *Bcam0581* coding sequence. To facilitate construction, *Bam*HI and *Hind*III sites were included in the upstream fragment, whereas *Hind*III 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 (d0581) were identified by colony PCR using the primer pair BCAM0581KO_LF and BCAM0581KO_RR described above.

2.2.6 Complementation of strains 8004dF and d0581

The coding region of *Bcam0581* was amplified via PCR using primers pair BCAM0581-F (50-ggatccatgcaactccaatcccatcc) and BCAM0581-R (50 aagcttttacaccgtgcgcagctt). 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 d0581 and 8004dF, respectively, by tri-parental mating. The transconjugants of 8004dF and d0581 were selected on YEB agar plates containing rifampicin and tetracycline and on LB agar plates supplemented with gentamycin and trimethoprim, respectively.

2.2.7 Extracellular polysaccharide and biofilm analysis of *Xcc*

For quantification of extracellular polysaccharide (EPS) production, 10 ml of overnight YEB cultures at OD_{600} of 3.0 were centrifuged at 12,000 rpm for 20 min. The supernatants were mixed with 2.5 volumes of absolute ethanol and the mixture was incubated at $4^{o}C$ for 30 min. The precipitated EPS was isolated by centrifugation and dried overnight at $55^{o}C$ before determination of dry weights.

For analysis of biofilm formation, a single colony of *Xcc* wild type and the DSF-minus mutant 8004dF was separately inoculated and grown overnight in 5 ml of YEB medium with or without signal molecule. Methanol was added to the corresponding wild-type strain as a solvent control. After overnight incubation, bacterial samples were visualized with a phase contrast microscope (Olympus BX50). Imaging was performed using an Olympus DP70 digital camera.

2.2.8 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^{o}C. For growth experiments, overnight cultures (OD_{600}≈1.0) were diluted to OD_{600}≈0.05 in GMM medium and cultured at 30^{o}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).

2.3 Results

2.3.1 Detection of DSF-like activity in *B. cenocepacia*

In our preliminary screening, several environmental isolates, belonging to *Burkholderia* spp. based on 16S rDNA and *recA* sequence 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 (Fig. 2-1a), suggesting that *B. cenocepacia* J2315 may secrete a DSF molecule, which was tentatively designated as BDSF.

2.3.2 *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

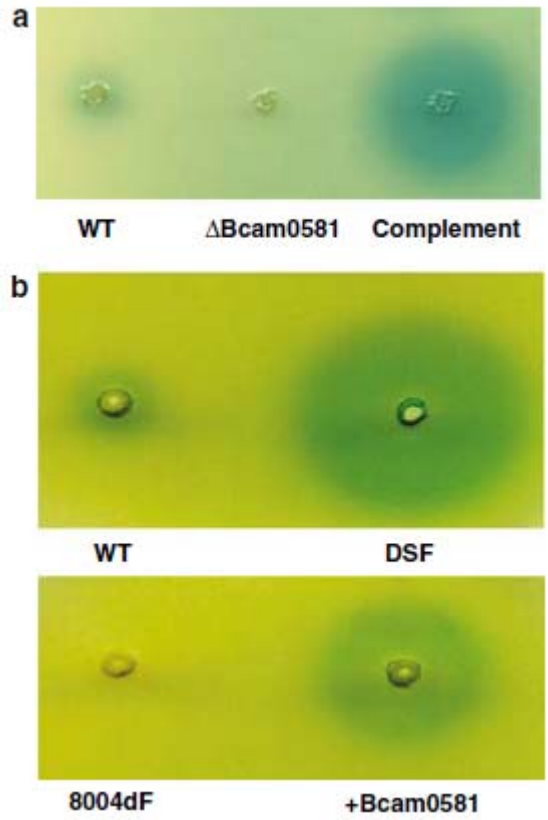


Fig. 2-1. *B. cenocepacia* produced diffusible signal factor (DSF)-like signals. (a) DSF bioassay with *B. cenocepacia* J2315 and derivatives. WT, wild-type *B. cenocepacia* J2315; complement, d0581(pMLS7-Bcam0581). (b) Expression of *Bcam0581* in the DSF-deficient mutant 8004dF of *X. campestris* pv. *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.

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* (Fig. 2-2a).

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* (Fig. 2-2b), 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.

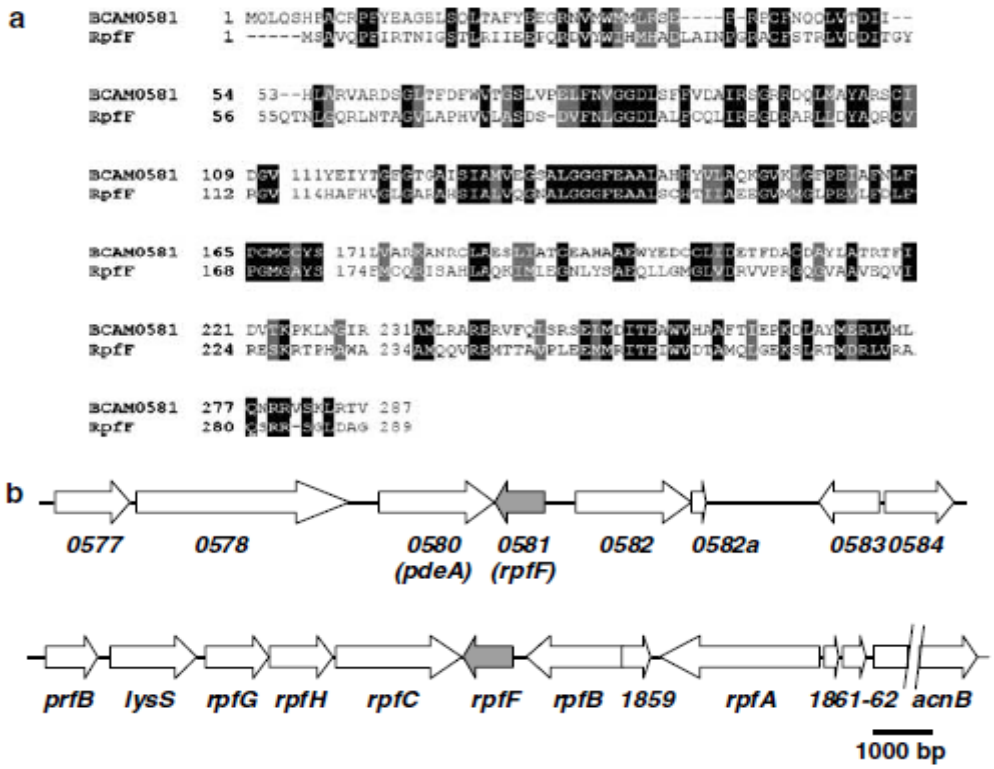


Fig. 2-2. Comparison of the peptide sequence and genomic organization of *rpfF* and its homologue from *B. cenocepacia*. (a) Alignment of protein sequences of Bcam0581 with RpfF from *Xcc*. The black and gray shading indicates the identical and similar residues, respectively. (b) Genomic organization 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*).

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 (Fig. 2-1a).

2.3.3 *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 *lac* 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 2-1b, 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* (Fig. 2-3). 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.

2.3.4 BDSF pfurification and structural analysis

The active component was collected from 70 liters of *B. cenocepacia* J2315 culture supernatants by ethyl acetate extraction and purified by flash column chromatography. About 7mg 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

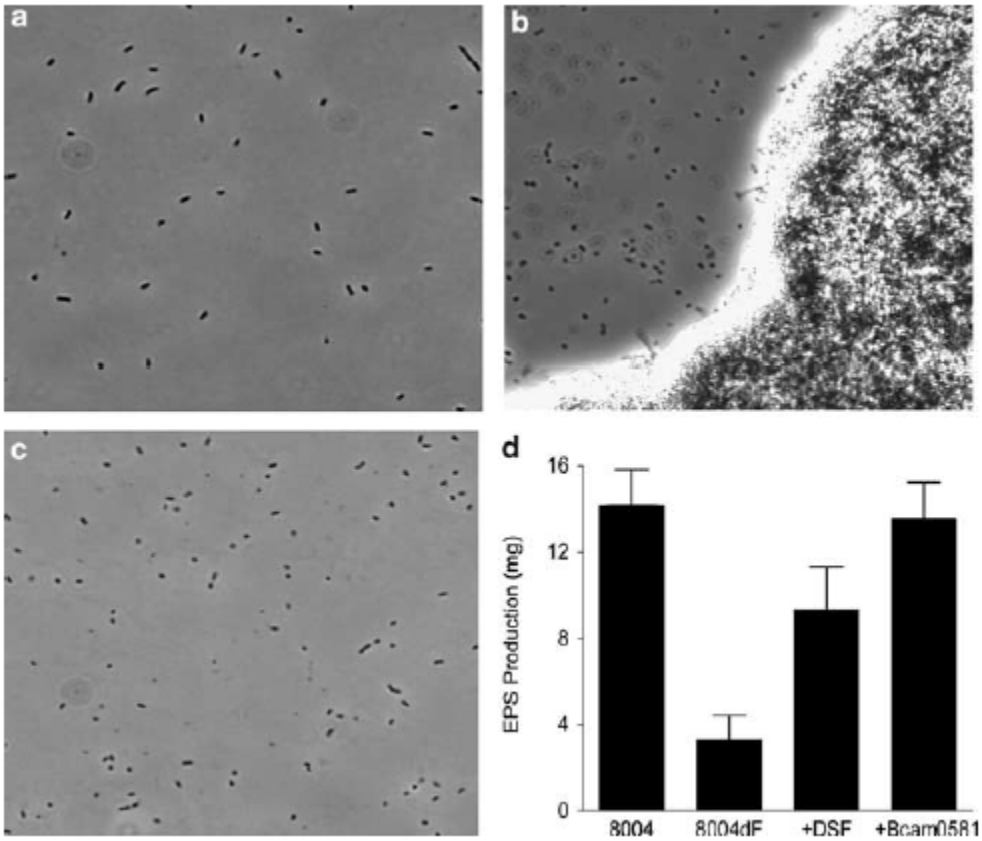


Fig. 2-3. Complementation of the *Xcc* diffusible signal factor (DSF)-deficient mutant 8004dF by expression of the *B. cenocepacia* gene *Bcam0581*. (a) The cells of wild-type *Xcc* strain 8004 grew in planktonic (free-floating) form. (b) The *rpfF* deletion mutant 8004dF derived from strain 8004 grew in biofilm (cell aggregates at the right-hand side of the photo) form. (c) *In trans* expression of *Bcam0581* 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.

were 12 peaks, which represent 12 carbons as stated below (Fig. 2-5B). 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 indicated that the peak at 14.08 p.p.m. was correlated with the three aliphatic protons at 0.88 p.p.m. (Fig. 2-5B), 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-H) with an m/z of 197.1532, suggesting a molecular formula of $C_{12}H_{21}O_2$ (197.1542) (Fig. 2-4). Taken together, these data indicate that BDSF is *cis*-2-dodecenoic acid (Fig. 2-5C), 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).

2.3.5 BDSF inhibited the germ tube formation by *C. albicans*

BDSF is structurally related to farnesol and highly similar to DSF. Both were shown to

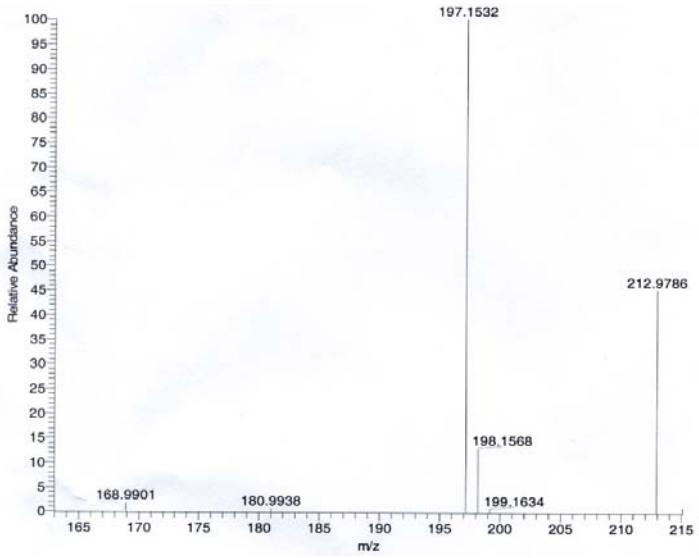


Fig. 2-4. ESI-MS analysis of purified DSF-like fractions from *B. cenocepacia*.

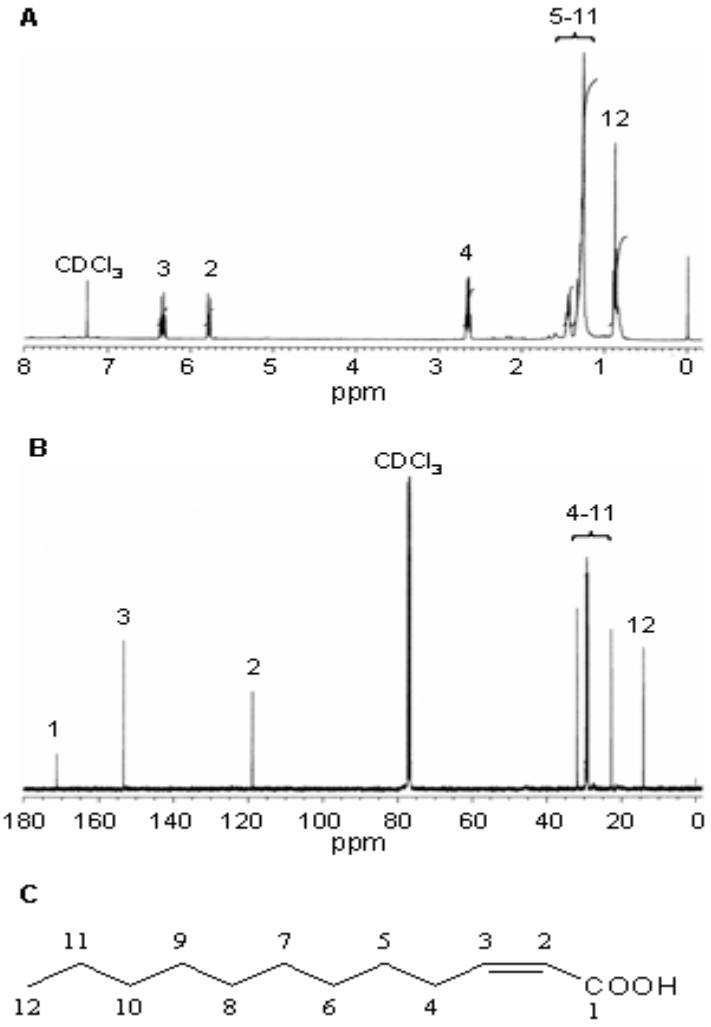


Fig. 2-5. Purification and structural characterization of BDSF. (A) ^{1}H NMR spectral of BDSF. (B) ^{13}C NMR spectra of BDSF. (C) The predicted chemical structure of BDSF, nuclear magnetic resonance.

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 (Fig. 2-6a). Farnesol and methanol were used as a positive and solvent control, respectively. After incubation at 37^{o}C for 3 h, more than 90% of *C. albicans* cells in solvent control formed germ tubes (Fig. 2-6b), and farnesol at a final concentration of 5 μM slightly reduced the length of germ tubes but seemed to have no effect on germination (Fig. 2-6c). In contrast, BDSF at a final concentration of 5 μM caused a marked reduction of germ tube germination and elongation (Fig. 2-6d). 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 (Fig. 2-6e and f), using the same medium andgrowth 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 (Fig. 2-6f). 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 (Fig. 2-6f). It is also interesting to note that farnesol, 3OC12HSL and DSF at 100μM did no affect the yeast cell growth, but strikingly, BDSF at the same concentration showed a detrimental effect on yeast cell growth (Fig. 2-6e). 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 (Fig. 2-6f). Interestingly, even at 0.5 μM, BDSF outperformed its close

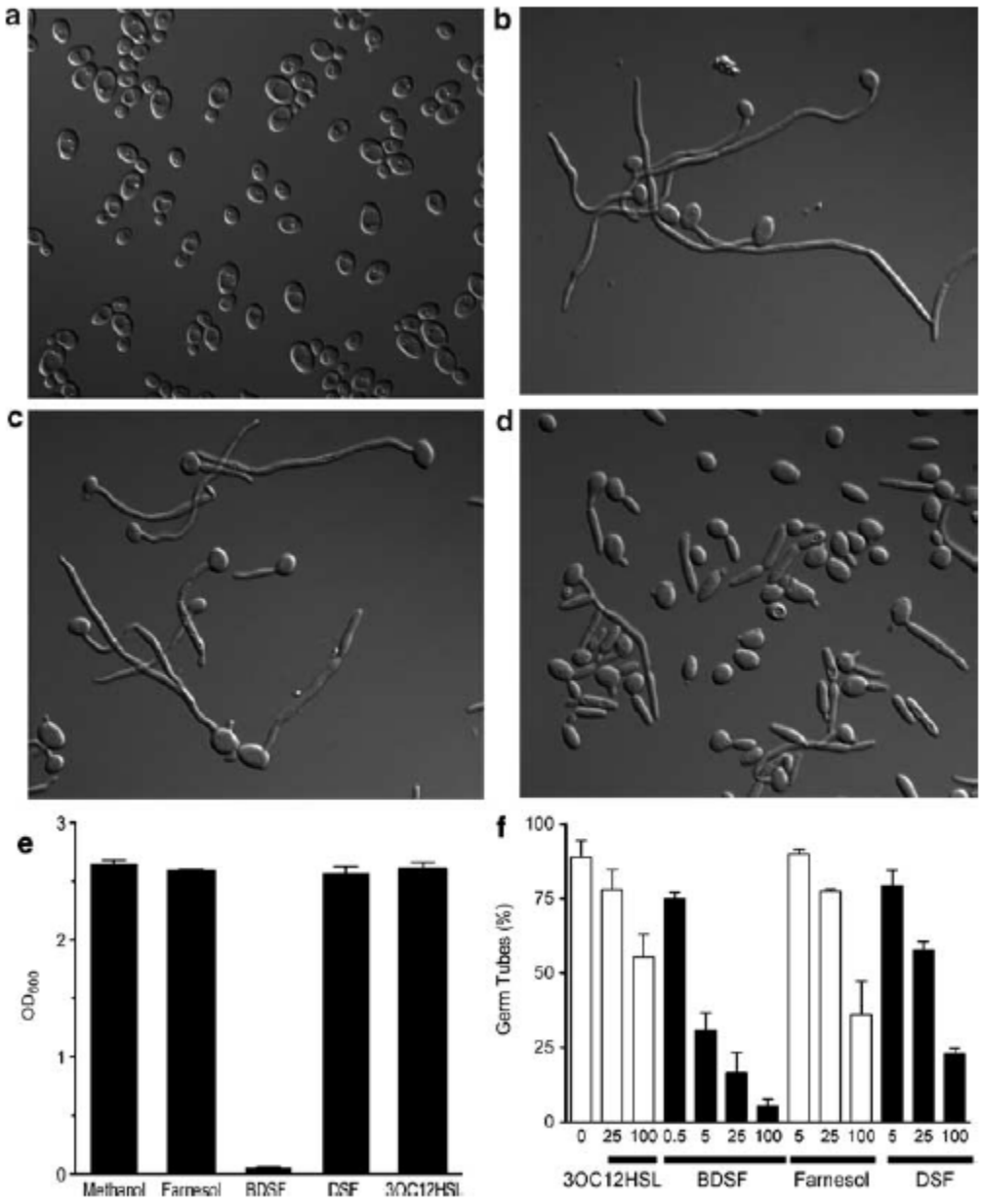

a
b
c
d
e
OD_{600}
3
2
1
0
Methanol
Farnesol
BDSF
DSF
3OC12HSL
f
Germ Tubes (%)
100
75
50
25
0
0 25 100 0.5 5 25 100 5 25 100 5 25 100
3OC12HSL
BDSF
Farnesol
DSF

Fig. 2-6. The effect of BDSF on *C. albicans* growth and germ tube formation. *C. albicans* cells were grown under non-induction conditions (30^{o}C) (a), or under induction conditions (serum extract, 37^{o}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^{o}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.

structural analogue DSF at 5 μM by reducing about 15% germ tube formation (Fig. 2-6f).

2.3.6 d0581 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 wildtype *B. cenocepacia* J2315 significantly decreased the fungal morphological transition as a large percentage of *C. albicans* cells grew in yeast-form (Fig. 2-7a). In contrast, majority of the fungal cells appeared as filaments when grown together with the BDSF-deficient mutant d0581 (Fig. 2-7b). Interestingly, the deletion mutant d0581 formed heavy cell aggregates together with *C. albicans* hyphal cells (Fig. 2-7b), whereas overexpression of *Bcam0581* in the mutant resumed the planktonic phenotype and abolished the yeast-to-hyphal transition of *C. albicans* (Fig. 2-7c). Quantitative analysis found that approximately 50% of the *C. albicans* cells counted were grown in hyphae-form when cocultured with d0581, 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% (Fig. 2-7d). The fungal hyphae cell level was further reduced down to only about 5% by the complementary strain that overproduced BDSF (Fig. 2-1a and 7d), highlighting a BDSF dosage-dependent effect.

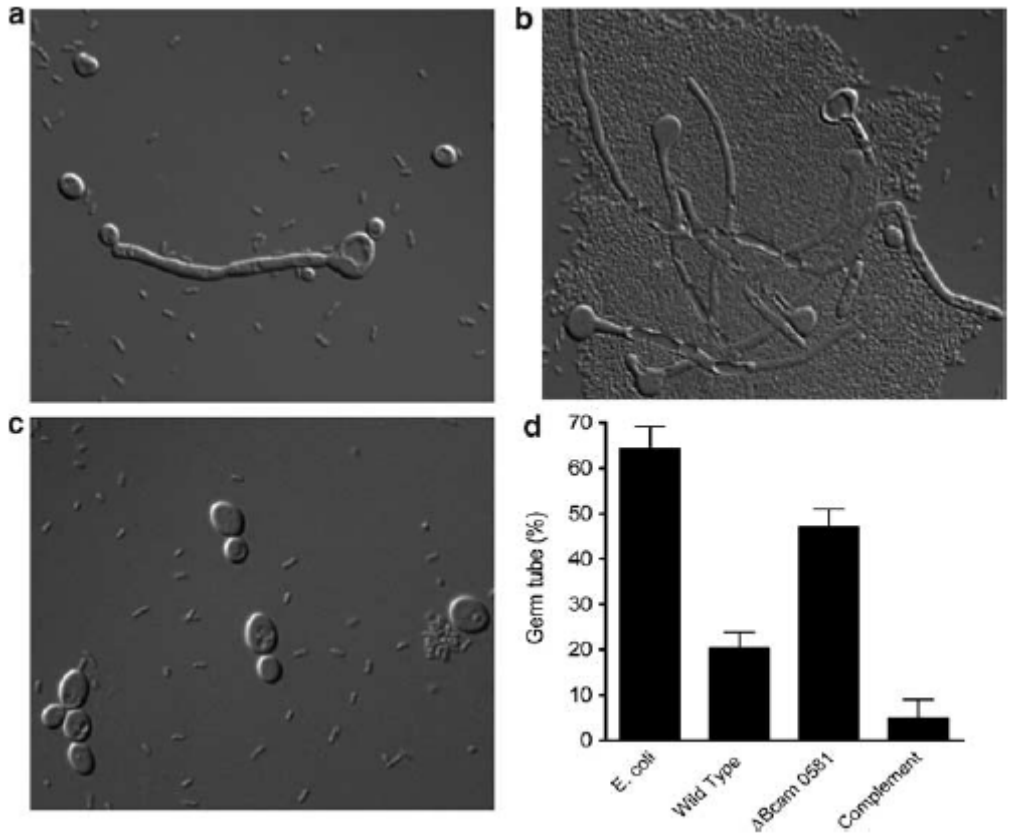


Fig. 2-7. The inhibitory effect of *B. cenocepacia* on *C. albicans* germ tube formation was dependent on the BDSF synthase gene *Bcam0581*. *C. albicans* cells cocultured with (a) wild-type *B. cenocepacia* J2315, (b) BDSF-deficient mutant d0581 and (c) the complemented strain d0581(pMLS7-Bcam0581). (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.

2.4 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 (Fig. 2-1, 2-4 and 2-5). 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 hundred 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, 2006b, 2007). *Xcc* belongs to the γ-subdivision of proteobacteria, whereas *B. cenocepacia* is a member of the distantly related b-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 (Fig. 2-2), the key enzyme known for DSF biosynthesis in *Xc*c (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 (Fig. 2-3). Third, deletion of *Bcam0581* in *B. cenocepacia* abolished the BDSF biosynthesis (Fig. 2-

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* (Fig. 2-5). 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 (Fig. 2-6f). 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 (Fig. 2-6e). 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.

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% (Fig. 2-6f), 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 (Fig. 2-7). 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 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.

CHAPTER 3

DIFFERENTIAL MODULATION OF *BURKHOLDERIA CENOCEPACIA* VIRULENCE AND ENERGY METABOLISM BY QUORUM SENSING SIGNAL BDSF AND ITS SYNTHASE

3.1 Introduction

The Burkholderia cepacia complex (Bcc) strains have emerged as problematic opportunistic pathogens in patients with cystic fibrosis and immunocompromised individuals (Coenye and Vandamne**,** 2003; Mahenthiralingam *et al*., 2002; Vandamme *et al*., 2003). Although all 17 Bcc species have been isolated from both environmental and clinical sources, *B. cenocepacia* and *B. multivorans* are most commonly found in clinical samples (Coenye and Vandamne**,** 2003; Mahenthiralingam *et al*., 2008). Apart from acquisition from the environment, patient-to-patient transmission and indirect nosocomial acquisition from contaminated surfaces have caused several outbreaks within and between regional CF centers (Saiman and Siegel, 2004).

B. cenocepacia strains are not only important opportunistic pathogens of humans but can also cause infections in a diverse range of species including rodents (Bernier *et al*., 2003; Speert *et al*., 1999), nematodes (Kothe *et al*., 2003), amoebae (Marolda *et al*., 1999), and plants (Bernier *et al*., 2003). The ability to survive and adapt to a wide range of habitats and to infect various host organisms suggests that *B. cenocepacia* is metabolically highly

adaptable and likely to produce multiple virulence factors. Presumably, the organism has evolved complex environmental sensing and regulatory mechanisms to coordinate cellular activities to thrive and survive in different environmental niches.

At least three types of chemical signals that are used by bacteria for cell-to-cell communication have been identified in *B. cenocepacia*. The most intensively characterized one is the AHL-type quorum sensing signals. The CepI/CepR system, which is a member of the conserved LuxI/LuxR-type QS system, is present in all members of the Bcc (Eberl, 2006; Sokol *et al*., 2007). Chemical analysis showed that the AHL synthase CepI catalyzes the production of *N*-octanoyl-L-homoserine (C8-HSL) and as a minor component *N*-hexanoyl-L-homoserine lactones (C6-HSL) (Gotschlich *et al*., 2001; Malott *et al*., 2005). The CepR/C8-HSL complex may activate or repress transcription of a wide range of biological functions, including virulence factor production, swarming motility and biofilm formation (Eberl, 2006; Sokol *et al*., 2007). In addition, *B. cenocepacia* as well as other *Burkholderia* species have been reported to produce 2-heptyl-4-quinolone (HHQ), the precursor of the *Pseudomonas aeruginosa* quinolone signal molecule (PQS), and it has been suggested that HHQ may also be used for cell-to-cell communication (Diggle *et al*., 2006). In the case of the human pathogen *Burkholderia pseudomallei*, loss of HHQ production was shown to affect colony morphology and increase elastase production (Diggle *et al*., 2006). More recently it has been demonstrated that *B. cenocepacia* produces, *cis*-2-dodecenoic acid (BDSF), which inhibits germ tube formation of the fungal pathogen *Candida albicans* (Boon *et al*., 2008). Given that germ tube formation is an essential trait for the fungal pathogen to establish an

infection, this finding may provide the molecular basis for the observation that *C. albicans* can normally not be isolated from cystic fibrosis patients who are infected with Bcc strains (Kerr, 1994). However, neither the BDSF signaling network nor the regulated functions in *B. cenocepacia* has been elucidated.

BDSF is a structural analogue of DSF, which is a QS signal molecule in the plant pathogen *Xanthomonas campestris* (Barber *et al*., 1997; Wang *et al*., 2004). Microarray analysis has shown that DSF regulates over 160 genes, many of which encode virulence factors (He *et al*., 2006b). As a consequence, DSF-deficient mutants are partially attenuated in virulence (Barber *et al*., 1997; He et al., 2006a; Wang *et al*., 2004). In this study, we have investigated the role of BDSF-signaling in the physiology and virulence of *B. cenocepacia*. Genetic analyses unveiled that BDSF plays a role in intraspecies cell-to-cell communication and regulates several virulence factors that are also controlled by the AHL-dependent CepIR QS system. Evidence is presented that the enzyme directing the biosynthesis of BDSF, Bcam0581, is also involved in the modulation of ATP biogenesis.

3.2 Methods and Materials

3.2.1 Bacteria strains and growth conditions

Bacterial strains used in this work are listed in Table 3-1. *E. coli* were grown at 37^{o}C with shaking at 250 x rpm in Luria-Bertani (LB) broth. *Burkholderia* strains were cultured at 37^{o}C in either LB or Anwar medium (Lonon *et al*., 1988), which contains 3 mM KCl, 12 mM $(NH_4)_2SO_4$, 20 mM glucose, 3.2 mM $MgSO_4$, 1.2 mM K_2HPO_4, 0.02 mM $FeSO_4$, 3

mM NaCl, and 50 mM Mops 50 with pH = 7.6. For cell aggregation and static biofilm formation assay, bacteria were cultured in Basal salt media (pH 7.2) containing 20 mM citrate and 0.5% Casamino acids (Boon *et al*., 2008). The following antibiotics were supplemented when necessary: gentamycin, 100 μg ml^{-1}; trimethoprim, 400 μg ml^{-1} (*B. cenocepacia*) and 1.5 mg ml^{-1} (*E. coli*).

3.2.2 Determination of the BDSF accumulation profile

Cells were harvested at different time points as indicated and BDSF was extracted as described previously with minor modification (Boon *et al*., 2008). Briefly, *B. cenocepacia* J2315 was grown in LB medium with agitation at 37°C. At each time point, 100 ml of culture supernatants were collected by centrifugation and extracted with an equal volume of ethyl acetate. The extracts were dried to dryness and the remainder was dissolved in 1 ml of methanol. Quantification of BDSF was achieved by using the *Xcc* biosensor strain FE58 (Wang *et al*., 2004). To this end, the biosensor was grown at 28°C in YEB medium (Zhang *et al*., 2002) to an OD_{600} of 0.6 prior to the addition of 100 μl of BDSF extracts to 5 ml of culture. Following incubation for 3 h at 28°C with shaking at 200 x rpm, 1 ml of culture was centrifuged and the bacterial pellets were lysed in 250 μl Cell Lytic™ reagent (Sigma) according to the recommendations of the manufacturer. Protein concentrations were determined using the Bradford Assay Kit (Bio-Rad). All assays were performed with equal amounts of proteins. GUS activities were determined according to Jefferson *et al*. (1987). BDSF concentrations were measured using a standard curve that was prepared with defined concentrations of chemically synthesized BDSF (Wang *et al*., 2004).

Table 3-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotypes and/or characteristics	Reference or source
B. cenocepacia		
J2315	Wild type strain, Genomovars III of *B. cepacia* complex	ATCC
d0581	BDSF-minus mutant derived from J2315 with *Bcam0581* being deleted	Boon *et al*., 2008
J2315(P0581-lacZ)	Wild type harboring the reporter construct pMLS7P0581-lacZ	This study
J2315(PzmpA-lacZ)	Wild type harboring the reporter construct pMLS7PzmpA-lacZ	This study
J2315(Plip-lacZ)	Wild type harboring the reporter construct pMLS7Plip-lacZ	This study
J2315(Porb-lacZ)	Wild type harboring the reporter construct pMLS7Porb-lacZ	This study
d0581(pMLS7-0581)	Mutant d0581 harboring the expression construct pMLS7-0581	Boon *et al*., 2008
d0581(P0581-lacZ)	Mutant d0581 harboring the reporter construct pMLS7P0581-lacZ	This study
d0581(PzmpA-lacZ)	Mutant d0581 harboring the reporter construct pMLS7PzmpA-lacZ	This study
d0581(Plip-lacZ)	Mutant d0581 harboring the reporter construct pMLS7Plip-lacZ	This study
d0581(Porb-lacZ)	Mutant d0581 harboring the reporter construct pMLS7Porb-lacZ	This study
d0581(cepI)	Mutant d0581 harboring the expression construct pMLS7-cepI	This study
d0581(cepR)	Mutant d0581 harboring the expression construct pMLS7-cepR	This study
cepR::Km^r	cepR insertion mutant derived from strain H111	Huber *et al*., 2001
cepR-pBAH27	cepR insertion mutant harboring *cepR* in the construct pBAH27	Huber *et al*., 2001
E. coli		
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir*	Laboratory collection
S17-1	pro res^- mod^+ integrated copy of RP4, mob^+	Laboratory collection
X. campestris		
FE58	Biosensor for DSF/BDSF	Wang *et al*., 2004
Plasmid		
pMLS7	pBBR1 ori, P_{S7} promoter, Tp^r *mob*$^+$	Lefebre and Valvano, 2002
pMLS7-0581	pMLS7 containing *Bcam0581*	Boon *et al*., 2008
pMLS7P0581-lacZ	pMLS7 containing the *Bcam0581* promoter-*lacZ* fusion	This study
pMLS7PzmpA-lacZ	pMLS7 containing the *zmpA* promoter-*lacZ* fusion	This study
pMLS7Plip-lacZ	pMLS7 containing the *lipA* and *lipB* promoter-*lacZ* fusion	This study
pMLS7Porb-lacZ	pMLS7 containing the *orb* operon promoter-*lacZ* fusion	This study
pMLS7-cepI	pMLS7 containing *cepI*	This study
pMLS7-cepR	pMLS7 containing *cepR*	This study
pMLS7-0207	pMLS7 containing *Bcas0207*	This study
pBAH27	pBBR1MCS-5 containing the *cepR* gene of *B. cepacia* H111	Huber *et al*., 2001

Table 3-2. PCR primers used in this study

Primer	Sequence
For reporter construction	
lacZF	5'- cccggaattcatgaccatgattacggattcactg
lacZR	5'-ctagctagcttatttttgacaccagaccaactgg
0581PF	5'-ctagctagctcgtgcggcccagtgtg
0581PR	5'-cggaattcggtatgtcctcgtgagatgtgg
PzmpAF	5'-cccaagcttctggagcgctcgcattcac
PzmpAR	5'-cgcggatccgggcagcagtcgagacagtttctt
PlipF	5'-cccaagctttcgatgcatgggtgtcggcg
PlipR	5'-cgcggatccggaacgcatcgatttggccat
PorbF	5'-cccaagctttgtgatagccgagcgagccg
PorbR	5'-cgcggatccgaaactcgtcatgtgcgtgaagtcctt
For in trans expression	
cepI-F	5'-cgcggatccatgcagaccttcgttcacgag
cepI-R	5'-cccaagctttcaggcggcgatagcttgc
cepR-F	5'-cgcggatccatggaactgcgctggcag
cepR-R	5'-cccaagctttcagggtgcttcgatgagcc
For RT-PCR analysis	
Bcas0207F	5'-cgctcgcgcatctgtggttc
Bcas0207R	5'-ccggcgagtcgtggcgtgtc
zmpAF	5'-gcggcggcggctcggtctac
zmpAR	5'-cgggatcgttcgggttgttcg
lipAF	5'-aaccgcgcccgccgacgactat
lipAR	5'-gccctggctgtgaccgacgagatt
lipBF	5'-gccggcgtcgcgatgtggag
lipBR	5'-gcgcggtcaggcaatagtcg
orbIF	5'- acgcgtgcattgctgggtctgttc
orbIR	5'- gcgcggccgtcgtatgct

3.2.3 Bacterial growth analysis and determination of cellular ATP levels

B. cenocepacia cultures grown overnight in LB medium were washed with Anwar medium and diluted to a final OD_{600} of 0.05 in Anwar medium. Citrate and other carbohydrates were supplemented at a final concentration of 20 mM as indicated. Bacterial growth was determined at 37^{o}C in a low intensity shaking model using the Bioscreen-C Automated Growth Curves Analysis System (OY Growth Curves AB Ltd, Finland). For determination of ATP and colony forming units (c.f.u), samples were taken at various time points for spreading onto LB plates after appropriate dilutions. Quantification of ATP was achieved using the BacTiter-Glo™ Microbial Cell Viability Assay kit (Promega, USA) according to manufacturer's protocols.

3.2.4 Construction of reporter strains and measurement of β-galactosidase activity

A promoterless vector was created after removing the promoter sequence upstream of the multiple cloning site in pMLS7 (Lefebre and Valvano, 2002) by digestion with *Nhe*I and *Eco*RI. The *lacZ* gene was amplified using the primer pair lacZF and lacZR (Table 3-2). The amplified *lacZ* was ligated to the linealized pMLS7 to generate the promoterless fusion construct pMLS7-*lacZ*. For generating transcriptional fusions the promoter regions of Bcam0581 and other virulence genes were amplified using the primers listed in Table 3-2. The PCR fragments were digested with *Bam*HI and *Hin*dIII, and then ligated into the same sites of pMLS7-lacZ, respectively. These constructs were verified by DNA sequencing before they were introduced into *B. cenocepacia* J2315 and d0581 by tri-parental mating. The *B. cenocepacia* transconjugants were selected on LB agar plates containing trimethoprim.

For measurement of *β-galactosidase activity,* strains were grown in LB medium at 37^{o}C with shaking at 250 rpm. When necessary, BDSF and C8HSL were added separately or in combination to a final concentration of 5 μM. Bacterial cells were harvested at various time points along the growth curve and β-galactosidase activities were assayed as described previously (Jeffrey, 1992).

3.2.5 RNA extraction and RT-PCR analysis

Cultures grown in LB medium at 37^{o}C with shaking (250 rpm) overnight were diluted to an OD_{600} of 0.05 in fresh LB medium or Anwar medium,as indicated and grown under the same conditions to an OD_{600} of 1.5 (LB medium) or 48 h (Anwar medium), respectively. RNA was isolated from one milliliter of culture using the RNeasy mini kit according to the manufacturer's instructions (Qiagen). The concentration and purity of RNA were determined by spectrometry and agarose gel electrophoresis. RT-PCR analysis was performed using the One-step RT-PCR kit according to the manufacturer's instructions (Qiagen).

3.2.6 Cell aggregation analysis

Bacteria stock cultures were inoculated to obtain an OD_{600} of less than 1.0 after overnight incubation at 37^{o}C and diluted to OD_{600} of 0.05 the next day. Subsequently, 3 ml of the diluted cultures were dispensed in sterile 12 well tissue culture plates in triplicates. They were incubated at 37^{o}C with agitation set at 150 rpm and observed visually every hour. Imaging was achieved using a HP5370C Flat bed scanner.

3.2.7 Generation of static biofilms

The bacteria cultures were grown 24 hours and the density were adjusted to OD_{600} of about 3.0. Five micro liters culture was inoculated in duplicates into sterile 6 well tissue culture plates containing 3 ml of culture media. They were subsequently incubated without agitation at 30^{o}C for 3 days. To observe the coarse surface of the resultant biofilm, the plates were then directly observed under a stereo microscope at 25X magnification equipped with a polarizer (Olympus) and imaged using an attached standard 35 mm film camera.

3.2.8 Virulence assays using a zebrafish infection model

B. cenocepacia virulence was tested by infecting 6 months old zebrafish (*Danio rerio*). To this end, thirty microliters of bacterial cultures grown to an OD_{600} of 1.0 were injected intraperitoneally into each fish using a 1-ml tuberculin syringe attached to a 30.5 gauge Precision Glide® needle (Becton Dickinson). Mortality was scored daily and dead fish were removed immediately. The experiment was repeated 3 times, each time using 15 fish per treatment. To determine bacterial survival *in vivo*, bacterial strains were inoculated as described above except that 30 fish were used for each treatment. Each day three fish were sacrificed, surface sterilzed with 70% ethanol for 1 min, washed with phosphate-buffered saline (PBS, pH 7.3) and homogenized. Following centrifugation at 500 rpm for 1 min to remove large debris, serial dilutions of the homogenates were prepared in PBS and plated in triplicates on LB agar containing kanamycin (200 μg ml^{-1})

and gentamycin (200 μg ml^{-1}). The bacterial cell numbers (c.f.u) were determined 2 days after incubation at 37°C.

3.3 Results

3.3.1 Accumulation of BDSF is cell density-dependent

To determine the time course of BDSF production, we extracted *B. cenocepacia* J2315 supernatants at various growth stages and measured BDSF concentrations by the aid of the biosensor strain FE58 (Wang *et al*., 2004). Detectable amounts of BDSF were first measured 6 h post inoculation (Fig. 3-1A). After this time point, BDSF levels increased steadily during the exponential and early stationary growth phase. BDSF accumulation peaked in the late stationary phase (36 h) followed by a significant decline in BDSF levels (Fig. 3-1A). We noted that the pH of bacterial supernatants reached approximately 8.6 at 40 h post inoculation. However, incubation of BDSF in solutions of similar alkaline pH overnight did not affect BDSF activity (data not shown), suggesting that a biological factor(s) may account for the decline of BDSF at the later stage of bacterial growth.

3.3.2 BDSF production is controlled at the level of transcription

The drastic decline of BDSF amounts in the late stationary phase prompted us to investigate the transcriptional profile of *Bcam0581,* which encodes BDSF production. A 613-bp DNA containing the *Bcam0581* promoter region was transcriptionally fused to a *lacZ* coding sequence and the construct was introduced into the wild type strain. As shown in Fig. 3-1B, the promoter activity of *Bcam0581* gradually increased and reached a stable plateau 12 h post inoculation, which was maintained for another 12 hours.

Thereafter, BDSF levels declined rapidly. The *Bcam0581* promoter activities coinciding very well with the BDSF accumulation profile: a rapid increase in BDSF levels following the stimulation of *Bcam0581* promoter activity and a sharp decline in activity prior to the drop in BDSF signal concentrations in the culture supernatants.

To test whether the *Bcam0581* promoter is autoregulated by BDSF, we determined the activity profile of the promoter in the *Bcam0581* deletion mutant d0581 genetic background. The enzyme assay data showed that the promoter activity in mutant d0581 (data not shown) is virtually indistinguishable from the one in the wild type background, suggesting that the production of BDSF may not be autoregulated.

3.3.3 Deletion of *Bcam0581* impaired growth and cellular ATP levels when grown in minimal medium

While deletion of *Bcam0581* did not have obvious effect on bacterial growth in LB medium, we noticed that the mutant grew much slower than the wild type in Anwar medium, which is a minimal medium containing 20 mM glucose as the sole carbon source. Growth experiments revealed that not only the growth rate of d0581 was reduced but that the mutant only reached an OD_{600} of 0.2 at 44 h after inoculation (Fig. 3-2A). In contrast, the wild type strain grew significantly faster than the mutant and reached a maximum OD_{600} of 0.45. *In trans* expression of *Bcam0581* in the deletion mutant d0581 resulted in a faster initial growth rate but then, surprisingly, a slower rate than the wild type strain after 28 h (Fig. 3-2A). Moreover, exogenous addition of BDSF up to a concentration of 25 μM was not able to rescue the growth defect of d0581 (Fig. 3-3C).

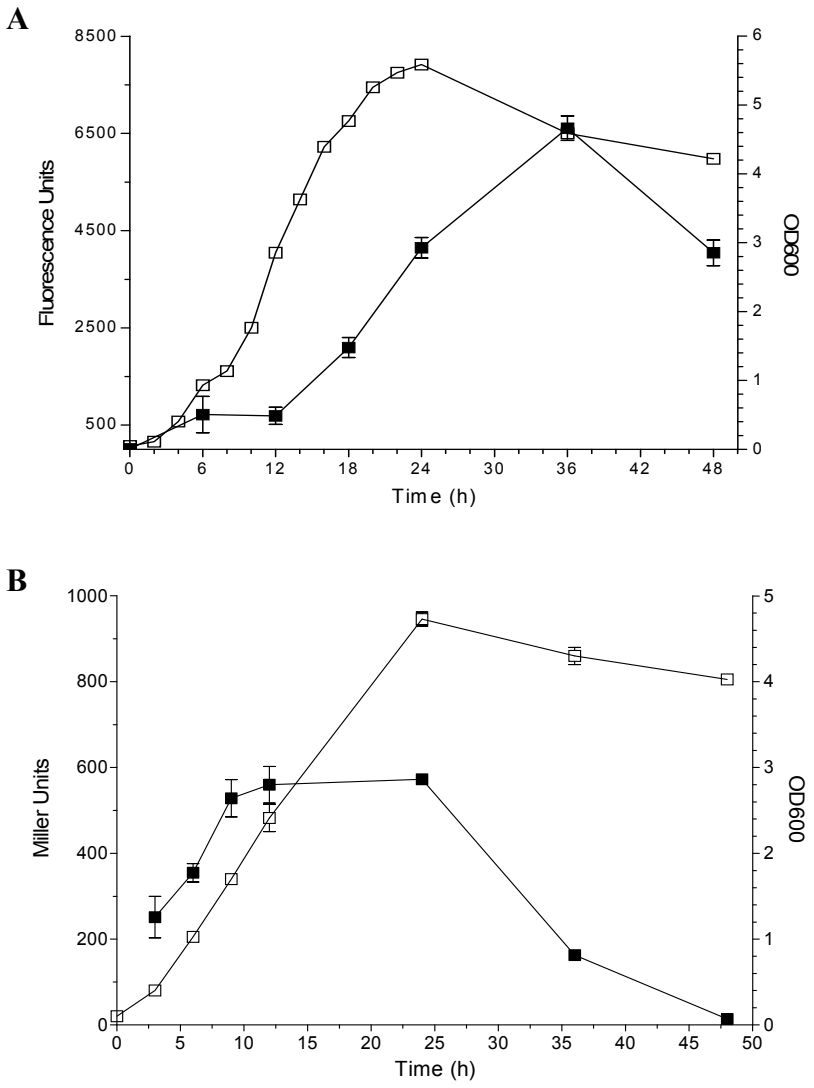


Fig. 3-1. BDSF production and *Bcam0581* transcriptional expression. (A) Time course analysis of *B. cenocepacia* growth in LB medium (□) and BDSF accumulation in culture supernatants (■). (B) β-galactosidase activity of a *Bcam0581-lacZ* transcriptional fusion (■) and bacterial growth (□). Strain J2315 was inoculated in flask containing LB medium and cultured at 37^{o}C with shaking at 220 x rpm. The data presented are the means of two independent experiments and the error bars represents the standard deviation.

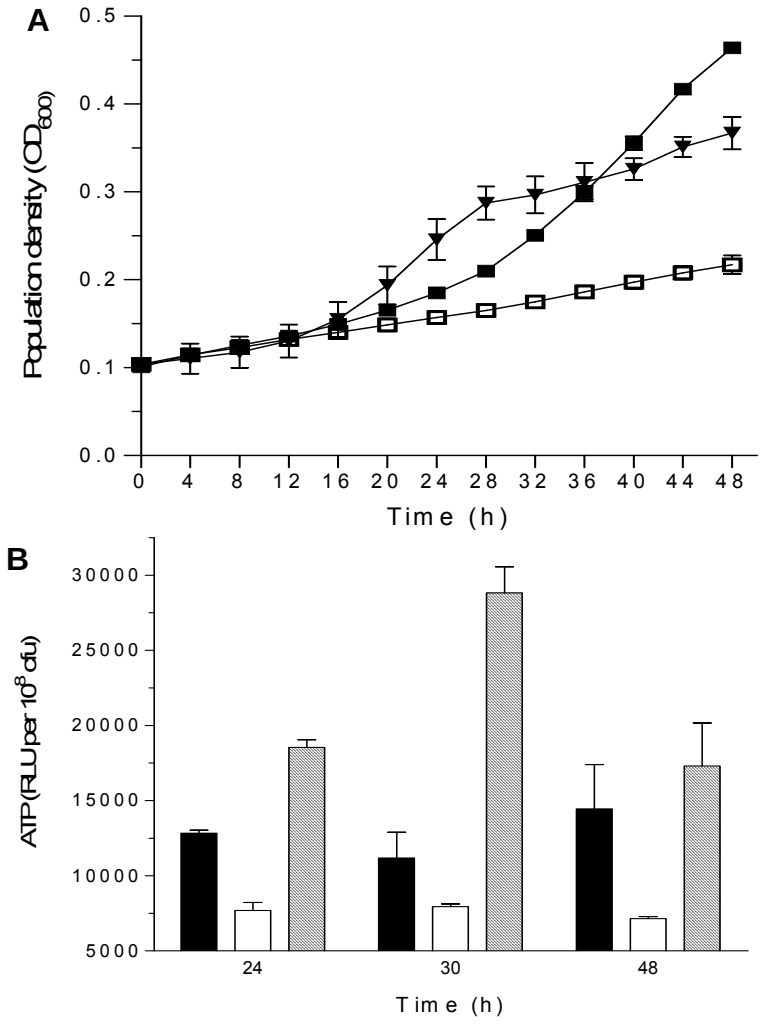


Fig 3-2. Effect of *Bcam0581* deletion on *B. cenocepacia* growth and energy biogenesis in minimal medium. (A) Growth pattens of the wild type strain J2315 (■), the mutant d0581 (□), and the complemented mutant d0581(pMLS7-0581) (▼). Bacterial cells were inoculated in Anwar medium with three duplicates for each strain and growth profiles at 37^{o}C were recorded by BIOSCREEN. (B) Cellular ATP levels expressed in RLU per 10^{8} c.f.u. Bacteria were grown under the same conditions as in (A) and samples were taken at different time points for ATP measurement as indicated. Symbol: Solid bar, wild type J2315; open bar, mutant d0581; slashed bar, complemented strain d0581(pMLS7-0581). The data shown are means of three repeats and error bars indicate the standard deviations.

These unexpected phenomena were confirmed in several independent experiments using both flask cultures and the Bioscreen-C Automated Growth Curves Analysis System. These data suggest that, in addition to its role in BDSF biosynthesis, Bcam0581 may also have a metabolic function. We next investigated the cellular ATP levels of the strains. Quantitative measurements of ATP concentrations at three time points showed that deletion of Bcam0581 resulted in significantly reduced ATP levels relative to the wild type strain (Fig. 3-2B). Consistent with its growth profile, the complement strain produced more ATP than the wild type strain, in particular 24 and 30 hours after inoculation.

3.3.4 The growth defect of mutant d0581 in minimal medium is rescued when citrate is supplemented or by *in trans* expression of citrate synthase

As the ATP measurements suggested that Bcam0581 may somehow influence energy metabolism of *B. cenocepacia* J2315, we tested whether supplementation of carbohydrates associated with citric acid cycle could rescue the growth defect of the deletion mutant d0581. To this end, Anwar medium containing 20 mM glucose as a sole carbon source, was amended with 20 mM of different carbohydrates, including citrate, malate and pyruvate. As shown in figure 3-3A, Anwar medium supplemented with 20 mM citrate not only enhanced growth of the wild type strain J2315 but also rescued the growth defect of mutant d0581. Interestingly, the complemented strain d0581(0581) also proliferated well in the Anwar medium supplemented with 20 mM citrate but at a lower rate than the wild type strain (Fig. 3-3A).

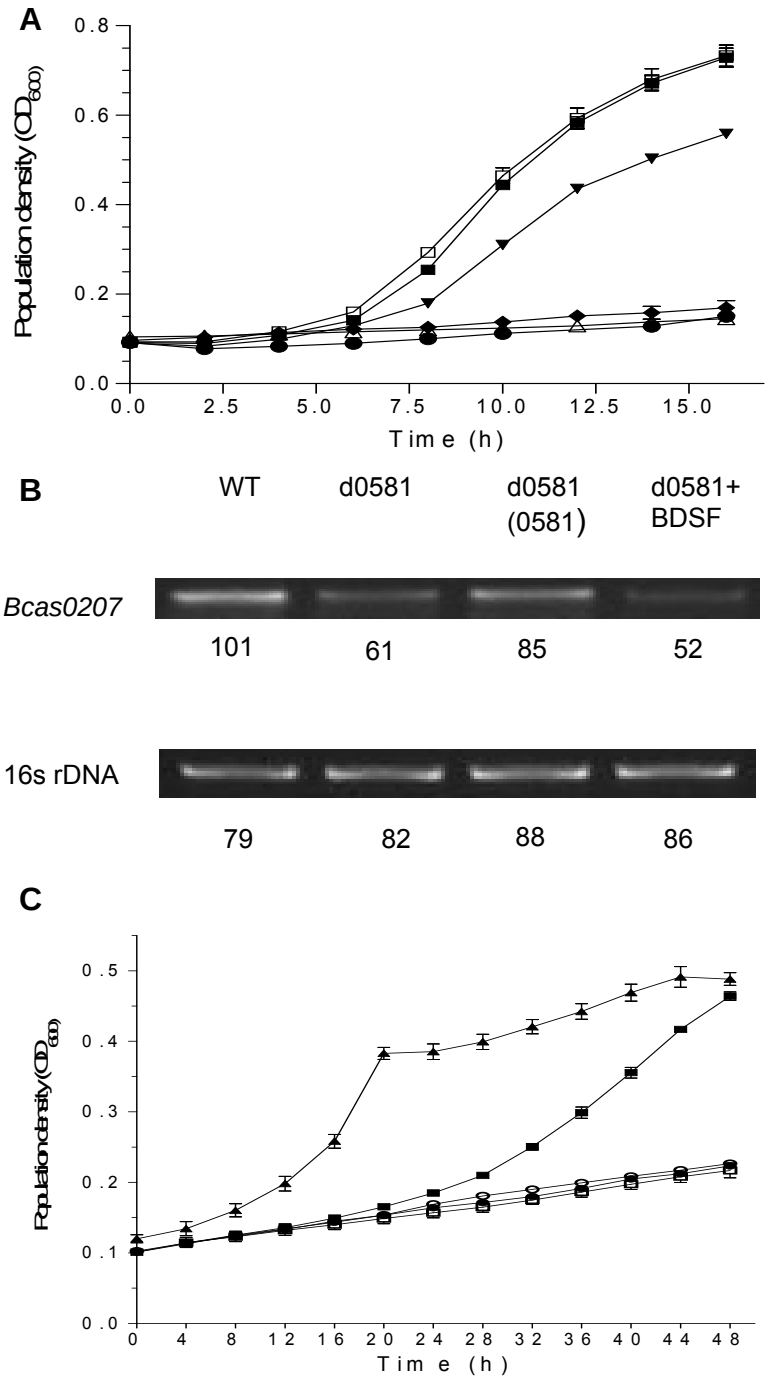

A
Population density (OD600)
0.0
0.2
0.4
0.6
0.8
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time (h)
B
WT
d0581
d0581 (0581)
d0581+ BDSF
Bcas0207
101
61
85
52
16s rDNA
79
82
88
86
C
Population density (OD600)
0.0
0.1
0.2
0.3
0.4
0.5
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (h)

Fig. 3-3. Addition of citrate to Anwar medium rescues the growth defect of the *Bcam0581* mutant. (A) Growth curve of the wild type J2315 (■) and the complemented mutant d0581(pMLS7-0581) (▼) in Anwar medium supplemented with 20 mM citrate; mutant d0581 in Anwar medium (Δ), in Anwar medium supplemented with 20 mM citrate (□), 20 mM malate (♦), and 20 mM pyruvate (●). The error bars show the standard deviations of three repeats. (B) RT-PCR analysis of the citrate synthase gene *Bcas0207* expression in the wild type J2315, the mutant d0581, the complemented mutant d0581(pMLS7-0581) and the mutant d0581 supplemented with 5 μM BDSF. The signal intensity (using the software Image J; http://rsb.info.nih.gov/ij/) determined for each RT-PCR band is indicated. For each RNA sample, two dilutions (5, 50 ng) were used as templates for RT-PCT reaction with similar results. The error bars show the standard deviations of three repeats. (C) Growth curve of wild type J2315 (■), the mutant d0581 (□), d0581(pMLS7-Bcas0207) (▲) and the mutant d0581 supplemented with 5 μM BDSF (○) and 25 μM BDSF (●) in Anwar medium. The error bars show the standard deviations of three repeats.

The involvement of Bcam0581 in citrate biosynthesis was further analyzed by semi-quantitative RT-PCR. *Bcas0207* of *B. cenocepacia* J2315 encodes a citrate synthase homologue, which contains an oxalacetate binding site, a citrylCoA binding site and a coenzyme A binding site involved in citrate biosynthesis of the TCA cycle (Holden *et al*., 2009). RT-PCR analyses showed that transcription of *Bcas0207* in the *Bcam0581* deletion mutant was significantly reduced, which was restored to the level of the wild type strain by complementation with the wild type *Bcam0581* gene, but exogenous addition of 5 μM BDSF to the mutant had not effect (Fig. 3-3B). We then tested whether *in trans* expression of *Bcas0207* could rescue the growth defect in the mutant d0581. The results showed that the resultant strain d0581(Bcas0207) grew faster initially than the wild type strain but its proliferation was slower down after 20 h, and
reached a similar population density as the wild type at about 48 h after inoculation (Fig. 3-3C).

3.3.5 d0581 displays enhance auto aggregation and altered surfaced structures in static biofilms

Previous study showed that BDSF is a structural homologue of DSF from *Xcc*. Deletion of *rpfF* in *Xcc* displayed enhance cell aggregation and biofilm formation (Boon *et al*., 2008; He *et al*., 2006a). This impelled us to examine the extent of cell aggregation and the nature of the biofilm formed by d0581. Indeed, it demonstrated increased auto aggregation as compared to the wild type which can be complemented by both the expression of *Bcam0581 in trans* (Fig. 3-4A) and exogenous addition of 5 μM BDSF. When *B. cenocepacia* is grown statically, it forms a thin layer of pellicle-like biofilm at

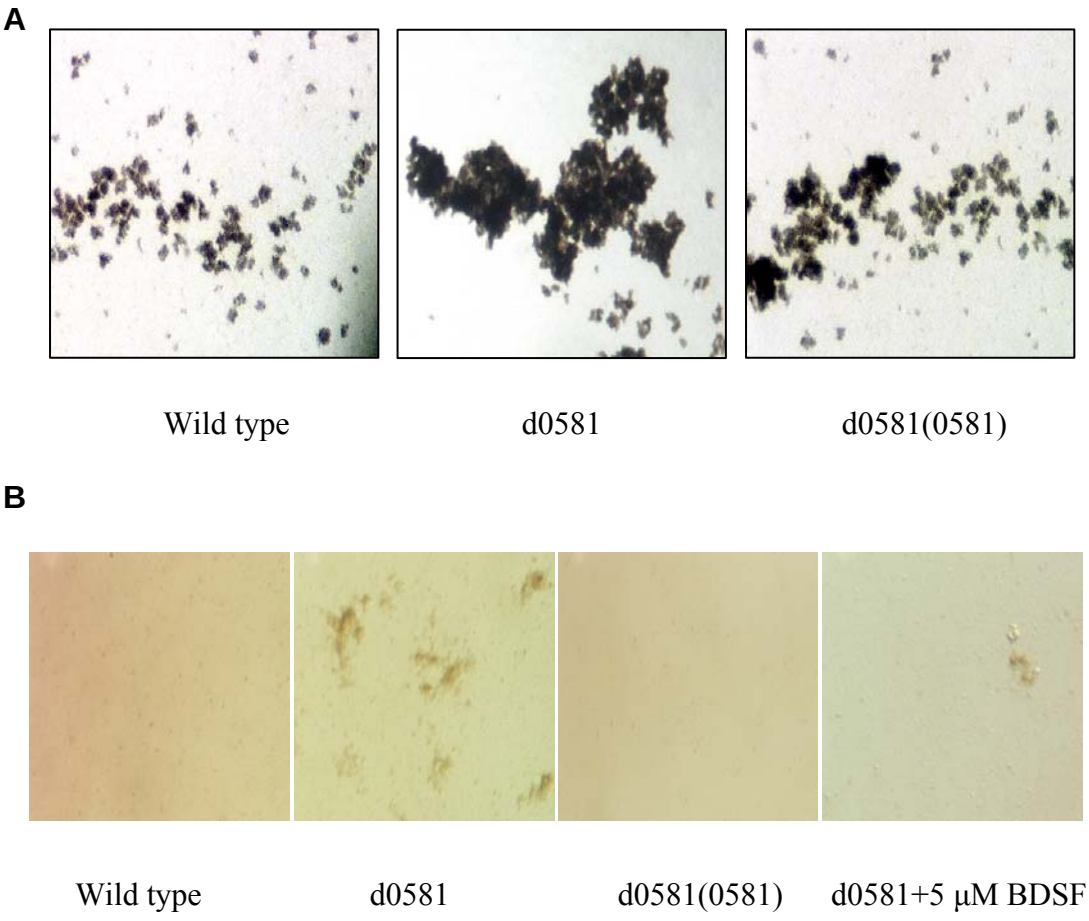


Fig. 3-4. The role of BDSF in cell aggregation and biofilm formation. (A) Extent of cell aggregation in the various *B. cenocepacia* strains. (B) Macro surface structures of static biofilms observed under a stereo microscope. Complementation was performed by the *in trans* expression of *Bcam0581* in (A, B) and addition of 5 μM of BDSF in (B).

the liquid to air interface after three days of incubation. Microscopic examination of the biofilm formed by the wild type revealed an uneven rough surface due to the presence of numerous pimple-like protrusions (Fig. 3-4B). Interestingly, the surface of the biofilm formed by the mutant contains moderately lesser amount of protrusions but they appear drastically larger in size. Importantly, the complemented strain which over produces BDSF or exogenous addition of BDSF displayed considerable reduction in the amount and size of these protrusions.

3.3.6 Deletion of *Bcam0581* affects expression of virulence genes

To test the role of BDSF signaling in modulation of virulence gene expression we selected a few previously characterized virulence factors for RT-PCR and gene fusion analyses, including *zmpA* (Bcas0409) encoding a metalloprotease (Gingues *et al*., 2005; Kooi *et al*., 2005), *lipA* (Bcam0949) and *lipB* (Bcam0950) encoding a lipase and a lipase chaperone (Holden *et al*., 2009), respectively, and the *orbI* gene (Bcal1696) required for the biosynthesis of the siderophore ornibactin (Holden *et al*., 2009). These investigations revealed that the transcript levels of *zmpA*, *lipA, lipB,* and to a lesser degree of *orbI,* were reduced in the mutant d0581 relative to levels of these genes in the parental strain (Fig. 3-5). *In trans* expression of the wild type *Bcam0581* gene rescued the defects, confirming the importance of Bcam0581 for the regulation of these virulence factors under normal growth conditions. Unlike the growth defect in minimal medium, the decreased transcription of these virulence genes could be fully restored by exogenous addition of 5 μM BDSF (Fig. 3-5). These data suggest that the BDSF signaling network affects virulence gene expression in *B. cenocepacia*.

We also fused the promoter regions of *zmpA*, *lipAB* and *orbIJK* to *lacZ* and measured reporter gene activities in the wild-type and the d0581 mutant background. Consistent with the RT-PCR analysis, null mutation of Bcam0581 caused a 30 - 50% reduction in transcriptional expression of these genes, which was almost fully rescued by the addition of 5 μM BDSF to the medium (Fig. 3-6A, 6B, 6C).

3.3.7 Co-regulation of virulence gene expression by BDSF and AHL signal molecules

Interestingly, ZmpA and ornibactin biosynthesis have been previously shown to be under control of CepIR QS system (Subsin *et al*., 2007). This prompted us to investigate whether deletion of *Bcam0581* affects transcriptional expression of *cepI* or *cepR*. However, no difference in *cepI or cepR* transcript levels between the wild type and the Bcam 0581 mutant could be observed using RT-PCR (data not shown). Quantification of AHL production of strain J2315 and its mutant d0581 showed that the mutant produced lower amounts of AHL signal molecules than the wild type when grown in Anwar minimal medium for a same time (data not shown). However, the difference became insignificant after normalization against bacterial cell density.

To test the possibility that the BDSF signaling system may act downstream of CepIR, we analyzed the effect of *cepI* and *cepR* on virulence gene expression in the d0581 mutant background. RT-PCR analysis showed that similar to *in trans* expression of *Bcam0581*, overexpression of *cepI* or *cepR* in the mutant d0581 increased the transcript levels of *zmpA* (*Bcas0409*), *lipA* (*Bcam0949*), *lipB* (*Bcam0950*) and *orbI* (*Bcal1696*) (Fig. 3-5A).

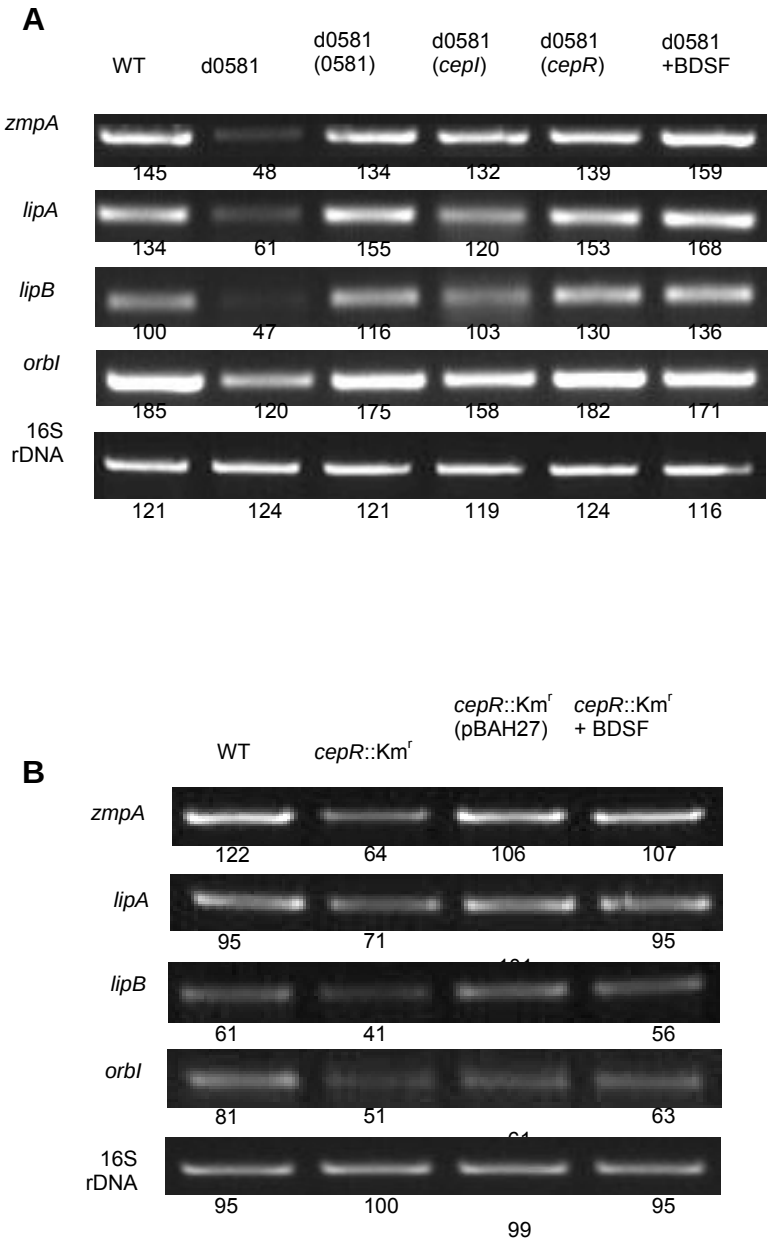


Fig.3-5. Analysis of the role of Bcam0581/BDSF and AHL system in the regulation of virulence gene expression. Bacterial strains were grown in LB medium to an OD_{600} of 1.5. BDSF was added at a final concentration of 5 μM as indicated. For each RNA sample, two dilutions (5, 50 ng) were used as templates for RT-PCT reaction with similar results.

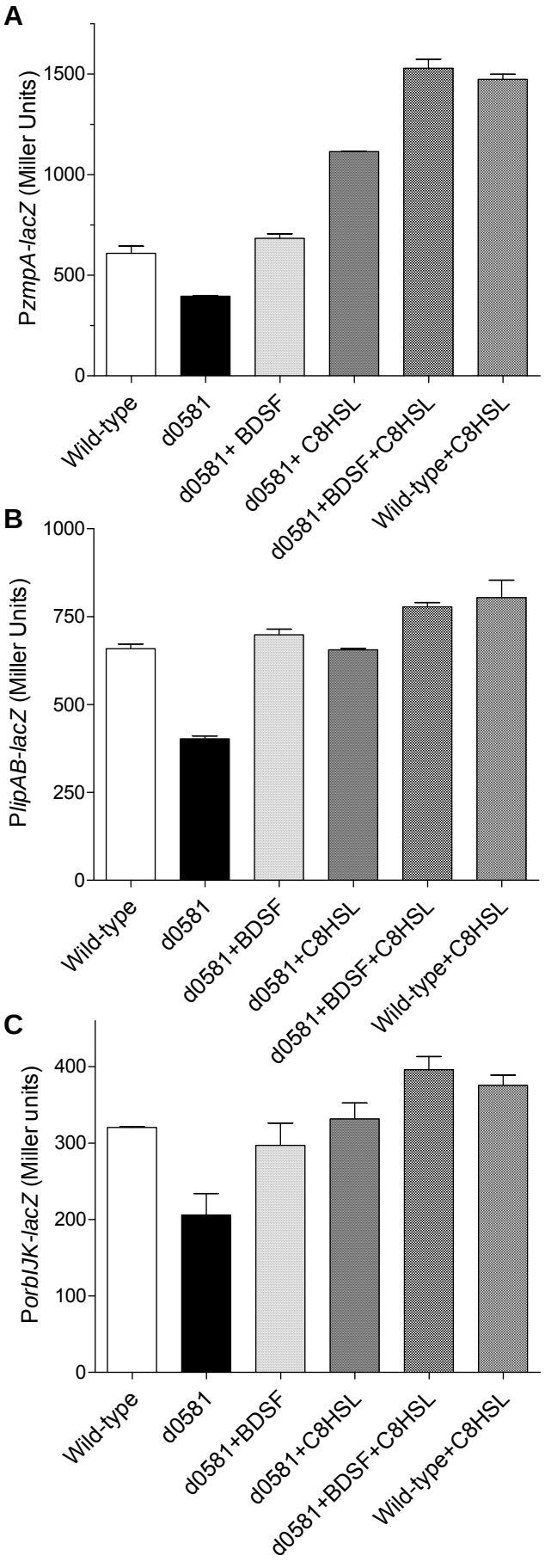

A
PzmpA-lacZ (Miller Units)
0
500
1000
1500
Wild-type
d0581
d0581+ BDSF
d0581+ C8HSL
d0581+BDSF+C8HSL
Wild-type+C8HSL
B
PlipAB-lacZ (Miller Units)
0
250
500
750
1000
Wild-type
d0581
d0581+BDSF
d0581+C8HSL
d0581+BDSF+C8HSL
Wild-type+C8HSL
C
PorbJK-lacZ (Miller units)
0
100
200
300
400
Wild-type
d0581
d0581+BDSF
d0581+C8HSL
d0581+BDSF+C8HSL
Wild-type+C8HSL

Fig. 3-6. Influence of *Bcam0581* on expression of virulence genes *zmpA* (A), *lipAB* (B), and *orbIJK* (C), as determined by using corresponding promoter-*lacZ* fusion reporter strains. Bacterial strains were grown in LB medium, and BDSF and C8HSL were added separately or in combination at a final concentration of 5 μM. The data shown are the means of three repeats and error bars indicate the standard deviations.

We then tested whether BDSF can further increase virulence gene expression in the AHL mutant background. The results showed that addition of 5 μM BDSF to the *cepR* mutant *cepR*::Km^r fully restored the transcriptional expression of above four virulence genes (Fig. 3-5B). In addition, we found that exogenous addition of C8-HSL at a final concentration of 5 μM to the growth medium significantly increased the promoter activities of *zmpA, lipAB,* and *orbIJK* in mutant d0581 (Fig. 3-6A, 6B, 6C). Furthermore, addition of BDSF together with C8HSL to the mutant d0581 further increased the promoter activity of these three genes to a level similar to addition of C8HSL to the wild type strain J2513 (Fig. 3-6A, 6B, 6C). These data suggest that the AHL- and BDSF-dependent QS systems regulate a similar set of virulence factors in parallel.

3.3.8 The pathogenicity of d0581 is reduced in a zebrafish infection model

We employed a zebrafish infection model to determine the role of Bcam0581 in *B. cenocepacia* pathogenesis. Cells of the wild type, the mutant d0581 and the complemented mutant were injected into zebrafish. Phosphate saline buffer was used as a mock control. While more than 90% of the fish infected with the wild type strain J2315 died within 6 days post inoculation, the *Bcam0581* mutant strain was much less virulent and only 40 % of the infected fish survived (Fig. 3-7A). *In trans* expression of the *Bcam0581* gene in d0581 fully restored virulence and none of the fish infected with the complemented mutant was alive at day 7 post infection (Fig. 3-7A).

Given that mutation of *Bcam0581* affects both virulence gene expression and bacterial growth in minimal medium, it was of interest to investigate whether inactivation of

Bcam0581 affects proliferation and survival of the mutant in the animal host. We therefore determined the number of bacterial cells within the fish body by plating serial dilutions of homogenates on LB plates. The data shown in Fig. 3-7B show that the c.f.u counts of the mutant during the first three days after infection were significantly lower than those of the wild type and the complemented mutant strain. While both the wild type and the mutant strain proliferated in a linear manner, proliferation of the complemented strain was stalled at day 2 past - infection (Fig. 3-7B). It is noteworthy that the *in vivo* growth trends of the three strains are similar to their *in vitro* growth patterns (Fig. 3-2A). On the 4^{th} day of infection, the bacterial cell number of the complemented strain was indistinguishable from that of the mutant d0581 (Fig. 3-7B), likely due to loss of the plasmid construct containing *Bcam0581*.

3.4 Discussion

Previous work has shown that *B. cenocepacia* utilizes a highly conserved AHL-dependent QS system for cell-to-cell communication (Gotschlich *et al*., 2001; Lewenza *et al*., 1999; Lutter *et al*., 2001). In this study, we present evidence that this bacterium also employs a BDSF-dependent communication system to coordinate virulence gene expression and biofilm formation. Genetic analysis demonstrated that a BDSF-null mutant was compromised in the expression of known virulence factors and attenuated in pathogenicity in a zebrafish infection model. Both RT-PCR and transcriptional analysis using promoter-*lacZ* gene fusion approach showed that the reduced expression levels of several virulence genes in the BDSF-null mutant could be fully restored by the exogenous addition of a physiological relevant amount of BDSF, indicating a positive regulatory role of this signal in modulation of bacterial virulence. Additionally, BDSF is

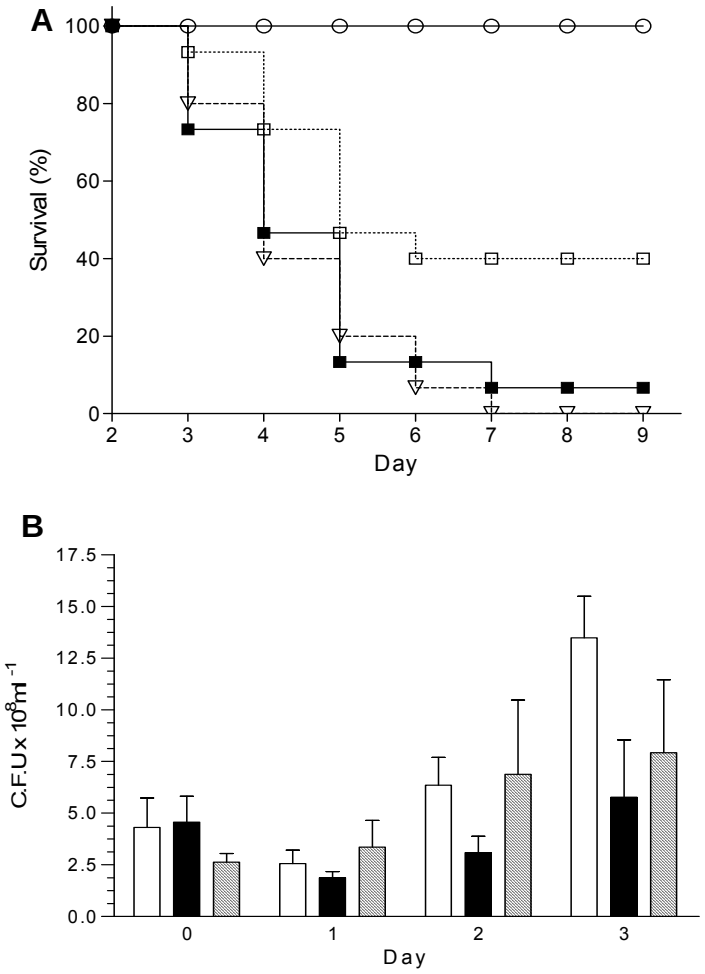


Fig. 3-7. BDSF is essential for full virulence of *B. cenocepacia* J2315 in a zebrafish infection model. (A) Survival rate of the fish after infection with the wild type strain J2315 (■); the BDSF-deficient mutant d0581 (□) and the complemented mutant d0581(pMLS7-0581) (Δ). Phosphate saline buffer was injected in the same way as a mock control (O). The experiment was repeated three times and a representative set of data is shown. (B) *In vivo* bacterial cell numbers of the strains in the host after infection. Symbol: open bar, J2315; solid bar, d0581; slashed bar, d0581(pMLS7-0581). The data presented are the mean of two independent experiments and the error bars represents the standard deviation.

negatively involved in the regulation of cell aggregation formation. BDSF-deficient mutant forms bigger cell aggregations not only in dynamic but also in static-biofilm formation.

Among the virulence genes positively regulated by BDSF, *zmpA* and the genes encoding ornibactin biosynthesis have been previously shown to be under the control of CepIR QS system (Subsin *et al*., 2007). The finding that deletion of *Bcam0581* did neither affect transcription of *cepI* nor *cepR* but addition of C8-HSL could restore virulence gene expression of the BDSF-null mutant suggests that the two signaling systems co-regulate expression of some virulence genes. Evidence is accumulating that a bacterial pathogen may have evolved several signaling pathways to regulate the same set of virulence genes. For example, *Pseudomonas aeruginosa* utilizes both an AHL- and a PQS-dependent QS system to modulate the production of a wide range of extracellular virulence factors (McKnight *et al*., 2000). Likewise, expression of the virulence regulon in *Xanthomonas campestris* is co-regulated by QS signal DSF and hypoxia cue (He *et al*., 2009). Identification of BDSF as another QS signal in *B. cenocepacia* presents a new addition to the expanding list of bacterial pathogens which recruit multiple signaling mechanisms for co-regulating virulence gene expression.

The population density-dependent accumulation of BDSF in the culture supernatant of *B. cenocepacia* J2315 is reminiscent of the one observed with AHL signal production (Kaplan and Greenberg, 1985; Kuo *et al*., 1994; von Bodman *et al*., 1998; Zhang *et al*., 1993). A rapid increase of the signal in culture supernatants was observed during the mid

exponential to late stationary growth phase (12 – 36 h post inoculation). This boost in BDSF level was preceded by a strong increase of promoter activity driving expression of *Bcam0581,* which encodes an enzyme required for BDSF biosynthesis (Boon *et al*., 2008), suggesting that BDSF production is controlled at the level of transcription. Given that maximum BDSF concentrations were observed one day after the peak of *Bcam0581* promoter activity, it is possible that biosynthesis of BDSF may also be controlled at the level of substrate availability as it is the case for AI-2 signal production in *Salmonella enterica* serovar. *typhimurium* (Beeston and Surette, 2002). The chemical structure of BDSF closely resembles the one of DSF produced by *X. campestris* (Boon *et al*., 2008; Wang *et al*., 2004), in which DSF biosynthesis is autoregulated by a signal sensor-synthase interaction mechanism (He *et al*., 2006a). So far, only one component of the *B. cenocepacia* BDSF-dependent signaling system, namely the BDSF synthase Bcam0581, has been identified (Boon *et al*., 2008). Additional work will be required to identify the BDSF sensor and the other components that constitute the signaling circuitry and to elucidate their interactions.

Biofilm formation is coupled to cell density and therefore it is regulated by quorum sensing signals. Disruption of the AHL QS system in *B. cenocepacia*, had been shown to result in defects in biofilm maturation (Huber *et al*., 2001). Similarly, we found that abolishing the BDSF signal results in significant changes to the biofilm surface architecture. Microscopy showed that the pellicle-like biofilm that forms at the liquid to air interface is considerably much more serrated in the mutant when compared to the wild type. Addition of BDSF or *in trans* expression of *Bcam0581* completely abolish this

phenomenon. These data thus implies a role for BDSF in the fine tuning of the biofilm structure. Since both AHL and BDSF signals can influence biofilm formation, this result enhances the interaction between AHL and BDSF systems in *B. cenocepacia* also involves in the regulation of biofilm formation

This study has also unveiled a novel function of Bcam0581 in addition to its role in the biosynthesis of BDSF. Our data suggest that Bcam0581 is essential for energy biogenesis during growth under unfavorable nutritional condition. The notion is supported by several lines of evidence. First, the growth of the deletion mutant d0581 was not affected in rich media, but was significantly retarded in minimal media where glucose is the sole carbon source. Second, although the growth defect was restored by *in trans* expression of *Bcam0581* in the mutant, exogenous addition of BDSF up to 25 times the physiologically relevant concentration did not rescue the growth defect. Third, inactivation of Bcam0581 resulted in decreased cellular ATP levels that could be restored by genetic complementation but not by BDSF. We further showed that minimal medium supplemented with citrate, but not malate or pyruvate, fully rescued the growth defect of the mutant. Consistently, *in trans* expression of the citrate synthase encoded by *Bcas0207* in the deletion mutant d0581, which was down-regulated by deletion of *Bcam0581*, resulted in a higher growth rate than the wild type strain. These data suggest that Bcam0581 may influence energy metabolism through affecting citric acid cycle. Such an 'additional' function of a signal molecule synthase has also been found in the case of the signal synthase LuxS, which in addition to the synthesis of AI-2, plays a key metabolic role in the activated methyl cycle through recycling the toxic intermediate S-

adenosylhomocysteine (Winzer *et al*., 2002). The detailed mechanism of Bcam0581 in energy metabolism in *B. cenocepacia* remains to be elucidated.

It was noticed that over-expression of *Bcam0581*in the mutant d0581 resulted in a higher initial growth rate than the wild type but then a growth-deceleration phenotype after 28 h. Coincidently, over-expression of the citrate synthase gene *Bcas0207* in d0581 also showed a similar growth-deceleration phenotype. Given that the expression of *Bcas0207* is positively modulated by Bcam0581, we speculate that this phenotype may be due to accumulation of a toxic metabolite(s) generated by the enzyme encoded by *Bcas0207*, which will be further investigated.

The virulence of mutant d0581 was significantly attenuated in zebrafish. While animals infected with the mutant strain had a survival rate of 40% only 10% of the fish infected with the wild type and none of those infected with the complemented mutant survived. Decreased virulence factor production in the mutant could partially account for the attenuated virulence, but the growth defect of the mutant under unfavorable nutritional conditions might also contribute to the compromised virulence, as it is likely that nutrient availability in the host is limited. Indeed, determination of bacterial cell numbers showed that the mutant's ability to survive in the zebrafish was significantly reduced. The dual role of Bcam0581 in QS-regulation of virulence and modulation of energy biogenesis may make this protein a very attractive drug target. Work is now in progress to map the entire BDSF regulon of *B. cenocepacia*, which would facilitate the characterization of

signaling pathway and shed light on the mechanism by which it cross talks with the AHL-dependent QS system.

CHAPTER 4

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE DSF-FAMILY QUORUM SENSING SIGNALS PRODUCED BY THE *BURKHOLDERIA CEPACIA* COMPLEX

4.1 Introduction

The *Burkholderia cepacia* complex (Bcc) is a family of important opportunist pathogens in patients with cystic fibrosis (CF) and chronic granulomatous disease (CGD) (Goldmann and Klinger, 1986; Isles *et al*., 1984; Mahenthiralingam *et al*., 2005). There are at least 9 species within the complex which are phenotypically similar but genetically distinct, and all the species are capable of causing infections (Coenye *et al*., 2001; Mahenthiralingam *et al*., 2005). *B. cepacia* infection usually causes cepacia syndrome, a necrotizing pneumonia with fever and occasional bacteremia, which may result in a rapid and fatal pulmonary decline and death (Isles *et al*., 1984; Mahenthiralingam *et al*., 2001, 2002). The genomovars of the *B. cepacia* complex could vary significantly in their pathogenic potentials and transmissibility (Mahenthiralingam *et al*., 2000; Vandamme *et al*., 1997). In addition, there are geographical differences in the prevalence of the *B. cepacia* complex, with *B. cenocepacia* and *B. multivorans* predominating North America and Europe, respectively (Govan *et al*., 2007).

Many bacterial pathogens have evolved a cell-cell communication mechanism known as

quorum sensing (QS) to coordinate the expression of their virulence genes. Disregarding their genetic differences, all the 9 genomovars of the *B. cepacia* complex are known to produce an acylhomoserine lactone-family QS signal, which is known for its role in modulation of virulence gene expression (Wopperer *et al*., 2006). Recently, another type of QS signal, i.e., *cis*-2-dodecenoic acid (BDSF), has been identified in *B. cenocepacia* (Boon *et al*., 2008). Subsequent studies showed that BDSF plays a role in regulation of bacterial virulence (Deng *et al*., 2009; Ryan *et al*., 2009). Interestingly, the two QS systems appear to act in conjunction in regulation of *B. cenocepacia* virulence as a set of the AHL-dependent virulence genes are also regulated positively by BDSF (Deng *et al*., 2009). Furthermore, mutation of the *Bcam0581* gene encoding BDSF biosynthesis results in retarded energy production and bacterial growth in minimal medium (Deng *et al*., 2009), highlighting the dual roles of the BDSF QS system in the bacterial physiology and infection.

BDSF is similar to the QS signal DSF (*cis*-11-methyl-2-dodecenoic acid) of *Xanthomonas campestris* pv. c*ampestris* (*Xcc*) (Barber *et al*., 1997; Wang *et al*., 2004). Evidence is accumulating that the DSF-family signals are widely conserved. For example, DSF and seven structural derivatives were found in *Stenotrophomonas maltophilia* (Huang and Wong, 2007), 12-methyl-tetradecanoic acid was produced by *Xylella fastidiosa* (Newman *et al*., 2004), and *cis*-10-2-decenocic acid was identified in *Pseudomonas aeruginosa* (Davies and Marques, 2009). In addition, DSF-like activity has also been reported in a range of *Xanthomonas* species, including *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas axonopodis* pv *citri* (Andrade *et al*., 2006; Barber *et al*., 1997;

Chatterjee and Sonti, 2002; Wang *et al*., 2004), but the chemical structures of DSF analogues in these bacterial pathogens remain to be determined.

The importance of the DSF-family signals is recognized due to their roles in regulation of virulence and biofilm formation in a range of bacterial pathogens (He and Zhang, 2008). In this study, we have investigated the ability of 9 genomovars of the *B. cepacia* complex in the production of the DSF-family signals. The results showed that BDSF is conserved in all the members of the *B. cepacia* complex. Interestingly, we showed that DSF and a novel member of the DSF-family signals were also produced by a few but not all the members of the *B. cepacia* complex. This new signal was purified and structurally characterized by nuclear magnetic resonance (NMR) and mass spectrometry analysis. Furthermore, we have determined the biological significance of the newly identified CDSF in intraspecies and interspecies signal communications.

4.2 Materials and Methods

4.2.1 Bacteria strains and growth conditions

The *B. cepacia* complex strains used in this work are listed in Table 4-1. These strains were grown at 28^{o}C or 37^{o}C as indicated with shaking at 250 rmp in Luria-Bertani (LB) broth. *Xanthomonas campestris* pv. *campestris* strain 8004 and its *rpfF* deletion mutant 8004dF were described previously (He *et al*., 2006; Wang *et al*., 2004). *Xcc* strains were maintained at 30^{o}C in YEB medium (Zhang *et al*., 2002). *B. cenocepacia* J2315 and its *Bcam0581* deletion mutant d0581 were described previously (Boon *et al*., 2008). For static biofilm formation assay of *B. cenocepacia*, bacteria were cultured in Basal salt

medium (pH 7.2) containing 20 mM citrate and 0.5% Casamino acids (Boon *et al*., 2008). The following antibiotics were supplemented when necessary: rafimpicin, 50 μg ml^{-1}; gentamycin, 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; trimethoprim, 400 μg ml^{-1} (*B. cenocepacia*) or 1.5 mg ml^{-1} (*E. coli*). *Candida albicans* SC5314 were grown in GMM medium consisting of 6.7 g of Bacto yeast nitrogen base (Difco) and 0.2% glucose (pH 7.2). The DSF-family signals were added to medium in a final concentration of 5 μM unless otherwise indicated.

4.2.2 Thin Layer Chromatography (TLC) and DSF bioassay analysis

The overnight bacterial culture supernatants (250 ml) were extracted with ethyl acetate in a 1:1 ratio. The organic phase was dried using a rotary evaporator and the residues were dissolved with 200 μl of methanol. An aliquot of 5 μl extracts was spotted onto a 20 × 20 cm silica gel TLC plate (MERCK) and separated with ethyl acetate-hexane (20:80 v/v) as running solvents. Subsequently, the plates were dried under air flow and overlaid with 100 ml of NYG medium (20 g Glycerol, 5 g peptone, 3 g yeast extract per liter), which was supplemented with 0.8 g agarose, 250 μg of 5-Bromo-4-chloro-3-indolyl-β-D-glucoside and 4 ml of the biosensor strain FE58 at an OD_{600} of 1.8 (Wang *et al*., 2004). TLC plate was incubated overnight at 28^{o}C and the DSF activity was indicated by the presence of a blue spot.

4.2.3 Purification and structural analysis of the DSF-family signals

To isolate and identify BDSF and its analogues from the supernatants of the *B. cepacia* complex, one liter culture of each strain was grown to an OD_{600} of about 3.0 and

Table 4-1. The bacteria strains used in this study

Strains	Characteristics	Growth Temperature	References/Source
B. cepacia 383	Genomovars I	37°C	Mahenthiralingam E. Laboratory
B. multivorans ATCC 17616	Genomovars II	37°C	Mahenthiralingam E. Laboratory
B. cenocepacia J2315	Genomovars III	37°C	ATCC
B. stabilis LMG 14086	Genomovars IV	28°C	BCCMTM
B. vietnamiensis G4	Genomovars V	37°C	Mahenthiralingam E. Laboratory
B. dolosa LMG 18941	Genomovars VI	28°C	BCCMTM
B. ambifaria AMMD	Genomovars VII	37°C	Mahenthiralingam E. Laboratory
B. anthina LMG 16670	Genomovars VIII	28°C	BCCMTM
B. pyrrocinia LMG 14191	Genomovars IX	37°C	BCCMTM
d0581	BDSF-minus mutant derived from J2315 with *Bcam0581* being deleted	37°C	Boon *et al*., 2008
d0581(0581)	Mutant d0581 harboring the expression construct pMLS7-Bcam0581	37°C	Boon *et al*., 2008
d0581(5121)	Mutant d0581 harboring the construct pMLS7-Bmul5121	37°C	This study
J2315 (egfp)	Wildtype harboring the expression construct pMLS7-egfp	30°C	This study
d0581(egfp)	Mutant d0581 harboring the expression construct pMLS7-egfp	30°C	This study
J2315(PzmpA-lacZ)	Wild type harboring the reporter construct pMLS7PzmpA-lacZ	37°C	Deng *et al*., 2009
d0581(PzmpA-lacZ)	Mutant d0581 harboring the construct pMLS7PzmpA-lacZ	37°C	Deng *et al*., 2009
8004	Wildtype strain of *Xanthomonas campestris* pv. *campestris*	30°C	He *et al*., 2006
8004dF	DSF-minus mutant derived from 8004 with *rpfF* being deleted	30°C	He *et al*., 2006
DH5α	*E.coli*	37°C	Laboratory collection
DH5α(0581)	DH5α harboring the expression construct pMSL7-Bcam0581	37°C	This study
DH5α(5121)	DH5α harboring the expression construct pMSL7-Bmul5121	37°C	This study
C. albicans SC5314	Clinical isolate	30°C	Gietz *et al*., 2007
FE58	Biosensor for DSF/BDSF	30°C	Wang *et al*., 2004

centrifuged. The supernatant was acidified to pH 4.0 by diluted HCl and extracted by ethyl acetate (1.0 v/v) twice. After removal of ethyl acetate by rotary evaporator, the residue was dissolved in methanol and subjected to flash chromatography on normal-phase silica gel, eluted consecutively with 2 bed volumes of hexane, 2 bed volumes of 10% ethyl acetate in hexane, and 4 bed volumes of 25% ethyl acetate in hexane. The active fractions, which were detected using the DSF sensor FE58 described previously (Wang *et al*., 2004), were combined for the HPLC profiling analysis on a reverse phase column (Phenomenex Luna 5 μ C_{18} 250 × 4.60 mm), eluted with 80% methanol in H_2O at a flow rate of 1 ml min^{-1}. Separation was monitored by UV detector with λ = 210 and 254 nm at a flow rate of 1 ml min^{-1}. Fractions at one minute interval were collected and assayed using the DSF biosensor FE58.

The ^{1}H, ^{13}C and heteronuclear multiple quantum coherence (HMQC) nuclear magnetic resonance (NMR) spectra in $CDCl_3$ solution were obtained using a Bruker DRX500 spectrometer operating at 500MHz for ^{1}H or 125MHz for ^{13}C. High-resolution electrospray ionization mass spectrometry was performed on a Finnigan/MAT MAT 95XL-T mass spectrometer using the conditions described previously (Wang *et al*., 2004).

4.2.4 Complementation of strains d0581 and heterologous expression of *Bcam0581* and *Bmul5121* in *E.coli*

The coding region of *Bmul5121* was amplified from *B. multivorans* via PCR using the primer pair BMUL5121-F (5'-tgctctagagcaatgcagctccaatcacatccc) and BMUL5121-R (5'-cccaagcttgggtcacaccgtgcgcaacttc). The product was digested with *Xba*I and *Hin*dIII and

ligated at the same enzyme sites under the control of the S7 ribosomal protein promoter in the plasmid vector pMSL7 (Lefebre and Valvano, 2002). After sequence verification, the resultant construct was introduced into the mutant d0581, by tri-parental mating. Transconjugants of d0581 were selected on the LB agar plates supplemented with gentamycin and trimethoprim. Complementation of the mutant d0581 with the gene *Bcam0581* of *B. cenocepacia* were followed the method described in Chapter 2. The donors used for the tri-parental mating, which harbor the expression construct pMSL7-Bcam0581 and pMSL7-Bmul5121, respectively, were used to analysis the heterologous expression of *Bcam0581* and *Bmul5121* in *E.coli*.

4.2.5 Extracellular polysaccharide and biofilm analysis

For quantification of the extracellular polysaccharide (EPS) production, 10 ml of overnight YEB cultures at OD_{600} of 3.0 were centrifuged at 12,000 rpm for 20 min. The supernatants were mixed with 2.5 volumes of absolute ethanol and the mixture was incubated at 4^{o}C for 30 min. The precipitated EPS was isolated by centrifugation and dried overnight at 55^{o}C before determination of dry weights.

For analysis of biofilm formation, a single colony of *Xcc* wild type and the DSF-minus mutant 8004dF was separately inoculated and grown overnight in 5 ml of YEB medium with or without signal molecule. Methanol was added to the corresponding wild-type strain as a solvent control. After overnight incubation, bacterial samples were visualized with a phase contrast microscope (Olympus BX50). Imaging was performed using an Olympus DP70 digital camera.

4.2.6 Static biofilm analysis and measurement of β-galactosidase activity

To fluorescently tag the cells, the plasmid pMLS7-egfp (Lefebre and Valvano, 2002) was conjugated into *B. cenocepacia* J2315 and its BDSF-minus mutant d0581. The bacterial cultures were grown overnight to an OD_{600} of about 3.0 and 5 µl cells were inoculated in duplicates into sterile 6-well tissue culture plates containing 3 ml of LB medium. The plates were incubated without agitation at 30^{o}C for 3 days. The biofilms formed in the air-liquid interface was sampled and visualized. Analysis and imaging of the static biofilm was performed using confocal scanning laser microscopy using a Carl Zeiss LSM510-Axiovert 100 M confocal microscope.

To test the effect of signal molecules on the expression of virulence genes, the previously generated *B. cenocepacia* containing the P*zmpA-lacZ* gene fusion was used as a reporter strain (Deng *et al*., 2009). For measurement of β-galactosidase activity, the bacterial cells were grown in LB medium at 37^{o}C with shaking at 250 rpm. When necessary, signal molecules were added separately to a final concentration of 5 µM as indicated. Bacterial cells were harvested and the β-galactosidase activities were assayed as described (Jeffrey, 1992).

4.2.7 Microscopic analysis and quantification of germ tube formation in *C. albicans*

To test the effect on *C. albicans* germ tube formation, the overnight culture of *C. albicans* strain SC5314 grown in GMM medium were diluted 20-fold in fresh GMM medium. Signal molecules were then added separately as indicated and the cells were induced for 3

h at 37^{o}C. Visualization and quantification of germ tube formation were performed using a phase contrast microscope (Olympus BX50) by counting about 400 fungal cells per sample. Imaging was done with an Olympus DP70 digital camera.

4.3 Results

4.3.1 BDSF-like signal molecules are found in all the genomovars of the *B. cepacia* complex

Our previous study showed that BCAM0581 of *B. cenocepatia* is the enzyme responsible for synthesis of BDSF (Boon *et al*., 2008). A BLAST search found that BCAM0581 is conserved in *B. cepacia* (Genomovars I), *B. multivorans* (Genomovars II), *B. cenocepacia* (Genomovars III), *B. vietnamiensis* (Genomovars V), *B. dolosa* (Genomovars VI), and *B. ambifaria* (Genomovars VII). The presence of BCAM0581 homologues in other Genomovars remains to be determined due to lack of genome sequence in public domain. Sequence alignment analysis of the BCAM0581 homologues showed that they are highly conserved with more than 94% amino acid identity, but none of them shares an identical sequence with its counterparts (Fig. 4-1A). The genome organization analysis showed that the neighboring region of *Bcam0581* was partly conserved, with a variable upstream section (left hand side) and a highly conserved down stream section (right hand side) (Fig. 4-1B). Sequence alignment and Blast search showed that *Bcam0580* encodes a PAS-GGDEF-EAL multidomain fusion protein, *Bcam0578* encodes a putative 5-oxoprolinase; and *Bcam0582* encodes a transglutaminase, which share about 80-91% identities in amino acids with their counterparts in other genomovars, respectively (Fig. 4-1B).

To test the ability of BDSF production, we analyzed the crude solvent extracts of nine strains from different genomovars of the *B. cepacia* complex using thin layer chromatography (TLC). After separation, the BDSF-like signals were detected by overlaying the TLC plate with DSF-sensor strain FE58, which contains a GUS gene under the control of a DSF-inducible promoter (Wang *et al*., 2004). A blue spot with the similar R_f value to BDSF was detected in all nine strains, suggesting that all of them produce a BDSF-like signal(s) (Fig. 4-1C).

4.3.2 Purification and structural analysis of the BDSF-like signals

For purification, the solvent extracts of supernatants were first subjected to flash chromatography. The active fractions identified by using bioassay were combined for reverse phase HPLC analysis. Bioassay of HPLC fractions and subsequent spectrometry analysis showed that similar to *B. cenocepacia* (Boon *et al*., 2008), BDSF was produced by other 8 genomovars of the *B. cepacia* complex (Fig. 4-2A). However, except for *B. cepacia, B. vietnamiensis, B. dolosa,* and *B. ambifaria,* which produced only BDSF (Fig. 4-2A), we found that the remaining *Burkholderia* species produced additional one or two molecules sharing DSF-like activity (Fig. 4-2A).

Identification of these unknown DSF-like molecules was illustrated using *B. multivorans,* which produced three UV absorbance peaks showing DSF-like activity (Fig. 4-2B). The three active fractions were collected separately and analysized using high-resolution electrospray ionization mass spectrometry (ESI-MS), which showed the *m/z* of fractions a, b and c are 211.27, 197.27, and 209.27, respectively (Fig. 4-3). These *m/z* values are

agreeable with the corresponding molecular formulas of $C_{13}H_{23}O_2$, $C_{12}H_{21}O_2$, and $C_{13}H_{21}O_2$, respectively. Combined with the NMR data and the biological activity analysis (Fig. 4-4), fraction a and b were characterized as *cis*-11-methyl-2-decenoic acid (DSF) and *cis*-2-dodecenoic acid (BDSF) (Fig. 4-4C), which were originally identified in *X. campestris* and *B. cenocepacia* (Wang *et al*., 2004; Boon *et al*., 2008), respectively.

For the fraction c, ^1H spectrum indicates that there are two pairs of ethylenic protons (Fig. 4-4A). The coupling constants between the protons in each pair are less than 11 Hz. This indicates that the two double bonds are both in *cis* configuration. The two methylene protons at δ_H 3.45 suggest that this methylene carbon connects with the two double bonds. The overlapped signals of two doublet methyl group at δ_H 0.87 indicate a branched structure the same as DSF (Wang *et al*., 2004). ^{13}C spectra reveal that one of the double bonds is conjugated with a carbolic acid (Fig. 4-4B). Therefore, the second double bond in the molecule should be at C-5 (Fig. 4-4B). Collectively, the ^1H, ^{13}C and HMQC data establish the structure of this active molecule as a novel DSF-family member, *cis*-11-methyldodeca-2, 5-dienoic acid, which is structurally identical to DSF except an extra double bond between C5 and C6 (Fig. 4-4). For consistency and convenience, this newly identified molecule was designated as CDSF hereafter. By combination of HPLC analysis and DSF bioassay, we showed that DSF was only produced by *B. multivorans,* whereas CDSF was also produced by three genomovars of the *B. cepacia* complex, i.e., *B. stabilis*, *B. anthina* and *B. pyrrocinia* (Fig. 4-2A).

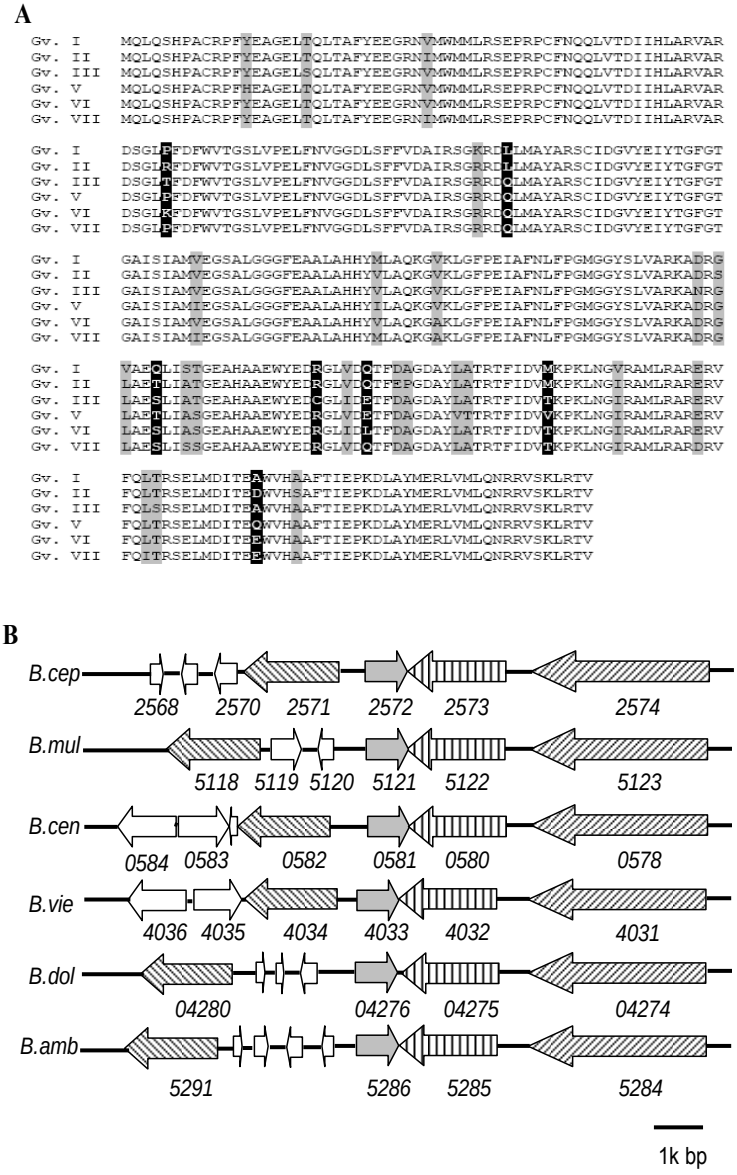
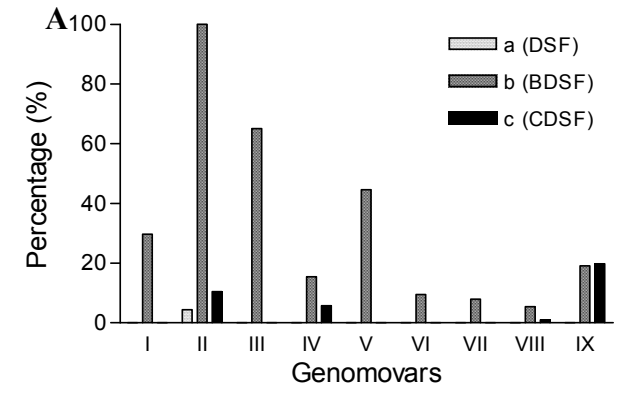
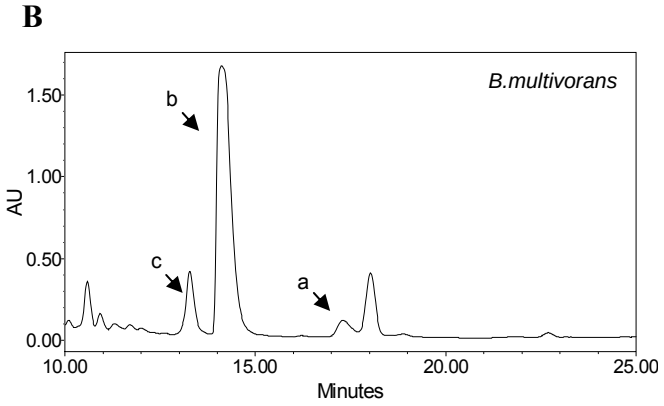
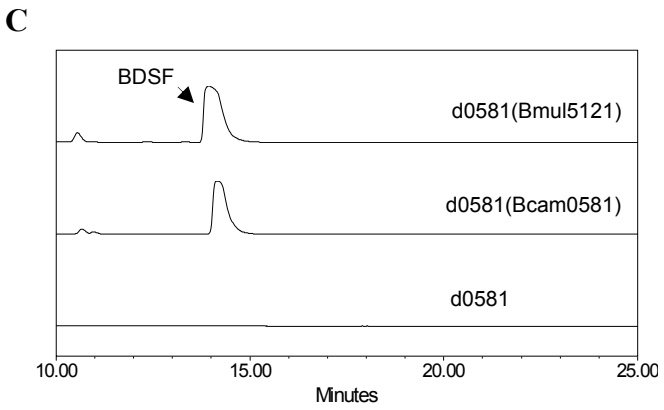

A
Gv. I MQLQSHPACRPFYEAGELTQLTAFYEEGRNVMWMMLRSEPRPCFNQQLVTDIIHLARVAR
Gv. II MQLQSHPACRPFYEAGELTQLTAFYEEGRNIMWMMLRSEPRPCFNQQLVTDIIHLARVAR
Gv. III MQLQSHPACRPFYEAGELSQLTAFYEEGRNVMWMMLRSEPRPCFNQQLVTDIIHLARVAR
Gv. V MQLQSHPACRPFHEAGELTQLTAFYEEGRNVMWMMLRSEPRPCFNQQLVTDIIHLARVAR
Gv. VI MQLQSHPACRPFYEAGELTQLTAFYEEGRNVMWMMLRSEPRPCFNQQLVTDIIHLARVAR
Gv. VII MQLQSHPACRPFYEAGELTQLTAFYEEGRNIMWMMLRSEPRPCFNQQLVTDIIHLARVAR
Gv. I DSGIPFDFDWVTGSLVPELFNVGGDLSFFVDAIRSGKRDLLMAYARSCIDGVYEIYTGFGT
Gv. II DSGIRFDFDWVTGSLVPELFNVGGDLSFFVDAIRSGRRDLLMAYARSCIDGVYEIYTGFGT
Gv. III DSGITFDFDWVTGSLVPELFNVGGDLSFFVDAIRSGRRDQLMAYARSCIDGVYEIYTGFGT
Gv. V DSGIPFDFDWVTGSLVPELFNVGGDLSFFVDAIRSGRRDQLMAYARSCIDGVYEIYTGFGT
Gv. VI DSGIKFDFDWVTGSLVPELFNVGGDLSFFVDAIRSGRRDQLMAYARSCIDGVYEIYTGFGT
Gv. VII DSGIPFDFDWVTGSLVPELFNVGGDLSFFVDAIRSGRRDQLMAYARSCIDGVYEIYTGFGT
Gv. I GAISIAMVEGSALGGGFEAALAHHYMLAQKGVKLGFPEIAFNLFPGMGGYSLVARKADRG
Gv. II GAISIAMVEGSALGGGFEAALAHHYVLAQKGVKLGFPEIAFNLFPGMGGYSLVARKADRS
Gv. III GAISIAMVEGSALGGGFEAALAHHYVLAQKGVKLGFPEIAFNLFPGMGGYSLVARKANRG
Gv. V GAISIAMIEGSALGGGFEAALAHHYILAQKGVKLGFPEIAFNLFPGMGGYSLVARKADRG
Gv. VI GAISIAMVEGSALGGGFEAALAHHYVLAQKGAKLGFPEIAFNLFPGMGGYSLVARKADRG
Gv. VII GAISIAMIEGSALGGGFEAALAHHYMLAQKGAKLGFPEIAFNLFPGMGGYSLVARKADRG
Gv. I VAEQLISTGEAHAAEWYEDRGLVDQTFDAGDAYLATRTFIDVMKPKLNGVRAMLRAREVV
Gv. II LAETLIATGEAHAAEWYEDRGLVDQTFEPGDAYLATRTFIDVMKPKLNGIRAMLRAREVV
Gv. III LAESLIATGEAHAAEWYEDCGLIDETFDAGDAYLATRTFIDVTKPKLNGIRAMLRAREVV
Gv. V LAETLIASGEAHAAEWYEDRGLVDETFDAGDAYVTTRTFIDVVKPKLNGIRAMLRAREVV
Gv. VI LAESLIASGEAHAAEWYEDRGLIDLTFDAGDAYLATRTFIDVTKPKLNGIRAMLRAREVV
Gv. VII LAESLISSGEAHAAEWYEDRGLVDQTFDAGDAYLATRTFIDVTKPKLNGIRAMLRARDRV
Gv. I FQLTRSELMDITEAVVHAAFTIEPKDLAYMERLVMLQNRRVSKLRTV
Gv. II FQLTRSELMDITEDVVHSAFTIEPKDLAYMERLVMLQNRRVSKLRTV
Gv. III FQLSRSELMDITEAVVHAAFTIEPKDLAYMERLVMLQNRRVSKLRTV
Gv. V FQLTRSELMDITEQVVHAAFTIEPKDLAYMERLVMLQNRRVSKLRTV
Gv. VI FQLTRSELMDITEEVVHAAFTIEPKDLAYMERLVMLQNRRVSKLRTV
Gv. VII FQITRSELMDITEEVVHAAFTIEPKDLAYMERLVMLQNRRVSKLRTV
B
B.cep
2568 2570 2571 2572 2573 2574
B.mul
5118 5119 5120 5121 5122 5123
B.cen
0584 0583 0582 0581 0580 0578
B.vie
4036 4035 4034 4033 4032 4031
B.dol
04280 04276 04275 04274
B.amb
5291 5286 5285 5284
1k bp
C
I II III IV V
VI VII VIII IX BDSF

Fig. 4-1. The DSF synthase and the DSF-like activity are conserved in the *B. cepacia* complex. (A) Sequence alignment of the Bcam0581 homologues of the *B. cepacia* complex. The strains and and the corresponding GeneBank accession numbers of the DSF synthases are: *B. cepacia* 383 (Gv. I), ABB12683; *B. multivorans* ATCC 17616 (Gv. II), ABX18791; *B. cenocepacia* J2315 (Gv. III), ABK10294; *B. vietnamiensis* G4 (Gv. V) ABO57014; *B. dolosa* AUO158 (Gv. VI), EAY71442; *B. ambifaria* AMMD (Gv. VII), ABI90833. The different amino acid residues are indicated by black shading, and the residues with similar physico-chemical properties are shown by gray shading. (B) Genomic organization of the *Bcam0581* homologues in the *B. cepacia* complex. *B.cep*, *B. cepacia* 383; *B.mul*, *B. multivorans* ATCC17616; *B.cen*, *B. cenocepacia* J2315; *B.vie*, *B. vietnamiensis* G4; *B.dol*, *B. dolosa* AUO158; *B.amb*, *B. ambifaria* AMMD. The gray shading arrows indicate the *Bcam0581* homologues. The other filled arrows indicate the genes flanking the *Bcam0581* homologues in each genomovars genome. (C) TLC analysis of the DSF-like activity from the crude extracts of the *B. cepacia complex* by using the DSF sensor strain FE58. The genomovars were denoted in roman numerals. Synthetic BDSF was included as the positive control.


A
100
80
60
40
20
0
Percentage (%)
a (DSF)
b (BDSF)
c (CDSF)
I
II
III
IV
V
VI
VII
VIII
IX
Genomovars


B
B.multivorans
b
c
a
1.50
1.00
0.50
0.00
AU
10.00
15.00
20.00
25.00
Minutes


C
BDSF
d0581(Bmul5121)
d0581(Bcam0581)
d0581
10.00
15.00
20.00
25.00
Minutes

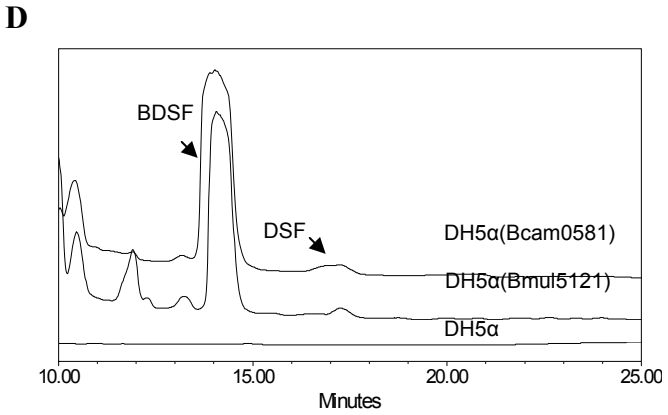

D
BDSF
DSF
DH5α(Bcam0581)
DH5α(Bmul5121)
DH5α
10.00
15.00
20.00
25.00
Minutes

Fig. 4-2. Characterization of the DSF-family signals produced by the different genomovars of the *B. cepacia* complex. (A) The DSF signal spectrum in the *B. cepacia* complex. The percentage was based the peak area of each signal molecule. For the convenience of comparison, the peak b (BDSF) of genomovars II was arbitrarily defined as 100%, which was used to normalize the signal ratios in different genomovars. (B) The HPLC spectrum of the *B. multivorans* supernatant extracts after flash chromatography. (C) Production of BDSF in the BDSF-minus mutant d0581 by *in trans* expression of the *Bcam0581* cloned from *B. cenocepacia* and its homologue *Bmul5121* from *B. multivorans.* (D) Production of DSF and BDSF in *E.coli* 5α by heterologous expression of *Bcam0581* cloned from *B. cenocepacia* and its homologue *Bmul5121* from *B. multivorans.*

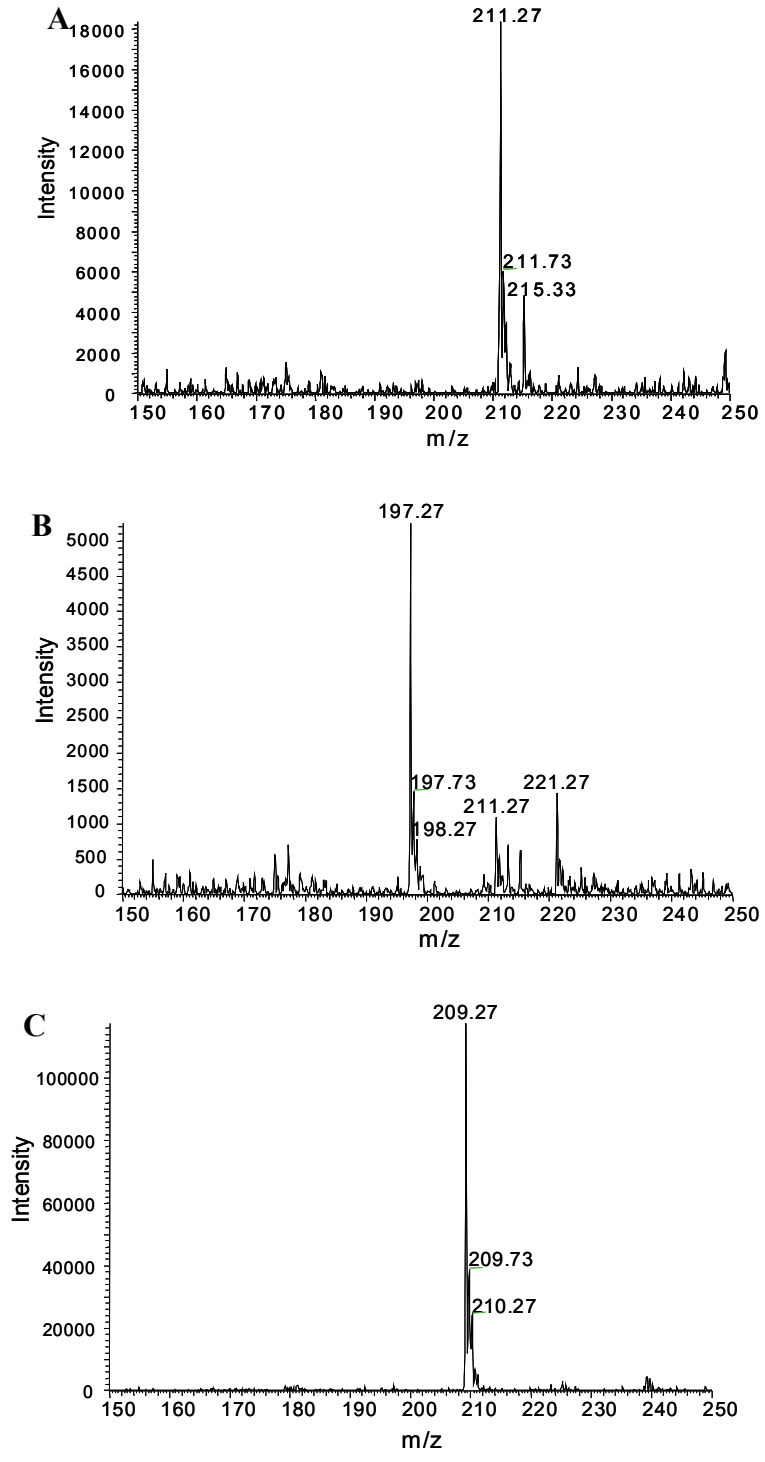


Fig. 4-3. ESI-MS analysis of purified fractions from *B. multivorans*. (A) ESI-MS spectrum of DSF. (B) ESI-MS spectrum of BDSF. (C) ESI-MS spectrum of CDSF

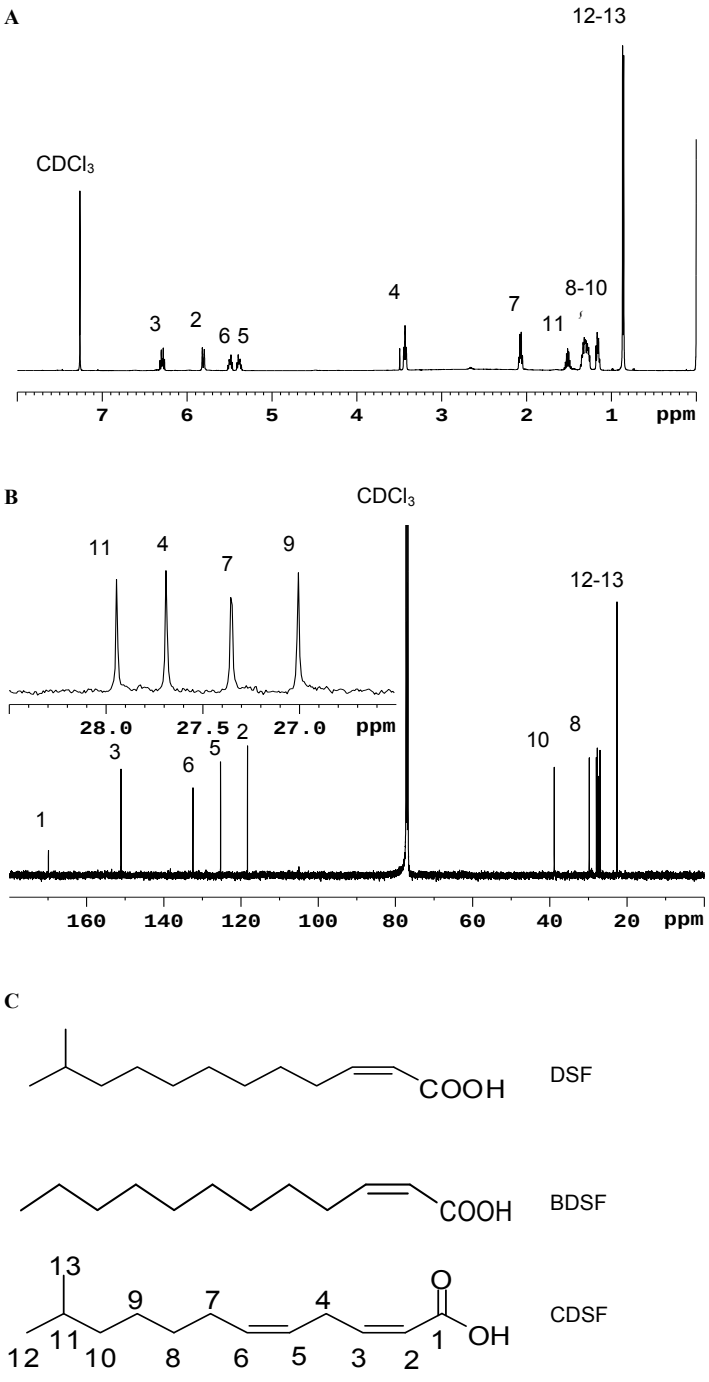


Fig. 4-4. NMR analysis of purified CDSF. (A) ^{1}H NMR spectral of CDSF. (B) ^{13}C NMR spectra of CDSF. (C) The predicted chemical structure of CDSF compared to DSF and BDSF. NMR, nuclear magnetic resonance.

4.3.3 The difference in DSF signal spectrum in the *B. cepacia* complex is not related to the variation of *Bcam0581* homologues

Quantitative analysis showed that the members of the *B. cepacia* complex were differed not only in the quantity of but also in the variety of DSF-like molecules they produced (Fig. 4-2A). Given that none of the genomovars shares an identical DSF synthase with the others (Fig 5-4A), it became intriguing to test whether the ability to produce different DSF-like signals is related to the variation in the DSF synthases. To this end, the DSF synthase gene *Bcam0581* of *B. cenocepacia* and its homologue *Bmul5121* of *B. multivorans* were cloned and expressed in the *Bcam0581* deletion mutant d0581. As expected, overexpression of *Bcam0581* in d0581 rescued the BDSF biosynthesis (Fig. 4-2C). Interestingly, in *trans* expression of *Bmul5121* in d0581 also led to the production of BDSF only but not DSF and CDSF (Fig. 4-2C). However, heterologous expression of *Bcam0581 and Bmul5121* in *E.coli* DH5α both caused the production of DSF and BDSF (Fig. 4-2D). The data indicate that it is the genetic background of *Burkholderia* strains but not the variation in the DSF synthases that governs their DSF signal spectrum.

4.3.4 CDSF is a functional analogue of DSF and BDSF

To evaluate the biological relevance of CDSF, we tested its ability in bacterial interspecies communication. In plant bacterial pathogen *X. campestris*, DSF is required for the maintenance of bacterial planktonic growth (Dow *et al*., 2003; He et al., 2006). Wild type strain 8004, which produces DSF, proliferated as planktonic cells (Fig. 4-5A), whereas the DSF-minus mutant 8004dF was grown in biofilm form (He *et al*., 2006) (Fig. 4-5B). Similar to the effect of DSF and BDSF (Fig. 4-5C, 5D), addition of 5 μM of

CDSF completely dispersed the cell aggregates formed by the mutant 8004dF (Fig. 4-5E).

We then quantitatively compared the biological activity of CDSF with its analogues on the induction of the extracellular polysaccharide (EPS) production in mutant 8004dF. The results showed that addition of 5 μM of DSF, BDSF and CDSF to the DSF-minus mutant 8004dF, increased the EPS productions to 78%, 69% and 78.9% of the wild type level, respectively (Fig. 4-5F). These results establish CDSF as an effective signal in the bacterial interspecies communication.

4.3.5 CDSF is a potent signal in bacterium-fungus interkingdom communication

Previous studies from our laboratory showed that BDSF and DSF are able to modulate the morphological transition of *C. albicans* (Boon *et al*., 2008; Wang *et al*., 2004). To test whether the extra double bond in CDSF might influence its potency in bacterium-fungus interkingdom communication, this newly identified DSF-like molecule and its analogues were added to the fresh fungal yeast cells, respectively, using methanol as a solvent control. After incubation at 37^{o}C for 3 h, majority of *C. albicans* cells in the solvent control formed germ tubes (Fig. 4-6A), the fungus grew mainly in the form of yeast cells with the treatment of 5 μM of CDSF, DSF, or BDSF (Fig. 4-6B-D). Quantitative analysis using a series of diluted signals showed that BDSF was the most potent signal followed by CDSF and DSF on the inhibition of the *C. albicans* germ tube formation (Fig. 4-6E).

4.3.6 CDSF is a functional analogue of BDSF in the regulation of *B. cenocepacia* biofilm formation and virulence factor production

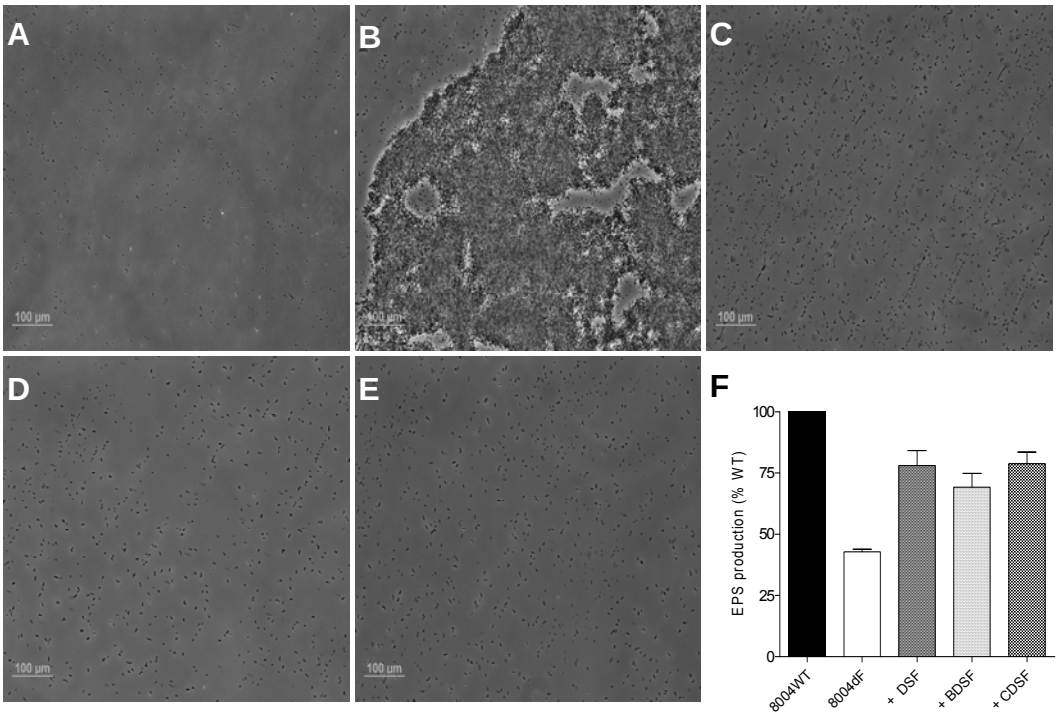


Fig. 4-5. The bacterial morphology of the *Xcc* wild type strain 8004 and the DSF-minus mutant 8004dF is shown in (A) and (B), respectively. Addition of DSF (C), BDSF (D), and CDSF (E) to 8004dF restored the planktonic grow form. (F) Exogenous addition of DSF, BDSF and CDSF in the mutant 8004dF restored the EPS production. The error bars show the standard deviations.

Previous studies showed that DSF plays a key role in negative regulation of the *Xcc* cell aggregate formation (Dow *et al*., 2003; He *et al*., 2006). This encouraged us to examine the role of the DSF-family signals in the biofilm formation of *B. cenocepacia*. When grown statically, *B. cenocepacia* formed a thin layer of pellicle-like biofilm on the liquid-air interface. Microscopic examination of the biofilms formed by the wild type *B. cenocepacia* revealed a smooth surface with a few small cell aggregates (Fig. 4-7A), whereas the surface of the biofilms formed by the BDSF-minus mutant was uneven, which showed the presence of large protrusions (Fig. 4-7B). The biofilm structure was restored to that of wild type strain when the mutant was grown in the presence of DSF, BDSF and CDSF (Fig. 4-7C-E), respectively.

Our previous study showed that the deletion of the BDSF synthase gene *Bcam0581* in *B. cenocepacia* resulted in the decreased expression of virulence genes, and this reduction was rescued by addition of BDSF (Deng *et al*., 2009). To test whether CDSF and DSF are the functional analogues of BDSF, the *B. cenocepacia* BDSF-minus mutant d0581 harboring the P*zmpA*-lacZ reporter gene fusion was used to test the effect of exogenous addition of signals on the regulation of the *zmpA* expression. Compared with the wild-type, the *zmpA* promoter activity in the mutant d0581 was decreased by 53% (Fig. 4-7F). This reduction was not only rescued by addition of BDSF, but also by supplementation of CDSF and DSF at the same concentration, respectively (Fig. 4-7F).

4.4 Discussion

The results of this study showed that BDSF is a conserved signal in the *B. cepacia*

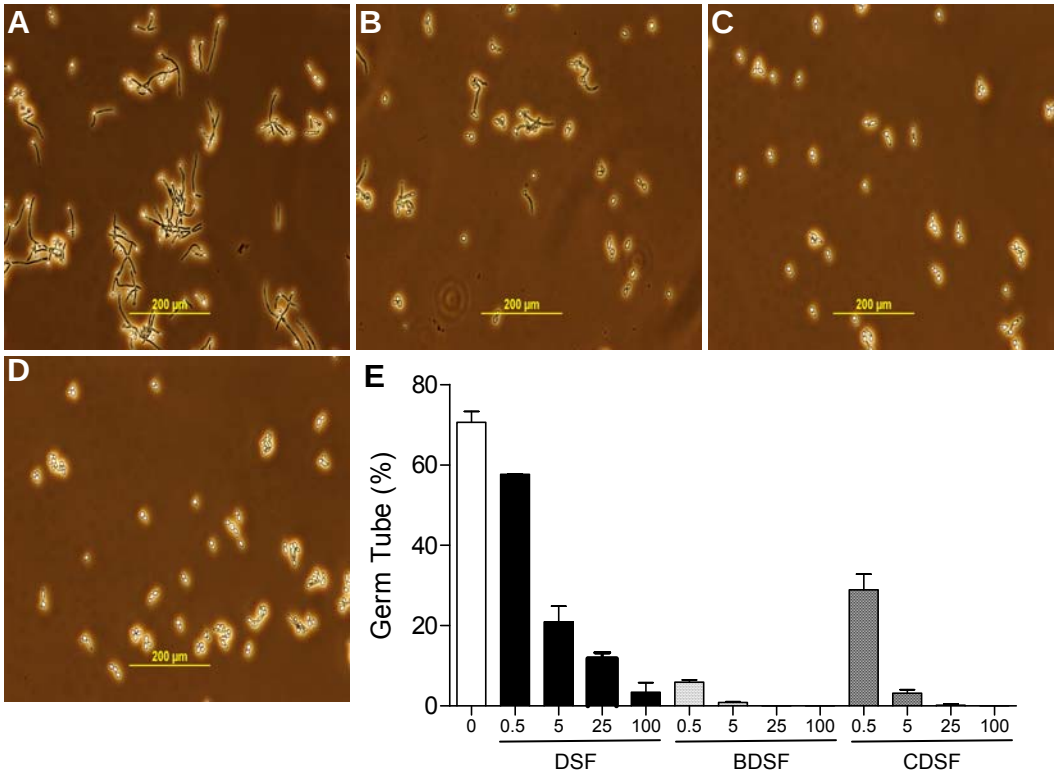


Fig. 4-6. The effect of the DSF-family signals on *C. albicans* germ tube formation. *C. albicans* cells were grown under induction conditions (37^{o}C), with the equal volume of methanol as control (A). In (B), (C) and (D), the cells were grown under the same condition as in (A) but supplemented with 5 μM of DSF, BDSF and CDSF, respectively. The photos were taken 3 h after induction. (E) Measurement of the inhibitory activity of the DSF-family signals on the germ tube formation of *C. albicans*. The experiment was performed twice and each time at least 400 cells were counted per treatment. The error bars show the standard deviations.

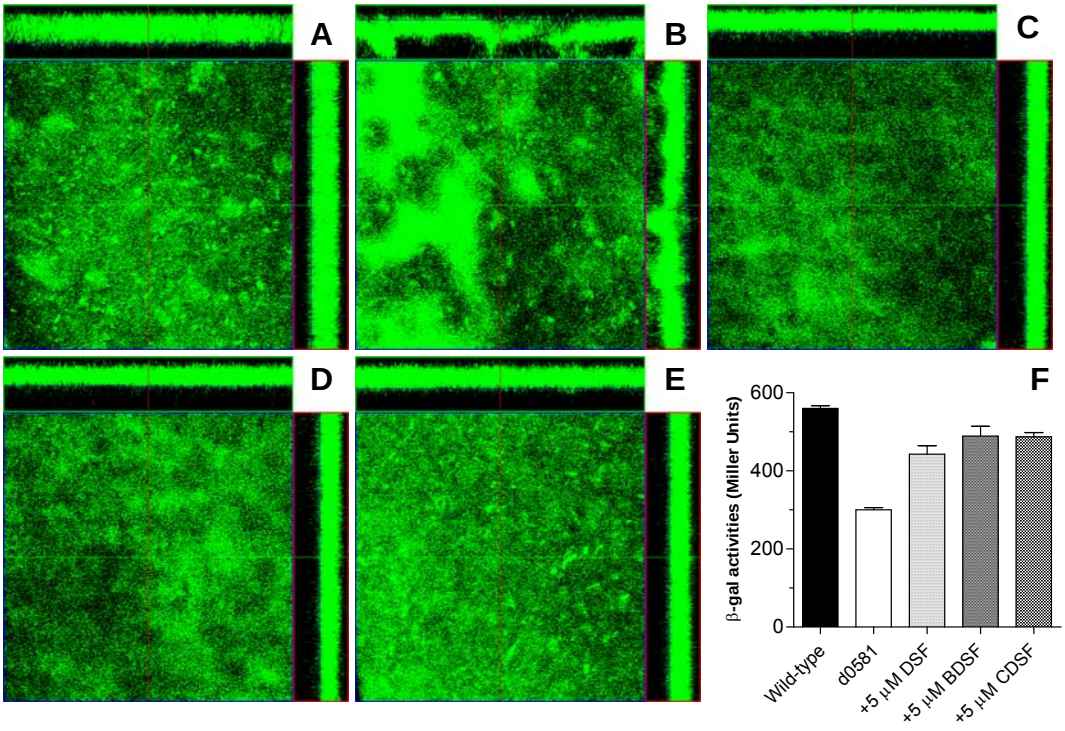


Fig. 4-7. Influence of exogenous addition of the DSF-family signals on static biofilm and virulence gene expression. The surface image of the static biofilm of wild type *B. cenocepacia* J2315 (A), its BDSF-minus mutant d0581 (B), d0581 supplemented with DSF (C), d0581 supplemented with BDSF (D), d0581 supplemented with CDSF (E). Scanning confocal images of the surface of static biofilms was using a 40X objective. (F) Virulence gene expression determined by using strain d0581 (P*zmpA-lacZ*). Bacterial strains were grown in LB medium, and the DSF-family signals were added separately at a final concentration of 5 μM. The data shown are the means of three repeats and error bars indicate the standard deviations.

complex with all the 9 genomovars producing BDSF as the major DSF-family signal molecule (Fig. 4-2A). In addition to its role in the interspecies signal communication (Boon *et al*., 2008), BDSF has recently been shown to play a role in the regulation of *B. cenocepacia* virulence genes expression (Deng *et al*., 2009; Ryan *et al*., 2009). In addition, evidence is emerging that *B. cenocepacia* appears to recruits both the AHL-type QS system and BDSF in coordination of the virulence gene expression (Deng *et al*., 2009). Interestingly, similar to BDSF, the AHL-type QS signal C8HSL is also produced by all the members of the *B. cepacia* complex (Wopperer *et al*., 2006). These findings, together with the results presented in this study, suggest that the BDSF QS system and the AHL-type QS system are likley co-evolved in the *B. cepacia* complex for the modulation of the bacterial physiology and virulence.

Surprisingly, while 5 genomovars, i.e., *B. cepacia*, *B. cenocepacia*, *B. vietnamiensis*, *B. dolosa*, and *B. ambifaria*, only produced BDSF, the remaining 4 members of the *B. cepacia* complex including *B. multivorans*, *B. stabilis, B. anthina* and *B. pyrrocinia* were shown to synthesize CDSF (Fig. 4-2A), which is a new member of the DSF-family signals. Among them, *B. multivorans* also produced another signal molecule, i.e., DSF (Fig. 4-2A; 2B), which is the first identified member of the DSF-family signals from the plant bacterial pathogen *X. campestris* pv. *campestris* (He and Zhang, 2008; Wang *et al*., 2004). What may account for this variation in the diversity of the DSF-family signals in these bacterial species? The findings from this study preclude the possibility that the variations in DSF synthases may be responsible for the difference in their product spectrum. Firstly, null mutation of the DSF synthase in *B. cenocepacia* abrogated its

ability to synthesize BDSF (Fig. 4-2C). Secondly, only the BDSF molecule was detected when the corresponding DSF synthase genes from both *B. cenocepacia* and *B. multivorans* were separately overexpressed in the BDSF-minus mutant of *B. cenocepacia* (Fig. 4-2C). Thirdly, heterologous expression of *Bcam0581 and Bmul5121* in *E.coli* DH5α both caused the production of DSF and BDSF (Fig. 4-2D). In addition, interrogation of the *B. multivorans* genome sequence with the protein sequence of the DSF synthase Bmul5121 did not reveal the presence of paralogues. Taken together, these data suggest that the variations in the production of different DSF analogues by the members of the *B. cepacia* complex is likely related to the availability of different precursors in each genomovars.

Structural analysis characterized CDSF as *cis*-11-methyldodeca-2, 5-dienoic acid (Fig. 4-4), which differs from DSF by an extra double bond in *cis*-configuration at the C5-C6 position (Fig. 4-4C). Functional characterization of this newly identified signal molecule showed that CDSF is not only active in the interspecies communication (Fig. 4-5, Fig. 4-6), but is also potent in the regulation of the virulence gene expression and biofilm development by *B. cenocepacia* (Fig. 4-7). Agreeable with the previous finding that the methyl group substitution at C11 contributes to the biological activity in the regulation of virulence gene expression (Wang *et al*., 2004), we found that CDSF was superior than BDSF in the induction of the EPS production in *X. campestris* pv. *campestris* (Fig. 4-5F). The conserved distribution of the DSF-family siganls in the *B. cepacia* complex has further strengthened the notion that DSF represents a new class of widely conserved signals for bacterial cell-cell communications (He and Zhang, 2008).

CHAPTER 5

ATTENUATION OF *PSEUDOMONAS AERUGINOSA* VIRULENCE BY BDSF THROUGH INHIBITION ON THE QUORUM SENSING AND TYPE III SECRETION SYSTEMS

5.1 Introduction

The human pathogen *Pseudomonas aeruginosa* usually shares the same niche as *B. cenocepacia* in the cystic fibrosis patients. *P. aeruginosa* is a ubiquitous environmental organism capable of infecting a wide variety of animals, plants, and insects. As a human pathogen, this bacterium is a major agent of opportunistic infections in immunocompromised individuals and cystic fibrosis patients (Bodey *et al*., 1983; Richards *et al*., 2000). *P. aeruginosa* has evolved three types of quorum sensing systems, i.e., *las*, *pqs* and *rhl*, which are implicated in regulation of several aspects of pathogenesis, including virulence factor production, biofilm development, and antimicrobial resistance. In *P. aeruginosa*, the *las* quorum-sensing system consists of the transcriptional activator LasR and the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), the synthesis of which is directed by the LasI autoinducer synthase (Gambello and Iglewski, 1991; Passador *et al*., 1993; Pearson *et al*., 1994). Similarly, the *rhl* system consists of the transcriptional activator RhlR and the autoinducer *N*-butyryl-L-homoserine lactone (C4-HSL), the synthesis of which is directed by the RhlI autoinducer synthase (Ochsner *et al*., 1994; Ochsner and Reiser, 1995; Pearson *et al*., 1994).

Moreover, there is the third intercellular signal, which was recently identified as 2-heptyl-3-hydroxy-4-quinolone (PQS) (Pesci *et al*., 1999). It was shown that LasR is required for PQS production and that RhlR is important for PQS bioactivity, indicating that PQS is intertwined in the quorum sensing hierarchy (Pesci *et al*., 1999).

Type III secretion system (T3SS) is a key virulence determinant in a wide range of animal and plant pathogens. Defects in T3SS may cause the bacterium to be non-pathogenic. T3SS plays diverse roles in host-pathogen interactions, such as promoting bacteria internalization in mammalian cells (Hayward and Koronakis, 1999), induction of macrophage apoptosis (Mills *et al*., 1997), inhibition of phagocytosis by changing macrophage actin structures (Frithz-Lindsten *et al*., 1997), and generation of pores in host cells (Lee *et al*., 2001). Diseases caused by the T3SS-bacteria kill hundreds of thousands of people every year. It was shown that most clinical isolates of *P. aeruginosa* use the type III secretion system to evade phagocytosis and facilitate infection (Feltman *et al*., 2001; Moss *et al*., 2001; Sato and Frank, 2004; Yahr *et al*., 1996; 1998). This system delivers cytotoxins, which have been identified as ExoS, ExoT, ExoY and ExoU, directly into eukaryotic cells (Caron and Hall, 1998).

Some synthetic compounds such as synthetic derivate of natural furanone can act as the antagonists of quorum sensing of *P. aeruginosa* and attenuate its virulence (Hentzer *et al*., 2003a; 2003b; Wu *et al*., 2004). Moreover, a group of salicylidene acylhydrazides were identified as inhibitors of T3SS in some human pathogens (Veenendaal *et al*., 2009). These results indicate that interference and inhibition on the quorum sensing and T3SS

can be specifically utilized as favorable therapeutic methods on *P. aeruginosa* infection (Wu *et al*., 2004; Hentzer and Givskov, 2003).

The DSF-family signals not only control a range of biological functions through intraspecies signaling; they can also interfere with other microorganisms through inter-species communication. DSF and BDSF were shown to inhibit the morphological transition of *Candida albicans* (Wang *et al*., 2004; Boon *et al*., 2008). Ryan *et al.* (2008) reported that DSF produced by *S. maltophilia* influenced the biofilm formation and tolerance to polymyxins B and E of *P. aeruginosa*. *B. cenocepacia* and *P. aeruginosa* are usually found to co-infect in the same niche of cystic fibrosis patients. In this chapter, we will study the role of BDSF plays on the inter-species communication between *B. cenocepacia* and *P. aeruginosa*, by focusing on its effect on the quorum sensing systems and T3SS of *P. aeruginosa*.

5.2 Methods and Materials

5.2.1 Bacterial strains and growth conditions

P. aeruginosa strains were maintained in Luria-Bertani (LB) broth at 37^{o}C. For analysis of the type III secretion system, bacteria were grown in LB medium supplemented with 10 mM NTA. For pyocyanin analysis, bacteria were grown in PA medium (per liter contains Bacto-peptone 20g, Glycerol 10g, K_2SO_4 10g, $MgCl_2$ 1.4g, PH 7.0). The following antibiotics were supplemented when necessary: tetracycline 100 μg ml^{-1}; kanamycin 50 μg ml^{-1}. Growth curves were performed at 37^{o}C in LB medium supplemented with or without BDSF using Bioscreen-C Automated Growth Curves

Analysis System. BDSF were synthesized as described previously (Wang *et al*., 2004). It was added to the medium as indicated.

Table 5-1. PCR primers used in this study

Primer	Sequence
For reporter construction	
pC-F	5'- gctctagacggtgatccagtccttc
pC-R	5'- ggggcgcctcctaaagctc
F-pLasR-HindIII	5'- cgatgggccgacagtgaacc
R-pLasR-EcoRI	5'- ctgcaggatggcgctccactc
F-pRhlR2-HindIII	5'- ggtgccgcaggtgctgctg
R-pRhlR-EcoRI	5'- gtggatcggctgcatctcgc
F-pRhlI-HindIII	5'- cgacgcgccgaacaagacg
R-pRhlI-EcoRI	5'- cagctcggcgatcatggcg
F-MvfR-pro-HindIII	5'- gtgcgtcatagtcgctacacctgaag
R-MvfR-pro-EcoRI	5'- ccgacggaccagctccacg
For RT-PCR analysis	
exsA-F	5'- ggcggcgatagctctgggtgaaat
exsA-R	5'- cgccgcggaagctatgtcgtaagt
exsC-F	5'- tggatttaacgagcaaggtcaa
exsC-R	5'- cgagaatctgcgcatacaactg
exoS-F	5'- ctcggccgtcgtgttcaagcagat
exoS-R	5'- ccggggttcagggaggtggaga
exoU-F	5'- gcggcgcaacgacaacctgat
exoU-R	5'- gaaaagccaccgccccgtctgt
exoT-F	5'-caggcgccgctctcccgtcag
exoT-R	5'-ctccgcctccagcccgaagtgc
exoY-F	5'-gcagggccccagcggtaaac
exoY-R	5'-gtcgggatgggcggtgaagtgata

5.2.2 Construction of reporter strains and measurement of *β*-galactosidase activity

The promoters of *lusI*, *lusR*, *rhlI*, *rhlR* and *pqsR* were amplified using the primer pairs listed in Table 5-1 with *Hin*dIII and *Eco*RI restriction sites attached. The resulting products were digested with *Hin*dIII and *Eco*RI, and ligated to the similarly digested vector pME2-lacZ. These constructs, verified by DNA sequencing, were introduced into *P. aeruginosa* by electroporation. Transconjugants were then selected on LB agar plates containing tetracycline. Bacteria were grown in LB medium supplemented with or without BDSF at 37°C to an OD_{600} of about 1.5. The bacterial cells were harvested to measure *β*-galactosidase activities following the methods as described previously (Jeffrey, 1992).

For the construction of reporter strain of T3SS, the promoter of *exsCEBA* was amplified by PCR using the primer pairs listed in Table 5-1, and cloned into the integration vector mini-CTX-lacZ (Zhou *et al*., 2007). The construct was introduced into *E. coli* S17-1(λpir) and then integrated into the chromosome of *P. aeruginosa* as described previously (Hoang *et al*., 2000). The engineered strain was then selected on the LB agar plates containing 100 μg/ml tetracycline and used as the T3SS reporter strain. For determination of the inhibitory activity against T3SS, BDSF was added to the LB medium supplemented with 10 mM NTA before inoculation of the T3SS reporter strain. After incubation at 37°C to an OD_{600} of 1.5, the bacterial cells were harvested by centrifugation to measure the *β*-galactosidase activities.

5.2.3 PQS assay

PQS production was assayed following the method described previously (Fletcher *et al*., 2007). In brief, overnight starter culture was inoculated in LB medium with or without BDSF to an OD_{600} of 0.05 and then grown at 37°C for 16 hours. The culture was centrifuged and 5 ml of supernatants were taken out and added with equal volume of acidified ethyl acetate. After mixing vigorously for 5 min, the top organic layer was collected. The organic solvent was removed by rotary evaporation, and the residue was dissolved in 100 µl methanol as the PQS extract for further analysis.

The normal phase silica gel TLC plates (MERCK) were activated by soaking in a 5% (w/v) solution of KH_2PO_4 for 30 min, and then dried at 100°C for 2 hours. For each sample, 10 µl of extract was spotted onto a TLC plate with synthetic PQS used as a positive control. The TLC plate was placed in a developing tank containing a mixture of dichloromethane: methanol (95:5) as the mobile phase until the solvent front reached about 1-2 cm from the top of the plate. The TLC plate was visualized using a UV transilluminator at 312 nm. The signal density of PQS was determined using ImageJ (http://rsb.info.nih.gov/ij/).

5.2.4 C4HSL and 3-oxo-C12-HSL assay

Bacteria were grown overnight at 37°C in LB liquid medium with or without BDSF. Culture supernatants (25 ml) were collected by centrifugation when it grew to an OD_{600} of about 2.2, and extracted with equal volume of the acidified ethyl acetate. The extracts were dried using a rotary evaporator and dissolved in 50 µl methanol. *N*-butyryl-L-homoserine lactone (C4-HSL) production was assayed using its biosensor strain CV026

(McClean *et al*., 1997). Bioassay plates were prepared by growing CV026 overnight at 28^{o}C in LB liquid medium and diluted in the ratio of 1:100 in melted LB agar (42^{o}C). The wells about 4-mm diameter were punched in the middle of the plates and 2.5 μl extracts was added to each well. The plates were incubated at 28^{o}C overnight. C4-HSL was indicated by the presence of purple halo around the well. The amount of production was quantified by measuring of the diffuse diameter of the purple halo.

3-oxo-C12-HSL was determined quantitatively with a reporter strain *A. tumefaciens* NT1 (Zhang *et al*., 2002). In brief, the reporter strain were grown at 28 ^{o}C in minimal medium supplemented with the extracts used for C4HSL analysis (Dong *et al*., 2000). When it grew to an OD_{600} of about 1.5, the bacterial cells were harvested by centrifugation to measure the *β*-galactosidase activities.

5.2.5 Proteolytic activity assay

Protease activity was quantified following the method described by Denkin *et al*. (2004) with minor modifications. Briefly, bacteria were cultured at 37^{o}C for about 12 hours with or without BDSF as indicated. After measuring the optical density at 600 nm, cultures were centrifuged at 13,000 rpm for 5 min and the supernatants were taken out and filtered through a 0.2 μm pore size cellulose-acetate filter. One hundred microliter of supernatants were incubated at 30^{o}C with equal volume of azocasein dissolved in proteolytic buffer B for 30 min. The reaction was stopped by addition of 406 μl of 10% (w/v) TCA buffer. After incubation for 2 min at room temperature, the mixture was centrifuged at 13,000 rpm for 1 min to remove the remaining azocasein. Supernatants

were taken out and mixed with 700 μl of 525 mM NaOH. Absorbance of the azopeptide supernatant was measured at the wavelength of 442 nm. Protease activity was obtained after normalization the absorbance against the corresponding cell density.

5.2.6 Pyocyanin assay

Bacteria were grown overnight at 37^{o}C in the PA medium with or without BDSF. After measuring the absorbance at 600 nm, the supernatants of bacterial culture were collected for extraction of pyocyanin with the method described previously (Essar *et al*., 1990). Briefly, cultures were centrifuged at 13,000 rpm for 1 min and 1.5 ml supernatants were collected and extracted with double volume chloroform with vigorous shaking at room temperature for 30 min. The solvent phase was transferred to a new tube containing 1 ml of 1N HCl. The mixture was shaken gently to transfer pyocyanin to aqueous phase. The quantity of pyocyanin was determined by measurement of absorbance at 520 nm and normalization against the cell density.

5.2.7 RNA extraction, RT-PCR and microarray analysis

P. aeruginosa was grown in LB medium supplemented with NTA till OD_{600} of 1.5. Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of RNA were determined by agarose gel electrophoresis and spectrometry. RT-PCR analysis was performed using the One-step RT-PCR kit according to the manufacturer's instructions (Qiagen). For microarray assay, cDNA was synthesized from total RNA samples by using random primers (Invitrogen). SuperScript II (Invitrogen) and biotin-ddUTP was used to label the product according to

the protocol from Affymetrix (Affymetrix). Target hybridization, washing and staining were performed according to the manufacture's instructions. Genechip arrays were scanned with an Affymetrix probe array scanner. The microarray analysis for each bacterial strain was repeated for two times and the data were analyzed using a statistics software MAS-5.0 from Affymetrix.

5.2.8 Protein isolation and western blotting analysis

Overnight bacterial cultures were inoculated in LB medium supplemented with NTA. After having been cultured at 37^{o}C to an OD_{600} of 1.5, 10 ml of each bacterial culture were collected and centrifuged. The supernatants were then filtered with 0.2 μm syringe filter and precipitated with trichloroacetic acid (TCA) at a final concentration of 10%. The precipitates were pelleted by centrifugation, washed twice with acetone, dried, and re-suspended in SDS sampling buffer. The protein samples were denatured by boiling for 5 min and separated by 10% SDS-PAGE. Western blot analysis was performed following the standard protocols (Sambrook *et al*., 1987).

5.2.9 Cytotoxicity assays in HeLa cell model

BDSF effect on the cytotoxicity of *P. aeruginosa* was assayed using HeLa cells. HeLa cells were seeded in 24-well tissue culture plates containing Dulbecco's Modified Eagle Medium (DMEM) and allowed to grow at 37^{o}C in CO_2 for about 18 hours to obtain 80-90% monolayer confluency ($5.0x10^5$ cells/well). Culture supernatants were removed and the monolayer was washed once with PBS buffer. Fresh bacterial cells were diluted in DMEM to a concentration about $5x10^7$ CFU per ml. Thereafter, 0.5 ml of bacteria

dilutions in the absence or presence of BDSF were applied to the HeLa cell monolayers at a multiplicity of infection (MOI) about 50. Cytotoxicity was determined by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) into supernatants using the cytotoxicity detection kit (Roche).

5.3 Results

5.3.1 BDSF inhibits the transcriptional expression of the QS systems of *P. aeruginosa*

There are two AHL-dependent quorum sensing systems in *P. aeruginosa*, the *las* and *rhl* systems, which control the expression of numerous genes (Schuster *et al*., 2003; Hentzer *et al*., 2003; Wagner *et al*., 2003). These two QS systems constitute a hierarchy regulatory network where the *las* quorum-sensing system is at the upstream of the *rhl* system (Latifi *et al*., 1996; Pesci *et al*., 1997). Moreover, it was identified that LasR was required for the *Pseudomonas* quinolone signal (PQS) production and PQS has a positive effect on the *rhl* system by intertwining in the quorum sensing hierarchy (Pesci *et al*., 1999; Susan *et al*., 2000).

To test the effect of BDSF on the quorum sensing systems of *P. aeruginosa*, the promoter-lacZ fusion reporters of *lasI*, *lasR*, *pqsR*, *rhlI* and *rhlR* were used to test the expression of QS systems at the transcriptional level. Results showed that addition of BDSF did not show obvious effect on bacterial growth (Fig. 5-1A), but the expressions of *lasR*, *pqsR*, *rhlI* and *rhlR* were repressed by treatment with BDSF in a dosage-dependent manner. Addition of 0.25 mM BDSF resulted in 38%, 35%, 23% and 48% reduction of

the expression of *lasR*, *pqsR*, *rhlI* and *rhlR*, respectively (Fig. 5-1C, Fig. 5-2A, Fig. 5-3), while the expression of *lasI* was increased about 37% (Fig. 5-1B).

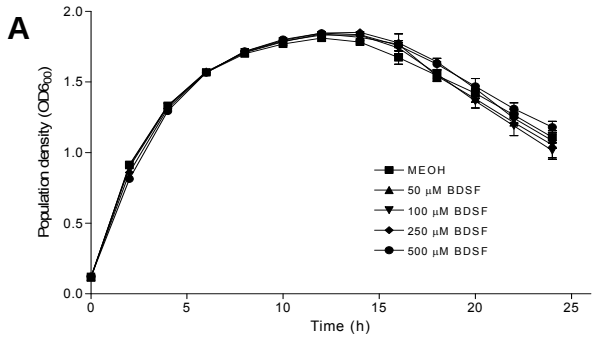
5.3.2 The effect of BDSF on the production of QS signals

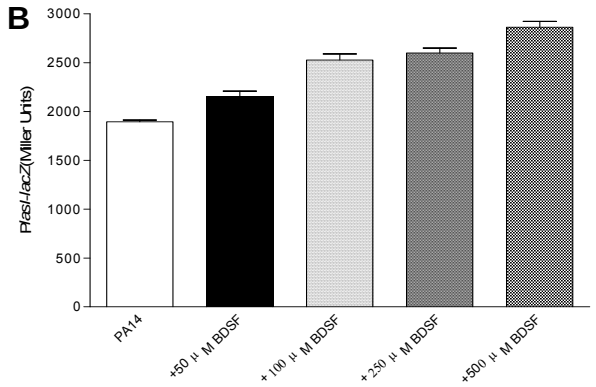
PQS was separated using the activated TLC plate, and visualized by UV transilluminator at the wavelength of 312 nm. The signal density of PQS spot was measured to quantify the production. Result showed that treatment with BDSF reduced the production of PQS. As shown in Fig. 5-2B, addition of 0.05, 0.1, 0.25 and 0.5 mM BDSF caused 12.5%, 16.7%, 18.8% and 25% reduction of the signal density, respectively.

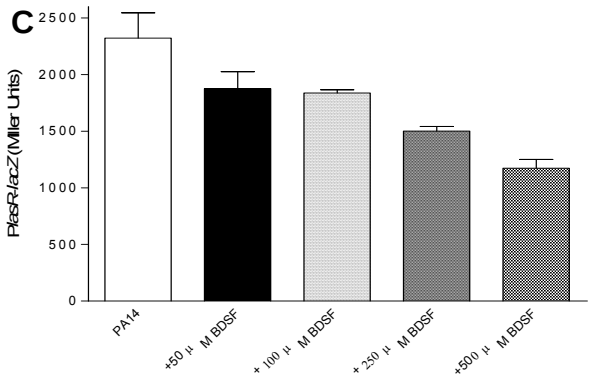
C4HSL (BHL) production was measured using BHL bioassay plate, which contains BHL biosensor AV026 (McClean *et al*., 1997). After inoculation at 28^{o}C overnight, BHL activity was determined by the presence of purple halo, which is proportional to the amount of BHL. It was shown that in the BHL bioassay plate, the purple halo became smaller and shallower when *P. aeruginosa* was treated with BDSF (Fig. 5-3), which indicated the reduction of BHL production. However, addition of BDSF caused no detectable reduction of the production of 3-oxo-C12HSL (Fig. 5-1D), may be possibly due to the opposite effects of BDSF on the transcriptional expression of *lasI* and *lasR* (Fig. 5-1B, Fig. 5-1C).

5.3.3 BDSF decreases the production of protease and pyocyanin

P. aeruginosa usually utilizes exoenzyme to induce its pathogenesis (Holder and Neely, 1991; Mahajan-Miklos *et al*., 1999). Previous studies found that proteases are potent


A
Population density (OD_{600})
Time (h)
MEOH
50 μM BDSF
100 μM BDSF
250 μM BDSF
500 μM BDSF


B
PlasI-lacZ (Miller Units)
PA14
+50 μ M BDSF
+ 100 μ M BDSF
+ 250 μ M BDSF
+500 μ M BDSF


C
PlasR-lacZ (Miller Units)
PA14
+50 μ M BDSF
+ 100 μ M BDSF
+ 250 μ M BDSF
+500 μ M BDSF

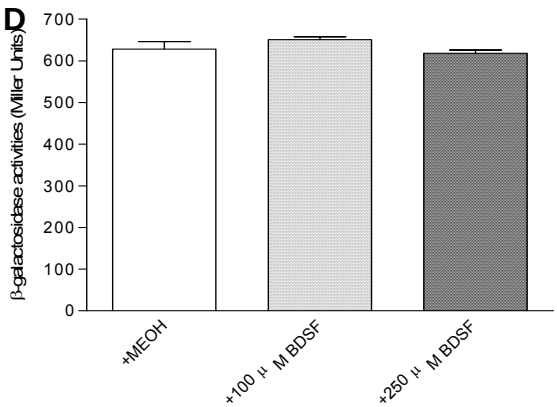

D
β-galactosidase activities (Miller Units)
+MEOH
+100 μ M BDSF
+250 μ M BDSF

Fig. 5-1. Influence of BDSF on the *las* system of *P. aeruginosa*. Effect of BDSF on the growth curve of *P. aeruginosa* (A); the transcriptional expression of *lasI* (B) and *lasR* (C), as determined by using corresponding promoter-*lacZ* fusion reporter strains; and the production of 3-oxo-C12-HSL (D). The data are the means of three repeats and error bars indicate the standard deviations.

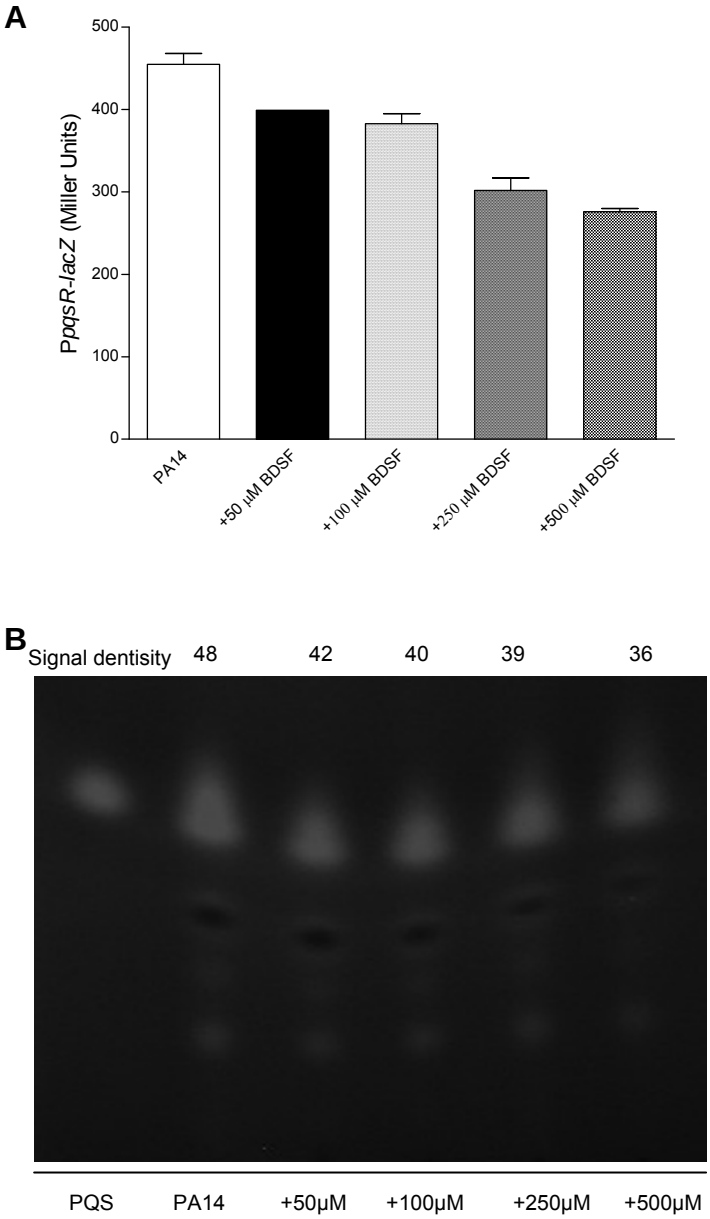
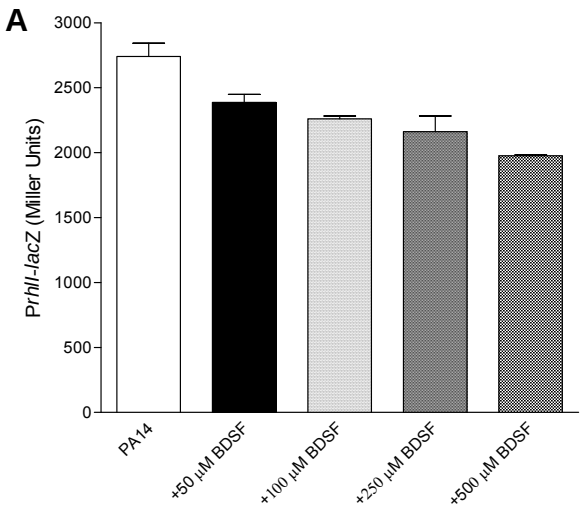
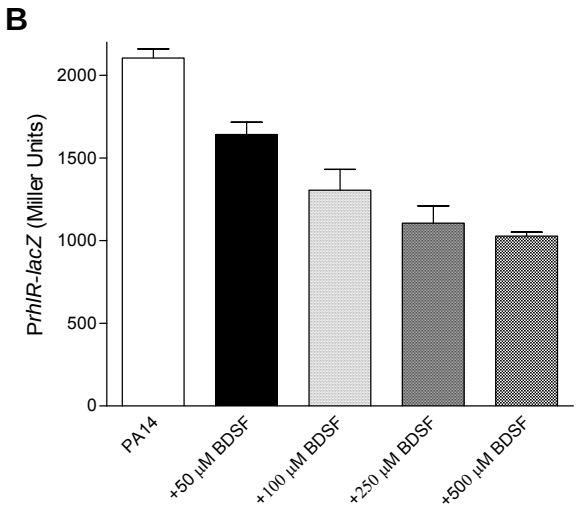
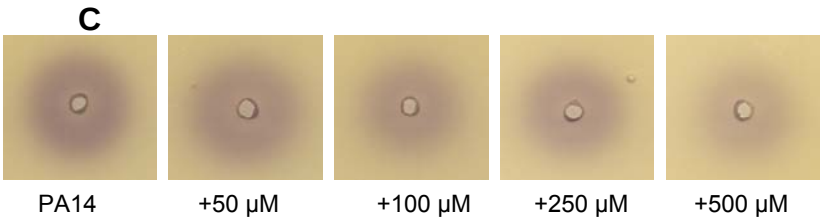


Fig. 5-2. Inhibition of BDSF on the *pqs* system of *P. aeruginosa*. BDSF inhibited the transcriptional expression of *pqsR* (A), as determined by using corresponding promoter-*lacZ* fusion reporter strain; and the production of PQS (B). The data are the means of three repeats and error bars indicate the standard deviations.


A
3000
2500
2000
1500
1000
500
0
PrhI-lacZ (Miller Units)
PA14
+50 μM BDSF
+100 μM BDSF
+250 μM BDSF
+500 μM BDSF


B
2000
1500
1000
500
0
PrhIR-lacZ (Miller Units)
PA14
+50 μM BDSF
+100 μM BDSF
+250 μM BDSF
+500 μM BDSF


C
PA14
+50 μM
+100 μM
+250 μM
+500 μM

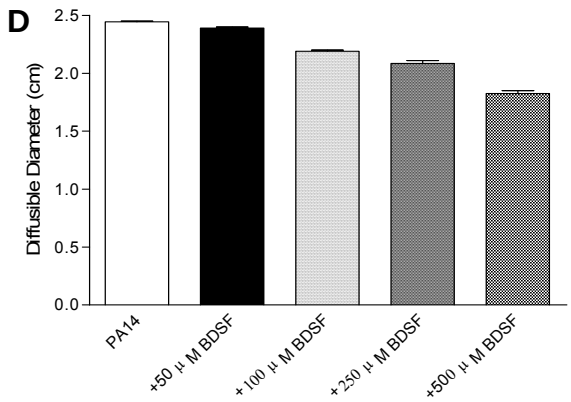


Fig. 5-3. Inhibitory effect of BDSF on the *rhl* system of *P. aeruginosa*. Treatment with BDSF reduced the transcriptional expression of *rhlI* (A) and *rhlR* (B), as determined by using corresponding promoter-*lacZ* fusion reporter strains; and the production of BHL, as shown by the presence of reduction of density (C) and diffusible diameters (D) of purple halo on the BHL bioassay plates. The data are the means of three repeats and error bars indicate the standard deviations.

virulence factors of *P. aeruginosa*. To study the effect of BDSF on exoproteases production, BDSF was added to the growth medium of *P. aeruginosa* as indicated to test its effect on the secreted proteases in the supernatants. It was found that addition of 0.25 mM BDSF casued 30% reduction of the protease activity (Fig. 5-4A). Moreover, when the BDSF concentration was increased to 0.5 mM, it caused almost 50% reduction of the protease activity (Fig. 5-4A).

During the growth process, it was observed that BDSF reduced the pigment accumulation of *P. aeruginosa* in the medium. *P. aeruginosa* produces a number of colored secondary metabolities; one of them is pyocyanin, which is a potent virulence factor (Rahme *et al*., 1997; Ran *et al*., 2003). To determine whether BDSF affects this virulence factor production, we measured pyocyanin accumulation in PA medium with or without BDSF. As shown in Fig. 5-4B, addition of BDSF to the bacterial medium reduced substantially the production of pyocyanin. Compared with the control, treatment with 0.1 and 0.5 mM BDSF reduced the pyocyanin production by about 55% and 70%, respectively (Fig. 5-4B).

5.3.4 BDSF inhibits the expression of master regulators of T3SS

Type III secretion system (T3SS) is a major virulence determinant conserved in many Gram-negative bacterial pathogens. To test the ability of BDSF on regulation of T3SS gene expression in *Pseudomonas aeruginosa*, the T3SS reporter construct P*exsCEBA-lacZ* (Zhou *et al*., 2007) was used. The promoter P*exsCEBA* directs the expression of the T3SS master regulator ExsA and ExsC, which positively controls the expression of all

T3SS genes in *P. aeruginosa* (Yahr and Wolfgang, 2006; Frank, 1997). The bioassay results showed that the expression of *exsCEBA* was inhibited by treatment with BDSF. Addition of 5 μM, 10 μM and 25 μM of BDSF to the bacterial culture resulted in 64.1%, 68.5% and 73.4% reduction of *β*-galactosidase activity, respectively (Fig. 5-5A).

The effect of BDSF on inhibition of the T3SS master regulator was further analyzed by semi-quantitative RT-PCR. At the panel of 5 ng RNA, results showed that treatment of 100 μM BDSF to *P. aeruginosa* led to about 30% and 50% reduction in the signal density of *exsC* and *exsA* (Fig. 5-5B), which are consistent with the results measured using the T3SS reporter (Fig. 5-5A).

5.3.5 BDSF represses the expression of T3SS effectors

Considering that the activator ExsA is required for the secretion of T3SS effectors (Hovey and Frank, 1995), we further determined the effect of BDSF on the four effectors of T3SS. Semi-quantitative RT-PCR analysis showed that treatment of *P. aeruginosa* with 100 μM BDSF led to about 39%, 17%, 24% and 22% reduction in transcripts levels of *exoS*, *exoT*, *exoU* and *exoY*, respectively (Fig. 5-6A). The results demonstrated that BDSF inhibits the transcriptional expression of all the effectors, which was regulated by the master regulators of T3SS. Furthermore, western blotting assay was used to analysis the effect of BDSF on ExoS at the translational level. As shown in Fig. 5-6B, addition of 100 μM BDSF reduced substantially the amount of ExoS secreted in the supernatants. When the final concentration of BDSF was increased to 500 μM; there was no detectable protein band of ExoS.

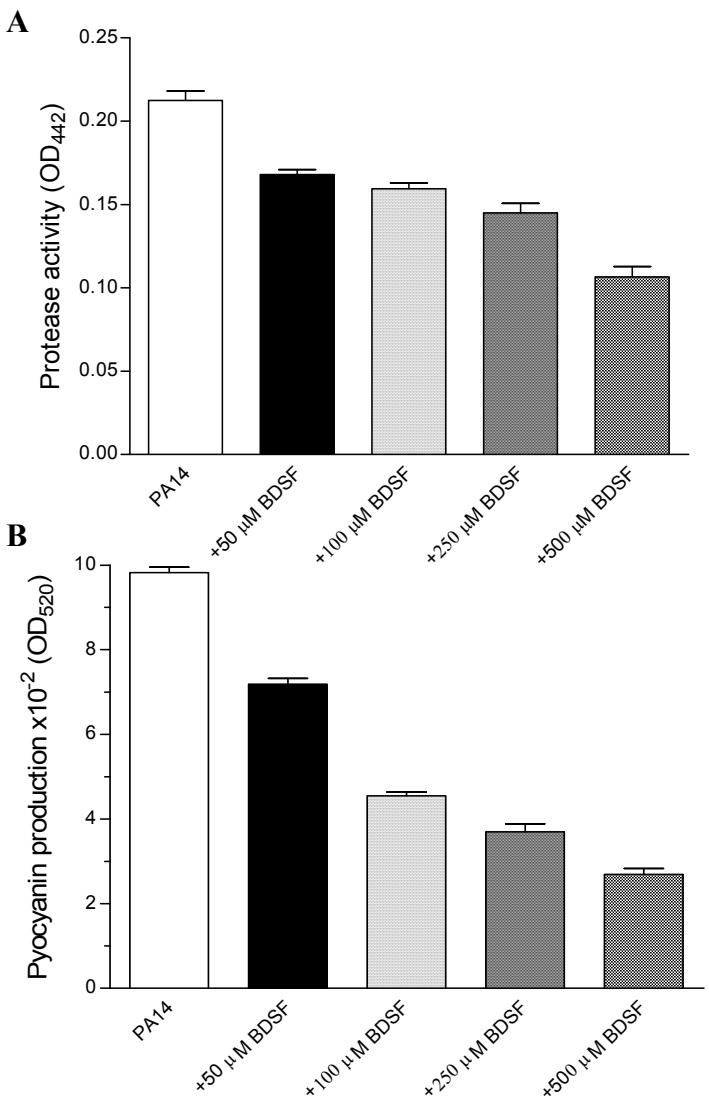


Fig. 5-4. Inhibitory effect of BDSF on the production of extracellular protease (A) and pyocyanin (B) of *P. aeruginosa*. The data are the means of three repeats and error bars indicate the standard deviations.

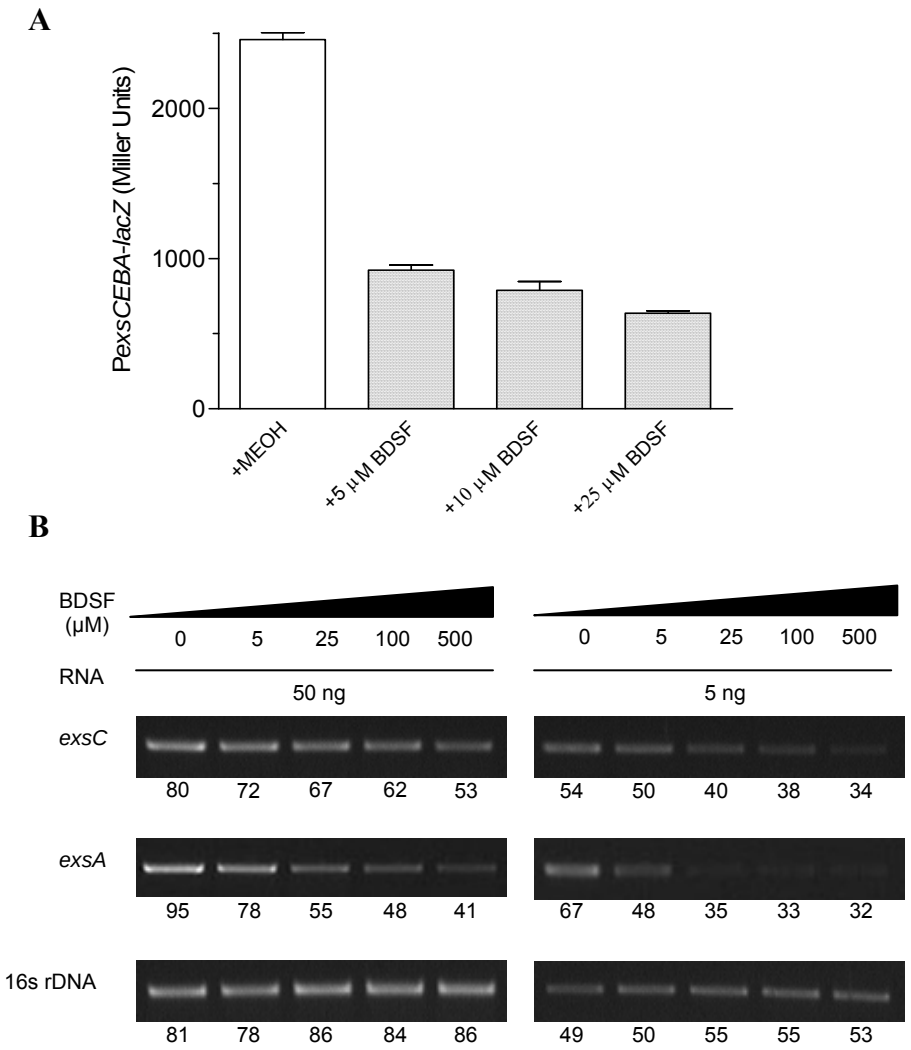


Fig. 5-5. Influence of BDSF on the T3SS master regulators gene expression of *P. aeruginosa*. Addition of BDSF caused the reduction of the transcriptional expression of T3SS master regulators, as determined by using P*exsCEBA-lacZ* fusion reporter strain (A) and RT-PCR analysis (B). Bacteria were grown in LB medium supplemented with 10 mM NTA to an OD_{600} of 1.5. BDSF was added at a series of final concentrations as indicated. For each RNA sample, two dilutions (5, 50 ng) were used as templates for RT-PCT reaction. The data are the means of three repeats and error bars indicate the standard deviations.

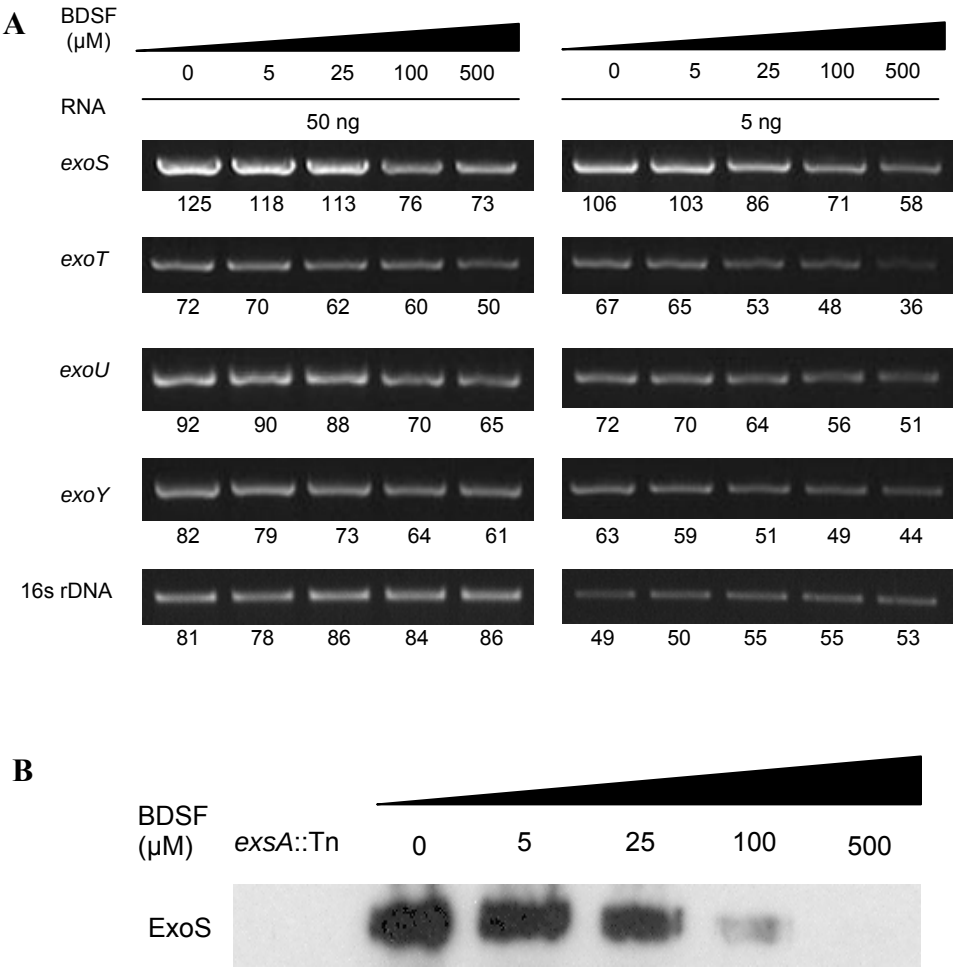


Fig. 5-6. Analysis of the effect of BDSF on the T3SS effectors gene expression in *P. aeruginosa*. (A) RT-PCR analysis of the inhibitory effect of BDSF on the T3SS effectors gene expression. For each RNA sample, two dilutions (5, 50 ng) were used as templates for RT-PCT reaction. (B) Inhibitory effect of BDSF on the secretion of ExoS. Bacteria were grown in LB medium supplemented with 10 mM NTA to an OD_{600} of 1.5. BDSF was added at a series of final concentrations as indicated. The extra-cellular proteins in supernatants were collected by trichloroacetic acid precipitation and separated by 10% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and blotted with anti-ExoS antibody.

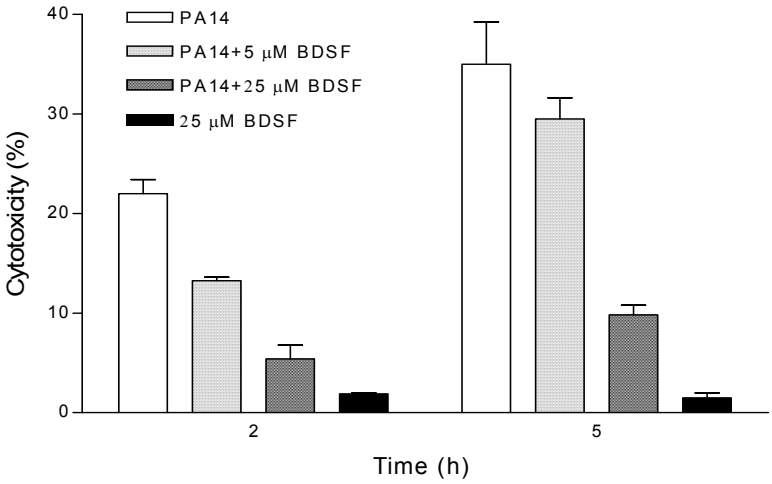


Fig. 5-7. Attenuation of the T3SS-mediated cytotoxicity on HeLa cell model by BDSF. Cytotoxicity was assayed by monitoring LDH release by the HeLa cells infected with a MOI of about 50. Experiments were performed with DMEM medium supplemented with BDSF. The data were the means of three replicates. The data are the means of three repeats and error bars indicate the standard deviations.

5.3.6 BDSF attenuates the T3SS-mediated cytotoxicity of PA14 on HeLa cell model

To test the effect of BDSF on T3SS-mediated cytotoxicity, we used human epithelial cell line HeLa, and the cytotoxicity of *P. aeruginosa* was measured by quantitative determination of lactate dehydrogenase (LDH) released at 2 hours and 5 hours post infection. Results showed that compared with the control, addition of BDSF decreased noticibly the cytotoxicity of *P. aeruginosa* to HeLa cell. For 2 hours inoculation, the cytotoxicity was reduced 41% and 75% with the treatment of 5 μM BDSF and 25 μM BDSF, respectively (Fig. 5-7). While for 5 hours inoculation, the reduction was 16% and 73%, respectively (Fig. 5-7).

5.3.7 The global effect of BDSF on PA14

To study the global effect of BDSF on *P. aeruginosa*, we used Affymetrix Genechip microarray technology to analysis the genes affected by BDSF on the genome level. Results showed that treatment with 250 μM of BDSF up-regulated 66 genes more than 2-fold, which is 1.38% to the total number of genes in genome. Meanwhile, a total of 120 genes were down-regulated by more than 2-fold (Table 5-2). Except for those genes encoding hypothetical proteins, the remaining 129 genes belong to the functional groups of metabolism, secretion, motility and cell wall, transcription regulation, protection, enzymes and carbon compound catabolism (Table 5-2).

Identification of a range of genes encoding secreted toxin in microarray analyses, which were mostly down-regulated by BDSF, is highly consistent with the previous findings that the bacterial virulence to HeLa cell was repressed with treatment of BDSF. More

interestingly, some genes related with motility and cell-wall are inhibited by BDSF, implicating that BDSF may affect the biofilm formation or motility activity of *P. aeruginosa*, which is consistent with the previous results reported by Ryan *et al*. (2008).

5.4 Discussion

The results in this chapter showed that besides working as an intraspecies signal, BDSF can also mediate inter-species communication between *B. cenocepacia* and *P. aeruginosa*, which leads to inhibition of the *P. aeruginosa* QS systems and T3SS. Previous studies found that there is some cross-talk between *B. cepacia* and *P. aeruginosa*, which is mediated by AHL (Riedel *et al*., 2001; Mckenney *et al*., 1995). It was reported that addition of cell-free exoproducts of PAO1 to *B. cepacia* enhanced the production of siderophore, lipase and protease. While addition of the supernatants of PAO1 with attenuated production of AHL only had slight effect on the production of these virulence factors (Mckenney *et al*., 1995). Taken together, the findings suggest that there may be a mutual communications between these two pathogens, which usually share the same niche in cystic fibrosis patients.

In *P. aeruginosa*, there are three QS systems, which consist of a hierarchy regulatory network. These QS systems regulate a broad range of genes important for the metabolism and virulence of *P. aeruginosa*. Antibiotics have been used for a long time to treat the bacterial infection of *P. aeruginosa*, but resistance to antibiotics can be evolved during clinic treatment. Interestingly, recently studies found that synthetic derivate of natural furanone successfully interfered with *N*-acyl homoserine lactone of *P. aeruginosa* and

suppressed bacterial quorum-sensing in lungs, finally caused accelerated lung bacterial clearance and reduced the severity of lung pathology (Wu *et al*., 2004). Based on the results of this study, we found that BDSF can be used as a new potential inhibitor on the QS systems of *P. aeruginosa*. It was shown that addition of BDSF reduced both the production of BHL and PQS (Fig. 5-2, 5-3), which was consistent with the decreased production of some virulence factors such as protease and pyocyanin (Fig. 5-4). However, the detailed mechanism of BDSF on interference of the QS systems of *P. aeruginosa* still needs further investigation.

Recently study reported that long-chain fatty acid sensor modulates the expression of *rpoS* and the type III *exsCEBA* operon in *P. aeruginosa* (Kang *et al*., 2009). T3SS is a key virulence determinant in a wide range of animal and plant pathogens and plays diverse roles in host-pathogen interactions. In *P. aeruginosa*, the transcriptional expression of effector genes of T3SS is coordinated by ExsA encoded by the *exsCEBA* operon. Our results showed that BDSF inhibits the expression of *exsCEBA*, which is consistent with the result of semi-quantitative RT-PCR analysis of BDSF inhibitory effect on the master regulators and effectors gene expression. Moreover, the results were further supported by western blotting analysis of the BDSF inhibitory effect on the production of ExoS. These results indicate that BDSF is an effective inhibitor on the T3SS of *P. aeruginosa*, and may be utilized as a potential antimicrobial agent against *P. aeruginosa* infection. Intriguingly, compared to QS systems, T3SS is significantly easier to be inhibited by BDSF, demonstrating that BDSF affects them possibly through two different pathways, for the detailed information, we need further investigation.

Table 5-2. The gene expression of *P. aeruginosa* when grown in LB versus LB+BDSF identified using microarrays (Fold change ≥ 2).

Accession number	Gene name	Fold change	Description
(i)			**Metabolism**
PA4888	desB	-7.0	Fatty acid and phospholipid metabolism ;
PA0782	putA	-6.5	Amino acid biosynthesis and metabolism ;
PA0132		-5.3	Amino acid biosynthesis and metabolism ;
PA0430	metF	-3.0	Amino acid biosynthesis and metabolism ; Central intermediary metabolism ;
PA0546	metK	-3.0	Amino acid biosynthesis and metabolism ; Central intermediary metabolism ;
PA2482		-2.8	Energy metabolism ;
PA3924		-2.3	Fatty acid and phospholipid metabolism ;
PA0432	sahH	-2.1	Amino acid biosynthesis and metabolism ;
PA3537	argF	-2.1	Amino acid biosynthesis and metabolism ;
PA3452	mqoA	-2.1	Central intermediary metabolism ; Energy metabolism ;
PA1523	xdhB	-2.0	Nucleotide biosynthesis and metabolism ;
PA1217		-2.0	Amino acid biosynthesis and metabolism ;
PA0895	aruC	2.0	Amino acid biosynthesis and metabolism ;
PA0897	aruG	2.0	Amino acid biosynthesis and metabolism ;
PA0899	aruB	2.0	Amino acid biosynthesis and metabolism ;
PA3182	pgl	2.0	Central intermediary metabolism ;
PA4429		2.0	Energy metabolism ;
PA0516	nirF	2.1	Energy metabolism ; Biosynthesis of cofactors, prosthetic groups and carriers ;
PA3183	zwf	2.1	Energy metabolism ; Carbon compound catabolism ;
PA3193	glk	2.1	Energy metabolism ; Carbon compound catabolism ;
PA3194	edd	2.1	Energy metabolism ; Carbon compound catabolism ;
PA0518	nirM	2.3	Biosynthesis of cofactors, prosthetic groups and carriers ; Energy metabolism ;
PA4430		2.3	Energy metabolism ;
PA0517	nirC	2.5	Biosynthesis of cofactors, prosthetic groups and carriers ; Energy metabolism ;
PA0519	nirS	2.5	Energy metabolism ;
PA0896	aruF	2.6	Amino acid biosynthesis and metabolism ;
PA4442	cysN	3.0	Central intermediary metabolism ; Amino acid biosynthesis and metabolism ;
PA0523	norC	3.0	Energy metabolism ;
PA3195	gapA	3.5	Energy metabolism ; Carbon compound catabolism ;
PA0437	codA	3.7	Nucleotide biosynthesis and metabolism ;
PA3395	nosY	4.0	Membrane proteins ; Energy metabolism ;
PA3392	nosZ	4.6	Energy metabolism ;
(ii)			**Secretion, transport and export apparatus**
PA4230	pchB	-14.9	Secreted Factors (toxins, enzymes, alginate) ; Transport of small molecules ;
PA1708	popB	-6.5	Protein secretion/export apparatus ;
PA4206	mexH	-5.7	Transport of small molecules ;
PA1707	pcrH	-4.9	Secreted Factors (toxins, enzymes, alginate) ; Protein secretion/export apparatus ;

PA1709	popD	-4.6	Protein secretion/export apparatus ;
PA0263	hcpC	-4.3	Secreted Factors (toxins, enzymes, alginate) ;
PA4207	mexI	-4.3	Membrane proteins ; Transport of small molecules ;
PA1706	pcrV	-3.7	Protein secretion/export apparatus ;
PA3877	narK1	-3.5	Membrane proteins ; Transport of small molecules ;
PA0044	exoT	-3.2	Secreted Factors (toxins, enzymes, alginate) ;
PA2068		-3.2	Membrane proteins ; Transport of small molecules ;
PA1183	dctA	-3.2	Transport of small molecules ;
PA1715	pscB	-3.0	Protein secretion/export apparatus ;
PA0783	putP	-3.0	Membrane proteins ; Transport of small molecules ;
PA5217		-3.0	Transport of small molecules ;
PA1696	pscO	-2.8	Protein secretion/export apparatus ;
PA1904	phzF2	-2.8	Secreted Factors (toxins, enzymes, alginate) ;
PA4208	opmD	-2.8	Membrane proteins ; Transport of small molecules ;
PA1905	phzG2	-2.8	Secreted Factors (toxins, enzymes, alginate) ;
PA1710	exsC	-2.8	Translation, post-translational modification, degradation ; Protein secretion/export apparatus ;
PA4064		-2.8	Transport of small molecules ;
PA4210	phzA1	-2.8	Secreted Factors (toxins, enzymes, alginate) ;
PA1719	pscF	-2.6	Protein secretion/export apparatus ;
PA1712	exsB	-2.6	Translation, post-translational modification, degradation ; Protein secretion/export apparatus ;
PA1718	pscE	-2.5	Protein secretion/export apparatus ;
PA1903	phzE2	-2.5	Secreted Factors (toxins, enzymes, alginate) ;
PA5500	znuC	-2.5	Transport of small molecules ;
PA4211	phzB1	-2.3	Secreted Factors (toxins, enzymes, alginate) ;
PA0866	aroP2	-2.1	Transport of small molecules ;
PA4628	lysP	-2.1	Membrane proteins ; Transport of small molecules ;
PA5231		-2.1	Membrane proteins ; Transport of small molecules ;
PA1717	pscD	-2.1	Protein secretion/export apparatus ;
PA1901	phzC2	-2.1	Secreted Factors (toxins, enzymes, alginate) ;
PA1902	phzD2	-2.1	Secreted Factors (toxins, enzymes, alginate) ;
PA1964		-2.1	Transport of small molecules ;
PA4142		-2.1	Protein secretion/export apparatus ;
PA4143		-2.0	Protein secretion/export apparatus ; Membrane proteins ; Transport of small molecules ;
PA1694	pscQ	-2.0	Protein secretion/export apparatus ;
PA1703	pcrD	-2.0	Protein secretion/export apparatus ;
PA5230		-2.0	Membrane proteins ; Transport of small molecules ;
PA4292		-2.0	Membrane proteins ; Transport of small molecules ;
PA5501	znuB	-2.0	Membrane proteins ; Transport of small molecules ;
PA2259	ptxS	2.0	Secreted Factors (toxins, enzymes, alginate) ; Transcriptional regulators ;
PA4502		2.0	Transport of small molecules ;
PA0280	cysA	2.1	Transport of small molecules ;
PA0602		2.5	Transport of small molecules ;
PA5369	pstS	2.5	Transport of small molecules ;
PA0281	cysW	2.6	Membrane proteins ; Transport of small molecules ;
PA5368	pstC	2.6	Membrane proteins ; Transport of small molecules ;
PA2114		2.8	Membrane proteins ; Transport of small molecules ;
PA2204		6.1	Transport of small molecules ;
(iii)			**Motility and cell wall**
PA2570	lecA	-2.5	Adaptation, Protection ; Motility & Attachment ; Cell wall / LPS / capsule ;

PA3361	lecB	-2.3	Motility & Attachment ;
PA0869	pbpG	-2.3	Cell wall / LPS / capsule ;
PA3337	rfaD	-2.0	Cell wall / LPS / capsule ;
PA4306	flp	-2.0	Motility & Attachment ;
PA4480	mreC	-2.0	Cell wall / LPS / capsule ; Cell division ;
PA3706	wspC	2.8	Chemotaxis ; Adaptation, Protection ; Motility & Attachment ;
(iv)			**Transcription regulation**
PA2885	atuR	-3.7	Transcriptional regulators ;
PA0797		-3.2	Transcriptional regulators ;
PA0547		-3.0	Transcriptional regulators ;
PA3006	psrA	-2.8	Transcriptional regulators ;
PA5239	rho	-2.5	Transcription, RNA processing and degradation ;
PA5499	np20	-2.5	Transcriptional regulators ;
PA5374	betI	-2.3	Transcriptional regulators ;
PA3266	capB	-2.1	Transcriptional regulators ; Adaptation, Protection ;
PA3587	metR	-2.1	Transcriptional regulators ;
PA3721		-2.1	Transcriptional regulators ;
PA0367		2.1	Transcriptional regulators ;
PA0479		2.5	Transcriptional regulators ;
PA2849		2.6	Transcriptional regulators ;
(v)			**Adaptation, Protection ; Chemotaxis ;**
PA1608		-4.3	Adaptation, Protection ; Chemotaxis ;
PA1561	aer	-2.3	Adaptation, Protection ; Chemotaxis ;
PA2788		-2.0	Adaptation, Protection ; Chemotaxis ;
PA2920		-2.0	Adaptation, Protection ; Chemotaxis ;
PA3327		3.0	Adaptation, Protection ;
(vi)			**Putative enzymes**
PA4889		-4.6	Putative enzymes ;
PA0730		-4.3	Putative enzymes ;
PA2069		-4.3	Putative enzymes ;
PA5181		-3.0	Putative enzymes ;
PA2067		-2.8	Putative enzymes ;
PA4217	phzS	-2.8	Putative enzymes ;
PA0506		-2.6	Putative enzymes ;
PA2889	atuD	-2.6	Putative enzymes ;
PA4209	phzM	-2.6	Putative enzymes ;
PA2237	pslG	-2.5	Putative enzymes ;
PA3768		-2.3	Putative enzymes ;
PA0130		-2.0	Putative enzymes ;
PA3437		-2.0	Putative enzymes ;
PA3035		2.0	Putative enzymes ;
PA0366		2.8	Putative enzymes ;
PA2197		2.8	Putative enzymes ;
PA0364		3.0	Putative enzymes ;
PA2099		5.7	Putative enzymes ;
(vii)			**Carbon compound catabolism ;**
PA2300	chiC	-3.5	Carbon compound catabolism ;
PA3192	gltR	2.0	Carbon compound catabolism ; Transcriptional regulators ; Two-component regulatory systems ;
PA3366	amiE	2.3	Carbon compound catabolism ;
PA2098		3.7	Carbon compound catabolism ;
PA2097		4.6	Carbon compound catabolism ;

Microarray results showed that BDSF affects *P. aeruginosa* at a global level. Expression of a total of 186 genes was changed more than 2-fold by treatment with 250 μM BDSF. A number of genes belong to the group of protein secretion and export apparatus were repressed obviously with treatment of BDSF. In addition, some secreted factors classified to toxins and enzymes were also inhibited by the addition of BDSF, which is possible the major reason for the reduction of virulence of *P. aeruginosa*. Moreover, BDSF has a negative effect on the amino acid metabolism, such as the biosynthesis of glutamate, arginine, proline, methane, and methionine. Although the function of many hypothetical genes is unknown, this study shows the possibility of inter-species communication between *B. cenocepacia* and *P. aeruginosa* mediated by BDSF. It may also shed some new light in the host-pathogen interaction between *P. aeruginosa* and its host. Because in the body of human, there are many different fatty acids, it is possible that some of them may cross-talk with *P. aeruginosa*.

CHAPTER 6

GENERAL CONCLUSIONS AND FURTHER STUDY

6.1 General conclusions

6.1.1 BDSF is a novel cell-cell communication signal

In this study, BDSF was characterized as a novel signal, which is a structural homologue of DSF (*cis*-11-methyl- 2-decenoic acid) produced by *X. campestris*. The only difference between them is at the C-11 position where BDSF lacks a methyl group. DSF is a well-characterized quorum-sensing signal that regulates a few hundred genes encoding diverse biological functions such as biofilm disperse and virulence through a signaling network (Ryan *et al*., 2006; He *et al*., 2006a; 2006b; 2007; He and Zhang, 2008; Tao *et al*., 2009). Interestingly, *in trans* expression of *Bcam0581*, which is the gene encoding BDSF synthase, or addition of BDSF can restore biofilm disperse and virulence factor production by the *Xcc rpfF* deletion mutant, demonstrating that *Bcam0581*/BDSF are the functional homologue/analogue of *rpfF*/DSF. Functional characterization of BDSF revealed that it is involved in regulation of biofilm formation and virulence in *B. cenocepacia*. Furthermore, it was shown that production of BDSF is under stringent transcriptional control and the molecule accumulates in a cell density-dependent manner, typically found with quorum sensing (QS) signals. Taken together, the results from this study demonstrate that BDSF is a novel cell-cell communication signal in *B. cenocepacia* that plays a vital role in the regulation of the bacterial physiological functions.

A blast analysis using Bcam0581 protein sequence to the NCBI non-redundant database detected the presence of Bcam0581 homologues in at least 32 species with protein identity levels ranging from 31% to 99% and a minimum e value of $3e^{-31}$ (Table 6-1). This suggests that Bcam0581 homologues are conserved in many bacteria. Especially, amino acids sequence alignment of the Bcam0581 homologues from the six sequenced genomovars of the *B. cepacia* complex revealed more than 94% high similarity. Subsequently, TLC and DSF bioassay results discovered that all nine species of the *B. cepacia* complex produce DSF-family signals, which were characterized as DSF, BDSF and CDSF by using MS and NMR analysis. Although only some members of the *B. cepacia* complex produce DSF and CDSF, all nine species produce BDSF, which indicates that BDSF is the key QS signal in the *B. cepacia* complex. Identification of BDSF provides further evidence to support the notion that DSF represents a family of widely conserved bacterial QS signals.

6.1.2 Virulence gene expression and biofilm formation are co-regulated by BDSF and AHL QS systems

Studies showed that *B. cenocepacia* utilizes *N*-acyl homoserine lactones (AHL)-dependent quorum-sensing system to regulate particular genes in a cell density-dependent mechanism. In *B. cenocepacia*, CepIR is the major AHL QS system, which is widely distributed in the *B. cepacia* complex and controls virulence factors production, swarming motility and biofilm formation (Lewenza *et al*., 1999; Gotschlich *et al*., 2001; Lutter *et al*., 2001; Huber *et al*., 2001; Hacker and Kaper, 2000; Mahenthiralingam, *et al*., 2005).

Table 6-1. BCAM0581 homologues in various bacteria.

Bacteria	Strain	Accession No.	Identity (%)	E Value
Burkholderia				
B. cenocepacia	J2315	ABK10294	100	0
B. dolosa	AU0158	EAY71442	96	1e-162
B. ambifaria	MC40-6	EAV51426	95	8e-162
B. vietnamiensis	G4	ABO57014	94	4e-161
B. cepacia	383	ABB12683	94	8e-162
B. multivorans	ATCC17616	ABX18791	94	1e-159
B. phymatum	STM815	EAU98797	71	1e-125
B. phytofirmans	PsJN	EAV07876	71	3e-121
B. xenovorans	LB400	ABE34805	71	6e-121
Enterobacteriaceae				
Serratia proteamaculans	568	ABV43836	69	8e-120
Yersinia bercovieri	ATCC43970	ZP_00822470	69	8e-117
Yersinia mollaretti	ATCC43969	ZP_00825829	67	7e-116
Y. enterocolitica pv *enterocolitica*	8081	CAL10196	66	9e-114
Yersinia frederiksenii	ATCC33641	ZP_00827993	66	1e-113
Yersinia intermedia	ATCC29909	ZP_00832221	66	2e-113
Enterobacter sp.	638	ABP60857A	62	2e-104
Enterobacter sakazakii	ATCC BAA-894	BU76841	61	8e-104
Xanthomonadaceae				
X. oryzae pv *oryzae*	KACC10331	AAW76123	37	7e-44
X. campestris pv *vesicatoria*	85-10	CAJ23597	36	9e-43
X. campestris pv *campestris*	8004	AAY49385A	36	2e-43
X. axonopodis pv. *Citri*	306	AM36741	37	1e-40
Stenotrophomonas maltophilia	R551-3	EAX22217A	36	2e-41
Xylella fastidiosa	Temecula1	AO28287	35	8e-41
Erythrobacter				
Erythrobacter litoralis	HTCC2594	ABC63454	34	3e-39
Erythrobacter sp.	NAP1	EAQ30316	32	3e-36
Other				
Methylobacillus flagellatus	KT	ABE50920	37	4e-49
Leptospirillum sp.	Group II UBA	EAY57477	37	4e-47
Sulfurovum sp.	NBC37-1	BAF71518	36	9e-45
Thiobacillus denitrificans	ATCC25259	AAZ98625	38	2e-44
Mariprofundus ferrooxydans	PV-1	EAU55268	36	2e-44
Sphingomonas sp.	SKA58	EAT06971	31	8e-35
Methylibium petroleiphilum	PM1	ABM97021	36	3e-31

Previous studies reported that ZmpA, lipase and the ornibactin biosynthesis were under the control of CepIR system (Subsin *et al*., 2007; Lewenza *et al*., 1999). In this study, RT-PCR analysis showed that the related virulence genes expression, such as *zmpA*, *lipA* and *orbI*, were positively controlled by BDSF in *B. cenocepacia*. These results demonstrate that BDSF system conjuncts with CepIR system to co-regulate the virulence of *B. cenocepacia*. Further study showed that there was no difference in *cepI* or *cepR* transcript levels and AHL production between the wild type strain and the d0581 mutant. However, overexpression of *cepI* or *cepR* in the mutant d0581, and addition of BDSF to the *cepR* mutant, fully restored the transcriptional expression of these virulence genes (Fig. 3-4B). In addition, exogenous addition of C8-HSL to the d0581 mutant, and addition of BDSF together with C8HSL to the mutant d0581 further increased the promoter activity of these three genes to a level similar to addition of C8HSL to the wild type strain (Fig. 3-5A, 5B, 5C). Cumulatively, the findings suggest that AHL- and BDSF-dependent QS systems regulate the virulence genes expression in parallel.

Disruption of the AHL QS system in *B. cenocepacia* had been shown to result in defects in biofilm maturation (Huber *et al*., 2001). Similarly, we found that abolishment of BDSF signal caused enhanced formation of cell aggregations. However, most aggregations were dispersed by *in trans* expression of *Bcam0581* or exogenous addition of BDSF. Although their effect on the biofilm formation is not exactly the same, it still can be concluded that the biofilm formation is co-regulated by AHL system and BDSF system in *B. cenocepacia*. However, much more work remains to be done to understand the mechanism of regulation.

6.1.3 BDSF controls bacterial biological functions likely through a novel mechanism

DSF-dependent QS in *Xcc* modulates its targets via a two-component system. After perceiving DSF signal, RpfC sensor protein is activated by autophosphorylation and phosphotransfer occurs to induce the downstream regulator RpfG, which acts as a phosphordiesterase to degrade cyclic-diGMP into GMP. Another key factor of the circuit is the DNA-binding protein Clp, which is a c-di-GMP effector. C-di-GMP specifically binds to Clp with high affinity and induces allosteric conformational changes that abolish the interaction between Clp and its target gene promoter. (He *et al*., 2007; Tao *et al*., 2009) (Fig. 6-1).

In *B. cenocepacia*, amino acid sequence alignment showed that Bcam0581 shares a 37.2% identity with RpfF. Domain analysis showed that Bcam0581 contained an enoyl-CoA hydratase domain similar to that of RpfF enzyme of *Xcc* (Fig. 2-2). *In trans* expression of *Bcam0581* in *Xcc rpfF* deletion mutant restores DSF activity, biofilm disperse and EPS production to wild type level, indicating that Bcam0581 is a functional homologue of RpfF. However, *rpfF* of *Xcc* is located within the same locus as *rpfC* and *rpfG*, while there are no *rpfC* or *rpfG* homologue in the vicinity of *Bcam0581* (Fig. 2-2). 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.

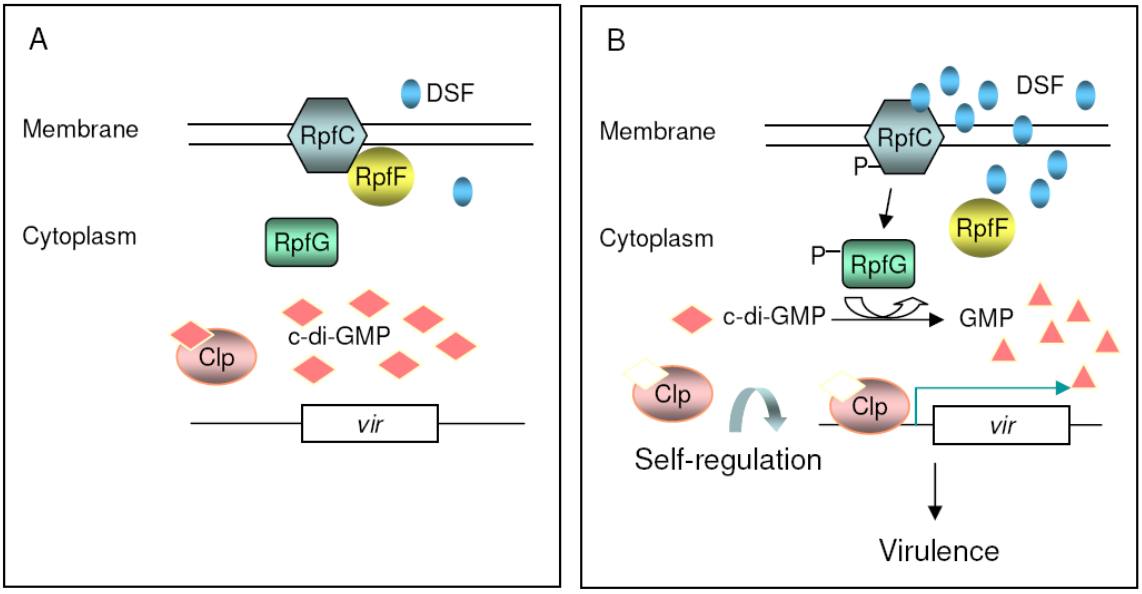


Fig. 6-1. DSF signaling pathway in *Xcc* in regulation of virulence at (A) low and (B) high population density. The Clp-dependent virulence regulon is represented by *vir*, which includes *engXCA* and other virulence genes as depicted in our previous study (copied from Tao *et al*., 2009).

To further investigate the signaling pathway of BDSF, *rpfC* and *rpfG* homologues were searched in the genome level of *B. cenocepacia*. Different from *Xcc*, no *rpfC* homologue could be found. Meanwhile, although *Bcas0263* has about 31% identity to *rpfG*, and with a similar domain structure containing REC and HD domains, deletion of *Bcas0263* caused no change in biofilm formation and in *zmpA* expression (data not shown). Moreover, the genes flanking *Bcas0263* are absolutely different from those flanking *rpfG*, demonstrating that *Bcas0263* is not the real functional homologue of *rpfG*. The findings suggest that the BDSF system in *B. cenocepacia* and the DSF system in *Xcc* do not share the same origin of evolution, and BDSF may use a new mechanism in regulation of the bacterial biological functions.

6.1.4 Dual functions of Bcam0581 in the biosynthesis of BDSF and energy metabolism

Besides abolishment of BDSF production, deletion of *Bcam0581* also caused a growth defect of *B. cenocepacia* in a nutrient-limited medium. Surprisingly, exogenous addition of BDSF up to a concentration of 25 μM was not able to rescue the growth defect of d0581 (Fig. 3-3C). These data suggest that, in addition to its role in BDSF biosynthesis, Bcam0581 may also have a metabolic function. Quantitative measurements of ATP concentrations showed that deletion of *Bcam0581* resulted in significantly reduced ATP levels relative to the wild type strain (Fig. 3-2B). Consistent with its growth profile, the complement strain produced more ATP than the wild type strain. Interestingly, addition of citrate or *in trans* expression of citrate synthase *Bcas0207*, which shows a reduced expression in d0581 mutant, can rescue the growth defect of mutant d0581. However, the

reduction of *Bcas0207* expression in d0581 can only be restored to wild type level by *in trans* expression of *Bcam0581* but not by exogenous addition of 5 μM BDSF (Fig. 3-3B). Taken together, these results have unveiled dual functions of Bcam0581 in addition to its role in the biosynthesis of BDSF, Bcam0581 also plays an essential role for energy biogenesis during growth under unfavorable nutritional condition, possibly through influencing energy metabolism by affecting citric acid cycle. The detailed mechanism of Bcam0581 in energy metabolism in *B. cenocepacia* remains to be elucidated.

6.1.5 BDSF signal shows the ecological significance via inter-species and inter-kingdom signal interference

Recently studies showed that DSF not only controls many biological functions; it also plays an important role in the microbial cross-talk through inter-species and inter-kingdom communication (Wang *et al*., 2004; Ryan *et al*., 2008). Our current work discovered that BDSF produced by *B. cenocepacia* interfered with *P. aeruginosa* through inhibition on its QS systems and T3SS, and resulted in attenuation of the T3SS-meidated cytotoxicity of *P. aeruginosa* on HeLa cell. Furthermore, BDSF was shown to be more effective than DSF in inhibition of germ tube formation by *C. albicans* (Boon *et al*., 2008). The significance of BDSF in microbial ecology became obvious as its repression on *C. albicans* germ tube formation occurs at the physiologically relevant concentrations.

6.2 Prospects of further study

6.2.1 BDSF signaling pathway

Based on our current results, BDSF was identified to regulate the biofilm formation and virulence gene expression in *B. cenocepacia*. This regulation profile is something similar to the DSF signaling pathway in *Xcc*. However, there may be significant difference between them. In *Xcc*, DSF cell-cell communication system regulates its targets through the RpfC/RpfG two-component system; while there are no any RpfC/RpfG homologues in *B. cenocepacia*. To further investigate the BDSF signaling pathway, one target gene of the BDSF signaling system, *zmpA*, can be used to construct the reporter system for screening the BDSF sensors and regulators.

6.2.2 Mechanism of co-regulation by BDSF and AHL

The BDSF system and AHL QS system in *B. cenocepacia* were identified to co-regulate the expression of a set of virulence genes. In addition, both of them are involved in biofilm formation of *B. cenocepacia*. Current knowledge supports that these two systems act in parallel to regulate these virulence genes expression. To identify the co-operation mechanisms of BDSF and AHL systems, *Bcam0581* deletion mutant, *cepI* deletion mutant and *Bcam0581cepI* double deletion mutant will be used to do microarray analysis to study the detailed relationship between the BDSF system and the AHL QS system in *B. cenocepacia*.

6.2.3 Regulatory mechanism of BDSF production

The promoter of *Bcam0581* will be fused with *lacZ* ORF to screen for the regulators controlling the expression of *Bcam0581*. Given that Bcam0581 is essential for the signal production in *B. cenocepacia*, the study of BDSF production will be focused on the

screening of the transcriptional regulators which control the expression of *Bcam0581*. Moreover, the two-hybrid methods will be used to find out whether there are proteins that regulate Bcam0581 by protein-protein or protein-DNA interactions.

6.2.4 The detailed mechanism of BDSF in interference of *P. aeruginosa*

BDSF was identified to interfere with *P. aeruginosa* through repression on its QS systems and T3SS, which finally caused the decreased production of virulence factors. In our future plan, we will study the detailed mechanisms of BDSF signaling in *P. aeruginosa*, to find out the sensor and regulator of BDSF, and the downstream transcriptional factors.

APPENDIX

PUBLICATION AND PATENT

Publicaions:

Yinyue Deng[#], Ji'en Wu[#], Leo Eberl and Lian-Hui Zhang*. 2010. Structural and functional characterization of DSF-family quorum sensing signals produced by members of the *Burkholderia cepacia* complex. *Applied and Environmental Microbiology*. Vol 76:4675-83.

Yinyue Deng[#], Calvin Boon[#], Leo Eberl and Lian-Hui Zhang*. 2009. Differential modulation of *Burkholderia cenocepacia* virulence and energy metabolism by quorum sensing singal BDSF and its synthase. *Journal of Bacteriology*. Vol 191: 7270-7278.

Calvin Boon[#], **Yinyue Deng**[#], Lian-Hui Wang, Yawen He, Fang Yang, Jin-Ling Xu, Shen. Q Pan and Lian-Hui Zhang*. 2008. A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *The ISME Journal*. Vol 2: 27-36. (#: Co-first authors)

Patent:

Yinyue Deng, Ji'en Wu, and Lian-Hui Zhang. Novel antimicrobial compounds and uses thereof. (US, Application no.: 61/334,727 Filing date: 14 May 2010).

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