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Differential Modulation of *Burkholderia cenocepacia* Virulence and Energy Metabolism by the Quorum-Sensing Signal BDSF and Its Synthase[▽]

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Burkholderia cenocepacia **produces the molecule *cis*-2-dodecenoic acid (BDSF), which was previously shown to play a role in antagonism against the fungal pathogen *Candida albicans* by interfering with its morphological transition. In this study, we show 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. *B. cenocepacia* mutant strain J2315 with a deleted Bcam0581 gene, which encodes an enzyme essential for BDSF production, exhibited a growth defect in minimal medium 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. We show that Bcam0581, but not BDSF, is associated with *B. cenocepacia* ATP biogenesis. We also provide evidence that some of the BDSF-regulated genes are also controlled by the acyl-homoserine-lactone-dependent QS system and are thus coregulated by two cell-to-cell signaling systems. These data demonstrate that in addition to the role in cross-kingdom signal interference, BDSF and its synthase are also important for the virulence and physiology of *B. cenocepacia*.**

Burkholderia cepacia complex strains have emerged as problematic opportunistic pathogens in patients with cystic fibrosis and in immunocompromised individuals (5, 10, 28, 37). Although all 17 *B. cepacia* complex species have been isolated from both environmental and clinical sources, *B. cenocepacia* and *Burkholderia multivorans* are most commonly found in clinical samples (5, 27). 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 cystic fibrosis centers (33).

Not only are *B. cenocepacia* strains important opportunistic pathogens of humans, they can also cause infections in a diverse range of species, including rodents (3, 34), nematodes (21), amoebae (30), and plants (3). The ability to survive and adapt to a wide range of habitats and 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 acyl-homoserine-lactone (AHL)-type quorum-sensing (QS) signals. The CepI/CepR system, which is a member of the conserved LuxI/LuxR-type QS system, is present in all members of the *B. cepacia* complex (7, 35). 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) (9, 29). 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 (7, 35). 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, and it has been suggested that HHQ may also be used for cell-to-cell communication (6). In the case of the human pathogen *Burkholderia pseudomallei*, loss of HHQ production was shown to affect colony morphology and increase elastase production (6). 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* (4). 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 *B. cepacia* complex strains (19). 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* (1, 39). Microarray analysis has shown that DSF regulates over 160 genes, many of which encode virulence factors (13). As a

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotype and/or characteristic(s)	Source or reference
Strain		
B. cenocepacia		
J2315	Wild-type strain, genomovar III of *B. cepacia* complex	ATCC
d0581	BDSF mutant strain derived from J2315 with Bcam0581 being deleted	4
J2315(P0581-lacZ)	Wild-type strain harboring the reporter construct pMLS7P0581-lacZ	This study
J2315(PzmpA-lacZ)	Wild-type strain harboring the reporter construct pMLS7PzmpA-lacZ	This study
J2315(Plip-lacZ)	Wild-type strain harboring the reporter construct pMLS7Plip-lacZ	This study
J2315(Porb-lacZ)	Wild-type strain harboring the reporter construct pMLS7Porb-lacZ	This study
d0581(0581)	Mutant strain d0581 harboring the expression construct pMLS7-0581	4
d0581(P0581-lacZ)	Mutant strain d0581 harboring the reporter construct pMLS7P0581-lacZ	This study
d0581(PzmpA-lacZ)	Mutant strain d0581 harboring the reporter construct pMLS7PzmpA-lacZ	This study
d0581(Plip-lacZ)	Mutant strain d0581 harboring the reporter construct pMLS7Plip-lacZ	This study
d0581(Porb-lacZ)	Mutant strain d0581 harboring the reporter construct pMLS7Porb-lacZ	This study
d0581(cepI)	Mutant strain d0581 harboring the expression construct pMLS7-cepI	This study
d0581(cepR)	Mutant strain d0581 harboring the expression construct pMLS7-cepR	This study
cepR::Km^r	*cepR* insertion mutant derived from strain H111	15
cepR::Km^r (pBAH27)	*cepR* insertion mutant harboring *cepR* in construct pBAH27	15
d0581(0207)	Mutant strain d0581 harboring the expression construct pMLS7-0207	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* λ*pir*	Laboratory collection
S17-1	*pro res* mutant *mod*$^+$-integrated copy of RP4, *mob*$^+$	Laboratory collection
X. campestris		
FE58	Biosensor for DSF/BDSF	39
Plasmid		
pMLS7	pBBR1 *ori*, P_{S7} promoter, Tp^r *mob*$^+$	23
pMLS7-0581	pMLS7 containing Bcam0581	4
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* strain H111	15

consequence, DSF-deficient mutants are partially attenuated in virulence (1, 12, 39). 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.

MATERIALS AND METHODS

Bacteria strains and growth conditions. Bacterial strains used in this work are listed in Table 1. *E. coli* were grown at 37°C with shaking at 250 rpm in Luria-Bertani (LB) broth. *Burkholderia* species strains were cultured at 37°C in either LB or Anwar medium (25), 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 morpholineethanesulfonic acid (MOPS) 50 with a pH of 7.6. The following antibiotics were supplemented when necessary: gentamicin (100 μg ml^{-1}) and trimethoprim (400 μg ml^{-1} for *B. cenocepacia*; 1.5 mg ml^{-1} for *E. coli*).

Determination of the BDSF accumulation profile. Cells were harvested at different time points as indicated, and BDSF was extracted as described previously with minor modifications (4). Briefly, *B. cenocepacia* J2315 was grown in LB medium with agitation at 37°C. At each time point, 100 ml of culture supernatant was 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 *Xanthomonas campestris* pv. *campestris* biosensor strain FE58 (39). To this end, the biosensor was grown at 28°C in YEB medium (41) 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 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. β-Glucuronidase (GUS) activities were determined according to the methods of Jefferson et al. (16). BDSF concentrations were measured using a standard curve that was prepared with defined concentrations of chemically synthesized BDSF (39).

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 optical density at 600 nm (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°C in a low-intensity shaking model using the Bioscreen-C automated growth curves analysis system (OY Growth Curves; AB Ltd., Helsinki, Finland). For determination of ATP and CFU, samples were taken at various time points and spread onto LB plates after appropriate dilutions. Quantification of ATP was achieved using the BacTiter-Glo microbial cell viability assay kit (Promega) according to the manufacturer's protocols.

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 (23) by digestion with NheI and EcoRI. The *lacZ* gene was amplified using the primer pair lacZF and lacZR (Table 2). The amplified *lacZ* was ligated to the linealized pMLS7 to generate

TABLE 2. PCR primers used in this study

Primer and purpose	Sequence
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
In *trans* expression	
cepI-F	5′-CGCGGATCCATGCAGACCTTCGTTCACGAG
cepI-R	5′-CCCAAGCTTTCAGGCGGCGATAGCTTGC
cepR-F	5′-CGCGGATCCATGGAACTGCGCTGGCAG
cepR-R	5′-CCCAAGCTTTCAGGGTGCTTCGATGAGCC
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

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 2. The PCR fragments were digested with BamHI and HindIII 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 triparental 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°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 (17).

RNA extraction and RT-PCR analysis. Cultures grown in LB medium at 37°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 after 48 h (Anwar medium), respectively. RNA was isolated from 1 ml 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. Reverse transcription-PCR (RT-PCR) analysis was performed using the One-Step RT-PCR kit according to the manufacturer's instructions (Qiagen). The signal density of the PCR band was determined using ImageJ (http://rsb.info.nih.gov/ij/).

Virulence assays using a zebrafish infection model. *B. cenocepacia* virulence was tested by infecting 6-month-old zebrafish (*Danio rerio*). To this end, 30 μl 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 three 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 sterilized 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 gentamicin (200 μg ml^{-1}). The bacterial cell numbers (CFU) were determined 2 days after incubation at 37°C.

RESULTS

Accumulation of BDSF is cell density dependent. To determine the time course of BDSF production, we extracted *B. cenocepacia* strain J2315 supernatants at various growth stages and measured BDSF concentrations with the aid of the biosensor strain FE58, in which the GUS reporter gene was driven by the promoter of the DSF-inducible endoglucanase gene *engXCA*. Detectable amounts of BDSF were first measured at 6 h postinoculation (Fig. 1A). After this time point, BDSF levels increased steadily during the exponential and early stationary growth phases. BDSF accumulation peaked in the late stationary phase (36 h), followed by a significant decline in BDSF levels (Fig. 1A). We noted that the pH of bacterial supernatants reached approximately 8.6 at 40 h postinoculation. 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.

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. 1B, the promoter activity of Bcam0581 gradually increased and reached a plateau at 12 h postinoculation, which was maintained for another 12 h. Thereafter, BDSF levels declined rapidly. The Bcam0581 promoter activities correlated well with the BDSF accumulation profile as follows: 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 at the transcriptional level.

Deletion of Bcam0581 impaired growth and cellular ATP levels when grown in minimal medium. While deletion of Bcam0581 did not have an obvious effect on bacterial growth in LB medium, we noticed that the mutant grew much more

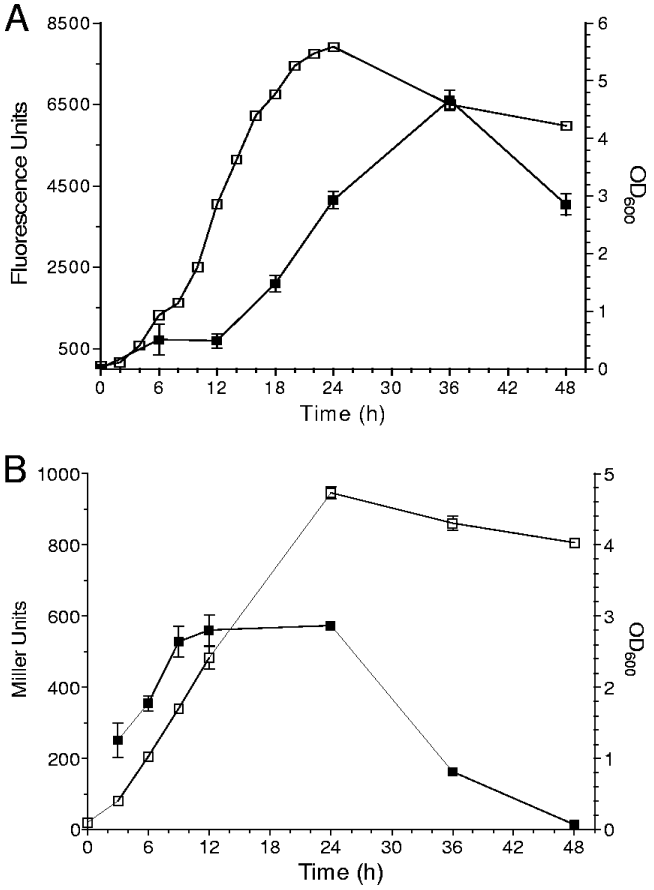

FIG. 1. BDSF production and Bcam0581 transcriptional expression. (A) Time course analysis of *B. cenocepacia* growth in LB medium (□) and BDSF accumulation in culture supernatants (■). (B) β-Galactosidase activity of a Bcam0581-*lacZ* transcriptional fusion (■) and bacterial growth (□). Strain J2315 was inoculated in a flask containing LB medium and cultured at 37°C with shaking at 220 rpm. The data presented are the means of two independent experiments, and the error bars represent standard deviations.

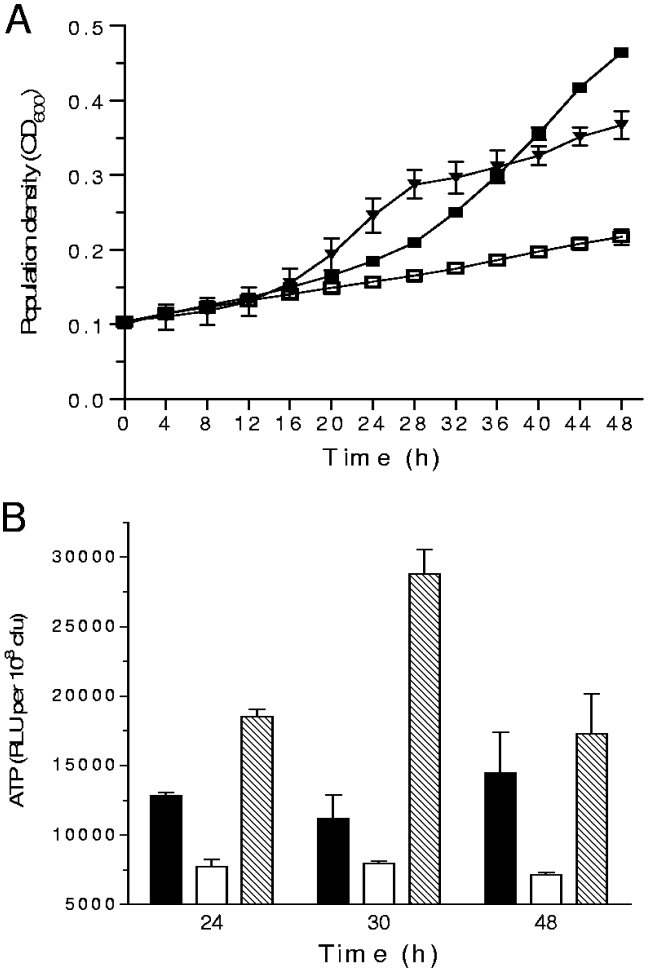

FIG. 2. Effect of Bcam0581 deletion on *B. cenocepacia* growth and energy biogenesis in minimal medium. (A) Growth patterns of the wild-type strain J2315 (■), the mutant strain d0581 (□), and the complemented mutant strain d0581(0581) (▼). Bacterial cells were inoculated in Anwar medium with three duplicates for each strain, and growth profiles at 37°C were recorded by Bioscreen. (B) Cellular ATP levels expressed in relative light units (RLU) per 10^8 CFU. Bacteria were grown under the same conditions as described for panel A, and samples for ATP measurement were taken at different time points as indicated. Shown are results for wild-type strain J2315 (solid bars), mutant strain d0581 (open bars), and complemented strain d0581(0581) (striped bars). The data shown are the means of three repeats, and error bars indicate standard deviations.

slowly 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 was the growth rate of d0581 reduced, but the mutant reached an OD_{600} of only 0.2 at 44 h after inoculation (Fig. 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 higher initial growth rate but then, surprisingly, a lower rate than the wild-type strain after 28 h (Fig. 2A). Moreover, exogenous addition of BDSF up to a concentration of 25 μM was not able to rescue the growth defect of d0581 (Fig. 3C). 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. 2B). Consistent with its growth profile, the complement strain produced more ATP than the wild-type strain, in particular 24 and 30 h after inoculation.

The growth defect of mutant Δ0581 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 the energy metabolism of *B. cenocepacia* strain J2315, we tested whether supplementation of carbohydrates associated with the 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 carbohy-

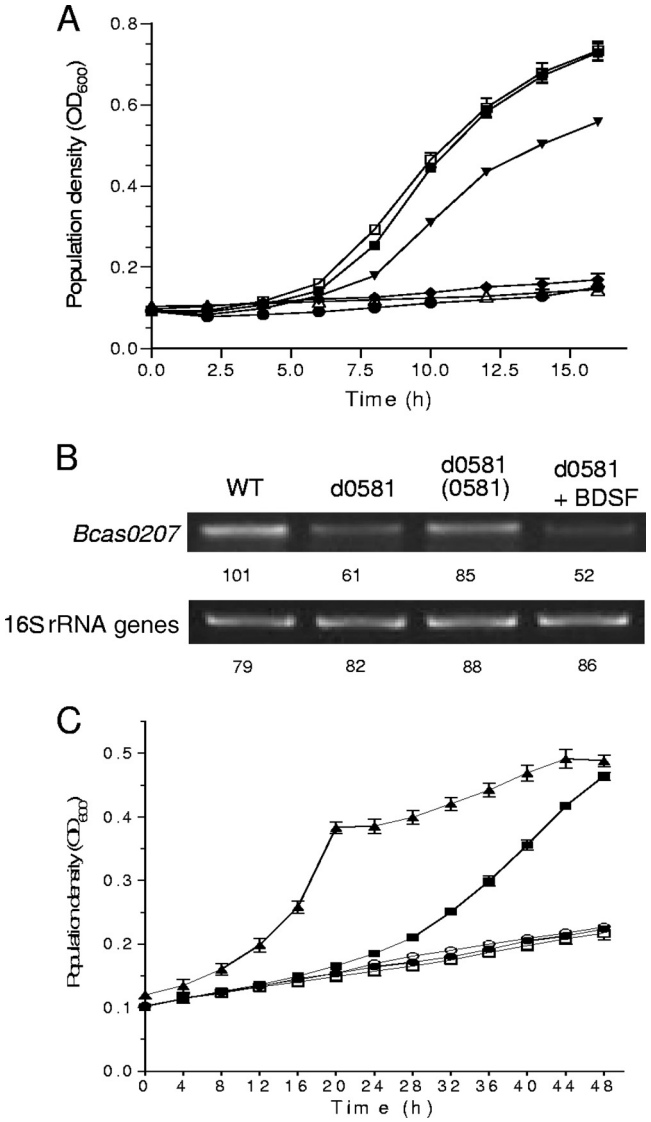

FIG. 3. Addition of citrate or in *trans* expression of citrate synthase rescues the growth defect of the Bcam0581 mutant in Anwar medium. (A) Shown are growth curves of the following strains: wild-type strain J2315 (■) and the complemented mutant strain d0581(0581) (▼) in Anwar medium supplemented with 20 mM citrate, and mutant strain d0581 in Anwar medium (△) and 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 strain J2315 (WT), the mutant strain d0581, the complemented mutant strain d0581(0581), and the mutant strain d0581 supplemented with 5 μM BDSF. The signal intensity (using the software ImageJ [http://rsb.info.nih.gov/ij/]) determined for each RT-PCR band is indicated. For each RNA sample, two dilutions (5 and 50 ng) were used as templates for RT-PCR reactions with a similar pattern of results. (C) Shown are growth curves of the following strains: wild-type strain J2315 (■) and the mutant strains d0581 (□), d0581(0207) (▲), and d0581 supplemented with 5 μM (●) or 25 μM (○) BDSF in Anwar medium. The error bars show the standard deviations of three repeats.

drates, including citrate, malate, and pyruvate. As shown in Fig. 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 strain d0581. 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. 3A).

The involvement of Bcam0581 in citrate biosynthesis was further analyzed by semiquantitative RT-PCR. Bcas0207 of *B. cenocepacia* strain J2315 encodes a citrate synthase homologue, which contains an oxalacetate binding site, a citryl-coenzyme A binding site, and a coenzyme A binding site involved in the citrate biosynthesis of the tricarboxylic acid cycle (14). 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 no effect (Fig. 3B). We then tested whether in *trans* expression of Bcas0207 could rescue the growth defect in the mutant strain d0581. The results showed that the resultant strain d0581(0207) initially grew faster than the wild-type strain, but its proliferation was slowed down after 20 h and reached a population density similar to that of the wild type at about 48 h after inoculation (Fig. 3C).

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 (8, 20), *lipA* (Bcam0949) and *lipB* (Bcam0950) encoding a lipase and a lipase chaperone (14), respectively, and the *orbI* gene (Bcal1696) required for the biosynthesis of the siderophore ornibactin (14). These investigations revealed that the transcript levels of *zmpA*, *lipA*, *lipB*, and to a lesser degree *orbI* were reduced in the mutant strain d0581 relative to levels of these genes in the parental strain (Fig. 4). 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 the exogenous addition of 5 μM BDSF (Fig. 4). 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, a null mutation of Bcam0581 caused a 30 to 50% reduction in transcriptional expression of these genes, which was almost fully rescued by the addition of 5 μM BDSF to the medium (Fig. 5A to C).

Coregulation of virulence gene expression by BDSF and AHL signal molecules. Interestingly, ZmpA and ornibactin biosynthesis have been previously shown to be under the control of the CepIR QS system (36). 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 Bcam0581 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 smaller amounts of AHL signal molecules than the wild type when grown in Anwar minimal medium for the same length of time (data not

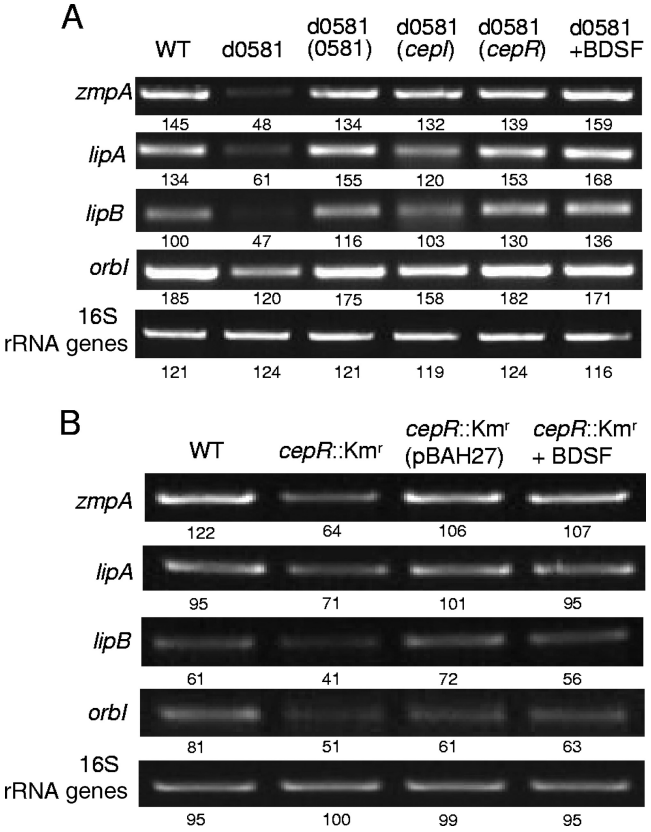

FIG. 4. Analysis of the role of Bcam0581/BDSF and the AHL system in the regulation of virulence gene expression. Bacterial strains were grown in LB medium to an OD_{600} of 1.5. BDSF was added at a final concentration of 5 μM as indicated. (A) Effect of the loss of Bcam0581 in strain J2315. (B) Effect of the *cepR* mutation in strain H111. The *cepR* mutant of strain J2315 was also tested with similar results (data not shown). For each RNA sample, two dilutions (5 and 50 ng) were used as templates for RT-PCR reactions and resulted in a similar pattern of results. WT, wild type.

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. 4A). We then tested whether BDSF can further increase virulence gene expression in the AHL mutant background. The results showed that the addition of 5 μM BDSF to the *cepR* mutant *cepR*::Km^r fully restored the transcriptional expression of the four virulence genes described above (Fig. 4B). 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 the mutant d0581 (Fig. 5A to C). 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. 5A to C). These data suggest that

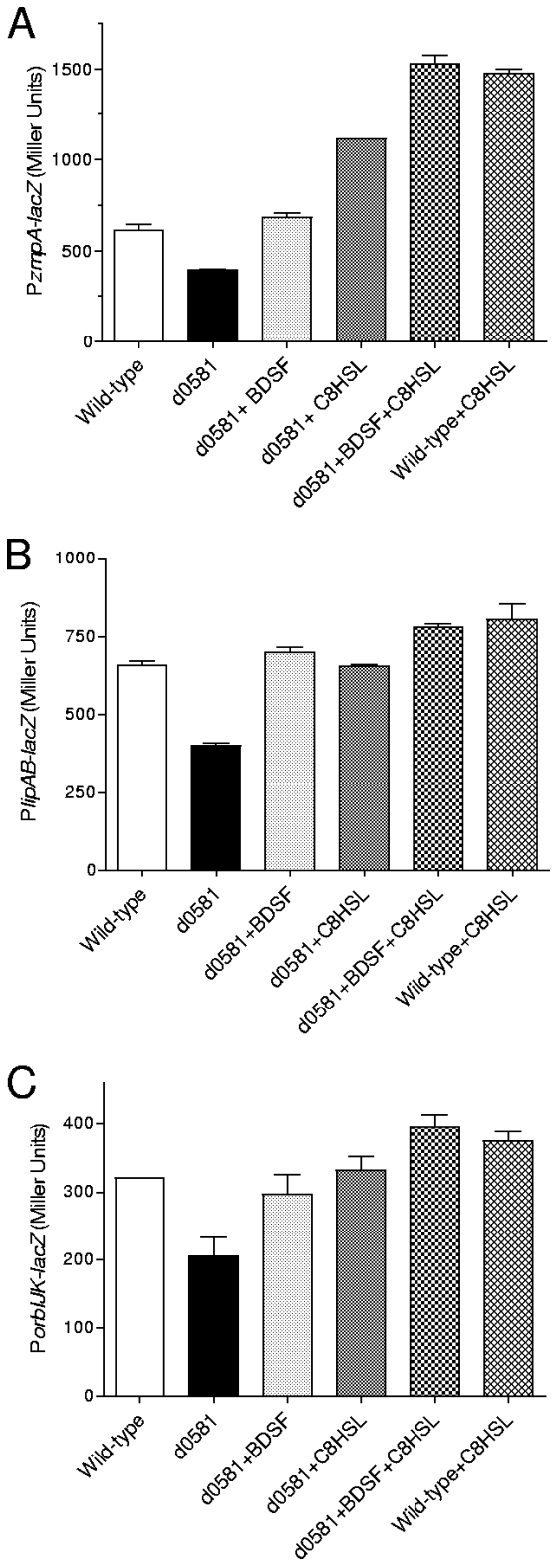

FIG. 5. Influence of Bcam0581 on expression of virulence genes *zmpA* (A), *lipAB* (B), and *orbIJK* (C) as determined by using corresponding promoter-*lacZ* fusion reporter strains. Bacterial strains were grown in LB medium, and BDSF and C8HSL were added separately or in combination at a final concentration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

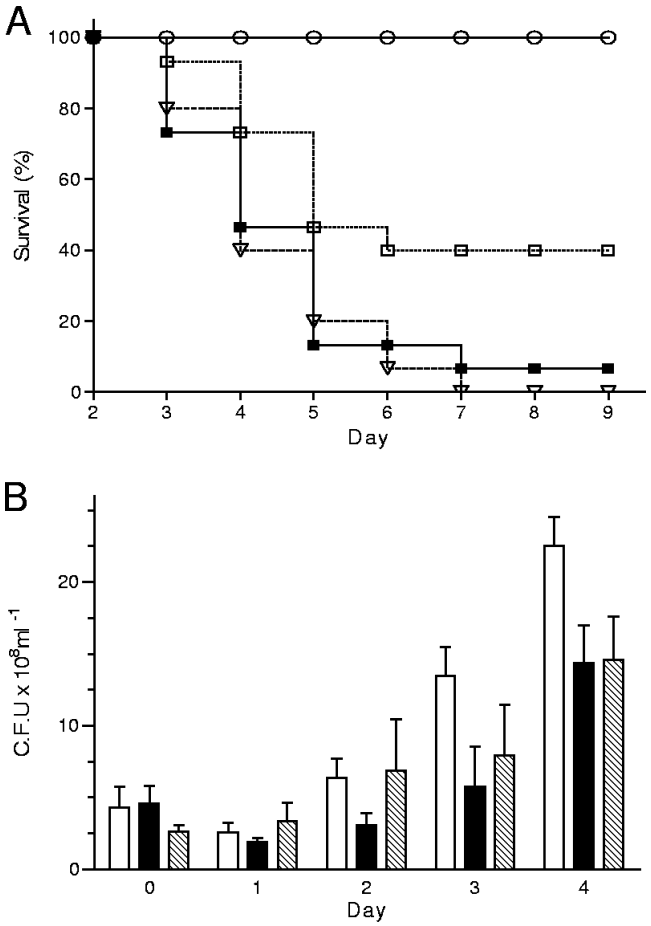

FIG. 6. BDSF is essential for full virulence of *B. cenocepacia* strain J2315 in a zebrafish infection model. (A) Survival rate of the fish after infection with the wild-type strain J2315 (■), the BDSF-deficient mutant d0581 (□), and the complemented mutant d0581(0581) (▽). PBS was injected in the same way as for a mock control (○). 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. The following strains are shown: J2315 (open bars), d0581 (solid bars), and d0581(0581) (striped bars). The data presented are the means of two independent experiments, and the error bars represent standard deviations.

the AHL- and BDSF-dependent QS systems regulate a similar set of virulence factors in parallel.

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. PBS was used as a mock control. While more than 90% of the fish infected with the wild-type strain J2315 died within 6 days postinoculation, the Bcam0581 mutant strain was much less virulent and only 40% of the infected fish survived (Fig. 6A). In *trans* expression of the Bcam0581 gene in strain d0581 fully restored virulence, and none of the fish infected with the complemented mutant was alive at day 7 postinfection (Fig. 6A).

Given that the 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. 6B show that the CFU counts of the mutant during the first 3 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 postinfection in comparison with the wild type (Fig. 6B). It is noteworthy that the in vivo growth trends of the three strains are similar to their in vitro growth patterns (Fig. 2A). On the fourth day of infection, the number of bacterial cells of the complemented strain was indistinguishable from that of the mutant d0581 (Fig. 6B), likely due to loss of the plasmid construct containing Bcam0581.

DISCUSSION

Previous work has shown that *B. cenocepacia* utilizes a highly conserved AHL-dependent QS system for cell-to-cell communication (9, 24, 26). In this study, we present evidence that this bacterium also employs a BDSF-dependent communication system to coordinate virulence gene expression. 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 the 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 physiologically relevant amount of BDSF, indicating a positive regulatory role of this signal in modulation of bacterial virulence.

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 the CepIR QS system (36). The finding that deletion of Bcam0581 affected transcription of neither *cepI* nor *cepR* but that addition of C8-HSL could restore virulence gene expression of the BDSF-null mutant suggests that the two signaling systems coregulate 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-dependent and a *P. aeruginosa* quinolone signal molecule-dependent QS system to modulate the production of a wide range of extracellular virulence factors (31). Likewise, expression of the virulence regulon in *Xanthomonas campestris* is coregulated by the QS signal DSF and hypoxia cue (11). Interestingly, a recent study suggested that BDSF regulates virulence gene expression in *B. cenocepacia* independent of the AHL-type QS system (32). Given that the BDSF-dependent genes identified in that study are different from the virulence genes characterized this paper, it is a possibility that in addition to coregulation of a common set of virulence genes, BDSF and AHL may also have their own separate regulons. 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 coregulating 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 (18, 22, 38, 42). A rapid increase of the signal in culture supernatants was observed during the mid exponential to late stationary growth phases (12 to 36 h postinoculation). This boost in BDSF level was preceded by a strong increase in promoter activity driving expression of Bcam0581, which encodes an enzyme required for BDSF biosynthesis (4), suggesting that BDSF production is controlled at the level of transcription. Given that maximum BDSF concentrations were observed 1 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 is the case for AI-2 signal production in *Salmonella enterica* serovar Typhimurium (2). The chemical structure of BDSF closely resembles that of DSF produced by *X. campestris* (4, 39), in which DSF biosynthesis is autoregulated by a signal sensor-synthase interaction mechanism (12). So far, only one component of the *B. cenocepacia* BDSF-dependent signaling system, namely the BDSF synthase Bcam0581, has been identified (4). Additional work will be required to identify the BDSF sensor and the other components that constitute the signaling circuitry as well as elucidate their interactions.

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 conditions. 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 at 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 downregulated by deletion of Bcam0581, resulted in a higher growth rate than the wild-type strain. These data suggest that Bcam0581 may influence energy metabolism by affecting the 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 by recycling the toxic intermediate *S*-adenosylhomocysteine (40). The detailed mechanism of Bcam0581 in energy metabolism in *B. cenocepacia* remains to be elucidated.

It was noticed that overexpression of Bcam0581 in the mutant d0581 initially resulted in an growth rate that was higher than the wild type but then showed a growth deceleration phenotype after 28 h. Coincidently, overexpression of the citrate synthase gene Bcas0207 in strain 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 strain 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 the signaling pathway and shed light on the mechanism by which it cross-talks with the AHL-dependent QS system.

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