Role of *Burkholderia cenocepacia* *afcE* and *afcF* genes in determining lipid-metabolism-associated phenotypes

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*Burkholderia cenocepacia* is an opportunistic pathogen that primarily infects cystic fibrosis patients. Previously we have reported that mutations in *shvR*, a LysR-type transcriptional regulator, and *ShwR*-regulated genes BCAS0208 and BCAS0201 (designated *afcE* and *afcF*, respectively) affect colony morphology, biofilm and pellicle formation and virulence in *B. cenocepacia*. In this study we investigated the role of *afcE* and *afcF* in influencing lipid-metabolism-associated phenotypes. As previously reported for K56-2ΔshvR, the Δ2afcE and ΔafcF::lux mutants had no antifungal activity against *Fusarium* and *Rhizoctonia solani*, suggesting that these genes are involved in synthesis of a membrane-associated antifungal lipopeptide. Strains Δ2afcE and ΔafcF::lux had reduced swarming motility and altered cell membrane morphology, both of which were restored to wild-type levels upon providing these genes in trans. Both K56-2ΔshvR and Δ2afcE showed increased uptake of the hydrophobic fluorescent probe N-phenylnaphthylamine (NPN), indicating altered outer membrane properties. Total lipid profiles determined by TLC revealed distinct differences in cellular lipid compositions of K56-2ΔshvR, Δ2afcE and ΔafcF::lux compared with K56-2. Taken together, these results indicate that *afcE* and *afcF* are involved in metabolic pathway(s) influencing lipid profiles and affect both cell surface and antifungal properties of *B. cenocepacia*.

INTRODUCTION

*Burkholderia cenocepacia* belongs to the *Burkholderia cepacia* complex (Bcc), a group of at least 17 closely related and genetically distinct *Burkholderia* species with a widespread distribution in diverse environments (Drevinek & Mahenthiralingam, 2010; Mahenthiralingam et al., 2008; Sousa et al., 2011; Vanlaere et al., 2008, 2009). Bcc species cause multi-host infections, fix nitrogen and promote plant growth, produce antifungal compounds and degrade recalcitrant pollutants, including aromatic hydrocarbons (Sousa et al., 2011). *B. cenocepacia*, one of the most common Bcc species, is also an opportunistic pathogen of immunocompromised individuals and cystic fibrosis (CF) patients (LiPuma et al., 2001; Speert et al., 2002). The clinical outcome of Bcc infections can range from chronic to acute infections, sometimes leading to rapid lung deterioration and death by what has been termed cepacia syndrome (Mahenthiralingam et al., 2008). Although transmission of *B. cenocepacia* infections between CF patients has decreased over time due to implementation of improved infection control procedures, hospital-acquired infections are becoming more common and the high resistance of these bacteria to most antibiotics remains problematic (Chernish & Aaron, 2003; Saiman & Siegel, 2004).

The colony morphology of *B. cenocepacia* K56-2 on solid medium is typically rough (Bernier et al., 2008). ShwR was initially identified as a gene involved in the determination of rough colony morphology since its mutation resulted in a shiny (shv) morphotype (Bernier et al., 2008). K56-2ΔshvR is defective in biofilm formation, antifungal activity, extracellular matrix formation and virulence (Bernier et al., 2008; O’Grady et al., 2011). Although ShvR is a global regulator, the most stringently regulated genes are in two divergently transcribed putative operons adjacent to *shvR*, designated the *afcA* and *afcC* operons based on the first gene in each transcriptional unit (O’Grady et al., 2011).

In a previous study, we reported the characterization of the BCAS0208–S0201 genes located at the 3′ end of the *afcA* operon and determined that some of these genes have...
important roles in colony morphology, biofilm formation and virulence in both chronic rat lung infection and alfalfa models of infection (Subramoni et al., 2011). The putative acyl-CoA dehydrogenase encoded by afeE is required for rough colony morphology, biofilm formation and virulence in the alfalfa infection model. The putative FAD-dependent oxidoreductase encoded by afeF is required for optimum biofilm formation and alfalfa virulence but not colony morphotype. BCAS0208::Tn, a strain with a polar mutation affecting both afeE and afeF expression, was defective in pellicle formation, although strains Δ2afeE or afeF::lux with individual mutations did not show defects in pellicle formation. Cell surface hydrophobicity assays revealed that the Δ2afeE or afeF::lux mutants were much more hydrophilic than the K56-2 wild-type strain. These studies demonstrated a role for these genes in influencing surface properties. Biolog and growth assays suggested a role for afeE in utilization of the branched-chain fatty acids α-ketobutyric acid, α-hydroxybutyric acid and isovaleric acid, and a role for afeF in utilization of the branched-chain amino acids (BCAAs) leucine, isoleucine and valine. FAD-dependent oxidoreductase is part of the branched-chain ketoacid dehydrogenase enzyme complex of the BCAAs degradation pathway and it is required to generate branched-chain acyl-CoA derivatives. Acyl-CoA dehydrogenases use these as substrates to carry out a β-oxidation reaction, which is similar to the first step in fatty acid oxidation. We proposed that the enzymes encoded by afeF and afeE are part of a metabolic pathway that influences cellular lipid metabolism.

In the previous study, we also analysed the genes between afeE and afeF; BCAS0207, which encodes a citrate synthase, and BCAS0204, which encodes an ATP-binding component of a transporter complex, were not important for colony morphotype (Subramoni et al., 2011). BCAS0207::lux had a partial defect in biofilm formation and virulence while BCAS0204::lux was partially defective in biofilm formation without affecting virulence. Biolog assays showed that BCAS0207 has a role in utilization of substrates of intermediary metabolism including acetate, citrate, succinate and fumarate. BCAS0204 has a role in utilization of L-arabinose. Neither BCAS0207::lux nor BCAS0204::lux showed a defect in utilization of fatty acids.

Although functions of other genes in the afe operons remain unknown, mutations in afeA, afeB and afeC homologues in Burkholderia pyrrhocina BC11 (formerly B. cepacia BC11) drastically reduced production of the antifungal lipopeptide AFC-BC11 (Kang et al., 1998). AFC-BC11 has been characterized as a non-ribosomally synthesized lipopeptide that was largely associated with the membrane (Kang et al., 1998). AFC-BC11 had antifungal activity against Rhizoctonia solani and protected cotton plants from damping-off disease. Several antifungal compounds, including pyoluteorin in Pseudomonas fluorescens Pf-5 (Nowak-Thompson et al., 1999), kutznerides in actinomycete Kutzneria sp. (Fujimori et al., 2007), fengycin in Bacillus subtilis (Tosato et al., 1997) as well as the antimicrobial compounds fumulimicin in Actinoplanes friulienisi (Heinzelmanna et al., 2005) and avermectin in Streptomyces avermitilis (Zhang et al., 1999), contain genes coding for an acyl-CoA dehydrogenase as part of their biosynthetic cluster. FAD-dependent oxidoreductase-encoding genes occur as part of the biosynthetic clusters of the antifungal metabolites pyrrolnitrin in Pseudomonas fluorescens Pf-5 (Lee & Zhao, 2007), endophenazines in Streptomyces anulatus (Saleh et al., 2012) and ML-449 in Streptomyces sp. (Jørgensen et al., 2010). Biosynthesis of antifungal compounds is under strict regulation and depends on carbon sources, environmental conditions and regulatory systems including quorum sensing (Mahenthiralingam et al., 2011; O’Grady et al., 2011; Schmidt et al., 2009).

Since the results of growth assays and annotations of afeE and afeF suggested a link to lipid metabolic pathways, we looked at phenotypes that are known to be influenced by lipid levels in order to further investigate the role of these genes in virulence. Swarming motility involves rapid movement of a group of bacteria over moist surfaces using flagella. Bacteria often produce extracellular biosurfactants that act as wetting agents to reduce surface tension (Kearns, 2010; Verstraeten et al., 2008). Biosurfactants are amphiphilic and may be lipopeptides, glycolipids, fatty acids, neutral lipids, phospholipids or polymeric in nature and may have potent antimicrobial activity (Desai & Banat, 1997). We carried out experiments to detect changes in swarming motility and cell membrane properties, as cellular lipids are important for both these traits. We further show that both afeE and afeF function in production of the AFC antifungal activity and influence total lipid profiles.

**METHODS**

**Strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were routinely grown at 37 °C in Miller’s Luria broth (LB) (Invitrogen) with shaking or on 1.5% Lennox LB agar plates. B. cepacia strains were grown on yeast extract–mannitol (YEM) agar consisting of 0.05% (w/v) yeast extract, 0.4% (w/v) mannitol and 1.5% (w/v) agar or King A agar, consisting of 2% (w/v) gelatin peptone, 0.14% (w/v) magnesium chloride, 1% (w/v) potassium sulphate, 1% (w/v) agar and 1% (w/v) glycerol for exopolysaccharide detection or potato dextrose agar (PDA; Difco) for lipid analysis. When appropriate, the following concentrations of antibiotics were used: 100 µg trimethoprim ml⁻¹ (Tp) and 200 µg tetracycline ml⁻¹ (Tc) for B. cepacia and 100 µg Tp ml⁻¹ and 50 µg kanamycin ml⁻¹ (Km) for Escherichia coli. Antibiotics were purchased from Sigma–Aldrich Canada.

**Antifungal assays.** Analyses of antagonistic activities against Rhizoctonia solani Kühn strain 160 and Fusarium solani strain D185 (obtained from the culture collection of the phytopathology group of the Institute of Plant Sciences, Federal Institute of Technology, Zurich, Switzerland) were carried out as previously described (O’Grady et al., 2011).

**Swarming motility.** For swarming motility assays, cultures were grown on yeast extract–mannitol (YEM) agar consisting of 2% (w/v) gelatin peptone, 0.14% (w/v) magnesium chloride, 1% (w/v) potassium sulphate, 1% (w/v) agar and 1% (w/v) glycerol for exopolysaccharide detection or potato dextrose agar (PDA; Difco) for lipid analysis. When appropriate, the following concentrations of antibiotics were used: 100 µg trimethoprim ml⁻¹ (Tp) and 200 µg tetracycline ml⁻¹ (Tc) for B. cepacia and 100 µg Tp ml⁻¹ and 50 µg kanamycin ml⁻¹ (Km) for Escherichia coli. Antibiotics were purchased from Sigma–Aldrich Canada.

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Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>B. cenocepacia</td>
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<tr>
<td>K56-2</td>
<td>CF sputum isolate (Canada); ET12 lineage</td>
<td>Mahenthiralingam <em>et al.</em> (2000)</td>
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<td>K56-ΔshvR</td>
<td>shv derivative of K56-2</td>
<td>O’Grady <em>et al.</em> (2011)</td>
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<tr>
<td>K56-2BCAS0225::Tn</td>
<td>BCAS0225::TnRhaBout derivative of K56-2; Tp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Bernier <em>et al.</em> (2008)</td>
</tr>
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<td>Δ2afcE</td>
<td>K56-2 Δ2BCAS0208 deletion mutant, 1.2 kb of BCAS0208 deleted, non-polar</td>
<td>Subramoni <em>et al.</em> (2011)</td>
</tr>
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<td>BCAS0207::lux</td>
<td>K56-2 with pGSVTP-luxCDABE inserted in BCAS0207; Tp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
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<tr>
<td>BCAS0204::lux</td>
<td>K56-2 with pGSVTP-luxCDABE inserted in BCAS0204; Tp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
</tr>
<tr>
<td>afcF::lux</td>
<td>BCAS0201::lux, K56-2 with pGSVTP-luxCDABE inserted in BCAS0201; Tp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
</tr>
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<td>K56-2S15</td>
<td>Spontaneous shv morphotype mutant, virulent in alfalfa</td>
<td>Bernier <em>et al.</em> (2008)</td>
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<td>K56-2S86</td>
<td>Spontaneous shv morphotype mutant, avirulent in alfalfa</td>
<td>Bernier <em>et al.</em> (2008)</td>
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<td>K56-2S92</td>
<td>Spontaneous shv morphotype mutant, avirulent in alfalfa</td>
<td>Bernier <em>et al.</em> (2008)</td>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-3</td>
<td>Broad-host-range, mobilizable plasmid pBBR1CM Kovach <em>et al.</em> (1995) with pBluescript II KS-lacZa-polylinker; Tc&lt;sup&gt;i&lt;/sup&gt;; abbreviated as pBBR</td>
<td>Kovach <em>et al.</em> (1995)</td>
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<td>pBBRafcE</td>
<td>pBRS0208, pBBR1MCS-3 with a 1.91 kb KpnI-XbaI fragment containing BCAS0208; Tc&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
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<td>pBRS0207-S0205</td>
<td>pBBR1MCS-3 with a 3.1 kb Apal-XbaI fragment containing BCAS0205, BCAS0206 and BCAS0207; Tc&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
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<td>pBRS0204-S0202</td>
<td>pBBR1MCS-3 with a 2.9 kb KpnI-XbaI fragment containing BCAS0202, BCAS0203 and BCAS0204; Tc&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
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<tr>
<td>pBBRafF</td>
<td>pBRS0201, pBBR1MCS-3 with a 1.6 kb KpnI-XbaI fragment containing BCAS0201; Tc&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Subramoni <em>et al.</em> (2011)</td>
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<td>pUCP28T</td>
<td>Broad-host-range vector, Tp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Schweizer <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>p28T-shvR</td>
<td>pUCP28T with a 1.7 kb PstI–BanHI fragment containing BCAS0225 and upstream region, Tc&lt;sup&gt;i&lt;/sup&gt;</td>
<td>O’Grady <em>et al.</em> (2011)</td>
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Strains and plasmids used in this study.

B. cenocepacia cells was measured using the 1-N-phenylthiophenylamine (NPN) fluorescence assay (Hancock & Wong, 1984). Briefly, NPN was dissolved in acetone at a concentration of 500 μM and added to cells in HEPES buffer at a final concentration of 10 pM. Cultures were grown overnight with antibiotics and further subcultured without antibiotics to obtain cells in exponential phase (OD<sub>600</sub> 0.4-0.6). Cells were washed with buffer and normalized to OD<sub>600</sub> 0.5. Fluorescence spectra and emission intensities were measured with a PTI fluorescence spectrophotometer at room temperature using a 420 nm wavelength emission filter. For the assay, 1 ml normalized culture in buffer was transferred to a quartz cuvette and fluorescence measured for 25 s. Twenty microlitres of NPN was added and fluorescence measured for another 25 s, followed by the addition of 10 μl polymyxin B (0.64 mg ml<sup>-1</sup> H<sub>2</sub>O stock solution) and measurement for 75 s. Polymyxin B was added to increase potential differences in permeability to NPN. All measurements were continuous and the culture within the cuvette was stirred for the entire duration. Each strain was assayed at least three times independently.

Polymyxin B minimum inhibitory concentration (MIC<sub>50</sub>). Bacterial cultures were grown to exponential phase and diluted to obtain an OD<sub>600</sub> of 0.01. A 50 μl sample of this culture was added to 2.5 ml LB. Polymyxin B (Sigma) was dissolved in 0.2 % BSA–0.01 % acetic acid buffer to a concentration of 5.12 mg ml<sup>-1</sup>. Cultures diluted as described above were mixed with the polymyxin solution at a 1:1 ratio in 96-well plates and incubated at 37 °C for 16 h. Growth was determined by measuring OD<sub>600</sub>. Control cultures were mixed with 0.2 % BSA–0.01 % acetic acid buffer without polymyxin B. The MIC<sub>50</sub> was defined as the lowest polymyxin B concentration at which growth was inhibited by 50 % (Loutet *et al.*, 2006). Each culture and polymyxin B concentration was assayed in triplicate.

Cellular fatty acid analysis. Fatty acid extraction was carried out from 40 mg cells scraped from agar plates by saponification, methylation and extraction (MIDI, Microbial ID). The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (MIS) (MIDI) which consisted of an Agilent model 6890N gas chromatograph fitted with a phenyl-methyl silicone capillary column (0.2 mm × 25 m), a flame-ionization detector, automatic sampler and a computer with MIDI database. Peaks are automatically integrated and fatty acid names and percentages...
calculated by the MIS Standard Software (Microbial ID). *Stenotrophomonas maltophilia* ATCC 13637 and *Staphylococcus aureus* ATCC 25923 were used as control strains. Extraction and analysis by GC were done twice for each strain.

**Total lipid analysis.** Total cell lipids were extracted from cultures that were grown overnight on PDA as previously described with minor modifications (Taylor *et al.*, 1998). Briefly, cells were resuspended in PBS (NaCl, 8g; KCl, 0.2g; Na₂HPO₄, 1.44g; KH₂PO₄, 0.24g per litre; pH 7.5), normalized by OD₆₆₀ and were pelleted by centrifugation. Cell pellets were extracted with chloroform : methanol (2 : 1) and incubated again with an equal volume of chloroform : methanol (2 : 1) mixture. The extracts were pooled and one quarter volume was resuspended in PBS (NaCl, 8g; KCl, 0.2g; Na₂HPO₄, 1.44g; KH₂PO₄, 0.24g per litre; pH 7.5), normalized by OD₆₆₀ and were pelleted by centrifugation. Cell pellets were extracted with chloroform : methanol (2 : 1) and incubated for 2 h at 37 °C, centrifuged and extracted again with an equal volume of chloroform : methanol (2 : 1) mixture. The extracts were pooled and one quarter volume 0.04 % CaCl₂ was added for phase separation (Folch *et al.*, 1957). The organic phase was collected and polyhydroxybutyrate was precipitated by the addition of four volumes diethyl ether. The organic phase was evaporated dry and the pellet was dissolved in 100 µl chloroform : methanol (2 : 1) and incubated for 2 h at 37 °C, centrifuged and extracted again with an equal volume of chloroform : methanol (2 : 1) mixture. The extracts were pooled and one quarter volume 0.04 % CaCl₂ was added for phase separation (Folch *et al.*, 1957). The organic phase was collected and polyhydroxybutyrate was precipitated by the addition of four volumes diethyl ether. The organic phase was evaporated dry and the pellet was dissolved in 100 µl chloroform : methanol (2 : 1). The organic extract was spotted onto Al SigG/ UVTLC plates (Whatman) or Silica gel G TLC plates (Analtech). TLC was performed in a solvent consisting of chloroform : methanol : water (60 : 16 : 2, v/v). Total lipids were visualized by exposing the TLC plate to iodine vapour and amino-group-containing lipids were visualized by spraying with the ninhydrin reagent (Sigma). For each strain lipid extraction and analyses by TLC were carried out three independent times.

**RESULTS**

*afcE and afcF are required for antifungal activity of B. cenocepacia*

It has previously been reported that *afcA*, *afcB*, *afcC* and *afcD* are required for biosynthesis of AFC-BC11, a membrane-associated lipopeptide (Kang *et al.*, 1998). The *afcA*, *afcC*, *afcD* and partial *afcB* nucleotide sequence of *B. cepacia* BC11 (accession number AF076477) (Kang *et al.*, 1998) was 94 % identical to those genes in the *B. cenocepacia* J2315 genome suggesting that they are closely related and likely to code for proteins with similar functions. Since synthesis of antifungal lipopeptides typically requires several genes and our previous studies indicate that *afcE* and *afcF* are involved in lipid metabolism, we asked whether *afcE*–*afcF* are involved in the synthesis of the AFC antifungal lipopeptide. Antifungal assays were carried out against *R. solani* and *F. solani* (Table 1). Mutant strains Δ*afcE*(pBBR) and *afcF*::lux(pBBR) exhibited no activity against either fungal species, but this was restored by providing wild-type (WT) copies of the respective genes in trans (Fig. 1). Mutations in either BCAS0207 or BCAS0204 did not influence antifungal activity in these assays (Fig. 1). These results show that in addition to the previously described *afcA*, *afcB*, *afcC* and *afcD* genes, *afcE* and *afcF* function in the biosynthesis of an AFC antifungal lipopeptide.

*afcE and afcF influence swarming motility*

Mutations influencing membrane lipid composition or biosurfactant production are known to affect swarming motility, probably through the control of membrane fluidity and surface tension (Daniels *et al.*, 2004; Lai *et al.*, 2005). In order to determine whether *afcE* and *afcF* influence swarming motility, strains K56-2(pBBR), Δ*afcE*(pBBR), Δ*afcE*(pBBRafcE), *afcF*::lux(pBBR) and *afcF*::lux(pBBRafcF) were spotted on semi-solid agar and their migration was measured over time. K56-2(pBBR) swarmed up to 60 mm radially from the point of inoculation (Fig. 2). The Δ*afcE*(pBBR) mutant had 50 % of the swarming motility of K56-2(pBBR), which was restored by providing *afcE* in trans (Fig. 2a). The *afcF*::lux(pBBR) mutant exhibited only 60 % swarming motility compared with K56-2(pBBR) at 24 h (Fig. 2b). No difference could be detected by 48 h suggesting different mechanisms of action for the two genes concerning swarming motility. This swarming motility defect of the *afcF*::lux(pBBR) mutant was restored by providing *afcF* in trans. Similar to our observations with the antifungal assays, BCAS0207::lux and BCAS0204::lux did not show any change in swarming motility compared with K56-2.

*afcE and afcF contribute to cell membrane morphology*

In order to determine whether there were any structural differences in the cell membrane, K56-2(pBBR), Δ*afcE*(pBBR), Δ*afcE*(pBBRafcE), *afcF*::lux(pBBR) and *afcF*::lux(pBBRafcF) were analysed by TEM. Distinct differences were found in the cell membranes of strains Δ*afcE*(pBBR) and *afcF*::lux(pBBR) compared with K56-2(pBBR) (Fig. 3). The K56-2(pBBR) cell membrane had a wavy appearance and two distinct layers with the outer and cytoplasmic membranes clearly separated by the periplasmic space. However, the Δ*afcE*(pBBR) mutant had a smooth appearance and loss of separation of the two membranes (Fig. 3). The wavy appearance and membrane separation were restored by providing *afcE* in trans (Fig. 3). The *afcF*::lux mutant also had a smoother appearance although without the loss of the two distinct membrane layers. The wavy appearance was restored by providing *afcF* in trans (Fig. 3). These observations suggested that mutations in *afcE* and *afcF* might alter membrane lipids, resulting in membrane changes.

*afcE influences membrane properties*

We next determined whether the altered cell envelope morphology of the Δ*afcE*(pBBR) and *afcF*::lux(pBBR) mutants was associated with changes in membrane integrity. Changes in cell membrane integrity were measured by incubating cultures with NPN, a hydrophobic membrane probe. Upon excitation, NPN exhibits fluorescence emission at 420 nm in a hydrophobic environment, such as within the cell membrane. When K56-2(pBBR) was incubated with NPN, fluorescence emission remained steady at approximately 150,000 counts per second (c.p.s.) (Fig. 4a). Upon treatment with NPN, the Δ*afcE*(pBBR) mutant emitted fluorescence that was at least fourfold higher than that obtained with K56-2(pBBR). When *afcE* was provided in trans, strain Δ*afcE*(pBBRafcE) showed a 60 % reduction in...
fluorescence emission in the presence of NPN compared with strain Δ2afcE(pBBR) although it was still almost twofold higher than K56-2(pBBR), indicating partial complementation (Fig. 4a). This suggested that the Δ2afcE mutant has altered membrane integrity which results in increased entry of NPN into the membrane. The afcF::lux(pBBR) mutant did not show any difference in NPN fluorescence emission compared with K56-2(pBBR) (data not shown). K56-2BCAS0225::Tn(pBBR), a strain with a mutation in shvR, was also assessed for permeability to NPN as mutations in shvR resulted in smooth cell morphology similar to that of the Δ2afcE(pBBR) mutant (Bernier et al., 2008). K56-2BCAS0225::Tn(pBBR) also had increased fluorescence in the presence of NPN compared with K56-2(pBBR) but less than that observed with the Δ2afcE(pBBR) mutant (Fig. 4b). These results suggested a correlation between permeability to NPN and the shv morphotype characteristic of shvR and afcE mutants.

**Fig. 1.** Antifungal activity of K56-2, Δ2afcE, BCAS0207::lux, BCAS0204::lux and afcF::lux and their complemented derivatives. Antagonistic activities against (a) *R. solani* and (b) *F. solani* were measured by spotting *B. cenocepacia* in triplicate and measuring zones of growth inhibition of the fungal strain after 3 and 8 days of incubation respectively. V=pBBR vector control, C=constructs containing wild-type copies of respective genes. Values were significantly different between K56-2 and mutants compared at *P*<0.001 (one-way ANOVA with Bonferroni multiple comparisons post-test).
Since the NPN assay results suggested compromised membrane integrity in strain Δ2afcE(pBBR), we determined susceptibility to polymyxin B, an antimicrobial compound that binds to and alters outer membrane properties. There was no significant growth defect for strain Δ2afcE(pBBR) in polymyxin B concentrations up to 2 mg ml⁻¹ compared with control cultures (data not shown). Since the concentration of polymyxin B used for NPN assay was only 6.4 µg ml⁻¹, it is likely that increased permeability to NPN may reflect a subtle sensitivity to polymyxin B that is not detectable by MIC determination and this might be due to a general defect in membrane assembly.

afcE, afcF and shvR mutants have altered lipid profiles

Because of the membrane alterations and loss of biosynthesis of a membrane-associated antifungal lipopeptide in both the Δ2afcE and afcF::lux mutants, we examined their cellular fatty acid and lipid profiles. The fatty acid profiles expressed as a percentage of total cellular fatty acids of strains K562(pBBR), Δ2afcE(pBBR), Δ2afcE(pBBRafcE), Δ2afcE(pBBRafC), afcF::lux(pBBR), afcF::lux(pBBRafC) and Δ2afcE(pBBRafC) were significantly different (P<0.001; one-way ANOVA with Bonferroni multiple comparisons post-test).

Fig. 2. Swarming motility of K56-2, Δ2afcE and afcF::lux and their complemented derivatives. Swarming motility of (a) Δ2afcE and (b) afcF::lux strains compared with K56-2. Strains were spotted on swarm agar plates and the extent of the swarm area was measured from the centre after 24, 48 and 60 h of incubation. Values were significantly different between K56-2 and mutants (P<0.001; one-way ANOVA with Bonferroni multiple comparisons post-test).

Fig. 3. Transmission electron micrographs showing cell membrane morphology changes in (a) K56-2(pBBR), (b) Δ2afcE(pBBR), (c) Δ2afcE(pBBRafC), (d) afcF::lux(pBBR) and (e) afcF::lux(pBBRafC). The left panels show electron micrographs at ×20 000 magnification and the right panels show magnified views of a portion of this field. Bars, 100 nm.
Table 2. Fatty acid profiles of Δ2afcE determined by fatty acid methyl ester GC

<table>
<thead>
<tr>
<th>Fatty acid*</th>
<th>K56-2</th>
<th>Δ2afcE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>Complemented</td>
</tr>
<tr>
<td>n-Hexadecanoic acid (C_{16:0})</td>
<td>24.26</td>
<td>21.15</td>
</tr>
<tr>
<td>cyclo-Heptadecanoic acid (C_{17:0 cyclo})</td>
<td>11.56</td>
<td>9.91</td>
</tr>
<tr>
<td>n-Heptadecanoic acid (C_{17:0})</td>
<td>0.18</td>
<td>–</td>
</tr>
<tr>
<td>cis-11-Octadecanoic acid (C_{18:1 cyclo})</td>
<td>27.45</td>
<td>30.03</td>
</tr>
</tbody>
</table>

*The amount of each fatty acid is expressed as a percentage and the value given is the average of two independent experiments. Fatty acid percentages unchanged between strains are not shown.
alterations in lipid profiles, which cause changes in membrane morphology and eliminate antifungal peptide production.

Other shv morphotype mutants with loss of antifungal activity exhibit changes in lipid profile

Previously we had described spontaneous shv morphotype mutants that had wild-type shvR and afcE sequences (Bernier et al., 2008; Subramoni et al., 2011). Since our results suggested a correlation between altered cell surface properties and antifungal activity, we wanted to determine if these shv morphotype mutants had any changes in antifungal activity and lipid profile. Four shv morphotype mutants with wild-type shvR and afcE, K56-H15, S15, S86 and S92, were chosen for analysis (Table 1). Both K56-H15 and S92 showed no antifungal activity and had altered lipid profiles (Fig. 6; compare lanes 1, 2 and 5). S86 had reduced antifungal activity, but no major differences in lipid profile, whereas S15 had no changes in either antifungal activity or lipid profile (Fig. 6; compare lanes 1, 3 and 4). These results suggest that there is a correlation between loss of antifungal activity and the altered lipid profile, and that the

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**Fig. 5.** TLC of total lipids from K56-2 and mutants. (a) Lipid profile of K56-2, Δ2afcE, afcF::lux and their complemented derivatives detected by iodine (left panel) and by ninhydrin (right panel). Lanes: 1, K56-2(pBBR); 2, Δ2afcE(pBBR); 3, Δ2afcE(pBBR::lux); 4, afcF::lux(pBBR); 5, afcF::lux(pBBR::lux). Differences in ninhydrin-positive lipids (⁎) and iodine-positive lipids (⁎⁎) are indicated. PE1 and PE2 denote two forms of PEs, while OL1 and OL2 denote two forms of OL.

**Fig. 6.** Antifungal activity and TLC of total lipids from *B. cenocepacia* K56-2 and shv mutants. Antagonistic activities against (a) *R. solani* and (b) *F. solani* were measured by spotting *B. cenocepacia* in triplicate and measuring zones of growth inhibition of the fungal strain after 3 and 8 days of incubation, respectively. Values were significantly different between K56-2 and mutants compared at \( P < 0.001 \) (one-way ANOVA with Bonferroni multiple comparisons post-test). (c) Lipid profiles of K56-2, K56-H15, S15, S86 and S92 detected by iodine (left panel) and by ninhydrin (right panel). Lanes: 1, K56-2(pBBR); 2, K56-H15; 3, S15; 4, S86; 5, S92.
DISCUSSION

In this study we have shown that a ShvR-regulated acyl-CoA dehydrogenase and a FAD-dependent oxidoreductase influence synthesis of a membrane-associated antifungal lipopeptide, cell surface properties, including membrane morphology and permeability, surface motility and total cellular lipid profile. These phenotypes were restored when wild-type copies of either afcE or afcF were provided in trans to the respective mutants, suggesting that afcE and afcF affect these phenotypes by influencing pathways that affect lipid levels. Mutations in BCAS0207 and BCAS0204, two other genes in the afcE–F region, did not show altered cell surface properties or antifungal activity suggesting these genes are not involved in lipid synthesis. K56-2ΔshvR has a cellular lipid profile with partial similarity to both the Δ2afcE and afcF::lux mutant lipid profiles. Taken together these results suggest that ShvR-regulated genes afcE and afcF play a key role in determining these cell-surface properties of B. cenocepacia.

Several studies have shown that Bcc species produce antifungal metabolites including pyrroltinrin, a phenylpyrrole (Costa et al., 2009; el-Banna & Winkelmann, 1998; Schmidt et al., 2009); 4-quinoilinones produced by B. cepacia (Moon et al., 1996); cepalexins, haemolytic antifungals produced by B. cepacia (Abe & Nakazawa, 1994); cepacidine, a glycopeptide produced by B. cepacia (Lim et al., 1994); and burkholdines, cyclic lipopeptides produced by Burkholderia ambifaria (Tawfik et al., 2010). The afc genomic region is different from the NRPS biosynthetic gene cluster for occidiofungin and the PKS gene cluster for enacyloxin biosynthesis reported in B. ambifaria AMMD (Mahenthiralingam et al., 2011; Thomson & Dennis, 2012). B. cenocepacia K56-2 lacks the occidiofungin genes based on PCR analyses and enacyloxin biosynthetic gene homologues are absent in the genome sequence of B. cenocepacia J2315 (Mahenthiralingam et al., 2011; Thomson & Dennis, 2012).

Although afcAB and afcCD have been previously shown to be required for AFC-BC11 biosynthesis (Kang et al., 1998), the size of the complete afc biosynthetic cluster was not clear prior to this study. Most genes in the afcA operon are annotated as encoding proteins involved in lipid metabolism or cell envelope biogenesis (Holden et al., 2009; Winsor et al., 2008). Antifungal metabolite biosynthesis is usually carried out by modular polyketide synthetase (PKS) enzymes by addition of two-carbon ketide (-CH2-CO-) units composed of thioesters of acetate or short carboxylic acids or by non-ribosomal peptide synthetase (NRPS) enzymes by sequential addition of amino acid monomers (Ansari et al., 2004; Grünewald & Marahiel, 2006; Weissman & Leadlay, 2005). An NRPS module consists of an adenylation (A) domain, condensation (C) domain and thiolation or peptidyl carrier protein (PCP) domain whereas a PKS module consists of an acyltransferase (AT) domain, an acyl carrier protein (ACP) domain and ketoacylsynthase (KS) domain as obligatory or core domains (Ansari et al., 2004). Sequence analyses of proteins encoded by the afc region using Conserved Domain Database (CDD) (Marchler-Bauer et al., 2005) and by NRPS-PKS, a web based software for predicting NRPS–PKS megasynthases (Ansari et al., 2004) (http://www.nii.res.in/nrps-pks.html) indicated that the afcA operon codes for proteins with an A domain, ACP domain and KS domain, suggesting that this region probably codes for enzymes of a hybrid NRPS–PKS system.

The afcE- and afcF-encoded acyl-CoA dehydrogenase and FAD-dependent oxidoreductase, respectively, are probably not involved in formation of the core domain of this NRPS–PKS megasynthase complex, but may act as modifying enzymes. A gene encoding acyl-CoA dehydrogenase functions to introduce an unusual Δ5s double bond in the acyl residue of the lipopeptide antibiotic fruiliuminic in Actinoplanes fruiliensis (Heinzelmann et al., 2005). The biosynthetic gene cluster of fruiliuminic consists of NRPs, amino acid and lipid metabolism and transport genes (Müller et al., 2007). Several studies suggest that the BCAA catabolic pathway can provide acyl-CoA substrates, which are used by PKS to build the carbon backbone of macrolide antibiotics (Omura et al., 1983; Tang et al., 1994). Since the Δ2afcE and afcF::lux mutants were defective in utilizing BCAAs as a carbon source, it is likely that the role of afcE and afcF in the BCAA and/or lipid metabolic pathways is important for AFC lipopeptide biosynthesis.

In addition to their role in AFC biosynthesis, the acyl-CoA dehydrogenase and FAD-dependent oxidoreductase might be involved in a metabolic pathway leading to membrane lipid or biosurfactant biosynthesis which is important for swarming motility. Biosurfactants are wetting agents and include lipid derivatives. Well-characterized biosurfactants include rhamnolipid in Pseudomonas and serrawettin in Serratia which are glycolipids and lipopeptides respectively (Abdel-Mawgoud et al., 2010; Eberl et al., 1999). Biosurfactant production by B. cenocepacia has not been described. It is possible that products encoded by the afc region act as biosurfactants.

The alterations in cell envelope morphology in the afcE and afcF mutants might be caused by defects in biosynthesis of the membrane-associated AFC lipopeptide or other membrane lipids. The TEM images showed abundant extracellular matrix in the form of a fibrous material between K56-2 cells while the afcE and afcF mutant strains did not contain this matrix material, similar to the phenotype that has been previously reported for a shvR mutant, suggesting changes in extracellular matrix and its association with the cell envelope (data not shown) (Bernier et al., 2008). Estimation of extracellular polysaccharide (EPS) amounts from colonies grown on YEM or King A agar, however, did not reveal any differences
between strains Δ2afcE, afcF::lux and K56-2 (data not shown).

Surface-associated phenotypes influenced by afcE and afcF might be important during infection since infections with the mutant BCA2028::Tn resulted in lungs with reduced histopathology and inflammatory changes compared with K56-2 infections in the rat agar-bead model (Subramoni et al., 2011). Changes in membrane fatty acid composition, especially the extent of fatty acid saturation, have been detected as one of the adaptive changes to severely oxygen limited conditions that occur during long-term colonization of a CF patient (Coutinho et al., 2011).

Comparison of total lipid profiles of strains Δ2afcE, afcF::lux and K56-2ΔshvR showed distinct differences in the quantities of some lipids, which were restored to wild-type levels by complementation. B. cepacia surface lipids have been described as highly polar and are composed of two forms of phosphatidylethanolamines (PEs) and ornithine aminolipids (OLs) that differ in the presence or absence of 2-hydroxy fatty acids, phosphatidylglycerol (PG) and/or bis(phosphatidyl) glycerol (DPG) and an uncharacterized glycolipid (Cox & Wilkinson, 1989; Taylor et al., 1998; Yabuuchi et al., 1992). The four lipids observed in the chromatogram developed with ninhydrin represent the polar lipids PE1, PE2, OL1 and OL2 previously described (Fig. 5) (Cox & Wilkinson, 1989). The ninyhydrin-positive lipids with altered band intensities in strains Δ2afcE, afcF::lux and K56-2ΔshvR most probably correspond to OL and PE. We do not know the nature of the other ninyhydrin-positive and ninyhydrin-negative lipids of lower mobility detected on the TLC plates. Lipids were extracted from strains grown on PDA plates at 28 °C since these conditions were reported to favour biosynthesis of AFC lipopeptide. Therefore, the differences observed might reflect disruption of biosynthesis of AFC lipopeptide in the Δ2afcE, afcF::lux and K56-2ΔshvR mutants.

Spontaneous shv morphotype mutants with loss of antifungal activity had altered lipid profiles. S86 and S92 are spontaneous shv morphotype mutants which were avirulent on alfalfa seedlings; S15 was not affected in virulence in alfalfa although it was less virulent in the rat lung infection model (Bernier et al., 2008). K56-H15 is a transposon mutant in which the shv morphotype is due to an unidentified secondary mutation and this mutant also shows significant reduction in virulence on alfalfa (Bernier et al., 2003). Our results suggest that mutants deficient for antifungal activity are also avirulent in the alfalfa seeding infection model. In a study on clinical and environmental isolates of B. cepacia, all isolates recovered from onion rhizosphere or onion field soil had moderate levels of antifungal activity against R. solani (Springman et al., 2009). Many of the clinical isolates which exhibited nematode pathogenicity also had moderate antifungal activity, while isolates with low nematode pathogenicity had poor antifungal activity (Springman et al., 2009). Similarly, mutations affecting afcE and afcF resulted in reduced lung histopathological changes in a murine infection model (Subramoni et al., 2011). It is possible that AFC antifungal activity may be a factor that promotes B. cepacia survival in the soil environment and multi-host pathogenicity. Future studies of afcE and afcF in different hosts will be useful in understanding this aspect of B. cepacia pathogenicity.

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