The suhB gene of Burkholderia cenocepacia is required for protein secretion, biofilm formation, motility and polymyxin B resistance

Roberto Rosales-Reyes,1,2 María Soledad Saldías,1 Daniel F. Aubert,1 Omar M. El-Halfawy1,3 and Miguel A. Valvano1

Correspondence Miguel A. Valvano mvalvano@uwo.ca

1Centre for Human Immunology, Department of Microbiology and Immunology, University of Western Ontario, London, ON N6A 5C1, Canada
2Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F., C.P. 02200 México
3Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

INTRODUCTION

Burkholderia cenocepacia is a member of the Burkholderia cepacia complex (Bcc), a group of closely related Gram-negative opportunistic pathogens that cause severe lung infections in patients with cystic fibrosis and display extreme intrinsic resistance to antibiotics, including antimicrobial peptides. B. cenocepacia BCAL2157 encodes a protein homologous to SuhB, an inositol-1-monophosphatase from Escherichia coli, which was suggested to participate in post-transcriptional control of gene expression. In this work we show that a deletion of the suhB-like gene in B. cenocepacia (ΔsuhBΔc) was associated with pleiotropic phenotypes. The ΔsuhBΔc mutant had a growth defect manifested by an almost twofold increase in the generation time relative to the parental strain. The mutant also had a general defect in protein secretion, motility and biofilm formation. Further analysis of the type II and type VI secretion systems (T2SS and T6SS) activities revealed that these secretion systems were inactive in the ΔsuhBΔc mutant. In addition, the mutant exhibited increased susceptibility to polymyxin B but not to aminoglycosides such as gentamicin and kanamycin. Together, our results demonstrate that suhBΔc deletion compromises general protein secretion, including the activity of the T2SS and the T6SS, and affects polymyxin B resistance, motility and biofilm formation. The pleiotropic effects observed upon suhBΔc deletion demonstrate that suhBΔc plays a critical role in the physiology of B. cenocepacia.

Abbreviations: BcCV, Burkholderia cenocepacia-containing vacuole; CF, cystic fibrosis; IMPase, inositol monophosphatase; T2SS, type II secretion system; T6SS, type VI secretion system; TMR, tetramethylrhodamine.

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1967) prior to accepting the phosphatidic acid moiety from CDP-diglyceride for the generation of PtdIns (Paulus & Kennedy, 1960). In mammals and plants, inositol phosphates and inositol phospholipids are involved in signal transduction (Munnik & Nielsen, 2011; Shewan et al., 2011). PtdIns play a critical structural role in the cell wall architecture of mycobacteria, acting as the lipid anchor for lipooligosamomannan (Movahedzadeh et al., 2010), and in some species lipooligosamomannan is also capped with phosphoinositol groups (Parish et al., 1997). In Archaea, phosphoinositol is used to make membrane phospholipids and in some species phosphoinositol derivatives serve as protective, compatible solutes (Michell, 2011). In contrast to mycobacteria and Archaea, Escherichia coli, Schizosaccharomyces pombe and human membranes may have only small amounts of PtdIns and their role in bacterial function is unclear (Kozloff et al., 1991; Michell, 2011). suhB_Ec mutants were initially isolated as suppressors of secY, rpoH and dnaB mutations, suggesting that SuhB_Ec has an effect on multiple cell processes, including protein export, the heat shock stress response, and DNA replication (Chang et al., 1991; Inada & Nakamura, 1995; Ito et al., 1983; Shiba et al., 1986). In addition, suhB_Ec mutants display a cold-sensitive phenotype and a defect in the rate of protein synthesis (Shiba et al., 1984a, 1984b). The mechanism of action of SuhB_Ec remains unclear. In vitro, purified SuhB_Ec has IMPase activity (Matsuhisa et al., 1995). However, a SuhB_Ec mutant protein D87N that lacks IMPase activity remains fully functional in complementing a defective suhB mutant (Chen & Roberts, 2000). Therefore, SuhB_Ec may participate in post-transcriptional control of gene expression by physically interacting with and modulating RNA III activity (Chen & Roberts, 2000; Wang et al., 2007).

In this work, we describe that a B. cenocepacia mutant with a deletion of BCAL2157 (ΔsuhB_Ec) had compromised protein secretion, decreased biofilm formation and motility, and reduced resistance to polymyxin B and SDS. Surprisingly, the mutant was not impaired in its abilities to delay the phagolysosomal fusion and survive within macrophages. As in E. coli, the lack of SuhB_Ec function causes pleiotropic phenotypes that together suggest a compromise of the cell envelope integrity, paving the way to target this protein for the design of novel antimicrobials.

### METHODS

#### Antibodies and reagents.
Dulbecco’s modified Eagle’s medium (DMEM), PBS and FBS were purchased from Wisent. Mouse monoclonal anti-FLAG (M2 clone) was from Sigma. Anti-56-2 rabbit IgG was from Rockland Immunochemicals and goat anti-rabbit conjugated to Alexa Fluor 488 and goat anti-mouse conjugated to Alexa Fluor 680 were from Invitrogen. All other chemicals were purchased from Sigma unless otherwise indicated.

#### Growth conditions.
Bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown in Luria–Bertani (LB) medium (Difco) at 30 or 37 °C. Trimethoprim was used at final concentrations of 50 µg ml⁻¹ for E. coli and 100 µg ml⁻¹ for B. cenocepacia. Tetracycline was used at final concentrations of 20 µg ml⁻¹ for E. coli and 100 µg ml⁻¹ for B. cenocepacia. Other antibiotics used were: 40 µg kanamycin ml⁻¹ to maintain the helper plasmid pRK2013, and 50 µg gentamicin ml⁻¹ to select against the E. coli donor and helper strains in triparental matings. Growth curves were determined with a Bioscreen C Automated Microbiology Growth Curve Analysis System (MTX Lab Systems). For these experiments, fresh LB medium was inoculated with aliquots of overnight cultures to give a starting OD₆₀₀ of 0.03. Doubling times were calculated on the basis of OD₆₀₀ values measured during exponential growth.

#### General molecular techniques.
Routine DNA manipulations were performed as described by Maniatis et al. (1982). Restriction enzymes; T4 DNA ligase and alkaline phosphatase were obtained from Roche Diagnostics. Conjugations were performed by triparental mating (Craig et al., 1989) with the pRK2013 helper plasmid (Figurski & Helinski, 1979). DNA amplifications by PCR were done with the PTC-0200 or PTC-221 DNA engine (MJ Research) with Taq or HotStar HiFidelity DNA polymerases (Qiagen), and DNA sequencing was performed at the DNA Sequencing Facility, York University (Toronto, Canada).

#### Mutagenesis of B. cenocepacia K56-2 and complementing plasmid.
Unmarked and non-polar deletions were performed as described previously (Flannagan et al., 2008). PCR amplifications of the 5’ and 3’ regions flanking BCAL2157 were performed with primers pairs 4907 (5’-GCTCTAGGCTACATGCTGCTGACGG-3’) and 4908 (5’-GGCCGATGATGATCACCAGTTTCG-3’; Clal site underlined), and 4909 (5’-TTTTATCGATGATCAAGCTGTT-GGGAAC-3’; Clal site underlined) and 4910 (5’-GCTTACTCCG-CCTCGCGGATGC-3’). The amplicons were digested with Clal and ligated together into pGPl-Scl digested with SmaI, giving rise to pSM66 (BCAL2157 mutagenesis plasmid; Table 1). The mutant MSS40 (K56-2ΔsuhB_Ec Table 1) was confirmed by PCR. The BCAL2157 gene was PCR-amplified using primers 5052 (5’-TTTTATCGATGATCAAGCTGTT-GGGAAC-3’; Ndel site underlined) and 5053 (5’-TTTTATCGATGATCGCTGCTGCTGACGG-3’; XbaI site underlined). The PCR product was digested with restriction enzymes NdeI/ XbaI and cloned into similarly digested plasmid pDA17, giving rise to pSM67 encoding SuhB_Ec fused with a FLAG epitope at the C terminus (Table 1).

#### Protease assays.
Protease assays were performed as described by Sokol et al. (1979). The protease activity was recorded by measuring the radius of the zones of clearing surrounding the bacterial spot.

#### Motility assays and biofilm formation.
Swarming motility was analysed on nutrient agar plates (0.5 %, v/v, agar supplemented with 0.2 %, v/v, glucose), inoculated with 2 µl of culture at OD₆₀₀ 1 and incubated at 37 °C. The diameter of the motility zone was measured after 24 h. Biofilm formation was quantified as previously described (Aubert et al., 2008). Briefly, 5 ml overnight cultures grown at 37 °C were diluted to OD₆₀₀ 0.003 in LB, and triplicate 500 µl aliquots were dispensed into polystyrene tubes. Following 24 h of static incubation at 30 or 37 °C, the medium was removed and the tubes were washed gently once with distilled water. Adherent bacteria were stained with 0.1 % (w/v) crystal violet and washed three times with distilled water. The bound crystal violet was dissolved in 1 ml of 100 % methanol and quantified by measuring A₅₄₀.

#### Electrophoretic analysis of B. cenocepacia lipopolysaccharide (LPS).
LPS samples were extracted, processed and visualized by silver staining as previously described (Marolda et al., 2006).
Table 1. Bacterial strains and plasmids used in this study

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*B. cepacia Research and Referral Repository for Canadian CF Clinics.

**Antibiotic sensitivity assays.** Antibiotic sensitivity assays were performed as previously described (Loutet et al., 2006). Cultures were incubated at 37 °C for 24 h in the Bioscreen C system, and the final density was determined by determining the OD<sub>600</sub>. All dilutions were tested in triplicate. Disk diffusion assays were used to test the susceptibility of *B. cenocepacia* strains to SDS. Briefly, 100-fold diluted overnight cultures in LB were spread on LB agar plates, and dry disks were applied to the surfaces. Eight microlitres of a solution containing 10% SDS or distilled H<sub>2</sub>O was added to the disks in triplicate. The plates were incubated for 24 h at 37 °C, and zones of growth inhibition were measured.

**Macrophage infection, lysosomal labelling and immunostaining.** *B. cenocepacia* MH1K (Hamad et al., 2010) and MH1K-ΔsuhB<sub>hc</sub> were grown overnight in LB at 37 °C with shaking. One millilitre was washed twice with PBS and resuspended in DMEM-10% FBS, ANA-1 macrophages (Cox et al., 1989) were seeded onto 12-well tissue culture plates. Bacteria were added at a m.o.i. of 50, centrifuged for 1 min at 700 r.p.m., and incubated at 37 °C under 5% CO<sub>2</sub>. At the appropriate times, infected macrophages were washed three times with PBS to remove non-adherent bacteria. Extracellular bacteria were killed by incubation with DMEM-10% FBS-Gentamicin40 (40 µg gentamicin ml<sup>−1</sup>) for 30 min. Then medium was removed and macrophages were maintained in DMEM-10% FBS-Gentamicin20 (20 µg gentamicin ml<sup>−1</sup>) thereafter. To enumerate intracellular bacteria, infected macrophages were lysed with 1% Triton X-100 in PBS, and serial dilutions were made and plated on LB agar plates. To label lysosomes, macrophages were seeded onto coverslips in six-well tissue culture plates in the presence of 50 µg ml<sup>−1</sup> Dextran–TMR 10 kDa (tetramethylrhodamine conjugate; Invitrogen) for 24 h. One hour prior to infection, macrophages were washed three times with PBS and incubated in DMEM-10% FBS, and infected as described above. Four hours post-infection, the coverslips were washed three times with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X in PBS. Cells were first incubated with the blocking solution (3% BSA, 2% PBS in PBS) for 30 min and with anti-*B. cenocepacia* polyclonal antibody for 45 min at room temperature. After washing twice with PBS, cells were subsequently incubated with goat anti-rabbit conjugated to Alexa Fluor 488 for 30 min at room temperature in the dark. Coverslips were washed three times with PBS and mounted on glass slides using fluorescent mounting medium (Dako Cytomation). Images were acquired using an Axioscope 2 (Carl Zeiss) microscope with a ×100 oil immersion objective.

**Analysis of secreted proteins and western blotting.** The purification of *B. cenocepacia* secreted proteins was done as previously described (Aubert et al., 2008) with a few modifications. Briefly, overnight cultures were diluted to an OD<sub>600</sub> 0.03 in 50 ml pre-warmed LB. After 4–5 h incubation at 37 °C with shaking, the cultures were adjusted to the same OD<sub>600</sub> and centrifuged for 15 min at 10,000 r.p.m. at 4 °C. Culture supernatants were sterilized by filtration through a 0.22 µm pore-size filter (Millipore), and proteins were precipitated overnight at 4 °C with 10% (v/v) TCA (final concentration). The precipitates were isolated by centrifugation at 20,000 r.p.m. for 30 min at 4 °C, and the pellets were washed with ice-cold acetone. Another centrifugation was performed at 20,000 r.p.m. for 30 min. The pellets were air-dried and then resuspended in 0.1 M sodium phosphate buffer, pH 7.0. Protein concentration was determined by Bradford assay (Bio-Rad), and aliquots containing 10 µg protein were loaded on a 16% SDS-PAGE gel. Detection was performed with a Brilliant Blue G-Colloidal staining according to the manufacturer’s recommendations (Sigma). The fusion protein (SuhB<sub>hc</sub>–FLAG) was detected by Western blotting, using a monoclonal anti-FLAG antibody (Sigma) and the appropriate secondary antibody. Membranes were analysed by infrared imaging using an Odyssey imager (Licor).

**Statistics.** Statistical analysis was carried out using GraphPad Prism version 4.03 (GraphPad Software) Student’s t test, with a 95% confidence limit; a probability value of P<0.05 was considered significant.
RESULTS AND DISCUSSION

Deletion of suhB<sub>Bc</sub> causes a growth defect

The <i>B. cenocepacia</i> suhB<sub>Bc</sub> gene (BCAL2157) encodes a protein that shares 50% amino acid identity with SuhB<sub>Bc</sub>. A motif search against the Pfam database clearly identified SuhB<sub>Bc</sub> as a member of the IMPase family with an E-value of 5.7e-69. In addition, SuhBB<sub>C</sub> carries the conserved motif for Mg<sup>2+</sup> or Li<sup>2+</sup> binding typically found in human IMPase sequences (Fig. 1a, boxed sequence) (Matsuhisa et al., 1995). We investigated whether the suhB<sub>Bc</sub> deletion mutant (herein ΔsuhB<sub>Bc</sub>) has a growth defect. At 37 °C, ΔsuhB<sub>Bc</sub> and ΔsuhB<sub>Ec</sub>-pDA17 (carrying the vector control) showed reduced ability to replicate during the exponential growth phase in comparison with the wild-type strain, such that the generation time was 2.5 h for K56-2-pDA17 and 4.3 h for ΔsuhB<sub>Bc</sub> (Fig. 1b), but these differences disappeared at 24 h. The growth defect of ΔsuhB<sub>Bc</sub> was rescued by introducing pSM67, a plasmid expressing suhB<sub>Bc</sub> (Fig. 1b). At 30 °C (Fig. 1c), the generation times for K56-2-pDA17 and ΔsuhB<sub>Bc</sub> were 5 and 9.2 h, respectively. The normalized generation times of the mutant (t<sub>mutant</sub>/t<sub>wt</sub>) at 37 and 30 °C were 1.72 and 1.84, demonstrating that the growth defect is similar, irrespective of the temperature, in contrast to reports for some suhB<sub>Bc</sub> mutant alleles, which display a cold-sensitive growth impairment (Inada & Nakamura, 1995). We also noticed that the growth defect was more pronounced in strains grown at 37 °C under static conditions, where the generation time was 5.9 h for K56-2-pDA17 and 26 h for ΔsuhB<sub>Bc</sub>, respectively (T<sub>mutant</sub>/T<sub>wt</sub>=4.4; Fig. 1d). Experiments with the LIVE/DEAD staining kit demonstrated that the slow growth rate under static conditions was not due to cell death, since culture samples had similar proportions of live and dead cells at 24 h of growth (data not shown). These results suggest that reduced oxygen availability exacerbates the growth defect phenotype of ΔsuhB<sub>Bc</sub>.

The <i>B. cenocepacia</i> ΔsuhB<sub>Bc</sub> mutant has a defect in protein secretion, swarming motility and biofilm formation

Since the suhB<sub>Bc</sub> gene product is involved in general protein synthesis and export (Ito et al., 1983; Shiba et al., 1984a), we investigated whether the suhB<sub>Bc</sub> deletion could affect protein secretion in <i>B. cenocepacia</i>. Secreted proteins were purified from <i>B. cenocepacia</i> K56-2-pDA17, ΔsuhB<sub>Bc</sub>-pDA17 and ΔsuhB<sub>Bc</sub>-pSM67 culture supernatants as described in Methods, and the protein profiles were compared. The results show that <i>B. cenocepacia</i>-ΔsuhB<sub>Bc</sub>-pDA17 has an observable defect in protein secretion (Fig. 2a), which was restored to parental levels upon complementation with pSM67 (Fig. 2a). In addition, the SuhB<sub>Bc</sub>-FLAG fusion protein was detected only in the total cell extract and not in the secreted protein fraction (Fig. 2b), indicating that this protein is not secreted.

The role of suhB<sub>Bc</sub> in protein secretion was further confirmed by testing specifically the activities of two secretion

![Fig. 1. suhB<sub>Bc</sub> deletion causes a growth defect in <i>B. cenocepacia</i>. (a) Sequence alignment of SuhB from <i>B. cenocepacia</i> and <i>E. coli</i> (amino acids 31–120). The box indicates the IMPase active site; conserved amino acids are indicated with asterisks. (b) Growth curves over 24 h of <i>B. cenocepacia</i> K56-2-pDA17 (wild-type), ΔsuhB<sub>Bc</sub>, ΔsuhB<sub>Bc</sub>-pDA17 and ΔsuhB<sub>Bc</sub>-pSM67 at 37 °C with shaking. (c) Same as in (b) but at 30 °C. (d) Same as in (b) but at 37 °C under static conditions. The graphs shown are representative of three independent experiments.](https://www.microbiologyresearch.org/content/W158/Fig1.jpg)
systems from \textit{B. cenocepacia}. One of these systems is the type II secretion system (T2SS), which mediates the secretion of two zinc metalloproteases, ZmpA and ZmpB, that are responsible for the extracellular proteolytic activity in this bacterium (Kooi \textit{et al.}, 2005, 2006; Kooi & Sokol, 2009). We investigated the proteolytic activity of \textit{B. cenocepacia} under conditions where the activity of ZmpA and ZmpB is readily detected, which serves also to assess the functionality of the T2SS. In contrast to the parental K56-2-pDA17 strain or the complemented D\textsubscript{suhB\textsubscript{Bc}}-pSM67 strains, D\textsubscript{suhB\textsubscript{Bc}} or D\textsubscript{suhB\textsubscript{Bc}}-pDA17 did not show any casein proteolytic activity (Fig. 3a), even after 5 days of incubation at 37 °C. A similar phenotype was observed at 30 °C (data not shown). That the casein proteolytic activity is due to ZmpA and ZmpB production was confirmed by the absence of a halo in the spot inoculated with the D\textsubscript{zmpA\textsubscript{Bc}}\textsubscript{zmpB\textsubscript{Bc}} double mutant.

Another important secretion system is the type VI secretion system (T6SS), which is widely conserved in pathogenic and non-pathogenic Gram-negative bacteria (Cascales, 2008; Jani & Cotter, 2010; Pukatzki \textit{et al.}, 2009). Previous work in our laboratory demonstrated that the \textit{B. cenocepacia} T6SS is essential for virulence \textit{in vivo} (Hunt \textit{et al.}, 2004), and also required for the formation of actin-rich protrusions in infected macrophages (Aubert \textit{et al.}, 2010, 2008; Rosales-Reyes \textit{et al.}, 2012) through inactivation of Rac1 (Flannagan \textit{et al.}, 2012; Rosales-Reyes \textit{et al.}, 2012). To determine whether the \textit{suhB\textsubscript{Bc}} deletion affects T6SS function, we infected macrophages with \textit{B. cenocepacia}-K56-2, D\textsubscript{suhB\textsubscript{Bc}}, D\textsubscript{suhB\textsubscript{Bc}}-pDA17, D\textsubscript{suhB\textsubscript{Bc}}-pSM67 and the control strain D\textsubscript{zmpA\textsubscript{Bc}zmpB\textsubscript{Bc}} (Table 1) were tested for casein proteolytic activity on dialysed brain heart infusion-milk agar plates (Sokol \textit{et al.}, 1979). Zones of clearing around the colonies were measured after 48 h of incubation at 37 °C. Values are mean radius ± SD in millimetres of two experiments performed in triplicate.

(b) ANA-1 macrophages were infected at an m.o.i. of 50 with \textit{B. cenocepacia} K56-2, D\textsubscript{suhB\textsubscript{Bc}} and D\textsubscript{suhB\textsubscript{Bc}}-pSM67 for 6 h. Representative images are presented; macrophage protrusions are indicated by a white arrow. Uninfected macrophages were used as a negative control. Bars, 50 μm.

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\textbf{Fig. 2.} \textit{suhB\textsubscript{Bc}} deletion affects protein secretion. (a) Profile of secreted proteins from \textit{B. cenocepacia} K56-2-pDA17, D\textsubscript{suhB\textsubscript{Bc}}-pDA17 and D\textsubscript{suhB\textsubscript{Bc}}-pSM67 analysed by SDS-PAGE. (b) Detection of SuhB\textsubscript{Bc}–FLAG fusion protein (arrow) by Western blotting using total and secreted proteins of \textit{B. cenocepacia} K56-2-pDA17, D\textsubscript{suhB\textsubscript{Bc}}-pDA17 and D\textsubscript{suhB\textsubscript{Bc}}-pSM67.

\textbf{Fig. 3.} Functional assays for the T2SS and T6SS. (a) \textit{B. cenocepacia} K56-2-pDA17, ΔsuhB\textsubscript{Bc}, ΔsuhB\textsubscript{Bc}-pDA17, ΔsuhB\textsubscript{Bc}-pSM67 and the control strain ΔzmpAΔzmpB (Table 1) were tested for casein proteolytic activity on dialysed brain heart infusion-milk agar plates (Sokol \textit{et al.}, 1979). Zones of clearing around the colonies were measured after 48 h of incubation at 37 °C. Values are mean radius ± SD in millimetres of two experiments performed in triplicate. (b) ANA-1 macrophages were infected at an m.o.i. of 50 with \textit{B. cenocepacia} K56-2, ΔsuhB\textsubscript{Bc} and ΔsuhB\textsubscript{Bc}-pSM67 for 6 h. Representative images are presented; macrophage protrusions are indicated by a white arrow. Uninfected macrophages were used as a negative control. Bars, 50 μm.
normal function of the T2SS and T6SS. This suggests a general defect in the assembly of multiprotein complexes in the cell envelope. To provide further evidence for this notion, we examined whether the suhB gene deletion affects bacterial motility. These experiments revealed that ΔsuhBbc cells are unable to swarm, but the defect can be rescued by the introduction of the complementing plasmid pSM67 (Fig. 4). Also, the suhBbc deletion compromised the ability of the mutant to form biofilm at 37 °C (Fig. 5a), a defect that was even more pronounced at 30 °C (Fig. 5b). These results agree with a report showing that a suhB transposon mutant in the unrelated B. cenocepacia strain H111 is also defective in biofilm formation (Huber et al., 2002).

The ΔsuhBbc mutant shows increased sensitivity to polymyxin B and SDS

We reasoned that the pleiotropic effects observed in ΔsuhBbc could also be explained by compromised membrane biogenesis, which could be altered in the absence of suhBbc function, and account for the observed defects in the functionality of the T2SS, T6SS, motility and biofilm formation, as the composition of membrane phospholipids is important in the formation of multiprotein complexes. Therefore, we also investigated whether the mutant has a defect in membrane permeability by assessing resistance to the cationic antimicrobial peptide polymyxin B (Loutet et al., 2006, 2009). Fig. 6a shows that B. cenocepacia K56-2-pDA17 is highly resistant to polymyxin B (MIC50 > 1024 μg ml⁻¹), while ΔsuhBbc or ΔsuhBbc-pDA17 (containing the vector control) showed reduced resistance to polymyxin B (MIC50 ≤ 512 μg ml⁻¹). Resistance to polymyxin B was restored to parental levels upon complementation with pSM67 (MIC50 > 1024 μg ml⁻¹). Furthermore, ΔsuhBbc showed increased sensitivity to the anionic detergent SDS (Fig. 6b), which was rescued by the introduction of pSM67 (Fig. 6b). Together, increased susceptibility to polymyxin B and SDS suggests that ΔsuhBbc has an altered outer membrane. This alteration is not due to gross defects in the LPS of the mutant compared with the parental strain (data not shown) and its mechanism is not clear. B. cenocepacia also exhibits high resistance to aminoglycosides (Chen & Roberts, 2000; Hamad et al., 2010; Waters & Ratjen, 2006), which depends for the most part on efflux pumps (Buroni et al., 2009; Hamad et al., 2010). ΔsuhBbc does not show any significant differences in resistance to kanamycin or gentamicin in comparison with the parental strain (Fig. 6c, d). These results, together with the observed defects in the T2SS and T6SS functionality (Fig. 3a, b), further suggest that the outer-membrane organization or stability in ΔsuhBbc is compromised.

**Fig. 4.** Deletion of suhBbc affects swarming. B. cenocepacia K56-2-pDA17, ΔsuhBbc, ΔsuhBbc-pDA17 and ΔsuhBbc-pSM67 were assayed on swarm-agar plates. Bacterial swarm zones were measured after 24 h of incubation at 37 °C. Values are means of swarm zones ± SD in centimetres of two experiments performed in triplicate.

**Fig. 5.** Deletion of suhBbc in B. cenocepacia affects biofilm formation. B. cenocepacia K56-2-pDA17, ΔsuhBbc-pDA17 and ΔsuhBbc-pSM67 were tested by their ability to form biofilms at 37 °C (a) or 30 °C (b) for 24 h. A representative image of biofilm formation is presented at the top of (a). The graphs show the quantification of crystal violet associated to biofilms (A540). The graphs show data from one representative experiment of three independent repeats in triplicate; error bars, SD.
suhB<sub>Bc</sub> does not affect the ability of <i>B. cenocepacia</i> to infect and survive in macrophages

The ability of <i>B. cenocepacia</i> to infect and persist in macrophages is associated with delayed phagolysosomal fusion (Huynh <i>et al.</i>, 2010; Keith <i>et al.</i>, 2009; Lamothe <i>et al.</i>, 2007; Rosales-Reyes <i>et al.</i>, 2012). To identify whether suhB<sub>Bc</sub> plays a role in the ability of <i>B. cenocepacia</i> to survive intracellularly we deleted the suhB<sub>Bc</sub> gene in the MH1K strain, an isogenic derivative of strain K56-2 that exhibits high susceptibility to gentamicin (Hamad <i>et al.</i>, 2010). A gentamicin protection assay was performed on infected macrophages with MH1K and MH1K<sup>Δ</sup>suhB<sub>Bc</sub>. Infected cells were stained with an anti-<i>B. cenocepacia</i> and anti-LAMP-1 (marker for late endosomal and lysosomal compartments) to identify whether the suhB<sub>Bc</sub> deletion causes a defect in trafficking resulting in loss of the phagolysosomal fusion arrest and therefore a higher number of BcCVs acquiring LAMP-1. The results show that ΔsuhB<sub>Bc</sub> does not mediate an increased LAMP-1 association with BcCVs (Fig. 7a). To confirm this result, we infected macrophages preloaded with TMR–Dextran to quantify the lysosomal fusion at BcCV. The results show that the suhB<sub>Bc</sub> deletion does not affect the ability of <i>B. cenocepacia</i> to delay the lysosomal fusion at BcCV (Fig. 7b). Finally, we performed a conventional gentamicin protection assay to quantify the intracellular infection. The results show that both MH1K and MH1KΔsuhB<sub>Bc</sub> infect and survive in macrophages (Fig. 7c), indicating that the suhB<sub>Bc</sub> gene deletion does not alter the ability of intracellular <i>B. cenocepacia</i> to delay the phagolysosomal fusion and its survival in macrophages.

**Concluding remarks**

Our results show that the suhB<sub>Bc</sub> deletion causes dramatic effects on the function of protein secretion systems, a growth defect and also various defects in motility and biofilm formation. Given the level of amino acid identity in
the SuhB proteins from *E. coli* and *B. cenocepacia* it is possible that SuhB<sub>Bc</sub> works in conjunction with RNA polymerase III and affects protein expression of multiple genes and gene products, as described for the *E. coli* counterpart (Wang et al., 2007). However, the cold-sensitive phenotype described in *E. coli suhB* mutants is not evident in ΔsuhB<sub>Bc</sub>, suggesting that despite the similarities between the *E. coli* and *Burkholderia* proteins, their cellular functions may not be identical. *B. cenocepacia* possesses novel lipids and also cholesterol-like molecules in its cell envelope (González-Silva et al., 2011; Schmerk et al., 2011). It is therefore possible that SuhB<sub>Bc</sub> may be involved in the synthesis of minor PtdINs that play a key role in membrane organization. The results of macrophage infections indicate that the loss of SuhB<sub>Bc</sub> affects the T6SS-mediated actin rearrangements but does not impair intracellular survival and the arrested maturation of the BcCV, both of which are T6SS-independent traits. At present, we cannot rule out that the availability of myo-inositol in the host cell may provide intracellular *B. cenocepacia* with the product of the IMPase reaction and chemically rescue the loss of SuhB<sub>Bc</sub>. Further experiments are under way in our laboratory to investigate the specific mechanism of SuhB<sub>Bc</sub> function and determine whether inhibitory strategies may be an alternative to reduce at least in part the extreme antimicrobial resistance burden of *B. cenocepacia*.

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**Fig. 7.** Deletion of suhB<sub>Bc</sub> does not affect the ability of *B. cenocepacia* to infect and survive in macrophages. ANA-1 macrophages were infected with *B. cenocepacia* MH1K and MH1K-ΔsuhB<sub>Bc</sub> for 4 h. Infected macrophages were fixed and stained using anti-LAMP1 antibody (a). The arrow indicates bacteria in vacuoles associated with LAMP1. (b) Macrophages preloaded with Dextran–TMR were infected with *B. cenocepacia* MH1K and MH1K-ΔsuhB<sub>Bc</sub> for 4 h. The arrow indicates bacteria in vacuoles associated with Dextran–TMR. Quantitative analysis was performed by counting over 100 vacuoles per experiment. Values represent mean ± SEM. (a, b) Bars, 10 μm. (c) Macrophages were infected with *B. cenocepacia* MH1K and MH1K-ΔsuhB<sub>Bc</sub> at an m.o.i. of 80 for 30 min. Intracellular bacteria were recovered and quantified by plating serial dilutions on LB agar plates at 1, 6 and 24 h post-infection.
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