Burkholderia cenocepacia requires RpoE for growth under stress conditions and delay of phagolysosomal fusion in macrophages

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**INTRODUCTION**

The *Burkholderia cepacia* complex (Bcc) comprises a group of nine closely related bacterial species that are phenotypically very similar but genetically distinct (Coenye *et al.*, 2003; Mahenthiralingam *et al.*, 2000). These bacteria are opportunistic pathogens that cause infections in immunocompromised individuals and often infect cystic fibrosis (CF) patients (Mahenthiralingam & Vandamme, 2005). *Burkholderia cenocepacia*, a member of the Bcc, is one of the species most often recovered from CF patients worldwide and is also associated with the most severe infections (Brisse *et al.*, 2004; LiPuma, 2005; Manno *et al.*, 2004; Reik *et al.*, 2005; Speert *et al.*, 2002). Furthermore, *B. cenocepacia* infections in CF patients are associated with an accelerated decline in lung function as compared to other CF-related pathogens such as *Pseudomonas aeruginosa* (Courtney *et al.*, 2004; Jones *et al.*, 2004). The treatment of infection is difficult because this bacterium is multi-drug resistant (Zhou *et al.*, 2007) and can be transmitted from person to person (Govan *et al.*, 1993; LiPuma *et al.*, 1990; Smith *et al.*, 1993). Infections are further complicated by the ‘cepacia syndrome’, a potentially fatal necrotizing pneumonia that occurs in a subset of patients (Isles *et al.*, 1984; Thomassen *et al.*, 1985).

Although *B. cenocepacia* can be transmitted between patients, it is also acquired from environmental sources (Coenye & Vandamme, 2003) as these bacteria occupy many different niches, including the rhizosphere, plants and humans. Therefore, it is likely that *B. cenocepacia* can readily adapt to many different stresses. One way by which bacteria adapt to stress is through the activity of the alternative sigma factor RpoE, a key regulator of the extra-cytoplasmic stress response that has been extensively characterized in the enteric bacterium *Escherichia coli* (De

Abbreviations: Bcc, *Burkholderia cepacia* complex; BcCV, *B. cenocepacia*-containing vacuole; CF, cystic fibrosis; OM, outer membrane; Tp, trimethoprim.
Las Peñas et al., 1997b; Raina et al., 1995). RpoE is normally sequestered to the cytoplasmic face of the inner membrane by an anti-sigma factor, RseA, and a periplasmic protein, RseB (De Las Peñas et al., 1997a; Missiakas et al., 1997). RseA is degraded under certain stress conditions by the concerted activities of the proteases DegS, YaeL and ClpXP (Alba et al., 2002; Chaba et al., 2007; Flynn et al., 2004; Kanehara et al., 2002), resulting in the release of RpoE into the cytosol.

In E. coli, where rpoE is essential (De Las Peñas et al., 1997b), a great deal of work has been done to determine which genes belong to the RpoE regulon (Dartigalongue et al., 2001; Rezucha et al., 2003). Many of the genes identified are involved in membrane biogenesis or repair, protein folding or degradation, and they include rpoE itself along with its regulatory proteins (Dartigalongue et al., 2001). An example of an RpoE-regulated gene in E. coli is DegP, an HtrA-like protease that functions to degrade misfolded proteins in the periplasm (Dartigalongue et al., 2001; Lipinska et al., 2003). Previously, we characterized an HtrA protease from B. cenocepacia that is required for growth under certain stress conditions and for virulence (Flannagan et al., 2007) and this was the first evidence that the B. cenocepacia stress response is important for pathogenesis. Very little is known about which genes are required for the survival of B. cenocepacia under stress and the contribution of these genes to virulence. As a next step we wanted to characterize the master regulator of the extracytoplasmic stress response, RpoE, in B. cenocepacia. In this report we describe the creation and characterization of an rpoE mutant and show that this gene is required for the maturation delay of the B. cenocepacia-containing vacuole (BcCV) in a macrophage model of infection.

METHODS

Bacterial culture conditions and conjugations. Bacteria and plasmids used in this study are listed in Table 1. B. cenocepacia and E. coli were cultured at 30 and 37 °C in Luria–Bertani (LB) broth or LB agar. For growth under nutrient-limited conditions a modified M9 minimal medium was prepared from 5 × M9 salts (Difco) supplemented with 0.2 % (w/v; final concentrations) glycerol, 0.2 % (w/v) glucose, 0.2 % (w/v) Casamino acids, 2.0 μg vitamin B1 ml⁻¹ and 20 μg tryptophan ml⁻¹. For antibiotic selection in E. coli 50 μg trimethoprim (Tp) ml⁻¹, 20 μg tetracycline ml⁻¹ and 40 μg kanamycin ml⁻¹ were used, while 100 μg Tp ml⁻¹ and 100 μg tetracycline ml⁻¹ were used for selection in B. cenocepacia. Plasmids were transferred into B. cenocepacia by tri-parental mating at 30 °C using E. coli DH5α carrying the helper plasmid pRK2013 (Figurski & Helinski, 1979). Gentamicin (50 μg ml⁻¹) was used to counter-select against the E. coli donor and helper strains.

General molecular techniques. DNA manipulations were performed as described by Sambrook, et al. (1990). Restriction enzymes and T4 DNA ligase (Roche Diagnostics) and Antarctic phosophatase (New England Biolabs) were used as recommended by the suppliers. E. coli DH5α and E. coli SY327 cells were transformed by the calcium chloride protocol (Cohen et al., 1972). DNA was amplified by PCR using the PTC-0200 DNA engine (MJ Research) with Taq DNA polymerase or Proof Start DNA polymerase (Qiagen). PCR reactions to amplify B. cenocepacia DNA were supplemented with Qiagen Q solution. DNA sequencing was performed at the DNA sequencing Facility at York University, Toronto, Ontario, Canada. The genome sequence of B. cenocepacia strain J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) was analysed with the BLAST program.

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference†</th>
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<tbody>
<tr>
<td><strong>B. cenocepacia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J2315</td>
<td>CF clinical isolate</td>
<td>BCRRC</td>
</tr>
<tr>
<td>K56-2</td>
<td>ET12 clone related to J2315</td>
<td>BCRRC</td>
</tr>
<tr>
<td>RSF24</td>
<td>K56-2, mucD::pRFint-mucD, Tp^R</td>
<td>This study</td>
</tr>
<tr>
<td>RSF25</td>
<td>K56-2, rpoE::pRFint-rpoE, Tp^R</td>
<td>This study</td>
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<td><strong>E. coli</strong></td>
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<td></td>
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<tr>
<td>DH5α</td>
<td>F^−, φ80 lacZAM15 (∆lacZYA–argF)U169 endA1 recA1 hsdR17(r^g m^k+) supE44 thi-1 ΔggyA96 relA1</td>
<td>Laboratory stock</td>
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<tr>
<td>SY327</td>
<td>araD, Δ(lac pro) argE(Am) recA56 rif^R naldA Δ pir</td>
<td>Miller &amp; Mekalanos (1988)</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pDA17</td>
<td>oripB3R1, Tet^R, mob^+, P_f_2, FLAG epitope</td>
<td>D. Aubert</td>
</tr>
<tr>
<td>pRK2013</td>
<td>oripB3R1, RK2 derivative, Kan^R, mob^+, tra^+</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pMLBAD</td>
<td>oripB3R1, Tp^R, mob^+, araC-PBAD</td>
<td>Lefebre &amp; Valzano (2002)</td>
</tr>
<tr>
<td>pGPAP1p</td>
<td>pGP704, Tp^R, Ap^R, mob^+</td>
<td>This study</td>
</tr>
<tr>
<td>pRFint-rpoE</td>
<td>pGPAP1p, 219 bp internal fragment from rpoE</td>
<td>This study</td>
</tr>
<tr>
<td>pRFint-mucD</td>
<td>pGPAP1p, 291 bp internal fragment from mucD</td>
<td>This study</td>
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<tr>
<td>pRF132</td>
<td>pDA17, BCAL2829 FLAG</td>
<td>Flannagan et al. (2007)</td>
</tr>
<tr>
<td>pRF135</td>
<td>pDA17, rpoE</td>
<td>This study</td>
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*Tp, trimethoprim; Kan, kanamycin; Ap, ampicillin; Tet, tetracycline.
†BCRC, B. cepacia Complex Research and Referral Repository for Canadian CF Clinics; D. Aubert, University of Western Ontario.
Construction of the plasmid pGPAPtp and inactivation of the mucD and rpoE genes. The mutagenesis plasmid pGPAPtp was built to inactivate mucD and rpoE in B. cenocepacia (Fig. 1). pGPAPtp carries the R6K origin of replication and cannot replicate in the absence of the Pi protein (Filutowicz & Rakowski, 1998). The dfrB2 gene from pMLBAD was PCR amplified using primers 548 (5'-TCTACGCGGCCGTCGCTGAGCAGCC-3') and 549 (5'-AGGGATCCTAAGATATCGCTTAGGCCACACGTTCAAG-3'). The resulting 675 bp amplicon was subsequently cloned into the EcoRV site of pGP704, giving rise to pGPAPtp. The orientation of the dfrB2 gene was confirmed by PCR analysis using the R6K ori primer 1300 (5'-TAACGGTTGGAGAACAAGCCAGG-3') and primer 549. To inactivate mucD an internal fragment of the gene was PCR amplified using primers 2215 (5'-TTTTCTAGATGACGGTGCAGGAGCCACACGTTCAAG-3') and 2217 (5'-TTTTCTAGACTACGGTCGCTGAGCAGCC-3') with the same thermal cycling conditions: 95 °C for 3 min 30 s followed by 30 cycles of 95 °C for 40 s, 62 °C for 40 s and 72 °C for 1 min 10 s. The amplicon was digested with XbaI (sites underlined in primer sequences) and cloned into pGPAPtp treated with the same enzyme and Antarctic phosphatase. The orientation of the cloned mucD internal fragment was the same as that of the dfrB2 gene and was confirmed by PCR using primers 612 (5'-TCAAGGATCTACGGTCGCTGAGCAGCC-3') and 2217. The resultant plasmid was named pRFint-mucD. This mutagenesis plasmid was conjugated into B. cenocepacia K56-2 and Tp-resistant exconjugants were selected. Targeted integration of pRFint-mucD into the mucD gene was confirmed by PCR and Southern blot hybridization using a digoxigenin-labelled internal fragment from mucD. The resultant strain was named B. cenocepacia RSF24. To mutate the rpoE gene an internal fragment was PCR amplified using primers 2215 (5'-TTTTCTAGATGACGGTGCAGGAGCCACACGTTCAAG-3') and 2217 (5'-TTTTCTAGACTACGGTCGCTGAGCAGCC-3') and the thermal cycling conditions described above. The resultant amplicon was digested with EcoRI and XbaI (restriction sites are underlined in the primer sequences) and cloned into pDA17 that had been similarly digested. The resulting plasmid was named pRFintE. The rpoE gene was cloned in-frame with and fused to the FLAG epitope coded for in pDA17. Expression of RpoE was confirmed by Western blot analysis of whole-cell lysates as described previously (Flannagan et al., 2007).

Southern blot hybridization. Chromosomal DNA was isolated from RSF25 and digested with XhoI. Digested DNA was separated by electrophoresis in a 0.7 % (w/v) agarose gel and was transferred to positively charged nylon membrane by capillary transfer. The rpoE internal fragment was labelled with digoxigenin-11-UTP by PCR using primers 2215 and 2216, a digoxigenin labelling nucleotide mix (Roche) and Taq DNA polymerase. The membrane was hybridized with the probe under high-stringency conditions and hybridization was detected by chemiluminescence with disodium 3-(4-methoxyxypyrro[1,2-dioxetane-3,2'-5'-chloro[tricyclo[3.3.1.1(7)]decane]-4-yl]phenyl phosphate (CSPD) as recommended by the manufacturer (Roche). The same procedure was followed for RSF24 except that the chromosome was digested with NolI and the probe used was the internal fragment from mucD labelled with digoxigenin.

RpoE expression plasmid. For complementation experiments the entire rpoE gene was PCR amplified from B. cenocepacia K56-2 genomic DNA using primers 2417 (5'-TCTACGCGGCCGTCGCTGAGCAGCC-3') and 2418 (5'-TTTTCTAGACTACGGTCGCTGAGCAGCC-3'). The resultant PCR product was digested with EcoRI and XbaI (restriction sites are underlined in the primer sequences) and cloned into pDA17 that had been similarly digested. The resulting plasmid was named pRF135. The rpoE gene was cloned in-frame with and fused to the FLAG epitope coded for in pDA17. Expression of RpoE was confirmed by Western blot analysis of whole-cell lysates as described previously (Flannagan et al., 2007).

RT-PCR. RT-PCR was performed as described previously (Ortega et al., 2005) to investigate the transcriptional organization of the rpoE operon. Total RNA was isolated from B. cenocepacia K56-2 using the RNeasy Mini kit (Qiagen) following the manufacturers’ protocol. Isolated RNA was treated with DNase (Qiagen) for 30 min at 37 °C and for 15 min at 75 °C. To amplify the intergenic regions, RT reactions using RNA treated with reverse transcriptase and without reverse transcriptase (negative control) were performed at 55 °C for 30 min followed by 5 min at 85 °C. These were used as templates for PCR using DNA as a positive control. The primers used for each gene are listed in Table 2.

Osmotic sensitivity analysis. To expose B. cenocepacia to osmotic stress, bacteria were cultured at 30 °C in LB [1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 86 mM NaCl] or a modified LB medium (LB with 426 mM NaCl) with an increased NaCl concentration. Cultures were started at OD600 0.005 (~1.3 × 106 bacteria) and growth was determined in a 100-well disposable plate using a Bioscreen C automated microbiology growth curve analysis system (MTX Lab Systems). Growth was analysed using the low continuous shaking setting.

Table 2. RT-PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
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<tr>
<td>2757 (rseA RT F)</td>
<td>5'-GCAATTCGGCCGACGCACGCCG-3'</td>
</tr>
<tr>
<td>2758 (rseB RT R)</td>
<td>5'-CAGAAGCTTTCGTTGACTGCTGCTG-3'</td>
</tr>
<tr>
<td>2759 (rseB RT F)</td>
<td>5'-GTTGAGCACTTTCGTTGACTGCTGCTG-3'</td>
</tr>
<tr>
<td>2760 (mucD RT R)</td>
<td>5'-GACATGCGGCCGACGCCGACGCCG-3'</td>
</tr>
<tr>
<td>2761 (mucD RT F)</td>
<td>5'-CGATGCGGCCGACGCCGACGCCG-3'</td>
</tr>
<tr>
<td>2762 (orf5 RT R)</td>
<td>5'-CACACATTTGCGGCCGACGCCGACGCCG-3'</td>
</tr>
<tr>
<td>2776 (rpoE RT F)</td>
<td>5'-CGATGCGGCCGACGCCGACGCCGACGCCG-3'</td>
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**Temperature sensitivity.** Temperature sensitivity was assessed by drop plating serially diluted (10^2–10^-5) cell suspensions onto LB agar or modified M9 minimal agar. Bacteria from overnight cultures were used to set up cell suspensions at a starting OD_{600} 0.1 (~2.6 x 10^7 bacteria). The plates were allowed to dry and then incubated at 30 or 44 °C for 24 h.

**Calcofluor binding.** Bacteria were cultured overnight in LB at 30 °C with shaking. Overnight cultures were used to inoculate fresh LB and the cells were cultured again at 30 °C with shaking until they entered exponential phase (OD_{600} 0.15–0.25). From these cultures cell suspensions were set up at OD_{600} 0.1 (~2.6 x 10^7 bacteria) and 10 µl volumes of the suspensions were drop plated onto LB agar with 0.02 % (w/v) calcofluor white. Plates were incubated for 24 h at 30 °C then photographed under UV and white light using a ChemiDoc XRS system (Bio-Rad) with Quantity-One 1D analysis software (Bio-Rad). Light intensities of zones of growth were quantified using Odyssey 2.1 system (Bio-Rad) with Odyssey Infrared Imager (Li-Cor Biosciences). Statistical analyses were performed using GraphPad Prism 4.0 software.

**Isolation of outer membrane (OM) proteins.** OM proteins were isolated from *B. cenocepacia* following a modified version of the Sarkosyl insolubility protocol of Carlone et al. (1986). Bacteria were cultured overnight at 30 °C in LB and the cells were pelleted. The bacterial pellet was resuspended in 5 ml 10 mM Tris/HCl (pH 8.0) and lysed by sonication until clear (approximately 40 s pulses with a 40 % amplitude). The lysate was pelleted in Eppendorf tubes and then centrifuged at 31 191 g for 30 min at 4 °C. The supernatant was removed and the pellet suspended in 500 µl buffer [1.5 % (w/v) Sarkosyl, 20 mM Tris/HCl pH 8.0] and incubated at room temperature for 20 min. The samples were centrifuged as described above, the supernatant was removed and the pellet containing OM proteins was resuspended in sterile water. Proteins (40 µg ml^-1) were boiled and separated in a 14 % SDS-polyacrylamide gel and visualized following Coomassie staining with 0.1 % (w/v) Coomassie blue, 10 % (v/v) acetic acid and 50 % (v/v) methanol.

**Macrophage infections.** Macrophage infections were performed as described by Lamotte et al. (2007). The murine macrophage-like cell line RAW 264.7 (TIB-71) was obtained from the American Type Culture Collection (Manassas, VA). Macrophage cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). Cell culture reagents were purchased from Wisent unless otherwise indicated. For macrophage infections, cells were trypsinized and used to seed six-well plates containing glass coverslips and DMEM with 10 % (v/v) FBS. Plates were then incubated overnight at 37 °C in the presence of 5 % CO₂. Bacterial strains for infections were cultured overnight and then washed twice with HEPES-buffered RPMI 1640 and resuspended in DMEM with 10 % (v/v) FBS. For control experiments bacteria were heat inactivated at 60 °C for 25 min prior to infection. Bacteria were added to macrophages cultured on glass coverslips at a m.o.i. of 40 : 1, centrifuged for 1 min at 300 g and incubated at 37 °C in the presence of 5 % CO₂. To label lysosomes for colocalization experiments, tetramethylrhodamine (TMR)-dextran (250 µg ml⁻¹; Molecular Probes) was added to wells containing macrophages prior to infection, incubated at 37 °C with 5 % CO₂ for 2 h and chased for 1 h in DMEM with 10 % (v/v) FBS. Bacteria were used to infect dextran-labelled macrophages as described above and were incubated for 4 h at 37 °C with 5 % CO₂. At 4 h post-infection the external bacteria were removed by three washes with RPMI 1640 pre-warmed to 37 °C. Infected macrophages were visualized by phase-contrast and fluorescence microscopy using a Quantitative Retiga1300 cooled mono 12-bit camera on an Axioscope 2 microscope (Carl Zeiss) with a 100 x objective. Images were acquired and processed using Northern Eclipse version 6.0 software (Empix Imaging).

## Results

**Identification and mutagenesis of rpoE and mucD in *B. cenocepacia* K56-2**

We previously characterized an HtrA-like serine protease that is required for growth under stress and for virulence in the rat agar bead model of pulmonary infection (Flannagan et al., 2007). In *E. coli* and other enteric bacteria HtrA is, in part, regulated by RpoE and both proteins are required for adaptation to stress (Dartigalongue et al., 2001). Searching for other *htra* genes in the sequenced genome of *B. cenocepacia* J2315 we identified BCAL1001 and BCAL2869, which have identical DNA sequences encoding MucD, an HtrA-like serine protease. The *mucD* gene is the fourth gene in an operon encoding proteins homologous to RpoE, RseA, RseB and a protein of unknown function (Fig. 2a). The genetic organization of the *rpoE* operon in *B. cenocepacia* differs from that in both *E. coli* and *P. aeruginosa*. In these bacteria *rseC* is located downstream of *rseB* (Boucher et al., 1996; Missiakas et al., 1997); however, *rseC* is not present in the *B. cenocepacia* *rpoE* operon. The function of *rseC* is not clear, as there are conflicting reports describing its function in the literature (De Las Peñas et al., 1997a; Missiakas et al., 1997). Furthermore, both *P. aeruginosa* and *B. cenocepacia* carry a *mucD* gene in the *rpoE* operon but *E. coli* does not (De Las Peñas et al., 1997a; Missiakas et al., 1997). *B. cenocepacia* K56-2, which was used in this study, is clonally related to J2315 but is easier to manipulate genetically (Mahenthiralingam et al., 2000). In *B. cenocepacia* J2315 the entire *rpoE* operon and flanking DNA sequences are duplicated. PCR analysis of *B. cenocepacia* K56-2 genomic DNA confirmed that the genes encoding RpoE, RseA, RseB and MucD were present and shared the same genetic organization as in J2315. RT-PCR analysis confirmed that in K56-2 these genes are co-transcribed and constitute an operon (Fig. 2b).

*A. cenocepacia* K56-2 strain with an inactivated *rpoE* gene was created and named RSF25 (Fig. 2a). The *rpoE* gene was mutated in a non-polar manner by targeted integration of the suicide plasmid pGPApTp. This mutagenesis plasmid was designed to prevent polar effects on downstream genes since the orientation of the *dfbR2* gene and its promoter permits read-through transcription. Targeted integration was confirmed by PCR analysis and Southern blot hybridization (data not shown and Fig. 2c). In the genomic DNA from the parental strain K56-2 a band of ~3 kb was detected, while a higher molecular mass band of ~7.6 kb was obtained with the genomic DNA of RSF25, which was expected after integration of the suicide plasmid. Similarly, the *mucD* gene was inactivated by integration of a suicide plasmid as described in Methods. The *mucD* mutation in this strain, named RSF24, was confirmed by Southern blot hybridization, which also revealed a single higher molecular mass band.
in the mutant due to the integration of the mutagenesis plasmid (data not shown). Together these data show that rpoE and mucD were mutated by the integration of each respective mutagenesis plasmid. In B. cenocepacia J2315, the predicted rpoE operon is duplicated. However, the Southern blot banding patterns in the genomic DNA from K56-2 confirmed that in contrast to J2315, single copies of the rpoE and mucD genes exist in K56-2. These data are consistent with the results of Menard et al. (2007), who reported that B. cenocepacia K56-2 carries only one copy of the rpoE operon, which spans the genes BCAL0998 to BCAL1002.

**rpoE is required for growth at elevated temperature**

To characterize the role of the predicted rpoE gene in growth of B. cenocepacia under stress we tested a variety of conditions known to adversely affect other bacteria that have a mutated rpoE. No differences in efficiency of plating between the parental strain K56-2 and the rpoE mutant RSF25 were found in rich and minimal media at 30 and 37 °C (Figs 3a and 4, inset, and data not shown). Also, no differences were detected in the ability of RSF25 and K56-2 to form biofilm or to grow in the presence of 500 µg polymyxin B ml⁻¹, oxidative stress (100 µM H₂O₂, 100 µM paraquat and xanthine/xanthine oxidase), 0.005 % (w/v) SDS, cold stress (4, 15 and 21 °C), and acid conditions (pH 7.6 vs 5.5 and 4.5) (data not shown). In contrast, when RSF25 was cultured on LB plates at 44 °C the efficiency of plating of the mutant was reduced by ~1000-fold as compared to K56-2 (Fig. 3b). In this case, both RSF25 and K56-2 strains carried the control plasmid pDA17. RSF25 carrying a pDA17 derivative expressing rpoE (pRF135) displayed increased temperature sensitivity as compared to the same strain carrying a vector control. Consistent with the notion that increased expression of rpoE exacerbates the temperature-sensitive phenotype, B. cenocepacia K56-2 carrying plasmid pRF135 also became temperature-sensitive at 44 °C (Fig. 3b). The rpoE mutant and parental strains carrying pRF135 also showed reduced growth at 37 °C as compared to the same strains carrying the vector control (pDA17), indicating that the bacteria are stressed even under these conditions when rpoE is expressed from a plasmid (data not shown). To further characterize the temperature-sensitive phenotype K56-2(pDA17), RSF25(pDA17), RSF25(pRF135) and K56-2(pRF135) were plated on minimal media and incubated at 30 and 44 °C. Under these conditions these strains grew equally well at both temperatures, indicating that the temperature-sensitive phenotype is only apparent when bacterial cells are presumably rapidly dividing in rich growth medium (data not shown). Mutant RSF24 did not demonstrate any growth defects, indicating that mucD is not required for growth under the stress conditions tested (data not shown).
Inactivation of rpoE compromises growth of B. cenocepacia under conditions of osmotic stress

Previous work in our laboratory has shown that predicted stress response genes contribute to the growth of B. cenocepacia under conditions of osmotic stress (Flannagan et al., 2007). Similarly, growth of the rpoE mutant, RSF25(pDA17), was inhibited upon culture in media containing excess salt (426 mM NaCl) as compared to K56-2(pDA17) (Fig. 4). Expression of rpoE from pRF135 restored the wild-type growth phenotype to RSF25 under the same conditions, indicating that the observed defect was due to inactivation of rpoE alone. Growth of RSF25 was also reduced in the presence of 750 mM sucrose (data not shown). In some instances the addition of an osmoprotectant to the culture medium can rescue the growth of bacteria under conditions of osmotic stress (Le Rudulier & Bouillard, 1983). The addition of betaine (1–200 mM) to cultures of either RSF25(pDA17) or K56-2(pDA17) under osmotic stress had no effect on growth (data not shown). Given that the protein HtrABCAL2829 is also required for growth under conditions of osmotic stress (Flannagan et al., 2007), we tried to complement the RSF25 growth defect by providing HtrABCAL2829 in trans. Expression of HtrABCAL2829 from plasmid pRF132 did not affect the growth of RSF25, indicating that the reduced growth of RSF25 is due to factors other than HtrABCAL2829 (data not shown). In contrast to the mucD mutant, RSF24 grew similarly to the parental strain K56-2.

The B. cenocepacia rpoE mutant has an altered cell envelope

RpoE in E. coli regulates many genes involved in the maintenance and biogenesis of the outer membrane (Dartigalongue et al., 2001). The rpoE mutant RSF25 was observed to ‘sink’ in liquid culture, which is reminiscent of strains lacking O-antigen expression. When LPS isolated from RSF25 and K56-2 was analysed by SDS-PAGE with silver staining the O-antigen banding patterns were

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**Fig. 4.** Growth of B. cenocepacia RSF25 under osmotic stress. B. cenocepacia K56-2(pDA17) (■), RSF25(pDA17) (▲) and RSF25(pRF135) (▼) were cultured at 30 °C in LB medium containing 426 mM NaCl. In the inset, growth of K56-2 and RSF25 in LB with 86 mM NaCl at 30 °C is illustrated. The experiment was performed three times, with each strain analysed in quadruplicate. Error bars indicate the SD.
identical (data not shown). Given that RpoE regulates outer membrane assembly and stability in other bacteria, we hypothesized that some aspect of the outer membrane must be altered in *B. cenocepacia* upon inactivation of *rpoE*. To test this, the sensitivity of RSF25 and K56-2 to SDS was analysed. These experiments revealed no differences between these strains, indicating that the membrane barrier must be intact (data not shown). It has been shown previously that *E. coli* cells under stress produce vesicles that contain periplasmic and OM proteins (McBroom & Kuehn, 2007). However, TCA precipitation and SDS-PAGE analysis of culture supernatants recovered from RS25 and K56-2 did not reveal any differences with respect to the amount or presence/absence of proteins (data not shown). In other bacteria, expression of exopolysaccharide and capsule can also be under the control of alternative sigma factors (Kaufusi et al., 2004; Yu et al., 1995). To look for the presence of surface-associated carbohydrates, RSF25 and K56-2 were stained with the dye Congo red, which binds β-D-glucans (Wood & Fulcher, 1983). However, TCA precipitation and SDS-PAGE analysis of culture supernatants recovered from RS25 and K56-2 did not reveal any differences with respect to the amount or presence/absence of proteins (data not shown).

In contrast, when RSF25(pDA17) was cultured in the presence of the dye calcofluor white, which also interacts with β-D-glucans (Wood & Fulcher, 1983), we observed an approximately ninefold increase in binding of the dye (*P* < 0.0001) compared to the parental strain K56-2(pDA17) (Fig. 5a–c). Expression of *rpoE* in trans restored calcofluor white binding almost back to wild-type levels (0.3500 ± 0.039) (Fig. 5a–c). These data suggest that inactivation of *rpoE* leads to the expression or unmasking of carbohydrates on the cell surface. To determine if calcofluor white binding was due to the synthesis of capsule we used India ink staining (Richardson & Sadoff, 1977). *Klebsiella pneumoniae* expresses capsule that can be detected by India ink staining and was used as a positive control. However, staining with India ink did not reveal any differences between K56-2 and RSF25. Although these experiments cannot rule out the absence of capsule, we conclude that calcofluor white binds a surface carbohydrate structure whose expression is modulated by RpoE. We also looked for altered patterns of OM protein expression in RSF25 and K56-2 by SDS-PAGE analysis. OM proteins from RSF25(pDA17) showed differences in protein banding as compared to K56-2(pDA17) and RSF25(pRF135), which disappeared after complementation of the *rpoE* defect (data not shown). Together, these results suggest that inactivation of *rpoE* leads to alterations in the cell envelope of *B. cenocepacia* K56-2.

**Intracellular rpoE-deficient *B. cenocepacia* cannot delay phagolysosomal fusion in macrophages**

Previous work in our laboratory has demonstrated that in macrophages *B. cenocepacia* resides in a membrane-bound compartment that does not fuse with lysosomes, at least during the first few hours post-infection (Lamothe et al., 2007). We have also shown that the delay in phagolysosomal fusion requires viable bacteria (Lamothe et al., 2007) and does not take place in *B. cenocepacia* mutants showing a defect in intracellular survival (Keith & Valvano, 2007; Maloney & Valvano, 2006). To assess whether RpoE controls functions required for the intracellular lifestyle of *B. cenocepacia* we performed infection experiments in RAW264.7 macrophages with the *rpoE* mutant RSF25. Since *B. cenocepacia* cannot replicate in macrophages and is extremely resistant to most antibiotics, classical methods to assess intracellular bacterial survival based on the selective killing of extracellular bacteria with host-cell-impermeable antibiotics are not possible (Lamothe et al., 2007).
Therefore, we use single-cell analysis by fluorescence microscopy, in macrophages preloaded with a fluid-phase marker. Dextran, a fluid-phase probe for the endocytic pathway, is commonly used to pre-label the lysosomal compartment (Eissenberg et al., 1988; Rodriguez et al., 2006; Wiater et al., 1998). Macrophages were exposed to fluorescent dextran for 3 h and then infected with either K56-2 or RSF25. In both cases, bacteria can be seen within membrane-bound compartments (Fig. 6a, b). However, in contrast to the parental strain, K56-2, where 41.1 % ± 3.5 of bacteria-containing vacuoles labelled red with dextran, 89.2 % ± 6.8 of the vacuoles containing RSF25 were fluorescent indicating dextran colocalization (Fig. 6c). As a control, heat-inactivated K56-2 cells were used to infect macrophages and at 2 h post-infection 94.8 % ± 2.4 of the bacteria-containing vacuoles were fluorescent, indicating fusion of the membrane-bound compartment with lysosomes. Complementation of RSF25 was not performed in these experiments because, as stated above, in B. cenocepacia expression of rpoE from a plasmid even at 37 °C adversely affects growth. Nevertheless, these experiments show that, similar to heat-killed K56-2, the RSF25 rpoE mutant is less able to promote a delay in the fusion of lysosomes with BcCVs. Although these experiments do not directly demonstrate a defect in intracellular survival, previous work in our laboratory has demonstrated that failure to delay phagolysosomal fusion causes rapid degradation of bacterial cells in the lysosomes (Keith & Valvano, 2007; Maloney & Valvano, 2006).

**DISCUSSION**

A search for HtrA-like serine proteases in the B. cenocepacia J2315 genome allowed us to identify an operon encoding the alternative sigma factor RpoE, and its regulatory proteins RseA and RseB. This operon is in a duplicated genomic fragment in strain J2315, but it is present as a single copy in strain K56-2, which we used for the genetic manipulations in this study. The third ORF downstream of rpoE, mucD, encodes a predicted HtrA protease, and it is followed by a gene of unknown function. While inactivation of mucD produced no phenotype, mutagenesis of rpoE resulted in a strain that was compromised for growth under certain stress conditions including elevated temperature and high osmolarity. Previous work that characterized an rpoE mutant in B. cepacia, another member of the Burkholderia cepacia complex, suggested that RpoE in this bacterium does not contribute significantly to the stress response (Devescovi & Venturi, 2006). However, our results showed that the B. cenocepacia RSF25 rpoE mutant exhibited growth defects under elevated temperature and osmolarity, and displayed cell envelope alterations. Previously, we have shown that B. cenocepacia requires the HtrA protein, BCAL2829, for growth under stress and

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**Fig. 6.** The rpoE mutant RSF25 co-localizes with dextran-labelled lysosomes. RAW 264.7 macrophages were labelled with 250 μg TMR-dextran ml⁻¹ and then infected with either wild-type (K56-2) or mutant (RSF25) bacteria at a m.o.i. of 40. Infected macrophages were analysed at 4 h post-infection. (a) K56-2 bacteria are found within membrane-bound compartments that do not co-localize with dextran. (b) RSF25 bacteria are found within membrane-bound vacuoles that are fluorescent. White arrows indicate bacteria within a membrane-bound compartment. (a) and (b) are representative images of those obtained from at least three independent experiments. Phase, phase-contrast microscopy; UV, fluorescence microscopy of the same field; Merge, merged images. (c) Bar graph representing the average percentage of BcCVs that co-localize with TMR-dextran for K56-2, RSF25 and a heat-inactivated (HI) K56-2 control. Error bars indicate SE. Significant differences were determined using the unpaired t-test from at least three independent experiments. *, P < 0.0005; **, P < 0.0001 when compared to wild-type K56-2. RSF25 and K56-2 (HI) did not differ significantly.
for survival in vivo (Flannagan et al., 2007). However, inactivation of the mucD gene did not compromise growth of *B. cenocepacia* under the conditions tested. Bioinformatic analysis of the *B. cenocepacia* J2315 genome reveals that this bacterium encodes five different HtrA-like serine proteases and it is conceivable that these HtrA proteins may have redundant functions that compensate for a mucD mutation. Although our experiments do not rule out the possibility that MucD functions in some capacity in the *B. cenocepacia* stress response they do indicate that this gene is dispensable in the presence of the remaining *htrA* genes.

The mechanism for activating RpoE-mediated transcription is well conserved in other bacteria (Cezairliyan & Sauer, 2007; Craig et al., 2002; Korbsrisate et al., 2005; Martinez-Salazar et al., 1996; Mathur et al., 2007; Missiakas & Raina, 1998; Yu et al. 1995) and follows the misfolding of porin protein precursors in the periplasm, which trigger a proteolytic cascade involving DegS (Walsh et al., 2003) and YaeL (Alba et al., 2002; Kanehara et al., 2002) resulting in cleavage of the anti-sigma factor RseA followed by release of RpoE (Kanehara et al., 2002). The mechanism of RpoE activation in *B. cenocepacia* remains to be characterized, but given that genes encoding RseA and RseB are present in the genome within the *rpoE* operon, it is likely that the mechanism of RpoE activation is also conserved in *B. cenocepacia*. In *E. coli*, degP is an RpoE-regulated gene that encodes an HtrA-like serine protease and is transcribed upon exposure to certain stress conditions (Dartigalongue et al., 2001; Lipinska et al., 1988). DegP degrades misfolded proteins that accumulate in the periplasmic space (Clausen et al., 2002). Previously we characterized an HtrA protease (BCAL2829) in *B. cenocepacia* that was required for growth under heat and osmotic stress, mirroring the phenotype of the *rpoE* mutant (Flannagan et al., 2007). This suggested that RpoE could in fact regulate BCAL2829, but attempts to complement the growth defects of RSF25 by expression of BCAL2829 failed. This indicates either that *htrA* is not part of the *rpoE* regulon or that other genes required for growth under stress, in addition to BCAL2829, are not expressed in the *rpoE* mutant.

In other bacteria, such as *Salmonella enterica*, RpoE is critical for intracellular survival and virulence in mice (Humphreys et al., 1999). Previous work has shown that *B. cenocepacia* can survive within amoebae and macrophages and has suggested that intracellular survival may contribute to the persistence of *B. cenocepacia* during infection (Lamothe et al., 2007; Saini et al., 1999). To determine if the *rpoE* gene plays any role in the intracellular survival of *B. cenocepacia* we turned to a macrophage model of infection. After phagocytosis by murine macrophages, wild-type *B. cenocepacia* delay the maturation of the BcCV and at 4 h post-infection intracellular wild-type bacteria that are within a membrane-bound compartment do not fuse with lysosomes (Lamothe et al., 2007). The dextran colocalization experiments performed in our study demonstrate the requirement for RpoE for *B. cenocepacia* to cause this delay. Although not directly demonstrated, the rapid trafficking of *rpoE*-defective *B. cenocepacia* to lysosomes is likely to be associated with loss of bacterial viability. This notion is supported by our previous observations demonstrating that every mutant created thus far in *B. cenocepacia* that fails to delay the maturation of the BcCV is destroyed in the lysosomes (Keith & Valvano, 2007; Maloney & Valvano, 2006). The specific mechanism employed by *B. cenocepacia* to delay maturation of the BcCV is currently unknown. However, our data suggest that it may require the expression of a gene or genes that are under the control of RpoE, and therefore respond to extracytoplasmic stress.

In summary, our data show that RpoE in *B. cenocepacia* is required for growth under stress and regulates some of the biological properties of the bacterial cell envelope. We have also shown that RpoE is required for the normal maturation of BcCVs in macrophages. The bacterial stress response in *B. cenocepacia* has only recently begun to be elucidated and further studies, currently under way in our laboratory, should shed light on the components of the RpoE regulon, and more importantly on the molecular mechanism explaining how this bacterium can persist in many different environments including the respiratory tract of CF patients.

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Characterization of B. cenocepacia rpoE


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