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

Ronald S. Flannagan¹ and Miguel A. Valvano^{1,2}

Correspondence

Miguel A. Valvano
mvalvano@uwo.ca

¹Infectious Disease Research Group, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada

²Medicine, Siebens–Drake Research Institute, University of Western Ontario, London, Ontario, Canada

Burkholderia cenocepacia is an opportunistic pathogen causing serious infections in patients with cystic fibrosis. The widespread distribution of this bacterium in the environment suggests that it must adapt to stress to be able to survive. We identified in *B. cenocepacia* K56-2 a gene predicted to encode RpoE, the extra-cytoplasmic stress response regulator. The *rpoE* gene is the first gene of a predicted operon encoding proteins homologous to RseA, RseB, MucD and a protein of unknown function. The genomic organization and the co-transcription of these genes were confirmed by PCR and RT-PCR. The *mucD* and *rpoE* genes were mutated, giving rise to *B. cenocepacia* RSF24 and RSF25, respectively. While mutant RSF24 did not demonstrate any growth defects under the conditions tested, RSF25 was compromised for growth under temperature (44 °C) and osmotic stress (426 mM NaCl). Expression of RpoE *in trans* could complement the osmotic growth defect but exacerbated temperature sensitivity in both RSF25 and wild-type K56-2. Inactivation of *rpoE* altered the bacterial cell surface, as indicated by increased binding of the fluorescent dye calcofluor white and by an altered outer-membrane protein profile. These cell surface changes were restored by complementation with a plasmid encoding *rpoE*. Macrophage infections in which bacterial colocalization with fluorescent dextran was examined demonstrated that the *rpoE* mutant could not delay the fusion of *B. cenocepacia*-containing vacuoles with lysosomes, in contrast to the parental strain K56-2. These data show that *B. cenocepacia* *rpoE* is required for bacterial growth under certain stress conditions and for the ability of intracellular bacteria to delay 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; Rezuchova *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.*, 1988). 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 extra-cytoplasmic 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 phosphatase (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

Strain/plasmid	Relevant characteristics*	Source/reference†
<i>B. cenocepacia</i>		
J2315	CF clinical isolate	BCRRC
K56-2	ET12 clone related to J2315	BCRRC
RSF24	K56-2, <i>mucD</i> ::pRFint- <i>mucD</i> , Tp ^R	This study
RSF25	K56-2, <i>rpoE</i> ::pRFint- <i>rpoE</i> , Tp ^R	This study
<i>E. coli</i>		
DH5α	F ⁻ , φ80 <i>lacZ</i> ΔM15 (Δ <i>lacZ</i> YA- <i>argF</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1</i> Δ <i>gyrA96 relA1</i>	Laboratory stock
SY327	<i>araD</i> , Δ(<i>lac pro</i>) <i>argE</i> (<i>Am</i>) <i>recA56 rif^R nalA λ pir</i>	Miller & Mekalanos (1988)
Plasmids		
pGP704	<i>ori</i> _{R6K} , Ap ^R , <i>mob</i> ⁺	Miller & Mekalanos (1988)
pDA17	<i>ori</i> _{pBBR1} , Tet ^R , <i>mob</i> ⁺ , <i>P</i> _{dfpB2} , FLAG epitope	D. Aubert
pRK2013	<i>ori</i> _{colE1} , RK2 derivative, Kan ^R , <i>mob</i> ⁺ , <i>tra</i> ⁺	Figurski & Helinski (1979)
pMLBAD	<i>ori</i> _{pBBR1} , Tp ^R , <i>mob</i> ⁺ , <i>araC-P</i> _{BAD}	Lefebvre & Valvano (2002)
pGPAPTp	pGP704, Tp ^R , Ap ^R , <i>mob</i> ⁺	This study
pRFint- <i>rpoE</i>	pGPAPTp, 219 bp internal fragment from <i>rpoE</i>	This study
pRFint- <i>mucD</i>	pGPAPTp, 291 bp internal fragment from <i>mucD</i>	This study
pRF132	pDA17, BCAL2829 FLAG	Flannagan <i>et al.</i> (2007)
pRF135	pDA17, <i>rpoE</i>	This study

*Tp, trimethoprim; Kan, kanamycin; Ap, ampicillin; Tet, tetracycline.

†BCRRC, *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'-TCTACGGGGTCTGACGCTCAGTGAACG-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'-TAACGGTTGTGGACAACAAGCCAGGG-3') and primer 549. To inactivate *mucD* an internal fragment of the gene was PCR amplified using primers 2217 (5'-TTTTCTAGATCGACGATGCGGACACCA-TCTAC-3') and 2218 (5'-TTTTCTAGACAGGGTTCACGGCCAC-GTCGGTC-3') with the following 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'-TCAAGGATCTTACCGCTGTTG-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'-TTTGTCGACCGTGGCGCTGCCGCAATTCCGCG-3') and 2216 (5'-TTTGTCGACCGTGGCGCTGCCGCAATTCTGCTTG-3') and the thermal cycling conditions described above. The resultant amplicon was digested with *SalI* and cloned into pGPApTp that had been similarly digested and treated with Antarctic phosphatase. The orientation of the *rpoE* internal fragment was the same as that of the *dfrB2* gene and was confirmed by PCR using primers 612 and 2215. The resultant plasmid was named pRFint-*rpoE*. This mutagenesis plasmid was conjugated into *B. cenocepacia* K56-2 and mutants were confirmed

as above except that a digoxigenin-labelled internal fragment from *rpoE* was used. The resultant strain was named *B. cenocepacia* RSF25.

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-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan]-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 *NotI* 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'-TTGAATTCGTGAGTGA-AAAAGAAATCGATCAGGC-3') and 2418 (5'-TTTCTAGACCA-GCGCTTGCCTTCGGGCGTATC-3'). The resulting 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.

Fig. 1. Schematic map of the mutagenesis plasmid pGPApTp. The suicide plasmid pGPApTp carries the Pir-dependent R6K origin (*oriR6K*) of replication. The β -lactamase (*bla*) gene confers resistance to ampicillin and the dihydrofolate reductase (*dfrB2*) gene confers resistance to trimethoprim. The *mob* element is required for conjugal transfer. The unique restriction sites for *SalI*, *HindIII*, *KpnI*, *XbaI*, *SmaI* and *EcoRI* are shown.

Table 2. RT-PCR primers

Primer	Nucleotide sequence
2757 (<i>rseA</i> RT F)	5'-GCAATTCGCCAGCAGCCCG-3'
2758 (<i>rseB</i> RT R)	5'-CGAACGTACCTTCGTAGCTCTGCTG-3'
2759 (<i>rseB</i> RT F)	5'-GTTGCAGCAGTTCGCGTCTGCC-3'
2760 (<i>mucD</i> RT R)	5'-GCACGTTGGCGGTGGTCCG-3'
2761 (<i>mucD</i> RT F)	5'-CGTGGCGACAACACGCAAGTTC-3'
2762 (<i>orf5</i> RT R)	5'-CACACTTCCGCGCCGTCCAG-3'
2776 (<i>rpoE</i> RT F)	5'-CGATGCGGAGGAAGCGGAAAC-3'

Temperature sensitivity. Temperature sensitivity was assessed by drop plating serially diluted (10^0 – 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 OD₆₀₀ 0.1 ($\sim 2.6 \times 10^7$ 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 (OD₆₀₀ 0.15–0.25). From these cultures cell suspensions were set up at OD₆₀₀ 0.1 ($\sim 2.6 \times 10^7$ 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 ChemoDoc XRS system (Bio-Rad) with Quantity-One 1D analysis software (Bio-Rad). Light intensities of zones of growth were quantified using Odyssey 2.1 software from an 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 sonic disruption until clear (approximately four 30 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 Lamothe *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 Qimaging Retiga1300 cooled mono 12-bit camera on an Axioscope 2 microscope (Carl Zeiss) with a

100 × 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 *B. 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 *dfrB2* 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

Fig. 2. Genetic organization of the *rpoE* operon in *B. cenocepacia* K56-2. (a) Schematic representation of the genomic region encoding the *rpoE* operon. Genes that are part of the *rpoE* operon are shown in black with the gene names listed above the ORFs. *hyp* indicates a gene encoding a conserved hypothetical protein. *orf5* also encodes a protein of unknown function. Solid black lines under intergenic regions indicate the areas analysed by RT-PCR, and denote co-transcription. The strain names, RSF25 and RSF24, are written below the genes that were insertionally mutated in these bacteria. (b) RT-PCR analysis of the *rpoE* genomic region in *B. cenocepacia* K56-2. The intergenic regions analysed are as follows: 1, *rpoE* and *rseA*; 2, *rseA* and *rseB*; 3, *rseB* and *mucD*; 4, *mucD* and *orf5*. The black lines in (a) correspond to regions 1–4 from left to right. For each region, reactions were performed with DNA (positive control for amplification), –RT (negative control) and +RT (after reverse transcription). White arrows indicate the expected amplicons obtained from DNA and RT reactions. The intergenic region between *orf5* and *lepA* was negative by RT-PCR. (c) Southern blot analysis of the *rpoE* mutant RSF25. Chromosomal DNA from K56-2 (lane 1) and from four independent colonies of RSF25 (lanes 2–5) was digested with *Xho*I and probed with a digoxigenin-labelled internal fragment from *rpoE*. M, molecular size markers in kb.

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).

Fig. 3. Growth of *B. cenocepacia* RSF25 under conditions of thermal stress. Serial dilutions (10^0 – 10^{-4}) of K56-2 or RSF25 carrying either pDA17 (vector control) or pRF135 (*rpoE*⁺) were drop-plated onto LB agar and incubated at either 30 °C (a) or 44 °C (b). Two independent K56-2 exconjugants carrying pRF135 were analysed. Similar results were obtained in at least three independent experiments.

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

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.

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 HtrA_{BCAL2829} is also required for growth under conditions of osmotic stress (Flannagan *et al.*, 2007), we tried to complement the RSF25 growth defect by providing HtrA_{BCAL2829} *in trans*. Expression of HtrA_{BCAL2829} from plasmid pRF132 did not affect the growth of RSF25, indicating that the reduced growth of RSF25 is due to factors other than HtrA_{BCAL2829} (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

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, the two strains were indistinguishable in these experiments (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.*,

Fig. 5. Binding of calcofluor white to *B. cenocepacia* RSF25. Cell suspensions of *B. cenocepacia* K56-2 (carrying pDA17) and RSF25 (carrying pDA17 or pRF135) were drop-plated onto LB agar containing 0.002% (w/v) calcofluor white and incubated for 24 h at 30 °C. Plates were imaged with exposure to white light (WL) (a) and UV light (b). (c) Bar graph showing the densitometric ratio of calcofluor white (CW) binding. Values were obtained by dividing UV light pixel intensities by WL pixel intensities for individual spots. Error bars denote the SEM obtained from three independent experiments with each strain analysed in quadruplicate.

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 (Eisenberg *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 $41.1\% \pm 3.5$ of bacteria-containing vacuoles labelled red with dextran, $89.2\% \pm 6.8$ 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 $94.8\% \pm 2.4$ 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

Fig. 6. The *rpoE* mutant RSF25 co-localizes with dextran-labelled lysosomes. RAW 264.7 macrophages were labelled with $250\ \mu\text{g TMR-dextran ml}^{-1}$ 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 through expression of BCAL2829 failed. This indicates either that *htrA*_{BCAL2829} 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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