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## Role of *Bordetella pertussis* RseA in the cell envelope stress response and adenylate cyclase toxin release

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### Abstract

*Bordetella pertussis* is the bacterial agent of the human disease, whooping cough. In many bacteria, the extracellular function sigma factor  $\sigma^E$  is central to the response to envelope stress, and its activity is negatively controlled by the RseA anti-sigma factor. In this study, the role of RseA in *B. pertussis* envelope stress responses was investigated. Compared with the wild-type strain, an *rseA* mutant showed elevated resistance to envelope stress and enhanced growth at 25°C. *rpoH* and other predicted  $\sigma^E$  target genes demonstrated increased transcription in the *rseA* mutant compared with the wild type parent. Transcription of those genes was also increased in wild type *B. pertussis* and *Escherichia coli* under envelope stress, whereas no stress-induced increase in transcription was observed in the *rseA* mutant. *rseA* inactivation was also associated with altered levels of certain proteins in culture supernatant fluids, which showed increased adenylate cyclase toxin (CyaA) levels. The increased CyaA in the mutant was correlated with an apparent increased stability of the extracellular toxin and increased production of CyaA-containing outer membrane vesicles. Consistent with this, compared with the wild type strain, *rseA* mutant cells produced increased numbers of large surface-associated vesicles.

### Keywords

*Bordetella pertussis*; envelope stress; RseA;  $\sigma^E$

## INTRODUCTION

*Bordetella pertussis* is a Gram negative bacterium that is an obligate pathogen of humans, causing the acute respiratory disease pertussis (whooping cough) that manifests as a prolonged coughing illness that is more severe in infants (von Konig *et al.*, 2002; Hewlett &

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Edwards, 2005; Mattoo & Cherry, 2005). *B. pertussis* produces multiple virulence factors, including a variety of adhesins and toxins (Locht *et al.*, 2001; Mattoo & Cherry, 2005), which contribute to the complex pathology of pertussis. Most of the major virulence factors, including adhesins, pertussis toxin, and adenylate cyclase toxin (CyaA), are positively and coordinately regulated at the transcriptional level by the BvgAS two-component phosphorelay system (Weiss *et al.*, 1983; Arico *et al.*, 1989; Mattoo & Cherry, 2005).

Bacterial alternative sigma factors of RNA polymerase play important roles in the transcriptional response to a variety of environmental stimuli and stresses (Raivio & Silhavy, 2001).  $\sigma^E$  (RpoE), an extracytoplasmic function (ECF) sub-family sigma factor present in many bacteria, is involved in the cell's stress response to perturbations in the outer membrane and the presence of misfolded proteins in the periplasm (Raivio & Silhavy, 2001; Alba & Gross, 2004; Hayden & Ades, 2008). Proteins encoded by  $\sigma^E$  regulon genes are diverse and have been linked to bacterial processes such as the synthesis, assembly and homeostasis of lipopolysaccharides and outer membrane proteins, maintenance of periplasmic proteases and outer membrane proteins, and energy metabolism (Humphreys *et al.*, 1999; Alba & Gross, 2004; Rhodius *et al.*, 2006).

The molecular mechanisms of  $\sigma^E$  activation by cell envelope stress have been elucidated in *Escherichia coli*, a species in which *rpoE* is apparently essential (De Las Penas *et al.*, 1997). In the absence of stress,  $\sigma^E$  is inactivated *via* binding by RseA, an anti-sigma factor located in the cytoplasmic membrane. Upon detection of envelope stress in the periplasm, the DegS protease cleaves the periplasmic region of RseA, initiating what is known as regulated intramembrane proteolysis (Akiyama *et al.*, 2004; Chaba *et al.*, 2007). Subsequently, RseP proteolytically cleaves RseA in its transmembrane region, releasing the N-terminal RseA fragment with its associated  $\sigma^E$ . RseA is then completely digested by the ClpPX cytoplasmic protease, freeing  $\sigma^E$ , allowing it to function in transcription (Flynn *et al.*, 2004).

In other bacteria,  $\sigma^E$  has been implicated in the regulation of genes involved in pathogenesis. For example, *htrA* (also known as *degP*), which encodes a periplasmic serine protease, is transcribed in a  $\sigma^E$ -dependent manner (Clausen *et al.*, 2002) and is known to be required for survival of *Salmonella enterica* subgroup Typhimurium (Johnson *et al.*, 1991), *Yersinia enterocolitica* (Yamamoto *et al.*, 1996) and *Legionella pneumophila* (Pedersen *et al.*, 2001) in macrophages. Furthermore,  $\sigma^E$  has been shown to be important for the growth of *S. Typhimurium* in mice (Humphreys *et al.*, 1999) and macrophages (Yoon *et al.*, 2009), and is necessary for *Vibrio cholerae* survival in the intestine of experimentally infected mice (Kovacikova & Skorupski, 2002).

*B. pertussis* typically colonizes the ciliated respiratory epithelium and the bacteria have also been observed within epithelial cells and leukocytes (Hellwig *et al.*, 1999; Mattoo & Cherry, 2005). Little is known about *B. pertussis* stress responses that may be activated in the host environment. A recent study examined  $\sigma^E$  function of *Bordetella bronchiseptica*, which is primarily a respiratory pathogen of nonhuman mammals (Barchinger *et al.*, 2012). The authors demonstrated that mutation of *rpoE* in *B. bronchiseptica* resulted in increased sensitivity to heat, certain antibiotics and other stressors. A *B. bronchiseptica rpoE* mutant was more susceptible to being killed by polymorphonuclear neutrophils. In *E. coli*, the *B.*

*bronchiseptica rpoE* gene functioned to increase expression of an *E. coli rpoHP3-lacZ* fusion, and it complemented an *rpoE* mutation, which is normally lethal in *E. coli*.

In the present study, an *rseA* mutant in the human-pathogenic species, *B. pertussis*, was constructed. Consistent with the role of RseA as an anti-sigma factor that antagonizes  $\sigma^E$  function, the *rseA* mutant exhibited increased resistance to envelope stressors, elevated transcription levels of *rpoH* and other genes, and also showed an enhanced capacity to grow at low temperature when compared with wild-type cells. The mutant was altered in its profile of extracellular proteins; in particular, levels of CyaA were highly elevated. The *rseA* mutant produced more numerous outer membrane vesicles than wild type cells and exhibited a remarkable number of large surface-associated vesicles.

## MATERIALS AND METHODS

### Bacterial strains and cultivation

The bacterial strains used in this study are listed in Table 1. *B. pertussis* strains were grown for two days at 35°C on Bordet Gengou agar (Bordet J & Gengou O, 1906). Liquid culture of *B. pertussis* used modified Stainer-Scholte (SS) medium (Stainer & Scholte, 1970; Schneider & Parker, 1982) supplemented with 0.5% casamino acids at 35°C with shaking at 300 rpm. SS cultures for  $\beta$ -galactosidase measurements also contained 500  $\mu\text{g ml}^{-1}$  of heptakis(2,6-di-O-methyl)- $\beta$ -cyclodextrin (Imaizumi *et al.*, 1983). *E. coli* strains were cultured on Luria Bertani agar or broth. As necessary, 15  $\mu\text{g ml}^{-1}$  of tetracycline and 10  $\mu\text{g ml}^{-1}$  of gentamicin were added to the media for plasmid selection. For selection of *B. pertussis* strains, streptomycin was used at 200  $\mu\text{g ml}^{-1}$ .

### DNA manipulation

Standard molecular biological techniques were performed as described previously (Sambrook *et al.* 1989). *B. pertussis* DNA fragments were amplified using PrimeSTAR® GXL DNA Polymerase (Takara Bio Inc., Otsu, Japan). TaKaRa Ex Taq™ (Takara Bio Inc., Otsu, Japan) was used for the polymerase chain reaction (PCR) when the template DNA was from *E. coli*. Conjugal transfer of plasmids was performed as described by Brickman & Armstrong (1996). The sequences of oligonucleotide primers used for PCR in this study are shown in Table 2.

### Sequence analysis

Nucleotide sequence data were from the genome sequence of *B. pertussis* Tohama I produced by the *Bordetella* Sequencing Group at The Wellcome Trust Sanger Institute ([http://www.sanger.ac.uk/Projects/B\\_pertussis](http://www.sanger.ac.uk/Projects/B_pertussis)) and analyzed using GeneDB (<http://www.genedb.org>) (Parkhill *et al.*, 2003). Sequence analyses were also conducted using the Lasergene sequence analysis software package (DNASTAR, Inc., Madison, WI). Database searches were accomplished using the BLAST servers provided by the Sanger Centre and the National Center for Biotechnology Information at the National Library of Medicine and Gene Context Tool from the Computational Genomic Group, Instituto de Biotecnologia, Universidad Nacional Autonoma de Mexico (<http://bioinfo.ibt.unam.mx/gecont/index.cgi?second>).

## Construction of *B. pertussis* mutant strains and complementation plasmids

An *rpoE* in-frame deletion mutation of 381 nt was constructed by overlap extension PCR as described (Ho *et al.*, 1989; Horton *et al.*, 1989) using the *rpoE1*, *rpoE2*, *rpoE3* and *rpoE4* primers listed in Table 2. The DNA fragment was cloned into allelic exchange plasmid pSS1129 (Stibitz, 1994) and the resulting *rpoE* plasmid, pSS22, was conjugally transferred to *B. pertussis* UT25Sm1, with selection on gentamicin. Transconjugants were scored for streptomycin sensitivity, and the modest number of subsequent streptomycin resistant and gentamicin sensitive allelic exchange mutant candidates were analyzed by PCR for the presence of the *rpoE* allele. Of the candidates obtained, all retained the wild-type *rpoE* gene. The conjugation and allelic exchange experiment was performed three times (assessing over 100 candidates in total), with similar negative results. Other allelic exchange attempts, using a different DNA fragment where both *rpoE* and *rseA* were deleted, also failed to yield mutants.

To construct the *B. pertussis* *rseA* mutant strain PM18, a 1.86-kb DNA fragment containing a 459-bp in-frame *rseA* deletion mutation was synthesized using the *rseA1* and *rseA2*, and *rseA3* and *rseA4* primer pairs by overlap extension PCR. The fragment was cloned into the pSS1129 suicide vector using the *EcoRI* and *BamHI* restriction sites. The resulting plasmid, pSS20, was transferred to *B. pertussis* UT25Sm1 by conjugation. After allelic exchange, the *B. pertussis* strain carrying the *rseA* mutation was confirmed by PCR using the primers *rseA-BamHI* and *rseA-EcoRI*.

For use in genetic complementation experiments, the 632-bp DNA fragment containing the wild type *rseA* gene from *B. pertussis* UT25Sm1 was amplified by PCR with the primer pair *rseA-BamHI* and *rseA-EcoRI*, and cloned into pRK415 downstream of the *lac* promoter, resulting in plasmid pRK59. Additionally, the wild type *rseA* allele on a 2.0-kb DNA fragment synthesized by PCR using the *rseA1* and *rseA4* primers was cloned into pSS1129 to produce pSSK26. The plasmid was conjugated to the *B. pertussis* *rseA* strain PM18 to reconstruct the wild type *rseA* allele by allelic exchange, resulting in strain PMK22. PCR was performed to confirm the allele replacement. To construct a plasmid containing the *rseA* gene expressed from a promoter inducible by isopropylthio- $\beta$ -galactoside (IPTG), the 1.82-kb DNA fragment of pMALc2™ (New England Biolabs Inc., Ipswich, MA, USA) containing *lacI* and *Ptac* promoter was synthesized by PCR using the pMal-EcoRI/F and pMal-EcoRI/R primers, and cloned into pRK415 resulting in plasmid pRK60. The 632-bp DNA fragment containing the *rseA* gene from *B. pertussis* UT25Sm1 was amplified by PCR with the primer pair *rseA-BamHI* and *rseA-HindIII*, and cloned into pRK60 downstream of the *Ptac* promoter, resulting in plasmid pRK601. **Stress sensitivity assays.** An overnight *B. pertussis* SS culture was diluted to an OD<sub>600</sub> of 0.1 with fresh SS medium and then sub-cultured to obtain bacteria in the exponential growth phase. When the turbidity reached an OD<sub>600</sub> of approximately 0.4, either H<sub>2</sub>O<sub>2</sub> or polymyxin B was added to the final concentrations indicated in the text. Parallel control cultures were untreated. After incubation at 35°C at the indicated times, the number of viable bacteria was determined by plate count on Bordet Gengou agar and percent survival was calculated. Means and standard deviations of a representative of three experiments are shown. The data were analyzed by Student's t test (*p* values are shown).

## Construction of transcriptional fusions with putative $\sigma^E$ -dependent promoters and *cyaA* and *cyaB* promoters

The DNA regions upstream of the BP0893, BP2014 (*acnA*), BP3218, and BP3748 (*rpoH*) genes of *B. pertussis* UT25Sm1 were amplified by PCR from genomic DNA using the primer pairs: BP0893-EcoRI and BP0893-XbaI, BP2014-EcoRI and BP2014-XbaI, BP3218-EcoRI and BP3218-XbaI, and *rpoH*-EcoRI and *rpoH*-XbaI, respectively (Table 2). The primer pairs: *cyaA*-EcpRI and *cyaA*-Xba, and *cyaB*-EcoRI and *cyaB*-XbaI were used for PCR reactions generating the fragments containing the *cyaA* and *cyaB* promoters. The resulting fragments were digested with *EcoRI* and *XbaI* and subsequently ligated into the pMP220 *lacZ* fusion plasmid (Spaink *et al.*, 1987).

## Measurement of $\beta$ -galactosidase activity

*E. coli* and *B. pertussis* strains carrying transcriptional fusion plasmids were grown in defined M9 and SS media with selection, respectively. To assess transcription under stress conditions, overnight cultures of *E. coli* strains were subcultured and grown for 6.5 h to an OD<sub>600</sub> of approximately 0.4 and treated with the indicated concentrations of polymyxin B for 30 min prior to  $\beta$ -galactosidase measurement, as described previously (Miller, 1972; Brickman *et al.*, 1990). *B. pertussis* 15-hr SS cultures were treated for 3 h with either H<sub>2</sub>O<sub>2</sub>, polymyxin B or sucrose, added to the indicated concentrations, prior to measurement of  $\beta$ -galactosidase. The assays were performed in triplicate and the results reported are representative of at least three experiments.

## SDS-PAGE and immunoblotting

Proteins were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% polyacrylamide gels (Laemmli *et al.*, 1970). For standard protein gels, each lane was loaded with culture fluid proteins corresponding to a culture with an OD<sub>600</sub> of 1.5, and periplasmic protein samples corresponding to a culture with an OD<sub>600</sub> of 7.0, and equivalent amounts of membrane protein fractions (0.1 units of OD<sub>280</sub>). For immunoblotting, the amount of protein sample that was loaded was doubled; the proteins were electrophoretically separated and transferred to PVDF membranes (Clear Blot Membrane-p; ATTO Co., Tokyo, Japan). Immunoblot membranes were probed with a 1:200 dilution of mouse monoclonal antibody specific for mouse monoclonal antibody to the *B. pertussis* CyaA (sc-13582; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After reaction with a goat anti-mouse IgG and IgM-alkaline phosphatase conjugate (Sigma, St. Louis, MO) at a dilution of 1:10,000, the blots were washed and developed using ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

## Preparation of *B. pertussis* protein samples

To obtain proteins from culture fluids, spent culture supernatant was obtained from a 24-h culture with an OD<sub>600</sub> of approximately 1.2 by centrifugation (8,000  $\times$  g, 10 min) and passed through a 0.45- $\mu$ m sterile membrane filter (Milex®GV, Millipore, Carrigtwohill, Ireland) to remove residual bacterial cells. Proteins in the supernatant fluids were

precipitated with 10% trichloroacetic acid (TCA) in acetone and resuspended in loading buffer for SDS-PAGE.

Bacterial cells were disrupted by ultrasonication and the lysate centrifuged at  $100,000 \times g$  for 1 h to obtain the soluble (cytosol and periplasm) and membrane fractions. After the addition of Triton X-100 to a final concentration of 2 %, solubilized inner membrane proteins were isolated from the insoluble outer membrane fraction by ultracentrifugation. Proteins in the inner membrane fraction were concentrated by methanol precipitation. Periplasmic fractions were prepared using osmotic shock and separation from spheroplasts. Briefly, *B. pertussis* strains were grown in SS medium to an  $OD_{600}$  of 1. Cells were harvested, washed twice with a solution containing 30 mM NaCl, 10 mM Tris HCl (pH 7.5) and resuspended in 2.5 ml of a solution of 20% sucrose and 30 mM Tris-HCl (pH 7.5) at room temperature. After addition of EDTA to a final concentration of 1 mM, the cell suspensions were incubated at room temperature for 10 min. Bacterial cells were collected by centrifugation at  $8,000 \times g$  at  $4^{\circ}C$  for 10 min and resuspended in 10 ml of ice cold 0.5 mM  $MgCl_2$ . After 10 min of incubation on ice, the cells were removed by centrifugation for 10 min at  $8,000 \times g$ , followed by filtration. The periplasmic proteins were concentrated by precipitation with 10% TCA in acetone.

To prepare outer membrane vesicles, cell-free culture fluids were concentrated by ultrafiltration (Millipore Amicon Ultra 50K, Millipore). Proteins in the ultrafiltrate were concentrated 100-fold by precipitation using 10% TCA in acetone (fraction 1). The concentrated samples retained by the membrane were fractionated by ultracentrifugation at  $150,000 \times g$  for 3 h. Proteins in the resulting supernatant fraction were precipitated by 10% TCA in acetone (fraction 2); the pellets contained crude outer membrane vesicles. Samples were analyzed by SDS-PAGE and immunoblotting. Protein amounts corresponding to a culture with an  $OD_{600}$  of 6.0 were applied to each lane.

### Scanning electron microscopy

*B. pertussis* strains were grown on a layer of BG agar solidified onto a sterile glass slide. The colonies were fixed in situ using 2.5% glutaraldehyde prior to examination by scanning electron microscopy using a JSM 6330F microscope (JEOL Ltd. Tokyo, Japan).

## RESULTS

### Identification of the *B. pertussis rpoE* gene cluster and construction of an *rseA* deletion mutant

Barchinger and colleagues reported recently that the genomes of *B. bronchiseptica*, *Bordetella parapertussis* and *B. pertussis* contain gene clusters predicted to encode RpoE-related functions (Barchinger *et al.*, 2012). In the present study, we specifically identified in the *B. pertussis* Tohama I genome sequence the genes BP2437, BP2436, BP2435 and BP2434 which are predicted orthologs of the known bacterial genes *rpoE*, *rseA*, *rseB*, and *mucD*, respectively (Fig. 1). Upstream of *rpoE* are genes encoding a putative 3-oxoacyl-[acyl-carrier-protein] synthase II (BP2439, FabF) and BP2438, encoding a membrane protein of unknown function. The *mucD* (BP2434) product is a predicted member of the

HtrA family of serine proteases that includes MucD of *Pseudomonas aeruginosa* and *E. coli* DegP, which are involved in degrading damaged proteins; *degP* transcription is dependent on  $\sigma^E$  and enhanced under heat shock stress (Clausen *et al.*, 2002). No obvious *rseC* homolog was identified in the *B. pertussis* genome sequence; however a gene annotated as *degQ* (BP0280) specifies a protein that exhibits significant similarity to *E. coli* DegS and DegQ. Based on the predicted transcriptional organization (Cummings *et al.*, 2006), as well as short intergenic regions and overlapping coding sequences suggestive of transcriptional coupling, the *B. pertussis* *rpoE*, *rseA*, *rseB* and *mucD* genes are likely cotranscribed from the *fabF* promoter. This gene organization is conserved in *B. parapertussis*, *B. bronchiseptica* and *Bordetella avium*. Bioinformatic analysis revealed that other  $\beta$ -proteobacteria, including *Burkholderia* species, possess *rpoE* gene clusters that are also adjacent to *fabF* and other fatty acid biosynthesis genes.

In contrast to the *B. bronchiseptica* *rpoE* mutant results recently described (Barchinger *et al.*, 2012), in this study, multiple efforts to construct a *B. pertussis* *rpoE* null mutant were unsuccessful. To facilitate the study of stress responses in this human-adapted *Bordetella* species, the gene encoding the predicted RseA anti-sigma factor was mutated. An *rseA* mutant would be expected to exhibit a  $\sigma^E$ -constitutive phenotype, regardless of envelope stress. An in-frame *rseA* deletion mutation in *B. pertussis* was constructed that resulted in the removal of 153 amino acids, comprising the majority of RseA polypeptide.

### Stress sensitivity of *B. pertussis*

Both wild-type *B. pertussis* strain UT25Sm1 and its isogenic *rseA* mutant derivative PM18 were assessed for susceptibility to hydrogen peroxide stress and the bactericidal cationic peptide, polymyxin B, which are known activators of the  $\sigma^E$ -dependent envelope stress response (Humphreys *et al.*, 1999; Testerman *et al.*, 2002; Korbsrisate *et al.*, 2005; Mathur *et al.*, 2007). Exposure of the wild-type strain to 40 mM hydrogen peroxide for 1 h decreased its survival to 43%, relative to untreated cells, whereas the percent survival of the *rseA* mutant was 76% (Fig. 2A). Similarly, the *rseA* mutant showed moderately decreased sensitivity to polymyxin B (Fig. 2B). Sensitivity to both hydrogen peroxide and polymyxin B approaching wild-type levels was restored to mutant PM18 by complementation in *trans* using a wild type copy of the *rseA* gene (pRK59). Overexpression of *rseA* from an IPTG-inducible promoter (pRK601) in mutant PM18 led to markedly decreased resistance to hydrogen peroxide and polymyxin B (Fig. 2C, D). This decreased survival was not due to *rseA* overexpression itself since, in the absence of stress compounds, there was no difference in viable cell counts of uninduced cultures versus those induced with IPTG for 6 h (data not shown). Overexpression of *rseA* on multicopy plasmid pRK59 in wild-type *B. pertussis* UT25Sm1 increased its sensitivity to stress (Fig. 2E, F). In sum, these results suggest that the activation of  $\sigma^E$  caused by the loss of *rseA*, encoding the cognate anti-sigma, leads to increased tolerance to envelope stress. These results are also consistent with the idea that *rseA* overexpression leads to unrelieved cell envelope stress, since excess RseA may overwhelm the predicted protease that normally cleaves it; this would allow RseA to bind the available  $\sigma^E$ , preventing it from activating stress regulon genes.

### Effect of *rseA* deletion on growth

Since the  $\sigma^E$  system is reported to influence bacterial growth at different temperatures (Chi & Bartlett, 1995; Miticka *et al.*, 2003), the effect of *rseA* mutation on *B. pertussis* growth at a range of temperatures was examined (Fig. 3). The optimal temperature for growth of *B. pertussis* is 35°C - 37°C. Both the wild-type and *rseA* mutant strains grew equally well at 30°C, 35°C, 39°C and 42°C and both demonstrated similarly poor growth at 45°C. As expected, at 25°C, both the wild-type strain and *rseA* mutant PM18 exhibited a slow growth rate. However, later during the culture period, PM18 consistently exhibited an increased growth rate compared with the parent strain, with a significantly higher final growth yield. Using a plasmid-borne *rseA* gene (pRK59), PM18 was genetically complemented, restoring the lower wild-type growth level (Fig. 3F). Replacement of the PM18 chromosomal *rseA* gene with a copy of wild-type *rseA* via allelic exchange (“knock-in” strain PMK22) also restored the lower, wild-type growth level (Fig. 3G). These results indicate that mutation of *rseA* did not affect *B. pertussis* growth at higher temperatures but rather, it suppressed the slow growth phenotype observed in wild type cells at 25°C.

### Induction of transcription by envelope stress and the effect of RseA

In *E. coli* and other bacteria,  $\sigma^E$  recognizes promoters with the conserved -35/-10 sequence motif [GAAC[A/T][A/T]X(16,17)TCXXA] (Lane & Darst, 2006; Rhodius & Mutalik, 2010). An *in silico* analysis of the *B. pertussis* genome revealed several candidate  $\sigma^E$ -dependent promoter sequences upstream of the genes of unknown function, BP0893 and BP3218, as well as BP2014 (annotated as *acnA*, encoding a putative aconitase A) and BP3748, an ortholog of *rpoH*. In characterized bacterial systems, *rpoH* expression is regulated by  $\sigma^E$  (Raivio & Silhavy, 2001). Since RpoH is known to be involved in bacterial heat shock and stress responses and the recent *B. bronchiseptica*  $\sigma^E$  analysis demonstrated its role in *rpoH* transcription (Barchinger *et al.*, 2012), we examined whether the *B. pertussis* *rpoH* gene was also subject to RseA/ $\sigma^E$ -mediated regulation.

To analyze the putative promoter activities in *B. pertussis*, *lacZ* transcriptional fusions were constructed and the  $\beta$ -galactosidase activity of wild type and *rseA* mutant strains carrying the fusion plasmids was measured (Fig. 4 A-D). In the wild type strain UT25Sm1, transcription of all four fusions was modest. In contrast, in the *rseA* mutant PM18, significantly higher levels of LacZ activity were measured, especially for the BP0893 transcriptional fusion (Fig. 4 A). When the same plasmid-borne fusions were expressed in *E. coli* that was subjected to polymyxin B stress, a dose responsive increase in transcriptional activity was observed (Fig. 5 A-D). These results demonstrate that these promoter activities were elevated in the *B. pertussis* *rseA* mutant and could also be activated in a heterologous host background under relevant stress conditions. A similar trend was observed when the *rpoH-lacZ* fusion was expressed in *B. pertussis* exposed to cell envelope stress (Fig. 6). In the wild type strain, *rpoH* expression was modestly increased when the cells were treated with hydrogen peroxide, polymyxin B or 0.4 M sucrose. In contrast, *rpoH-lacZ* transcription in *rseA* mutant PM18 was high regardless of the presence or absence of any of the stressors. These results indicate that in wild-type *B. pertussis*, *rpoH* transcription is responsive to three types of cell envelope stress and in the absence of RseA, there is an increase in *rpoH*



transcription (presumably  $\sigma^E$ -mediated). Loss of RseA function also appeared to result in constitutive  $\sigma^E$  activity and a constitutive envelope stress response.

### Effect of *rseA* mutation on protein localization

Since cell envelope stress is associated with protein misfolding and mislocalization and with membrane perturbation, the cellular protein profiles of wild-type *B. pertussis* UT25Sm1 and *rseA* mutant PM18 were compared. In general, the numbers and relative abundance of proteins in the culture supernatant and those found in the periplasm were increased in mutant PM18, relative to UT25Sm1 (Fig. 7A). In the outer and inner membrane fractions, several proteins of unknown identity detected in the wild-type membrane fractions were less abundant or not visible in the PM18 samples. To determine if the higher abundance of proteins observed in the PM18 culture supernatant could be attributed to PM18 cell lysis during culture, cytoplasmic malate dehydrogenase activity was measured in cell-free culture fluids. No malate dehydrogenase activity was detected in either wild-type or PM18 culture supernatant fluids, compared with positive control cell fractions, indicating that lysis of PM18 cells was not responsible for the increase in proteins in the culture fluids (data not shown). Furthermore, the turbidity and viable counts of the cultures were nearly identical for both the wild type and mutant strains (data not shown).

*B. pertussis* produces and secretes several virulence factors including CyaA, which is considered crucial for pathogenesis (Weiss *et al.*, 1984; Carbonetti, 2010). It was reasoned that RseA and the  $\sigma^E$  system might affect the localization of CyaA. CyaA is primarily localized to the bacterial cell surface (Glaser *et al.*, 1988), although that which is secreted is the form that primarily intoxicates eukaryotic cells (Gray *et al.*, 2004). The *Bordetella* protein fractions shown in Fig. 7A were subjected to immunoblotting to detect CyaA (Fig. 7B). Significantly, in this representative immunoblot and in replicate experiments, more CyaA was detected in the culture supernatant of mutant PM18 than that of the parental strain. Complementation of PM18 with the *rseA* gene supplied in *trans* appeared to restore the lower wild type levels of CyaA in the culture fluid, compared with PM18 carrying the plasmid vector control (Fig. 7C). This result was further supported by the demonstration of reduced amounts of released CyaA using the *B. pertussis* PMK22 “knock-in” strain, with *rseA* in single copy, compared with the *rseA* PM18 parent derivative (data not shown).

### Extracellular CyaA levels are increased by stress

Since the absence of RseA led to an increased amount of CyaA in the culture fluids of the *rseA* mutant, it was hypothesized that the amount of CyaA in the culture supernatants from the wild type strain would be increased by cell envelope stress. The wild type *B. pertussis* strain UT25Sm1 was exposed to three concentrations of hydrogen peroxide prior to CyaA detection in the cell-free culture supernatants (Fig. 8). There was no evidence of bacterial cell lysis on exposure to hydrogen peroxide. The levels of CyaA in the culture fluids were increased proportionally to the concentration of hydrogen peroxide used in the culture (Fig. 8A). In addition, the overall amounts of protein in the culture fluids were increased upon exposure of the bacteria to hydrogen peroxide (Fig. 8B). A similar increase in CyaA in the culture fluid following treatment with polymyxin B was also observed (data not shown).

## Analysis of extracellular CyaA

It was possible that the increased amount of extracellular CyaA from the *rseA* mutant was due to increased *cyaA* expression. Transcription studies using the wild type and *rseA* mutant strains carrying a *cyaA-lacZ* fusion showed that *cyaA* expression in both strains was similar and could also be decreased using the established method of phenotypic modulation from Bvg<sup>+</sup> to Bvg<sup>-</sup> phase using 40 mM MgSO<sub>4</sub> (data not shown) (Lacey, 1960; Melton & Weiss, 1989). Since CyaB is involved in the secretion of CyaA, the *rseA* mutant and wild type strains were tested and shown to express a *cyaB-lacZ* fusion at equivalent levels. These results indicate that the increased extracellular level of CyaA in the *rseA* mutant strain was not due to increased transcription of *cyaA* or *cyaB*.

During these studies, it was noted that in highly concentrated protein samples prepared from cell-free culture supernatant fluids, CyaA from the wild type strain appeared to be degraded to lower ~30-50 kDa molecular mass fragments. Interestingly, the *rseA* mutant samples did not exhibit this degradation. Extracellular proteins from both strains were subjected to ultrafiltration using a 100 kDa molecular mass cutoff filter and examined by immunoblot analysis to detect CyaA (Fig. 9A). In the retentate (> 100 kDa lanes), samples from wild type cells showed little immunoreactive high M<sub>r</sub> CyaA, whereas the PM18 *rseA* mutant sample primarily contained apparently intact ~180 kDa CyaA. The majority of the immunoreactive proteins from wild type cells that passed through the ultrafiltration membrane (< 100 kDa lanes) were of lower molecular mass (38-52 kDa), but a small amount of high M<sub>r</sub> CyaA was also observed. The < 100 kDa sample from the *rseA* mutant contained no obvious CyaA degradation products, and apparently intact CyaA that was in a conformation capable of transiting the membrane. In sum, these results suggested that the extracellular CyaA of wild type cells was more susceptible to degradation, compared with that of the *rseA* mutant strain.

Since *B. pertussis* has been reported to produce outer membrane vesicles containing CyaA (Hozbor *et al.*, 1999; Donato *et al.*, 2012), outer membrane vesicles from cell-free culture supernatant fluids were prepared and examined by immunoblotting (Fig. 9B). Analysis of extracellular proteins that passed through the initial 50 kDa M<sub>r</sub> cutoff ultrafiltration membrane revealed virtually no immunoreactive proteins in either the wild type or *rseA* mutant strains (Fig. 9B, Fr. 1). The extracellular protein fractions retained by the membrane were subjected to ultracentrifugation, with the resulting supernatants (Fr. 2) containing soluble proteins, and the pellet material containing crude outer membrane vesicles (OMV). The Fr. 2 soluble protein samples from wild-type cells revealed many immunoreactive bands suggesting proteolytic degradation, while samples from the *rseA* mutant exhibited what appeared to be intact CyaA. Intact CyaA was detected in the outer membrane vesicle samples from the *rseA* mutant, but no immunoreactive CyaA was observed in the vesicle samples from the wild type strain.

Examination by scanning electron microscopy showed that the *rseA* mutant cells produced more numerous and larger surface-associated vesicles, compared with the wild-type UT25Sm1 parent cells (Fig. 10). Quantitation of vesicles from four fields of view, showed there were 282 vesicles on the surfaces of 266 wild-type cells, yielding an average of 1.1 vesicles per cell. 646 vesicles were counted on 234 *rseA* mutant cells, averaging 2.8 vesicles

per cell. The vesicles on the *rseA* cells also had larger diameters than those of wild-type cells: an 82.9 nm average, versus 54.7 nm, respectively. The mutant cells also appeared to be somewhat longer than wild type cells. Overall, these results suggest that the extracellular CyaA of the *rseA* mutant is more stable than the wild type strain CyaA, which appears to be proteolytically degraded, at least under the conditions used in this study. This apparent stability may also be related to differences in the abundance or content of outer membrane vesicles, which contain CyaA.

## DISCUSSION

Orthologs of the *rpoE* gene encoding  $\sigma^E$  are widely found in bacteria, and  $\sigma^E$  function is negatively regulated by RseA-type anti-sigma factors (Raivio & Silhavy, 2001). In *E. coli*, the  $\sigma^E$  regulon includes essential genes, which may explain why *rpoE* is essential in that organism (De Las Penas *et al.*, 1997; Hayden & Ades, 2008). A recent report described the construction and characterization of a *B. bronchiseptica rpoE* mutant and noted that the *B. bronchiseptica rpoE* gene could complement an *E. coli rpoE* mutant (Barchinger *et al.*, 2012). Our inability to obtain a *B. pertussis rpoE* mutant suggests that  $\sigma^E$  is essential in this human-adapted *Bordetella* species, which is a possibility that requires further investigation. *B. pertussis* has the *rpoE*, *rseA*, and *rseB* genes as well as a predicted *mucD* ortholog. *rseA* and *rseB* orthologs have been identified in the *rpoE* gene clusters of  $\gamma$ -proteobacteria and several  $\beta$ -proteobacteria. In *E. coli* and other organisms, RseB is a periplasmic protein that binds to  $\sigma^E$  and negatively influences its function (Alba & Gross, 2004; Brown & Gulig, 2009). For  $\sigma^E$  activation, the DegS serine protease requires unassembled outer membrane protein ligands as an activating signal, while RseB function is inhibited by an undefined mechanism; both proteins play a role in sensing signals from the cell envelope. MucD, encoded in the *Pseudomonas aeruginosa* AlgU (RpoE) genetic system, is a periplasmic serine protease of the HtrA family that is involved in degrading misfolded proteins (Clausen *et al.*, 2002). The *B. pertussis rpoE*, *rseA*, *rseB* and *mucD* genes are predicted to be cotranscribed from a promoter upstream of *fabF*. In bacteria, FabF is the 3-oxoacyl-[acyl carrier protein] synthase II involved in fatty acid biosynthesis. Upstream of *B. pertussis fabF* lies the *acpP* gene, encoding a predicted acyl carrier protein, and other genes related to fatty acid synthesis including *fabH*, *fabD* and *fabG* as well as a gene similar to *maf*, annotated as encoding a protein that inhibits septum formation. Other  $\beta$ -proteobacteria such as *Burkholderia* spp. also have *fab* genes adjacent to their *rpoE* system genes. This organization of the fatty acid biosynthesis genes adjacent to the *rpoE* stress genes may reflect the involvement of the  $\sigma^E$  system in responding to cell membrane stress. That our *B. pertussis rseA* mutant produces excessive numbers of large outer membrane vesicles may also indicate a functional relationship between stress responses and membrane homeostasis. Compared with the wild-type parent, the *B. pertussis rseA* mutant PM18 exhibited an enhanced ability to grow at 25°C. Interestingly, in the barophilic *Photobacterium profundum* strain SS9, growth at low temperatures and high pressure is controlled by an  $\sigma^E$  system (Chi & Bartlett, 1995) and involves FabF-mediated alteration of membrane phospholipids to increase the amounts of unsaturated fatty acids (Allen & Bartlett, 2000).

Many functions of core members of the  $\sigma^E$  regulons appear to be conserved in bacteria (Miticka *et al.*, 2003; Rhodius *et al.*, 2006; Flannagan & Valvano, 2008; Brown & Gulig,

2009). In general, many of these gene products help to maintain the integrity of the bacterial cell envelope. In the present study, mutation of *B. pertussis rseA* resulted in a moderate increase in resistance to chemical stress, compared with the wild type strain. These differences in stress susceptibility are similar to the differences observed between *B. bronchiseptica* wild type and *rpoE* strains subjected to chemical stressors, where the mutant exhibited modest but significant increases in susceptibility to compounds such as ampicillin and SDS-EDTA (Barchinger *et al.*, 2012). The *B. bronchiseptica rpoE* mutant was no more sensitive to polymyxin B, hydrogen peroxide or osmotic stress than the wild type parent strain. These results seem to differ from our findings of increased survival of the *B. pertussis rseA* strain (over the wild type) with either hydrogen peroxide or polymyxin B treatment. However, until parallel studies using a *B. pertussis rpoE* mutant strain are performed, interpretation of these apparently contrasting results is not possible.

Our studies tested the involvement of RseA on the expression of four candidate RpoE-controlled promoters in *B. pertussis* and demonstrated that those promoters were stress-activated in both *E. coli* and *B. pertussis*. Although expression of the *B. pertussis acnA* gene encoding aconitase A was influenced by RseA, we could find no reports in the literature of *acnA* as a member of any  $\sigma^E$  regulon. The heat shock sigma factor gene, *rpoH*, is a member of multiple bacterial  $\sigma^E$  regulons (Raivio & Silhavy, 2001; Alba & Gross, 2004), and the report from Barchinger *et al.* (2012) and the present analysis indicate that this is also true for *B. bronchiseptica* and *B. pertussis*, respectively. The *Bordetella rpoH* promoter region contains a region bearing high similarity with the *E. coli*  $\sigma^E$  consensus recognition sequence, and the *B. bronchiseptica rpoE* gene was shown to function in *E. coli* to activate transcription of an *E. coli rpoH* gene fusion (Barchinger *et al.*, 2012). In the present study, the presumed activation of  $\sigma^E$  by mutation of *rseA* in *B. pertussis* resulted in constitutive *rpoH* expression.

Our results showed that in the *rseA* mutant, proteins were found at increased levels in culture supernatants and at decreased levels in the outer membrane fraction. Notably, the amount of CyaA in cell-free culture supernatants was increased by both *rseA* mutation and by envelope stress. CyaA is an important virulence factor for *B. pertussis* and has both hemolytic and cytolytic activities (Hewlett *et al.*, 1976; Glaser *et al.*, 1988; Mattoo & Cherry, 2005). Upon translocation of the catalytic domain into a eukaryotic cell, host calmodulin activates the toxin, allowing it to catalyze production of supraphysiologic levels of cAMP, leading to cytotoxicity and inhibition of cellular functions such as phagocyte chemotaxis. CyaA is secreted to the extracellular environment *via* a Type I secretion apparatus formed by the CyaB, CyaD and CyaE proteins (Glaser *et al.*, 1988). *Bordetella* CyaA can be found associated with the cell surface (Hewlett *et al.*, 1976), but Gray *et al.* reported that it was the soluble, newly secreted form of CyaA that intoxicates eukaryotic target cells (Gray *et al.*, 2004). A recent report noted that *B. pertussis* produces outer membrane vesicles in humans during infection, and experiments showed that *B. pertussis* outer membrane vesicles contain CyaA (Donato *et al.*, 2012). These investigators also reported that the CyaA within vesicles represents about 1.2 % of the total amount of CyaA released into *B. pertussis* culture supernatant fluids. In that report, as well as another (Hozbor *et al.*, 1999) *B. pertussis* cells were noted to naturally produce rather low levels of outer membrane vesicles. Additionally in those studies, suspensions of *B. pertussis* cells were sonicated to artificially generate

greater numbers of vesicles that were shown to contain CyaA; sonically generated vesicles also contain pertussis toxin and other proteins (Hozbor *et al.*, 1999). Our findings confirmed that, compared with the *rseA* mutant, wild type *B. pertussis* naturally produces low numbers of outer membrane vesicles (data not shown) which contained little CyaA (Fig. 9B). Scanning electron microscopy revealed that the *rseA* mutant not only produced more numerous cell surface-associated vesicles than the wild type parent, but those vesicles were larger in size. In unfractionated extracellular culture fluids, a large proportion of wild type CyaA appeared to be degraded to 38-50 kDa fragments, which has also been previously described in the literature (Ladant *et al.*, 1986). If the vesicle-associated CyaA is only a small fraction of the total found in cell-free supernatants, as reported by Donato *et al.* (2012) and supported by our findings (Fig. 9B), then the majority of it that is in the culture supernatant may be susceptible to proteolysis. However, this hypothesized proteolysis was not observed for the extracellular CyaA of the *B. pertussis rseA* mutant. The CyaA in sonically manufactured *B. pertussis* vesicles was shown to be sensitive to exogenously added trypsin, indicating surface exposure (Donato *et al.*, 2012). Our results suggest that the *rseA* mutant extracellular CyaA, whether associated with naturally produced vesicles or free in the culture supernatant, is either sequestered within the vesicles or is otherwise resistant to proteolytic attack. It is also possible that the loss of RseA function interferes with the production or activity of one or more proteases that may act on CyaA.

Outer membrane vesicle formation has been reported as a  $\sigma^E$ -mediated stress response in Gram negative bacteria (McBroom & Kuehn, 2007). Vesicle formation may be one way for cells to rid the periplasm of excess protein that may induce envelope stress. During growth on the ciliated respiratory epithelial surface, *B. pertussis* may be subjected to changes in osmolarity and pH, or the presence of complement components and cationic antimicrobial peptides. *B. pertussis* would also be exposed to significant oxidative stress upon phagocytosis. The *Bordetella*  $\sigma^E$  system may play a role in the deflection of, and recovery from, such stresses and may also control the release of virulence factors such as CyaA, which is known to dysregulate phagocytic cell function.

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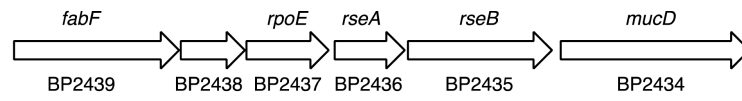
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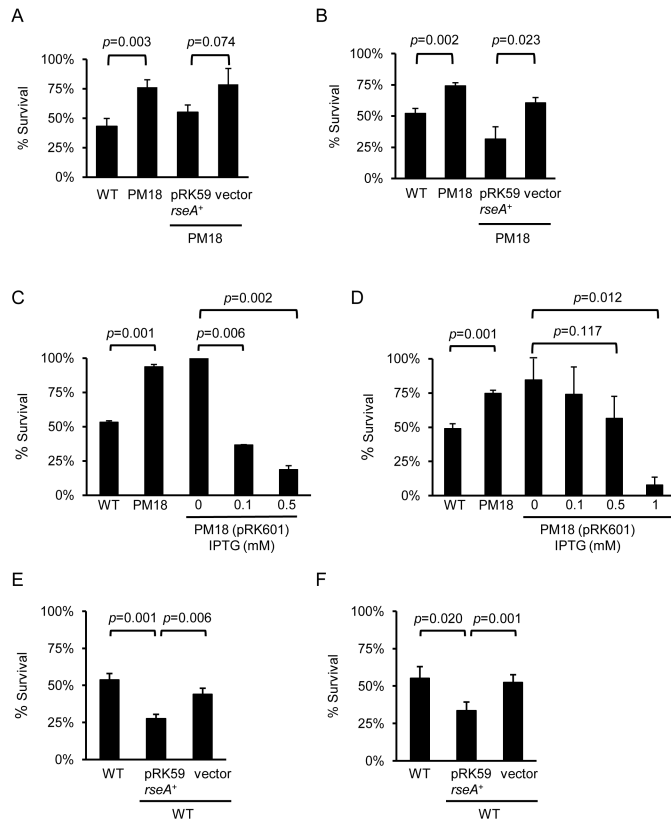
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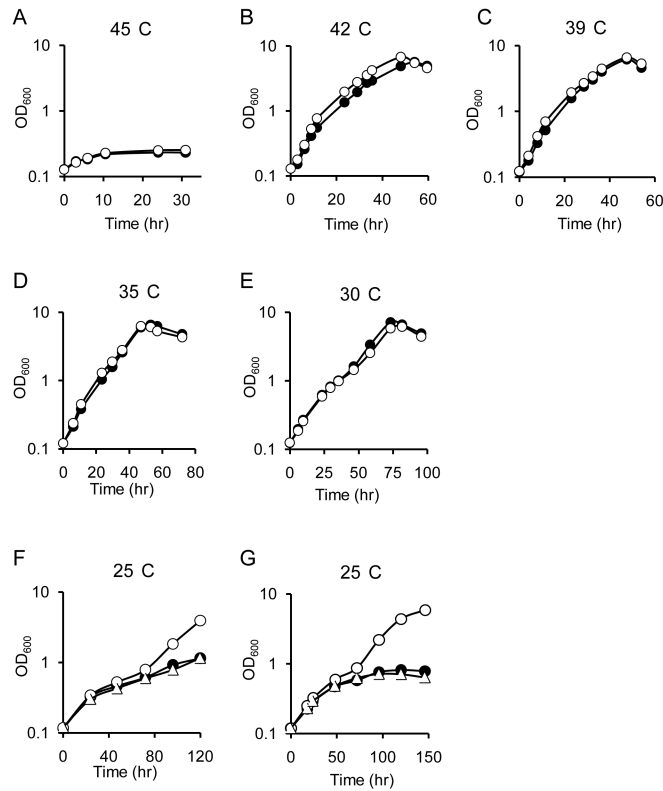


**Figure 1. Genetic organization of the *B. pertussis* *rseA* gene cluster**

A schematic of the *B. pertussis* strain Tohama I DNA region is shown; arrows denote the spatial limits and transcriptional orientation of the genes. Gene locus tags are indicated.

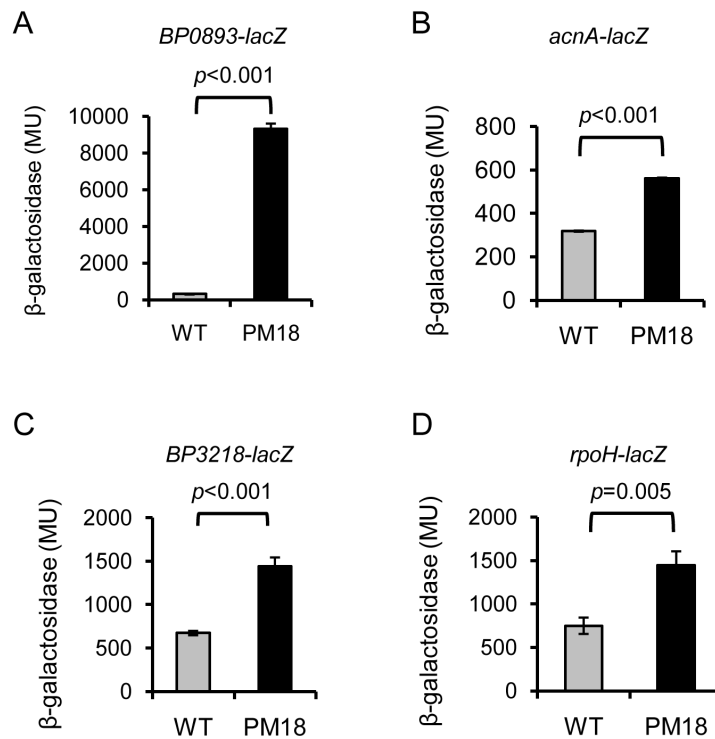


**Figure 2. Sensitivity of *B. pertussis* strains to stress conditions and effect of *rseA* overexpression**  
 Cells were untreated or treated for 1 h with 40 mM hydrogen peroxide (A) or 5  $\mu\text{g ml}^{-1}$  polymyxin B (B). Wild-type UT25Sm1; WT, isogenic *rseA* mutant strain PM18, and PM18 carrying the *rseA*<sup>+</sup> plasmid pRK59 or the pRK415 vector control were cultured as described in Materials and Methods. Wild-type strain UT25Sm1 (WT), *rseA* mutant PM18, strain PM18 carrying the IPTG-inducible *rseA*<sup>+</sup> plasmid pRK601, and UT25Sm1 carrying the pRK59 (*rseA*<sup>+</sup>) or the pRK415 vector control plasmid were cultured as described in Materials and Methods. Cells were treated with hydrogen peroxide (C, D) and polymyxin B (E, F). Panels C, D: pRK601-carrying cells were supplemented with the denoted concentrations of IPTG or were unsupplemented (control). Percent survival indicates the ratio of the numbers of stressed bacterial cells per ml compared with the cell numbers from untreated cultures. Means and standard deviations of a representative of three experiments are shown. The data were analyzed by Student's t test (*p* values are shown).

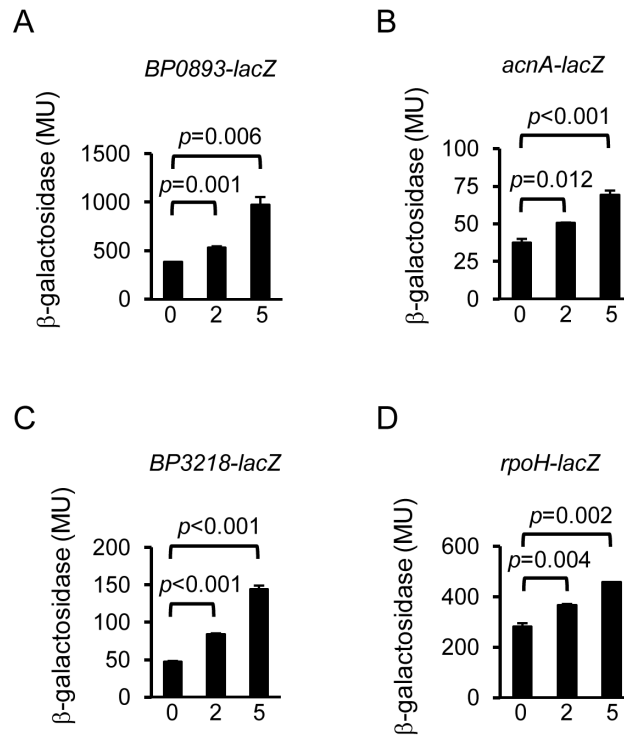


**Figure 3. Influence of temperature on growth of *B. pertussis* strains**

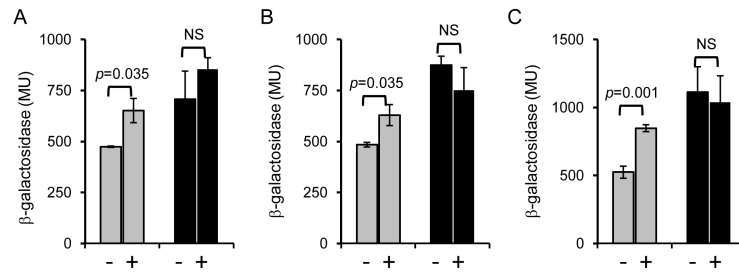
Strains were grown in SS medium at the indicated temperature and the OD<sub>600</sub> values of the cultures were measured over time. Panels A – E: wild-type UT25Sm1 (closed circles), *rseA* mutant PM18 (open circles). Panel F, wild-type UT25Sm1 (closed circles), PM18 (open circles), PM18(pRK59)(RseA<sup>+</sup>) (open triangles). Panel G, wild-type UT25Sm1 (closed circles), PM18 (open circles), PMK22 (RseA<sup>+</sup>) (open triangles). The result of a representative experiment that was repeated independently three times is shown.



**Figure 4. Effect of RseA on the expression of predicted RpoE-regulated genes in *B. pertussis*** *lacZ* transcriptional fusions to predicted RpoE target gene promoter regions were constructed and the plasmids transferred to *B. pertussis* wild-type strain UT25Sm1 (gray bars) and isogenic *rseA* mutant strain PM18 (black bars). Strains were grown in SS medium and  $\beta$ -galactosidase activity was measured as described in Materials and Methods. Gene fusions are indicated above each graph. Results are reported in Miller units; means and standard deviations of a representative of three experiments are shown. The data were analyzed by Student's *t* test (*p* values are shown).

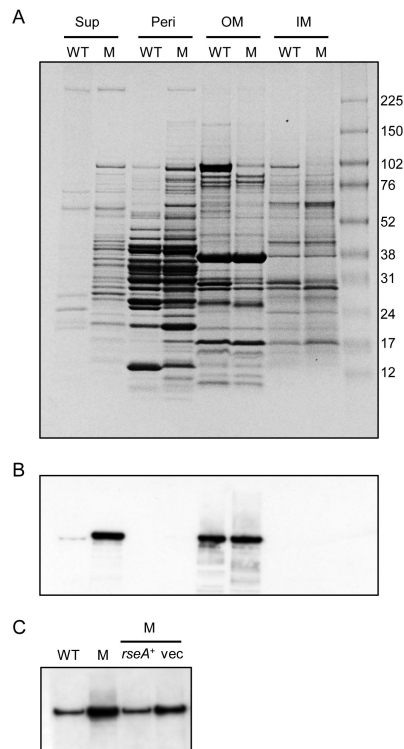


**Figure 5. Stress-induced expression of predicted *B. pertussis*  $\sigma^E$ -regulated genes in *E. coli*** *lacZ* transcriptional fusion plasmids were transferred to *E. coli* strain DH5 $\alpha$ . Bacteria were grown in M9 medium for 6.5 h, then treated for 30 min. with 0, 2 or 5  $\mu\text{g ml}^{-1}$  of polymyxin B.  $\beta$ -galactosidase activity was measured as described in Materials and Methods and the results are indicated in Miller units. Gene fusions are indicated above each graph. Means and standard deviations of a representative of three experiments are shown. The data were analyzed by Student's t test (*p* values are shown).



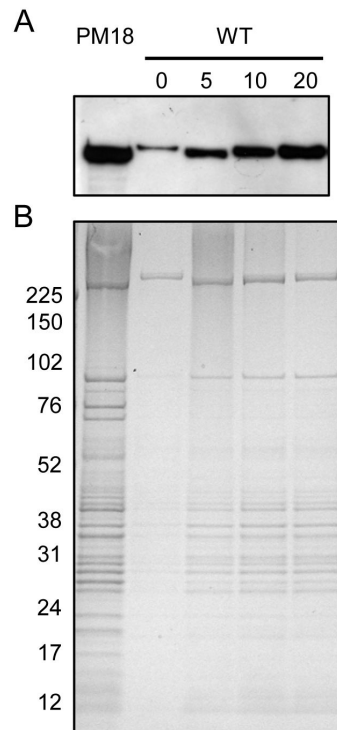
**Figure 6. Analysis of *rpoH* expression in *B. pertussis***

Wild-type strain UT25Sm1 (gray bars) and isogenic *rseA* mutant PM18 (black bars), each carrying the *rpoH-lacZ* fusion plasmid pM02, were assessed for  $\beta$ -galactosidase activity after 3h exposure to cell envelope stress (+) or in the absence of stress (-). A, treatment with 20 mM hydrogen peroxide; B, treatment with 2  $\mu\text{g ml}^{-1}$  ml polymyxin B; C, treatment with 0.4 M sucrose.  $\beta$ -galactosidase activity was measured as described in Materials and Methods and the results indicated in Miller units. Means and standard deviations of a representative of three experiments are shown. The data were analyzed by Student's t test ( $p$  values are shown; NS, not significant).



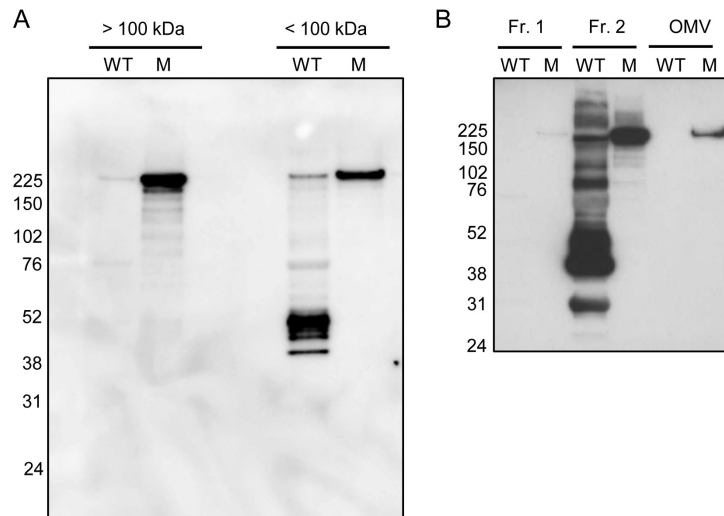
**Figure 7. *rseA* mutation alters *B. pertussis* protein localization and CyaA release**

Cell-free culture supernatant (Sup), periplasmic (Peri), outer membrane (OM) and inner membrane fractions (IM) were prepared as described in the Materials and Methods. Panel A: Normalized protein loads from wild-type UT25Sm1 (WT) and *rseA* mutant PM18 (M) were subjected to SDS-PAGE. Panel B: CyaA was detected in the *Bordetella* fractions by immunoblotting a replicate gel and using specific monoclonal antibody. Panel C: proteins in culture supernatant fluids were prepared from *B. pertussis* UT25Sm1 (WT), *rseA* mutant PM18 (M), and PM18 carrying plasmid pRK59 (*rseA*<sup>+</sup>) or the pRK415 plasmid vector control (vec); CyaA was detected by immunoblotting. Protein amounts corresponding to the supernatant of a culture with an OD<sub>600</sub> of 1.5 were loaded onto gels. Molecular mass markers in panel A are indicated in kDa to the right. Panels B, C: immunoreactive CyaA migrated with an apparent mass of approximately 200 kDa.



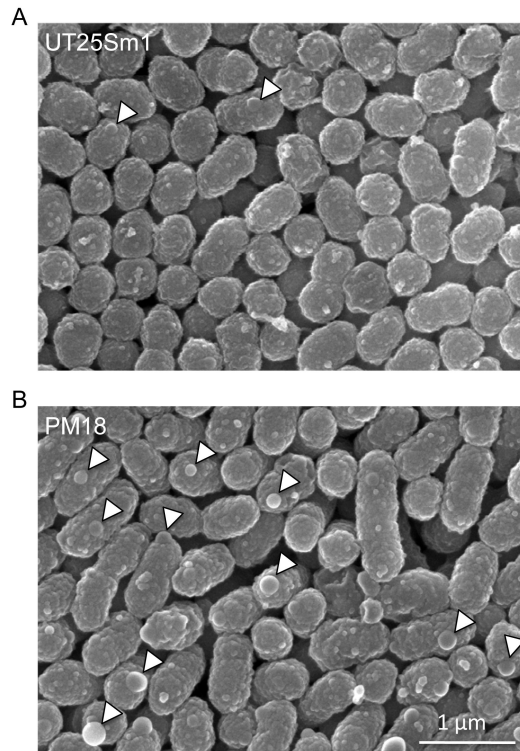
**Figure 8. Hydrogen peroxide-mediated stress induces release of CyaA into the culture medium**  
Cell-free culture fluids from wild-type *B. pertussis* UT25Sm1 (WT) were prepared 6 h after addition of hydrogen peroxide, at the indicated mM concentrations, to exponentially growing bacterial cultures. Sample preparation and immunoblotting with anti-CyaA antibody (A) and SDS-PAGE and staining with Coomassie Brilliant Blue (B) were performed as described in the Materials and Methods. Untreated PM18 *rseA* mutant cell samples are shown in the left lanes. Migration positions of the molecular mass markers are indicated, in kDa.





**Figure 9. Enhanced stability of extracellular CyaA from the *rseA* mutant**

*B. pertussis* UT25Sm1 (WT) and *rseA* mutant PM18 (M) were grown in SS medium and cell-free extracellular fluids were obtained, fractionated, and analyzed by SDS-PAGE and immunoblotting to detect CyaA. Panel A: > 100 kDa, protein fractions retained by a 100 kDa molecular weight cutoff filter; < 100 kDa, proteins that passed through the filter. Protein amounts corresponding to the supernatant of a culture with an OD<sub>600</sub> of 4.0 for samples were loaded onto gels. Panel B: extracellular proteins were subjected to ultrafiltration using a 50 kDa molecular weight cutoff filter. Fr. 1, proteins that passed through the filter. Proteins retained by the filter were then fractionated by ultracentrifugation: Fr. 2, proteins in the supernatant fluids; OMV, pelleted material enriched in outer membrane vesicles. Protein amounts corresponding to the supernatant of a culture with an OD<sub>600</sub> of 6.0 were loaded onto gels.



**Figure 10. Cellular morphology of *B. pertussis* wild-type strain UT25Sm1 (A) and *rseA* mutant strain PM18 (B)**  
Bacterial cells were prepared for scanning electron microscopy as described in Materials and Methods. Arrowheads denote large vesicular structures. Magnification: 20,000 ×.

**Table 1**

## Bacterial strains and plasmids used in this study

Bacterial strain	Relevant genotype or description	Reference
<i>B. pertussis</i> UT25Sm1	Spontaneous streptomycin-resistant derivative of wild type isolate <i>B. pertussis</i> UT25	(Field & Parker, 1978; Brickman & Armstrong, 1996)
<i>B. pertussis</i> PM18	UT25Sm1; <i>rseA</i>	This study
<i>B. pertussis</i> PMK22	PM18; wild type <i>rseA</i> allele reconstructed by allelic exchange	This study
<i>E. coli</i> DH5 $\alpha$	Host strain for general genetic methods	Invitrogen
Plasmids		
pRK2013	Helper plasmid for conjugation, kan <sup>r</sup>	(Figurski & Helinski, 1979)
pRK415	Low copy broad-host-range cloning vector, tet <sup>r</sup>	(Keen <i>et al.</i> , 1988)
pRK59	pRK415 with BamHI and EcoRI insert fragment containing <i>B. pertussis rseA</i>	This study
pRK60	pRK415 with 1.8-kb fragment containing <i>lacI</i> and <i>Ptac-malE</i>	This study
pRK601	pRK60 with promoterless <i>B. pertussis rseA</i> for IPTG-inducible expression construction	This study
pMAL-c2	Source of 1.82-kb fragment containing <i>lacI</i> and <i>Ptac-malE</i>	New England Biolabs Inc.
pSS1129	Allelic exchange plasmid gen <sup>r</sup> , amp <sup>r</sup>	(Stibitz, 1994)
pSSK26	pSS1129 with 2.0-kb DNA fragment encoding wild type <i>rseA</i>	This study
pSS20	pSS1129 with 1.86-kb DNA fragment encoding <i>rseA</i> allele; gen <sup>r</sup>	This study
pSS22	pSS1129 with 1.43-kb DNA fragment encoding <i>rseA</i> allele; gen <sup>r</sup>	This study
pMP220	Contains promoterless <i>lacZ</i> for transcriptional fusion construction	(Spaink <i>et al.</i> , 1987)

**Table 2**

Oligonucleotide primers used for PCR amplification in this study

PCR primer	Nucleotide sequence
rpoE1	5'-GGCC <u>AAGCTT</u> CCGGAGTGCACCTGGATTAC-3'
rpoE2	5'-AACCGACTGGCAATGGCGGCTTCTCGCGACATCCT-3'
rpoE3	5'-ATGTCGCGCAGGAAGCCGCCATTGCCAGTCGGTTGC-3'
rpoE4	5'-GGCGCCTGCTGGATGCTGGAGA-3'
rseA1	5'-GGCC <u>GAATTC</u> AGGCGGGTCTCGGGTTAC-3'
rseA2	5'-TCAACGTCCTGCTCCGGCGAGGGACTTGGCTGCGGTTTG-3'
rseA3	5'-ACCGCAGCCAAGTCCCTCGCCGAGCAGGACGTTGATGAC-3'
rseA4	5'-GGCC <u>GGATCCC</u> CTTTGCGGGCTTATTGTTGTTT-3'
rseA-BamHI	5'-GGCC <u>GGATCC</u> GGTTCGTTTCGCGCATATTCAGAGC-3'
rseA-EcoRI	5'-GGCC <u>GAATTC</u> GGCGCATGTAAAGCAACGGTGTCA-3'
rseA-HindIII	5'-GGCC <u>AAGCTT</u> GGCGCATGTAAAGCAACGGTGTCA-3'
pMal-EcoRI/F	5'-GGCC <u>GAATTC</u> CCAGCCTAGCCGGTCTCTCAAC-3'
pMal-EcoRI/R	5'-GGCC <u>GAATTC</u> CAGCGTCGTGTGCCAGAAGATAA-3'
cyaA-EcoRI	5'-GGCC <u>GAATTC</u> CGCTTGCTCGCTTATTATC-3'
cyaA-XbaI	5'-GGCC <u>TCTAGAT</u> GTGTAGCGCTCAGAACCTC-3'
cyaB-EcoRI	5'-GGCC <u>GAATTC</u> GGTCGAGGCAATGGCGCAGTATCC-3'
cyaB-XbaI	5'-GGCC <u>TCTAGAC</u> CCAGCATGACCAGGCAGAGCAACC-3'
rpoH-EcoRI	5'-GGCC <u>GAATTC</u> GGCTGGTAAATAAGGGACGCATAG-3'
rpoH-XbaI	5'-GGCC <u>TCTAGAC</u> CGGAAAGGGCCAACGAAC-3'
BP0893-EcoRI	5'-GGCC <u>GAATTC</u> CATCGCCAATAACAGTTTCTTTTC-3'
BP0893-XbaI	5'-GGCC <u>TCTAGAT</u> GTCTCGCGCAGCCAATCC-3'
BP2014-EcoRI	5'-GGCC <u>GAATTC</u> CGCGCGGCTGGCTGTTCTGAGTC-3'
BP2014-XbaI	5'-GGCC <u>TCTAGAC</u> CCAGGGCCGCGCAGGAATAGA-3'
BP3218-EcoRI	5'-GGCC <u>GAATTC</u> CCCTGACAACCGGCTGAACTATT-3'
BP3218-XbaI	5'-GGCC <u>TCTAGAT</u> CTGGCCGACTGCAACAACCTGAT-3'

Restriction site adapter sequences used for cloning are underlined.