Dissection of σ^E-dependent cell lysis in *Escherichia coli*: roles of RpoE regulators RseA, RseB and periplasmic folding catalyst PpiD

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To understand the mechanism of σ^E-dependent cell lysis, we examined the consequences of deletion derivatives of *rpoE* regulators *rseA*, *rseB* and *rseC* on σ^E transcription, on levels of free versus membrane-bound σ^E and on OMP-biogenesis limiting factor(s) that could impact cell lysis. RT-PCR showed that individual nonpolar Δ*rseA* and Δ*rseB* increased the *rpoE* expression to varying extents, with pronounced induction in Δ*rseA*. Significantly the ratio of soluble (free) versus membrane-bound form of RpoE increased in Δ*rseA*, however without increase of its total amount, unraveling furthermore complexity in RpoE regulation. Significant characteristics of cell lysis, accompanied by a severe reduction in the levels of periplasmic OMP-folding factor (PpiD), were observed in Δ*rseA*. The cell-lysis phenotype of Δ*rseA* was suppressed by either *rseA* or *ppiD* plasmids, but neither by *rseB* nor by *rseC* clones. However, the cell lysis of the wild-type strain was almost completely repressed not only by the *rseA* clone but also by the *rseB* clone, suggesting RseB might be limiting *in vivo*. Thus, increase in the ratio of free σ^E in *rseA* mutants with a concomitant reduction in PpiD levels can account for σ^E-dependent lysis in concert with a potential role of small RNAs on the lysis process.

Introduction

σ^E is a heat-shock sigma factor that was first discovered in *Escherichia coli* as a positive regulator for σ^H, another heat-shock sigma factor (Erickson & Gross 1989; Wang & Kaguni 1989; Raina *et al*. 1995; Rouvière *et al*. 1995). *rpoE*, encoding σ^E, is an essential gene required for growth at both high and low temperatures (Hiratsu *et al*. 1995; De Las Peñas *et al*. 1997a). The gene is located in the *rpoE-rseABC* operon with two promoters P1 and P2 located upstream of the *rpoE* ORF, which are recognized presumably by σ^D, the main sigma factor for the exponential growth, and σ^E, respectively (Raina *et al*. 1995; Rouvière *et al*. 1995). Such a promoter organization allows a positive auto-regulation of the operon and its sustained transcription under variety of growth conditions. σ^E is involved in the cellular response to extracytoplasmic stresses and appears to express over 100 genes, including *rpoH* encoding σ^H and *rpoD* encoding σ^D genes (Missiakas & Raina 1997; Dartigalongue *et al*. 2001; Kabir *et al*. 2005). To perform the task, an ingenious regulatory system for σ^E activity seems to have evolved. Most of the σ^E molecules usually remain sequestered by the interaction with the inner membrane protein RseA (Missiakas *et al*. 1997; De Las Peñas *et al*. 1997b; Collinet *et al*. 2000).

With the current evidence, it is established that RseA primarily functions as an antisigma factor for RpoE by sequestering it to the inner membrane thereby preventing the formation of σ^E-RNA polymerase core complex. RseB is known to interact with periplasmic C-terminal domain of RseA and this interaction is supposed to furthermore fine-tune the negative regulation by RseA antisigma factor (De Las Peñas *et al*. 1997b; Missiakas *et al*. 1997). *In vitro*, it has been shown that RseB inhibits proteolysis of RseA by DegS (Cezairliyan & Sauer 2006). In parallel, RseB also seems to be required for the function of essential RseP (EcfE) protease that acts as a site 2 specific protease, cleaving RseA in the inner membrane after DegS protease action (Grigorova *et al*. 2004). The extent to which

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RseB plays role in the induction of σ^E and its physiological significance in controlling σ^E function is not clear. This is not surprising given the lack of any described robust phenotype of *rseB* mutants. However, RseC may exert a subtle but positive modulating effect on σ^E (Missiakas *et al.* 1997) or function in the reducing system of the superoxide sensor SoxR, which is involved in protection of cells against superoxide and nitric oxide (Koo *et al.* 2003).

The programmed cell death (PCD) is a mechanism for the removal of damaged or unnecessary cells. In *E. coli*, σ^E-dependent cell lysis has been demonstrated (Kabir & Yamada 2005) to be akin to the addiction-module (toxin–antitoxin)-directed (Hazan *et al.* 2004) and mutagen-directed PCDs (Walker 1996). A large number of cells defective in colony forming ability, that is, viable but nonculturable (VBNC) cells (Nitta *et al.* 2000; Desnues *et al.* 2003; Nyström 2003; Cuny *et al.* 2005), which accumulate in the *E. coli* early stationary phase, have been shown to be significantly lysed in the *rseA*::Tn*10*Kan background with increased expression of the *rpoE* encoding σ^E. However, culturable cells capable of forming colonies are not affected (Nitta *et al.* 2000). The expression of *rpoE* increases in the wild-type strain in the early stationary phase (Nitta *et al.* 2000; Costanzo & Ades 2006), even though its cell lysis occurs to a lower extent than that of the *rseA* mutant. The VBNC population at the stationary phase appears to contain damaged cells, since the lysis is enhanced in an *rpoS* background (Kabir *et al.* 2004b). The global gene expression along with the process toward the lysis has been analyzed (Kabir *et al.* 2004a, 2005). However, the molecular mechanism of σ^E-dependent cell lysis has not been elucidated yet.

Majority of the genes belonging to the RpoE regulon, whose transcription is positively regulated by $E\sigma^E$ polymerase, seem to be dedicated to function for maintaining the integrity of outer membrane (OM) or maturation of outer membrane proteins (OMPs) (Missiakas *et al.* 1996a; Dartigalongue *et al.* 2001; Kabir *et al.* 2005). This includes genes like *surA*, *skp*, *htrA*, *ecfK* (*yaeT*) and some of the genes involved in LPS biosynthesis and its translocation. Collectively, they perform important functions for assembly and maturation of OMPs. However, none of the tested regulon members were found directly to be involved in cell lysis (Kabir *et al.* 2005). Paradoxically, the synthesis of major OMPs is negatively regulated upon RpoE induction, which is ascribed to RpoE-dependent induction of synthesis of noncoding small RNAs, which in turn repress the OMP synthesis at post-transcriptional level, presumably to maintain a tight control of OMP levels (Valentin-Hansen *et al.* 2007).

Since σ^E-dependent cell lysis is a highly reproducible and quantifiable phenotype associated with σ^E induction, we examined roles of *rseABC* genes and any additional limiting factors in its control. The fine-tuned regulation of concentration of the active σ^E in response to cellular damage levels might decide either repairing damaged proteins or killing cells. In this work, we first focused on constituents of the *rpoE-rseABC* operon and examined the effects of deletion mutants and plasmid clones of the individual gene on the damaged cell lysis, since their relative contributions to σ^E-dependent cell lysis are not known at present. Next, we found the levels of PpiD are severely diminished in *rseA* mutants, and the *ppiD* gene, when expressed from a plasmid, can efficiently suppress the cell lysis of *rseA* mutants, suggesting that cell lysis in Δ*rseA* mutants due to RpoE activation results from defects in maturation and synthesis of OMPs. Presented data suggest that RseA and PpiD are capable of repressing the σ^E-dependent cell lysis, that RseB might be limiting *in vivo* and that RseC protects a cell lysis phenomenon distinct from the σ^E-dependent cell lysis at 45 °C.

Results

Effect of the deletion mutations of *rseA*, *rseB* and *rseC* on the σ^E-dependent cell lysis

We previously demonstrated that σ^E-dependent cell lysis phenotype is characterized by decrease in cell density without significant influence in colony forming unit (CFU) and by protein accumulation in the medium under growth conditions of 37 °C (Nitta *et al.* 2000; Kabir *et al.* 2005). Since σ^E was first identified as a heat-shock sigma factor (Erickson & Gross 1989; Wang & Kaguni 1989; Raina *et al.* 1995; Rouvière *et al.* 1995), we examined whether the deletion mutants of *rseA, rseB* and *rseC* showed altered cell lysis phenotype at different temperatures (Fig. 1). At all temperatures tested, Δ*rseA* showed a significant decrease in optical density at 600 nm (OD_{600}), but Δ*rseB* and Δ*rseC* showed nearly similar OD_{600} as compared to that of the wild-type strain (Fig. 1A). The reduction in OD_{600} was more at higher temperature in Δ*rseA*, accompanied by decrease in CFU. However, at 45 °C, further reduction in the OD_{600} of Δ*rseA* bacteria stopped after 48 h of incubation, where cells might cease the σ^E-dependent lysis and presumably became completely defective cells, which were indicated by a large reduction of CFU upon prolonged incubation (60 or 72 h) (Fig. 1B). Notably, Δ*rseA*, as well as the other two mutants, showed nearly similar patterns of CFU curves to that of the wild-type strain (Fig. 1B).

Next, lysis protein accumulation in culture medium of the three deletion mutants as well as of the wild-type strain was analyzed (Fig. 1C). At 30 °C, proteins appeared in the medium fractions of Δ*rseA* after 36 h, and amounts

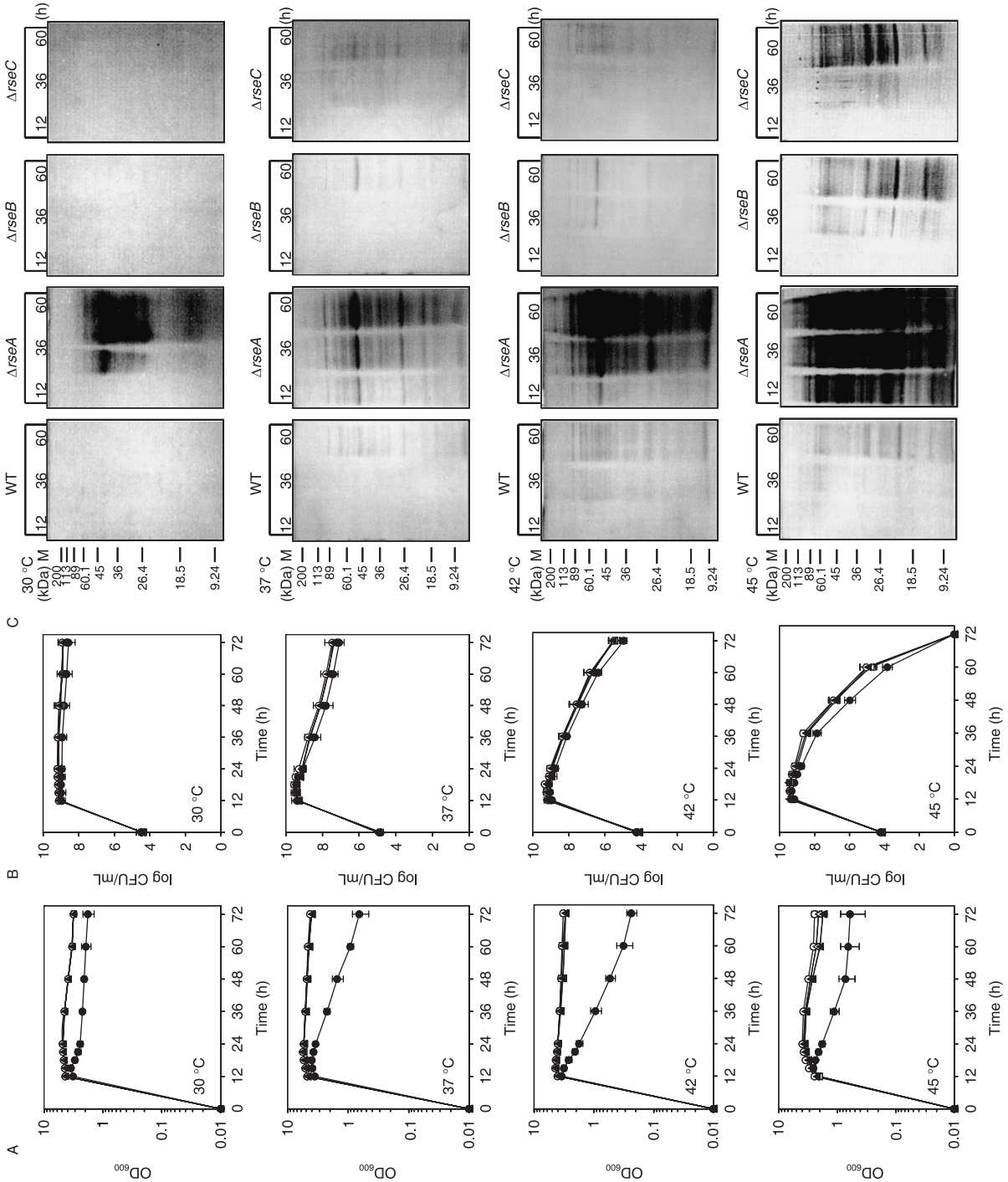

Figure 1 Effects of the Δ*rseA*, Δ*rseB* or Δ*rseC* mutation on cell turbidity, CFU and protein accumulation in medium at different temperatures. Wild-type (open circles), Δ*rseA* (closed circles), Δ*rseB* (open triangles) and Δ*rseC* (closed triangles) strains were grown in LB medium at 30, 37, 42 and 45 °C with shaking. (A) Cell turbidity at 600 nm (OD_{600}) and (B) CFU were measured at various times as indicated. The experiments were carried out three times. Error bars represent standard deviation ± mean. (C) Detection of cell lysis through protein accumulation in medium fractions. Aliquots were removed from the culture at 12, 36 and 60 h, and were fractionated as described in Experimental procedures. Proteins in medium fractions, corresponding to 0.6 mL culture, were separated by 12% SDS–PAGE and stained with Coomassie brilliant blue. M represents prestained protein markers. The experiments were carried out three times, and the patterns were shown to be reproducible. Data from one representative experiment are shown.

of proteins, as visualized by the intensity of the bands, increased with the cultivation time, whereas the Δ*rseB*, Δ*rseC* and wild-type bacteria showed no such appearance of proteins at all times tested. At the temperatures above 37 °C, further significant increase in the amounts of proteins were found in the Δ*rseA* strain at each time tested compared with those at 30 °C. Few proteins, although lower in abundance, were detected at 60 h at 37 °C and 42 °C in the wild-type and Δ*rseB* strains as compared to that in Δ*rseC*. These data clearly showed that the stationary phase proceeded, the OD_{600} of Δ*rseA* further decreased with a gradual increase in lysis protein accumulation in the medium at 30–42 °C.

At 45 °C, protein accumulation in Δ*rseA* increased at 12 h compared to those at other temperatures. Interestingly, at this temperature, the decrease in OD_{600} and the increase in accumulation of proteins in Δ*rseA* appeared to cease around 48 h (Fig. 1A,C), presumably due to the high temperature stress, which resulted in drastic reduction in CFU upon furthermore incubation and may hamper the σ^E-dependent lysis. An increase in protein accumulation in Δ*rseB* and Δ*rseC* strains at 45 °C over other temperatures was observed after 36 h as compared to the wild-type strain. Therefore, lack of RseB and RseC appears to have a minor effect for the protein accumulation in the σ^E-dependent cell lysis up to 42 °C but may prevent cell lysis at higher temperature such as 45 °C. However, the cell lysis phenotype at 45 °C in case of Δ*rseC* might be σ^E-independent (see below). As a test for cell lysis, we measured β-galactosidase activity using the wild-type, Δ*rseA*, Δ*rseB* and Δ*rseC* strains. The β-galactosidase activity in the medium fractions of wild type, Δ*rseA*, Δ*rseB* and Δ*rseC* were estimated around 19%, 85%, 23% and 16%, respectively, of the sum of that of medium and remaining fractions. The significant increase in β-galactosidase activity in Δ*rseA* medium fraction indicates the accumulation of cytoplasmic proteins in medium due to cell lysis.

Complementation of the Δ*rseA* phenotype by the *rseA* plasmid clone

Although the deleted regions in Δ*rseA*, Δ*rseB* and Δ*rseC* encompassed from the second codon of each gene to the front of the Shine–Dalgarno sequence for the just downstream gene, we examined the complementary effect of the *rseA, rseB* or *rseC* plasmid clone on the Δ*rseA* strain. Examination of the colony morphology on LB plates after 48 h incubation showed that the Δ*rseA* strain formed flat and thin colonies at temperatures above 37 °C and slightly flat colonies at 30 °C, which seems to be consistent with the accumulated protein level in liquid medium. The formation of flat colonies seems to reflect the lysis of cells defective in colony formation. In contrast, both Δ*rseB* and Δ*rseC* showed round colonies, thus being indistinguishable from those of the parental strain. Introduction of the *rseA* plasmid clone, but not the *rseB* or *rseC* plasmid clone, changed the Δ*rseA* strain to form round colonies similar to those of the wild-type strain. The effect of these plasmid clones on the OD_{600} in growth or accumulation of proteins in the Δ*rseA* strain was also tested. Only the *rseA* plasmid clone recovered the OD_{600} (data not shown) and reduced protein accumulation (Fig. 2A) in medium to the level of the wild-type strain.

Effect of *rseA*, *rseB* or *rseC* plasmid clones on the σ^E-dependent cell lysis in the wild-type strain

As reported previously, the wild-type strain also showed accumulation of proteins in liquid medium in early stationary phase, consistent with increased expression of the *rpoE* gene in this phase (Nitta *et al.* 2000). If the σ^E-dependent cell lysis was responsible for this protein accumulation, the control factors for the *rpoE* expression were assumed to repress the protein accumulation of the wild-type cells. The *rseA*, *rseB* and *rseC* plasmid clones were thus introduced into the wild-type strain and examined for protein accumulation in the culture medium. Samples of medium fractions of the wild type, equivalent to a 20-fold volume of that of the *rseA* mutant strain used in experiments for which results are shown in Figs 1(C) and 2(A), were applied on 12% SDS–PAGE. This analysis showed that not only *rseA* clone, but also *rseB* clone repressed the protein accumulation (Fig. 2B). The amount of proteins observed at every time of sampling shows this phenomenon of reduction in the accumulated proteins by introduction of either *rseA* or *rseB* plasmids (Fig. 2B). The expression of these genes from the plasmid clones was assumed to be very low because arabinose as an inducer was not added in the culture medium. Given the suppression observed by RseB plasmid in the wild type only but not in Δ*rseA*, suggest that RseB-mediated suppression of cell lysis requires *rseA* wild-type copy. Consistent with these results, introduction of *rse*A plasmid clone in Δ*rseB* also caused repression of cell lysis phenotype as was observed in the wild type (Fig. 2C). Taken together, it can be concluded that RseA suppresses the lysis in the wild-type strain independent of RseB. In contrast, RseC was found to slightly increase the protein accumulation in the wild type. This result is consistent with the minor protein bands in Δ*rseC* compared to the wild-type or Δ*rseA* or Δ*rseB* strains at 37 °C and 42 °C as observed in Fig. 1(C). Therefore, these data suggest a minor positive role for RseC in the regulation of cell lysis control by *rpoE-rseABC* operon, supporting a similar previously proposed role for RpoE regulation (Missiakas *et al.* 1997).

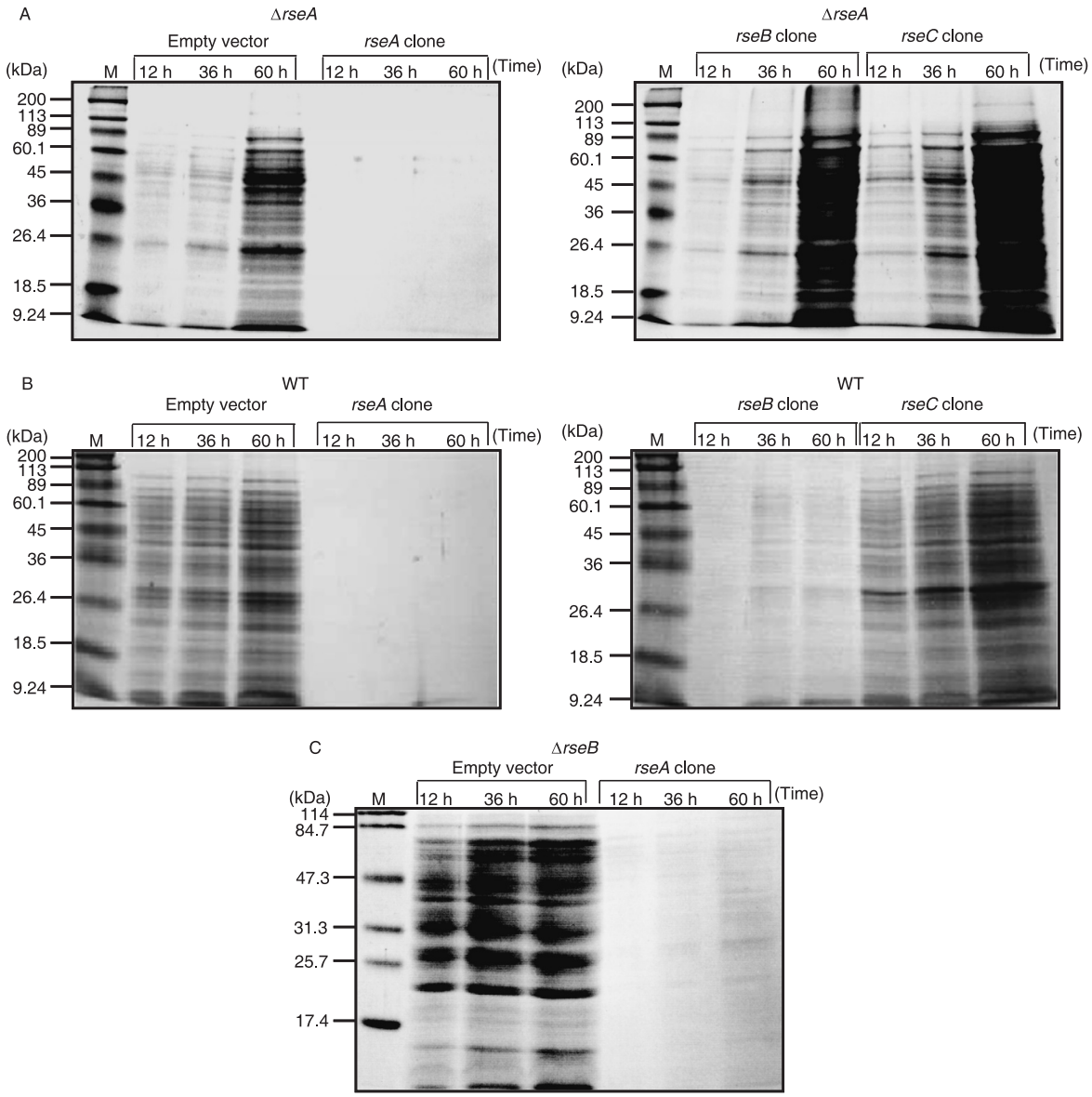

Figure 2 Effects of introduction of *rseA*, *rseB* or *rseC* plasmid clone on the cell lysis in Δ*rseA* and the wild-type (WT) strains. (A) Δ*rseA*/pBAD24 (empty vector), Δ*rseA*/pBADRSEA, Δ*rseA*/pBADRSEB and Δ*rseA*/pBADRSEC cells, (B) W3110/pBAD24, W3110/pBADRSEA, W3110/pBADRSEB and W3110/pBADRSEC cells or (C) Δ*rseB*/pBAD24, Δ*rseB* /pBADRSEA cells were grown in LB medium at 37 °C with shaking. Aliquots were removed from the culture at 12, 36 and 60 h, and were fractionated as described in Experimental procedures. Proteins in medium fractions from 0.6 mL culture of Δ*rseA* strain and from 12 mL culture of the wild-type or Δ*rseB* strain were examined as in Fig. 1C. M represents prestained protein markers. The experiments were carried out three times, and the patterns were shown to be reproducible. Data from one representative experiment are shown.

Effect of *rseA* and *rseB* plasmid clones on the transcription from *rpoE*P2 promoter in Δ*rseA* and the wild-type strain

As showed from data presented in Fig. 2, the suppression of lysis by RseB in the wild-type strain, but not in Δ*rseA*, led us to assume that RseB function requires RseA to negatively regulate *rpoE-rseABC* operon. Therefore, the *rpoE* activity was assayed by measuring the activity of *rpoE*-dependent promoter fusions both in *rseA* null mutant and the wild-type strain, harboring either RseA or RseB plasmid clones. From the transcriptional assay using single-copy chromosomal *rpoE*P2 promoter fused with *lacZ*, in *rseA* null mutant containing either *rseA* or *rseB* plasmids

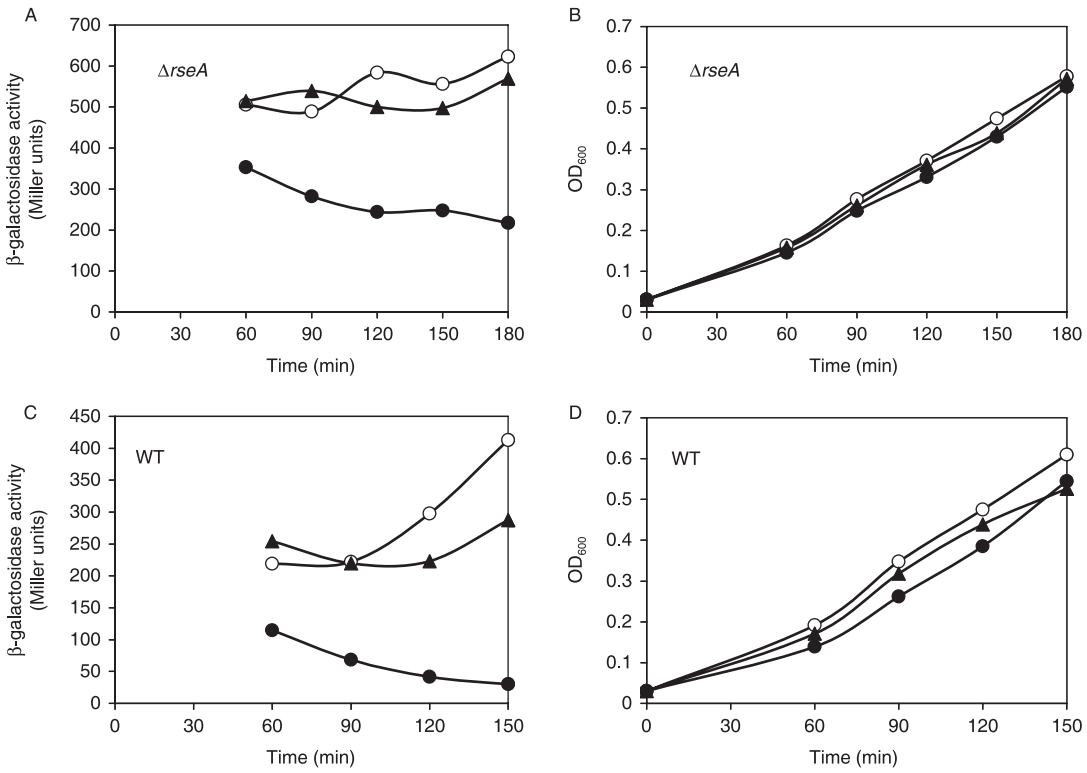

Figure 3 Effects of *rseA* and *rseB* plasmid clones on the expression of *rpoE*P2 promoter in (A) Δ*rseA* and (C) wild-type (WT) strains. Empty vector (open circles), RseA plasmid clone (closed circles) or RseB plasmid clone (triangles) were electroporated to the cells carrying *rpoE*P2-*lacZ* promoter fusion on the genome. Selected transformants were grown at 37 °C, and samples taken at the times indicated were subjected to a β-galactosidase assay as described in Experimental procedures. The growth of Δ*rseA* (B) and the wild type (D) was measured by the absorbance at 600 nm at specific time points used simultaneously for β-galactosidase measurements. The experiments were carried out on four independently obtained transformants and one representative data set has been shown.

showed that RseA severely repressed the *rpoE*P2 promoter activity, whereas the repression by RseB was insignificant in this mutant as compared to that in the empty vector (Fig. 3A). In contrast, the transcriptional assay in the wild-type strain showed that both RseA and RseB suppressed *rpoE*P2 promoter activity, although the magnitude of repression was higher with RseA than RseB (Fig. 3C). Taken together, with the results from Fig. 2, these data suggest that σ^E response and consequent control of cell lysis by RseB acts primarily via RseA.

Effect of the deletion mutations of *rseA*, *rseB* and *rseC* on the transcription of *rpoE*

To furthermore examine the effects of the three deletion mutations on the σ^E-dependent cell lysis, the expression of *rpoE* was compared with the Δ*rseA*, Δ*rseB*, Δ*rseC* and wild-type strains. Total RNAs from cells that were grown until early stationary phase at 37–45 °C were subjected to RT-PCR. The mRNA level of *rpoE* was quantified from the intensities of PCR products after agarose gel electrophoresis (Supporting Information/Supplementary Material Fig. S1). Considering the number of the PCR cycle, the *rpoE* expression in Δ*rseA* was estimated to be 5- to 20-fold higher than that in the wild type at different temperatures. Approximately twofold higher expression of *rpoE* was observed in Δ*rseB*, and no significant difference in Δ*rseC* was found. These results furthermore establish that functional transcriptional activity is primarily enhanced in Δ*rseA* and modestly in Δ*rseB*.

Effect of the deletion mutations of *rseA*, *rseB* and *rseC* on the amounts of σ^E and its association with inner membrane

Next, the expression level and cellular fractionation of σ^E protein were compared with the three deletion mutants (Fig. 4A–C), since the extent of influence by defined individual nonpolar deletion mutants is not clear enough. The total cell extracts or fractionated samples from cells

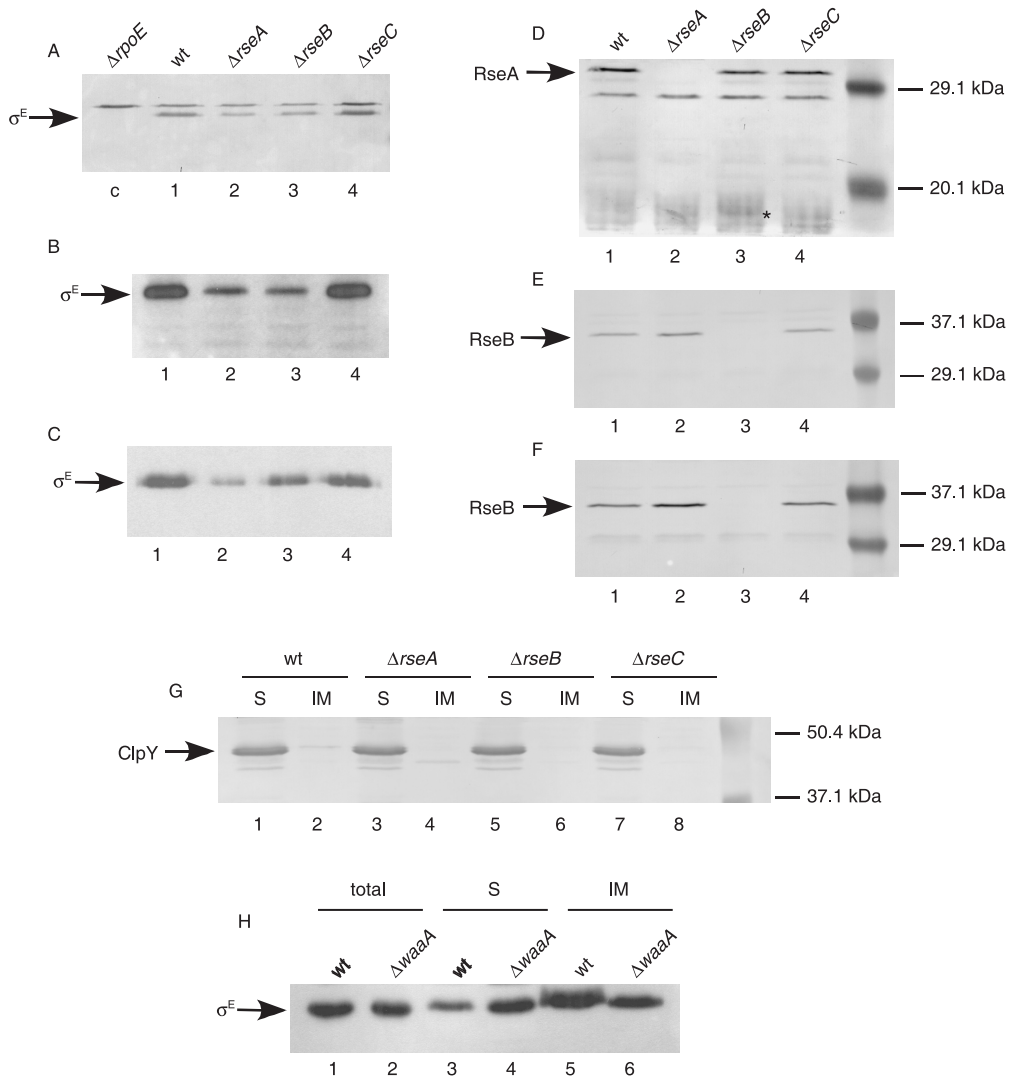

Figure 4 σ^E level and its intracellular location in Δ*rseA*, Δ*rseB* and Δ*rseC* mutants. Cells were grown in LB medium at 37 °C with shaking until early stationary phase. (A) Total, (B) soluble and (C) inner membrane fractions were prepared as described in Experimental procedures. The samples were then subjected to 15% SDS–PAGE, followed by Western blotting with an antibody against σ^E. As controls, (D) RseA- and (E) RseB-antibodies were used against the total fractions. (F) Represents Western blot from soluble fractions using antibody against RseB. (G) Shows the presence of ClpY in soluble (S) and inner membrane (IM) fractions from wild-type, *rseA*, *rseB* and *rseC* mutants used in the above panels. (H) Immunoblot showing detection of RpoE in the isogenic wild-type and Δ*waaA* mutant in total crude cell extract, soluble fraction (S) and in the inner membrane fraction (IM), using equivalent amounts of proteins from each fraction. Arrows indicate the position of σ^E, RseA, RseB and ClpY. The star symbol in (D) indicates cross-reacting RseA-specific cleavage product(s) in *rseB* mutant. Migrated prestained Molecular weight standards are shown.

grown until early stationary phase, which was the same condition as for the study of the *rpoE* transcription, were subjected to Western blot analysis. As a control for fractionation of soluble and inner membrane proteins, we analyzed the corresponding samples for the presence of relatively abundant known cytosolic heat-shock protease subunit ClpY (Missiakas *et al.* 1996b). As can be seen, no ClpY could be detected in the inner membrane (IM) fractions of either wild type or those from *rseA/B/C* mutants (Fig. 4G), but was present only in the soluble fractions. Furthermore, PpiD served as a perfect marker for IM fractionation given its exclusive inner membrane localization due to its single N-terminal inner membrane anchor (Dartigalongue & Raina 1998) (see Fig. 5A). To ensure the authenticity of RpoE recognition, total cell extracts from Δ*rpoE* were applied as additional control (Fig. 4A).

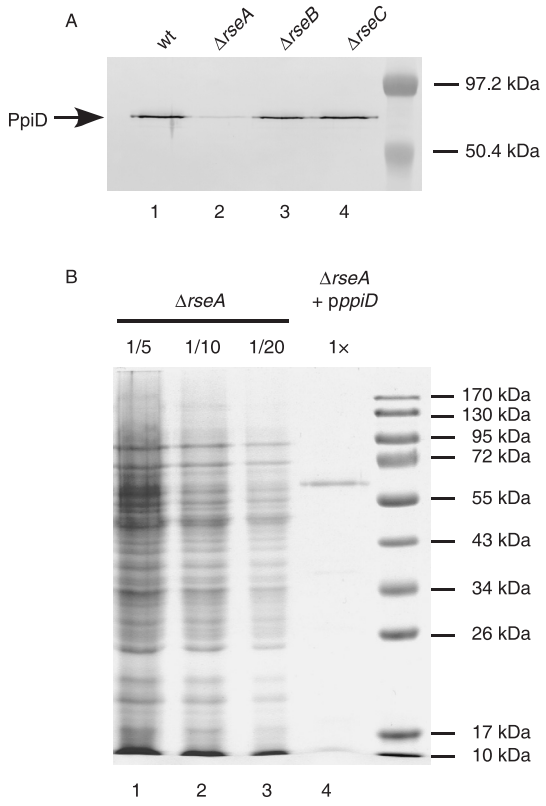

Figure 5 Reduction in PpiD levels in Δ*rseA* and suppression of cell lysis upon PpiD over-expression. (A) Equivalent amounts of inner membrane solubilized fractions from wild type (wt), Δ*rseA*, Δ*rseB* and Δ*rseC* were applied to 12% SDS–PAGE and analyzed by Western blotting. Proteins blotted to nitrocellulose membrane were probed for the amounts of PpiD using anti-PpiD antibodies. (B) Logarithmic grown cultures of Δ*rseA* transformed with empty vector pSE420 and isogenic Δ*rseA* carrying cloned *ppiD*$^{+}$ in pSE420 were adjusted to OD 0.05 and furthermore incubated with shaking at 37 °C. Cell lysis was measured as described in Fig. 1. The culture volume applied in case of Δ*rseA* (empty vector) is 1 : 5, 1 : 10 and 1 : 20 as compared to that used for corresponding *ppiD*+ over-expressing in Δ*rseA*, which was undiluted.

The analyses of fractionation experiments showed that the total RpoE amounts in cell extracts did not increase in either Δ*rseA* or Δ*rseB* mutants, rather only the ratio between soluble versus IM-associated forms increased (Fig. 4A,B). However, RpoE amount in soluble fraction was found to be lower in *rseA* and *rseB* mutants than the wild type. Of significance, the Δ*rseA* and the Δ*rseB* mutants showed decrease in IM association of RpoE was much more pronounced in Δ*rseA* (Fig. 4C). These results together with those of RT-PCR can explain constitutive induction of the *rpoE* regulon in Δ*rseA* mutants with only a minor induction in Δ*rseB* mutants. Thus, the key to induction of RpoE regulon seems to be extent of its decrease in IM association and increased ratio of soluble versus IM form, rather than overall amounts of RpoE. Interestingly, a fraction of RpoE was still found to be present in the IM of Δ*rseA* (Fig. 4C). These results thus show that despite a decrease in total σ^E amounts in Δ*rseA*, there is an increase in the ratio of free σ^E, as seen by changes in soluble versus membrane-bound fractions of Δ*rseA*, rather than totally abolishing membrane association of RpoE.

To validate that the activation of RpoE arises mostly due to increased ratio of soluble versus IM RpoE forms and a decrease in its IM association, we examined RpoE amount in suppressor-free Δ*waaA* mutants, grown under permissive conditions. Δ*waaA* mutants synthesize minimal LPS structure, composed of only free lipid IV_A precursor without any glycosylation, due to the deletion of Kdo transferase encoding gene. The rationale for using Δ*waaA* mutants was due to their ~6- to 8-fold constitutive induction of the RpoE regulon even under permissive growth conditions of 21–30 °C and decreased OMP content (Klein *et al*. 2009). The analyses of cellular fractions obtained from Δ*waaA* mutants grown under permissive conditions did not show any significant increase in the total RpoE amount as compared to the wild type. Importantly, quite pronounced decrease in IM-associated form of RpoE, with a concomitant increase in the ratio of soluble versus IM of RpoE in Δ*waaA* is obvious (Fig. 4H). However, Δ*waaA* mutants also led to a small increase in the soluble RpoE form as compared to the wild type. However, this minor increase alone cannot explain 6- to 8-fold basal increase in the activity of RpoE-dependent promoters. Thus, the results from Δ*waaA* and Δ*rseA* argue that increased RpoE activity can be accounted by two-fold events, due to increase in the ratio of soluble versus IM forms and by its decreased IM association. This would consequently result into increased availability of transcriptionally active $E\sigma^E$ complex leading to enhanced transcription from σ^E-dependent promoters.

Control experiments were carried out using specific antibodies against RseA and RseB. Extracts from Δ*rseB* showed accumulation of small anti-RseA cross-reacting species (marked with the star sign), which is consistent with RseB function in interaction with RseA, to protect it or alter its proteolysis (Fig. 4D). These data provide *in vivo* evidence of accumulation of RseA cleavage product(s), presumably due to its increased turnover in *rseB* mutants. Furthermore, RseB amounts seem to be increased only in Δ*rseA* in both crude extracts and in soluble fractions, consistent with the negative regulation of whole *rpoE* operon by RseA (Fig. 4E,F). Moreover, no significant amounts of RseB in the IM fractions of the wild type, *rseA* or *rseC* could be detected to be physiologically relevant (data not shown).

Finally, our data demonstrate the accumulation of RseA cleavage product(s) in *rseB* mutants, explaining role for RseB acting as a modulator for RseA proteolysis *in vivo*.

Reduction in the amounts of PpiD in Δ*rseA* and suppression of lysis in *rseA* mutants upon increased expression of PpiD, a peptidyl-prolyl isomerase with OMP folding function

To provide molecular basis of cell lysis phenotype, we examined if any OMP biogenesis factors in *rseA* mutants are limiting, which might explain this phenotype. The rationale being that *rseA* mutants or over-expression of RpoE in general leads to reduction in the levels of OMPs (Kabir *et al*. 2005). However, since the levels of HtrA, SurA, FkpA and Skp are increased in *rseA* mutants (Dartigalongue *et al*. 2001; Kabir *et al*. 2005), we looked for effect on other OMP biogenesis factors, which could be limiting and cause cell lysis. In this context role of the *ppiD* gene was examined, since its product recognizes early OMP folding intermediates and its over-expression suppresses OMP biogenesis defects of *surA* mutants restoring them to near wild-type levels (Dartigalongue & Raina 1998; Antonoaea *et al*. 2008). However, its levels have not been examined under the conditions of RpoE induction up to now. Thus, total cell extracts or fractionated samples from soluble and inner membrane fractions from wild-type or *rseA/B/C* mutants were analyzed. As shown, the levels of PpiD were severely reduced in *rseA* mutants if either total or in octyl-β-D-glucopyranoside solublized inner membrane (Fig. 5A). However, the levels of PpiD were unaffected by *rseB* or *rseC* mutations and were comparable to that in the wild type. Such a reduction in the levels of PpiD is up to now unprecedented in any genetic background and could partly account for OMP reduction and hence cell lysis in Δ*rseA* mutants.

PpiD amounts were uniquely reduced only in *rseA* mutants, but not in *rseB* mutants. This reduction in the levels of PpiD in *rseA* mutants seems to mirror the cell lysis phenotype. Hence, we examined the influence of increased expression *in trans* of PpiD from a plasmid. The supernatant fractions from *rseA* mutant with empty vector and isogenic *rseA* construct, carrying the *ppiD* gene on the plasmid, were subjected to SDS–PAGE. Quite interestingly, the accumulation of protein was dramatically suppressed in the presence of PpiD plasmid (Fig. 5B). These results clearly suggest that upon multicopy plasmid-born expression of the *ppiD* gene, the cell lysis phenotype of *rseA* mutants is significantly suppressed and restored to nearly wild-type levels. PpiD multicopy suppression of cell lysis is reminiscent of suppression of OMP defects in a defined *surA* mutant by PpiD over-expression (Dartigalongue & Raina 1998). Thus, we can assume that cell lysis phenotype of *rseA* mutants arises mainly due to known reduction in the amounts of major OMPs, and PpiD over-expression causes suppression of this phenotype due to acceleration of OMP folding. These results indicate an integrated in built mechanism of cell lysis association with integrity of OM, which is presumably impaired in *rseA* mutants.

Reduction in VBNC population in Δ*rseA*

The σ^E-dependent cell lysis appears to occur specifically for VBNC cells that are defective in colony forming ability. Such cells become major in early stationary phase under usual growth conditions (Nitta *et al*. 2000; Kabir *et al*. 2004b). To attempt discrimination of the defective cells from the cells capable of forming colonies, morphological observation was carried out. The difference between the total count and the viable count shows the number of dead cells, and the difference between the viable count and CFU number shows the number of VBNC cells (Desnues *et al*. 2003; Abe *et al*. 2007). As depicted in Fig. 6, after the late log phase, cells loose the colony forming ability. The total cell count in the Δ*rseA* was found to be decreased over days whereas that in wild type (WT), Δ*rseB* or Δ*rseC* did not show such significant reduction. The ratio between the total cell count and the viable cell count in Δ*rseA* remained nearly constant over days. In contrast, the other strains increased this ratio. However, no major difference in the number of CFU was observed among all the strains. Therefore, Δ*rseA* apparently reduced in the number of dead cells all along the incubation over days in contrast to other mutants and WT.

Discussion

To understand the mechanism of σ^E-dependent cell lysis, using nonpolar disruptions of *rseA, rseB* and *rseC* genes, we show that (i) only Δ*rseA* show significant enhanced cell lysis phenotype, but not Δ*rseB* or Δ*rseC*. (ii) This cell lysis phenotype of Δ*rseA* could be suppressed by plasmid-born *rseA*, but neither by *rseB* nor by *rseC* genes. However, cell lysis of the wild type can be repressed by both *rseA* and *rseB*, arguing RseB suppression requires functional presence of RseA. (iii) Δ*rseA* showed increased ratio of soluble versus membrane-associated RpoE, without increasing the total or soluble RpoE amounts, which seems to be sufficient to induce *rpoE* transcription. (iv) RpoE association with the inner membrane was significantly reduced in *rseA* mutants, but still a minor fraction of σ^E was present in such mutants. (v) Δ*rseA* have severely repressed levels of PpiD, an inner membrane-anchored periplasmic OMP biogenesis factor with a robust peptidyl-prolyl isomerase

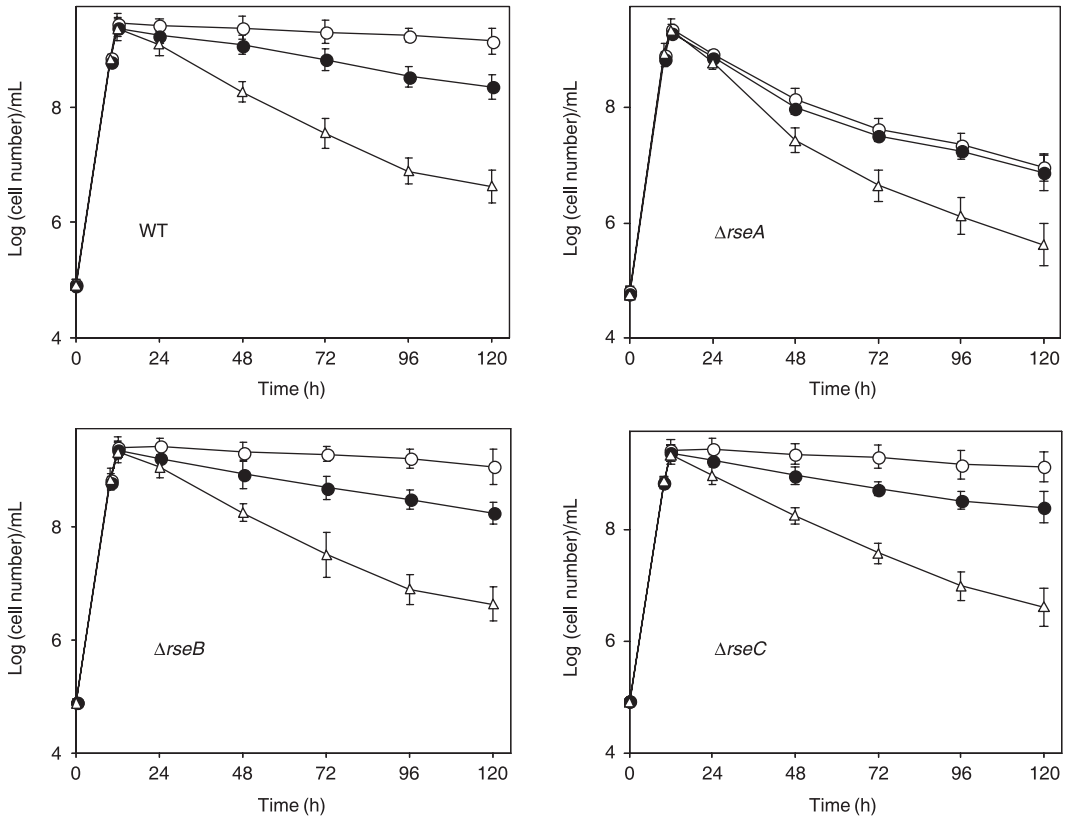

Figure 6 Determination of VBNC population in W3110 (WT), Δ*rseA*, Δ*rseB* and Δ*rseC* strains. Cells were grown in LB medium at 37 °C with shaking. Cells were collected at times indicated and DAPI-stained all cells (open circles), CTC-stained viable cells (closed circles) and culturable cells (triangles) capable of forming a colony were determined as described in Experimental procedures. The experiments were carried out three times. Error bars represent standard deviation ± mean.

activity, and (vi) consistent with reduced levels of PpiD in Δ*rseA*, over-expression of PpiD dramatically suppressed the cell lysis phenotype in Δ*rseA*. These results imply that cell lysis phenotype of Δ*rseA* can be primarily explained by defects in OMP maturation and their synthesis. The observed reduced levels of OMPs in Δ*rseA* has thus far been only ascribed to translational repression of OMPs by RpoE-dependent synthesis of small noncoding RNAs, namely *micA* and *rybB* (Valentin-Hansen *et al.* 2007). These results have manifold implications in our understanding of σ^E-dependent cell lysis, OMP biogenesis limiting factors that might lead to cell lysis upon σ^E induction and overall σ^E regulation.

The significant effect of the Δ*rseA* mutation on cell lysis appears to be due to the increase in transcription of *rpoE*, and a significant decrease in the amounts of OMP biogenesis folding catalyst PpiD in Δ*rseA*. Consistent with the lack of significant cell lysis in *rseB* mutants, only a minor decrease in the membrane association of σ^E was observed. Lack of cell lysis phenotype of Δ*rseB* is further supported by the absence of any major effect on PpiD levels and hence on OMP maturation. The *ppiD* gene was originally identified as a multicopy suppressor of *surA* mutants, which resulted in restoration of OMP biogenesis and maturation defects in such bacteria (Dartigalongue & Raina 1998). PpiD has recently been shown to be the first periplasmic factor that interacts with nascent secretory chains emerging from Sec machinery (Antonoaea *et al.* 2008). Δ*rseA* have constitutively increased levels of RpoE-regulated genes, including negatively regulating OMP synthesis noncoding RNAs, accounting in part reduction in OMP levels upon RpoE induction (Valentin-Hansen *et al.* 2007). Furthermore, inactivation of the *rseA* gene concomitantly resulted in severe reduction of PpiD. Hence, the overall cell lysis phenotype in Δ*rseA* due to membrane lysis would thus stem from coupled *micA*- and *rybB*-mediated repression, with further defective OMP folding due to reduced/limiting amounts of PpiD, explaining the cell lysis phenotype of Δ*rseA*. The observed reduction in the levels of PpiD in Δ*rseA* and repression of cell lysis in such mutants

by PpiD over-expression provides substantial evidence that cell lysis arises due to OMP perturbations. PpiD-mediated suppression of cell lysis defect mimics similar suppression of OMP folding defects of *surA* mutants. However, this reduction in PpiD amounts is not unique to Δ*rseA*, but is found in all conditions that induce RpoE activity significantly, particularly upon defects in OMP maturation, such as in deep-rough mutants and their under-acylated derivatives (Klein *et al*. 2009). However, the magnitude of reduction in Δ*rseA* is most severe. Our overall conclusion about OMP reduction due to reduced PpiD levels and induction of small non-coding RNA is further supported by a separate study, where we found that the knockout mutation of *micA*, encoding MicA for a regulator of *ompA* gene or *rybB*, encoding RybB for a regulator of *ompC* and *ompW* genes, also suppressed the σ^E-dependent lysis and that of *ompA*, *ompC* or *ompW* caused cell lysis at a significant level (our unpublished results). Moreover, the lysis was enhanced when *micA* or *rybB* was over-expressed in the wild-type background. Therefore, we assumed that σ^E, when at a high level, directs cell lysis by expressing *micA* and *rybB*, leading to reduction in OMP amounts, which is furthermore accentuated by accompanied reduction in PpiD assisted OMP folding and hence disintegration of the outer membrane.

The present study showed that despite increase in the *rpoE* transcription and RpoE activity, the overall amounts of RpoE do not increase in Δ*rseA* mutants. To test the model that the primary cause and magnitude of induction of σ^E-dependent promoters was due to increased ratio of soluble (accessible to RNA polymerase) versus IM form (sequestered), RpoE amounts were measured in Δ*waaA* mutants. Δ*waaA* mutants lack Kdo transferase and hence synthesize nonglycosylated free lipid IV_A minimal LPS, and show six- to eightfold increase in transcription from σ^E-dependent promoters even under permissive growth conditions between 21 °C and 30 °C (Klein *et al*. 2009). Although Δ*waaA* and *rseA* mutants are not exactly comparable, since the *waaA* gene is essential for bacterial growth above 30 °C, however, both the mutants have reduced OMP content and show high RpoE activity. Quite like *rseA* mutants, *waaA* mutants showed significantly decreased amounts of IM-associated form of RpoE, without any significant increase in total RpoE amount. Taken together, our results from *rseA* and *waaA* mutants provide a convincing mechanism of RpoE induction. It should be however noted that in *waaA* mutants soluble RpoE also showed a small increase, but that does not seem to correspond to six- to eightfold increase in the activity of σ^E-dependent promoters. Thus, decreased inner membrane association, resulting in increase in ratio of soluble (free) versus IM form explains RpoE induction without increase of the total RpoE amounts. However, additional mechanisms implying IM association altering RpoE proteolysis or such an association causing abortive RNA polymerase association, which could be inhibitory cannot be ruled out and needs furthermore investigation.

Since the introduction of the *rseB* plasmid clone into the wild-type strain repressed both the accumulation of proteins in the medium in the wild-type strain as well *rpoE*P2 promoter activity, argues for a function for RseB as a modulator of σ^E-dependent lysis in an RseA-dependent manner. However, this RseB repression required the functional presence of RseA, since neither of these processes could be repressed in Δ*rseA* by RseB plasmid clone. Hence, when RseB was provided from the plasmid in the wild type, increase in RseB amounts could render RseA resistant to proteolytic action of DegS and RseP, leading to higher inhibition of σ^E by its further sequestration than by the free RseA alone and consequent repression of cell lysis. Our *in vivo* results of accumulation of RseA proteolytic product(s) in *rseB* mutant thus provide a support for our conclusions and suggested models of RseB function based on *in vitro* results (Grigorova *et al*. 2004; Cezairliyan & Sauer 2006). Thus, it seems the function of RseB is primarily to modulate the RseA function and hence the effect on σ^E.

Concerning RseC, significant amount of proteins accumulated in the Δ*rseC* strain at 45 °C after 36-h incubation, which however may not be due to the σ^E-dependent lysis, because increase in lysis seems to stop under the same condition in the Δ*rseA* strain. Furthermore experiments are required to unravel the mechanism of the lysis in the Δ*rseC* strain at the higher temperature as well as at intermediate temperature.

Although the VBNC populations in all the strains were nearly to the similar level, only Δ*rseA* showed a significant reduction in dead cells compared to others. We assume that the VBNC population in Δ*rseA* is directly subjected to cell lysis, rather than being the dead cell state. As a consequence, Δ*rseA* showed a significant reduction in total cell count. However, in the WT, Δ*rseB* or Δ*rseC* mutant, both the dead cells and the VBNC cells increased gradually. At the higher temperature, further reduction of OD_{600} and more amounts of proteins accumulated in the medium were observed in Δ*rseA*, consistently with decrease in CFU, suggesting that damaged cells are accumulated as temperature increases.

Finally, it will be interesting to further understand the molecular basis of reduction of RpoE and PpiD amounts in Δ*rseA*. It is particularly intriguing in case of RpoE, since the *rpoE* specific mRNA increases without corresponding increase in the total protein level.

Table 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant properties	Source/reference
Escherichia coli strains		
W3110	IN (*rrnD-rrnE*) *rph-1rpoS*(33Q)	Kabir *et al.* (2004b)
WK3	W3110 *rseA*::Tn*10*Kan	Nitta *et al.* (2000)
YU689	BW25113 *rseA::kan*	H. Mori
YU690	BW25113 *rseB::kan*	H. Mori
YU691	BW25113 *rseC::kan*	H. Mori
YU681	W3110 *rseA*::Tn*10*Kan	This work
YU692	W3110 Δ*rseA*	This work
YU693	W3110 Δ*rseB*	This work
YU694	W3110 Δ*rseC*	This work
GK2085	W3110 Δ*rseA* Δ*lac*	This work
GK2086	W3110 Δ*rseB* Δ*lac*	This work
GK2087	W3110 Δ*rseC* Δ*lac*	This work
SR8621	W3110 Δ*waaA*::*kan*	Klein *et al.* (2009)
SR8689	W3110 Δ*rpoE*::*cat*	This work
Plasmids		
pCP20	amp^R, cml^R	Cherepanov & Wackernagel (1995)
pBAD24	*araBAD* promoter, amp^r	J. Beckwith
pBADRSEA	pBAD24 with 0.65-kb DNA fragment bearing the *rseA* gene	This work
pBADRSEB	pBAD24 with 0.96-kb DNA fragment bearing the *rseB* gene	This work
pBADRSEC	pBAD24 with 0.48-kb DNA fragment bearing the *rseC* gene	This work
pSR4067	pSE420 carrying the minimal *ppiD*$^+$ coding sequence (1.9-kbp *NdeI–Eco*RI)	This work

Experimental procedures

Materials

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs. DNA sequencing kit (ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit) was obtained from Applied Biosystems Japan. Oligonucleotide primers were synthesized by Proligo Japan K. K. (Tokyo, Japan). Other chemicals were all of analytical grade and obtained from commercial sources.

Bacterial strains, medium and culture conditions

The bacterial strains used in this study were derivatives of *E. coli* K-12. Their relevant genotypes and plasmids are presented in Table 1. Null mutations in the *rseA, rseB, rseC* genes with coding sequences replaced by kan cassette in the strain BW25113 were obtained from H. Mori (NIG Japan) and used as donors for bacteriophage P1-mediated transduction (Miller 1992) into W3110 genetic background. The removal of the kan cassette from each transduced strain was carried out as described below, resulting into strains YU692 (Δ*rseA*), YU693 (Δ*rseB*) and YU694 (Δ*rseC*). Precultured cells were transferred into LB [1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl] medium, adjusted by dilution to an OD_{600} of 0.1. Cells were further incubated for appropriate time at required temperatures under aerobic conditions by reciprocal shaking (100 times/min). When required, antibiotics were added as appropriate, at the following concentrations: ampicillin 50 μg/mL, tetracycline 8 μg/mL, kanamycin 25 μg/mL.

Construction of deletion mutants

pCP20 is an Amp^R and Cml^R plasmid with a temperature-sensitive replication and thermal induction of FLP synthesis (Cherepanov & Wackernagel 1995). W3110 *rseA*::*kan*, W3110 *rseB*::*kan* or W3110 *rseC*::*kan* was transformed with pCP20, and the resultant ampicillin-resistant transformants were obtained at 30 °C, from which a few were colony-purified once nonselectively at 43 °C. Resultant derivatives were tested by PCR in addition to checking for the loss of all antibiotic resistances. The majority of deletion mutants lost simultaneously the FRT-flanked resistance gene and the FLP helper plasmid as described previously (Datsenko & Wanner 2000). A chromosomal deletion in the *rpoE* gene was constructed by substitution of its coding sequence by *cat* cassette from pKD3 (Datsenko & Wanner 2000).

DNA manipulation

Conventional recombinant DNA techniques were followed (Sambrook & Russell 2001). The DNA fragments bearing the coding region of *rseA* was amplified by PCR using the primers for rseA-5′ (5′-GGGGGTACCGATAGCGGGATA-3′) and rseA-3′ (5′-GGGAAGCTTCCAAAGTTGCTTCATTACT-3′), *rseB* was amplified with the primers for rseB-5′ (5′-GGGGGTACCCTG TACAGGTGCCAGGAATT-3′) and rseB-3′ (5′-GGGAAGCTT GATCATTGCGCTGCCCCGAA-3′), and *rseC* was amplified with the primers for rseC-5′(5′-GGGGGTACCCAAACGGCGA AACGCATTGC-3′) and rseC-3′ (5′-GGGCTGCAGATCACTG GCTCGCGTCTTCC-3′) by PCR as described previously (Yamada *et al.* 1993), with *Kpn*I or *Hin*dIII sites and W3110 genomic DNA as a template. The amplified DNA fragments bearing the *rseA* (650 bp), *rseB* (956 bp) or *rseC* (479 bp) genes were digested with *Kpn*I and *Hin*dIII, and inserted between the *Kpn*I and *Hin*dIII sites on pBAD24, generating pBADRSEA, pBADRSEB and pBADRSEC. The integrity of the cloned *rseA*, *rseB* or *rseC* genes was verified by nucleotide sequencing (Sanger *et al.* 1977). Each of the three genes was cloned under the control of *araBAD* promoter.

Monitoring of cell lysis

Cells were grown at various temperatures in the range of 30–45 °C as described above. The cell growth was monitored by measuring OD_{600}, and CFUs were determined by counting the colony number on LB plates after 24 h of incubation. Protein accumulation in medium was tested as described previously (Nitta *et al.* 2000). A portion of the culture was centrifuged at 3000 ***g*** for 10 min to separate the supernatant (medium fraction) and the pellet (remaining fraction). Proteins in the medium fraction were recovered by the addition of trichloroacetic acid at the final concentration of 5%, treated with ether and resolved in 20 mM Tris–HCl (pH 7.0). The pellet was resolved in 20 mM Tris–HCl (pH 7.0) and subjected to sonic oscillation. Proteins from both the fractions were resolved by 12% SDS–PAGE.

For the test for lysis, wild-type, Δ*rseA*, Δ*rseB* and Δ*rseC* cells were grown at 42 °C as described above. A portion of the culture was centrifuged at 5000 ***g*** for 2 min to separate the supernatant (medium fraction) and the pellet (remaining fraction). The pellet fraction was suspended with Z-buffer and the β-galactosidase activity of both of the medium- and the remaining fractions were measured according to the procedure described by Miller (1992). The β-galactosidase activity in the medium fractions for each strain was divided by the sum of that of medium and remaining fractions.

Complementation studies

The *rseA*, *rseB* or *rseC* cloned in pBAD24 and the empty plasmid vector as a control were introduced into W3110 (wild type), W3110 *rseA*::Tn*10*Kan or W3110 Δ*rseA* cells. The *rseA* plasmid clone or the empty plasmid vector was also introduced to W3110 Δ*rseB* cells. The transformants were tested on LB plates containing ampicillin 50 μg/mL. Precultured cells were transferred into LB medium diluted at a turbidity corresponding to OD_{600} of 0.1, and then the cells were further grown for appropriate times at 37 °C under aerobic conditions by reciprocal shaking (100 times/min). Protein accumulation in medium was measured as described above.

Transcriptional assay

For assay with the *lacZ* operon fusion, empty vector (as a control), *rseA* plasmid clone or *rseB* plasmid clone were transformed with the *rpoE-lacZ* fusion on the genome. Transformants were grown at 37 °C in LB media containing appropriate antibiotics. Exponentially grown culture was diluted with the same medium and adjusted to OD_{600} of 0.02, and further incubated for the appropriate time. Samples were then taken from the culture, and β-galactosidase activity was measured according to the procedure described by Miller (1992).

RT-PCR analysis

Cells were grown in 30 mL of LB medium at different temperatures (37, 42 and 45 °C) for 10 h (early stationary phase), and total RNA was immediately extracted using the hot phenol method (Aiba *et al.* 1981). RT-PCR analysis was carried out using an mRNA selective RT-PCR Kit (Takara Shuzo) to examine the expression of *rpoE* (Tsunedomi *et al.* 2003). The primers used for *rpoE* expression were rpoE-5′ (5′-TGGGGAGACTTTACCTC-3′) and rpoE-3′ (5′-TCGTCAACGCCTGATAA-3′). The RT reaction was carried out at 45 °C for 15 min using 0.1 μg of total RNA and each downstream primer, followed by PCR consisting of denaturing at 85 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 2 min was carried out using the two primers for each gene. After the completion of 25, 30, 35 and 40 cycles, the PCR products were analyzed by 0.9% agarose gel electrophoresis and stained with ethidium bromide. The relative amounts of RT-PCR products on the gel were compared by measuring the band density after the color of the image taken had been reversed using model GS-700 Imaging Densitometer (Bio-Rad) (Nitta *et al.* 2000). Linearity of the amplification was observed up to 30th or 35th cycle. In our conditions, the RNA-selective RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

Western blot analysis

Isogenic W3110, GK 2085 (Δ*rseA*), GK 2086 (Δ*rseB*) and GK 2087 (Δ*rseC*) cells were grown at 37 °C for 10 h in 200 mL LB medium and harvested by centrifugation at 3000 ***g*** for 10 min. Cells were washed, resuspended in 10 mM Tris–HCl buffer (pH 8.0) and French pressed twice. The cell extracts were fractionated into the membrane and the soluble fractions by centrifugation at 17 000 ***g*** for 30 min (Yamada *et al.* 1993). To obtain inner membrane, proteins were extracted by incubation in 2% octyl-β-D-glucopyranoside. The solubilized inner membrane fraction was recovered by centrifugation at 17 000 ***g*** for 30 min. Crude extracts, membrane fractions and soluble fractions were applied to 15% SDS–PAGE. After the electrophoresis, proteins in the gel were blotted to nitrocellulose membrane. σ^E, RseA, RseB, PpiD and ClpY proteins were

detected with an antibody against σ^E, RseA, RseB and ClpY as described previously (Yamada *et al.* 1993; Missiakas *et al.* 1996b; Dartigalongue & Raina 1998; Klein *et al.* 2003). For experiments with Δ*waaA* mutant and its isogenic parent, cultures were grown at 21 °C in minimal M9 medium, washed and resuspended in LB medium at 30 °C and allowed to grow till OD of 0.4. Cells were harvested, fractionated as described above and analyzed by Western blot analysis for the presence of RpoE.

Determination of VBNC populations

The VBNC cell population was determined by the method described previously (Abe *et al.* 2007) with slight modification. Cells were grown as described above. The cell culturability (CFU) was measured by using LB agar plates. CFUs were enumerated with samples diluted at three different concentrations as described above. The staining procedure with DNA-binding fluorochrome, 4′,6-Diaminido-2-phenylindole, dihydrochloride (DAPI, Dojindo Laboratories, Kumamoto, Japan) staining was used to count total bacterial without distinguishing between live and dead bacteria by using an epifluorescence microscope (Nikon, Eclipse, E-600) (Porter & Fieg 1980). Two per cent formalin-fixed samples were filtered through 0.2-μM-pore-size polycarbonate black filters (Toyo Roshi Kaisha, Ltd, Tokyo, Japan) and stained for 20 min with a 2.5 μg/mL DAPI solution. Viable cell counts were determined using the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Dojindo Laboratories) method (Rodriguez *et al.* 1992); that is, bacteria with a functioning electron transport chain reduce the CTC in CTC-formazan, forming a red fluorescent precipitate in the cell membrane. Samples were incubated with 3.0 mM CTC, in the presence of 0.025% yeast extract, overnight at room temperature, and then fixed with formalin (2% final concentration). Samples were stained with DAPI, as described above. Cells showing red precipitate under green excitation were counted. Development of VBNC was monitored using CFU enumeration and total and viable counts, as described above.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Expression of *rpoE* in Δ*rseA*, Δ*rseB* and Δ*rseC* mutants.

Additional Supporting Information may be found in the online version of this article.

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