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New envelope stress factors involved in σ^E activation and conditional lethality of *rpoE* mutations in *Salmonella enterica*

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Abstract

Salmonella enterica serovar Typhimurium (*S. typhimurium*) can cause food- and water-borne illness with diverse clinical manifestations. One key factor for *S. typhimurium* pathogenesis is the alternative sigma factor σ^E, which is encoded by the *rpoE* gene and controls the transcription of genes required for outer-membrane integrity in response to alterations in the bacterial envelope. The canonical pathway for σ^E activation involves proteolysis of the antisigma factor RseA, which is triggered by unfolded outer-membrane porins (OMPs) and lipopolysaccharides (LPS) that have accumulated in the periplasm. This study reports new stress factors that are able to activate σ^E expression. We demonstrate that UVA radiation induces σ^E activity in a pathway that is dependent on the stringent response regulator ppGpp. Survival assays revealed that *rpoE* has a role in the defence against lethal UVA doses that is mediated by functions that are dependent on and independent of the alternative sigma factor RpoS. We also report that the envelope stress generated by phage infection requires a functional *rpoE* gene for optimal bacterial tolerance and that it is able to induce σ^E activity in an RseA-dependent fashion. σ^E activity is also induced by hypo-osmotic shock in the absence of osmoregulated periplasmic glucans (OPGs). It is known that the *rpoE* gene is not essential in *S. typhimurium*. However, we report here two cases of the conditional lethality of *rpoE* mutations in this micro-organism. We demonstrate that *rpoE* mutations are not tolerated in the absence of OPGs (at low to moderate osmolarity) or LPS O-antigen. The latter case resembles that of the prototypic *Escherichia coli* strain K12, which neither synthesizes a complete LPS nor tolerates null *rpoE* mutations.

INTRODUCTION

Bacteria adapt to changes in the environment through the regulation of their gene expression. Alternative sigma factors are crucial in this phenomenon: they can be activated by molecular signals generated by environmental changes and, as a result, sets of specific genes needed to counteract stress are transcribed. Among these, extracytoplasmic function (ECF) sigma factors control the transcription of genes required for outer-membrane integrity in response to alterations in the bacterial envelope [1]. These include genes that encode proteins involved in the folding or degradation of periplasmic polypeptides, the biogenesis and/or modification of lipopolysaccharides (LPS), primary metabolism and the regulation of other sigma factors [2–5]. The best characterized ECF sigma factor is the *Escherichia coli* σ^E factor, which is encoded by *rpoE*, an essential gene under non-stress conditions in this micro-organism [5–7]. When envelope stress is absent, σ^E function is prevented by the action of the antisigma factor RseA [8]. RseA is an inner-membrane protein whose cytoplasmic domain binds tightly to σ^E, thus preventing its binding to core RNA polymerase. Under conditions of envelope stress, the canonical pathway of σ^E activation involves RseA sequential cleavage by DegS and RseP proteases, with the consequent release of σ^E into the cytoplasm to initiate the transcription of σ^E-dependent genes [9]. Two stress sensor proteins are involved in this mechanism, DegS and RseB, which recognize alterations of outer-membrane porins (OMPs) and LPS in the periplasm, respectively. The C-terminal motif of unfolded or unassembled OMPs binds to the DegS PDZ domain, converting DegS into its active form [10, 11]. Active DegS cleaves the periplasmic C terminus of RseA, which in turn is the substrate for RseP, which cleaves the transmembrane segment of RseA. The binding of RcsB to RseA prevents activated DegS from cleaving RseA, and so LPS that have accumulated in the periplasm are required to dissociate RseB from RseA to facilitate the release of σ^E [12]. It should be noted that the biogenesis of OMPs and LPS are closely linked, and

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Abbreviations: DOC, sodium deoxycholate; ECF, extracytoplasmic function; KDO, 3-deoxy-D-manno-2-octulosonic acid; LPS, lipopolysaccharides; OPGs, osmoregulated periplasmic glucans; ROS, reactive oxygen species; SHX, serine hydroxamate; *S. typhimurium*, *Salmonella enterica* serovar Typhimurium; UVA, ultraviolet A; WT, wild-type.

Two supplementary figures are available with the online version of this article.

then alterations of either OMPs or LPS can initiate RseA proteolysis [13]. Alternative mechanisms of σ^E activation that are independent of DegS cleaving of RseA have also been demonstrated in cells exposed to acid stress or arriving at stationary growth phase [14–16].

Several environmental stress factors, such as heat shock, oxidative stress, hyperosmotic stress, acid stress and starvation, have been proposed or are known to activate the σ^E-dependent envelope stress response [5]. This study investigated the role of σ^E in the response of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) to other environmental stress factors. We report here that exposure to ultraviolet A (UVA) radiation, P22-phage and hypo-osmotic shock activate σ^E expression by different molecular mechanisms, and demonstrate the importance of the *rpoE* gene in the defence against these envelope stress factors. In addition, we describe two conditions that make the *rpoE* gene essential in *S. typhimurium*: the absence of LPS O-antigen or osmoregulated periplasmic glucans (OPGs) at low to moderate osmolarity. We suggest that *rpoE* may not be an essential gene in *S. typhimurium* because of the presence of a complete LPS O-antigen playing a protective role for the cell envelope.

METHODS

Bacterial strains, phage and growth conditions

All the bacterial strains used were derivatives of *S. typhimurium* LT2, unless otherwise specified; their origins and relevant genetic properties are listed in Table 1. Strain DA1468, an *argC95* mutant used as the standard strain in this laboratory [17], was employed for the construction of all the *S. typhimurium* strains described in this paper. Transductions were mediated by phage P22 HT105/1 *int*-201. All the strains constructed by transduction were obtained free of phage by successive single-colony isolations. The strains were *de novo* constructed prior each set of experiments; after each transduction, the phenotype of at least six derivatives was analysed (survival assays, β-galactosidase assays and growth curves) in preliminary assays, and a representative strain was chosen for the experiments shown in the paper. The *galE* strain was transduced in selective medium containing 0.5 % each of galactose and glucose to allow the

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
S. typhimurium		
IB2	*rpoE*::Cm	[50]
IB332	*rseA*::Cm	[50]
MA6999	*ompC390*::Tn*10*	N. Figueroa Bossi
MA8771	*ompF88*::FRT	N. Figueroa Bossi
SF1005	*rpoS*::Ap	[89]
DA1468	*argC95*, wild-type	[17]
DA1667	*galE1922*	Laboratory strain
DA2143	*relA21*::Tn*10* Δ*spoT281*::Cm	[90]
DA2172	*opgH2*::Mu*dJ*	[20]
DA2241	*rpoE*::Cm	DA1468×IB2 phage
DA2242	*rseA*::Cm	DA1468×IB332 phage
DA2245	*rpoS*::Ap	DA1468×SF1005 phage
DA2266	*rpoS*::Ap *rpoE*::Cm	DA2245×DA2241 phage
DA2280	*opgH2*::Mu*dJ rpoE*::Cm	DA2172×DA2241 phage
DA2281	*opgH2*::Mu*dJ ompC390*::Tn*10*	DA2172×MA6999 phage
DA2282	*opgH2*::Mu*dJ ompF88*::FRT	DA2172×MA8771 phage
DA2283	*galE1922 rpoE*::Cm	DA1667×DA2241 phage
DA2284	*galE1922 rseA*::Cm	DA1667×DA2242 phage
DA2285	*rpoS*::Ap *rseA*::Cm	DA2245×DA2242 phage
E.coli		
DH5α	F⁻ Φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*) *endA1 recA1 hsdR17*	Gibco
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pRS550	Transcriptional *lacZ* fusion vector	[23]
p*rpoEp3-lacZ*	Transcriptional fusion *rpoEp3–lacZ*	This work

All of the DA strains are derivatives of strain DA1468. For the β-galactosidase assays, the p*rpoEp3-lacZ* reporter plasmid was transduced to the corresponding recipient strain by employing a lysate of DA1468 p*rpoEp3-lacZ* and selecting ampicillin or kanamycin resistance.

synthesis of normal LPS [18]. The strains were grown routinely at 37 °C with shaking in rich medium Lysogeny broth (LB), composed of 10 g tryptone, 5 g yeast extract and 5 g NaCl bringing the volume up to 1000 ml in distilled water. Hereafter, this medium was referred to as standard LB (257 mosM) [19]. The low-osmolarity medium was LB with no NaCl added (100 mosM) and high-osmolarity medium was LB containing 0.3 M NaCl (700 mosM) [20]. For solid medium, 15 g l^{-1} agar was added. When required, the media were supplemented with ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; spectinomycin, 80 μg ml^{-1}; 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 40 μg ml^{-1}.

Construction of transcriptional fusion *rpoEp3–lacZ*

Conventional recombinant DNA techniques were employed to construct a transcriptional fusion, *rpoEp3–lacZ* [21]. The plasmids employed are listed in Table 1. To this purpose, genomic DNA of the DA1468 strain was amplified by PCR using Q5 high-fidelity DNA polymerase (New England Biolabs) and the primers RPES3 (5′-CCTCTAGATAAGACCTGTCTACAACATGAC-3′) and RPES4 (5′- CCC TCGAGGTAGCGCACTACCAGTAAGTTA-3′) [22]. The PCR amplification product (240 bp), containing the *p3* promoter of the *rpoE* gene, was first cloned into pGEM-T Easy (Promega) by previous A-tailing of the PCR product with *Taq* DNA polymerase (New England Biolabs). The resulting plasmid was introduced by transformation into *E. coli* DH5α (Bethesda Research Laboratories). The *Eco*RI fragment containing the *rpoEp3* promoter was removed from this plasmid and cloned into the *Eco*RI site of the cloning vector for operon fusions pRS550 [23], using DH5α as the recipient strain. The resulting new plasmid carrying the fusion *rpoEp3–lacZ* was transferred to DA1468 by electroporation. A lysate of P22 phage was obtained in this recombinant *S. typhimurium* strain to transfer by transduction this plasmid to the strains employed in this study .

β-galactosidase assay

β-galactosidase activity was assayed as described by Miller [24] in cells treated with sodium dodecyl sulfate (SDS) and chloroform. Specific activities were expressed in Miller units referred to OD_{650}.

Irradiation source

Cell suspensions were irradiated with a bench with two Philips TDL 18W/08 tubes (more than 95 % of the UVA emission at 365 nm), mounted on aluminium anodiser reflectors to enhance the fluence rate on the section to be irradiated. The incident fluence was measured at the surface of the suspension with a 9811.58 Cole-Parmer Radiometer (Cole-Parmer Instruments Co., Chicago, IL, USA). Two types of experiments were performed using this irradiation source at a fluence rate of 28 W m^{-2}: expression assays in LB medium and survival assays in saline solution (NaCl 0.1 M). This fluence rate resulted in sublethal doses in LB and lethal doses in saline because the culture medium absorbs the radiation at 365 nm, lowering the effective total UVA dose (Fig. S1, available in the online version of this article).

Growth under sublethal UVA radiation

Mid-exponential cultures were diluted to OD_{650} 0.05 in LB medium. The suspensions were divided into two 30 ml fractions, each of which was placed in a glass beaker. The beakers were placed in a multichamber coupled to a water circulator to ensure that the temperature of the suspensions was maintained at 37 °C. One of the fractions was irradiated from above at a fluence rate of 28 W m^{-2} at the level of the free surface of the suspension, whilst the other one was covered with a black plastic sheet (dark control). The cell suspensions were stirred continuously with a magnetic bar. Cell growth was followed by measuring the OD_{650}. Samples for chemiluminescence or β-galactosidase assays were taken at intervals.

Survival UVA assays

Bacteria were grown to stationary growth phase, washed once and suspended in saline solution (NaCl 0.1 M) at OD_{650} 0.4. The suspensions were divided into two 30 ml fractions, each of which was placed in a glass beaker. The beakers were maintained at 20 °C by using a water circulator, as described above. One of the fractions was irradiated from above at a fluence rate of 28 W m^{-2}, whilst the other fraction was covered with a black plastic sheet (dark control). The cell suspensions were stirred continuously with a magnetic bar. Samples were taken at intervals and plated on LB solid medium after dilution. Plates were incubated in the dark immediately after irradiation to prevent light-induced DNA repair and the colonies were counted after 24–48 h incubation at 37 °C. Survival was expressed as a fraction of the number of colony-forming units per ml (c.f.u. ml^{-1}) at time 0.

Treatment with serine hydroxamate

The induction of the stringent response with serine hydroxamate (SHX) was based on the work of Song *et al.* [25]. To this purpose, overnight cultures grown in LB with 160 μg ml^{-1} DL-serine added were diluted to OD_{650} 0.05 in the same medium and grown to OD_{650} 0.3. These cultures were diluted again in LB plus DL-serine to OD_{650} 0.05 and divided into two fractions: one had SHX 240 μg ml^{-1} added and the other was the control. Cells were incubated for 60 min and samples were withdrawn at different times for OD_{650} and β-galactosidase measurements.

Phage challenge tests

For phage challenge tests, overnight cultures were diluted to OD_{650} 0.05 in LB and split into two fractions: P22-HT phage was added to one [at a multiplicity of infection (m.o.i.) of about 100] and the other was the control fraction. Bacterial growth was followed spectrophotometrically. To analyse the induction of σ^E activity, control and test samples prepared as described above were grown for 24 h and β-galactosidase activity was measured.

Hypo-osmotic shock assays

Overnight cultures obtained in LB containing 0.3 M NaCl were diluted to OD_{650} 0.05 in standard LB, LB without NaCl or LB with 0.3 M NaCl added. Cell growth was followed by measuring the OD_{650}. To determine the effect on σ^E activity, strains submitted to the same treatment were grown for 24 h at 37 °C and β-galactosidase activity was measured.

LPS extraction and gel electrophoresis

LPS was extracted by the aqueous butanol method [26] and samples containing similar amounts of 3-deoxy-D-manno-2-octulosonic acid (KDO) were submitted to electrophoresis in 12 % polyacrylamide gels containing sodium deoxycholate (DOC-PAGE) [27]. The KDO was estimated as described by Dröge *et al.* [28]. LPS gels were silver-stained following the procedure of Tsai and Frasch [29].

Chemiluminescence assays

The production of photoemissive species was followed by means of a liquid scintillation system in the out-of-coincidence mode [30]. For this purpose, 1 ml aliquots were taken during bacterial growth and quickly transferred to the scintillation system, which was equipped with photomultipliers that are sensitive in the blue region up to 600–650 nm (Tri-Carb 1500, Packard Instruments Co.). Chemiluminescence values were expressed as counts min^{-1}/ OD_{650} unit.

Statistical analysis

The significance of each treatment was evaluated by Student's *t*-test; $P<0.05$ was considered significant.

RESULTS

Low UVA doses induce σ^E activity in a ppGpp-dependent pathway

One of the most stressing agents for bacteria present in aquatic or terrestrial environments is solar UVA radiation (400–315 nm), the major fraction of ultraviolet radiation reaching the Earth's surface. High doses of UVA lead to lethal effects indirectly via reactive oxygen species (ROS) (singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl anion), which produce oxidative damage to proteins and lipids, and finally cause cell inactivation [31–33]. On the other hand, it has been demonstrated that low (non-lethal) UVA doses also produce some oxidative damage and induction of adaptive defensive mechanisms [34–37]. In *S. typhimurium*, tolerance to oxidant agents such as hydrogen peroxide or paraquat requires σ^E activity, and alterations in the redox conditions can stimulate the σ^E regulon [38]. Taking this into account, we investigated the ability of UVA radiation to induce σ^E activity in cultures of *S. typhimurium* exposed to sublethal doses of this light. σ^E activity was evaluated by employing a reporter plasmid carrying the *rpoEp3* promoter fused to the *lacZ* gene. The *rpoEp3* promoter is one of the three promoters of the *rpoE* gene and its activity depends exclusively on σ^E [22].

Fig. 1a shows that exposure to UVA significantly induced σ^E activity in the wild-type (DA1468), increasing it by about twofold. In order to evaluate whether the RseA pathway is involved this response, a mutant lacking the *rseA* gene (DA2242 strain) was exposed to UVA and the β-galactosidase activity was quantified. σ^E activity was also induced in this strain (Fig. 1a), suggesting an alternative mechanism that is independent of RseA.

The transcriptional regulator ppGpp is elicited in response to nutritional stress. The presence of uncharged tRNAs through amino acid deprivation or a carbon source deficit lead to an increase in the level of this compound, a mechanism that is known as stringent response [39]. The regulator ppGpp represses the transcription of genes involved in RNA and protein biosynthesis, with the consequent arrest of bacterial growth, and induces other genes involved in virulence and protective functions [40]. A similar phenomenon was described in bacteria exposed to low UVA doses. Under this condition, certain photosensitive tRNAs are damaged, the aminoacylation capacity is restricted and ppGpp synthesis is induced by the direct (oxygen-independent) action of UVA [41–43].

It has been demonstrated in *E. coli* that, in addition to the direct regulation of RpoE by RseA, the transcriptional regulator ppGpp regulates the activity of σ^E [15]. On the one hand, it was demonstrated in *in vitro* and *in vivo* assays that ppGpp (in concert with the cofactor protein DksA) activates the transcription of sensitive promoters directly by binding to the σ^E RNA polymerase complex. On the other hand, it was proposed that ppGpp activates σ^E activity indirectly by altering the competition among sigma factors for core RNA polymerase in favour of alternative sigma factors [16]. To analyse whether the activation of σ^E by UVA depends on ppGpp, we assayed a strain that is unable to synthesize it by inactivation of the genes *spoT* and *relA* (DA2143, hereafter $ppGpp^0$) . In contrast to the results observed for the wild-type and *rseA* strains, exposure to UVA radiation did not induce σ^E activity in the $ppGpp^0$ strain (control, 56±13 Miller units; UVA, 49±17 Miller units) (Fig. 1a). It is worth noting the different levels of σ^E activity in the three strains: *rseA*>wild-type>$ppGpp^0$. In the *rpoE* mutant, the basal values of β-galactosidase (19±1 Miller units) were not affected by the UVA exposure, demonstrating that induction is due to the action of ppGpp on σ^E. Fig. 1(b) shows the growth curves for the three assayed strains under UVA and the dark controls. UVA produced a slight growth delay compared to the control cultures, but the viable cell count was not affected by the treatment (data not shown). The ultra-weak chemiluminescence procedure was employed to evaluate *in vivo* whether UVA exposure produced any oxidative damage. A peak of light production was observed in the three strains at the beginning of the exposure, indicating that in addition to the production of ppGpp by the direct effect of UVA, indirect oxygen-dependent effects were present under the conditions employed (Fig. 1c).

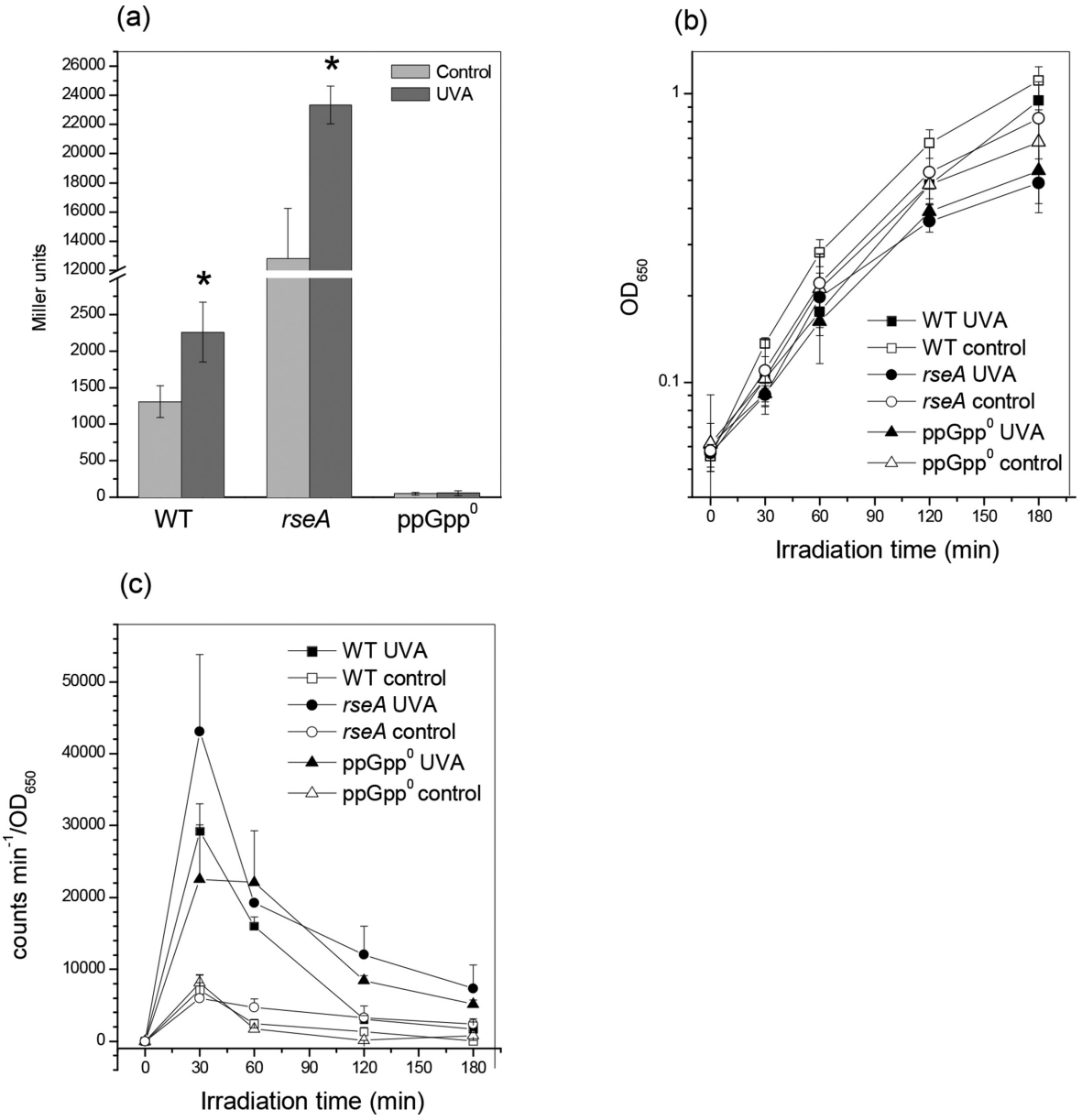

Fig. 1. Induction of σ^E activity, growth and oxidative damage by sublethal UVA exposure. The β-galactosidase activity of wild-type (WT), *rseA* and ppGpp^0 strains carrying the plasmid p*rpoEp3-lacZ* was measured after 270 min of growth under UVA (total UVA dose, 460 kJ m^{-2}) or in the dark (control) (a). The wild-type, *rseA* and ppGpp^0 strains were grown in LB under sublethal UVA doses (fluence rate 28 W m^{-2}) and growth (b) and chemiluminescence values (c) are shown. The control cells were grown under similar conditions but kept in the dark. The data are presented as the mean±SE of at least three independent experiments. *, P<0.05.

To confirm the role of ppGpp in σ^E activity in *S. typhimurium*, the effect of the serine analogue SHX was assayed. SHX resembles the bacterial response to amino acid starvation, triggering a *relA*-dependent ppGpp accumulation. The addition of SHX to the culture medium induced a marked growth delay in the wild-type, possibly due to an increase in the amount of ppGpp in the cells in response to this treatment; as expected, this growth delay was not observed in the ppGpp^0 strain because it does not produce ppGpp (Fig. 2a). As shown in Fig. 2(b), SHX-induced starvation caused the induction of σ^E activity in the wild-type after 30 and 60 min incubation; this effect was not observed in the ppGpp^0 strain. It was noted that in the ppGpp^0 strain the β-galactosidase activity was greater in the SHX assays (Fig. 2b) compared to that observed in UVA assays (Fig. 1b). The fact that the β-galactosidase measurements correspond to different incubation times and the experimental conditions were not the same should be taken into account; the cultures

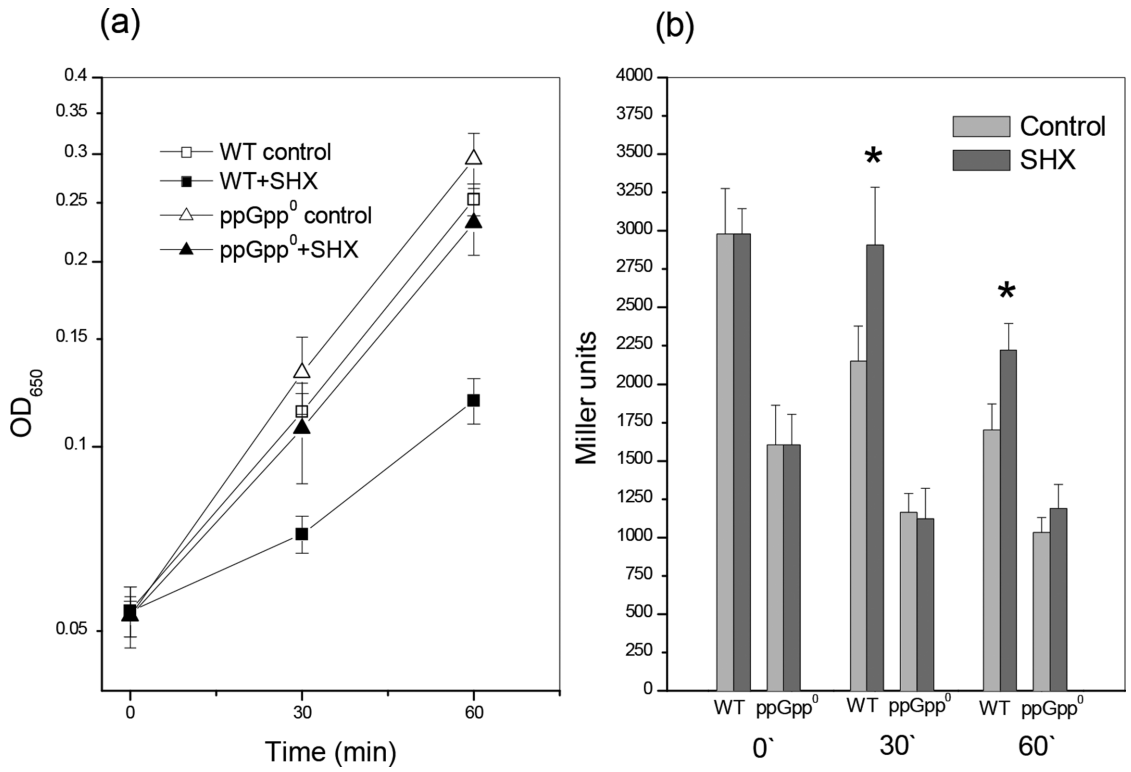

Fig. 2. Induction of σ^E activity by SHX. Logarithmic cultures of the wild-type (WT) and ppGpp^0 strains carrying the plasmid p*rpoEp3-lacZ* were grown in LB plus 160 µg ml^{-1} DL-serine or LB plus 160 µg ml^{-1} DL-serine and 240 µg ml^{-1} SHX. The cell growth (a) and β-galactosidase activity (b) were measured at 0, 30 and 60 min. The data are presented as the mean±SE of at least three independent assays. *, P<0.05.

were grown in LB in glass beakers open to air (UVA assays) or LB+serine in Erlenmeyer flasks capped with cotton plugs (SHX assays). These differences could explain the discrepancies in the β-galactosidase values of the ppGpp^0 strain; however, this is very striking, particularly in view of the fact that in the wild-type such differences were not observed.

Taken as a whole, the results indicating that UVA increases transcription from the *rpoEp3* promoter in the wild-type and the *rseA* strain but not in the ppGpp^0 strain suggest that this radiation promotes σ^E activity in a pathway that is dependent on ppGpp.

RpoE and RpoS are required for optimal resistance to lethal UVA doses

Since σ^E activity is induced by UVA exposure, we analysed the role of RpoE in the defence against the high UVA doses that *S. typhimurium* normally encounters in the environment [44]. Suspensions of stationary phase cells were exposed to a fluence rate of 28 W m^{-2} or maintained in the dark under similar conditions, and the survival curves were evaluated. As shown in Fig. 3, after 300 min exposure the *rpoE* strain (DA2241) was seen to be significantly more sensitive to UVA than the wild-type. By contrast, the *rseA* strain demonstrated greater resistance to UVA than the parental strain, possibly due to its high levels of σ^E activity.

It has been demonstrated that *S. typhimurium* has high resistance to natural sunlight and artificial UVA radiation compared to other micro-organisms ([45, 46], unpublished results). This resistance is mainly due to the action of the alternative sigma factor σ^S [47, 48], which is encoded for the *rpoS* gene and directs transcription during the stationary phase of genes related to resistance to diverse environmental stresses [49]. It has been demonstrated in *S. typhimurium* that σ^E promotes resistance to hydrogen peroxide by increasing σ^S levels, demonstrating the dominant role of the *rpoS* regulon in resistance to oxidative stress [50]. To investigate whether the role of *rpoE* in UVA defence is also ascribed to RpoS activity, we analysed the effect of UVA on an *rpoS* derivative and the double mutant *rpoE rpoS* (DA2245 and DA2266 strains, respectively). The viability of the *rpoS* mutant decreased by approximately 2–3 logs with respect to the wild-type, confirming the essential role of this gene in UVA defence (Fig. 3). The double mutant *rpoE rpoS* was more sensitive than the strain carrying the *rpoS* mutation alone, suggesting that *rpoE* participates in defence against UVA through functions that are dependent on and independent of RpoS. On the other hand, the *rpoS rseA*

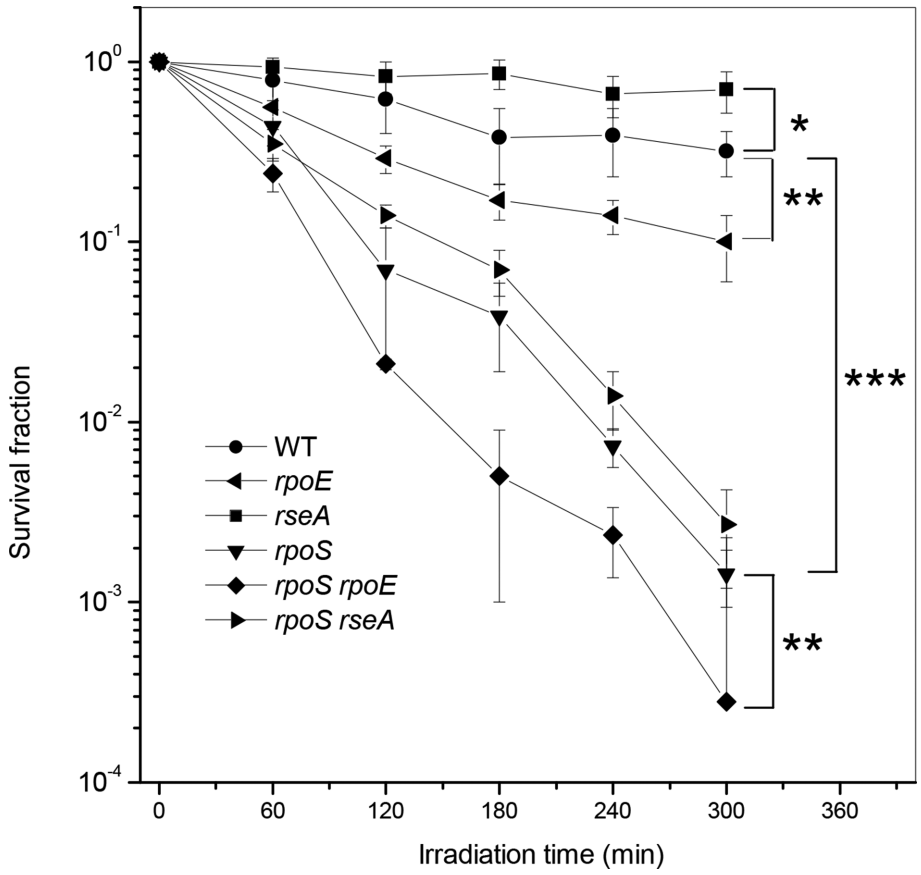

Fig. 3. Role of RpoE in survival against lethal UVA doses. Suspensions of stationary phase cells of the wild-type (WT) and its derivatives *rpoE*, *rseA*, *rpoS*, *rpoE rpoS* and *rpoS rseA* were exposed to a fluence rate of 28 W m^{-2} for 300 min (total UVA dose, 510 kJ m^{-2}) or kept in the dark. Samples were taken at different times and plated to determine survival. The data are presented as the mean±SE of at least three independent assays. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$.

double mutant was apparently more resistant than the *rpoS* single mutant, possibly due to an increase of σ^E activity; however a non-significant difference between both strains was observed after 300 min of UVA exposure (Fig. 3). The dark controls did not present changes in cell viability (data not shown).

P22 bacteriophage inhibits cell growth in *rpoE* mutants and induces σ^E activity in an RseA-dependent fashion

In attempts to construct the double mutant *rpoS rpoE* by transduction it was observed that the *rpoS*::Ap marker could not be transduced to the *rpoE* recipient strain, but *rpoS rpoE* derivatives could be obtained normally when the *rpoS* strain was used as a recipient. We also noted that the multiplication of the temperate phage HT-P22 onto solid medium in an *rpoE* recipient strain produced a significant higher number of clear plaques (92±2 %) compared to the wild-type (76±6 %). These observations led us to think that a strain lacking the *rpoE* gene could be more sensitive to phage infection. To investigate the role of σ^E in the response to this stress agent, the effect of P22 phage on bacterial growth of the wild-type and *rpoE* and *rseA* derivatives was evaluated. Fig. 4(a) shows that the presence of phage in the culture medium produced marked growth inhibition in the wild-type compared to the control culture without phage. This inhibition was stronger in a strain that was deficient for the *rpoE* gene, which barely duplicated its cell mass during the experiment. By contrast, the *rseA* mutant was more resistant to phage attack than the wild-type, possibly through its higher level of σ^E activity. A *galE* strain (DA1667) that was deficient for LPS antigen O-chains and resistant to P22 phage was tested as a control and showed no growth inhibition in the presence of phage (Fig. 4a, inset). The effect of P22 on the expression of β-galactosidase from the reporter plasmid p*rpoEp3-lacZ* was analysed in the wild-type and its derivatives. Fig. 4(b) shows significant induction of β-galactosidase activity in the wild-type. As expected, high β-galactosidase values were observed in the *rseA* strain; however, the presence of phage did not induce σ^E activity in this strain. Higher β-galactosidase

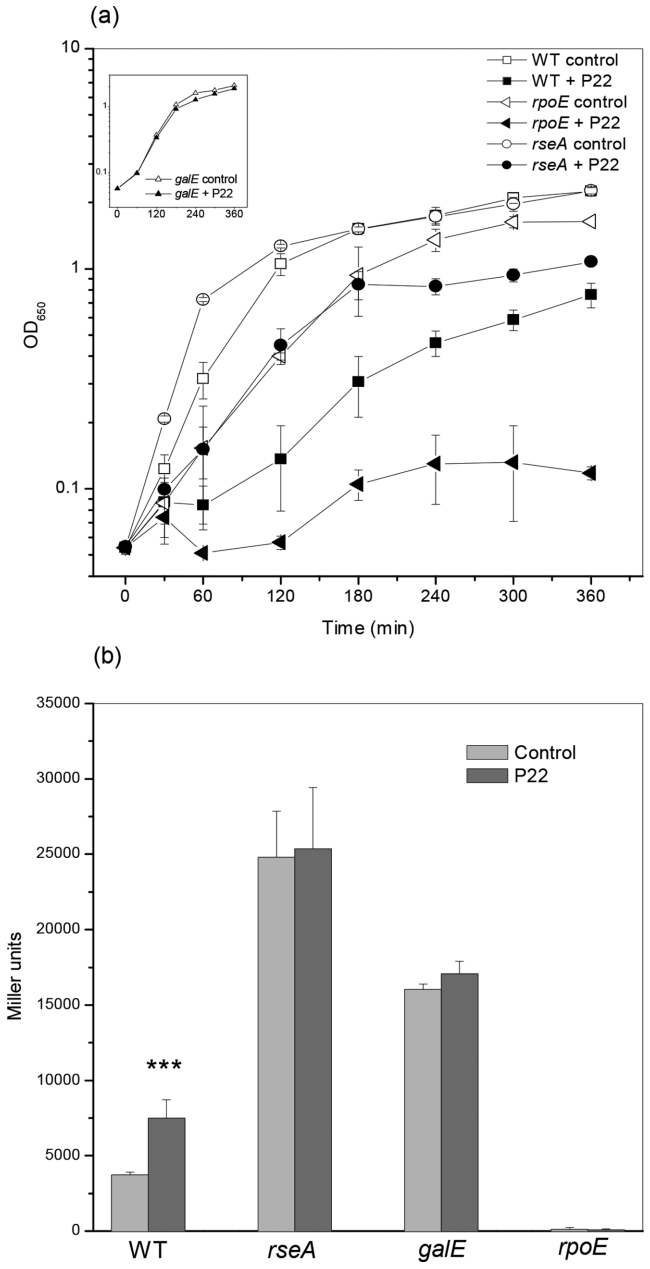

Fig. 4. Role of *rpoE* and the induction of σ^E activity in response to phage infection. Stationary phase cultures of the wild-type (WT) and its derivatives *rpoE*, *rseA* and *galE* were diluted to OD_{650} 0.05 and grown in LB or LB with phage P22 added [at a multiplicity of infection (m.o.i.) of about 100]. Growth was followed by measuring the OD_{650} (a). To evaluate the effect of phage on σ^E activity, the same strains carrying the plasmid p*rpoEp3-lacZ* were grown in LB or LB with phage P22 added and β-galactosidase activity was measured after 24 h incubation (b). The data are presented as the mean±SE of at least three independent assays. ***, $P<0.0005$.

values were observed in the *galE* strain compared to the wild-type; however, σ^E activity was not affected by the phage, which was an expected result because this is a resistant strain. The *rpoE* strain was tested as a negative control. It was concluded that the envelope stress generated by phage infection induces σ^E activity in *S. typhimurium* in an RseA-dependent fashion.

Induction of σ^E activity by hypo-osmotic shock: role of osmoregulated periplasmic glucans (OPGs)

One important component of the cell envelope of most Gram-negative bacteria is osmoregulated periplasmic glucans (OPGs). OPGs are periplasmic oligosaccharides composed of D-glucose synthesized by the products of the *opgGH* operon in response to a decrease in the osmolarity of the culture medium [51]. Previously, we observed that the *rpoE*::Cm marker was not transducible to *opg* mutants in standard LB agar (unpublished data). Taking the relationship between OPGs and medium osmolarity into account [20, 51], we analysed this incompatibility by plating the transduction mixes simultaneously in plates of LB with no salt, standard LB and LB containing 0.3 M NaCl. The wild-type strain was also assayed as a control. As shown in Fig. 5(a), the *rpoE*::Cm insertion could be transduced normally to the wild-type in all the tested media, but, surprisingly, *opgH rpoE* double mutants were only obtained in high osmolarity medium, suggesting an essential role for *rpoE* in the absence of OPGs at low to moderate osmolarity. Similar results were obtained with an *opgG* strain (data not shown). Control transductions were performed with other insertions, and the *opg* strains were demonstrated to be transducible in both standard and low-osmolarity media (data not shown).

Since *opg* mutants show alterations in the expression of the osmoregulated porins OmpC and OmpF when grown in low-osmolarity medium [52, 53] and σ^E activity is induced by alterations in porin levels [54], we analysed whether the lack of viability in *opg rpoE* derivatives could be reversed by removing OmpC or OmpF. To this purpose, we constructed *opgH* derivatives carrying *ompC* or *ompF* mutations (DA2281 and DA2282 strains, respectively) and analysed their tolerance to the *rpoE*::Cam insertion at different osmolarities. As shown in Fig. 5(b), the absence of *rpoE* is still lethal in these strains in low-osmolarity medium. However, in the absence of OmpC, the *rpoE* insertion was tolerated in standard LB, suggesting that this porin may be at least partially responsible for the observed phenomenon.

It has been reported that *rpoE* is critical to survival in highly hyperosmotic environments [55, 56]; however, it has not been reported that *rpoE* has a role in tolerance to hypo-osmotic shock. In order to analyse the role of *rpoE* (and its relationship with OPGs) in the bacterial adaptation to low-osmolarity medium, we compared the growth of the wild-type and its derivatives *rpoE*, *opgH* and *opgH rpoE* (DA2241, DA2172 and DA2280) when they were exposed to hypo-osmotic shock. Fig. 7(a, b) showed no significant effect in either the wild-type or the *opgH* strain; conversely, when the *rpoE* strain was changed to medium without NaCl, it suffered a growth delay, although after 120 min no difference was observed compared to the wild-type (Fig. 5b). The growth curves of the *opgH rpoE* derivative

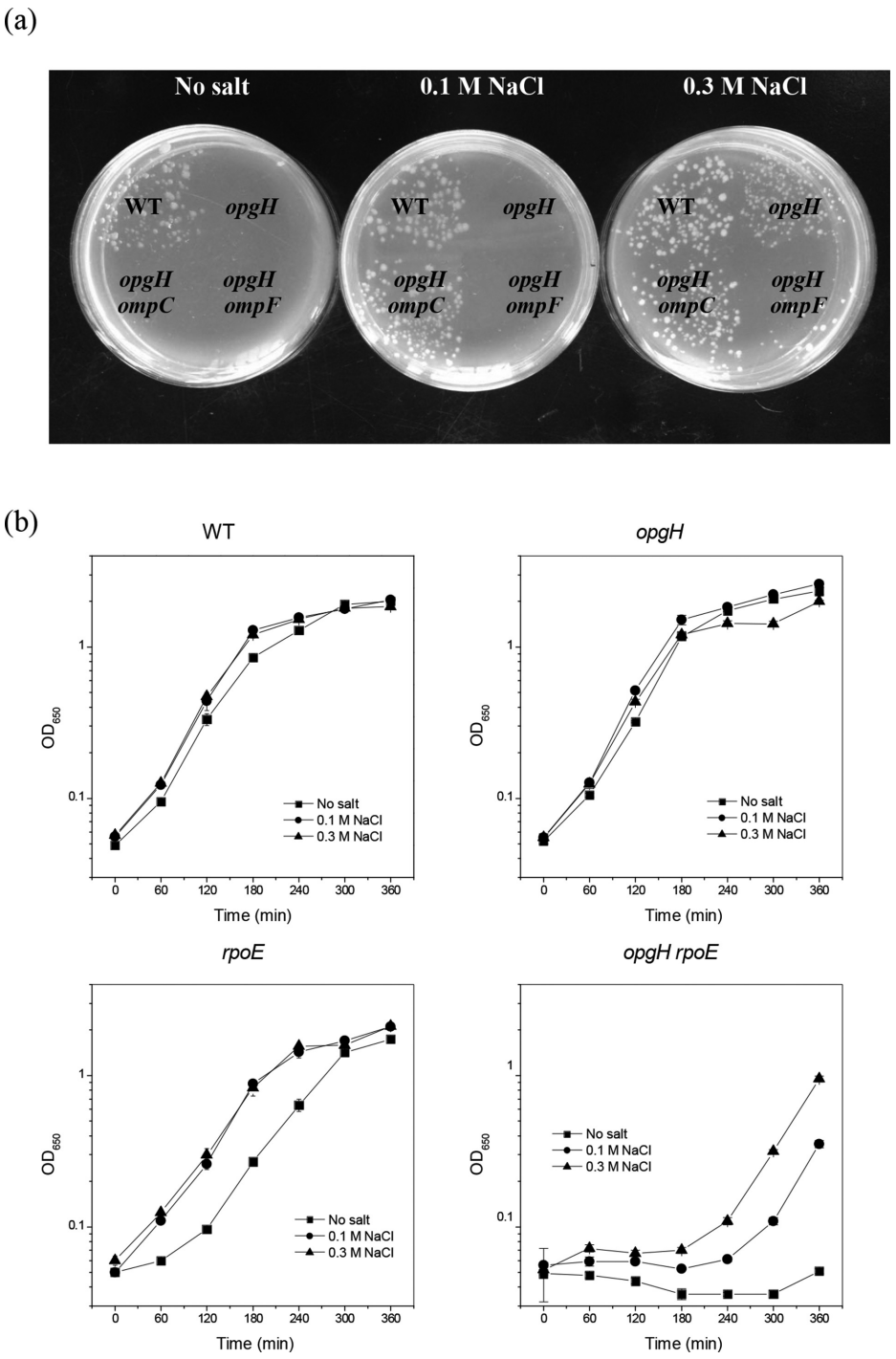

Fig. 5. Lethality of an *rpoE* mutation in an *opg* mutant under hypo-osmotic shock. Phage grown on the strain DA2241 was used to transduce the *rpoE*::Cam marker to the recipient strains: wild-type (WT), *opgH*, *opgH ompC* and *opgH ompF*. Equal volumes of transduction mixtures were spread on LB without NaCl (no salt), standard LB (0.1 M NaCl) or LB containing 0.3 M NaCl; all the plates contained 10 µg ml^{-1} cloramphenicol for the selection of the *rpoE*::Cam marker (a). Stationary phase cultures of the wild-type and its derivatives *opgH*, *rpoE* and *opgH rpoE* that had been grown in LB with 0.3 M NaCl added were diluted to OD_{650} 0.05 in LB without NaCl (no salt), or with standard LB (0.1 M NaCl) or LB with 0.3 M NaCl added. Growth was followed by measurement of the OD_{650} for 360 min. The data are presented as the mean±SE of at least three independent assays (b).

showed that the change to medium without NaCl completely inhibited the growth of this strain; by contrast, although a marked lag was observed in standard LB and in high-osmolarity media, after 240 min incubation the growth in the double mutant was restored (Fig. 5b). Even though the methodology was very different, these results were consistent with those presented in Fig. 5(a).

Taken as a whole, these results led us to propose that in *S. typhimurium* the absence of OPGs in low to moderate osmolarity media generate lethal disturbances in an *rpoE* context. To go further in this proposal, we compared the transcription from the *rpoEp3–lacZ* fusion in the wild-type and an *opgH* mutant transferred from high osmolarity to media of different osmolarities. As shown in Fig. 6, no significant difference was observed in the wild-type, but the *opgH* mutant exhibited a fivefold increase in β-galactosidase activity when changed to low-osmolarity medium in comparison to the parental strain. This induction decreased in the absence of OmpC or OmpF porins (fourfold compared to the wild-type) (Fig. 6). The results indicate that hypo-osmotic shock in the absence of OPGs generates envelope disturbances that are able to induce *rpoE*; this phenomenon could be attributed, at least in part, to OmpC and OmpF alterations.

In order to investigate whether changes in LPS are responsible for σ^E activation at low osmolarity in the absence of OPGs, LPS profiles from the wild-type and an *opgH* derivative grown at different osmolarities were compared. DOC-PAGE electrophoresis showed no significant differences between the banding patterns (Fig. S2), suggesting that the induction of σ^E activity in *opg* mutants exposed to hypo-osmotic shock is not related to LPS alterations.

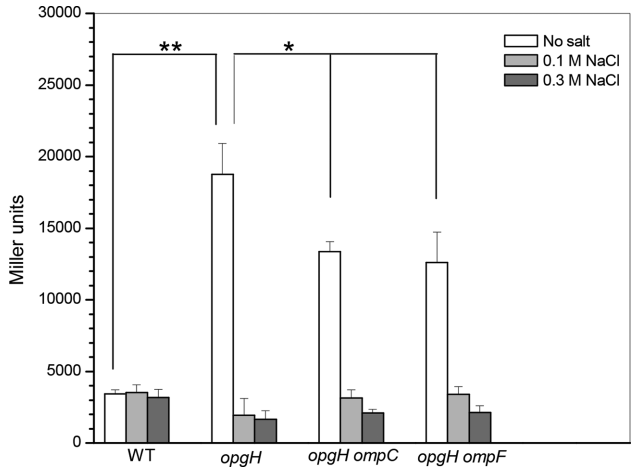

Fig. 6. Induction of σ^E activity in the absence of OPGs by hypo-osmotic shock. Stationary phase cultures of the wild-type and its derivatives *opgH*, *opgH ompC* and *opgH ompF* carrying the plasmid p*rpoEp3*-lacZ that had been grown in LB with 0.3 M NaCl added were diluted to OD_{650} 0.05 in LB without NaCl (no salt), standard LB (0.1 M NaCl) or LB with 0.3 M NaCl added. After 24 h incubation, the β-galactosidase activity was measured. The data are presented as the mean±SE of at least three independent assays. *, $P<0.05$; **, $P<0.005$.

rpoE gene is essential in the absence of LPS O-antigen in *S. typhimurium*

It has been reported that in *E. coli* changes in LPS due to mutations in the LPS– biosynthetic pathway or the addition of ammonium metavanadate enhance σ^E activity [12, 57, 58]. In agreement with these results, we showed that elimination of the LPS O-antigen by a *galE* mutation is able to induce an increase in the transcription from the *rpoEp3* promoter in *S. typhimurium* (Fig. 4b). This finding led us to hypothesize that the lack of LPS O-antigens could be harmful in an *rpoE*-deficient context in *S. typhimurium*. To test this hypothesis, the survival of a double mutant *galE rpoE* was analysed. A *galE* strain has defective LPS due to the absence of UDP-galactose 4-epimerase, an enzyme that converts glucose into galactose, which is required for the production of normal LPS O-antigen chains. The absence of O-antigen chains makes this strain resistant to P22 phage, but the addition of small amounts of galactose enables the synthesis of normal LPS in *galE* mutants and make it transducible. A *galE rpoE* (DA2283) double mutant was constructed by transduction of the DA1667 strain with phage grown on DA2241, and the *rpoE*::Cm marker was selected in LB plates supplied with chloramphenicol, galactose and glucose. A *galE rseA* derivative (DA2284) was constructed in the same way as control. Once double mutants were obtained, their behaviour in solid and liquid medium was analysed in the absence or presence of glucose and galactose, in order to compare the $galE^+$ and *galE* phenotypes. Fig. 7 shows that when galactose and glucose were present, all the tested strains grew normally, both in solid (Fig. 7a) and liquid media (Fig. 7b). By contrast, when galactose and glucose were eliminated to restore the LPS-defective *galE* phenotype, the double mutant *rpoE galE* almost did not grow in solid medium (Fig. 7a) and suffered from growth delay in liquid medium (Fig. 7b), while the other strains grew normally. This result indicates that the *rpoE* gene could be essential in *S. typhimurium* in the absence of a complete LPS when it is growing in solid medium, while in liquid medium this phenomenon is attenuated.

DISCUSSION

A large number of genes distributed throughout the entire genome are involved in pathogenic processes in the facultative intracellular pathogen *S. typhimurium*. These genes include the transcriptional regulator σ^E, which responds to envelope stress. Here we report new stress factors that are able to promote its activity and present evidence for the conditional essentiality of its encoding gene *rpoE*, contributing to our understanding of the molecular basis for the tolerance of *S. typhimurium* to potentially lethal environments.

Solar UVA radiation is a common stress factor that bacteria must face in the environment. Moreover, natural and artificial UVA sources have been proposed in disinfection

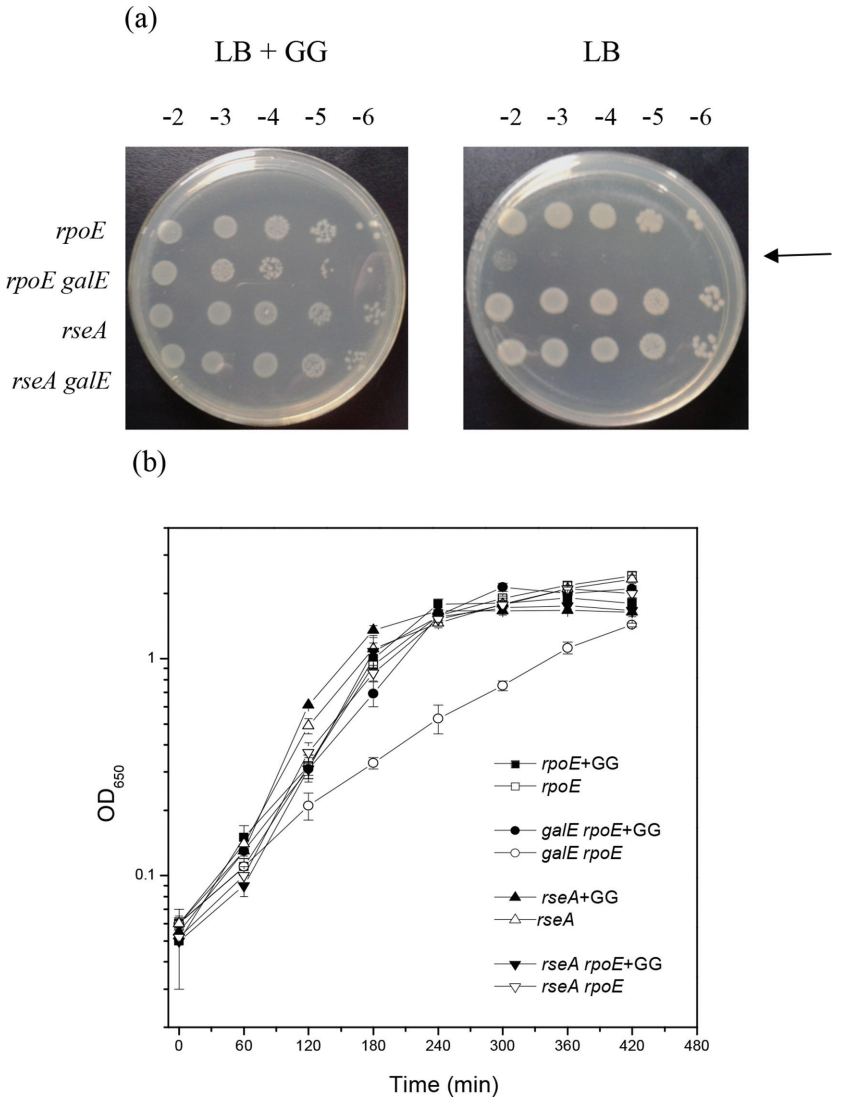

Fig. 7. Conditional lethality of an *rpoE* insertion in the absence of LPS O-antigen. Overnight cultures of strains *rpoE*, *rseA* and its derivatives *rpoE galE* and *rseA galE* were grown in LB to which 0.5 % each of galactose and glucose had been added. Ten microlitres of serial dilutions of these cultures were plated onto LB plates with and without these sugars (a). The same overnight cultures were diluted to OD_{650} 0.05 in LB or LB added with 0.5 % galactose and glucose and the growth was followed spectrophotometrically (b). The data are presented as the mean±SE of at least three independent assays.

strategies [59, 60]. In *S. typhimurium*, studies with mutants affected in genes encoding oxidative stress regulators (OxyR, RpoS) and antioxidant enzymes (*ahp*, *ghs*) have demonstrated that detoxification of ROS is a key factor in UVA defence [47, 48, 61]. We demonstrate here that σ^E is another component in *S. typhimurium*'s defensive response to UVA. A previous study with the unrelated micro-organism *Caulobacter crescentus* demonstrated the induction of σ^E activity by UVA and singlet oxygen [62], and it has been demonstrated in *Rhodobacter sphaeroides* that singlet oxygen (a product of UVA radiation) is able to dissociate the σ^E–antisigma ChrR complex, with the consequent expression of σ^E target genes [63]. Under the conditions employed in this study, we observed that the activation of σ^E depends on ppGpp production, generated by a direct effect (oxygen-independent) of the radiation. This sublethal effect has been investigated by several researchers and the phenomenon was proposed as an adaptive mechanism against the lethal and mutagenic effects of higher doses of UVA and solar irradiation [64]. To the best of our knowledge, the current study reports the first case of an environmental stress factor promoting σ^E activation independently of RseA proteolysis. Under sublethal UVA doses, we observed that some oxidative damage occurs (Fig. 1c), so the possibility that ROS produced by indirect (oxygen-dependent) effects can activate σ^E cannot be dismissed, as previously demonstrated in

R. sphaeroides [63]. However, if this process depends on RseA proteolysis, under our conditions we were unable to see it, because the *rseA* derivative still shows σ^E activation (Fig. 1a). The protective role of σ^E in UVA defence was confirmed by survival assays. Taking into account our results demonstrating that the double mutant *rpoE rpoS* is more sensitive to UVA than the single mutants *rpoE* and *rpoS*, the protective effect of *rpoE* against UVA radiation could be classified into two categories: RpoS-dependent and RpoS-independent. The former could be ascribed to the role of σ^E in the enhancement of RpoS concentration, with the consequent promotion of antioxidant defence, as proposed by Bang *et al.* [50]. On the other hand, RpoS-independent effects could be a consequence of the expression of genes related to the maintenance of envelope integrity or oxidative stress response [65].

In this study, we demonstrate that phage infection is able to induce σ^E activation in an RseA-dependent manner. Bacteriophages are the most abundant form of life in the biosphere and are key factors in modulating bacterial populations – they are crucial vectors of horizontal gene transfer, driving bacterial evolution [66]. Bacteriophage attachment to the bacterial surface is the first stage of phage infection and causes envelope perturbation [67]. *S. typhimurium* has several specific bacteriophages, which belong to different groups. P22, the prototypic P22-like phage, was employed in this study [68]. P22 adsorption is initiated by phage binding to the LPS O-side chains via the tailspike proteins of the viral particle [69]. These proteins show endorhamnosidase activity, which digests the O-antigen, enabling the diffusion of the phage through the LPS to the surface of the outer membrane [70, 71]. Previous studies have demonstrated that changes in the LPS structure caused by chemicals or mutations significantly induce the σ^E response [12, 57, 58], hence the induction of σ^E by phage infection could be attributed to LPS disruption. The role of *rpoE* in the relationship between bacteriophages and their hosts is relevant for the better understanding of microbial ecosystems and their exploitation.

It has also been demonstrated here for the first time that *rpoE* plays a role in optimal tolerance to the passage to low-osmolarity medium. Adaptation to hypo-osmotic shock requires bacterial mechanosensitive (MS) channels, membrane proteins that are capable of responding to mechanical stress [72]. The rapid transfer from high- to low-osmolarity environments produces an influx of water, which in turn increases the tension in the membrane that gates MS channels. Transient pores are then formed in the membrane and solute efflux through these pores reduces the rate of water influx, preventing cell lysis [73–75]. Stokes *et al.* [76] reported that channel expression belongs to the RpoS regulon. Since σ^E increases the level of RpoS concentration [50], it is possible that the role of *rpoE* in hypo-osmotic shock is due to the relationship between these alternative sigma factors.

In this study, two cases of *rpoE* mutations in *S. tyhimurium* being conditionally lethal are reported. Firstly, we have shown that the *rpoE* gene is essential in solid medium in the absence of OPGs at low and moderate osmolarity (Fig. 5a). This finding led us to conclude that OPGs play a role in the maintenance of envelope integrity under these conditions. At low osmolarity, this role seems to be unrelated to alterations in OmpC, OmpF or LPS. However, at moderate osmolarity, the lack of OmpC enables the survival of the double mutant *opgH rpoE*, indicating that the role of OPGs in envelope integrity is related to OmpC porin. Complementary studies in liquid cultures show that the absence of OPGs significantly induces σ^E activity at low osmolarity (Fig. 6), partially supporting the results in solid medium. Some differences can be seen between the two aspects of this phenomenon (tolerance to an *rpoE* mutation vs induction of σ^E activity), e.g. the role of porins, but these can be attributed to the use of different approaches. Nevertheless, this study opens up a new scenario regarding the role of OPGs. In *S. typhimurium*, OPGs are synthesized at low osmolarity and contribute to virulence, cell growth and motility in this condition through unknown mechanisms [77]. These functions could be related to the maintenance of cell envelope stability by these compounds, but further studies are needed to obtain more in-depth information regarding the underlying mechanisms involved in this role.

Secondly, we have demonstrated that *rpoE* mutations are not tolerated in a *S. typhimurium* derivative deprived of its normal O antigen; this phenomenon was absolute on agar plates, but attenuated in liquid cultures (Fig. 7). The difference between solid and liquid media could be explained by the more stringent conditions generated by solid medium, as demonstrated by Cuny *et al.* [78]. These authors reported that the passage of bacterial cultures to solid medium requires an adaptation that involves heat shock (RpoH, RpoE and CpxAR) and oxidative stress (SoxRS, OxyR and Fur) regulons, suggesting the involvement of multiple stresses. It was always striking that, unlike *S. tyhimurium*, *E. coli* does not accept absolute *rpoE* mutations [6, 7]. In order to explain this, a subsequent study showed that σ^E plays an essential function under non-stress conditions related to the maintenance of cell envelope integrity in this micro-organism [79]. On the basis of our results, this difference between these closely related bacteria may be a consequence of their particular envelopes. Outer-membrane LPS consist of three regions: a conserved lipid A, a short core carrying KDO and oligosaccharides, and the O-antigen polysaccharide assembled in a variable number of oligosaccharide repeating units. All derivatives of *E. coli* K12, the prototypic *E. coli* strain employed in laboratory assays, are unable to synthesize a complete O-antigen due to mutations within the *rfb* locus [80, 81]. By contrast, *S. typhimurium* possesses a complete O-antigen. This LPS fraction is essential in several functions related to resistance to stress factors and pathogenic processes, in addition to constituting the primary defence against serum complement activation [82–84]. An incomplete LPS might make the cell more sensitive to envelope stress factors and, in consequence, unable to tolerate the absence of the protective functions regulated by σ^E.

Pathogens' surfaces make first contact with the host and are a major target for its antibacterial strategies. Bacterial extracytoplasmic stress responses are essential in the defence against the injuries caused by these hosts, as demonstrated in the case of σ^E in *S. tyhimurium* [85–87]. Transcriptional studies have revealed that much of the *S. typhimurium* genome (approximately 58 %) is regulated by σ^E, probably indirectly through the modulation of multiple general regulators [88]. Thus, studies on the activation and role of σ^E are essential for a better understanding of the general biology and pathogenic processes of this microorganism.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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