



# Activation of the *Listeria monocytogenes* Stressosome in the Intracellular Eukaryotic Environment

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**ABSTRACT** *Listeria monocytogenes* is a ubiquitous environmental bacterium and intracellular pathogen that responds to stress using predominantly the alternative sigma factor SigB. Stress is sensed by a multiprotein complex, the stressosome, extensively studied in bacteria grown in nutrient media. Following signal perception, the stressosome triggers a phosphorylation cascade that releases SigB from its anti-sigma factor. Whether the stressosome is activated during the intracellular infection is unknown. Here, we analyzed the subcellular distribution of stressosome proteins in *L. monocytogenes* located inside epithelial cells following their immunodetection in membrane and cytosolic fractions prepared from intracellular bacteria. Unlike bacteria in laboratory media, intracellular bacteria have a large proportion of the core stressosome protein RsbR1 associated with the membrane. However, another core protein, RsbS, is undetectable. Despite the absence of RsbS, a SigB-dependent reporter revealed that SigB activity increases gradually from early (1 h) to late (6 h) postinfection times. We also found that RsbR1 paralogues attenuate the intensity of the SigB response and that the miniprotein Prli42, reported to tether the stressosome to the membrane in response to oxidative stress, plays no role in associating RsbR1 to the membrane of intracellular bacteria. Altogether, these data indicate that, once inside host cells, the *L. monocytogenes* stressosome may adopt a unique configuration to sense stress and to activate SigB in the intracellular eukaryotic niche.

**IMPORTANCE** The response to stress mediated by the alternative sigma factor SigB has been extensively characterized in *Bacillus subtilis* and *Listeria monocytogenes*. These bacteria sense stress using a supramacromolecular complex, the stressosome, which triggers a cascade that releases SigB from its anti-sigma factor. Despite the fact that many structural data on the complex are available and analyses have been performed in mutants lacking components of the stressosome or the signaling cascade, the integration of the stress signal and the dynamics of stressosome proteins following environmental changes remain poorly understood. Our study provides data at the protein level on essential stressosome components and SigB activity when *L. monocytogenes*, normally a saprophytic bacterium, adapts to an intracellular lifestyle. Our results support activation of the stressosome complex in intracellular bacteria. The apparent loss of the stressosome core protein RsbS in intracellular *L. monocytogenes* also challenges current models, favoring the idea of a unique stressosome architecture responding to intracellular host cues.

**KEYWORDS** *Listeria*, stressosome, SigB, intracellular, RsbR1, phosphorylation

**L** *isteria monocytogenes* is a ubiquitous Gram-positive bacterium that colonizes a large variety of environmental niches, including soil, decaying plant material, water, and industrial facilities (1, 2). This saprophytic lifestyle is shared with its capacity to

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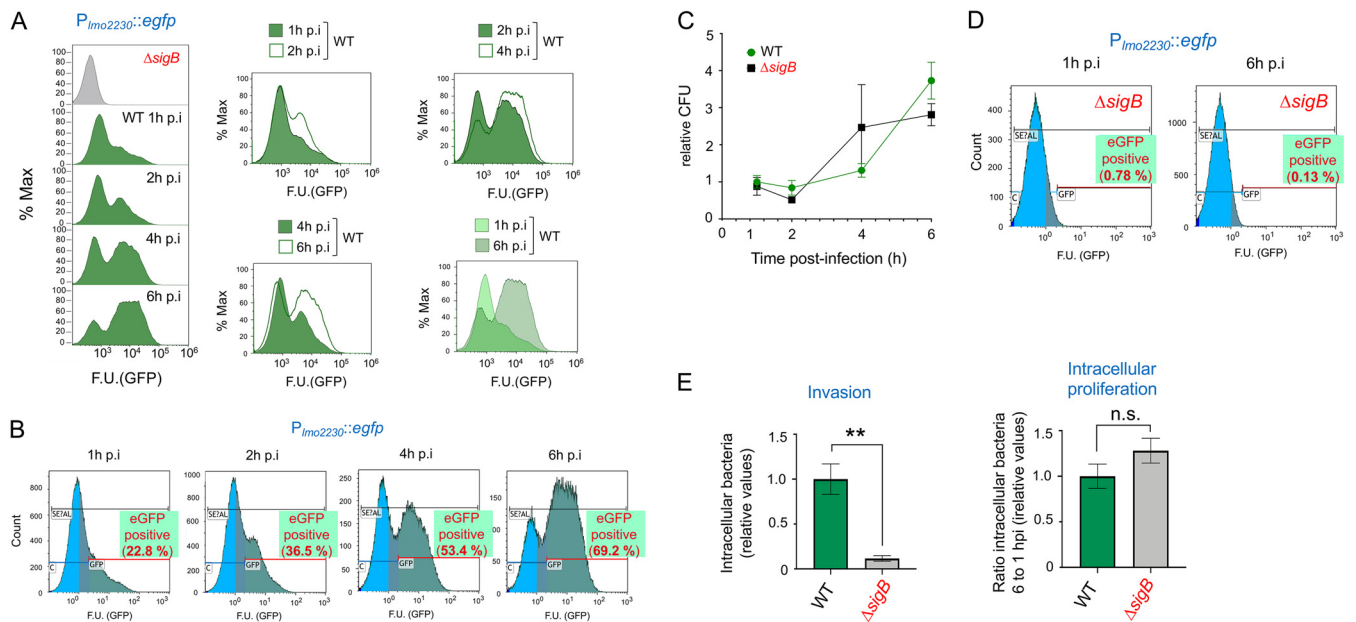
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infect animal hosts, causing severe foodborne diseases that progress with intracellular infections of phagocytic and nonphagocytic host cells (1, 3, 4). This pathogen withstands extreme stresses, including low pH, high osmolarity, low temperatures, and exposure to bile salts and antimicrobials, among others (5, 6). Such stress responses are fundamental to trigger a successful virulence program upon host contact (7). The stress experienced during this transition is relevant in the early infection stages, including passage through the acidic stomach, the high osmolality of the gut, and the competition with endogenous intestinal microbiota.

In *L. monocytogenes* and *Bacillus subtilis*, the alternative sigma factor SigB ( $\sigma^B$ ) mediates the response to stress by modulating the expression of hundreds of genes upon stress perception (6–10). SigB is normally bound to its anti-sigma factor, RsbW, which is itself regulated by the anti-anti-sigma factor RsbV. The association between RsbW and RsbV is favored when the latter is dephosphorylated by the RsbU phosphatase. The ratio between phosphorylated and unphosphorylated RsbV controls the levels of free SigB that associates with the RNA polymerase and, consequently, the intensity of the response (7, 11–13). RsbV phosphorylation is controlled in *B. subtilis* by two phosphatases, RsbU or RsbP, which respond to either environmental or energy-related stress signals (14–16), whereas in *L. monocytogenes* the stress signals are integrated in a single RsbU-dependent pathway (17).

Both *L. monocytogenes* and *B. subtilis* use a supramacromolecular complex, the stressosome, to perceive and process the external stress signals by yet-unknown mechanisms (18–21). An exception is sensing of blue light, which activates SigB via a stressosome-associated protein named RsbL (7). Although assembled and extensively characterized *in vitro* using purified recombinant proteins, the architecture of the stressosome in whole cells remains poorly understood. RsbR1 (RsbRA in *B. subtilis*) and RsbS are core proteins of the stressosome phosphorylated by the kinase RsbT following detection of the stress signal. Until recently, the most widely accepted model predicted that stress stimulates the RsbT kinase to phosphorylate one of the two phosphorylatable threonine residues in RsbR1 and a serine residue in RsbS (22–26). These phosphorylation steps culminate with RsbT release from the stressosome, allowing its interaction with RsbU and the stimulation of the SigB activation cascade. This model was mostly inferred from *in vitro* studies based on recombinant proteins. Thus, an RsbT variant of *B. subtilis* lacking its kinase activity does not complex with RsbRA and RsbS (27). Studies performed in *L. monocytogenes* exposed to oxidative stress identified Prli42, a miniprotein that tethers the stressosome to the membrane by interacting with RsbR1 (18). This work also showed that Prli42 interacts with the RsbR1 paralogues RsbR2 (Lmo0161), RsbL (Lmo0799), and RsbR3 (Lmo1642). A recent study based on subcellular fractionation and analysis of phosphorylation state of stressosome proteins revealed that *L. monocytogenes* RsbR1 and RsbS are mainly cytosolic and predominantly phosphorylated, regardless of exposure to osmotic stress (28). This work also showed that phosphorylated RsbR1 and its paralogues may play opposing roles in regulating SigB activity (28).

Despite the information obtained in *L. monocytogenes* exposed to different stresses under laboratory conditions, the role of SigB throughout the infection process remains controversial. Many studies suggest regulatory cross talk between SigB and the virulence regulator PrfA (6); the latter protein is essential for *L. monocytogenes* invasion and survival within eukaryotic cells (3). The available information assigns a major role to SigB in early events of the infection process mediating bacterial survival in the stomach and the intestinal tract (reviewed in reference 6). The fact that  $\Delta sigB$  mutants are attenuated by the oral route, although they are fully virulent when injected intravenously (29), provides further support for the apparent dispensability of SigB for virulence. Moreover,  $\Delta sigB$  mutants proliferate like wild-type bacteria inside eukaryotic cells (30). Nonetheless, other observations suggest that SigB could play a role in the interaction of *L. monocytogenes* with host cells. First, the SigB-dependent genes *opuCA* and *gadA*, which encode a glycine betaine transporter and a glutamate decarboxylase



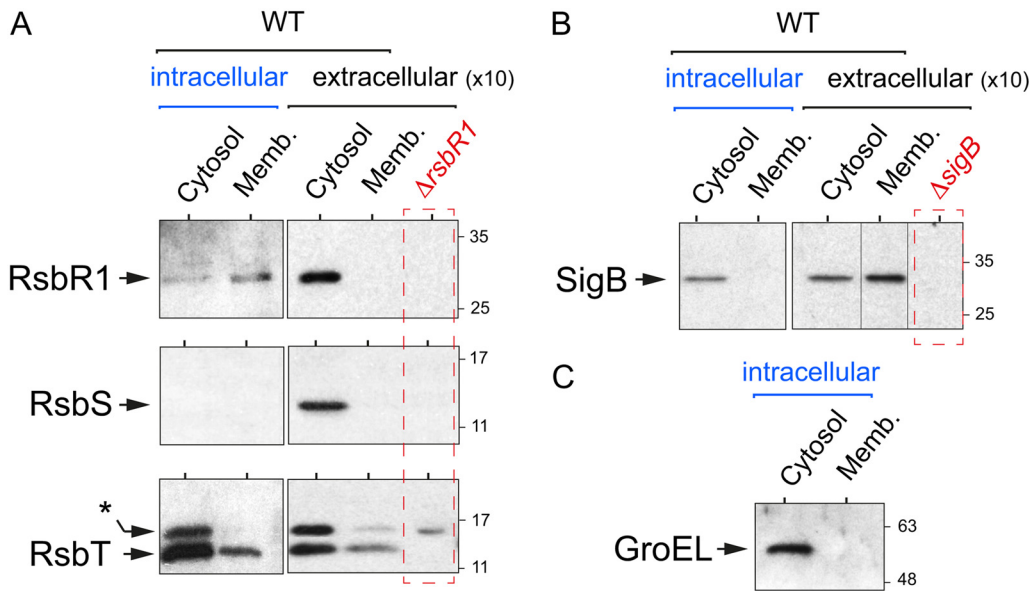
**FIG 1** *L. monocytogenes* increases SigB activity inside epithelial cells as infection progresses. Wild-type strain EGD-e (WT) and the isogenic  $\Delta sigB$  mutant bearing the  $P_{Imo2230}::egfp$  reporter fusion were used to infect JEG-3 epithelial cells. (A) Fluorescence intensity registered by flow cytometry at the indicated time points after infection with wild-type bacteria. The right panels show side-to-side comparison in the period from 1 h to 6 h, with a progressive increase in reporter expression. (B) Percentage of wild-type cells positive for expression of the  $P_{Imo2230}::egfp$  reporter at the indicated times. Data are representative of the analysis of three biological replicates with one technical replicate for each time point of infection. (C) Intracellular growth of *L. monocytogenes* wild-type and  $\Delta sigB$  reporter strains inside JEG-3 epithelial cells. The results are from three independent experiments and are shown as relative values (CFU values from WT and the  $\Delta sigB$  mutant at 1 hpi were set to 1 and corresponded to  $2.7 \times 10^4$  and  $3.1 \times 10^3$  CFU, respectively, in a representative experiment). (D) Signal derived from the  $P_{Imo2230}::egfp$  reporter in the  $\Delta sigB$  mutant at 1 h and 6 h postinfection. (E) Invasion and intracellular proliferation rates of wild-type and  $\Delta sigB$  reporter strains (CFU from WT at 1 h and 6 h postinfection were set to  $2.7 \times 10^4$  and  $6.7 \times 10^4$  CFU, respectively, in a representative experiment). One-tailed *P* values are indicated by asterisks for comparison between wild type and  $\Delta sigB$  strains by the *t* test (\*\*,  $P < 0.01$ ; n.s., not significant). F.U., fluorescence units.

subunit, respectively, are transcribed at higher levels in wild-type bacteria than in a  $\Delta sigB$  mutant in the cytosol of epithelial cells (30). This observation implies that functions of the SigB regulon known to be required for metabolic readjustments in response to extracellular stresses might also be involved in pathogen survival and/or growth inside host cells. Second, together with PrfA, SigB controls expression of the *inIA* and *inIB* genes, encoding surface proteins required for bacterial invasion (31, 32). Third, the  $P_{2prfA}$  promoter, which is active in intracellular bacteria, is partially dependent on SigB (30), and, fourth, *L. monocytogenes* represses motility inside eukaryotic cells, and the flagellar repressor gene *mogR* has a promoter that depends on SigB (33). This positive regulation agrees with the increased motility displayed by  $\Delta sigB$  mutants (34).

Taking these observations into account, we investigated *L. monocytogenes* SigB activity inside eukaryotic cells using approaches that involved monitoring in intracellular bacterial levels, activities, and subcellular distribution of SigB and core stressosome proteins. Consistent with the biochemical data, a SigB-dependent transcriptional reporter fusion confirmed a striking increment of SigB activity as the intracellular infection progressed to reach the pathogen replicative phase in the cytosol of the infected cell.

## RESULTS

***L. monocytogenes* activates SigB and alters localization of stressosome proteins inside epithelial cells.** To determine whether *L. monocytogenes* activates SigB inside host cells, we infected JEG-3 human epithelial cells with a strain bearing a  $P_{Imo2230}::egfp$  fluorescent reporter fusion integrated into the chromosome. *Imo2230* encodes a predicted arsenate reductase; it forms part of the SigB regulon and is highly dependent on this sigma factor for expression (31, 35, 36). The fluorescence signal derived from the  $P_{Imo2230}::egfp$  reporter was monitored by flow cytometry in intracellular bacteria at distinct postinfection times (Fig. 1A and B).  $P_{Imo2230}::egfp$  expression

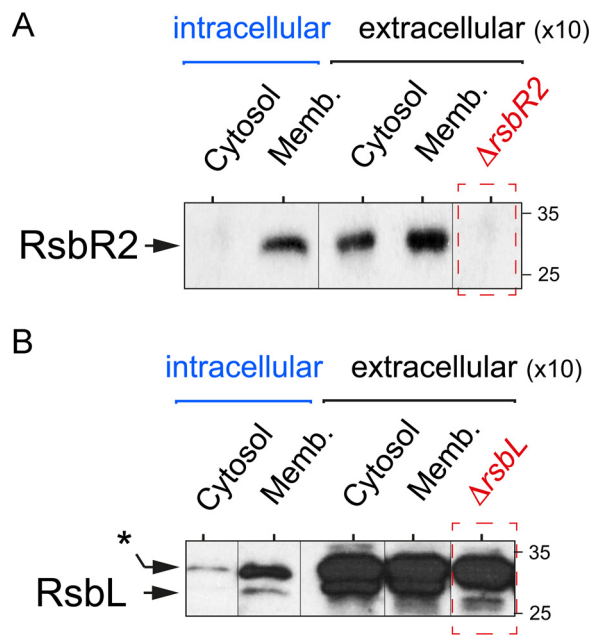


**FIG 2** The core stressosome protein RsbR1 is predominantly associated with the membrane in intracellular *L. monocytogenes*. JEG-3 epithelial cells were infected for 30 min with *L. monocytogenes* EGD-e wild type (WT) at a multiplicity of infection of 10:1 (bacteria to epithelial cells). Noninternalized bacteria were removed by washing steps, and 10 μg/ml gentamicin was added to the medium. Infected cells were incubated up to 6 h postinfection. At this time, intracellular bacteria were recovered in epithelial cell lysates by high-speed centrifugation. Cytosolic and membrane extracts were prepared from intracellular bacteria (6 hpi) and from extracellular bacteria grown in BHI medium (optical density at 600 nm [OD<sub>600</sub>] ≈ 0.4), as described elsewhere (28). The extracellular samples corresponded to 10-fold more bacteria. (A) Levels of the stressosome core proteins RsbR1 and RsbS and the kinase RsbT in intra- and extracellular bacteria. \*, unspecific band detected in the null *ΔrsbR1* polar mutant, as described in reference 28. (B) Levels of the sigma factor SigB. (C) Control assay for the relative enrichment of the cytosolic/membrane fractions based on the detection of the cytosolic chaperonin GroEL. Shown are immunoblots using the respective antibodies raised against each of the indicated proteins. Data correspond to a representative experiment from a minimum of three biological replicates.

increased gradually as intracellular bacteria replicated, from 22.8% of the bacterial population being positive for the reporter at 1 h postinfection (hpi) to 69.2% at 6 hpi (Fig. 1A and B). This postinfection time, 6 h, is coincidental to the maximal number of intracellular bacteria registered. Compared to the number of bacteria at 1 hpi, these numbers accounted for ~4- and ~3-fold increases in wild-type and *ΔsigB* strains, respectively (Fig. 1C). Importantly, no fluorescence signal derived from P<sub>Imo2230</sub>::*egfp* was detected in a *ΔsigB* mutant background at early (1 h) or late (6 h) postinfection times (Fig. 1D). This result demonstrated that the increase in the reporter signal observed in wild-type intracellular bacteria was fully dependent on SigB. In agreement with studies using other epithelial cell lines (13), the *ΔsigB* mutant replicated intracellularly in JEG-3 cells at similar rates to wild-type bacteria, although it displayed an ~10-fold-lower capacity to invade these epithelial cells (Fig. 1E).

We next sought to determine the levels and distribution of stressosome proteins in bacteria isolated from infected cells. In these bacteria, a large proportion of RsbR1 molecules associate with the membrane, whereas RsbS is undetectable (Fig. 2A). The RsbT kinase, however, does not alter its subcellular distribution in intracellular bacteria, being present in both the membrane and the cytosol but with an increased abundance in the cytosolic location (Fig. 2A). Unlike RsbR1 or RsbT, SigB is detected exclusively in the cytosol of intracellular bacteria (Fig. 2B). The lack of cross-contamination between the distinct subcellular fractions was confirmed in all cases by examining the distribution of the cytosolic chaperone GroEL (Fig. 2C).

Interestingly, immunoassays with antibodies against the paralogues RsbR2 and RsbL showed that intracellular bacteria have both proteins predominantly associated with the membrane (Fig. 3A and B). Taken together, these results indicated that *L. monocytogenes* increases SigB activity inside epithelial cells and that the architecture



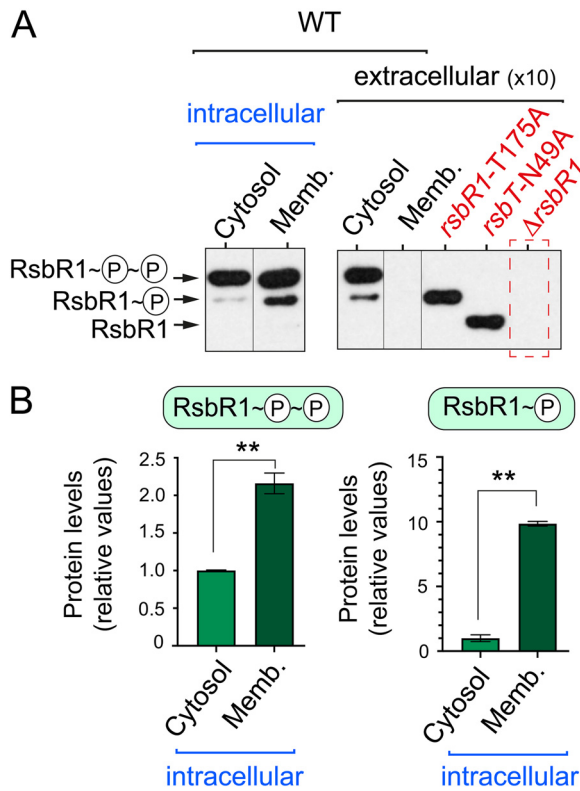
**FIG 3** The RsbR1 paralogues RsbR2 and RsbL are exclusively associated with the membrane in intracellular *L. monocytogenes*. Distribution of RsbR2 (A) and RsbL (B) in the cytosol and membrane (Memb.) fractions of intracellular wild-type *L. monocytogenes* EGD-e after infection of JEG-3 epithelial cells (6 h postinfection). In parallel, samples of extracellular bacteria grown to exponential phase ( $OD_{600} \sim 0.4$ ) in BHI medium corresponding to 10-fold more bacteria were analyzed. Control  $\Delta rsbR2$ - and  $\Delta rsbL$ -null mutants were also included for comparison. Shown are representative immunoblots obtained with anti-RsbR2 and anti-RsbL antibodies from a total of two biological replicates. \*, unspecific band detected in the  $\Delta rsbL$  mutant.

of the stressosome complex may differ in intracellular bacteria compared to bacteria grown in nutritional media.

**RsbR1 is in a fully phosphorylated state in intracellular *L. monocytogenes*.** The widely accepted models of stressosome activation include RsbR1 and RsbS phosphorylation by the kinase RsbT following exposure to stress (22–26). *L. monocytogenes* RsbR1 has two phosphorylatable threonine residues (T175 and T209) with T175 being phosphorylated under basal conditions (37). RsbS has one phosphorylatable serine residue (S56), which in *B. subtilis* was shown to be phosphorylated only upon stress (22, 26). In contrast to these data, our recent study in *L. monocytogenes* grown in nutrient medium revealed that RsbR1 is predominantly in a doubly phosphorylated state, regardless of exposure to hyperosmotic stress (28). The analysis in intracellular bacteria also showed predominance of doubly phosphorylated RsbR1, accounting for  $\sim 2$ -fold enrichment in the membrane relative to the cytosolic pool (Fig. 4A and B). Interestingly, monophosphorylated RsbR1 was highly enriched in the membranes of intracellular bacteria, with  $\sim 10$ -fold more of this isoform detected in the membrane pool (Fig. 4A and B). Two additional *L. monocytogenes* mutants, one expressing a kinase-inactive RsbT-N49A variant and the other expressing an RsbR1-T175A variant (the latter not phosphorylatable at residue 175), facilitated the differentiation of the distinct unphosphorylated and phosphorylated isoforms (Fig. 4A). Altogether, these data pointed to RsbR1 in intracellular *L. monocytogenes* staying in distinct phosphorylated states but with no unphosphorylated forms, a pattern consistent with the increased SigB activity detected under these same infection conditions with the  $P_{Imo2230}::egfp$  reporter fusion (Fig. 1).

**The miniprotein Prli42 is dispensable for RsbR1 association with the membrane of intracellular *L. monocytogenes*.** Prli42 has been proposed to tether the stressosome to the membrane in *L. monocytogenes* exposed to oxidative stress (18). RsbR1 levels associated with the membrane were, however, indistinguishable between wild-type and  $\Delta prli42$  strains following the infection of epithelial cells (Fig. 5A and B). This result ruled out a requirement for Prli42 in the stressosome to associate with the membrane

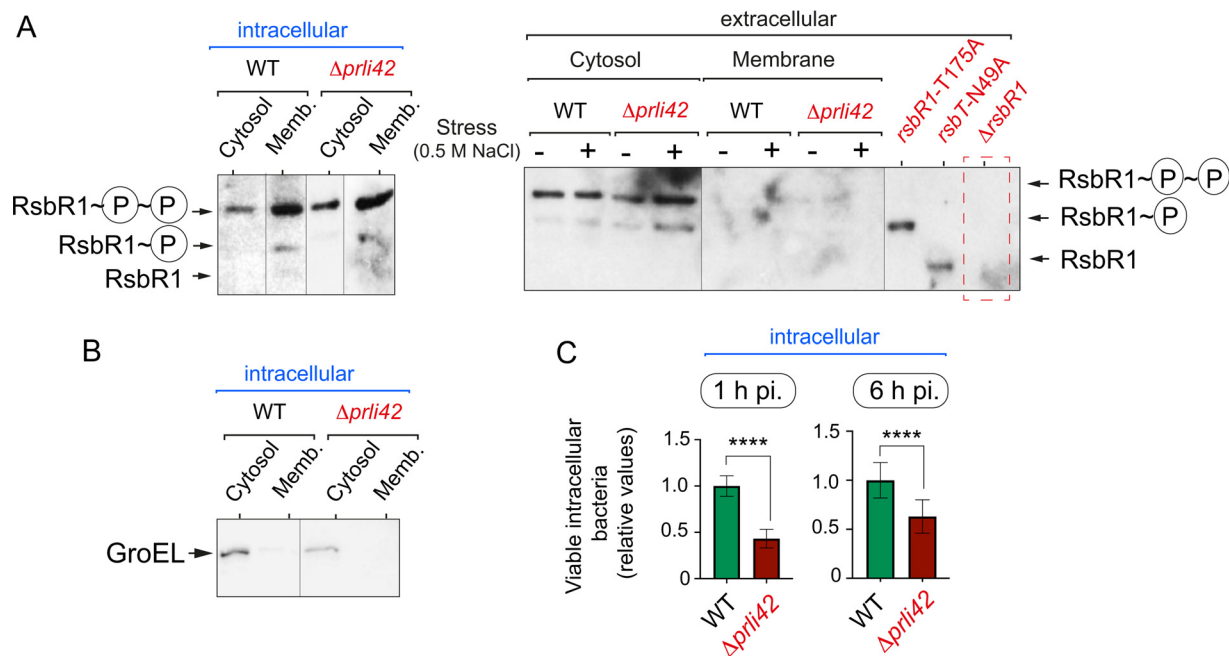




**FIG 4** RsbR1 is phosphorylated to a large extent by intracellular *L. monocytogenes*. (A) Phos-tag electrophoresis based on phosphate-Zn<sup>2+</sup> ion sequestration and immunoblots were used to analyze RsbR1 phosphorylation in cytosol/membrane extracts prepared from intracellular *L. monocytogenes* EGD-e wild type (6 h after infection of JEG-3 epithelial cells). In parallel, RsbR1 phospho-isoforms were detected in subcellular fractions from extracellular bacteria grown to exponential phase (OD<sub>500</sub> ~ 0.4) in BHI medium. Control cytosolic samples from *rsbR1-T175A*, *rsbT-N49A*, and  $\Delta$ *rsbR1* strains were included. An approximately similar number of bacteria (~2 × 10<sup>7</sup>) was analyzed in intra- and extracellular extracts. (B) Quantification of the signals obtained for RsbR1 in intracellular bacteria in three independent biological replicates. Data are means and standard deviations. Statistical analysis was performed by *t* test. \*\*, *P* < 0.01.

of intracellular bacteria. Nonetheless, the loss of Prli42 had effects on bacterial invasion (ca. 60% less in the absence of Prli42), although no phenotype was discernible in the rate of intracellular replication (Fig. 5C).

**RsbR1 association with the membrane of intracellular *L. monocytogenes* is modulated negatively by its paralogues.** The RsbR1 paralogues RsbR2, RsbR3, and RsbL were recently shown to be enriched in membrane fractions of *L. monocytogenes* grown in brain heart infusion (BHI) medium and were proposed to compete with RsbR1 for association with the membrane (28). In that study, a mutant having no functional RsbR1 paralogues (named  $\Delta$ 4) showed higher expression of the P<sub>Imo2230</sub>::*egfp* reporter fusion than wild-type bacteria, regardless of stress. This difference, although less than 2-fold for the  $\Delta$ 4 mutant, was statistically significant (28). This increase in SigB activity was correlated with higher association of RsbR1 with the membrane and, as consequence, of the stressosome. Considering that we observed substantial RsbR1 association with the membrane in intracellular *L. monocytogenes* (Fig. 2A), we infected epithelial cells with the  $\Delta$ 4 mutant lacking functional RsbR1 paralogues. Unlike in wild-type bacteria, RsbR1 was not detected in the cytosol of  $\Delta$ 4 mutant cells (Fig. 6A). Control assays with the chaperone GroEL validated the correct preparation of membrane and cytosolic fractions (Fig. 6B). Interestingly, the  $\Delta$ 4 mutant exhibited an ~2-fold lower invasion rate than wild-type bacteria, probably due to dysregulation of invasion factors controlled by SigB (Fig. 6C). However, no major differences were seen in the bacterial load at 6 hpi between the two strains (Fig. 6C), indicating that the  $\Delta$ 4 mutant could proliferate at a higher rate than wild-type bacteria inside host cells.



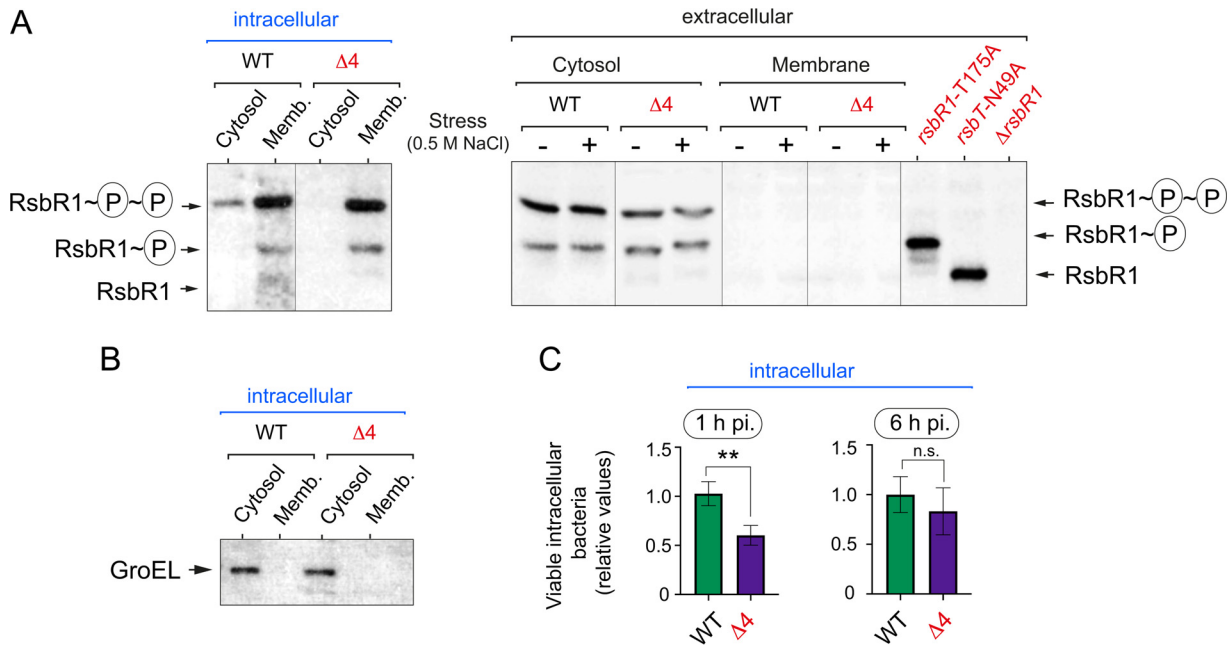
**FIG 5** Phosphorylated RsbR1 remains attached to the membrane of intracellular *L. monocytogenes* in the absence of Prli42. (A) A Phos-tag system was used for the analysis of phosphorylated RsbR1 in subcellular extracts prepared from intracellular *L. monocytogenes* EGD-e wild-type (WT) and  $\Delta prli42$  strains growing inside JEG-3 epithelial cells at 6 hpi. In parallel, subcellular fractions from extracellular bacteria subjected to osmotic stress (0.5 M NaCl for 30 min) were analyzed. Control cytosolic samples from extracellular  $rsbR1-T175A$ ,  $rsbT-N49A$ , and  $\Delta rsbR1$  strains are included. (B) Loading control with anti-GroEL for the samples prepared from intracellular bacteria. (C) Invasion (1 hpi) and proliferation (6 to 1 hpi) rates in JEG-3 epithelial cells of EGD-e wild-type and  $\Delta prli42$  strains. The results from three independent experiments are shown as ratios of the number of  $\Delta prli42$  to wild-type bacteria (the number of CFU from the WT at 1 hpi was arbitrarily set to 1 and corresponded to  $1.7 \times 10^5$  CFU; the number of WT CFU at 6 hpi was  $2.5 \times 10^6$ ). One-tailed *P* values are indicated by asterisks for comparison between wild-type and  $\Delta prli42$  strains by *t* test (\*\*\*\*,  $P < 0.0001$ ).

Overall, these results reinforced the idea of RsbR1 paralogues fine-tuning the activity of the stressosome.

**The RsbT kinase and RsbR1 paralogues are essential for adjusting SigB activation in intracellular *L. monocytogenes*.** The data obtained with the  $\Delta 4$  mutant suggested that a proper partitioning of RsbR1 between the membrane and cytosol of intracellular *L. monocytogenes* could modulate the progressive increase of SigB activity observed with the  $P_{Imo2230}::egfp$  reporter fusion (Fig. 1). If this hypothesis was true, an increase in SigB activity might be expected at all postinfection times in the absence of RsbR1 paralogues. This was the case for the  $\Delta 4$  mutant, which showed increased SigB activity even at the earliest time measured, 1 h postinfection, with 54.3% of bacteria being positive for expression of the reporter versus only 27.8% positivity observed for wild-type bacteria (Fig. 7A to C). The  $rsbT-N49A$  and  $rsbR1-T175A$  mutants, which are defective in RsbT kinase activity and not capable of full phosphorylation of RsbR1, respectively, showed residual SigB activity at 6 hpi that was much lower than that in wild-type or  $\Delta 4$  bacteria (Fig. 7A to C). These data proved that the SigB activation in intracellular *L. monocytogenes* requires perception of stress signals by the stressosome, involving RsbR1 phosphorylation at residue T175 by the kinase RsbT and attenuation of the output signal by RsbR1 paralogues.

## DISCUSSION

The interplay between stress signal perception and virulence is fundamental in most bacterial pathogens. This is especially relevant in *L. monocytogenes*, a bacterium that inhabits multiple environments. Many studies have shown that two regulators, SigB and PrfA, act as master regulators in the stress and virulence cross talk (reviewed in references 6 and 10). The most accepted view of this interplay suggests opposite activities for these regulators, with SigB being dedicated to coping with “extracellular”



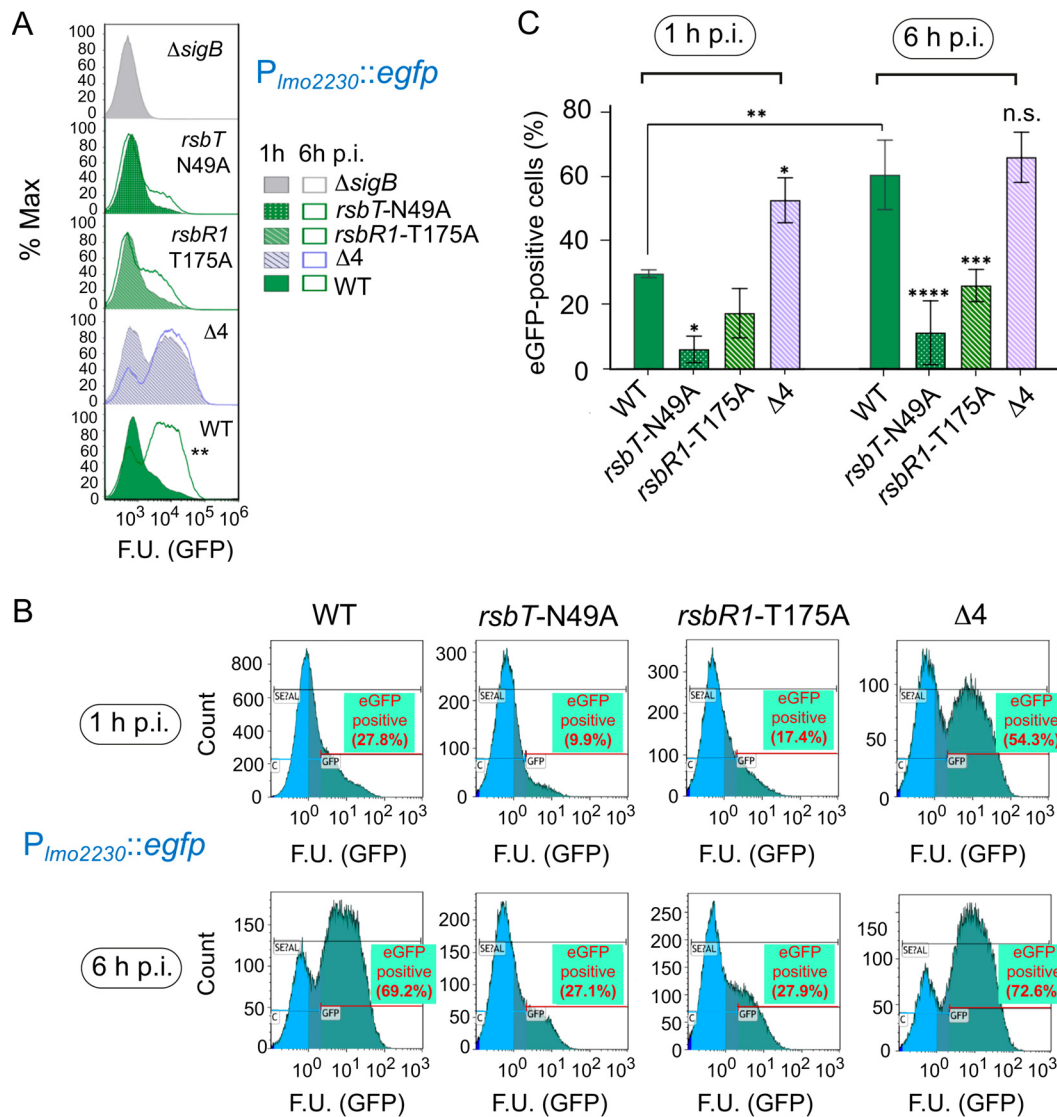
**FIG 6** Phosphorylated RsbR1 increases association with the membrane of intracellular *L. monocytogenes* in the absence of its paralogues. (A) A Phos-tag system was used for the analysis of phosphorylated RsbR1 in subcellular extracts prepared from intracellular *L. monocytogenes* EGD-e wild type (WT) and the Δ4 mutant growing inside JEG-3 epithelial cells at 6 hpi. In parallel, subcellular fractions from extracellular bacteria subjected to osmotic stress (0.5 M NaCl for 30 min) were analyzed. Control cytosolic samples from extracellular *rsbR1-T175A*, *rsbT-N49A*, and Δ*rsbR1* strains are included. (B) Loading control immunoblot with anti-GroEL for the samples prepared from intracellular bacteria. (C) Invasion (1 hpi) and proliferation (6 to 1 hpi) rates in JEG-3 epithelial cells of EGD-e wild-type and Δ4 strains. The results from three independent experiments are shown as ratios of the number of Δ4 to wild-type bacteria (the number of CFU from WT at 1 hpi was arbitrarily set to 1 and corresponded to  $1.7 \times 10^5$  CFU; the number of CFU from WT at 6 hpi was  $2.5 \times 10^6$ ). One-tailed *P* values are indicated by asterisks for comparison between wild-type and Δ4 bacteria by *t* test (\*\*, *P* < 0.01; ns, not significant).

stresses while PrfA is responsible for reprogramming gene expression upon perception of host cues, including those derived from the intracellular niche. However, this simple scenario does not reconcile all available information. In some instances, SigB facilitates the expression of PrfA, for example by increasing *prfA* transcription from the P2*prfA* promoter (30). Conversely, SigB has also been proposed to reduce PrfA activity or levels by yet-unknown mechanisms (38). This “attenuation” of PrfA activity has been postulated to occur in the intestinal tract, where the PrfA regulon might be minimally expressed, with SigB predominantly driving the expression of listeriolysin LLO and the InIA and InIB invasins (6).

To our knowledge, no previous study had addressed whether *L. monocytogenes* exploits SigB to cope with stresses encountered within eukaryotic cells. Stress signals could be sensed within the phagosome due to its acidic environment, nutrient limitation, and/or the presence of reactive oxidants in this compartment. Stress may also occur in the cytosolic phase, in which massive proliferation of the pathogen could lead to bacterial crowding and nutrient starvation at late infection times. It is in this cytosolic phase that *L. monocytogenes* also spreads to adjacent cells and is subsequently enclosed within a double-membrane vacuole, which is ruptured to initiate a new intracellular growth cycle (3). Despite these potential sources of stress, the fact that the lack of SigB does not affect *L. monocytogenes* fitness inside host cells is intriguing (30). This lack of a phenotype associated with the loss of SigB in the *in vitro* infection model has been claimed as proof for the absence of a SigB-mediated response in intracellular *L. monocytogenes*. In our opinion, the data presented here challenge this view with experimental evidence for a gradual increase of SigB activity in intracellular bacteria.

Our claim is sustained by three pieces of evidence. First, the expression of P<sub>Imo2230</sub>::*egfp* reporter increases in the population of intracellular bacteria as the infection progresses, reaching a maximum at 6 h postinfection. This reporter expression is fully





**FIG 7** The lack of RsbR1 phosphorylation or the absence of RsbR1 paralogues alter the activation pattern of SigB during *L. monocytogenes* growth inside epithelial cells. Expression of the  $P_{Imo2230}::egfp$  reporter fusion in the indicated strains at 1 h and 6 h postinfection of JEG-3 epithelial cells. (A) Side-to-side comparison of SigB activity from 1 h to 6 h of infection for each strain. (B) Representative experiment showing the percentage of eGFP-positive cells for the indicated mutants. (C) Quantification of eGFP-positive cells from three biological replicates. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant (one-way ANOVA with Tukey's multiple-comparison test). F.U., fluorescence units.

dependent on SigB, since it is abrogated in the  $\Delta sigB$  mutant irrespective of the infection time measured (Fig. 1D). Although an early transcriptomic study identified *Imo2230* as a gene that is putatively regulated by PrfA (39), this regulation might be indirect, since there is no PrfA box located in its upstream region. More recent transcriptome studies confirmed a SigB-dependent but PrfA-independent control of *Imo2230* expression (40). Second, the SigB protein is detected by Western blotting at high levels in extracts of intracellular bacteria at 6 h postinfection (Fig. 2B); and, third, the core protein RsbR1, essential for stressosome function, is likewise detected in intracellular bacteria associated with the membrane and in a fully phosphorylated state (Fig. 2 and 4). These observations shed light on the frequently discussed interplay between SigB and PrfA in *L. monocytogenes* upon host contact, opening the possibility of PrfA activity being fine-tuned by SigB not only in extracellular locations, such as the gut, but also inside host cells as the intracellular infection progresses from the phagosomal to the cytosolic phase.

How this cross talk may take place in intracellular bacteria is of much interest for future work. For now, we can only speculate on mechanisms, which are technically

challenging to demonstrate considering the scarce amounts of material that can be obtained from intracellular bacteria. For example, future experiments could address at different postinfection times whether there is correlation between the levels of PrfA and SigB produced by intracellular *L. monocytogenes* and whether the lack of one or the other results in compensatory effects. How PrfA orchestrates the production of the listeriolysin LLO and phospholipases in the phagosomal phase and further arrests such expression for stimulating expression of the actin-polymerizing protein ActA in the cytosolic phase is at present unknown and, based on the findings reported here, the putative contribution of SigB should be tested.

It is also important to note that a study based on transcriptome sequencing (RNA-Seq) and chromatin immunoprecipitation sequencing (ChIP-Seq) analyses showed that CodY, a regulatory protein that becomes active in bacteria starved for branched-chain amino acids (BCAA), binds directly to the upstream *sigB* region to repress its expression (41). In contrast, CodY was also reported to activate *prfA* expression from the P2*prfA* promoter (42), as SigB does (38). CodY has been postulated to sense the cytosolic niche during the intracellular proliferation phase, in which BCAA binding could result in repression of many metabolic and stress genes, including *sigB* (41, 42). However, a formal demonstration of CodY repressing *sigB* in intracellular *L. monocytogenes* is still lacking at both gene expression and protein levels. These apparently disparate data could be better integrated in future studies addressing in intracellular bacteria the dynamics of stressosome proteins and their phosphorylation status, as well as SigB protein levels in the presence/absence of CodY.

We believe that, taken together, our findings challenge the current view of SigB being active exclusively in extracellular *L. monocytogenes* by demonstrating a predominantly membrane-associated and fully phosphorylated stressosome core protein, RsbR1, that is detected when *L. monocytogenes* is intracellular. On the other hand, the apparent loss of RsbS in these bacteria is enigmatic, as there is no precedent for any *in vivo* study sustaining a stressosome complex that could be assembled in the absence of this core protein. We cannot rule out the possibility that the amount of RsbS decreases at such low levels in intracellular bacteria that it became undetectable by standard Western immunoassays. In the latter case, the RsbR1:RsbS stoichiometry, estimated to be 2:1 in stressosome isolated from bacteria (28, 43), should be necessarily compromised by such low levels of RsbS in intracellular *L. monocytogenes*. To the best of our knowledge, this study represents the first description at the protein level of the stressosome dynamics in *L. monocytogenes* and SigB activity in the intracellular eukaryotic environment. Future studies involving protein analyses in extracts of bacteria isolated from host cells will be fundamental for dissecting in detail the exact role that SigB plays in modulating virulence functions in the intracellular infection phase.

## MATERIALS AND METHODS

**Bacterial strains.** All *L. monocytogenes* strains used in this study are isogenic to the virulent parental wild-type EGD-e strain and are listed in Table 1. Construction and integration of the  $P_{Imo2230}::egfp$  transcriptional reporter fusion are described elsewhere (36).

**Preparation of intracellular bacterial extracts.** JEG-3 epithelial cells from human placenta (ATCC HTB-36) were propagated in 500-cm<sup>2</sup> Nunclon Delta treated square BioAssay dishes until reaching 80% confluence prior to bacterial infection. The cells were infected for 30 min with *L. monocytogenes* strains previously grown overnight at 37°C in static nonshaking conditions in brain heart infusion (BHI) medium (Becton Dickinson [BD]). The multiplicity of infection (bacteria to epithelial cells) was 10:1. Noninternalized bacteria were removed by three washes with prewarmed phosphate-buffered saline (PBS), pH 7.4, supplemented with 0.9 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>. The infected cells were then incubated during 30 min in fresh Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 100 μg/ml gentamicin (Sigma-Aldrich). From 1 h postinfection, the cells were maintained in DMEM–10% (vol/vol) FBS containing 10 μg/ml gentamicin. At the required postinfection times, the cells were washed twice in PBS, pH 7.4, and lysed with a scraper in 20 ml of lysis solution (1% phenol, 19% ethanol, 0.4% SDS, 0.1 mg/ml DNase A) per cell culture plate, as described elsewhere (44). The solutions obtained from the lysis were spun down (30,000 × *g*, 30 min, 4°C), and the supernatant containing epithelial cells debris was discarded. The pellet of intracellular bacteria was washed in 2 ml of PBS, pH 7.4 (30,000 × *g*, 30 min, 4°C), and kept at –80°C.

**Flow cytometry analysis of eGFP-expressing *L. monocytogenes*.** SigB activity was monitored with the  $P_{Imo2230}::egfp$  reporter (28, 36). The analysis of cells expressing enhanced green fluorescent protein (eGFP) at 1 h and 2 h postinfection required the isolation of bacteria from ~6 × 10<sup>7</sup> JEG-3 epithelial cells

**TABLE 1** *L. monocytogenes* strains used in this study<sup>a</sup>

Strain or genotype	Reference
EGD-e (wild type)	46
$\Delta sigB$	47
$\Delta rsbR1$	28
<i>rsbR1</i> -T175A	28
<i>rsbT</i> -N49A	28
$\Delta 4$ ( <i>rsbL</i> -C56A $\Delta rsbR2$ $\Delta rsbR3$ $\Delta rsbR4$ )	28
$\Delta rsbL$	48
$\Delta rsbR2$	This study
$\Delta prli42$	18
EGD-e pKSV7-P <sub>Imo2230</sub> :: <i>egfp</i>	36
$\Delta sigB$ pKSV7-P <sub>Imo2230</sub> :: <i>egfp</i>	36
<i>rsbR1</i> -T175A pKSV7-P <sub>Imo2230</sub> :: <i>egfp</i>	28
<i>rsbT</i> -N49A pKSV7-P <sub>Imo2230</sub> :: <i>egfp</i>	28
$\Delta 4$ pKSV7-P <sub>Imo2230</sub> :: <i>egfp</i>	28

<sup>a</sup>All strains are isogenic to EGD-e.

(two 500-cm<sup>2</sup> cell culture plates), whereas the analyses at 4 and 6 h postinfection were of  $\sim 1.2 \times 10^8$  cells. The pellet of intracellular bacteria was fixed with 4% (wt/vol) paraformaldehyde for 15 min at room temperature. Fixed cells were harvested by centrifugation (30,000  $\times g$ , 30 min, 4°C) and resuspended in 300  $\mu$ l of filtered PBS, pH 7.4. Fluorescence at the single-cell level was quantified by flow cytometry with a Beckman Coulter Gallios analyzer with 488-nm blue laser excitation and 50,000 events recorded for each sample. The collected data were processed with Kaluza software version 2.1 (Beckman Coulter) to plot side and forward scatter values, the percentage of eGFP-positive cells, and the mean fluorescence values.

**Bacterial invasion and proliferation rates in JEG-3 cells.** Epithelial JEG-3 cells were cultured in Nunc 24-well plates until 80% confluence ( $\sim 4 \times 10^5$  cells). At 1 h and 6 h postinfection, the infected cells were lysed in 100  $\mu$ l of lysis solution (PBS [pH 7.4]; 1% [vol/vol] Triton X-100, 0.1% [wt/vol] SDS) to which 400  $\mu$ l of PBS, pH 7.4, was added. The number of intracellular bacteria was determined by plating serial dilutions of host cell lysates on BHI agar plates and subsequent colony counting.

**Subcellular fractionation and Western blot analysis of stressosome proteins.** The pellet of intracellular bacteria obtained from  $\sim 3 \times 10^8$  JEG-3 epithelial cells (five 500-cm<sup>2</sup> cell culture plates) was treated with the peptidoglycan hydrolase mutanolysin from *Streptomyces globisporus* (Sigma-Aldrich), as described previously (28). The protoplasts were resuspended in 100  $\mu$ l PBS (pH 7.4), 1  $\mu$ g/ml DNase A, and protease inhibitor cocktail and lysed by sonication. Unbroken cells were removed by centrifugation (20,000  $\times g$ , 10 min, 4°C), and the supernatant was subjected to ultracentrifugation (100,000  $\times g$ , 1 h, 4°C) to separate cytosol and membrane fractions. The pellet containing membrane proteins was washed with PBS, pH 7.4, by centrifugation (100,000  $\times g$ , 1 h, 4°C). The pellet was resuspended in 100  $\mu$ l PBS, pH 7.4, to adjust the membrane extract to the same number of bacteria as in the cytosolic fraction. Cytosol and membrane fractions from exponential-phase bacteria were obtained as described previously (28, 45).

**Identification of phosphorylated RsbR1 with the Phos-tag system.** Phosphorylated RsbR1 was identified in cytosolic and membrane extracts from intracellular bacteria using the phosphate-Zn<sup>2+</sup> ion sequestration electrophoresis system with SuperSep Phos-tag precast gels (Fujifilm Wako Chemicals, U.S.A., Richmond, VA). In this system, the migration speed of phosphorylated proteins decreases due to the binding of the metallic ion, and the distinct phosphorylated isoforms are separated as different bands. Samples from exponential-phase bacteria were prepared as previously described (28).

**Statistical analyses and densitometry.** Statistical significance was analyzed with GraphPad Prism v8.4.3 software (GraphPad Inc.). The analysis by *t* test was selected for the comparison of invasion and proliferation rates as well as for evaluating the distribution of the phosphorylated RsbR1 isoforms between cytosolic and membrane extracts. To compare the percentage of positive cells for expression of the P<sub>Imo2230</sub>::*egfp* reporter, the statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison tests. A *P* value of  $\leq 0.05$  was considered significant. Densitometry on bands obtained in immunoblot assays was performed using Image J, available from the National Institutes of Health, USA.

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We declare no competing interests.

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