Journal of Bacteriology

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J. Bacteriol. **2012, 194(11):2949. DOI: 10.1128/JB.06820-11. Published Ahead of Print 30 March 2012.**

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The PhoP/PhoQ System and Its Role in *Serratia marcescens* Pathogenesis

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***Serratia marcescens* is able to invade, persist, and multiply inside nonphagocytic cells, residing in nonacidic, nondegradative, autophagosome-like vacuoles. In this work, we have examined the physiological role of the PhoP/PhoQ system and its function in the control of critical virulence phenotypes in *S. marcescens*. We have demonstrated the involvement of the PhoP/PhoQ system in the adaptation of this bacterium to growth on scarce environmental Mg^{2+}, at acidic pH, and in the presence of polymyxin B. We have also shown that these environmental conditions constitute signals that activate the PhoP/PhoQ system. We have found that the two *S. marcescens mgtE* orthologs present a conserved PhoP-binding motif and demonstrated that *mgtE1* expression is PhoP dependent, reinforcing the importance of PhoP control in magnesium homeostasis. Finally, we have demonstrated that *phoP* expression is activated intracellularly and that a *phoP* mutant strain is defective in survival inside epithelial cells. We have shown that the *Serratia* PhoP/PhoQ system is involved in prevention of the delivery to degradative/acidic compartments.**

Serratia marcescens is a wide-host-range enterobacterium that can be isolated from plants, insects, and nematodes, being also an opportunistic pathogen of mammals. *S. marcescens* is mainly isolated as an etiological agent of nosocomial infections and outbreaks in neonatal intensive care units. Even though *Serratia* is mainly considered a nosocomial opportunistic pathogen, recently published population-based studies indicate that a percentage of the identified *Serratia* infections are community acquired (43). In addition, the incidence of *Serratia* infections has lately been increased by the emergence of multidrug-resistant *Serratia* isolates (32, 33, 42, 43, 48). However, a comprehensive picture of the mechanisms used by this pathogen to invade its host has not yet emerged.

In vivo and *in vitro* experimental evidence shows that *Serratia* is able to invade nonphagocytic cells: it can be found invading midgut epithelial cells when it transverses the gut to reach the body cavity in a *Drosophila melanogaster* infection model (56) and several nonphagocytic cultured cell lines (34). More recently, we demonstrated that once inside epithelial cells, *Serratia* is able to inhabit and proliferate inside membrane-bound compartments, which we named *Serratia*-containing vacuoles (SeCVs) (16). A vast majority of these SeCVs exhibit autophagosome-like features, acquiring markers characteristically recruited throughout the progression of the autophagosome (29, 36, 58). Nevertheless, we have shown that this autophagic vacuole population is predominantly nonacidic and has no degradative properties, indicating that *Serratia* is capable of either delaying or preventing fusion with lysosomal compartments. These findings showed that this pathogen can alter the canonical progression of autophagosome maturation, manipulating the vesicular traffic in order to tailor a suitable niche for survival and proliferation within the host cell (16).

The ability of bacteria to interact with, colonize, and invade abiotic niches or live hosts relies on their capacity to cope with the changing and life-threatening conditions posed by the characteristics of each environment. Two-component systems (TCS) are one of the most ubiquitous mechanisms by which bacteria generate adaptive responses to environmental challenges. In these signal transduction systems, the activation of a sensor histidine kinase leads to autophosphorylation followed by transfer of the phosphoryl group to a cognate response regulator in an aspartate residue (21).

PhoP/PhoQ is a broadly conserved TCS among many pathogenic and nonpathogenic bacteria. Evolutionary analysis has shown that in most of these organisms the PhoP/PhoQ system preserves an ancient function in the control of the magnesium homeostasis, while in pathogenic bacteria it is also dedicated to governing the expression of critical virulence phenotypes (62). Despite the fact that in pathogens such as *Salmonella enterica*, *Mycobacterium tuberculosis*, and *Yersinia pestis* (8, 20, 35, 44, 60) the PhoP/PhoQ system converges in conferring survival capacity inside the host, the set of regulated genes recruited under its control, and therefore the mechanism engaged, varies in a species-specific manner.

In this work, we examined the physiological role of the PhoP/PhoQ system in *S. marcescens*. We have determined that the activity of the system allows *Serratia* to withstand scarce environmental Mg^{2+} availability, acidic pH, and the action of the cationic antimicrobial compound polymyxin B. We have demonstrated that these environmental challenges also constitute input signals that promote the activity of the system. By an *in silico* approach, we screened for PhoP-regulated genes in *Serratia*, and interestingly, we found that the two *S. marcescens mgtE* orthologs display a conserved PhoP-binding motif and demonstrated that *mgtE1* expression is PhoP dependent. *mgtE* is predicted to encode a magnesium transporter, and its identification among PhoP-regulated targets

Received 29 December 2011 **Accepted** 25 March 2012
Published ahead of print 30 March 2012
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doi:10.1128/JB.06820-11

TABLE 1 Bacterial strains, vectors, and plasmids used in this study

Strain/description, vector, or plasmid	Genotype and/or comments	Source or reference
Strains		
WT	*S. marcescens* RM66262; clinical isolate	9
phoP	*phoP*::pKNOCK-Gm	This study
phoP::*lacZ*	prom*phoP*::*lacZ*::pKNOCK-Cm	This study
mgtE1::*lacZ*	*mgtE*::mini-Tn*5*-*lacZ1* Km^r	This study
*Sm*Db11	*S. marcescens* Db11	19
*Sm*Db11*phoP*	*S. marcescens* Db11 *phoP*::pKNOCK-Gm	This study
Vectors and plasmids		
pJB	$lacI^q$ subcloned in the pBBR1-MCS1 vector; Cm^r	This study
pJB::*phoPQ*	pJB::prom*phoPQ*; Cm^r	This study
pGFP	pBBR1-MCS1::gfpmut3.1; Cm^r	Laboratory stock
pBBR1-MCS1	Cm^r; broad-host-range, mobilizable vector	41
pBBR1-MCS2	Km^r; broad-host-range, mobilizable vector	41
pUJ8	Amp^r; multicopy promoter probe vector to generate fusions with a promoterless *lacZ* gene	13
pKNOCK-Gm	Gm^r; mobilizable suicide plasmid	1
pKNOCK-Cm	Cm^r; mobilizable suicide plasmid	1
pUH	pUHE21-2$laqI^q$	67
pBluescript	Amp^r; high-copy-no. cloning vector	Stratagene

extends the possible array of magnesium transporters that have been evolutionarily recruited by the PhoP regulon.

We have also shown that *phoP* is activated intracellularly and that a *phoP* mutant strain is defective in survival inside epithelial cells. While we provide evidence that the PhoP/PhoQ system is required neither for the internalization of the bacteria inside the epithelial cell nor for the delivery of the SeCVs to the autophagic route, we have demonstrated that the *Serratia* PhoP/PhoQ system is involved in the prevention of the delivery to degradative/acidic compartments.

Cumulatively, our findings show that the PhoP/PhoQ system confers on this opportunistic pathogen traits for confronting environmental challenges that would be key in the switch between host- and non-host-associated lifestyles.

MATERIALS AND METHODS

Bacterial strains, cell culture, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in lysogeny broth-Miller (LB) medium or on LB agar plates overnight at 37°C. The antibiotics used were ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), and spectinomycin (100 μg/ml). All of the reagents, chemicals, and oligonucleotides used were from Sigma.

For cultured bacteria, β-galactosidase levels were determined as described previously (50). To assay Mg^{2+} regulation, strains were grown overnight in low Mg^{2+} (LB or N medium [0.1 M Tris-HCl {pH 7.4}, 0.1% {wt/vol} Casamino Acids, 38 mM glycerol, 0.37 g/liter KCl, 0.99 g/liter $(NH_4)_2SO_4$, 0.087 g/liter K_2SO_4, 0.14 g/liter KH_2PO_4] [57] plus 10 μM $MgCl_2$) or high Mg^{2+} (LB plus 50 mM $MgCl_2$ or N medium plus 1 and 10 mM $MgCl_2$). In the case of the pH signal, after exponential growth in N medium morpholineethanesulfonic acid (MES) [0.1 M MES, 0.1% (wt/vol) Casamino Acids, 38 mM glycerol, 0.37 g/liter KCl, 0.99 g/liter $(NH_4)_2SO_4$, 0.087 g/liter K_2SO_4, 0.14 g/liter KH_2PO_4] (63) (pH 7.7), strains were inoculated (1:50 dilution) in N medium MES (pH 7.7 or 5.5) and grown for 4 h with vigorous shaking.

To examine antimicrobial peptides as PhoP detected signal, after exponential growth in LB or N medium plus 1 mM $MgCl_2$, strains were inoculated (1:50 dilution) into LB and LB plus 100 μg/ml polymyxin B or N medium plus 1 mM $MgCl_2$ and N medium plus 1 mM $MgCl_2$ supplemented with 20 μg/ml polymyxin B and grown for 1 h with vigorous shaking. The optical density at 600 nm (OD_{600}) was determined using a 96-microwell plate reader, BioTek ELx808.

Measurement of β-galactosidase activity from intracellular bacteria was performed using the fluorescent substrate 4-methylumbelliferyl-β-D-galactoside as described previously (65). Chinese hamster ovary (CHO) cells were infected with the *S. marcescens phoP*::*lacZ* strain as described below. At 180 min postinfection (p.i.), cells were washed with phosphate-buffered saline (PBS) and treated for 10 min with 1 ml of 0.1% (vol/vol) Triton X-100. Two hundred microliters of each sample was used to determine the number of intracellular viable bacteria (as CFU/ml) by serial dilution and plating on LB. The remaining 800 μl was used to measure β-galactosidase activity as follows: epithelial cell lysates containing intact bacteria were centrifuged, the bacterial pellet was resuspended in 200 μl of M buffer (25 mM Tris-HCl [pH 7.5], 125 mM NaCl, 2 mM $MgCl_2$, and 0.3 mM 4-methylumbelliferyl-β-D-galactoside [Sigma]), and after addition of 14 μl 0.1% (wt/vol) SDS and 24 μl $CHCl_3$, the reaction mixtures were incubated for 60 min at 37°C. Reactions were stopped by adding, after centrifugation, 100 μl of supernatant to 1.9 ml of stop buffer (133 mM glycine and 83 mM Na_2CO_3 [pH 10.7]). Fluorescence of samples was analyzed in a Varian Cary Eclipse fluorescence spectrophotometer using excitation at 365 nm and emission at 460 nm. Each measurement was calibrated with a standard curve using 4-methylumbelliferone (Sigma). Intracellular β-galactosidase activity was expressed as nmol of 4-methylumbelliferone per CFU. Values correspond to the ratio between the β-galactosidase activity obtained under each condition and β-galactosidase activity for the same strain grown in LB at 30°C without agitation.

For cell culture, CHO cells or the derived stably transfected CHO cells overexpressing enhanced green fluorescent protein-LC3 fusion protein (EGFP-LC3) (CHO-EGFPLC3) (64) were grown in alpha minimal essential medium (α-MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) (both reagents obtained from Invitrogen [Argentina]) at 37°C with 5% (vol/vol) CO_2.

Genetic and molecular biology techniques. *S. marcescens phoPQ* was disrupted by integration of the suicide plasmid pKNOCK-Gm (1). Briefly, the *phoP* gene was amplified by PCR using the primers PhoP NTF and PhoP CTR (Table 2). The purified PCR product was digested with the PstI restriction enzyme, and the fragment of 408 bp was ligated into the PstI sites of pKNOCK-Gm. The resulting plasmid was mobilized into *S. marcescens* RM66262 (wild type [WT]) and into *S. marcescens* Db11 (*Sm*Db11)

TABLE 2 Oligonucleotide primers used in this study

Name	Sequence (5′ → 3′)
PhoP NTF	GAGGATCCTTGCGAGCCGGGC
PhoP CTR	TCCAAGCTTCACTTCGCGTCAAAAC
promphoP F	ACCGAATTCGCGCTTAACCCGCTCG
promphoP R	AGGGATCCCATGGCGAACTCCTGTG
phoQ R	CTTAAGCTTCCATCGCCAGCCC
lacIq F	GGTATTAATCACCGCATATGG
lacIq R	CGTATTAATCATGGGCAAATATTATACG
Tn5-I-end F	TTCGTCGACGGACTGCGGCC

strains by conjugation. Insertional mutants were confirmed by PCR analysis and Southern blotting. For complementation of the *S. marcescens phoP* mutant strain, *phoPQ* with its own promoter was amplified by PCR using the primers prom*phoP* F and *phoQ* R and digested with the EcoRI and HindIII restriction enzymes. The fragment was ligated into pBBR1-MCS2 (41). Then, this plasmid was digested with HindIII, and the released fragment was ligated into pJB. The resulting plasmid, pJB::*phoPQ*, was mobilized into the *S. marcescens phoP* strain by conjugation. The pJB vector was constructed by amplifying *lacI*^{q} from plasmid pUH by PCR using the primers *lacI*^{q} F and *lacI*^{q} R. The purified PCR product was digested with the AseI restriction enzyme and ligated into pBBR1-MCS1 (41).

A *phoP*::*lacZ* transcriptional fusion was constructed by integration of the suicide plasmid pKNOCK-Cm (1). The *phoP* promoter region (prom*phoP*) was amplified by PCR using the primers promphoP F and promphoP R (Table 2) and digested with the EcoRI and BamHI restriction enzymes, and the fragment of 500 bp was ligated into the EcoRI/BamHI sites of the pUJ8 vector (13). This construction was digested with NotI, and the fragment of 4,600 bp, including the *phoP* promoter and *lacZ* reporter transcriptional fusion, was ligated into the NotI site of pKNOCK-Cm. The resulting plasmid was mobilized into *S. marcescens* by conjugation, and the insertional mutant was confirmed by PCR analysis.

A random mini-Tn*5-lacZ1* gene fusion insertional library was obtained from the *S. marcescens* strain as described previously (13). The screening of the random insertion library was performed by replica plating on LB and LB plus 50 mM $MgCl_2$ agar plates. To identify the localization of the transposon insertions, chromosomal DNA from the selected clones was prepared, digested with SalI, ligated to SalI-digested pBluescript, and transformed into *Escherichia coli* XL1 Blue. Km- and Amp-resistant clones were selected and sequenced using the primer Tn5-I-end F.

Bacterial growth curves under acidic and low-Mg^{2+} conditions. Bacterial growth curves were monitored in a 96-microwell plate reader, Synergy2, and incubated with vigorous shaking and temperature control (37°C), over an 18-h period. The OD_{600} was determined every 60 min. For the low-magnesium growth assay, overnight cultures were diluted 1:50 into N medium supplemented with 10 μM or 10 mM $MgCl_2$. For the *Sm*Db11 and *Sm*Db11 *phoP* strains, the OD_{600} was determined overnight. To assay acid resistance, overnight cultures were diluted 1:50 in E glucose broth [0.2 g/liter $MgSO_4 \cdot 7H_2O$, 2 g/liter citric acid, 13.1 g/liter $K_2HPO_4 \cdot 3H_2O$, 3.5 g/liter $Na(NH_4)HPO_4 \cdot 4H_2O$, and 0.4% glucose] (5) at pH 7, pH 5, or pH 4.3. For the *Sm*Db11 and *Sm*Db11 *phoP* strains, the OD_{600} was determined after 6 h.

Polymyxin B susceptibility assays. Fifty microliters of a 1:50 dilution of overnight LB cultures was mixed with 50 μl of antibiotic solution dissolved in the same medium at final polymyxin B concentrations that ranged between 0 and 6 mg/ml. The mixtures were incubated in 96-well microtiter plates at 37°C with orbital agitation for 18 h. OD_{600} readings were determined with a BioTek ELx808 microplate reader. The mean and standard deviations for triplicate analysis were calculated.

Kanamycin protection and adherence assays. Both assays were performed as previously described (16) with modifications, as follows. CHO cells were cultured in 24-well plates until they reached confluence. *S. marcescens* was grown in LB, without shaking, overnight at 30°C (we have previously determined this growth temperature to be optimal for *Serratia* adherence and invasion assays using CHO cells [16]), and an appropriate volume was added to each well to reach a multiplicity of infection (MOI) of 2. Plates were centrifuged for 10 min at 1,000 rpm and incubated for 1 h at 37°C and 5% (vol/vol) CO_2. For the adherence assay, cells were then washed four times with PBS and lysed with 0.05% (vol/vol) Triton X-100. The resultant supernatants were serially diluted, and the numbers of CFU on LB agar plates were determined. For the kanamycin protection assay, cells were washed with PBS and incubated with medium supplemented with 400 μg/ml kanamycin. Cells were then washed with PBS and lysed with 0.05% (vol/vol) Triton X-100 at various time points. The resultant supernatants were serially diluted, and the numbers of CFU were determined on LB agar plates. The results for each experiment are the average for an assay performed in triplicate and independently repeated three times.

Indirect immunofluorescence and confocal microscopy. CHO cells infected with the WT/pGFP and *phoP*/pGFP strains (Table 1) were fixed with 0.5 ml of 3% (wt/vol) paraformaldehyde solution in PBS for 10 min and permeabilized with a 0.1% (vol/vol) Triton X-100 solution in PBS for 5 min. Subsequently, cells were mounted with SlowFade Antifade reagent in glycerol-PBS (Molecular Probes).

CHO cells overexpressing EGFP-LC3, infected with the WT and *phoP* strains, were fixed with paraformaldehyde and permeabilized with Triton X-100 solution, as described before. Subsequently, cells were incubated with primary polyclonal antibodies against *S. marcescens* (1:500) and detected by incubation with secondary antibodies conjugated with anti-rabbit Cy3 (1:150) or anti-rabbit Alexa Fluor 647 (1:300). Cells were mounted with SlowFade Antifade reagent in glycerol-PBS (Molecular Probes). Finally, cells were analyzed by confocal microscopy with a Nikon Eclipse TE-2000-E2 microscope and the EZ-C1 3.20 Free Viewer software program (Nikon, Japan) or with an Olympus FluoView FV1000 confocal microscope (Olympus, Argentina) and the FV10-ASW (version 01.07.00.16) software program, respectively.

Colocalization of intracellular bacteria with cathepsin D or Bodipy dye conjugated to BSA (DQ-BSA). At 360 min p.i., cells were fixed for 10 min in 3% (wt/vol) paraformaldehyde and permeabilized with 0.1% (vol/vol) Triton X-100 solution. Subsequently, cells were incubated with primary polyclonal antibodies against cathepsin D (1:200) (Dako) and detected by incubation with anti-rabbit Cy3-conjugated secondary antibodies (1:150).

On the other hand, the ability of CHO cells to endocytose and degrade the self-quenched red DQ-BSA (Molecular Probes) was used to measure degradative lysosomal function. Red DQ-BSA requires enzymatic cleavage in a degradative intracellular compartment to generate a highly fluorescent product which can be monitored by confocal microscopy. CHO cells were preincubated for 4 h at 37°C with DQ-BSA (10 μg/ml in α-MEM supplemented with 10% [vol/vol] FCS) to ensure that the probe reaches the lysosomal compartment. Subsequently, the cells were infected with *S. marcescens* as described above, and the cells were fixed postinfection.

In both cases, intracellular bacteria were detected by direct fluorescence and the cells were analyzed by confocal microscopy. At least 200 cells were scored from three independent experiments using confocal microscopy. The percentage of colocalization was calculated as the ratio between the number of vesicles containing bacteria that colocalize with cathepsin D or DQ-BSA and the total number of vesicles containing bacteria. For this purpose, either small vacuoles or large vacuoles containing bacteria were counted as one vesicle.

Detection of acidic compartments with LysoTracker. CHO and CHO-EGFP-LC3 cells were infected with the *S. marcescens* WT, *phoP*, WT/pGFP, and *phoP*/pGFP strains, respectively. At 60 min p.i., the acidic compartments were labeled by incubation of infected cells with 3 mM LysoTracker (Molecular Probes) in α-MEM supplemented with 10% (vol/vol) FCS at 37°C. Finally, at 360 min p.i., cells were fixed and intra-

cellular bacteria were detected by direct fluorescence in the case of CHO cells and indirect immunofluorescence for CHO-EGFP-LC3 cells. Cells were analyzed by confocal microscopy. The percentage of colocalization was calculated as the ratio between the number of vesicles containing bacteria that colocalize with LysoTracker and the total number of *Serratia*-containing vesicles.

Bacterial uptake assay. The intracellular versus extracellular immunofluorescence invasion assay was performed as described previously (7, 31). Briefly, cells were infected with the WT/pGFP and *phoP*/pGFP strains at an MOI of 10 for 30 min at 37°C. Then, cells were fixed in 3% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature, and extracellular *Serratia* bacteria were stained using a primary polyclonal antibody against *S. marcescens* (1:500) and detected by incubation with anti-rabbit Cy3-conjugated secondary antibodies (1:150). Under these conditions, both extracellular and intracellular bacteria are green, and only extracellular bacteria are red. Then, cells were mounted with SlowFade Antifade reagent in glycerol-PBS (Molecular Probes). Finally, cells were analyzed by confocal microscopy. At least 600 cells were scored from three independent experiments. Bacterial internalization was calculated as the number of intracellular bacteria per cell. Data represent the means for at least three independent experiments. *S. marcescens* strains harboring the pGFP plasmid have the same invasion efficiency as strains without the vector.

Statistics. All statistical calculations were performed by using the SigmaPlot software program (Jandel Scientific, Chicago, IL). Significance was determined by using Student's *t* test; a P value of <0.001 was considered significant.

RESULTS

Features of the PhoP/PhoQ regulatory system in *S. marcescens*. The *phoPQ* genes have been found in all analyzed members of the *Enterobacteriaceae* family. In the *Serratia* genus, the *phoPQ* ortholog has been previously identified in the *Serratia proteomaculans* 568 genome (62), and a search in the *S. marcescens* Db11 genome (http://www.sanger.ac.uk) also reveals a conserved *phoPQ* operon. *S. marcescens* PhoP exhibits 82% identity and 95% homology, while PhoQ exhibits 62% identity and 80% homology, to the *Salmonella enterica* PhoP and PhoQ proteins, respectively. Compared with *Yersinia pestis*, an enterobacterium phylogenetically more closely related to *S. marcescens* (25), PhoP displays 90% identity and 94% homology, and PhoQ shows 80% identity and 90% homology. This analysis allowed us to predict a highly conserved function for the PhoP/PhoQ system in *Serratia*.

In order to identify *S. marcescens* PhoP-regulated genes, we performed a bioinformatic search of genes that display putative PhoP-binding sites in their promoter regions using the MEME/MAST motif detection program (3, 4). We generated a PhoP recognition motif using as a database a collection of previously defined promoter regions of PhoP-regulated genes in bacteria where the PhoP regulons have been more extensively characterized, such as *Salmonella enterica* serovar Typhimurium and *E. coli* (45, 51). We searched the *S. marcescens* Db11 genome and detected putative PhoP recognition motifs in *S. marcescens phoP*, *mgtA*, *mgtCB*, *pmrG*, *slyB*, and *virK* orthologs. As depicted in Fig. 1A, motifs that have direct or inverse direction relative to each translational start site were found in the detected genes, a feature that has been demonstrated to be compatible with functional PhoP-dependent regulatory regions (30, 75). The aforementioned genes are known members of the PhoP/PhoQ regulon in other bacteria (75).

One of the most conspicuous roles of the PhoP/PhoQ system in other bacteria is the maintenance of magnesium homeostasis, and the control of the transcriptional expression of the MgtA and MgtB magnesium transporters is one of the main features of this regulatory function. Remarkably, in this search we found highly conserved motifs upstream the two *S. marcescens* chromosomally encoded *mgtE* orthologs (which we have named *mgtE1 and mgtE2*, corresponding to the assigned fig/615.1.peg.726 and fig/615.1.peg.2863, in the *S. marcescens* Db11 annotated database [http://theseed.uchicago.edu]). *mgtE* orthologs are predicted to code for magnesium transporters (66, 72); however, the MgtE family of proteins, although subjected to Mg^{2+}-dependent controlled expression, have not been found to be governed by the PhoP/PhoQ system in other bacterial species previously examined. Together, these observations anticipate that the *S. marcescens* PhoP/PhoQ system preserves its fundamental evolutionarily conserved function across bacterial species, mediating the adaptive response to counter the cellular imbalances in magnesium availability.

The presence of PhoP-binding motifs in the promoter region of the *S. marcescens phoPQ* operon allowed us to predict that the system would be autoregulated, as was previously determined for other bacteria, such as *E. coli*, *S. enterica*, and *Y. pestis* (39, 62, 68). To analyze the regulatory features of the PhoP/PhoQ system in *Serratia*, we obtained a *phoP* mutant using the suicide plasmid pKNOCK-Gm, and we also constructed a *phoP*::*lacZ* reporter transcriptional fusion as described in Materials and Methods. In addition, the complete *phoPQ* operon was cloned into the pJB vector (pJB::*phoPQ*).

As mentioned before, the responsiveness to fluctuations in extracellular Mg^{2+} is one of the hallmarks of the PhoP/PhoQ system. In brief, when high extracellular Mg^{2+} concentrations are detected by the bifunctional PhoQ sensor, the phosphatase activity of PhoQ is activated and PhoP becomes dephosphorylated, turning down the system. In contrast, PhoQ histidine kinase activity controls the transcriptional induction of the regulon when the bacterium encounters limiting concentrations of the cation (10). Taking into account the potential autoregulatory features of the *phoPQ* operon and in order to examine PhoP/PhoQ Mg^{2+}-dependent regulation in *S. marcescens*, we measured the β-galactosidase activity of the transcriptional fusion *phoP*::*lacZ* when the bacterium was grown in LB or in minimal N medium containing different Mg^{2+} concentrations. As shown in Fig. 1B, we determined that *phoP* transcription is repressed with increasing concentrations of Mg^{2+} in both media assayed.

In addition to Mg^{2+}, low pH, polymyxin B, and other cationic peptides have been described as input signals that can modulate the expression of PhoP-regulated genes. To explore whether these conditions are effective regulatory signals in *S. marcescens*, the β-galactosidase activity from the *phoP*::*lacZ* transcriptional fusion was measured when bacteria were grown under different pH conditions or with sublethal concentrations of polymyxin B. We found that *phoP* transcription was regulated by pH in *S. marcescens*, with its expression levels being 2.4-fold higher at pH 5.5 than at pH 7.7. In addition, *phoP* was 5.3- and 2.5-fold induced by sublethal concentrations of polymyxin B in N medium or LB, respectively (Fig. 1C and D). These results show that a limited extracellular Mg^{2+} concentration, acidic pH, and the presence of the cationic antimicrobial compound polymyxin B in the bacterial growth medium are signals that transcriptionally activate the *Serratia phoPQ* operon.

In parallel, we analyzed the role of the PhoP/PhoQ system in the ability of *Serratia* to withstand limiting environmental concentrations of Mg^{2+}, a low ambient pH, or the presence of the

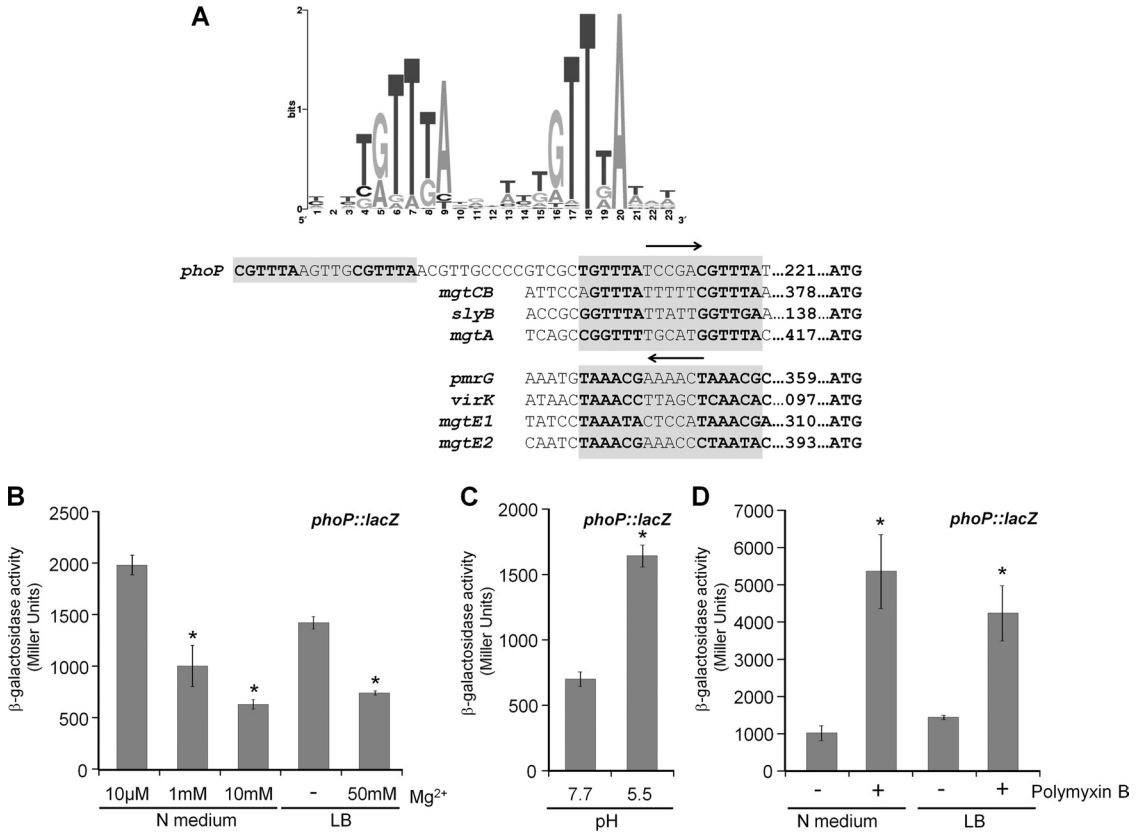

FIG 1 (A) PhoP binding sites in *Serratia marcescens* genome. A consensus motif for the PhoP binding site was generated using a collection of previously defined promoter regions of PhoP-regulated genes in *S.* Typhimurium and *E. coli* as a database and the MEME software tool (3). The logo shows the consensus motif for the PhoP binding site (weblogo.berkeley.edu), and the putative PhoP binding site sequences with high scores identified in the *S. marcescens* Db11 genome by MAST (4) are listed below. The PhoP boxes are shown in boldface; arrows indicate orientation relative to each translational start site. The name of the gene and the distance from the translational start site are also indicated. (B, C, and D) Mg^{2+}, pH, and polymyxin B control PhoP expression in *S. marcescens*. β-Galactosidase activity from the *phoP*::*lacZ* transcriptional fusion was determined. Bacteria were grown overnight in LB or N medium with 10 μM $MgCl_2$ for low Mg^{2+} and LB plus 50 mM $MgCl_2$ or N medium plus 1 and 10 mM $MgCl_2$ for high Mg^{2+} (B) in N medium MES (pH 7.7) to exponential phase and then inoculated into N medium MES (pH 7.7 or 5.5) and grown for an additional 4 h (C) or into LB or N medium with 1 mM $MgCl_2$ to exponential phase and then inoculated into LB and LB plus 100 μg/ml polymyxin B or N medium with 1 mM $MgCl_2$ and N medium with 1 mM $MgCl_2$ plus 20 μg/ml polymyxin B, grown for 1 h (D). The data correspond to mean values for four independent experiments performed in duplicate in each case. Error bars correspond to standard deviations. (*, $P < 0.001$).

antimicrobial cationic agent polymyxin B. To this aim, we compared the growth capacity of WT/pJB, the *phoP*/pJB mutant and complemented *phoP*/pJB::*phoPQ Serratia* strains in N-minimal medium containing a low (10 μM) or high (10 mM) Mg^{2+} concentration or in E glucose medium at pH 5 or at pH 7. As shown in Fig. 2A, in high Mg^{2+} medium the three strains showed a similar growth pattern, while the *phoP* mutant strain showed limited growth in low Mg^{2+} medium (i.e., an estimated 5-fold reduction at 18 h) compared to that of the WT strain. This growth deficiency could be rescued with the expression of the PhoP/PhoQ system from the pJB::*phoPQ* plasmid (Fig. 2A). While an acidic pH was detrimental either for the WT or for the complemented *phoP* mutant strains, which grew slowly and reached OD_{600}s of 0.60 ± 0.05 and 0.75 ± 0.07 at 18 h, respectively, which represent half the OD_{600} values reached by the strains at neutral pH, the *phoP* mutant strain was unable to grow under this condition (Fig. 2B). The susceptibility of the *phoP* mutant to polymyxin B in LB was assessed using concentrations between 0 and 2 mg/ml. As shown in Fig. 2C, concentrations of polymyxin that were ≥1.5 mg/ml totally inhibited the *phoP* mutant strain growth, while 0.5 mg/ml polymyxin was sufficient to lower its OD_{600} by 50% compared to the value reached by the WT strain, which withstood concentrations up to at least 5 mg/ml of the cationic polypeptide (for simplicity, the effect of up to 2 mg/ml polymyxin B is shown in the graphic). The polymyxin phenotype could not be complemented in the *phoP* mutant strain by pJB::*phoPQ* (not shown). This was not entirely unexpected, because finely tuned levels of PhoP are required to induce a specific gene according to the characteristics of each particular PhoP-promoter interaction. Therefore, we hypothesized that in this case we could not achieve the proper dose-response relationship (45). To verify that the observed phenotypes were not restricted to *S. marcescens* strain RM66262, we also obtained a *phoP* mutant from *S. marcescens* strain Db11 (*Sm*Db11). Interestingly, we observed that the wild-type *Sm*Db11 strain was less susceptible to equivalent Mg^{2+} extracellular concentrations, acidic pH, or high polymyxin B concentrations than *S. marcescens* strain RM66262 strain. We determined that the *Sm*Db11 *phoP* mutant showed a growth deficiency phenotype in low Mg^{2+}, acidic pH, and polymyxin B (the two last phenotypes were evident in this mutant strain when pH 4.3 and 6.0 mg/ml polymyxin were

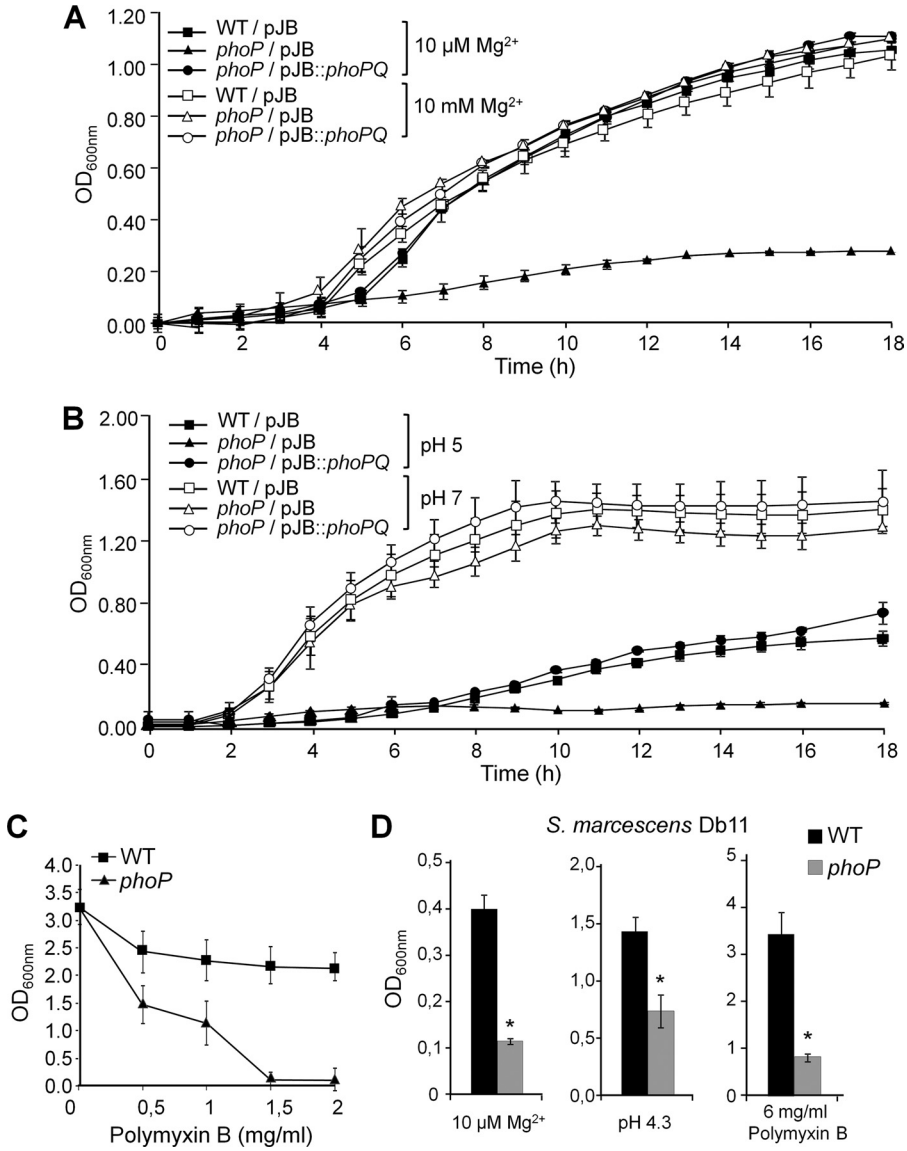

FIG 2 PhoP is required for *Serratia* to resist an environment deprived of Mg^{2+}, with low pH, or with the presence of polymyxin B. (A) WT and *phoP Serratia* strains harboring the pJB and pJB::*phoPQ* plasmids were grown in N minimal medium supplemented with 10 μM or 10 mM $MgCl_2$, and the OD_{600} was determined every 60 min. (B) WT and *phoP Serratia* cells harboring the pJB and pJB::*phoPQ* plasmids were grown in in E glucose broth at pH 7 or pH 5, and the OD_{600} was determined every 60 min. (C) The OD_{600} was determined for overnight cultures of the WT and *phoP Serratia* strains grown in LB in the presence of different concentrations of polymyxin B. (D) The OD_{600} was determined on overnight cultures of the *Sm*Db11 WT and *phoP* strains grown in N minimal medium supplemented with 10 μM $MgCl_2$ or LB with 6 mg/ml of polymyxin B. For the acid resistance assay, both strains were grown in E glucose broth at pH 4.3, and the OD_{600} was determined after 6 h. Results are averages from three independent assays performed in duplicate. Error bars correspond to standard deviations. (*, $P < 0.001$).

used) compared to results for the otherwise isogenic WT strain (Fig. 2D). In sum, these last results demonstrate that the integrity of the *phoPQ* operon is required for *Serratia* to tolerate the challenge of an environment deprived in Mg^{2+}, of acidic pH, or of the presence of polymyxin B.

***mgtE* is a PhoP-regulated gene and responds to the same environmental signals as *phoPQ*.** Consistent with our *in silico* screening of putative *Serratia* PhoP-regulated genes (Fig. 1), we also identified the *mgtE1* ortholog as result of a search for low/high-Mg^{2+}-differentially regulated genes using the reporter transposon mini-Tn5-*lacZ1* (see Materials and Methods for details).

CorA, MgtA/MgtB P-type ATPase, and MgtE are the three protein families of ion transporters involved in the achievement of homeostatic regulation of intracellular Mg^{2+} in bacteria (52, 66). In contrast to the other Mg^{2+} transporters, MgtE has been detected in all three kingdoms of life. While the expression of MgtA and MgtB is under transcriptional control of the PhoP/PhoQ system (22), CorA and MgtE have been found to be PhoP independent in other bacterial species. Moreover, *mgtE* translation has been found to be regulated by a Mg^{2+}-specific riboswitch that is responsive to the concentrations of the divalent ion inside the cell (12). The analysis of the nucleotide sequences of the *S. marcescens mgtE1* promoter region showed a potential 174-bp *ykoK* leader or M-box riboswitch aptamer (for Rfam analysis, see reference 23)

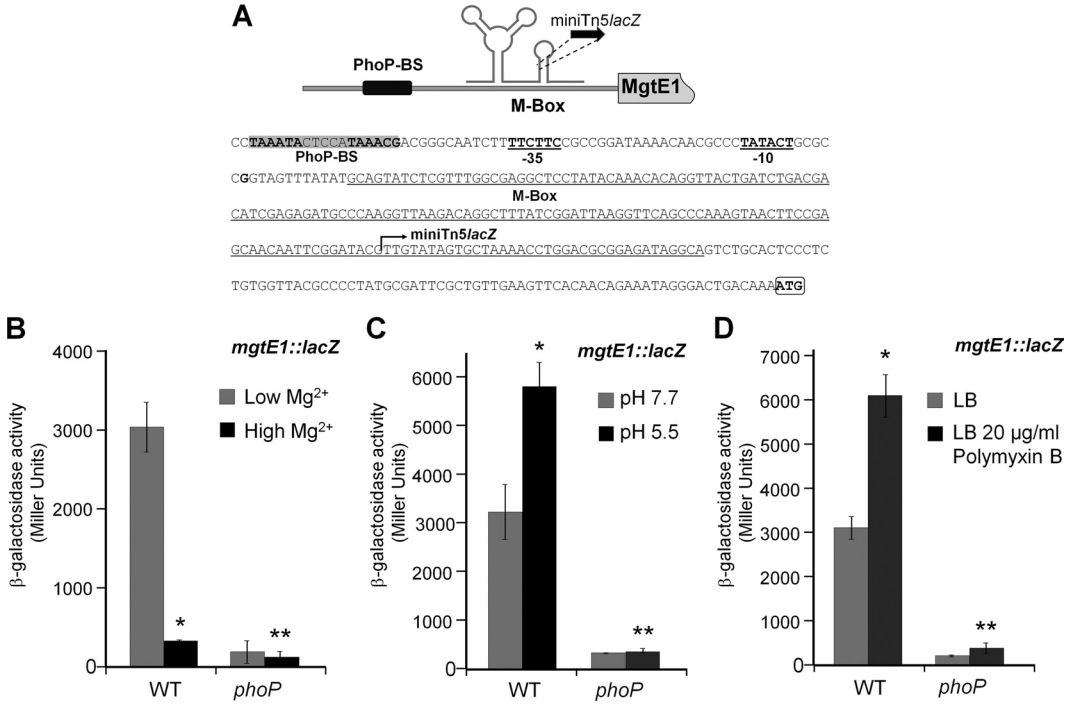

FIG 3 *mgtE1* responds to the same signals as *phoP*. (A) Schematic representation of the promoter region of *mgtE1*, including the predicted PhoP binding site (in black), the riboswitch-encompassed region (M-Box; the cartoon does not reflect an actual secondary structure but depicts only the localization of the predicted structure), the insertion site of mini-Tn5-*lacZ*, the predicted −35 and −10 regions (underlined), and the predicted transcriptional (bold G) and translational (boxed ATG) start site. (B, C, and D) β-Galactosidase activity from the *mgtE1*::*lacZ* transcriptional fusion was determined. Strains were grown overnight in LB for low Mg^{2+} and LB plus 50 mM $MgCl_2$ for high Mg^{2+} (B) and in N medium MES (pH 7.7) to exponential phase and then inoculated into N medium MES (pH 7.7 or 5.5) and grown additional 4 h (C) or in LB to exponential phase and then inoculated into LB and LB plus 20 μg/ml polymyxin B and grown for 1 h (D). Data correspond to mean values for four independent experiments performed in duplicate; error bars correspond to standard deviations (*, $P < 0.001$; **, $P > 0.2$).

and the predicted PhoP-recognition motif (Fig. 3A; the localization of the transposon insertion in the predicted riboswitch sequence relative to the PhoP-binding site [PhoP-BS] is also shown). The deduced amino acid sequence of *S. marcescens* MgtE1 shows 49% homology and 28% identity with *B. subtilis* subsp. *subtilis* strain 168 MgtE (12).

β-Galactosidase activity from the *mgtE1*::*lacZ* reporter in the *phoP* strain was 16-fold lower than that of the otherwise isogenic WT strain, and this value was not altered, irrespective of the Mg^{2+} concentration of the bacterial growth medium, for the *phoP* mutant (Fig. 3B). We also determined that *mgtE1* expression was regulated by pH and polymyxin B in a PhoP-dependent manner (Fig. 3C and D). It is worth highlighting that the mini-Tn5-*lacZ1* insertion localized disrupting the putative riboswitch sequence. Therefore, the expected intracellular Mg^{2+}-dependent regulation that would be modulated by the direct binding of the cation to the tridimensional structure of the mRNA would be interrupted, allowing only the detection of the transcription driven by the upstream putative PhoP-binding site.

These results demonstrate that *mgtE1* is a member of the *S. marcescens* PhoP regulon.

***phoP* transcription is induced within epithelial cells.** Previously, our group showed that *Serratia* is able to invade and persist and multiply within nonphagocytic cells (16). To examine whether *phoP* expression is modulated inside host cells, we carried out an invasion (kanamycin protection) assay using CHO epithelial cells. We then analyzed *phoP* transcription levels by measuring β-galactosidase activity from intracellular bacteria harboring the *phoP*::*lacZ* fusion recovered from the invaded CHO cells (65) and compared its expression with that obtained from bacteria grown in the α-MEM culture medium during the same time lapse. We determined that, at 180 min postinfection, *phoP* expression reached a value 1.8-fold ± 0.2-fold higher in intracellular bacteria than that attained by bacteria grown in α-MEM during the same period of time (Fig. 4A). This result indicates that the environment encountered by *S. marcescens* inside epithelial cells stimulates the activity of the PhoP/PhoQ system.

***Serratia marcescens phoP* mutant strain is deficient in survival within epithelial cells.** To evaluate the relevance of the PhoP/PhoQ system in the invasion and intracellular survival of *S. marcescens* in eukaryotic cells, we compared the survival of the WT/pJB, *phoP*/pJB, and *phoP*/pJB::*phoPQ* strains after invasion of cultured CHO cells.

At 120 min postinfection (p.i.), the intracellular CFU value reached by the *phoP* strain was 1.8-fold lower than the CFU values attained by the WT strain. At 360 min p.i., the WT strain showed a 2.6-fold increase in intracellular CFU relative to the number of intracellular bacteria at 120 min, while the *phoP* strain counts remained essentially unchanged. The complemented *phoP* strain (*phoP*/pJB::*phoPQ*) showed a behavior similar to that of the WT strain (Fig. 4B). This result shows that the *S. marcescens phoP* mutant strain has a defect in invasion, intracellular replication, and/or survival ability and points out that the PhoP/PhoQ system

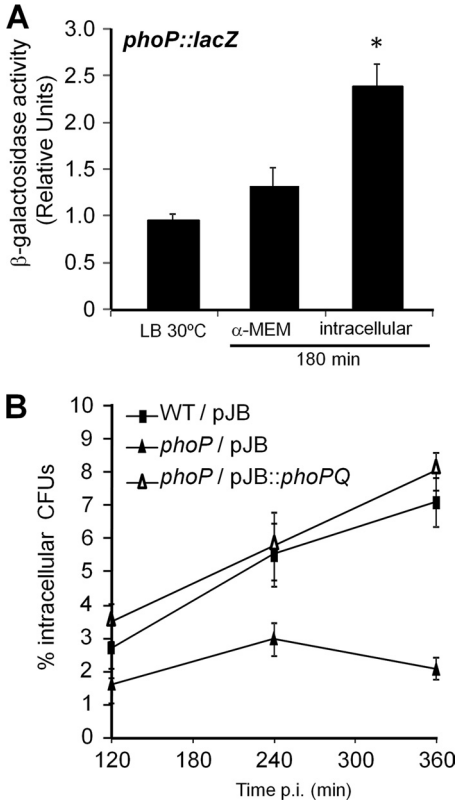

FIG 4 PhoP is involved in *S. marcescens* survival inside epithelial cells. (A) CHO cells were infected with the *phoP*::*lacZ* strain. At 180 min p.i., cells were disrupted, and β-galactosidase activity from intracellular bacteria was determined. β-Galactosidase values obtained from the *phoP*::*lacZ* strain grown in α-MEM culture medium and LB medium during the same time lapse were determined as the control. Values correspond to the ratio between the β-galactosidase activity obtained under each condition and the activity for the same strain grown in LB. Values are the means for triplicate wells from a representative of three independent experiments. Error bars correspond to standard deviations. β-Galactosidase activity values from intracellular bacteria and bacteria in α-MEM or LB were significantly different (*, $P < 0.001$). (B) CHO cells were infected with the WT/pJB, *phoP*/pJB, and *phoP*/pJB::*phoPQ* strains. Percentages of intracellular CFU calculated relative to CFU in the inoculum were determined at the indicated times. Results are averages from at least three independent assays performed in duplicate. Error bars correspond to standard deviations.

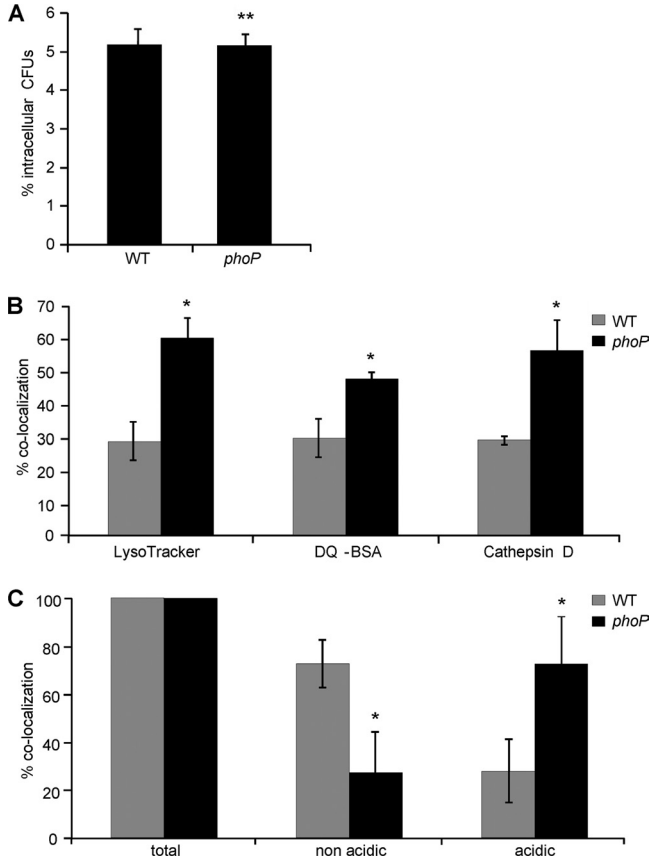

FIG 5 The *phoP* mutant is mainly delivered to an acidic and degradative autophagic compartment. (A) CHO cells were infected with the WT/pGFP and *phoP*/pGFP strains at an MOI of 10 for 30 min. Then, cells were fixed and extracellular *Serratia* was stained using anti-*Serratia* antibodies and detected by incubation with anti-rabbit Cy3-conjugated secondary antibodies. Bacterial internalization was calculated as the number of intracellular bacteria per cell. In each case, cells were analyzed by confocal microscopy. At least 200 infected cells (B and C) or 600 cells (A) were scored from three independent experiments. Data represent the means for three independent experiments. Error bars correspond to standard deviations (**, $P > 0.2$). (B) CHO cells were infected with the WT/pGFP and *phoP*/pGFP strains. After 360 min p.i., cells were fixed and intracellular bacteria were detected by fluorescence. To label acidic compartments, after 60 min p.i., cells were incubated with LysoTracker. To assay degradative compartments, CHO cells were preincubated with DQ-BSA. In a third independent assay, the lysosomal protease cathepsin D was detected using rabbit anti-human cathepsin D and secondary antibody goat anti-rabbit IgG conjugated with Cy3. The percentage of colocalization was calculated as the ratio between the number of vesicles containing bacteria that colocalize with the indicated fluorescently labeled marker and the total number of SeCVs. Error bars correspond to standard deviations (*, $P < 0.001$). (C) CHO-EGFP-LC3 cells were infected with WT or *phoP* strain. After 60 min p.i., cells were incubated with LysoTracker to label acidic compartments. Cells were fixed at 360 min p.i., and intracellular bacteria were detected with anti-*Serratia* antibodies coupled with a secondary antibody labeled with Alexa Fluor 647. The percentages of colocalization of bacteria with EGFP-LC3 and/or with LysoTracker were calculated as the ratio between the number of autophagic SeCVs that colocalize with LysoTracker (acidic) or not (nonacidic) and the total number of autophagic SeCVs. Error bars correspond to standard deviations (*, $P < 0.001$).

plays a relevant role in the pathogenesis mechanisms of this opportunistic pathogen.

To address these possibilities, we performed a bacterial adherence assay and detected no differential attachment to the CHO cells between the WT and the *phoP* strains (not shown; see Materials and Methods for details). Next, to determine whether *phoP* is required for bacterial entry into epithelial cells, we used a two-color immunofluorescence invasion assay, which distinguishes intracellular from extracellular bacteria by microscopic analysis. This bacterial uptake assay allowed us to quantitatively analyze the entry of the WT or *phoP* strains into CHO cells in a short term postinfection. Both strains showed an equivalent percentage of intracellular bacteria relative to total invaded cells at 30 min p.i. (Fig. 5A). These results demonstrate that the integrity of the *phoPQ* operon is not required for the early internalization process of *Serratia* into epithelial cells and suggest that the previously shown difference in intracellular counts between the WT and *phoP* strains might be attributed to a deficient replication and/or survival capacity of the mutant strain inside the cell.

The PhoP/PhoQ system is required for efficient avoidance of SeCV delivery to lysosomes. In previous work, we demonstrated

that at late times postinfection, a predominant population of internalized *Serratia* is contained in an autophagosome-like, nondegradative and nonacidic vacuole, indicating that the bacterium is capable of either delaying or hampering the fusion to lysosomal compartments (16).

To examine the intracellular fate of the *S. marcescens phoP* mutant strain, we analyzed whether the *Serratia*-containing vacuoles underwent acidification. For this purpose, we performed a colocalization assay using either WT or *phoP Serratia* strains that express the GFP protein from pGFP, and at 60 min p.i., we loaded CHO cells with LysoTracker, a fluorescent marker that labels acidic compartments. The ratio between the number of SeCVs that colocalized with the fluorescently labeled marker and the total number of SeCVs was calculated. At 360 min p.i., colocalization of *phoP* SeCVs and LysoTracker was estimated in 60% ± 7%, 2.1 times higher than the calculated value for the WT strain (28% ± 6%) (Fig. 5B, LysoTracker).

At the same time, we evaluated if *phoP* SeCVs displayed features of degradative compartments. With this aim, DQ-BSA was preloaded into CHO cells 4 h previous to infection. DQ-BSA is a self-quenched fluorophore that emits red fluorescence upon proteolytic digestion, useful for monitoring degradative vacuoles. In this assay, at 360 min p.i., for WT *Serratia*, colocalization of DQ-BSA with SeCVs was estimated in 29% ± 6%, while 47% ± 2% of *phoP* SeCVs showed colocalization (Fig. 5B, DQ-BSA). This result demonstrates that compared with the WT SeCVs, *phoP* SeCVs are preferentially delivered to degradative compartments.

Cathepsin D, an aspartyl protease involved in intracellular degradation of exogenous and endogenous proteins, is delivered to the lumen of late endosomes (40, 47, 54) and serves as a marker for phagolysosomes and autophagolysosomes (59). To further corroborate our previous result, in a third independent approach, we analyzed by immunodetection the presence of cathepsin D in SeCVs harboring either WT or *phoP* mutant strains. At 360 min p.i., cells were fixed, stained, and examined by confocal microscopy. At this time point, 29% ± 1% of WT SeCVs colocalized with cathepsin D, compared to 56% ± 9% of the *phoP* SeCVs (Fig. 5B, Cathepsin D), demonstrating that the deficiency in the expression of the PhoP/PhoQ system favors the delivery of SeCVs to lysosomal vesicles. Figure 6 shows representative captured images from 360 min p.i. of the colocalization assays with the lysosomal markers (Fig. 6, LysoTracker [A], d and h; DQ-BSA [B], d and h; and cathepsin D [C], d and h; magnified insets of vacuoles harboring *Serratia* are also provided for each colocalization assay).

We have previously demonstrated that at late times postinfection (i.e., 360 min p.i.), approximately 50% of the SeCVs relative to total infected cells were autophagic vesicles, decorated by LC3 and Rab7 markers, and that the vast majority of these vacuoles were nonacidic, nondegradative compartments (16). To assess whether the PhoP/PhoQ system contributes to directing *Serratia* to the autophagic pathway, we performed the colocalization assay of the WT and mutant *phoP* strains using CHO cells stably transfected with the EGFP-LC3 fusion protein and loaded with LysoTracker at 60 min p.i. We found that colocalization of SeCVs with the autophagic LC3 marker was indistinguishable for the WT and *phoP* strains, indicating that *Serratia* targeting by the autophagic machinery was not influenced by *phoPQ* (not shown). However, when we simultaneously quantified the autophagic SeCV colocalization with LysoTracker (i.e., decorated by LC3 and labeled by LysoTracker), the WT strain showed an estimated 3:1 distribution between nonacidic and acidic vesicles, while the *phoP* strain showed an inverted 1:3 ratio, indicating that autophagic SeCVs that contain the *phoP* mutant preferentially progressed into acidic compartments (Fig. 5C).

In addition, as determined by microscopic inspection at 360 min p.i. and in spite of the diminished quantity of total intracellular bacteria, the surviving *phoP* mutant strain localized in large SeCVs that could attain more than 20 bacteria/vesicle, similar to the SeCVs harboring the WT strain (not shown). This observation strongly indicates that irrespective of being a preferential target of intracellular eradication mechanisms, the *phoP* strain is able to replicate within the host cell.

Collectively, these results indicate that the PhoP/PhoQ system contributes to the intracellular survival strategy of the pathogen having a relevant role in the avoidance of the eukaryotic cell degradative machinery.

DISCUSSION

Characterization of regulatory and phenotypic features of PhoP/PhoQ in *S. marcescens*. The *phoPQ* operon encoding the signal transduction PhoP/PhoQ system has been identified in a wide variety of bacteria with very diverse lifestyles, ranging from ambient dwelling bacteria to plant and animal pathogens. However, in all these microorganisms, shared functional and physiological features of PhoP/PhoQ can be recognized. These essential characteristics comprise the involvement of the PhoP/PhoQ system in the control of the adaptive response to Mg^{2+}-restricted environments, and in those organisms that establish a relationship with a host, this TCS governs the expression of genes that are crucial for a successful interaction. *S. marcescens* can be isolated in host and nonhost environments. We have previously demonstrated that *S. marcescens* is able not only to invade but also to persist and proliferate inside epithelial cells, avoiding innate eradication mechanisms (16). Therefore, the occurrence of *phoPQ* in its genome allowed us to predict that the *S. marcescens* PhoP/PhoQ system will entail that this bacterium detects and responds to both ambient and host-associated signals.

In this work, we have demonstrated that *S. marcescens phoPQ* endows the bacterium with the ability to survive under an environmental Mg^{2+}-deprived condition and to tolerate acidic pH and also high concentrations of polymyxin B, a clinically extensively used antimicrobial agent effective against Gram-negative bacteria. We have also shown that under each of these three harsh conditions for bacterial growth (low Mg^{2+}, low pH, or presence of polymyxin B), the transcriptional expression of the *S. marcescens phoPQ* operon is activated (Fig. 1 and 3). As demonstrated for the first time in *Salmonella enterica* (68), in all other *phoPQ*-harboring bacteria examined up to date, including *Yersinia*, *Photorhabdus*, *Neisseria*, and *Mycobacterium* (14, 28, 62, 73, 74), the *phoPQ* operon is subjected to autoregulation. This implies a resting state in which no inducing signals are present and basal levels of the PhoP and PhoQ proteins are expressed in the bacteria due to the activity of a constitutively active, PhoP-independent promoter. When inducing signals promote the PhoQ-mediated increase in phosphorylated PhoP levels, the response is amplified via a positive feedback loop, which is warranted by the presence of a *phoP*-binding regulatory site upstream of the coding region in the operon. Consistent with this feature, conserved putative PhoP-binding motifs could be recognized by *in silico* examination of the *S. marcescens phoPQ* regulatory region (Fig. 1A). The presence of a

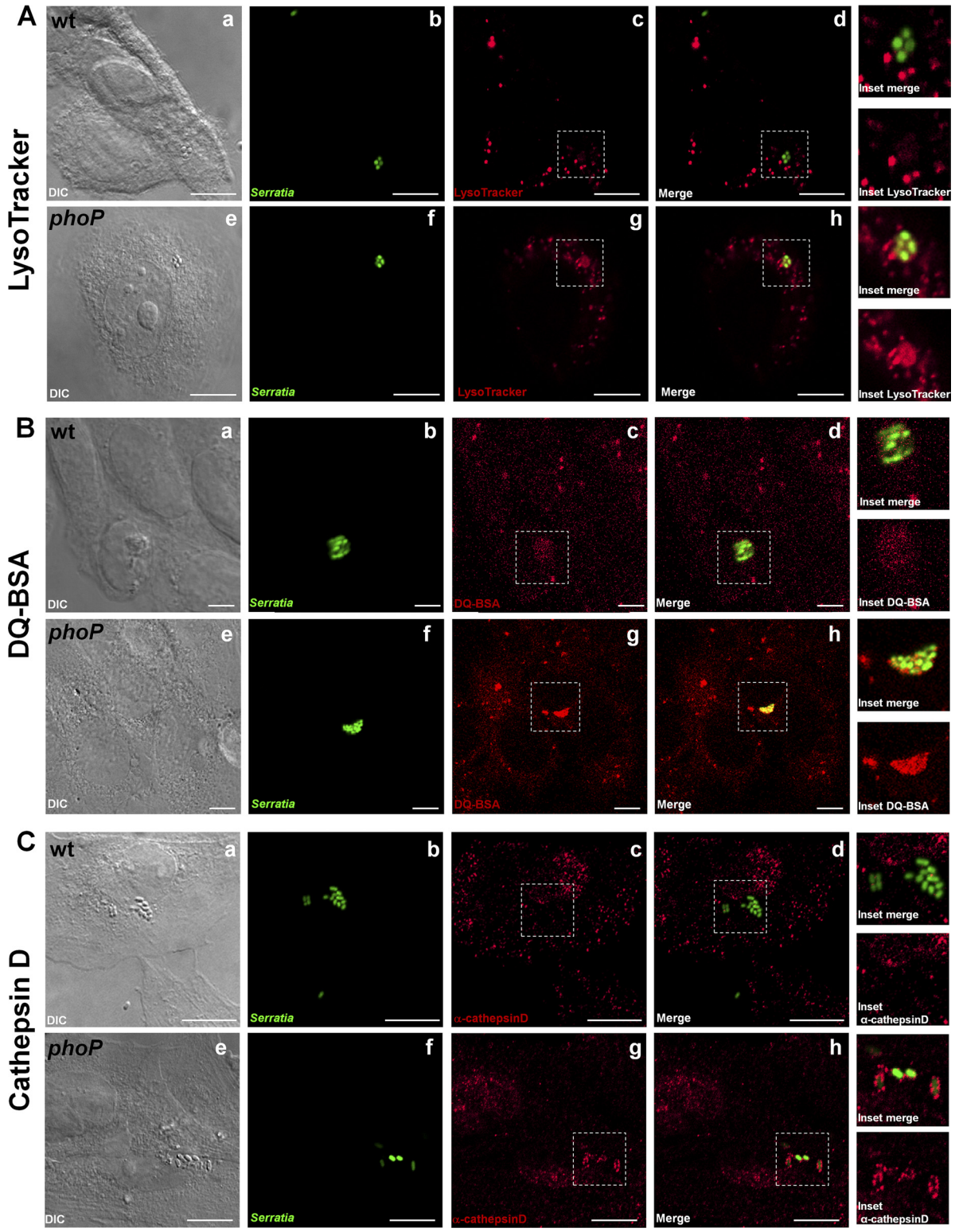

FIG 6 Representative confocal microscopy images of CHO cells infected with *S. marcescens* WT and *phoP* strains. (A) CHO cells infected with the WT/pGFP and *phoP*/pGFP strains (green fluorescence; b and f) and incubated with LysoTracker (red fluorescence, c and g) at 360 min p.i. are shown. Insets show higher magnification of the boxed area in the merged and LysoTracker images, respectively. The images highlight a nonacidic SeCV for the WT strain and an acidic SeCV for the *phoP* mutant strain. (B) CHO cells preincubated with DQ-BSA (red fluorescence; c and g) and infected with WT/pGFP and *phoP*/pGFP strains (green fluorescence; b and f) at 360 min p.i. are shown. Insets show higher magnification of the boxed area in the merged and DQ-BSA images, respectively. The images highlight a nondegradative SeCV for the WT strain and a degradative SeCV for the *phoP* mutant strain. (C) CHO cells infected with WT/pGFP and *phoP*/pGFP strains (green fluorescence; b and f) at 360 min p.i. are shown. Cathepsin D was detected with anti-cathepsin D antibodies coupled with a secondary antibody labeled with Cy3 (red fluorescence; c and g). Inset images show higher magnification of the boxed area in the merged and cathepsin D images, respectively, and highlight a SeCV without a lysosomal marker for the WT strain and a SeCV containing a lysosomal marker for the *phoP* mutant strain.

regulatory positive feedback loop that includes *phoPQ* within PhoP-regulated genes allows us to infer that low Mg^{2+}, acidic pH, and polymyxin B are inducing signals detected by the PhoPQ system in *Serratia*. These results are reinforced by the fact that transcription of *mgtE1*, which shows a conserved PhoP-binding motif in its promoter region, was strongly upregulated in a PhoP-dependent manner when the cells were grown under each *phoPQ*-activating condition assayed (Fig. 3).

Putative regulatory PhoP-binding motifs could be found in the regulatory regions not only of *mgtA* and *mgtCB*, as determined by our *in silico* predictive analysis, but also of the two *S. marcescens mgtE* orthologs, suggesting that the PhoP protein directly regulates their expression. This finding was unexpected, because no PhoP-dependent regulation has been detected for *mgtE* in other bacteria. We confirmed that *mgtE1* is part of the PhoP/PhoQ regulon in *Serratia* (Fig. 3). In this context, we demonstrated that Mg^{2+} availability shuts off the activity of the *S. marcescens* regulatory system when the bacterium is growing either in minimal or in rich LB medium. Downregulation of the PhoP/PhoQ system activity by high Mg^{2+} concentrations has been shown to be the evolutionarily acquired trait that allows PhoP/PhoQ-harboring bacteria to repress the production of Mg^{2+} transporters (MgtA and MgtB) when they are no longer necessary to preserve Mg^{2+} bacterial homeostasis (62). Therefore, the recruitment under PhoP-mediated regulation of *mgtE* ortholog genes in *Serratia* complies with the ancestral function of the system, unveiling a novel variation of an old theme. Besides, the analysis of the *S. marcescens mgtE1* promoter suggests that it combines the regulatory strategies that have been previously found in *mgtA* (11, 69), i.e., a predicted riboswitch that is expected to respond to the cytoplasmic availability of Mg^{2+}, defining posttranscriptionally the availability of the messenger, and the PhoP box, which commands transcription in response to the extracellular cation abundance.

Analysis of the *S. marcescens* MgtE1 protein shows 53% identity and 72% homology with MgtE of *Aeromonas hydrophila* ATCC 7966 and 24% identity and 43% homology with *Pseudomonas aeruginosa* PA1 MgtE. Interestingly, an *Aeromonas hydrophila mgtE* mutant leads to deficiency in virulence properties, including colonization of host sites, biofilm formation, and swarming (49). On the other hand, in *P. aeruginosa*, *mgtE* expression modulates the expression of type III secretion system (T3SS) genes, likely via inhibition of ExsA-dependent transcription. *P. aeruginosa* can utilize MgtE for magnesium transport and regulation of gene expression (2). Future studies in our laboratory will focus on determining the relationship between MgtE function and *Serratia* virulence.

The PhoP/PhoQ system has been extensively implicated in the modulation of virulence determinants in numerous bacteria that range from animal to plant pathogens, including *Salmonella*, *Yersinia*, *Mycobacterium*, *Neisseria*, *Pseudomonas*, *Erwinia*, and *Xanthomonas* (6, 18, 26, 27, 37, 38, 44, 46, 55, 60, 61). *phoPQ* mutant strains have shown severe attenuated virulence phenotypes in the diverse *in vitro* or *in vivo* infection models employed to characterize the role of the encoded signal transduction system in pathogenesis.

Role of the PhoP/PhoQ system in *Serratia* survival within epithelial cells. In pathogenic bacteria such as *Salmonella*, *Shigella*, *Yersinia*, and *Mycobacterium*, the PhoP/PhoQ system plays a crucial role in the bacterial capacity to survive inside macrophages, since the strains carrying an inactivated *phoP* gene are defective in this phenotype (6, 17, 24, 44, 53, 61, 70, 71). However, the precise mechanism controlled by PhoP/PhoQ that enables the bacteria to counteract the eradication processes of the invaded cell is still a matter of debate. In the more extensively analyzed *S.* Typhimurium macrophage infection model, earlier work showed that PhoP-regulated determinants would be implicated in the avoidance of the fusion between the *Salmonella*-containing vacuole and lysosomal compartments (24, 35). However, a recent report shows that *phoP*-deficient strains would have a replication defect that hinders proliferation after phagolysosomal fusion avoidance has taken place, by means of a PhoP/PhoQ-independent strategy, in a subpopulation of the SCVs (70). When tissue cultures other than macrophages have been challenged, complexity is added to this scenario. *Salmonella* supports limited growth in fibroblasts or dendritic cells (71); however, *phoP* mutants overgrow in fibroblasts, suggesting that in this cell line the PhoP/PhoQ system restricts intravacuolar proliferation. In epithelial cells, although the prevailing model also indicates that in its maturation process the SCV is able to avoid contact with late endosomes-lysosomes (20), live-cell imaging assays have demonstrated that the contents of these late endocytic compartments can be delivered to SCVs (15). In more distantly related bacteria, such as *M. tuberculosis*, a *phoP* mutant strain has shown impaired multiplication *in vitro* in mouse bone marrow-derived macrophages and *in vivo* in a mouse infection model (44, 61). More recent work has shown that this attenuation should be due to an inability to block phagosome-lysosome fusion compared with the parental strain (17). The *Salmonella* and *Mycobacterium* examples mentioned highlight the notion that in different pathogens the PhoP/PhoQ system has recruited under its control the expression of determinants that are key for the peculiar manipulation that, in order to survive intracellularly, bacteria exert on the host cell traffic pathways.

We have previously demonstrated that *S. marcescens* is able to tailor the features of its own replicative niche inside epithelial cells. After internalization, *Serratia* inhabits an autophagosome-like vacuole, where it survives and proliferates, avoiding or delaying the delivery to lysosomal compartments (16). In this work, we show that in our epithelial CHO cell invasion model, *phoPQ* transcription is intracellularly upregulated (Fig. 4A). This finding correlates with the fact that the PhoP/PhoQ-inducing cues, acidic pH, Mg^{2+} starvation, and the presence of antimicrobial peptides, are conditions that the bacteria would encounter within the host cell.

We also show that *phoPQ* was dispensable for *Serratia* to be internalized, to elicit the autophagic response, and to reside inside vacuoles with autophagic features in the CHO cell invasion model. However, by tracing acidic and/or degradative vacuoles, we showed that, in contrast with the WT strain, the *phoP* mutant was preferentially delivered to compartments with lysosomal features (Fig. 5 and 6). This result is in agreement with the estimated 3.3-fold-diminished intracellular survival of the *phoP* mutant in comparison to that of the WT strain (Fig. 4B), strongly suggesting that the PhoP/PhoQ system is engaged in the strategy that *Serratia* uses to circumvent the innate immune eradication responses of the cell. Conceivably, this could be due to the demonstrated PhoP/PhoQ-mediated conferred resistance of *Serratia* to the harsh intravacuolar milieu (i.e., acidic pH, presence of antimicrobial peptides, or Mg^{2+} deprivation), to the PhoP/PhoQ-regulated expression of yet-unknown effectors which might alter the recognition, tethering, or fusogenic capacity of the SeCVs for lysosomal

compartments, and/or to the involvement of the system in the control of intrinsic bacterial replication processes in response to intracellular cues. Further work is under way in our laboratory to identify and elucidate the mechanism of action of the implicated *S. marcescens* PhoP/PhoQ-regulated effectors.

ACKNOWLEDGMENTS

We are grateful to Rodrigo Vena (confocal laser microscopy) and to Dolores Campos (tissue culture) for excellent technical assistance. We thank M. F. Alexeyev for providing the pKNOCK plasmid series, L. Debarbieux for the kind gift of strains, and Diego Comerci for the kind gift of reagents required for specific assays.

E.G.V., M.E.C., and M.I.C. are supported by grants from Agencia Nacional de Promoción Científica y Tecnológica and from Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina. E.G.V., M.E.C., and M.I.C. are career investigators of CONICET, Argentina. J.B. and G.D.V. have fellowships from CONICET, Argentina.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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