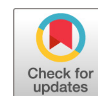
AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE



Serratia marcescens RamA Expression Is under PhoP-Dependent Control and Modulates Lipid A-Related Gene Transcription and Antibiotic Resistance Phenotypes

Javier F. Mariscotti,[a] Eleonora García Véscovi[a]

[a]Instituto de Biología Molecular y Celular de Rosario, Consejo Nacional de Investigaciones Científicas y Tecnológicas, Universidad Nacional de Rosario, Rosario, Santa Fe, Argentina

ABSTRACT *Serratia marcescens* is an enteric bacterium that can function as an opportunistic pathogen with increasing incidence in clinical settings. This is mainly due to the ability to express a wide range of virulence factors and the acquisition of antibiotic resistance mechanisms. For these reasons, *S. marcescens* has been declared by the World Health Organization (WHO) as a research priority to develop alternative antimicrobial strategies. In this study, we found a PhoP-binding motif in the promoter region of transcriptional regulator RamA of *S. marcescens* RM66262. We demonstrated that the expression of *ramA* is autoregulated and that *ramA* is also part of the PhoP/PhoQ regulon. We have also shown that PhoP binds directly and specifically to *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* promoter regions and that RamA binds to *ramA* and *lpxO1* but not to *mgtE1* and *lpxO2*, suggesting an indirect control for the latter genes. Finally, we have demonstrated that in *S. marcescens*, RamA overexpression induces the AcrAB-TolC efflux pump, required to reduce the susceptibility of the bacteria to tetracycline and nalidixic acid. In sum, we here provide the first report describing the regulation of *ramA* under the control of the PhoP/PhoQ regulon and the regulatory role of RamA in *S. marcescens*.

IMPORTANCE We demonstrate that in *S. marcescens*, the transcriptional regulator RamA is autoregulated and also controlled by the PhoP/PhoQ signal transduction system. We show that PhoP is able to directly and specifically bind to *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* promoter regions. In addition, RamA is able to directly interact with the promoter regions of *ramA* and *lpxO1* but indirectly regulates *mgtE1* and *lpxO2*. Finally, we found that in *S. marcescens*, RamA overexpression induces the AcrAB-TolC efflux pump, required to reduce susceptibility to tetracycline and nalidixic acid. Collectively, these results further our understanding of the PhoP/PhoQ regulon in *S. marcescens* and demonstrate the involvement of RamA in the protection against antibiotic challenges.

KEYWORDS *Serratia*, PhoP/PhoQ, RamA, LPS, antibiotics

Serratia marcescens is a Gram-negative bacterium that belongs to the *Enterobacteriaceae* family. *S. marcescens* is widely distributed in the environment and in a wide range of host organisms. In addition to its ubiquity, *S. marcescens* is an emergent health-threatening nosocomial pathogen, due to the acquisition of antibiotic resistance mechanisms, the ability to survive for months on inanimate surfaces, and its resistance to conventional disinfection procedures (1–3). This bacterium has been declared by the WHO as a research priority to develop alternative antimicrobial strategies (4). In the last years, multidrug resistance strains outbreaks and high incidence in intensive and neonatal care units have increasingly been reported (5–7). Moreover, recent work identified *S. marcescens* as one of the three most abundant microbial species that colonize dysbiotic gut in patients with Crohn's disease (8).

Citation Mariscotti JF, García Véscovi E. 2021. *Serratia marcescens* RamA expression is under PhoP-dependent control and modulates lipid A-related gene transcription and antibiotic resistance phenotypes. J Bacteriol 203:e00523-20. https://doi.org/10.1128/JB.00523-20.

Editor Michael Y. Galperin, NCBI, NLM, National Institutes of Health

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Javier F. Mariscotti, mariscotti@ibr-conicet.gov.ar, or Eleonora García Véscovi, garciavescovi@ibr-conicet.gov.ar.

Received 18 September 2020
Accepted 13 April 2021
Accepted manuscript posted online 29 April 2021
Published 8 June 2021

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

S. marcescens can also develop either symbiotic or pathogenic interactions with plants and insects (9). The ability of *S. marcescens* to produce myriad extracellular enzymes and various secondary metabolites allows it to adapt to and survive in both hostile and changing environments (9). Two-component systems (TCS) are one of the most ubiquitous mechanisms by which bacteria generate adaptive responses to environmental or intrahost 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 (10).

PhoP/PhoQ is a broadly conserved TCS among many pathogenic and nonpathogenic bacteria. *In vitro*, this system can be activated by acidic pH and antimicrobial peptides (APs) and is repressed by millimolar magnesium or calcium concentrations and by long-chain unsaturated free fatty acids (11–14). In various pathogenic bacteria it was demonstrated that PhoP/PhoQ has the ability to sense host intracellular signals and regulate bacterial lifestyle adaptation during infection (15). Although PhoP/PhoQ displays similar functions in regulating the virulence capacity of pathogenic bacteria, such as *Salmonella enterica* (16, 17), *Mycobacterium tuberculosis* (18), *Yersinia pestis* (19), *Shigella flexneri* (20), and extraintestinal pathogenic *Escherichia coli* (21), the set of regulated genes recruited under its control vary in a species-specific manner.

In our previous work, we have shown that in *S. marcescens* clinical isolate RM66262 (22), the PhoP/PhoQ system is involved in the adaptation of this bacterium to grow in scarce environmental Mg^{2+}, at acidic pH, and in the presence of polymyxin B. Furthermore, we have shown that the PhoP/PhoQ system is implicated in the avoidance strategy that allows *Serratia* to survive and multiply inside epithelial cells (23).

The lipopolysaccharide (LPS), composed of lipid A, core, and O antigen, contains a molecular pattern recognized by the innate immune system, thereby promoting host defense responses (24). The ability of Gram-negative bacteria to modify the LPS is implicated in the avoidance of the host immune system and the resistance to killing by APs. In many bacterial species, modifications in the LPS that confer resistance to antimicrobial peptides are regulated by the PhoP/PhoQ system (25–27). In *S. marcescens*, it was demonstrated that the expression of the *arn* operon, which is involved in LPS modification, is under PhoP control (28). On the other hand, in *Klebsiella pneumoniae*, the transcriptional regulator RamA (resistance antibiotic multiple A) functions as an alternative regulator to PhoP to modulate the expression of the *lpxC*, *lpxL-2*, and *lpxO* genes, which are associated with lipid A biosynthesis (29). It has been reported that *Klebsiella* remodels its lipid A *in vivo*, in the infected lung tissues of mice. The lipid A species found in the lungs have modifications dependent on the PhoP/PhoQ-regulated oxygenase LpxO. In addition, an *lpxO* mutant is attenuated *in vivo*, highlighting the importance of this lipid A modification for the *Klebsiella* infection process (30).

In this study, we identified a recognition site for the PhoP regulator in the putative promoter region of the *S. marcescens* RM66262 *ramA* homolog. Furthermore, we also found two *lpxO* orthologous genes (which we have named *lpxO1* and *lpxO2*). We characterized the regulation cascade that involves *S. marcescens ramA* and defined its participation in the regulation of the *lpxO* and *mgtE* genes and in the control of bacterial resistance to antibiotics. To our knowledge, this is the first report describing the recruitment of *ramA* under the PhoP/PhoQ regulon and the regulatory role of RamA in *S. marcescens*.

RESULTS AND DISCUSSION

***S. marcescens ramA* is a PhoP-regulated gene.** Previous reports demonstrated that the PhoP/PhoQ system is required for *S. marcescens* to tolerate the challenge of an environment deficient in Mg^{2+}, of acidic pH, or of the presence of polymyxin B (23). We have also shown that within the host, this TCS is involved in preventing the intracellular delivery of the bacteria to degradative/acidic compartments (23). In search of *S. marcescens* RM66262 PhoP-regulated genes, we previously performed a bioinformatic search by using the MEME/MAST motif detection program (31, 32) and identified

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

a set of genes that display putative PhoP-binding sites in their promoter regions. Among these genes, we found that the two *S. marcescens mgtE* orthologs displayed a conserved PhoP-binding motif and demonstrated that *mgtE1* expression is PhoP dependent (23). Following this strategy, we were able to additionally detect a conserved motif for PhoP recognition in the promoter regions of genes encoding an AraC-type transcriptional regulator (which we have named *ramA*) and two *lpxO* orthologs (which we have named *lpxO1* and *lpxO2*) (Fig. 1A). By sequence homology analysis, *lpxO* genes are predicted to encode proteins with oxygenase activity that would 2-hydroxylate specific lipid A acyl chains (33). In order to examine the PhoP-dependent expression of *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2*, we constructed reporter plasmids that harbor the *gfp* gene, which encodes the green fluorescent protein (GFP), under transcriptional control of the putative promoter regions (500 bp upstream of the translational ATG start) of each gene. Because *ramA* encodes a response regulator that belongs to the PhoP/PhoQ regulon, we carried out assays not only in the *phoP* background but also in the *ramA* and the double *phoP ramA* mutant background strains.

As shown in Fig. 1B, in the wild-type (WT) background, *ramA* transcriptional levels are not affected by the Mg^{2+} concentration of the bacterial growth medium. However, under low-Mg^{2+} conditions, *ramA* expression is circa 50% downregulated in the *phoP*, *ramA*, and *phoP ramA* strains, while under high-Mg^{2+} conditions, *ramA* expression is PhoP independent but dependent on *ramA* integrity. These results suggest that (i) *ramA* expression is autoregulated, (ii) under low-Mg^{2+} conditions, *ramA* transcriptional activity could be repressed by an unknown PhoP-independent mechanism, or (iii) under low-Mg^{2+} conditions, integrity of both *ramA* and *phoP* is required for full *ramA* transcriptional expression.

In enterobacteria, PhoP drives expression from an array of promoters with sequence diversity, and in doing so, it coordinates the expression of a variety of gene products that are required in different amounts and/or for different extents of time, according to environmental characteristics (34). In *S. marcescens* the *phoPQ* operon is autoregulated, as was previously reported (23), and *phoP* transcription is repressed with increasing concentrations of Mg^{2+}, and our results show that RamA is dispensable for *phoP* expression (Fig. 1B). As previously mentioned, we have shown that in *S. marcescens*, the transcription of *mgtE1* is PhoP regulated in a Mg^{2+}-dependent fashion (23) (Fig. 1B). In addition, our results show that *mgtE1* expression depends on RamA as a positive regulator, independent of the Mg^{2+} concentration of the growth medium (Fig. 1B), indicating that *mgtE1* transcriptional levels are dependent on the simultaneous presence of both PhoP and RamA.

Consistent with our *in silico* screening of putative *Serratia* PhoP-regulated genes, we also found that transcription of *mgtE2* is differentially regulated at low/high-Mg^{2+} concentrations. As shown in Fig. 1B, the transcriptional level of *mgtE2* was 2-fold higher at low Mg^{2+}, either in the WT or in the *ramA* strain, than the levels detected for either *phoP* or *ramA phoP* mutant strains. At high concentrations of Mg^{2+}, the transcription level of *mgtE2* was not altered in the *ramA phoP* or *ramA phoP* strains compared to the expression levels obtained for the WT strain. In sum, the transcriptional levels of *ramA* were downregulated in the *phoP* mutant, under either high- or low-Mg^{2+} conditions, while the expression of *ramA* did not affect *mgtE2* transcriptional levels. These results demonstrate that in contrast to *mgtE1*, *mgtE2* is a PhoP-dependent, RamA-independent gene.

In order to examine if these differentially expressed genes were under direct control of PhoP, we performed electrophoretic mobility shift assay (EMSA) using purified recombinant PhoP protein. When DNA fragments containing the promoter sequences of *ramA*, *mgtE1*, and *mgtE2* were incubated in the presence of PhoP, retarded bands were detected and their intensities were enhanced when the amount of PhoP was increased, with a concomitant intensity reduction in the band that corresponds to the unbound probe (Fig. 2A, left, and B). These results demonstrate that, as predicted, PhoP was able to directly interact with the promoter sequences of *ramA*, *mgtE1*, and

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

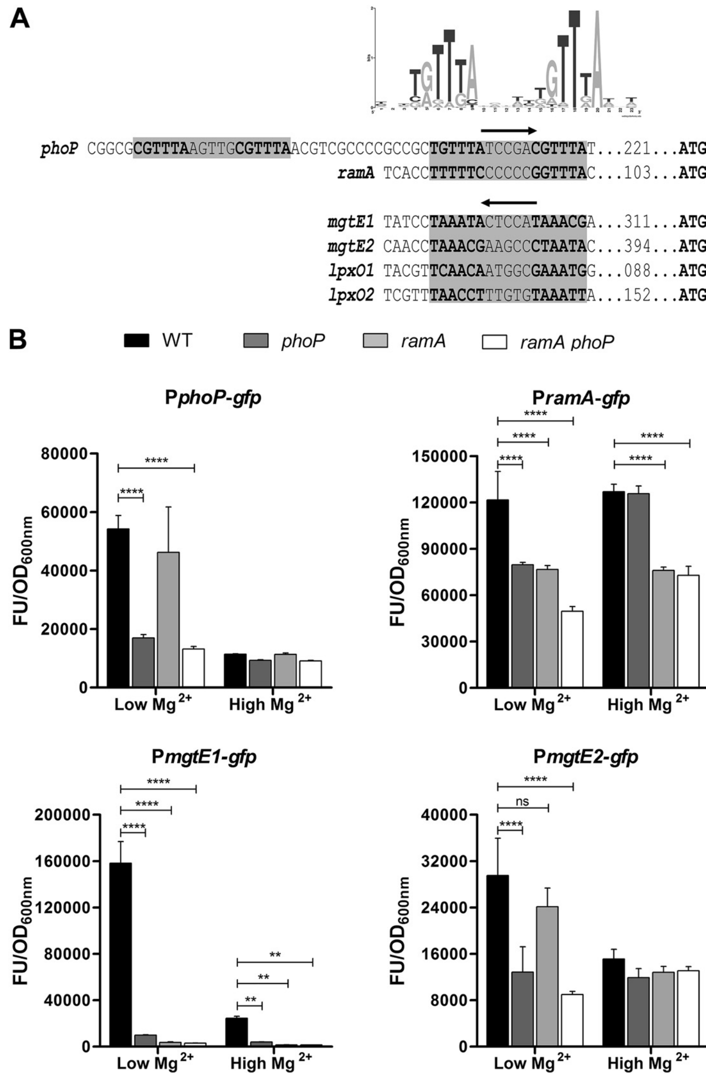


FIG 1 (A) PhoP-binding sites in *Serratia marcescens* RM66262 genome. A consensus motif for the PhoP-binding site was generated using a collection of previously defined promoter regions of PhoP-regulated genes in *S.* Typhimurium and *E. coli* as a database and the MEME software tool. The logo (obtained by using WebLogo [weblogo.berkeley.edu]) shows the consensus motif for the PhoP-binding site, and the putative PhoP-binding site sequences with high scores identified in the *S. marcescens* RM66262 genome by MAST are listed at the bottom. The PhoP boxes are shown in boldface; arrows indicate orientation relative to each translational start site. The name of the gene and the distance (in base pairs) from the translational start site are also indicated. (B) Transcriptional expression of *phoP*, *ramA*, *mgtE1*, and *mgtE2*. Bacteria were grown for 16 h in N medium with 10 μM $MgCl_2$ for low-Mg^{2+} conditions or 10 mM $MgCl_2$ for high-Mg^{2+} conditions, in 96-well microplates, at 37°C with agitation. Transcriptional activity was calculated as the ratio of GFP fluorescence values and OD_{600} (FU/OD_{600}) measured from the *S. marcescens* wild-type (WT), *phoP*, *ramA*, and *ramA phoP* strains carrying the P*phoP-gfp*, P*ramA-gfp*, P*mgtE1-gfp*, and P*mgtE2-gfp* reporter plasmids. Means ± SDs from three independent experiments performed in duplicate in each case are shown. Statistical analysis was performed using two-way analysis of variance with Bonferroni's posttest. **, $P < 0.01$; ****, $P < 0.0001$ (statistically significantly different from WT *S. marcescens*). ns, not significant.

mgtE2 that contain a PhoP-binding motif. A 25- to 100-fold excess of nonspecific *nucA* DNA fragment (a 441-bp DNA region that codes for the *S. marcescens* NucA nuclease) did not affect the interaction with the labeled probe (Fig. 2A, middle), while the shifted band was progressively lost when increasing amounts of unlabeled *ramA* promoter fragment were included in the mixture to compete with the labeled probe (Fig. 2A, right). This result indicates that the interaction of PhoP with the *ramA* promoter region was specific. In addition, the presence of competing nonlabeled *nucA* DNA fragment did not affect the interactions of PhoP with the labeled probes containing the

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

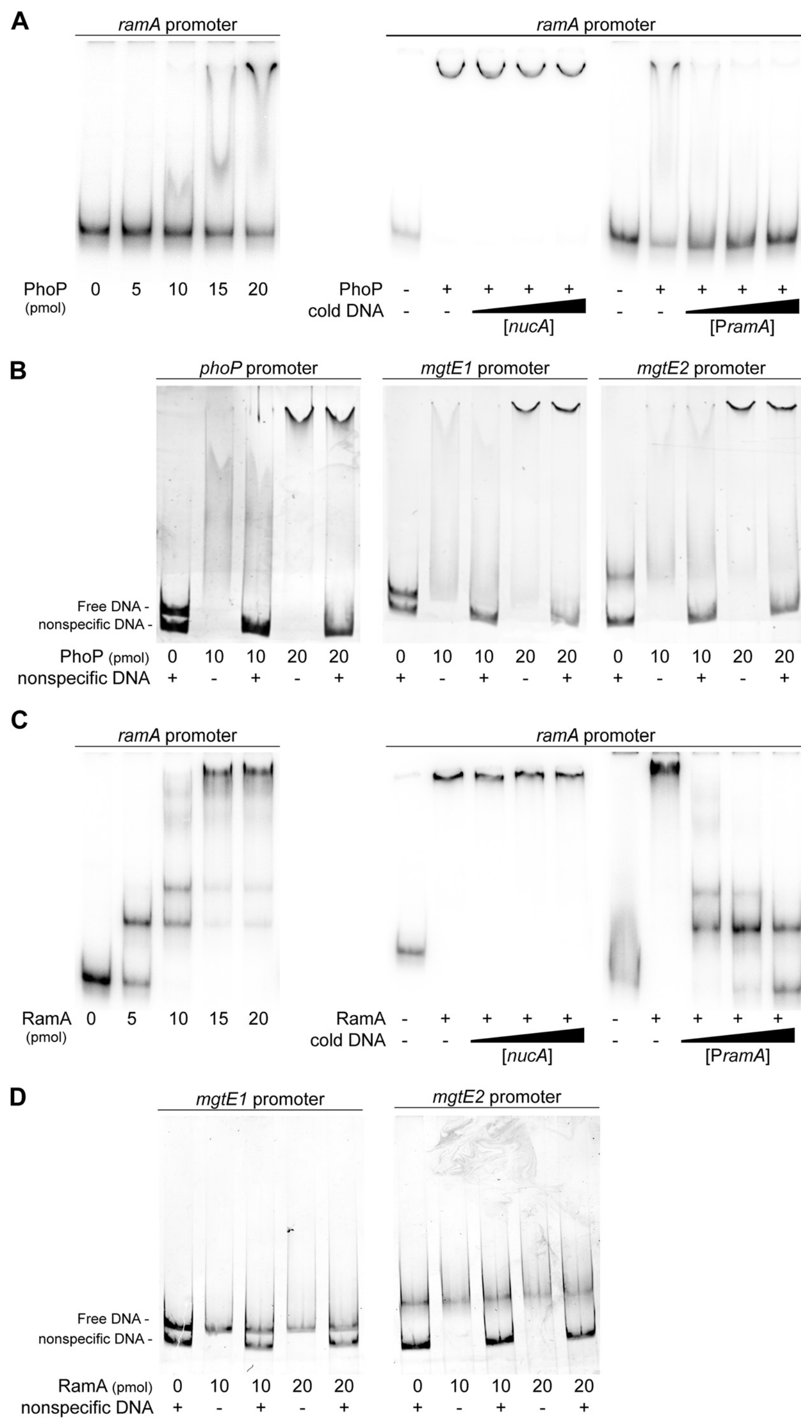


FIG 2 PhoP and RamA interactions with *ramA*, *mgtE1*, and *mgtE2* gene promoter regions of *S. marcescens*. (A and C) Electrophoretic mobility shift assays (EMSAs) were performed using different amounts of purified PhoP-6×His (A) or RamA-6×His (C). Target DNA was a ^{32}P-labeled PCR fragment that included the *ramA* promoter region (P*ramA*). Binding specificity was assessed by competition reactions using 20 pmol of purified PhoP-6×His (A) or RamA-6×His (C) in which increasing amounts (50, 100, and 200 ng) of nonspecific (*nucA*; middle) or specific (P*ramA*; right) unlabeled DNA template competed with labeled DNA for binding to PhoP-6×His (A) or RamA-6×His (C). (B and D) EMSAs were performed using nonlabeled PCR fragments carrying the complete *phoP*, *mgtE1*, and *mgtE2* promoter DNA sequences and purified protein PhoP-6×His (B) or RamA-6×His (D) (10 or 20 pmol, as indicated). When indicated, a 441-bp DNA fragment was used as a nonspecific competitor.

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

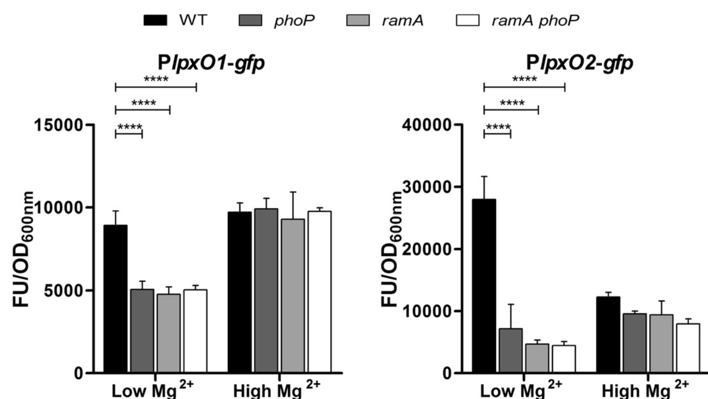


FIG 3 Transcriptional expression of *lpxO1* and *lpxO2*. Bacteria were grown for 16 h in N medium with 10 μM $MgCl_2$ for low-Mg^{2+} conditions or 10 mM $MgCl_2$ for high-Mg^{2+} conditions in 96-well microplates, at 37°C with agitation. Transcriptional activity was calculated as the ratio of GFP fluorescence values and OD_{600} (FU/OD_{600}) measured from the *S. marcescens* WT, *phoP*, *ramA*, and *ramA phoP* strains carrying the P*lpxO1-gfp* and P*lpxO2-gfp* reporter plasmids. Means ± SDs from four independent experiments performed in duplicate in each case are shown. Statistical analysis was performed using two-way analysis of variance with Bonferroni's posttest. ****, $P < 0.0001$ (statistically significantly different from WT *S. marcescens*).

promoter region of *phoP*, *mgtE1*, or *mgtE2* (Fig. 2B). This result indicates that the interaction of PhoP with these promoter regions was specific. Because *phoP* transcription is autoregulated, the interaction of PhoP with the promoter region of *phoP* was used as a positive control for EMSA.

To assess whether RamA is able to directly interact with the promoter regions upstream of *ramA* and *mgtE1*, EMSA was performed using purified recombinant RamA protein. When the probe containing the promoter region of *ramA* was incubated in the presence of RamA, a retarded band was detected and its intensity was enhanced with increasing amounts of RamA, with the concomitant intensity reduction in the lower band that corresponds to the unbound probe (Fig. 2C, left). A 25- to 100-fold excess of nonspecific *nucA* DNA fragment did not affect the interaction (Fig. 2C, middle), while the shifted band was progressively lost when increasing amounts of unlabeled *ramA* promoter fragment were included in the mixture (Fig. 2C, right), indicating that the interaction of RamA with the *ramA* promoter region was specific. These results show that RamA is able to directly bind to the promoter region of its own gene, *ramA*. When the promoter regions of *mgtE1* and *mgtE2* were incubated in the presence of RamA, no shift in the DNA probes was detected (Fig. 2D). This result suggests that *mgtE1* regulation is under indirect control of RamA.

Overall, these results show that *mgtE1*, *mgtE2*, and *ramA* are members of the *S. marcescens* PhoP regulon. They also indicate that RamA is able to control its own expression in an autoregulatory positive loop and that PhoP and RamA simultaneously control *mgtE1* transcription, while *mgtE2* expression depends only on PhoP.

The PhoP/PhoQ system and RamA regulate genes involved in lipid A modifications. In order to analyze the influence of PhoP and RamA on the expression of *lpxO1* and *lpxO2*, we determined their transcriptional expression levels detecting fluorescence over time from the P*lpxO1-gfp* or P*lpxO2-gfp* transcriptional reporter. As shown in Fig. 3, *lpxO1* transcript levels were not affected by the Mg^{2+} concentration of the bacterial growth medium. However, under low-Mg^{2+} conditions, *lpxO1* expression was circa 50% downregulated in the *phoP*, *ramA*, and *phoP ramA* strains, indicating that both PhoP and RamA are required for their full expression. In contrast, under high-Mg^{2+} conditions, *lpxO1* expression was independent of either PhoP or RamA. Under low-Mg^{2+} conditions, the *lpxO2* transcript level was at least 4-fold decreased in the *phoP*, *ramA*, and *ramA phoP* background strains compared to wild-type levels, indicating that both PhoP and RamA are required for their full expression. In contrast, at high Mg^{2+}

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

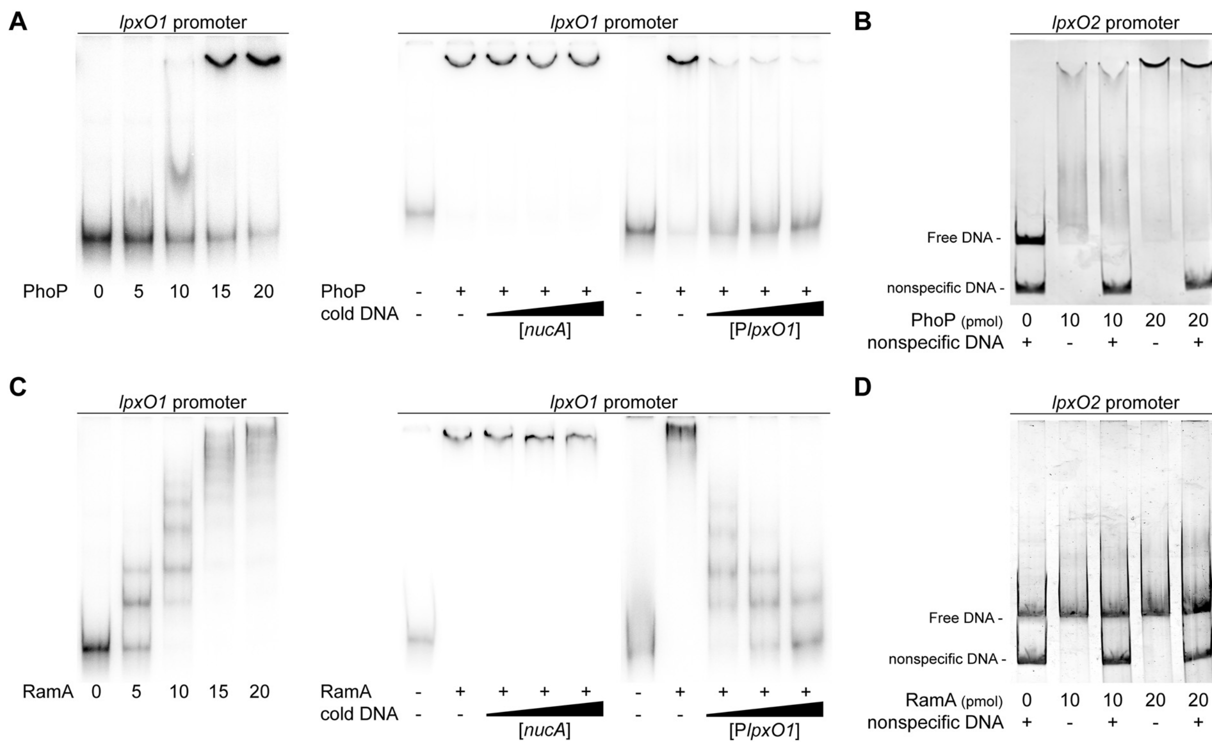


FIG 4 PhoP and RamA interactions with *lpxO1* and *lpxO2* promoter regions in *S. marcescens*. (A and C) EMSAs were performed using different amounts of purified PhoP-6×His (A) or RamA-6×His (C). Target DNA was a ^{32}P-labeled PCR fragment that included the *lpxO1* promoter region (P*lpxO1*). Binding specificity was assessed by competition reactions using 20 pmol of purified PhoP-6×His or RamA-6×His in which increasing amounts (50, 100, and 200 ng) of nonspecific (*nucA*; middle) or specific (P*lpxO1*; right) unlabeled DNA template competed with labeled DNA for binding to PhoP-6×His or RamA-6×His. (B and D) EMSAs were performed using nonlabeled PCR fragments carrying the complete *lpxO2* promoter and purified PhoP-6×His (B) or RamA-6×His (D) (10 or 20 pmol, as indicated). When indicated, a 441-bp DNA fragment was used as a nonspecific competitor.

concentrations, *lpxO2* transcription levels were independent of the integrity of either *phoP* or *ramA*.

In order to examine if these genes are under direct control of PhoP and RamA, we performed EMSA using purified recombinant PhoP and RamA proteins. When the promoter regions of *lpxO1* and *lpxO2* were incubated in the presence of PhoP, retarded bands were detected and their intensities were enhanced when the amount of PhoP was increased, with the concomitant intensity reduction in the band that corresponds to the unbound probe (Fig. 4A, left, and B). These results demonstrate that, as predicted, PhoP is able to directly interact with the promoter sequences that contain PhoP-binding motifs of *lpxO1* and *lpxO2*. A 25- to 100-fold excess of unlabeled nonspecific *nucA* DNA fragment did not affect the interaction (Fig. 4A, middle), while the shifted band was progressively lost when increasing amounts of the unlabeled *lpxO1* promoter fragment were included in the mixture (Fig. 4A, right), showing the specificity of the interaction. The presence of unlabeled nonspecific *nucA* DNA fragment did not affect the interactions (Fig. 4B), indicating that the interaction of PhoP with the *lpxO2* promoter region is specific.

On the other hand, when the promoter region of *lpxO1* was incubated in the presence of RamA, retarded bands were detected and their intensities were enhanced when the amount of RamA was increased, with concomitant intensity reduction in the band that corresponds to the unbound probe (Fig. 4C, left). The results show that RamA directly binds to the promoter region of the *lpxO1* gene. A 25- to 100-fold excess of competing nonspecific *nucA* DNA fragment did not affect the interaction (Fig. 4C, middle), while the shifted band was progressively lost when increasing amounts of unlabeled *lpxO1* promoter fragment were included in the mixture (Fig. 4C, right), indicating that the interaction of RamA with the *lpxO1* promoter region is specific. In

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

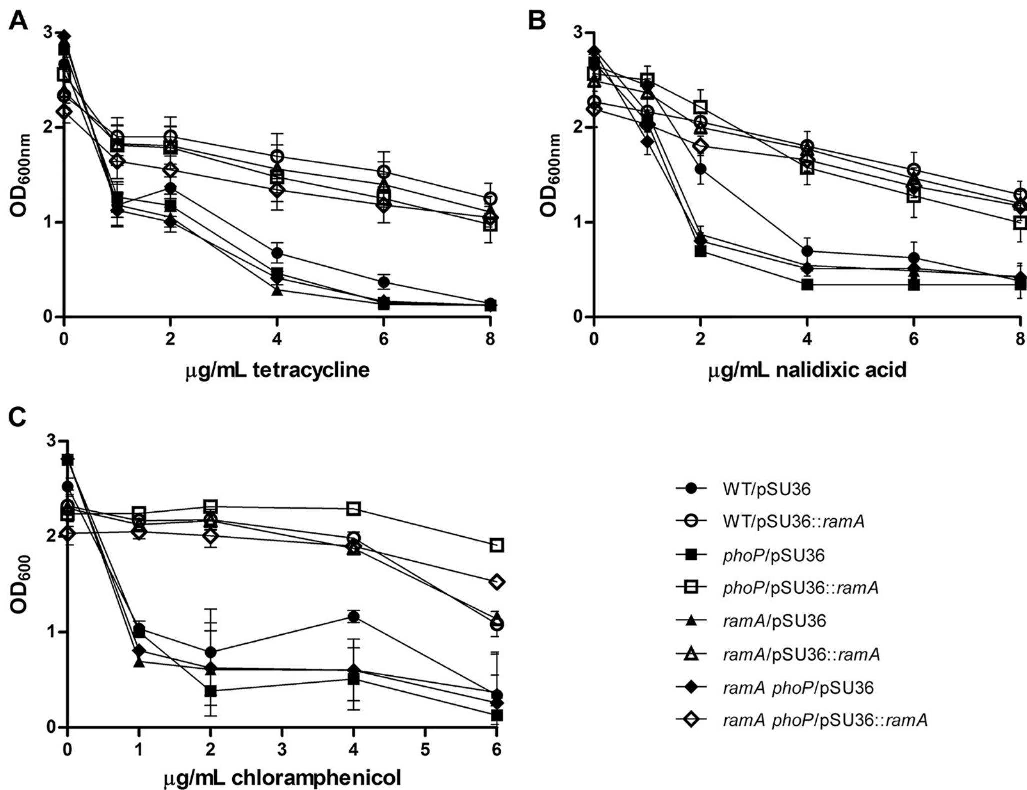


FIG 5 Susceptibility of *S. marcescens* to antibiotics. The OD_{600} was determined for overnight cultures of the WT, *phoP*, *ramA*, and *ramA phoP Serratia* strains carrying the pSU36 or pSU36::*ramA* plasmid grown in LB medium in the presence of different concentrations of tetracycline (A), nalidixic acid (B), or chloramphenicol (C). Results are averages from three independent assays performed in duplicate.

contrast, when the promoter region of *lpxO2* was incubated in the presence of RamA, no shift in the mobility of the DNA probe was detected (Fig. 4D). This result reinforces the notion that that *lpxO2* regulation would be under indirect control of RamA. Together, these results demonstrate that in *S. marcescens*, RamA functions together with PhoP as a regulator of genes that are predicted to be involved in lipid A modification.

RamA overexpression alters *S. marcescens* susceptibility to antibiotic compounds. In *Salmonella enterica* serovar Typhimurium (35, 36) as well as in other *Enterobacteriaceae*, including *Klebsiella* (37) and *Enterobacter* (38) spp., RamA was described to regulate the expression of the genes encoding the AcrAB-TolC resistance-nodulation-division multidrug efflux system. AcrAB-TolC multidrug efflux pumps restrict the intracellular concentrations of various antibiotics, including β-lactams, tetracyclines, chloramphenicol, and quinolones (39). Therefore, we sought to analyze whether RamA is involved in conferring tetracycline resistance to *S. marcescens*. To this end, we compared the growth capacities of WT, *phoP*, *ramA*, and *ramA phoP* strains in Luria-Bertani (LB) medium using concentrations of tetracycline between 0 and 8 μg/ml. No significant differences were obtained in susceptibility to tetracycline in these strains (Fig. 5, strains carrying an empty plasmid pSU36). Because for *K. pneumoniae*, *Enterobacter aerogenes*, and *S. enterica* serovar Typhimurium it has been previously reported that overexpression of RamA increases resistance to antibiotics (38, 40, 41) and we do not know the conditions that induce RamA expression or activation in *Serratia*, we conjectured that overexpression of the regulator could bypass or mimic inducing conditions. To assess whether RamA overexpression was able to increase the resistance of *S. marcescens* to tetracycline, nalidixic acid (a quinolone), or chloramphenicol, we compared the growth capacities of strains when we expressed RamA from pSU36::*ramA*, in the presence of

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

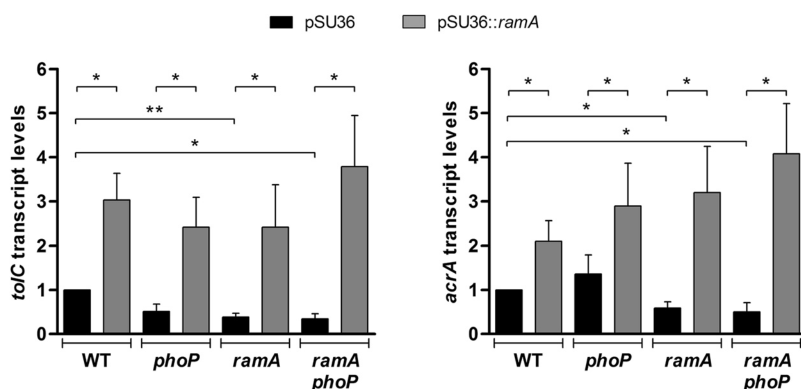


FIG 6 qRT-PCR assays showing relative expression data for the *tolC* and *acrA* genes analyzed. The strains carrying the pSU36 or pSU36::*ramA* plasmid were grown in LB medium for 4 h at 37°C. mRNA levels were normalized to the 16S rRNA gene, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. Means and standard errors of four independent experiments are shown. Significant differences versus reference condition calculated by paired *t* test are indicated as follows: *, $P < 0.05$, and **, $P < 0.01$.

increasing concentrations of these drugs. The half-inhibitory concentrations (IC_{50}) were estimated at 1.0 μg/ml for tetracycline, 1.7 μg/ml for nalidixic acid, and 0.7 μg/ml for chloramphenicol in the strains harboring the empty pSU36 vector. The IC_{50} values increased to 2.0 μg/ml for tetracycline, 3.3 μg/ml for nalidixic acid, and 4.4 μg/ml for chloramphenicol in the strains in which RamA was overexpressed (Fig. 5). The results showed that RamA overexpression reduced the susceptibility to tetracycline, nalidixic acid, and chloramphenicol.

To analyze whether the reduction in tetracycline, nalidixic acid, and chloramphenicol susceptibility observed by overexpressing RamA could be associated with an induced expression of the AcrAB-TolC efflux pump, we determined transcriptional levels of *acrA* and *tolC* by reverse transcription-quantitative PCR (qRT-PCR). As shown in Fig. 6, either *acrA* or *tolC* transcript levels were significantly lower in the *ramA* mutant than in the wild-type strain. Moreover, in the otherwise isogenic strains that overproduce RamA, we observed that the transcript levels of *acrA* or *tolC* increased more than 2-fold compared to those in strains carrying the empty plasmid. These results indicate that RamA is able to activate AcrAB-TolC efflux pump expression and suggest that in *S. marcescens*, the resistance to diverse antibiotics could be increased by RamA-mediated enhanced expression of AcrA/TolC. In order to confirm the role of the AcrAB-TolC efflux pump in resistance to these antibiotics, we constructed *tolC* and *acrA* mutant strains and compared the growth capacities of WT, *tolC*, and *acrA* strains in LB medium using concentrations of either tetracycline or nalidixic acid between 0 and 8 μg/ml. As expected, *tolC* and *acrA* mutants were less resistant to either tetracycline or nalidixic acid than the wild-type strain (Fig. 7A). The overexpression of RamA in *tolC* and *acrA* mutants was not able to restore the levels of wild-type resistance to either antibiotic (Fig. 7B), demonstrating that RamA-dependent upregulation of AcrAB-TolC expression is responsible for enhanced levels of antibiotic resistance in *S. marcescens*.

Overall, our results show that in *S. marcescens*, RamA expression has been recruited under PhoP-dependent regulation. In addition, although the identity of the inducing signal is unknown, increased RamA expression is able to induce the expression of the AcrA/TolC efflux pump, which, in turn, enhances antibiotic resistance levels by restricting the intracellular concentration of the assayed antibiotics.

Concluding remarks. *S. marcescens* can be isolated in host and nonhost environments. We have previously demonstrated that *S. marcescens* is able to invade, survive inside, and proliferate inside nonphagocytic cells (42). We have also shown that the PhoP/PhoQ system is implicated in the avoidance strategy that allows *Serratia* to survive and proliferate inside host cells. Furthermore, we have shown that the PhoP/PhoQ system is involved in the adaptation of this bacterium to growth in the context of scarce environmental Mg^{2+}, at acidic pH, and in the presence of polymyxin B (23).

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

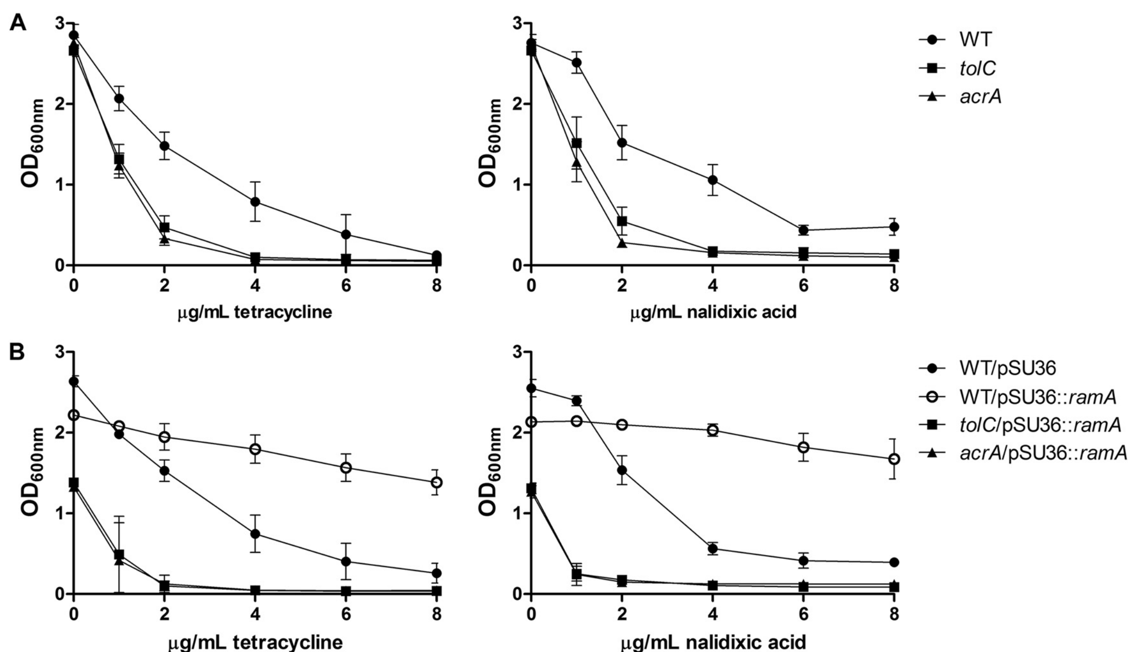


FIG 7 Susceptibility of *S. marcescens* to antibiotics. (A) The OD_{600} was determined for overnight-grown cultures in LB medium in the presence of different concentrations of tetracycline or nalidixic acid. (A) WT, *tolC*, and *acrA Serratia* strains. (B) WT, *tolC*, and *acrA Serratia* strains carrying the pSU36 or pSU36::*ramA* plasmid. Results are averages from four (A) or three (B) independent assays performed in duplicate.

Therefore, the PhoP/PhoQ system allows *S. marcescens* to detect and respond to both ambient and host-associated signals.

In this report, we show that the *S. marcescens ramA* gene, which codes for the AraC-type transcriptional regulator RamA, is autoregulated and that it is also part of the *S. marcescens* PhoP/PhoQ regulon. However, the *ramA* gene is atypically regulated by PhoP, because our results indicate that even high-Mg^{2+}-concentration conditions, which would imply low concentrations or inactive PhoP, would be sufficient to promote *ramA* transcription. Curiously, this is also the case for *lpxO1*. The facts that in the wild-type strain, *ramA* and *lpxO1* expression levels are not affected by the Mg^{2+} concentration of the growth medium and that PhoP integrity alters *ramA* and *lpxO1* transcriptional levels only under low-Mg^{2+} conditions indicate that the phosphorylation status of PhoP does not influence this regulation and suggest the involvement of an additional unknown Mg^{2+}-modulated factor. This unknown factor might contribute to activate *ramA* and *lpxO1* transcription under high-Mg^{2+} conditions and/or repress their expression under low-Mg^{2+} conditions (Fig. 8).

In addition, our results demonstrate that *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* are members of the *S. marcescens* PhoP regulon. While *mgtE2* is only PhoP dependent, *ramA*, *mgtE1*, *lpxO1*, and *lpxO2* are PhoP and RamA dependent. We also show that while PhoP is able to directly and specifically bind to *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* promoter regions, RamA is able to recognize *ramA* and *lpxO1* but not *mgtE1* and *lpxO2*, suggesting an indirect regulatory mechanism for the expression of the latter genes. LpxO has been identified as the oxygenase that 2-hydroxylates the acyl chains of lipid A (25). The addition of a 2-hydroxyl group in acyl chain would stabilize the outer membrane, contributing to resistance to antimicrobial peptides (30). According to the sequence homology of the *S. marcescens lpxO* genes with genes involved in lipid A remodeling in other enterobacteria, we can conjecture that PhoP and RamA would be relevant for the regulation of *S. marcescens* envelope properties.

It has been previously shown that in pathogenic bacteria, RamA regulates the expression of efflux pumps like AcrAB-TolC, responsible for the detoxification of a wide range of substrates, including antimicrobials, heavy metals, and detergents outside the cell

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

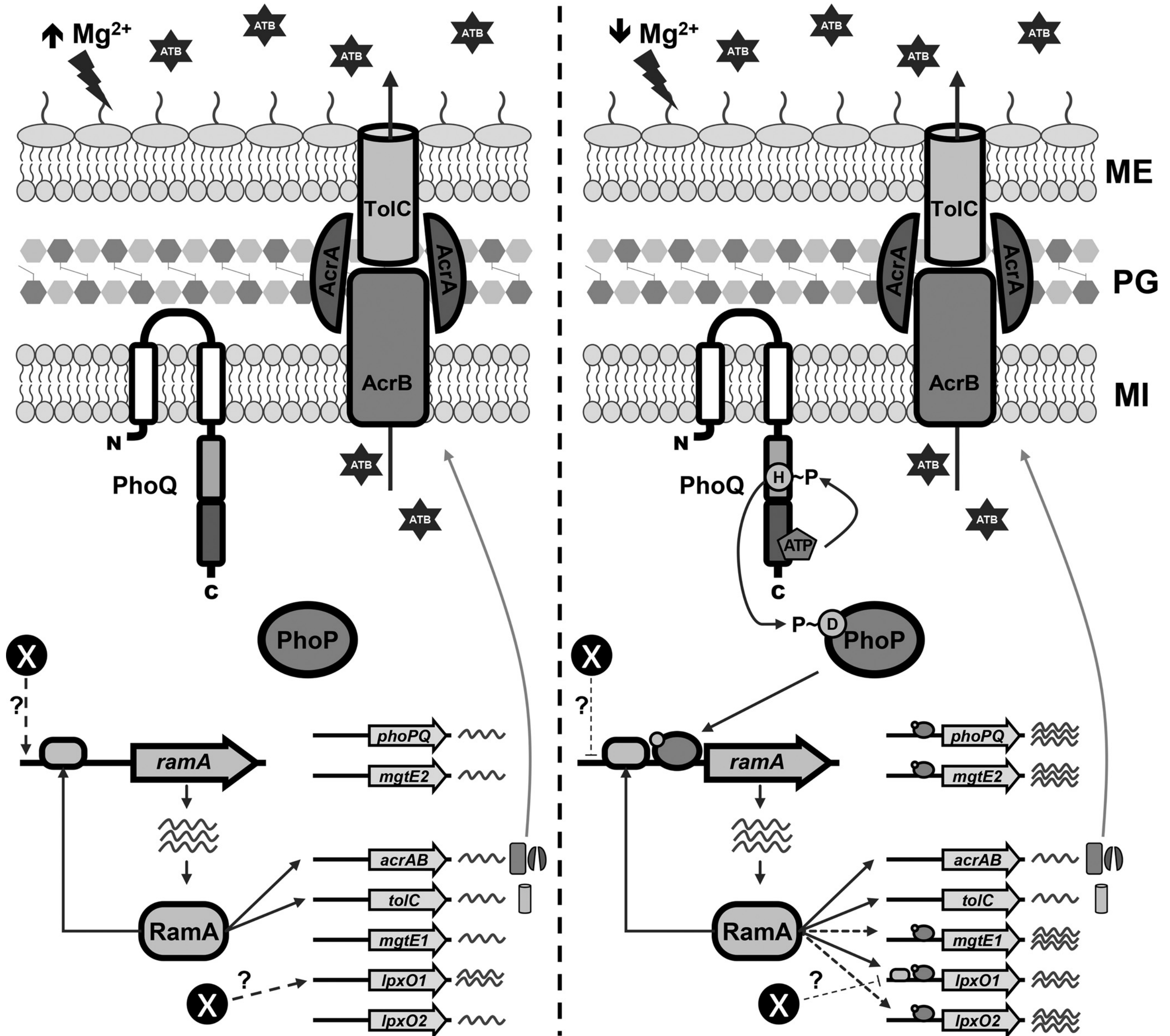


FIG 8 Proposed model for the *S. marcescens* PhoP/PhoQ-RamA regulatory cascade. Under high-Mg^{2+} conditions (left), *ramA* expression is PhoP independent but is dependent on *ramA* integrity. Under low-Mg^{2+} conditions (right), full *ramA* transcriptional expression is both RamA and PhoP dependent. RamA positively regulates the expression of the AcrAB-TolC efflux pump, responsible for detoxification of a wide range of substrates, including antimicrobials. *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* are members of the *S. marcescens* PhoP regulon. *mgtE2* is only PhoP dependent, while *ramA*, *mgtE1*, *lpxO1*, and *lpxO2* are PhoP and RamA dependent. PhoP is able to directly and specifically bind to *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* promoter regions, RamA is able to recognize *ramA* and *lpxO1* but not *mgtE1* and *lpxO2* promoter regions, suggesting an indirect regulatory mechanism for the expression of the latter genes. Still-uncovered regulatory mechanisms (represented by "X") might contribute to activate *ramA* and *lpxO1* genes transcription under high-Mg^{2+} conditions and/or repress its expression under low-Mg^{2+} conditions.

(43). Overexpression of RamA from a plasmid was found to reduce the susceptibility to antibiotics by increasing the levels of AcrAB-TolC efflux pump in *K. pneumoniae*, *E. aerogenes*, and *S. enterica* serovar Typhimurium (38, 40, 41). Furthermore, it was found that clinical isolates of multidrug-resistant (MDR) bacterial pathogens overexpress regulators such as RamA with subsequent overproduction of AcrAB-TolC, which confers higher resistance to antibiotics (44–48). It was recently shown that in *S. marcescens*, the MacAB efflux pump is essential for survival during oxidative stress and confers resistance to polymyxins and aminoglycoside antibiotics (49). These results show the crucial role played by efflux pumps in the intrinsic resistance of bacteria to antimicrobials.

Our results describe for the first time a regulatory cascade between the PhoP/PhoQ

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype and/or comments	Reference or source
S. marcescens strains		
RM66262	WT; clinical isolate	22
phoP strain	*phoP*::pKNOCK-Gm	23
lpxO1 strain	*lpxO1*::pKNOCK-Gm	This work
lpxO2 strain	*lpxO2*::pKNOCK-Gm	This work
Δ*ramA* strain	Δ*ramA*	This work
Δ*ramA phoP* strain	Δ*ramA phoP*::pKNOCK-Gm	This work
WT/pSU36	WT/pSU36	This work
WT/pSU36::*ramA*	WT/pSU36::*ramA*	This work
phoP/pSU36 strain	*phoP*::pKNOCK-Gm/pSU36	This work
phoP/pSU36::*ramA* strain	*phoP*::pKNOCK-Gm/pSU36::*ramA*	This work
Δ*ramA*/pSU36 strain	Δ*ramA*/pSU36	This work
Δ*ramA*/pSU36::*ramA* strain	Δ*ramA*/pSU36::*ramA*	This work
Δ*ramA phoP*/pSU36 strain	Δ*ramA phoP*::pKNOCK-Gm/pSU36	This work
Δ*ramA phoP*/pSU36::*ramA* strain	Δ*ramA phoP*::pKNOCK-Gm/pSU36::*ramA*	This work
tolC strain	*tolC*::pKNOCK-Gm	This work
acrA strain	*acrA*::pKNOCK-Gm	This work
tolC/pSU36::*ramA* strain	*tolC*::pKNOCK-Gm/pSU36::*ramA*	This work
acrA/pSU36::*ramA* strain	*acrA*::pKNOCK-Gm/pSU36::*ramA*	This work
E. coli strains		
One Shot Top10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL endA1* Sm^r	Invitrogen
SM10 λ_{pir}	*thi J thr leu tonA lacY61 lic recA*::*RP4-2*-Tc::Mu λ_{pir} Km^r	52
Plasmids		
pSU36	Km^r	55
pSU36::*ramA*	pSU36::*ramA*; Km^r	This work
pP*phoP-gfp*	pPROBE(NT)::prom*phoP* Km^r	Laboratory stock
pP*ramA-gfp*	pPROBE(NT)::prom*ramA* Km^r	This work
pP*lpxO1-gfp*	pPROBE(NT)::prom*lpxO1* Km^r	This work
pP*lpxO2-gfp*	pPROBE(NT)::prom*lpxO2* Km^r	This work
pP*mgtE1-gfp*	pPROBE(NT)::prom*mgtE1* Km^r	Laboratory stock
pP*mgtE2-gfp*	pPROBE(NT)::prom*mgtE2* Km^r	Laboratory stock
pET22b::*phoP*	Expression vector for PhoP-6×His	This work
pET22b::*ramA*	Expression vector for RamA-6×His	This work

system and the RamA regulator in *S. marcescens*. We here show that RamA is PhoP regulated and that RamA overexpression induces the AcrAB-TolC efflux pump, required to reduce the susceptibility of the bacteria to tetracycline, nalidixic acid, and chloramphenicol. The PhoP/PhoQ system in many bacteria regulates virulence genes and LPS modifications that give them resistance to antimicrobial cationic peptides. The fact that PhoP regulates RamA expression in *Serratia* suggests that the PhoP regulon could have adopted RamA regulation to simultaneously acquire the ability to resist the action of antibiotics used in the clinic for the treatment of bacterial infections and fight the effect of antimicrobial cationic peptides produced as defense by the host.

Overall, our work provides new insights into the PhoP/PhoQ signal transduction system regulon in *S. marcescens* and demonstrates the involvement of RamA in the protection against antibiotic challenges.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Serratia marcescens* RM66262 is a nonpigmented clinical isolate from a patient with a urinary tract infection (GenBank accession no. NZ_JWLO00000000.1) (22). The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Miller's Luria-Bertani (LB) medium or on LB agar plates overnight at 30°C or 37°C. To assay Mg^{2+} regulation, strains were grown overnight under low-Mg^{2+} conditions {N medium [0.1 M Tris-HCl (pH 7.4), 0.1% (wt/vol) Casamino Acids, 38 mM glycerol, 0.37 g/liter of KCl, 0.99 g/liter of $(NH_4)_2SO_4$, 0.087 g/liter of K_2SO_4, 0.14 g/liter of KH_2PO_4] [50] plus 10 μM $MgCl_2$} or high-Mg^{2+} conditions (N medium plus 10 mM $MgCl_2$). The antibiotics used were ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), streptomycin (100 μg/ml), and gentamicin (15 μg/ml).

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)[a]
lpxO1-fw.BamHI	CGGGATCCCCTGTTCGATCACTCCACC
lpxO1-rv.XhoI	CCGCTCGAGCGTTCTGCGCCCAGTG
lpxO1-fw[b]	CTATGGTTGCCGCGATCATT
lpxO1-rv[b]	AGCTGGCTGCCGACATCA
pKNOCK (ori) fwd[b]	TAAGGTTTAACGGTTGTGG
lpxO2-fw.BamHI	CGGGATCCCCCGTACCTGAAGCCGGAG
lpxO2-rv.XhoI	CCGCTCGAGCCAGTGGTTCACCGCCTG
lpxO2-fw[b]	CGCTACAACGTGTGGCGAC
lpxO2-rv[b]	TAACCGGTGCGGTTCCAG
tolC-fw.XbaI	TGCTCTAGAGATCACCGACGTGCAGAACG
tolC-rv.XhoI	CCGCTCGAGGGTGTGAGCTTTCCAACTGC
tolC-fw[b]	ATCAGCTGCAGTCGACCCG
tolC-rv[b]	GCCGTCGTTAATCAAGCGC
acrA-fw.XbaI	TGCTCTAGAAAACCGCTACAAGCCATTGC
acrA-rv.XhoI	CCGCTCGAGAGCGTATCGTTCGGGTTAGG
acrA-fw[b]	GCATCAAGGATTTGCTGTCG
acrA-rv[b]	TTGGCGGAAATACTCACCGC
ramA-A	CGCGGATCCGCGGCGTGCTGCAGTTCG
ramA-B	AAAAGGCCTCATGGTTTTAGTTCTCCC
ramA-C	AAAAGGCCTGCGCTGATAAACGCGG
ramA-D	CCGGAATTCGTGATCCAGTGCAATCCG
ramA-fw[b]	TGAACGACGACGATCGCTTC
ramA-rv[b]	GCTGTTCCAGATCGATGGTG
Prom 4911 Fw EcoRI	CTGAATTCGCGGCGTGCTGCAGTTCG
Prom 4911 Rv BamHI	CGGGATCCCATGGTTTTAGTTCTCCC
4911 Fw BamHI	CGGGATCCATGGATCGCGTCAATATC
4911 Rv HindIII	TCCAAGCTTTTAACGCAGCCCCATCCAG
Prom phoP serr fwd EcoRI	ACCGAATTCGCGCTTAACCCGCTCG
Prom phoP serr rev BamHI	AGGGATCCCATGGCGAACTCCTGTG
prommgtE EcoRI fwd	GCAGGAATTCCAGCAGGC
prommgtE rev BamHI	AGAGGATCCGCAGACTGCCTA
prom mgtE2 Fw EcoRI	CGGAATTCCAAAACGAACAGCGC
prom mgtE2 Rv BamHI	CGGGATCCGTTATGGGTTGCGGC
promlpxO1-Fw	CGGAATTCGTCCTGAGACTGGTGCAGCT
promlpxO1-Rv	CGGGATCCTGCGTAAAGGAAGCTGACGAT
promlpxO2-Fw	CGGAATTCCGGTCGTTATCCTGGATGAAG
promlpxO2-Rv	CGGGATCCCGTCCGCGGTAATGCACATAG
nuclease Fw (XbaI)	GCTCTAGAGGCAAGACGCGCAACTGG
nuclease Rv (XhoI)	CCGCTCGAGGAAATCGGCGCCCTTCGG
16S-F	AAACTGGAGGAAGGTGGGGATGAC
16S-R	ATGGTGTGACGGGCGGTGTG
RT-acrA-Fw	AAGCGCAACTTCGTTGAAGG
RT-acrA-Rv	ATGGCTTGTAGCGGTTTACC
RT-tolC-Fw	TGCTGCAGGTCTACAAACAG
RT-tolC-Rv	TGCTGTTAACGCCATTGCTG

[a]Restriction sites are underlined.
[b]Primer used to confirm the mutant by PCR.

Insertion mutations in *lpxO1* (RT90_RS21050), *lpxO2* (RT90_RS03150), *tolC* (RT90_RS20310), and *acrA* (RT90_RS11255) were constructed with the pKNOCK-Gm suicide plasmid (51). For each gene, an internal 500-bp region was amplified using primers lpxO1-fw.BamHI and lpxO1-rv.XhoI, lpxO2-fw.BamHI and lpxO2-rv.XhoI, tolC-fw.XbaI and tolC-rv.XhoI, and acrA-fw.XbaI and acrA-rv.XhoI (Table 2). The purified PCR products were digested with the restriction enzymes indicated in the primer names and cloned into the pKNOCK-Gm plasmid. The resulting plasmids were introduced into competent *E. coli* SM10 λ_{pir} (52) cells by electroporation and then mobilized into *S. marcescens* RM66262 by conjugation. Insertional mutants were confirmed by PCR analysis using primers.

S. marcescens Δ*ramA* was constructed as follows. PCR was used to generate 501 bp of DNA upstream of *ramA* (RT90_RS09790) using primers ramA-A and ramA-B (Table 2), and 520 bp of DNA downstream of *ramA* using primers ramA-C and ramA-D (Table 2). The resulting DNA fragments were digested with the BamHI-StuI and StuI-SpeI restriction enzymes, respectively, and the fragments were ligated into the BamHI and SpeI sites of pKNG101 (53). pKNG101::Δ*ramA* recombinant plasmid was then mobilized into *S. marcescens* RM66262 by conjugation. Mutant strains were selected on streptomycin, and then high

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

sucrose allowed isolation of mutants in which the deletion allele had replaced the wild-type copy. The *ramA* deletion was confirmed by PCR.

To analyze the transcriptional levels of *phoP* (RT90_RS07370), *ramA*, *lpxO1*, *lpxO2*, *mgtE1* (RT90_RS05070), and *mgtE2* (RT90_RS17415), the promoter regions of the genes were amplified by PCR using the primers Prom phoP serr fwd EcoRI and Prom phoP serr rev BamHI, Prom 4911 Fw EcoRI and Prom 4911 Rv BamHI, promlpxO1-Fw and promlpxO1-Rv, promlpxO2-Fw and promlpxO2-Rv, prommgtE EcoRI fwd and prommgtE rev BamHI, and prom mgtE2 Fw EcoRI and prom mgtE2 Rv BamHI (Table 2). The purified PCR products were digested with the EcoRI and BamHI restriction enzymes and were ligated into the same sites of pPROBE(NT′) (54). The resulting plasmids were introduced into competent *E. coli* Top10 cells by transformation. The plasmids P*phoP-gfp*, P*ramA-gfp*, P*lpxO1-gfp*, P*lpxO2-gfp*, P*mgtE1-gfp*, and P*mgtE2-gfp* were mobilized by conjugation into the *S. marcescens* wild-type strain and the *phoP*, *ramA*, and *ramA phoP* mutant strains.

For complementation of the *S. marcescens ramA* mutant strain, the *ramA* gene was amplified from the *S. marcescens* wild-type strain chromosome by PCR using primers 4911 Fw BamHI and 4911 Rv HindIII (Table 2). The PCR product was cloned into the pSU36 plasmid (55). The construction was then introduced into the *S. marcescens* strains by electroporation.

***phoP*, *ramA*, *mgtE1*, *mgtE2*, *lpxO1*, and *lpxO2* gene expression assays.** Cultures of *S. marcescens* wild-type (WT), *phoP*, *ramA*, and *ramA phoP* strains carrying the pP*phoP-gfp*, pP*ramA-gfp*, pP*mgtE1-gfp*, pP*mgtE2-gfp*, pP*lpxO1-gfp* or pP*lpxO2-gfp* reporter plasmids were grown with shaking overnight at 37°C. The bacterial cultures were washed two times with N medium, 10 μl was mixed with 1 ml of N medium supplemented with 10 μM or 10 mM $MgCl_2$ with kanamycin, and 100-μl volumes of the mixtures were incubated in a 96-well microtiter plate at 37°C with agitation for 16 h. Optical density at 600 nm (OD_{600}) and GFP fluorescence (excitation wavelength [λ_{exc}] of 485 nm and emission wavelength [λ_{em}] at 528 nm) were determined using a 96-microwell plate reader (Synergy 2). Transcriptional activity was calculated as the ratio of GFP fluorescence and OD_{600} (FU/OD_{600}) measured from strains carrying the pP*phoP-gfp*, pP*ramA-gfp*, pP*mgtE1-gfp*, pP*mgtE2-gfp*, pP*lpxO1-gfp*, and pP*lpxO2-gfp* reporter plasmids. The means and standard deviations for three independent assays performed in duplicate in each case were calculated.

Protein-DNA interaction analysis. Electrophoretic gel mobility shift assays (EMSAs) were performed using 6 fmol of ^{32}P-labeled DNA fragments containing the *ramA* promoter (P*ramA*) and *lpxO1* promoter (P*lpxO1*) with different amounts of purified PhoP-6×His and RamA-6×His proteins following basically previously described protocols (56). Prior to addition of the DNA probe, PhoP-6×His protein was phosphorylated by incubation with 25 mM acetyl phosphate at 30°C for 30 min. The specificity of binding was assayed using the unlabeled P*ramA* and P*lpxO1* probes or a 441-bp PCR fragment corresponding to the *nucA* gene from *S. marcescens* as a nonspecific competitor. To evaluated the interaction of PhoP or RamA with the promoter regions of the *mgtE1*, *mgtE2*, and *lpxO2* genes, EMSAs were performed using 30 ng of nonlabeled DNA fragments containing the complete promoter regions of the genes and 10 or 20 pmol of purified PhoP-6×His or RamA-6×His. The nonspecific competitor DNA was assayed using a 441-bp PCR fragment corresponding to the *nucA* gene from *S. marcescens*. The primers used to amplify the P*ramA*, P*lpxO1*, P*mgtE1*, P*mgtE2*, P*lpxO2*, and P*phoP* regions and *nucA* (RT90_RS08445) are listed in Table 2. After electrophoresis, the gels were either dried and exposed to autoradiography or stained with SYBR green (Invitrogen). DNA and protein-DNA complexes were detected and captured using a Typhoon FLA7000 laser scanner (GE Healthcare).

RT-PCR and qRT-PCR. cDNA synthesis was performed using random hexamers, 2 μg of total RNA, and 1 U of SuperScript II reverse transcriptase (Invitrogen). Five microliters of a 1/10 dilution of each cDNA was used as the template for DNA amplification in RT-PCR or quantitative RT-PCR (qRT-PCR) (20 μl), using primers *tolC and acrA* (Table 2). A primer set for the 16S rRNA was used as a control to confirm that equal amounts of total RNA were used in each reaction mixture. In every case, the amplified fragment was 250 bp. For RT-PCR, the number of cycles varied according to the level of expression of each mRNA to ensure that the comparison was performed in the linear range of the amplification. For the quantitative real-time PCR, the reactions were carried out in the presence of the double-stranded DNA-specific dye SYBR green (Molecular Probes) and monitored in real time with the Mastercycler ep realplex real-time PCR system (Eppendorf). The relative expression was calculated using the threshold cycle values (C_T) obtained from each sample as follows: relative expression = $2^{-\Delta\Delta}$, with $\Delta^{CT} = C_{T\ \mathrm{sample}} - C_{T\ 16S}$ and $\Delta\Delta C_T = \Delta C_{T\ \mathrm{sample}} - \Delta C_{T\ \mathrm{ref\ sample}}$ (where sample is the mutant strain transcript, 16S is the 16S rRNA transcript, and ref sample is the *S. marcescens* RM66262 transcript). The reference sample was *S. marcescens* RM66262. The average values were calculated from triplicate samples.

Tetracycline, nalidixic acid, and chloramphenicol 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 tetracycline, nalidixic acid, or chloramphenicol concentrations that ranged between 0 and 8 μg/ml. The mixtures were incubated in 96-well microtiter plates at 37°C without agitation for 16 h. OD_{600} readings were determined with a BioTek ELx808 microplate reader. The means and standard deviations for three independent assays performed in triplicate in each case were calculated.

Statistical analysis. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons with significance set at a *P* value of $<$0.05. Asterisks in figures indicate the values among the treatment groups in which a statistically significant difference was determined.

ACKNOWLEDGMENTS

E.G.V. and J.F.M. are Career Investigators of Consejo de Investigaciones Científicas y Tecnológicas (CONICET), Argentina. This work was supported by grants from Agencia

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, PICT 2016-1137 to E.G.V. and PICT 2013-0002 to J.F.M.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We are grateful to Marina Avecilla for excellent technical assistance.

REFERENCES

1. Yu VL. 1979. Serratia marcescens: historical perspective and clinical review. N Engl J Med 300:887–893. https://doi.org/10.1056/NEJM197904193001604.
2. Kramer A, Schwebke I, Kampf G. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 6:130. https://doi.org/10.1186/1471-2334-6-130.
3. Redondo-Bravo L, Gutiérrez-González E, San Juan-Sanz I, Fernández-Jiménez I, Ruiz-Carrascoso G, Gallego-Lombardo S, Sánchez-García L, Elorza-Fernández D, Pellicer-Martínez A, Omeñaca F, Robustillo-Rodela A. 2019. Serratia marcescens outbreak in a neonatology unit of a Spanish tertiary hospital: risk factors and control measures. Am J Infect Control 47:271–279. https://doi.org/10.1016/j.ajic.2018.08.026.
4. World Health Organization. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. https://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/.
5. Gastmeier P. 2014. Serratia marcescens: an outbreak experience. Front Microbiol 5:81. https://doi.org/10.3389/fmicb.2014.00081.
6. Grimont PAD, Grimont F. 1978. The genus Serratia. Annu Rev Microbiol 32:221–248. https://doi.org/10.1146/annurev.mi.32.100178.001253.
7. Mahlen SD. 2011. Serratia infections: from military experiments to current practice. Clin Microbiol Rev 24:755–791. https://doi.org/10.1128/CMR.00017-11.
8. Hoarau G, Mukherjee PK, Gower-Rousseau C, Hager C, Chandra J, Retuerto MA, Neut C, Vermeire S, Clemente J, Colombel JF, Fujioka H, Poulain D, Sendid B, Ghannoum MA. 2016. Bacteriome and mycobiome interactions underscore microbial dysbiosis in familial Crohn's disease. mBio 7:e01250-16. https://doi.org/10.1128/mBio.01250-16.
9. Petersen LM, Tisa LS. 2013. Friend or foe? A review of the mechanisms that drive Serratia towards diverse lifestyles. Can J Microbiol 59:627–640. https://doi.org/10.1139/cjm-2013-0343.
10. Alex LA, Simon MI. 1994. Protein histidine kinases and signal tranduction in prokaryotes and eukaryotes. Trends Genet 10:133–138. https://doi.org/10.1016/0168-9525(94)90215-1.
11. Bearson BL, Wilson L, Foster JW. 1998. A low pH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress. J Bacteriol 180:2409–2417. https://doi.org/10.1128/JB.180.9.2409-2417.1998.
12. Bader MW, Navarre WW, Shiau W, Nikaido H, Frye JG, McClelland M, Fang FC, Miller SI. 2003. Regulation of Salmonella typhimurium virulence gene expression by cationic antimicrobial peptides. Mol Microbiol 50:219–230. https://doi.org/10.1046/j.1365-2958.2003.03675.x.
13. García Véscovi E, Soncini FC, Groisman EA. 1996. Mg^{2+} as an extracellular signal: environmental regulation of Salmonella virulence. Cell 84:165–174. https://doi.org/10.1016/S0092-8674(00)81003-X.
14. Viarengo G, Sciara MI, Salazar MO, Kieffer PM, Furlán RLE, García Véscovi E. 2013. Unsaturated long chain free fatty acids are input signals of the Salmonella enterica PhoP/PhoQ regulatory system. J Biol Chem 288:22346–22358. https://doi.org/10.1074/jbc.M113.472829.
15. Dalebroux ZD, Miller SI. 2014. Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity. Curr Opin Microbiol 17:106–113. https://doi.org/10.1016/j.mib.2013.12.005.
16. Miller SI, Kukral AM, Mekalanos JJ. 1989. A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence. Proc Natl Acad Sci U S A 86:5054–5058. https://doi.org/10.1073/pnas.86.13.5054.
17. Carabajal MA, Viarengo G, Yim L, Martínez-Sanguiné A, Mariscotti JF, Chabalgoity JA, Rasia RM, Véscovi EG. 2020. PhoQ is an unsaturated fatty acid receptor that fine-tunes Salmonella pathogenic traits. Sci Signal 13:eaaz3334. https://doi.org/10.1126/scisignal.aaz3334.
18. Pérez E, Samper S, Bordas Y, Guilhot C, Gicquel B, Martín C. 2001. An essential role for phoP in Mycobacterium tuberculosis virulence. Mol Microbiol 41:179–187. https://doi.org/10.1046/j.1365-2958.2001.02500.x.
19. Oyston PCF, Dorrell N, Williams K, Li SR, Green M, Titball RW, Wren BW. 2000. The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in Yersinia pestis. Infect Immun 68:3419–3425. https://doi.org/10.1128/IAI.68.6.3419-3425.2000.
20. Lin Z, Cai X, Chen M, Ye L, Wu Y, Wang X, Lv Z, Shang Y, Qu D. 2018. Virulence and stress responses of Shigella flexneri regulated by PhoP/PhoQ. Front Microbiol 8:2689. https://doi.org/10.3389/fmicb.2017.02689.
21. Zhuge X, Sun Y, Xue F, Tang F, Ren J, Li D, Wang J, Jiang M, Dai J. 2018. A novel PhoP/PhoQ regulation pathway modulates the survival of extraintestinal pathogenic Escherichia coli in macrophages. Front Immunol 9:788. https://doi.org/10.3389/fimmu.2018.00788.
22. Bruna RE, Revale S, García Véscovi E, Mariscotti JF. 2015. Draft whole-genome sequence of Serratia marcescens strain RM66262, isolated from a patient with a urinary tract infection. Genome Announc 3:e01423-15. https://doi.org/10.1128/genomeA.01423-15.
23. Barchiesi J, Castelli ME, Di Venanzio G, Colombo MI, García Véscovi E. 2012. The PhoP/PhoQ system and its role in Serratia marcescens pathogenesis. J Bacteriol 194:2949–2961. https://doi.org/10.1128/JB.06820-11.
24. Maldonado RF, Sá-Correia I, Valvano MA. 2016. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. FEMS Microbiol Rev 40:480–493. https://doi.org/10.1093/femsre/fuw007.
25. Raetz CRH, Reynolds CM, Trent MS, Bishop RE. 2007. Lipid A modification systems in Gram-negative bacteria. Annu Rev Biochem 76:295–329. https://doi.org/10.1146/annurev.biochem.76.010307.145803.
26. Gunn JS. 2008. The Salmonella PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol 16:284–290. https://doi.org/10.1016/j.tim.2008.03.007.
27. Bertani B, Ruiz N. 2018. Function and biogenesis of lipopolysaccharides. EcoSal Plus 8:ESP-0001-2018. https://doi.org/10.1128/ecosalplus.ESP-0001-2018.
28. Lin QY, Tsai YL, Liu MC, Lin WC, Hsueh PR, Liaw SJ. 2014. Serratia marcescens arn, a PhoP-regulated locus necessary for polymyxin B resistance. Antimicrob Agents Chemother 58:5181–5190. https://doi.org/10.1128/AAC.00013-14.
29. De Majumdar S, Yu J, Fookes M, McAteer SP, Llobet E, Finn S, Spence S, Monaghan A, Kissenpfennig A, Ingram RJ, Bengoechea J, Gally DL, Fanning S, Elborn JS, Schneiders T. 2015. Elucidation of the RamA regulon in Klebsiella pneumoniae reveals a role in LPS regulation. PLoS Pathog 11:e1004627. https://doi.org/10.1371/journal.ppat.1004627.
30. Llobet E, Martínez-Moliner V, Moranta D, Dahlström KM, Regueiro V, Tomás A, Cano V, Pérez-Gutiérrez C, Frank CG, Fernández-Carrasco H, Insua JL, Salminen TA, Garmendia J, Bengoechea JA. 2015. Deciphering tissue-induced Klebsiella pneumoniae lipid A structure. Proc Natl Acad Sci U S A 112:E6369–E6378. https://doi.org/10.1073/pnas.1508820112.
31. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:W202–W208. https://doi.org/10.1093/nar/gkp335.
32. Bailey TL, Gribskov M. 1998. Combining evidence using p-values: application to sequence homology searches. Bioinformatics 14:48–54. https://doi.org/10.1093/bioinformatics/14.1.48.
33. Gibbons HS, Reynolds CM, Guan Z, Raetz CRH. 2008. An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of Salmonella lipid A. Biochemistry 47:2814–2825. https://doi.org/10.1021/bi702457c.
34. Zwir I, Shin D, Kato A, Nishino K, Latifi T, Solomon F, Hare JM, Huang H, Groisman EA. 2005. Dissecting the PhoP regulatory network of Escherichia coli and Salmonella enterica. Proc Natl Acad Sci U S A 102:2862–2867. https://doi.org/10.1073/pnas.0408238102.
35. Nikaido E, Yamaguchi A, Nishino K. 2008. AcrAB multidrug efflux pump regulation in Salmonella enterica serovar Typhimurium by RamA in response to environmental signals. J Biol Chem 283:24245–24253. https://doi.org/10.1074/jbc.M804544200.
36. Ricci V, Blair JMA, Piddock LJV. 2014. RamA, which controls expression of the MDR efflux pump AcrAB-Tolc, is regulated by the lon protease. J Antimicrob Chemother 69:643–650. https://doi.org/10.1093/jac/dkt432.

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.

37. Ruzin A, Visalli MA, Keeney D, Bradford PA. 2005. Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in Klebsiella pneumoniae. Antimicrob Agents Chemother 49:1017–1022. https://doi.org/10.1128/AAC.49.3.1017-1022.2005.
38. Chollet R, Chevalier J, Bollet C, Pages J-M, Davin-Regli A. 2004. RamA is an alternate activator of the multidrug resistance cascade in Enterobacter aerogenes. Antimicrob Agents Chemother 48:2518–2523. https://doi.org/10.1128/AAC.48.7.2518-2523.2004.
39. Blanco P, Hernando-Amado S, Reales-Calderon J, Corona F, Lira F, Alcalde-Rico M, Bernardini A, Sanchez M, Martinez J. 2016. Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms 4:14. https://doi.org/10.3390/microorganisms4010014.
40. Jiménez-Castellanos J-C, Wan Ahmad Kamil WNI, Cheung CHP, Tobin MS, Brown J, Isaac SG, Heesom KJ, Schneiders T, Avison MB. 2016. Comparative effects of overproducing the AraC-type transcriptional regulators MarA, SoxS, RarA and RamA on antimicrobial drug susceptibility in Klebsiella pneumoniae. J Antimicrob Chemother 71:1820–1825. https://doi.org/10.1093/jac/dkw088.
41. Grimsey EM, Weston N, Ricci V, Stone JW, Piddock LJV. 2020. Overexpression of RamA, which regulates production of the multidrug resistance efflux pump AcrAB-TolC, increases mutation rate and influences drug resistance phenotype. Antimicrob Agents Chemother 64:e02460-19. https://doi.org/10.1128/AAC.02460-19.
42. Fedrigo GV, Campoy EM, Di Venanzio G, Colombo MI, García Véscovi E. 2011. Serratia marcescens is able to survive and proliferate in autophagic-like vacuoles inside non-phagocytic cells. PLoS One 6:e24054. https://doi.org/10.1371/journal.pone.0024054.
43. Bailey AM, Ivens A, Kingsley R, Cottell JL, Wain J, Piddock LV. 2010. RamA, a member of the AraC/XylS family, influences both virulence and efflux in Salmonella enterica serovar Typhimurium. J Bacteriol 192:1607–1616. https://doi.org/10.1128/JB.01517-09.
44. Naha S, Sands K, Mukherjee S, Roy C, Rameez MJ, Saha B, Dutta S, Walsh TR, Basu S. 2020. KPC-2-producing Klebsiella pneumoniae ST147 in a neonatal unit: clonal isolates with differences in colistin susceptibility attributed to AcrAB-TolC pump. Int J Antimicrob Agents 55:105903. https://doi.org/10.1016/j.ijantimicag.2020.105903.
45. Majewski P, Gutowska A, Sacha P, Schneiders T, Talalaj M, Majewska P, Zebrowska A, Ojdana D, Wieczorek P, Hauschild T, Kowalczuk O, Niklinski J, Radziwon P, Tryniszewska E. 2020. Expression of AraC/XylS stress response regulators in two distinct carbapenem-resistant Enterobacter cloacae ST89 biotypes. J Antimicrob Chemother 75:1146–1150. https://doi.org/10.1093/jac/dkz569.
46. Molitor A, James CE, Fanning S, Pagès J-M, Davin-Regli A. 2018. Ram locus is a key regulator to trigger multidrug resistance in Enterobacter aerogenes. J Med Microbiol 67:148–159. https://doi.org/10.1099/jmm.0.000667.
47. Yuhan Y, Ziyun Y, Yongbo Z, Fuqiang L, Qinghua Z. 2016. Over expression of AdeABC and AcrAB-TolC efflux systems confers tigecycline resistance in clinical isolates of Acinetobacter baumannii and Klebsiella pneumoniae. Rev Soc Bras Med Trop 49:165–171. https://doi.org/10.1590/0037-8682-0411-2015.
48. Wang X, Chen H, Zhang Y, Wang Q, Zhao C, Li H, He W, Zhang F, Wang Z, Li S, Wang H. 2015. Genetic characterisation of clinical Klebsiella pneumoniae isolates with reduced susceptibility to tigecycline: role of the global regulator RamA and its local repressor RamR. Int J Antimicrob Agents 45:635–640. https://doi.org/10.1016/j.ijantimicag.2014.12.022.
49. Shirshikova TV, Sierra-Bakhshi CG, Kamaletdinova LK, Matrosova LE, Khabipova NN, Evtugyn VG, Khilyas IV, Danilova IV, Mardanova AM, Sharipova MR, Bogomolnaya LM. 2021. The ABC-type efflux pump MacAB is involved in protection of Serratia marcescens against aminoglycoside antibiotics, polymyxins, and oxidative stress. mSphere 6:e00033-21. https://doi.org/10.1128/mSphere.00033-21.
50. Nelson DL, Kennedy EP. 1971. Magnesium transport in Escherichia coli. Inhibition by cobaltous ion. J Biol Chem 246:3042–3049. https://doi.org/10.1016/S0021-9258(18)62288-4.
51. Alexeyev MF. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. Biotechniques 26:824–828. https://doi.org/10.2144/99265bm05.
52. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology 1:784–791. https://doi.org/10.1038/nbt1183-784.
53. Kaniga K, Delor I, Cornelis GR. 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the blaA gene of Yersinia enterocolitica. Gene 109:137–141. https://doi.org/10.1016/0378-1119(91)90599-7.
54. Miller WG, Leveau JH, Lindow SE. 2000. Improved gfp and inaZ broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13:1243–1250. https://doi.org/10.1094/MPMI.2000.13.11.1243.
55. Bartolomé B, Jubete Y, Martínez E, de la Cruz F. 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene 102:75–78. https://doi.org/10.1016/0378-1119(91)90541-I.
56. Di Venanzio G, Stepanenko TM, García Véscovi E. 2014. Serratia marcescens ShlA pore-forming toxin is responsible for early induction of autophagy in host cells and is transcriptionally regulated by RcsB. Infect Immun 82:3542–3554. https://doi.org/10.1128/IAI.01682-14.

Downloaded from https://journals.asm.org/journal/jb on 08 June 2021 by 181.199.144.13.