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Serratia marcescens arn, a PhoP-Regulated Locus Necessary for Polymyxin B Resistance

Quei Yen Lin,[a] Yi-Lin Tsai,[a] Ming-Che Liu,[a] Wei-Cheng Lin,[a] Po-Ren Hsueh,[b] Shwu-Jen Liaw[a,b]

Department and Graduate Institute of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China[a]; Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan, Republic of China[b]

Polymyxins, which are increasingly being used to treat infections caused by multidrug-resistant bacteria, perform poorly against *Serratia marcescens*. To investigate the underlying mechanisms, Tn*5* mutagenesis was performed and two mutants exhibiting increased polymyxin B (PB) susceptibility were isolated. The mutants were found to have Tn*5* inserted into the *arnB* and *arnC* genes. In other bacteria, *arnB* and *arnC* belong to the seven-gene *arn* operon, which is involved in lipopolysaccharide (LPS) modification. LPSs of *arn* mutants had greater PB-binding abilities than that of wild-type LPS. Further, we identified PhoP, a bacterial two-component response regulator, as a regulator of PB susceptibility in *S. marcescens*. By the reporter assay, we found PB- and low-Mg^{2+}-induced expression of *phoP* and *arn* in the wild-type strain but not in the *phoP* mutant. Complementation of the *phoP* mutant with the full-length *phoP* gene restored the PB MIC and induction by PB and low Mg^{2+} levels, as in the wild type. An electrophoretic mobility shift assay (EMSA) further demonstrated that PhoP bound directly to the *arn* promoter. The PB challenge test confirmed that pretreatment with PB and low Mg^{2+} levels protected *S. marcescens* from a PB challenge in the wild-type strain but not in the *phoP* mutant. Real-time reverse transcriptase-PCR also indicated that PB serves as a signal to regulate expression of *ugd*, a gene required for LPS modification, in *S. marcescens* through a PhoP-dependent pathway. Finally, we found that PB-resistant clinical isolates displayed greater expression of *arnA* upon exposure to PB than did susceptible isolates. This is the first report to describe the role of *S. marcescens arn* in PB resistance and its modulation by PB and Mg^{2+} through the PhoP protein.

Cationic antimicrobial polypeptides (CAPs) play an important role in host defenses against microbial infections by affecting membrane integrity and are key effectors of the host innate immune response (1). CAPs, having antimicrobial and immunomodulatory activities, are being developed as a promising new class of therapeutic drugs (2). Microbial pathogens have evolved distinct mechanisms to resist killing by CAPs (3, 4), including expelling CAPs through pumps and cleaving CAPs with proteases. One of the important mechanisms of resistance to CAPs in Gram-negative bacteria involves remodeling of the composition of the outer membrane through modification of lipopolysaccharide (LPS) with positively charged substituents, which leads to the repulsion of CAPs (4). In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, a seven-gene polycistronic unit (*arnBCADTEF* or *pmrHFIJKLM*, referred to as the *arn* or *pmr* operon, respectively) is involved in LPS modification; all genes of the *arn* operon except *arnF* are necessary for the biosynthesis and addition of 4-amino-arabinose (Ara4N) to the 4′ phosphate of lipid A, a modification that contributes to reduction of the net negative charge of LPS and consequently decreases the attraction and binding of CAPs to the outer membrane (5, 6).

In a large number of bacterial species, the genes conferring resistance to CAPs are regulated by bacterial two-component systems (7–12). In *Salmonella*, transcriptional activation of the *pmr* operon requires the PmrA-PmrB two-component regulatory system, where PmrB is the sensor kinase and PmrA is the cognate response regulator that controls *pmr* operon expression directly (7). Decreases in Mg^{2+} concentrations promote PmrA-dependent upregulation of the *pmr* operon. This process also requires the PhoP (regulator)-PhoQ (sensor kinase) two-component system, a master regulator of *S. enterica* virulence functions (8, 13). PhoP positively controls the *pmr* operon at the transcriptional level by increasing production of PmrD (8), which then activates the PmrA protein posttranslationally, resulting in modification of LPS. More recently, studies have shown that the transcription of PhoP-activated genes is upregulated by sublethal concentrations of CAPs (14) and that CAPs can bind to and activate the PhoQ sensor directly (15).

Serratia marcescens, which was first described in 1819, was thought for years to be a nonpathogen; *S. marcescens* is now an accepted clinical pathogen, causing nosocomial infections and a full spectrum of clinical diseases, including urinary tract infections and pneumonia (16). The significance of *S. marcescens* was further highlighted by the emergence of multidrug-resistant (MDR) isolates. Recently, polymyxins, a type of CAPs (17, 18), were being increasingly used for the treatment of infections caused by MDR bacteria (18, 19). Although polymyxin B (PB) demonstrated good activity against most *Enterobacteriaceae* species, the drug showed poor activity against *Proteus* and *Serratia* spp. (19, 20). Previously, we reported that a *Proteus mirabilis* two-component response regulator, RppA, is involved in PB resistance through regulation of the expression of its downstream genes, *ugd*

Received 4 January 2014 Returned for modification 16 February 2014
Accepted 14 June 2014
Published ahead of print 23 June 2014
Address correspondence to Shwu-Jen Liaw, sjliaw@ntu.edu.tw.
Q.Y.L. and Y.-L.T. contributed equally to this article.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00013-14.

doi:10.1128/AAC.00013-14

TABLE 1 Bacterial strains and plasmids used in this study

Strain, mutation, or plasmid	Genotype or relevant phenotype[a]	Reference or source
Serratia marcescens strains		
3927	Wild-type; Tet^r Cm^r Amp^r Sm^s Km^s	Clinical isolate
arnB(t)	3927 derivative; *arnB* Tn*5*-mutagenized mutant; PB^s Km^r	This study
arnC(t)	3927 derivative; *arnC* Tn*5*-mutagenized mutant; PB^s Km^r	This study
phoP	3927 derivative; *phoP*-knockout mutant; Km^r	This study
phoP(c)	*phoP* mutant containing pBAD33 (Sm^r)-*phoP*; *phoP*-complemented strain; Sm^r	This study
arnB	3927 derivative; *arnB*-knockout mutant; Km^r	This study
arnB(c)	*arnB* mutant containing pBAD33 (Sm^r)-*arnB*; *arnB*-complemented strain; Sm^r	This study
arnC(tc)	*arnC*(t) mutant containing pBAD33 (Sm^r)-*arnC*; *arnC*-complemented strain; Sm^r	This study
R1–R6	PB^r	Clinical isolate
S1–S6	PB^s	Clinical isolate
E. coli		
DH5α	*fhuA2 lac(del)U169 phoA glnV44* φ80′ *lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
S17-1 λ *pir*	λ *pir* lysogen of S17-1 (*thi pro* $hsdR^-$ $hsdM^+$ *recA* RP4 2-Tc::Mu-Km::Tn*7* [Tp^r Sm^r]); permissive host able to transfer suicide plasmids requiring Pir protein by conjugation to recipient cells	21
BL21(DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
Plasmids		
pGEM-T Easy	High-copy-number TA cloning vector; Amp^r	Promega
pGEM-4Z	High-copy-number cloning vector; Amp^r	Promega
yT&A::*xylE*	High-copy-number TA cloning vector containing *xylE* coding region; Amp^r	Provided by Yang
pET32a(+)	Expression vector containing T7 promoter; Amp^r	Novagen
pET32a(+)-*phoP*	pET32a(+) containing intact *phoP* sequence; Amp^r	This study
pUT/mini-Tn*5*-Km	Suicide plasmid requiring Pir protein for replication and containing mini-Tn*5* cassette containing Km^r gene	22
pBAD33	Low-copy-number cloning vector, P15A replicon; Cm^r	Addgene
pACYC184-Sm	Low-copy-number cloning vector, P15A replicon; Sm^r Tet^r	This study
pACYC184-Sm-*phoP-xylE*	pACYC184-Sm containing intact *phoP* promoter sequence and *xylE* ORF; Sm^r Tet^r	This study
pACYC184-Sm-*arn-xylE*	pACYC184-Sm containing intact *arn* promoter sequence and *xylE* ORF; Sm^r Tet^r	This study

[a] Tet, tetracycline; Amp, ampicillin; Km, kanamycin; Cm, chloramphenicol; Sm, streptomycin; Tp, trimethoprim.

and *pmrI*, which are responsible for the synthesis and/or modification of LPS (17, 21, 22). UDP-glucose dehydrogenase (Ugd) is an enzyme that converts UDP-glucose into UDP-glucuronic acid. Both UDP-glucose and UDP-glucuronic acid are precursors for the synthesis of extracellular polysaccharides and LPS in many pathogenic bacteria. *pmrI*, one gene of the *pmrHFIJKLM* operon that encodes UDP-glucuronic acid decarboxylase, is involved in LPS modification. Mutation of *rppA*, *ugd*, or *pmrI* in *P. mirabilis* leads to increased PB sensitivity (17, 21, 22).

In this study, by using a Tn*5* transposon mutagenesis approach, we identified two genes, *arnB* and *arnC*, that are involved in PB resistance in *S. marcescens*. We also found that expression of the *S. marcescens arn* operon is under the control of the two-component regulator PhoP. To our knowledge, this is the first report describing the role of the *arn* operon and its regulation by the PhoP response regulator in *S. marcescens*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium (17). Clinical isolates of *S. marcescens* were collected at the National Taiwan University Hospital. These isolates were obtained from sputum (R1, R2, S1, and S2), wounds (R3, S3, and S4), and urine (R4, R5, R6, S5, and S6). All of the isolates were identified by conventional biochemical identification methods, and results were confirmed by the Vitek automated system (bioMérieux). Random amplified polymorphic DNA (RAPD) analysis (Operon Technologies) was used to exclude repeat isolates.

Transposon mutagenesis and identification of the mutated gene. *S. marcescens* mini-Tn*5* kanamycin (Km) mutagenized mutants were isolated as described previously (17). PB-susceptible clones were identified by replica plating on LB plates containing 400 μg/ml PB. Chromosomal DNA was extracted from the mutants and partially digested with EcoRV, and fragments over 4 kb were cloned into SmaI-digested pGEM-4Z (Promega). Following transformation of *Escherichia coli* TOP10 cells, Km-resistant Tn*5* Km-containing clones were selected. Using I-out and O-out primers, the nucleotide sequences flanking the Tn*5* cassette were obtained and subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). We then searched the sequence in the released genome sequence of *S. marcescens* (https://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_marcescens) and cloned the full gene by PCR TA cloning using primers from highly conserved sequences around the Tn*5*-interrupted genes (*arnB* and *arnC*) in the released genome sequences of *S. marcescens*. The remaining genes of the *arn* operon were also cloned by PCR TA cloning, using primers from highly conserved sequences. The nucleotide sequence was determined using a 373A DNA sequencer (Applied Biosystems). Alignment of protein amino acids with counterpart proteins was performed using DNAman software (version 4.15).

Gene knockout by homologous recombination. For construction of the *arnB* and *phoP* mutants, the primer pairs *arnB*ko-F/*arnB*ko-R-XbaI and *phoP*ko-F/*phoP*ko-R-XbaI (see Table S1 in the supplemental material) were used to amplify the central regions of *arnB* and *phoP*, respec-

tively. PCR products were cloned into pGEM-T Easy (Promega) and ligated with a Km cassette by XbaI digestion. The *phoP*- and *arnB*-containing plasmids were excised as SalI/SphI and NotI fragments, respectively, and ligated into pUT vectors digested with SalI/SphI and NotI, respectively, to form pUT-*phoP* and pUT-*arnB*. For gene inactivation by homologous recombination, pUT-*phoP* or pUT-*arnB* was transferred to *S. marcescens* 3927 by conjugation. Transconjugants were spread on LB plates containing kanamycin (100 μg/ml). Mutant candidates were screened by colony PCR, and Southern blot hybridization was performed to confirm the mutant genotypes (data not shown). Results confirmed that a single crossover event had occurred (23) (data not shown).

Construction of *phoP*-, *arnB*-, and *arnC*-complemented strains. Full-length *phoP*, *arnB*, and *arnC* were amplified by PCR (primers are listed in Table S1 in the supplemental material) and cloned into pGEM-T Easy (Promega). The DNA fragments containing full-length *phoP*, *arnB*, and *arnC* were excised with XhoI/SacI and ligated into SalI/SacI-digested low-copy-number plasmid pBAD33 (also carrying a streptomycin resistance cassette for selection, since *S. marcescens* 3927 is resistant to chloramphenicol) to generate the *phoP*, *arnB*, and *arnC* complementation plasmids, respectively. Arabinose (0.02%)-induced expression of these genes was driven by the P_{BAD} promoter in the pBAD33 plasmid. The *phoP*, *arnB*, and *arnC* complementation plasmids were then transformed into the mutants to generate the respective complemented strains.

MIC assay. *In vitro* determination of MICs for PB was performed by the broth microdilution method, according to the guidelines proposed by the Clinical and Laboratory Standards Institute (24).

Preparation and analysis of LPS. The extraction and analysis of LPS were performed as described previously (17).

PB binding of LPS. The experiments to determine binding of PB by LPS were performed as described previously (17).

Binding of wild-type *S. marcescens* and *arnB* mutant with fluorescent polymyxin B. Overnight cultures of bacterial strains were washed with phosphate-buffered saline (PBS), followed by dilution in PBS to an optical density at 600 nm (OD_{600}) of 0.05. Oregon Green 514-conjugated polymyxin B (P13236; Invitrogen) was added to 50 μl of diluted cells at select concentrations, and the cells were incubated at 37°C for 10 min. Cells were then washed with PBS, resuspended in 50 μl of PBS, and placed in 96-well plates for analysis. The fluorescence (excitation, 480 nm; emission, 535 nm) and OD_{600} values for each well were determined using a microplate reader (SpectraMax M5; Molecular Devices) (25).

Reporter assay. The promoter region of the putative *phoP* or *arn* operon was amplified by SphI- and PstI-included primers (see Table S1 in the supplemental material) and cloned into pGEM-T Easy to create pGEMphoPp or pGEMarnp, respectively. pGEMphoPp and pGEMarnp were digested by SphI/PstI, and the promoter-containing fragments were ligated with *xylE*-containing pACYC184 digested by SphI/PstI to construct the *phoP*- and *arn-xylE* reporter plasmids. The reporter plasmids containing the wild-type, *phoP* mutant, and *phoP*-complemented strains were grown overnight in N minimal medium (NMM) [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% Casamino Acids, 0.1 M Tris-HCl, pH 7.4] with 2 mM $MgCl_2$ or LB broth with 100 μg/ml streptomycin, diluted 100-fold in the same medium, and grown to an OD_{600} value of 0.3. Then cells were incubated under conditions with or without 1 μg/ml PB (in LB broth) and with low or high Mg^{2+} levels (in NMM). The *xylE* activity was measured 1, 2, and 3 h after incubation (22).

Purification of His-tagged recombinant protein and electrophoretic mobility shift assay. The full-length *phoP* gene was cloned into the plasmid pET32a(+) with BamHI and XhoI to generate plasmid pET32a(+)-PhoP. Overexpression and purification of His-tagged PhoP were performed as described previously (22). The purity of the PhoP preparation was confirmed by SDS-PAGE. The amplified promoter DNA (0.1 μg each) was incubated with 0, 0.5, 1, or 2 μM His-tagged PhoP protein in 10 μl of binding buffer (22). After incubation for 30 min at room temperature, the reaction mixtures were loaded onto 5% nondenaturing polyacrylamide gels, and the gels were subjected to electrophoresis at 100 V for 1.5 to 2 h before being stained with ethidium bromide (22).

PB challenge test for *S. marcescens* after pretreatment with Mg^{2+} or PB. For Mg^{2+} pretreatment, cells were harvested from an overnight culture grown in NMM with 2 mM $MgCl_2$, washed with NMM without $MgCl_2$, and diluted 1:50 in NMM with 10 μM $MgCl_2$ (low Mg). Bacteria were grown at 37°C, with aeration, to an optical density at 600 nm (OD_{600}) of about 0.6 and were challenged with PB (1,000 μg/ml). Bacterial growth was monitored at 1-h intervals after incubation at 37°C with aeration. In addition, PB (1 μg/ml) was added to cultures at OD_{600} of 0.6 in NMM with 2 mM $MgCl_2$, cells were then induced for 1 h, and the PB challenge test was performed in the same way as described above. For PB pretreatment, overnight bacterial cultures were diluted, grown to an OD_{600} of 0.6 at 37°C in LB broth (containing about 0.1 mM Mg^{2+}) with or without $MgCl_2$ (20 mM), and challenged with the MIC dose of PB for the wild-type, *phoP* mutant, and *phoP*-complemented strains (2,048, 2, and 2,048 μg/ml, respectively). After being challenged with the respective MIC dose of PB for 1 h, bacteria were diluted and plated on LB agar plates, and the numbers of CFU were determined after overnight incubation. For PB induction, 1 μg/ml PB was added to cultures with an OD_{600} of 0.3, cells were grown to an OD_{600} of 0.6, and then the PB challenge was conducted. The percent survival was calculated as (CFU of PB-challenged culture/CFU of no-challenge culture) × 100% (26).

Reverse transcription assays. Total RNA was extracted from overnight LB cultures. Reverse transcriptase (RT)-PCR analyses were carried out by reverse transcription of total RNA using random primers or gene-specific primers and avian myeloblastosis virus reverse transcriptase (Roche). The resulting cDNA was amplified using the primer pairs of *arnBC*rt-F/*arnBC*rt-R and *arnCA*rt-F/*arnCA*rt-R (see Table S1 in the supplemental material) for the *arnBC* and *arnCA* intergenic regions, respectively (Fig. 1). For control reactions, RNA without RT or chromosomal DNA was used as a template. To study the effects of *phoP* mutation on the expression of *ugd* mRNA, overnight LB cultures of the wild-type, *phoP* knockout, and *phoP*-complemented strains were diluted 100-fold with LB broth and grown to an OD_{600} of 0.3, and the cultures were further grown at 37°C to an OD_{600} of 0.6, without or with 1 μg/ml PB. Then, total RNA was extracted and real-time RT-PCR was performed as described previously (17), to monitor the expression of *ugd* mRNA. *arnA* expression in clinical *S. marcescens* isolates (Table 2) was measured in the same way. The mRNA levels were normalized against 16S rRNA levels.

Nucleotide sequence accession numbers. The nucleotide sequences of the *S. marcescens phoPQ* and *arnBCADTEF* genes have been deposited in GenBank under accession no. KJ534564 and KJ560442, respectively.

RESULTS

Isolation of PB-sensitive *S. marcescens* mutants. In order to investigate the underlying mechanisms of PB resistance in *S. marcescens*, we used a mini-Tn*5* transposon mutagenesis approach, as described previously (17), to isolate PB-sensitive mutants of *S. marcescens*. Through characterization of these mutants, we identified two mutants, the *arnB*(t) and *arnC*(t) strains (Tn*5*-mutagenized mutants), that were 1,024-fold more susceptible to PB than wild-type *S. marcescens* (MICs, 2 versus 2,048 μg/ml) (Table 2). The nucleotide sequence was obtained from the cloned DNA fragment containing the mini-Tn*5* in the mutants. By searching the released genome sequence of *S. marcescens* using the sequence we obtained, we found that Tn*5* was inserted into two genes, named *arnB* and *arnC*. The genes were cloned and sequenced. The nucleotide sequences of *arnB* and *arnC* were found to be 95% and 96% identical, respectively, to the corresponding sequence of the sequenced *S. marcescens* Db11.

***arnB* and *arnC* are within a putative seven-gene *arn* operon.** The sequences of *arnB* and *arnC* were similar to the first and second genes of the *arn* (or *pmr*) operon (*arnBCADTEF*) in *Yer-*

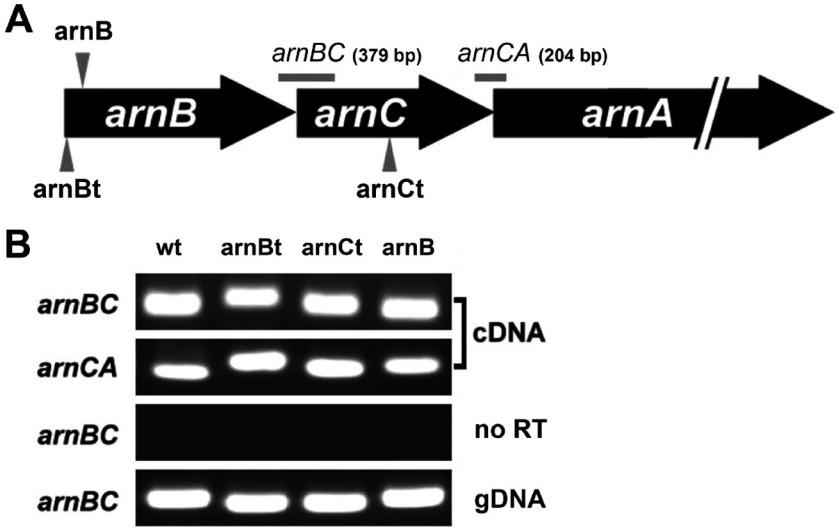

FIG 1 (A) Organization of *S. marcescens arnBCA*. Only *arnBCA* of the *arn* operon is shown. Arrow, knockout or Tn*5*-interrupted site for the *arnB*, *arnB*(t), or *arnC*(t) mutant; horizontal line, region amplified by PCR. The product sizes are indicated in parentheses. (B) RT-PCR analysis of *arn* transcription in wild-type *S. marcescens* and the *arnB*(t), *arnC*(t), and *arnB* mutants. RT-PCR using the primer pair annealing to *arnB/arnC* or *arnC/arnA* (the region shown in panel A) was performed with RNA isolated from overnight cultures of the wild-type and mutant strains. For control reactions, RNA without reverse transcriptase (no RT) or genomic DNA (gDNA) was used as a template. wt, wild-type strain; arnB, *arnB*-knockout mutant; arnBt, *arnB* Tn*5*-mutagenized mutant; arnCt, *arnC* Tn*5*-mutagenized mutant.

sinia pestis (http://www.ncbi.nlm.nih.gov/nuccore/NC_004088.1?report=genbank&from=2110265&to=2117676), an enterobacterium phylogenetically more closely related to *S. marcescens* (27), *E. coli*, and *Salmonella* (5, 28). Analysis of the amino acid sequences indicated that *arnB* and *arnC* may encode bacterial UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase and undecaprenyl phosphate-4-amino-4-deoxy-L-arabinose transferase, respectively. *S. marcescens* ArnB and ArnC proteins are homologous to those of *S. enterica* serovar Typhimurium (68 and 74% identity and 80 and 88% similarity, respectively), *E. coli* (70 and 76% identity and 81 and 87% similarity, respectively), and *Yersinia pestis* (76 and 86% identity and 84 and 92% similarity, respectively). Further analysis of the deduced amino acid sequences revealed that the Tn*5*-inserted *S. marcescens* locus may encode seven putative proteins, with 90%, 83%, 83%, 80%, and 82% similarities to *Y. pestis* ArnA, ArnD, ArnT, ArnE, and ArnF, respectively (GenBank protein accession numbers AAM85486 to AAM85490).

The *S. marcescens arnB*-knockout mutant exhibited increased susceptibility to PB. To demonstrate the role of the *arnB* gene in regulating PB susceptibility, we constructed the *arnB* mutant (see Materials and Methods). The MIC of PB for the *arnB* mutant was 2 μg/ml, compared with the wild-type value of 2,048 μg/ml (Table 2). The finding that complementation of the *arnB* and *arnC* genes in the *arnB* and *arnC*(t) mutants, respectively, restored the wild-type PB MIC (2,048 μg/ml) further confirms that *arnB* and *arnC* are determinants for PB susceptibility in *S. marcescens*. In addition, by RT-PCR using primer pairs annealing to the *arnB/arnC* and *arnC/arnA* genes, we detected amplicons of the expected sizes, i.e., approximately 0.4 kb (*arnBC*) and 0.2 kb (*arnCA*), from RNA samples from the wild-type strain and mutants, including the *arnB*(t), *arnC*(t), and *arnB* strains (Fig. 1). This implies that the transcript containing *arnB*, *arnC*, and *arnA* genes was produced in the *arnB*(t), *arnC*(t), and *arnB* mutants. The transcriptional readthrough may explain the restoration of wild-type PB susceptibility in the *arnB* and *arnC*(t) mutants complemented with *arnB* and *arnC* alone, respectively.

LPSs of *S. marcescens arnB* and *arnC* mutants had greater PB-binding abilities than that of wild type. LPS modifications play important roles in PB susceptibility in many Gram-negative

TABLE 2 PB MICs, *arnA* expression, and specimen types for wild-type *S. marcescens* and clinical isolates

Parameter	Wild-type	R1	R2	R3	R4	R5	R6	S1	S2	S3	S4	S5	S6
MIC (μg/ml)	2,048	4,096	>4,096	4,096	>4,096	4,096	>4,096	2	4	2	2	4	2
arnA expression[a]													
Nil	1	0.5 (0.2)	2.9 (1.2)	0.5 (0.2)	2.0 (0.2)	1.2 (0.4)	0.6 (0.2)	1.0 (0.4)	2.5 (0.9)	2.0 (1.0)	1.0 (0.4)	0.8 (0.2)	0.6 (0.1)
PB	4.1 (0.7)	3.7 (0.2)	8.8 (0.3)	2.0 (0.2)	10 (0.7)	5.0 (1.0)	3.0 (0.6)	0.9 (0.6)	2.8 (0.4)	0.8 (0.5)	1.2 (0.3)	0.9 (0.2)	0.6 (0.1)
Fold change[b]	4.1	7.4	3.0	4.0	5.0	4.2	5.0	0.9	1.1	0.4	1.2	1.1	1.0
Specimen type	Sputum	Sputum	Sputum	Wound	Urine	Urine	Urine	Sputum	Sputum	Wound	Wound	Urine	Urine

[a] The *arnA* mRNA level of *S. marcescens* in the presence of polymyxin B (PB) or not (nil) is shown. The value obtained for the wild-type strain in the absence of PB was set at 1, and other values (with standard deviations in parentheses) are relative to that value. R1 to R6, PB-resistant clinical isolates; S1 to S6, PB-susceptible clinical isolates.
[b] Fold change is *arnA* mRNA level in the presence of PB divided by that in the absence of PB.

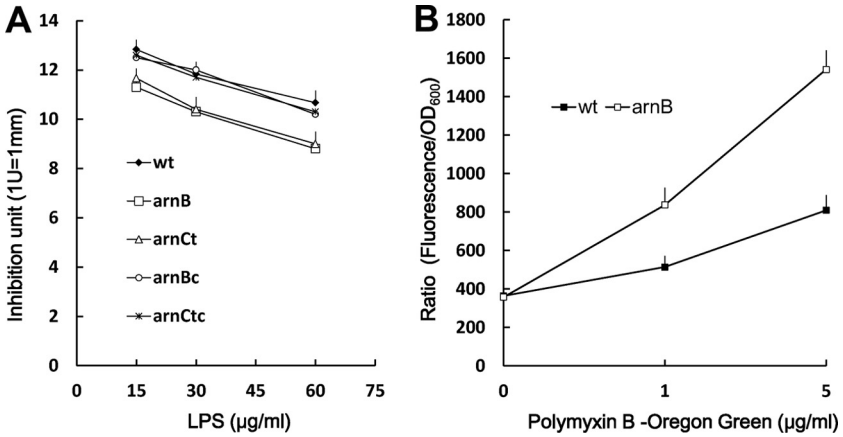

FIG 2 Polymyxin B-binding ability of wild-type *S. marcescens* and *arn* mutants. (A) PB-binding abilities of LPSs purified from the wild-type, *arnB*, *arnC*(t), *arnB*(c), and *arnC*(tc) strains. Various amounts of purified LPS were subjected to the PB-binding assay (see Materials and Methods). (B) Binding of wild-type and *arnB* mutant cells with fluorescent polymyxin B. The wild-type or *arnB* mutant cells were incubated for 10 min with Oregon Green 514-conjugated polymyxin B at the indicated concentrations and washed before being resuspended for analysis in 96-well plates. The fluorescence of each well was determined using a microplate reader. Data are reported as a ratio of fluorescence intensity to OD_{600}. Each experiment was repeated in triplicate, and the data are averages and standard deviations. wt, wild-type strain; arnB, *arnB*-knockout mutant; arnCt, *arnC* Tn*5*-mutagenized mutant; arnBc, *arnB*-complemented strain; arnCtc, *arnC*-complemented strain.

bacteria, including S*almonella*, *Yersinia*, *Pseudomonas*, *E. coli*, and *P. mirabilis* (9, 10, 22, 29). To investigate the underlying cause of PB sensitivity in the *arn* mutants, we compared the LPS profiles of the *arn* mutants with that of the wild-type strain. SDS-PAGE analysis of LPS extracted from equal amounts of wild-type or mutant cells revealed that the *arn* mutants had LPS profiles similar to that of the wild-type strain (data not shown). In addition, the *arnB* (*arnB*-knockout) and *arnC*(t) (Tn*5*-mutagenized) mutants synthesized similar amounts of LPS versus the wild-type strain (11.5 and 11.79 versus 12.95 mg/ml), using equal amounts of wild-type and mutant cells.

To explain why the *arn* mutants have increased PB susceptibility, we thus tested whether the LPS purified from the mutants and the wild-type strain had different binding activities with PB. Equal amounts of LPS from the mutants and the wild-type strain were incubated with PB, and the unbound fraction was subjected to the *E. coli* inhibition assay (17). As shown in Fig. 2A, LPS from the *arnB* or *arnC*(t) mutant bound larger amounts of PB than did LPS from the wild-type strain, and the *arnB*(c) and *arnC*(tc) strains displayed binding patterns similar to that of the wild-type strain. The whole-cell binding of the *arnB* mutant with fluorescent PB was also better than that of wild-type *S. marcescens* (Fig. 2B). Since identical LPS concentrations and cell densities were used in these binding assays, these data indicate that there was a qualitative change in the LPS of the *arn* mutants and that this change caused LPS from the *arn* mutants and *arnB* mutant cells to have higher binding activities with PB. The increased PB-binding activity of the *arn* mutants may have contributed to their sensitivity to PB.

Expression of the *S. marcescens arn* operon was induced by PB and low Mg^{2+} levels through the PhoP-dependent pathway. Knowing that PB can regulate the PB susceptibility of *P. mirabilis* in an Arn (Pmr)-dependent manner through the RppAB two-component system (21, 22) and that the *Salmonella* PhoPQ two-component signal transduction system can sense PB to control *pmr* gene expression (i.e., *arn* operon) (14, 30, 31), we searched for *phoPQ* homologues in *S. marcescens* Db11 and found the PhoP and PhoQ counterparts of *S. marcescens* Db11, with high levels of identity (90% for PhoP and 80% for PhoQ) and similarity (94%

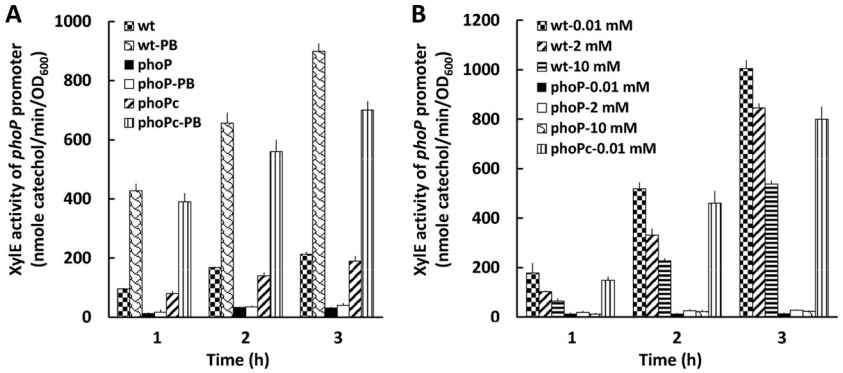

FIG 3 Activity of *xylE* in *phoP-xylE* reporter plasmid-transformed wild-type *S. marcescens*, *phoP* mutant, and *phoP*-complemented strains, in the presence or absence of 1 μg/ml polymyxin B (PB) (A) and with various concentrations of Mg^{2+} (0.01, 2, and 10 mM) (B), after induction for 1, 2, and 3 h. The data represent the averages of three independent experiments with standard deviations. wt, wild-type strain; phoP, *phoP* mutant; phoPc, *phoP*-complemented strain.

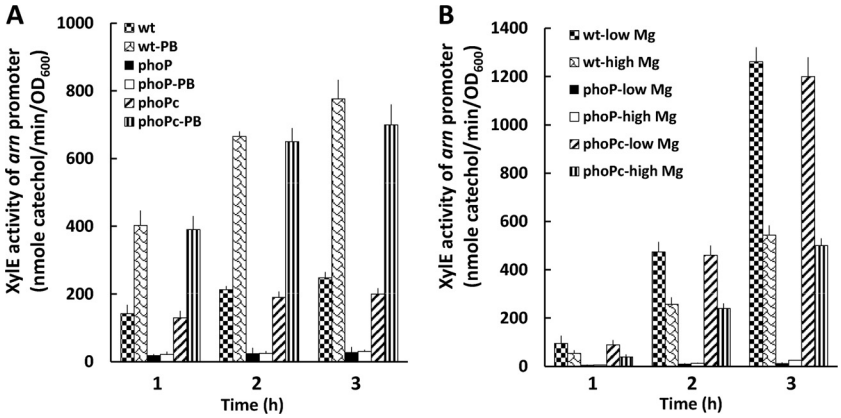

FIG 4 Activity of *xylE* in *arn-xylE* reporter plasmid-transformed wild-type *S. marcescens*, *phoP* mutant, and *phoP*-complemented strains, in the presence or absence of 1 μg/ml polymyxin B (PB) (A) and with low (0.01 mM) or high (10 mM) Mg^{2+} levels (B), after induction for 1, 2, and 3 h. The data represent the averages of three independent experiments with standard deviations. wt, wild-type strain; phoP, *phoP* mutant; phoPc, *phoP*-complemented strain.

for PhoP and 89% for PhoQ) to those of *Y. pestis*. In addition, expression of PhoPQ and RppAB, which are both involved in PB susceptibility, has been shown to be autoregulated and induced by PB and low Mg^{2+} levels in *Salmonella* (13, 32) and *Proteus* (17). Therefore, we constructed a *Serratia phoP* mutant and found its PB MIC to be 2 μg/ml. Then we investigated the expression of *arnB* and *phoP* in the wild-type strain and the *phoP*-knockout mutant in the presence of 1 μg/ml PB (in LB broth) or different concentrations of Mg^{2+} (in NMM medium) with the *xylE* reporter assay. RT-PCR using primer pairs annealing to the putative *arnB* and *arnC* genes and *arnC* and *arnA* genes, respectively, in wild-type *S. marcescens* demonstrated that the *arnB*, *arnC*, and *arnA* genes are cotranscribed (Fig. 1), indicating that they share the promoter upstream of the putative *arnB* gene. Therefore, the promoter region upstream of the putative *arnB* was used to construct the *xylE* transcriptional fusion (*arnB* promoter sequence fused with *xylE* gene with *xylE* ribosome binding site). We found that *phoP* and *arnB* promoter activities of the *phoP*-knockout mutant were low in comparison with the wild-type strain (Fig. 3A and 4A) during incubation in the absence of PB and that PB could induce the promoter activities of *phoP* and *arnB* in the wild-type strain but not in the *phoP*-knockout mutant after induction for 1, 2, and 3 h (Fig. 3A and 4A). Low Mg^{2+} levels induced expression of *phoP* and *arnB* in the wild-type strain but not in the *phoP*-knockout mutant (Fig. 3B and 4B). The results indicated that expression of the *S. marcescens arn* operon requires the *phoP* gene, which is positively autoregulated. The PB- or low-Mg^{2+}-induced promoter activities of *phoP* and *arnB* were restored to about the wild-type level in the *phoP*-complemented strain (Fig. 3 and 4). In order to determine whether PhoP could regulate the expression of *phoP* and *arnB* directly by binding the putative *phoP* and *arnB* promoters in *S. marcescens*, an electrophoretic mobility shift assay (EMSA) was conducted. Figure 5 shows that PhoP proteins can specifically bind the putative *phoP* and *arnB* promoter fragments but not the irrelevant 16S ribosomal DNA fragments (compare Fig. 5, lanes 1 to 4 and 8 to 11 with lanes 5 to 7). Taken together, these findings suggest that PB and low Mg^{2+} levels can modulate

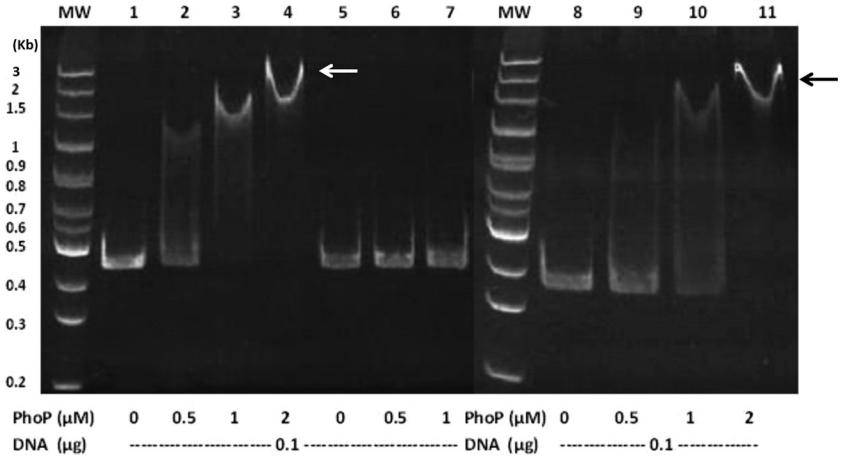

FIG 5 Electrophoretic mobility shift assay of purified *S. marcescens* PhoP with the *phoP* or *arn* promoter fragment. DNA fragments (0.1 μg) of the *phoP* promoter (431 bp), *arn* promoter (342 bp), or negative-control DNA (424 bp) obtained by PCR were incubated with the indicated concentrations (0, 0.5, 1, and 2 μM) of the PhoP protein. After protein-DNA complex formation, the fragments were resolved on a 5% nondenaturing polyacrylamide gel. Arrows, protein-DNA complex. Lane MW, size markers; lanes 1 to 4, *phoP* promoter with PhoP; lanes 8 to 11, *arn* promoter with PhoP; lanes 5 to 7, negative-control DNA with PhoP.

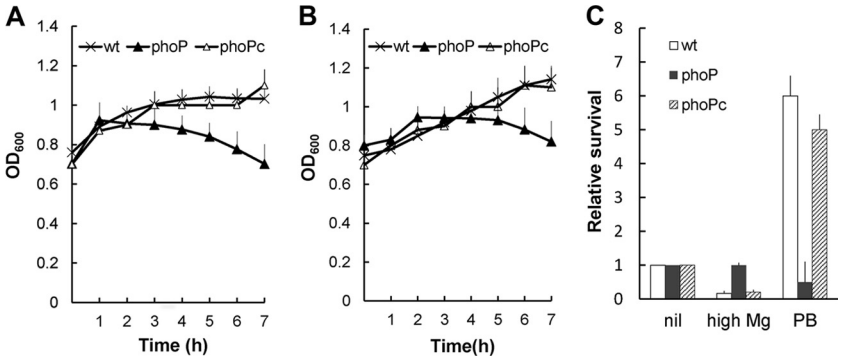

FIG 6 PB challenge test for *S. marcescens* after pretreatment with PB or Mg^{2+}. (A) Cells of wild-type, *phoP* mutant, and *phoP*-complemented strains were grown in N minimal medium with 2 mM $MgCl_2$ to an OD_{600} of 0.6, induced with 1 μg/ml PB for 1 h, and then challenged with PB (1,000 μg/ml). Bacterial growth (OD_{600}) was monitored at 1-h intervals. (B) Cells of wild-type, *phoP* mutant, and *phoP*-complemented strains were grown in N minimal medium with 10 μM $MgCl_2$ to an OD_{600} of 0.6 and then challenged with PB (1,000 μg/ml). Bacterial growth (OD_{600}) was monitored at 1-h intervals. (C) Cells of wild-type, *phoP* mutant, and *phoP*-complemented strains were grown in LB broth, with or without the addition of 20 mM $MgCl_2$ (high Mg^{2+}), to OD_{600} of 0.6 and then challenged for 1 h with the respective MIC dose of PB for wild-type, *phoP* mutant, and *phoP*-complemented strains. For PB pretreatment, 1 μg/ml PB was added to the cultures with OD_{600} of 0.3, cells were grown to OD_{600} of 0.6, and then the PB challenge was conducted as described above. After the challenge with PB, CFU were determined and the percent survival was calculated. The relative survival values were obtained, with the values for wild-type, *phoP* mutant, and *phoP*-complemented strains in the absence of PB set at 1. All data represent the averages of three independent experiments with standard deviations. wt, wild-type strain; phoP, *phoP* mutant; phoPc, *phoP*-complemented strain.

the expression of *phoP* and *arnB* through the PhoP-dependent pathway.

PB or Mg^{2+} pretreatment protected wild-type *S. marcescens* but not the *phoP*-knockout mutant from PB challenge. Knowing that *Serratia* PhoP regulated the expression of the *arn* operon in response to PB and Mg^{2+} levels, we tested the effects of pretreatment with PB or Mg^{2+} on the growth and survival of wild-type *S. marcescens*, *phoP* mutant, and *phoP*-complemented [*phoP*(c)] strains after challenge with PB. The *phoP* mutant pretreated with low-dose PB (1 μg/ml) or low Mg^{2+} levels (0.1 mM) in NMM (see Materials and Methods) displayed decreased OD_{600} values during the 7-h period after being challenged with 1,000 μg/ml PB, but the wild-type and *phoP*(c) strains exhibited increased growth (Fig. 6A and B). In addition, we performed the PB survival assay by treating bacterial strains with either 1 μg/ml PB or high Mg^{2+} levels (20 mM) in LB broth and then challenging bacterial cells with the respective MIC dose of PB for the wild-type, *phoP* mutant, and *phoP*(c) strains. The survival rates of the wild-type and *phoP*(c) strains were decreased to around 20% of the no-challenge control by pretreatment with 20 mM Mg^{2+} and were increased 6-fold, relative to the no-challenge control, by PB pretreatment (Fig. 6C). In contrast, treatment with 1 μg/ml PB or high Mg^{2+} levels had almost no effect on the survival of the *phoP* mutant (Fig. 6C). Together, these data indicate that 1 μg/ml PB or Mg^{2+} may serve as the signal to protect *S. marcescens* from the PB challenge through a PhoP-dependent pathway, which activates expression of the LPS-modifying *arn* operon.

Expression of *S. marcescens ugd* is regulated by PB through the PhoP-dependent pathway. The enzymes encoded by the *arn* operon are responsible for LPS modification with Ara4N, and the *ugd* gene is required for biosynthesis of Ara4N (5). The Ugd protein of *Salmonella*, *Yersinia*, and *Proteus* is under the control of the two-component pathway PhoPQ or RppAB in response to PB and/or Mg^{2+} (21, 31, 33). In this regard, *S. marcescens* Ugd has 79% identity and 86% similarity to that of *Y. pestis* KM10+. Knowing that *Serratia* PhoP regulated the expression of the *arn* operon in response to PB, we examined the effects of PB on the expression of *ugd* in the wild-type *S. marcescens*, *phoP* mutant, and *phoP*(c) strains by real-time RT-PCR. The results indicated that *ugd* mRNA expression was induced by PB in the wild-type and complemented strains but not in the *phoP* mutant (Fig. 7). Our data indicated that both *arn* and *ugd* genes may respond to the PB signal through the PhoP pathway to modify the LPS and give rise to PB resistance in *S. marcescens*.

Expression of the *arnA* gene was induced by PB in PB-resistant clinical isolates of *S. marcescens*. To further confirm the significance of the PhoP-dependent *arn* expression in PB resistance, we analyzed the expression of the *arnA* gene by real-time RT-PCR in clinical isolates of *S. marcescens*. We found that expres-

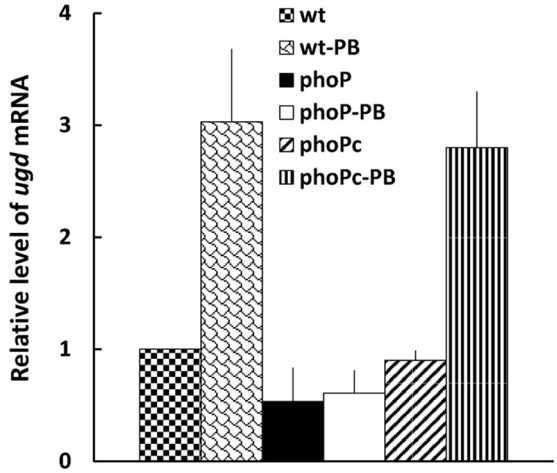

FIG 7 Effects of *S. marcescens phoP* mutation on the expression of *ugd* mRNA in the presence or absence of 1 μg/ml PB (see Materials and Methods). The *ugd* mRNA amounts in the wild-type, *phoP*-knockout mutant, and *phoP*-complemented strains were quantified by real-time RT-PCR. The value obtained for the wild-type cells in the absence of PB was set at 1. The data represent the averages of three independent experiments with standard deviations. wt, wild-type strain; phoP, *phoP* mutant; phoPc, *phoP*-complemented strain.

FIG 8 Conservation of the PhoP box in the *arn* promoter among different *Serratia* species. Underlining, −10 region; shading, PhoP binding sites, separated by 5 bp.

sion levels were comparable in the PB-resistant and -susceptible isolates in the absence of PB; however the expression of *arnA* was induced 3- to 7.4-fold in the presence of PB in the PB-resistant isolates but not in the susceptible ones (Table 2).

DISCUSSION

The ability of *S. marcescens* to survive the killing action of CAPs is clearly important in the pathogenesis of *S. marcescens* (27). Although some studies revealed that proteases and LPS modification appeared to be necessary for CAP resistance in other bacteria (3, 4), the molecular mechanisms underlying the CAP resistance of *S. marcescens* remain totally unknown. In this study, we isolated several mutants that were more sensitive to PB than the wild type. Two mutants were found to have Tn*5* inserted in the *arnB* and *arnC* genes of what appears to be the counterpart of the *pmrF* operon in *S. enterica* and *E. coli* (5, 28). The *arn* operon in *S. marcescens* and *Y. pestis* is flanked by the *btuD* (encoding a transporter of vitamin B_{12}) and *nlpC* (encoding a lipoprotein hydrolase) genes.

LPS modifications of Gram-negative organisms are regulated by two-component systems in response to environmental conditions (13, 33–36). In *Salmonella*, the LPS-modifying *arn* operon is induced by Fe^{3+}, which is sensed by the PmrA cognate sensor PmrB, and by low Mg^{2+} levels, in a way that requires PmrAB, the Mg^{2+}-responding PhoPQ, and PmrD protein (8, 31). *E. coli* induces PmrA-dependent PB resistance in response to Fe^{3+} but is blind to the low Mg^{2+} signal due to a highly divergent PmrD protein (37, 38). In *P. mirabilis*, we identified a two-component response regulator (RppA) as a regulator of PB susceptibility by directly controlling the expression of *pmrI* (*arnA*) in response to PB (22). Here we report that PB and Mg^{2+} can promote PhoP-dependent *arn* expression and PB resistance in *S. marcescens* through direct binding of PhoP in the *arn* promoter despite lacking a PmrD protein. Similarly, in PmrD-lacking *Yersinia pestis*, PhoP has been shown to regulate expression of the *arn* operon by directly binding the promoter region of the *arn* operon (33). In summary, bacterial species may use disparate regulatory pathways to control genes encoding conserved proteins. Moreover, the PhoP proteins of *Salmonella* and *Yersinia* trigger *ugd* expression by direct binding of the *ugd* promoter (31, 33). Accordingly, we found that *Serratia ugd* is under the control of PhoP in this study (Fig. 7).

Several lines of evidence indicate that the *S. marcescens* PhoPQ system, by sensing PB and Mg^{2+}, can regulate the expression of the *arn* operon to modify LPS and lead to PB resistance. First, all of the *phoP*, *arnB*, and *arnC*(t) mutants displayed a PB-sensitive phenotype, and the *phoP*(c), *arnB*(c), and *arnC*(tc) strains exhibited the wild-type MIC. PB and low Mg^{2+} levels protected *S. marcescens* from the PB challenge in the wild-type strain and the *phoP*-complemented strain but not in the *phoP* mutant (Fig. 6). Second, the PB binding assay demonstrated that LPS of *arnB* and *arnC*(t) mutants had increased PB-binding ability, which implied that certain LPS changes exist in the mutants (Fig. 2). Third, *phoP* and *arn* operon promoter activities were increased by PB and low Mg^{2+} levels in the wild-type and PhoP-complemented strains but not in the *phoP* mutant (Fig. 3 and 4). Fourth, EMSA indicated that PhoP can bind the *phoP* and *arn* promoter directly (Fig. 5). Fifth, expression of the *ugd* gene, encoding the Ara4N precursor for LPS modification, was also increased in response to PB in the wild-type and *phoP*(c) strains but not in the *phoP* mutant (Fig. 7). The significance of *arn*-mediated PB resistance was also demonstrated by the higher *arnA* expression of PB-resistant clinical isolates of *S. marcescens* versus susceptible isolates in response to PB (Table 2). The presence of a conserved PhoP binding site in the *arn* promoter among different *Serratia* species (Fig. 8) highlights the significance of the PhoP-regulated *arn* pathway in PB resistance.

S. marcescens PhoPQ has been demonstrated to control critical virulence phenotypes and be involved in the adaptation to growth with scarce Mg^{2+} and in the presence of PB, which constitute signals to activate the PhoPQ system (27). Here we found that *S. marcescens* PhoP can sense both Mg^{2+} and PB to activate the expression of *ugd* and *arn* genes, which are involved in LPS synthesis and modification. Given that LPS is involved in aspects of bacterial virulence such as adherence and subsequent invasion into host cells (39–41), we also found that *S. marcescens arnB* and *arnC* mutants exhibited significantly reduced ability for invasion into human epithelial cells (data not shown).

In this work, for the first time, we characterized an *arn* operon that is necessary for LPS modification and PB resistance, as well as its modulation by PB and Mg^{2+} through the PhoP protein, in *S. marcescens*. These data suggest that inhibition of the PhoP-regulated *arn* pathway can make *S. marcescens*, which is highly resistant to PB, become more vulnerable to PB treatment. It is tempting to suggest that the pathway is a potential target for drug development. In this regard, HQ17-2, a natural product from the lacquer

tree, has been shown to inhibit the expression of *rppA* and *pmrI* in *P. mirabilis* (42).

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Council and the National Taiwan University Hospital (Taipei, Taiwan).

We thank Yeong-Shiau Pu (National Taiwan University Hospital) for providing the NTUB1 cell line and Yang Tsuey-Ching (National Yang-Ming University) for giving us the yT&A::*xylE* plasmid.

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