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Role of *Salmonella enterica* Serovar Typhimurium Two-Component System PreA/PreB in Modulating PmrA-Regulated Gene Transcription

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The PmrA/PmrB two-component system encoded by the *pmrCAB* operon regulates the modification of *Salmonella enterica* serovar Typhimurium lipopolysaccharide leading to polymyxin B resistance. PmrA and PhoP are the only known activators of *pmrCAB*. A transposon mutagenesis screen for additional regulators of a *pmrC*::MudJ fusion led to the identification of a two-component system, termed PreA/PreB (*pmrCAB* regulators A and B), that controls the transcription of the *pmrCAB* operon in response to unknown signals. The initial observations indicated that insertions in, or a deletion of, the *preB* sensor, but not the *preA* response regulator, caused upregulation of *pmrCAB*. Interestingly, the expression of *pmrCAB* was not upregulated in a *preAB* mutant grown in LB broth, implicating PreA in the increased expression of *pmrCAB* in the *preB* strain. This was confirmed by overexpression of *preA*$^+$ in *preAB* or *preB* backgrounds, which resulted in significant upregulation or further upregulation of *pmrCAB*. No such effect was observed in any tested *preB*$^+$ backgrounds. Additionally, an ectopic construct expressing a *preA*[D51A] allele also failed to upregulate *pmrC* in any of the *pre* backgrounds tested, which implies that there is a need for phosphorylation in the activation of the target genes. The observed upregulation of *pmrCAB* occurred independently of the response regulators PmrA and PhoP. Although a *preB* mutation led to increased transcription of *pmrCAB*, this did not result in a measurable effect on polymyxin B resistance. Our genetic data support a model of regulation whereby, in response to unknown signals, the PreB sensor activates PreA, which in turn indirectly upregulates *pmrCAB* transcription.

Bacterial two-component systems (TCS) couple extracellular and intracellular signals to the transcriptional expression of genes or to the posttranslational regulation of molecular motors or enzymes (39). In particular, transcription of virulence genes in microbial pathogens is often tightly regulated and may involve TCS (8). TCS are typically composed of a sensor kinase, frequently membrane bound, and a response regulator. The sensor kinase responds to specific signals resulting in a net increase in the autophosphorylation rate of a conserved histidine residue of its C-terminal transmitter domain. The phosphate moiety is then transferred to a conserved aspartyl residue located in an acidic pocket on the N-terminal receiver domain of the cognate response regulator. The phosphorylated form of the response regulator is the effector of the signal transduction event, activating or repressing transcription or enzyme activities. The signaling is switched off by the unstable nature of the aspartyl phosphate moiety or by cellular phosphatases, including bifunctional sensors (19).

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Many *Salmonella enterica* serovar Typhimurium TCS have been shown to be important for virulence. For instance, SirA/BarA (21, 43) and EnvZ/OmpR (24) are all indirectly involved in regulating SPI-1 invasion genes. Also, the TCS SsrA/SsrB directly controls expression of SPI-2 intramacrophage survival genes (5, 9), while PhoP/PhoQ controls a vast regulon that includes invasion genes, macrophage survival genes, cation transporters, and genes involved in antimicrobial peptide (AP) resistance (10, 30). Often, TCS regulate other TCS at both transcriptional and posttranslational levels. For instance, PhoP has been recently shown to bind and regulate an internal promoter that drives the expression of the SPI-2 regulator *ssrB* (2), while at the same time, it controls dephosphorylation of the PmrA response regulator via transcriptional regulation of the gene encoding the small protein PmrD (22, 23).

PmrA/PmrB is another TCS both necessary for resistance to polymyxin B (PMB) in vitro and important for oral virulence in the mouse model of enteric fever (14, 15, 36). PmrA/PmrB regulates the expression of a complex regulon that includes *pmrCAB*, *pmrE*, *pmrG*, *pmrFHIJKLM*, *cptA*, and several other genes (15, 25, 40, 41). Some of these genes were shown to be involved in modifying the lipopolysaccharide (LPS), altering the surface charge and reducing the binding of cationic APs such as PMB (11, 13, 16). APs are important components of the host innate immune system. They are found at mucosal and skin surfaces and within professional phagocytes (18, 33). APs bind to the LPS in enterobacteria, gaining entry into the cell and most often causing perforation of the cytoplasmic mem-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
BW20339	F′ 128::Tn*10-12*(Kan) *lacI*^{q} Δ*lacZ*$_{M15}$ *pro*(*BA*)$^+$/DE3(*lac*)*X74 uidA* (Δ*Mlul*)::*pir*$^+$ *recA1* Δ*phoA532* Δ(*phnC*Δ*DEFGHIJKLMNOP*)*33-30*	28
DH5α	*supE44* Δ(*lacZYA-argF*) *U169* (ϕ80*lacZ* Δ*M15*) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Gibco
HB101	F$^-$ *thi-1 hsd20* (r_B^- m_B^-) *supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20* (Sm^r) *xyl-5 mtl-1*	3
SM10λ*pir*	*thi-1 thr leu tonA recA supE* (RP4-2 Km^r *tet*::Mu)	42
Salmonella enterica serovar Typhimurium		
CS019	ATCC 14028 *phoN*::Tn*10*d-*kan* (Kan^r)	30
JSG210	ATCC 14208 (CDC6516-60), wild type	ATCC
JSG206	JSG210 *phoP*::Tn*10*d-*cam* (aka CS015) (Cam^r)	30
JSG214	*pmrE214*::MudJ (Kan^r)	This study
JSG215	JSG210 *pmrC215*::MudJ (Kan^r)	30
JSG420	*pmrA421*::Tn*10*d *pmrC215*::MudJ (Tet^r Kan^r)	This study
JSG421	JSG210 *pmrA421*::Tn*10*d (Tet^r)	14
JSG542	CS019 *rpsL* (Str^r)	This study
JSG1038	JSG210 *preB1038*::Tn*10*d (Tet^r)	This study
JSG1039	JSG210 *preB1038*::Tn*10*d *pmrC215*::MudJ (Tet^r Kan^r)	This study
JSG1040	Δ*preB2343 pmrE214*::MudJ (Kan^r)	This study
JSG1051	JSG210 *pmrH1923*::MudJ (Kan^r)	This study
JSG1058	JSG210 *preB1038*::Tn*10*d *pmr1923*::MudJ (Tet^r Kan^r)	This study
JSG1060	*phoP206*::Tn*10*d *pmrC215*::MudJ (Tet^r Kan^r)	30
JSG1527	*preB1038*::Tn*10*d *yibD1525*::MudJ (Tet^r Kan^r)	This study
JSG1525	*yibD1525*::MudJ (Kan^r)	41
JSG1987	JSG210 *preA1987*::*kan* (Kan^r)	This study
JSG1998	JSG210 Δ*preA1998*	This study
JSG2003	JSG210 Δ*preA1998 pmrC215*::MudJ (Kan^r)	This study
JSG2115	Δ*preB2344 pmrC215*::MudJ (Kan^r)	This study
JSG2343	JSG210 Δ*preB2343*	This study
JSG2344	JSG210 Δ*preB2344*::*kan* (Kan^r)	This study
JSG2364	CS019 *preB2306*(H246G) (Cam^r)	This study
JSG2365	Δ*preB phoP206*::Tn*10*d *pmrC215*::MudJ (Tet^r Kan^r)	This study
JSG2366	Δ*preB pmrA421*::Tn*10*d *pmrC215*::MudJ (Tet^r Kan^r)	This study
JSG2422	*preB2306*[H246G] *pmrC215*::MudJ (Cam^r Kan^r)	This study
JSG2481	JSG210 Δ*preA1998 pmrA*[D51A]*2456 pmrC215*::MudJ (Kan^r)	This study
JSG2498	*preB1038*::Tn*10*d *pmrA*[D51A]*2456 pmrC215*::MudJ (Tet^r Kan^r)	This study
JSG2499	*pmrA*[D51A]*2456 pmrC215*::MudJ (Kan^r)	This study
JSG2523	Δ*preA1998 yibD2420*::MudJ (Kan^r)	This study
JSG2420	*pmrA421*::Tn*10*d Δ*preA1998 yibD2420*::MudJ (Tet^r Kan^r)	This study
JSG2527	Δ*preB2343 pmrA421*::Tn*10*d *yibD2420*::MudJ (Tet^r Kan^r)	This study
JSG2623	Δ*preAB*::*cat* (Cm^r)	This study
JSG2624	Δ*preAB2626 pmrC215*::MudJ (Kan^r)	This study
JSG2626	JSG210 Δ*preAB2626*	This study
Plasmids		
pWSK29	pSC101 *ori*, α*lacZ* (Ap^r)	46
pBAD18	ColE1 *ori*, P_{BAD} L(+) Ara inducible (Ap^r)	17
pCP20	FLP recombinase expression plasmid (Cm^r Ap^r)	6
pKD46	R101 *ori*, *rep101ts*, P_{BAD} expressing λ γ β *exo* (Ap^r)	6
pKD4	*ori*$_{R6K}$, *FRT*::*kan*::*FRT* template plasmid (Ap^r)	6
pKD3	*ori*$_{R6K}$, *FRT*::*cat*::*FRT* template plasmid (Ap^r)	6
pKAS46	*ori*$_{R6K}$, α*lacZ*, suicide vector carrying an *rpsL* locus (Ap^r Km^r)	35
pLD55	*ori*$_{R6K}$, α*lacZ tetAR* (Ap^r)	28
pRK2013::Tn*7*	ColE1 *mob*$^+$ *tra*$_{RK2}$ Δ*rep*$_{RK2}$ *repE kan*::Tn*7* (Tp^r Sm^r Sp^r)	7
pQseBC33	pBAD33 with the *E. coli* O157:H7 *qseBC* operon (Ap^r)	37
pJSG975	pWSK29 with a HindIII fragment carrying *tetAR* from Tn*10*d and upstream chromosomal DNA from JSG1038 (Tc^r Ap^r)	This study
pJSG2381	pKAS46 with a 1.2-kb insert containing *preB*[H246G] (Km^r Ap^r)	This study
pJSG2391	pBluescript with a 1.2-kb fragment containing *pmrA*[D51A] constructed by SOE-PCR (Ap^r)	This study
pJSG2456	pLD55 with a 0.7-kb SstI-XhoI PCR fragment containing *pmrA*[D51A] from pJSG2391 (Tc^r Ap^r)	This study
pJSG2558	pBAD18 with a 0.7-kb fragment containing *preA* expressed from P_{BAD} (Ap^r)	This study
pJSG2581	pBAD18 with a 1.5-kb fragment containing *preAB* expressed from P_{BAD} (Ap^r)	This study
pJSG2700	pJGS2558 derivative mutagenized to express a *preA*[D51A] allele (Ap^r)	This study

TABLE 2. Oligonucleotide primers

Name	5′ → 3′ sequence[a]	Purpose
JG489	ATGCGAATTTTACTGGTAGAAGATGAC*ACAGTGTAGGCTGGAGCTGCTTCG*	λ-Red deletion of *preA* or *preAB*
JG490	TCATGCGTCACCCAGGGTGTAGCCGATGCC*CATATGAATATCCTCCTTAG*	λ-Red deletion of *preA*
JG557	ATGAAATTGACGCAACGTCTCAGCCTG*ACAGTGTAGGCTGGAGCTGCTTCG*	λ-Red deletion of *preB*
JG558	TCGGTACGCCTTTGGCGTCGAGCGGCGTTT*CATATGAATATCCTCCTTAG*	λ-Red deletion of *preB* or *preAB*
JG634	ATAAGAATGCGGCCGCAGTTGATGTTGTTCGC	SOE-PCR *preB*[H246G]
JG635	CCGACGCCGCCGGCGAATTG	SOE-PCR *preB*[H246G]
JG636	CAATTCGCCGGCGGCGTCGG	SOE-PCR *preB*[H246G]
JG637	GAATTCTCCGCGGCGTTGCCAAACGA	SOE-PCR *preB*[H246G]
JG708	GGAATTCATGCGAATTTTACTGG	Amplify *preAB*
JG709	GGGGTACCTTACCAACTTACTACGGC	Amplify *preAB*
JG795	CGGGCAGCCCTAAAGCCTGCACCATCAGA	SOE-PCR *pmrA*[D51A]
JG796	TCTGATGGTGCAGGCTTTAGGGCTGCCCG	SOE-PCR *pmrA*[D51A]
JG797	CGCGTCGACTATTACAACCGTTATCCACCG	SOE-PCR *pmrA*[D51A]
JG798	GCTCTAGAGTACTGATTAACTGGAACACC	SOE-PCR *pmrA*[D51A]
JG1055	CCGGAATTCCGCGAGTTACCGCAAGGAAGAACAGATGCG	Amplify *preA*
JG1056	CCGGAATTCTCATGCGTCACCCAGGGTGTAGCCGATGC	Amplify *preA*
JG1190	CTTATGATGCGGTTATTTTAGCGTTGACGCTGCCAGGC	Mutagenize *preA*[D51] to A
JG1191	TAAAATAACCGCATCATAAGGCGCGCTGTAAAGCG	Mutagenize *preA*[D51] to A

[a] Restriction enzyme sites are underlined; P1 and P2 priming sites in pKD3 and pKD4 are in italics.

brane and eventual death (45). Besides being indirectly regulated by PhoP/PhoQ via the mechanism mentioned above, some PmrA-regulated genes such as *ugd*/*pmrE* are also transcriptionally controlled by the RcsB/RcsC two-component system (31). The fact that multiple signals/signaling pathways converge to modulate the *Salmonella* polymyxin resistance regulon suggests its critical importance for the adaptation/survival in both extracellular and intracellular environments. Additionally, previous work from our laboratory suggests that in vivo, regulators other than PmrA might be involved in controlling the expression of the *pmrFHIJKLM* operon (15).

In this study, we describe a *Salmonella* TCS, named PreA/PreB, that was identified in a transposon mutagenesis screen for regulators of *pmrCAB*. PreA/PreB is similar to the *luxS*-dependent quorum-sensing regulatory system QseB/QseC in enterohemorrhagic *Escherichia coli* (37). We demonstrate that PreA activates the transcription of *pmrCAB* in a PhoP- and PmrA response regulator-independent fashion. The results suggest that sensor kinase PreB inactivates PreA during growth in Luria-Bertani (LB) broth and that PreA is indirectly involved in *pmrCAB* regulation. Furthermore, the observed increase in *pmrCAB* transcription does not lead to observable transcriptional activation of most of the PmrA/PmrB regulon or to the alteration of the polymyxin resistance phenotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* and *Salmonella enterica* serovar Typhimurium strains and plasmids used in this study are listed in Table 1. LB broth and agar were used for strain maintenance, cloning, and expression experiments. For gene expression experiments, strains were grown in a rotating drum to mid- to late exponential phase (optical density at 600 nm [OD_{600}] of 0.6 to 0.9). When appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 25 μg/ml; tetracycline, 25 μg/ml; streptomycin, 100 μg/ml.

Molecular biology and genetic techniques. DNA purification, molecular cloning, and PCR were performed according to standard procedures (1). Plasmids were mobilized by electroporation. Transposon mutagenesis was performed with Tn*10*d and MudJ transposons as previously described (12). Marked mutations were transferred between *Salmonella enterica* serovar Typhimurium strains by P22 HT105 *int*-102-mediated generalized transduction as described previously (20, 34).

DNA sequencing and bioinformatics. DNA sequencing was performed using a Big Dye fluorescent terminator and an ABI3770 capillary sequencer at the Nucleic Acid Core Facility, University of Texas Health Science Center at San Antonio, and the Plant Microbe Genomic Facility at The Ohio State University. DNA sequences were analyzed by Blastx at the NCBI (27). Domain structure was analyzed by CDD searches (26). ClustalW was used to produce global progressive alignments (44).

Construction of mutants and plasmids. Nonpolar deletions of *preA* and *preB* were created using λ-Red mutagenesis (6). In particular, primers JG489 and JG490 or JG557 and JG558 (Table 2) were designed to amplify *preA′-kan-′preA* and *preB′-kan-′preB* cassettes, respectively, while primers JG489 and JG558 amplified *preA′-cat-′preB* using pKD3 or pKD4 as a template. The recombinant antibiotic cassettes were exchanged into the chromosome of *Salmonella enterica* serovar Typhimurium 14028s(pKD46) to generate strains JSG1987 Δ*preA*::*kan*, JSG2344 Δ*preB*::*kan*, and JSG2626 Δ*preAB*::*cat* (Table 1). The antibiotic cassettes were resolved by introducing pCP20, and the resulting unmarked strains were named JSG1998 Δ*preA*, JSG2626 Δ*preAB*, and JSG2343 Δ*preB*. A missense mutation to glycine was generated at the PreB predicted autophosphorylation site H246. Spliced overlap extension (SOE)-PCR with JG637 and JG634 as flanking primers and JG635 and JG636 as mutagenic primers was used to construct the mutant allele in vitro. The *preB*[H246G] allele was ligated into the suicide vector pKAS46 as a 1.2-kb EcoRI/NotI fragment to produce plasmid pJSG2381 in *E. coli* SM10λ*pir*. Allele exchange with pJSG2381 was performed using JSG542, a *Salmonella enterica* serovar Typhimurium 14028 Str^r derivative, as the recipient strain. The correct recombinant was confirmed by sequencing and was labeled JSG2364. The phosphorylation site at residue D51 of *pmrA* was mutated to alanine by SOE-PCR using primers JG797 and JG798 (flanking primers) with JG795 and JG796 (internal primers). The *pmrA*[D51A] allele was cloned as an SstI-XhoI fragment into the suicide vector pLD55 (28) to produce plasmid pJSG2456 in *E. coli* BW20339. Allele exchange into strain JSG2003 *preA pmrC*::MudJ was performed using Bochner selection, as described previously (28), to produce strain JSG2481. The *pmrC*::MudJ *pmrA*[D51A] locus was further mobilized in several genetic backgrounds by P22 transduction selecting for the Kan^r marker of MudJ linked to the *pmrA* mutation. The correct recombinant was verified by PCR and restriction analysis of the *pmrA*[D51A] allele at the engineered NheI site.

The entire *preAB* operon or the *preA* open reading frame was cloned into pBAD18 for expression from P_{BAD} as a JG708 and JG709 or a JG1055 and JG1056 PCR fragment to produce plasmids pJSG2581 and pJSG2558, respectively. Plasmid pJSG2558 was mutagenized at the conserved D51 residue of *preA* by oligomutagenesis with primers JG1190 and JG1191 using the Gene Tailor kit (Invitrogen) to generate pJSG2700.

Western blot analysis of His$_6$-PreA proteins. Whole-cell lysates of *Salmonella* strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto HyBond nitrocellulose (Amersham). Proteins were detected using a primary polyclonal antibody raised in rabbit against recombinant His$_6$-PreA (1:2,000 dilution), a donkey anti-rabbit immunoglobulin G

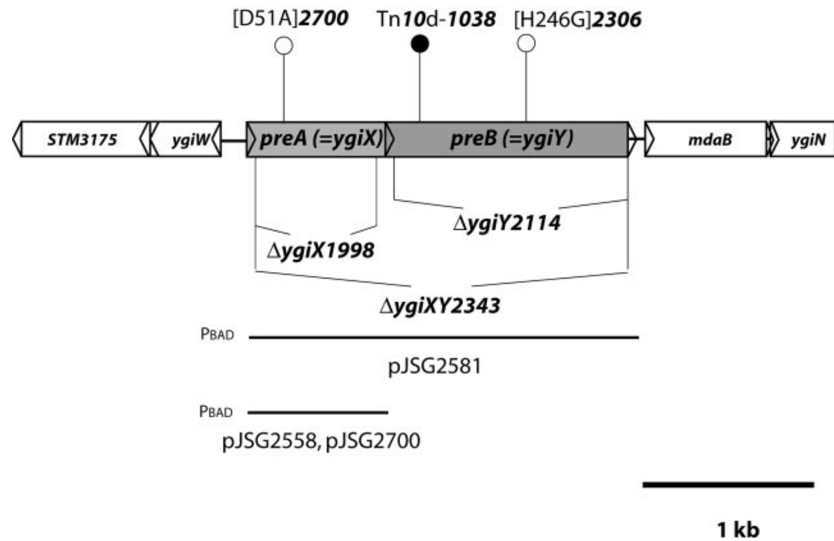

FIG. 1. Map of *preAB* genes, mutations, and plasmids. Boxes represent open reading frames. The solid circle indicates a Tn*10*d insertion. Locations of the *preA* and *preB* deletions are noted below the gene map, and a solid line delimiting the region cloned into the expression plasmids pJSG2581 and pJSG2558 is shown.

secondary antibody conjugated to alkaline phosphatase (1:4,000 dilution) (Amersham), and the chemiluminescent substrate CPD-Star (Roche) according to a standard Western blot protocol (1).

Enzyme assays. β-Galactosidase assays were carried out using either a spectrophotometric method with *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate or a modified fluorometric method with 4-methyl-umbelliferyl-β-galactopyranoside (MUGal) (29). Assays were performed in triplicate. Specific enzyme activities are reported in Miller units (ONPG assays) or in picomoles of released 4-methyl-umbelliferone per second per OD unit per milliliter of culture (MUGal assays).

MIC assays. Assays were carried out as previously described (38). Briefly, strains were grown in Mueller-Hinton broth overnight, and 10^4 cells per well were added to polypropylene microtiter plates. Each strain was tested for growth and pellet accumulation against serial dilutions of PMB in 0.2% bovine serum albumin–0.01% acetic acid for 16 h.

RESULTS

Identification and sequence analysis of the *preAB* operon. In an effort to identify additional putative regulators of the *pmrCAB* locus, a Tn*10*d mutant pool constructed in *Salmonella enterica* serovar Typhimurium 14028s was transduced into the reporter strain JSG215 (*pmrC*::MudJ). Out of 30,000 transductants, a mutant colony producing a strong blue color on LB plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside) was isolated. The mutant phenotype was named Pre (for *pmrCAB* regulator), while the mutant strain was named JSG1038. The mutation was transduced back into the parent strain to rule out second-site mutations (creating strain JSG1039), and the phenotype was confirmed. A HindIII fragment containing the region upstream of the transposon, as well as the transposon tetracycline resistance cassette, was shotgun cloned into pWSK29, producing pJSG975. DNA sequencing from the transposon 5′ end and sequence analyses revealed that the Tn*10*d insertion mapped to a gene corresponding to the *Salmonella enterica* serovar Typhimurium LT2 locus *ygiY* (STM3178), which has homology to orthodox bacterial sensor kinases (Fig. 1), most notably to *E. coli* K-12 and O157:H7 QseC (87 to 88% similarity; E = 0.0). YgiY is predicted to be a 51-kDa protein with two transmembrane segments between residues 8 and 56 and residues 149 and 199. CDD searches and alignments with known sensor kinases predicted a putative autophosphorylation site at residue H246. Upstream of *ygiY* is *ygiX* (STM3177), which encodes a 24-kDa putative response regulator of the OmpR subfamily with high similarity to *E. coli* O157:H7 QseB (93% similarity; E = 1×10^{-107}), *Pectobacterium carotovorum* PmrA (64% similarity; E = 8×10^{-49}), *E. coli* CFT073 PmrA (63% similarity; E =

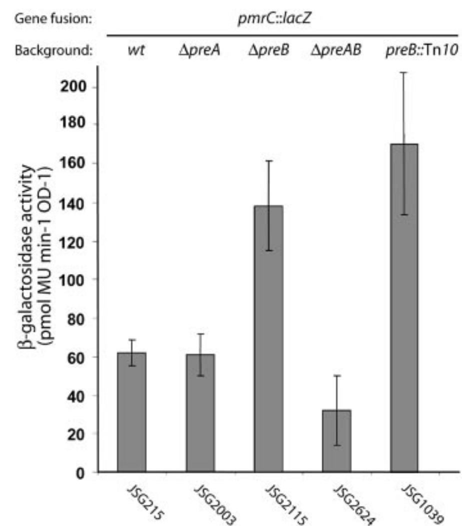

FIG. 2. Effect of *preA* or *preB* mutations on *pmrCAB* transcription. *Salmonella* strains were grown in LB medium to an OD_{600} of 0.6 before β-galactosidase assays were performed. Activities are expressed in picomoles of 4-methylumbelliferone (MU) per minute per OD unit. Error bars indicate the standard deviations. The following strains were used: JSG215 (*pmrC-lacZ*), JSG1039 (*preB*::Tn*10*d *pmrC-lacZ*), JSG2003 (Δ*preA pmrC-lacZ*), JSG2115 (Δ*preB pmrC-lacZ*), and JSG2624 (Δ*preAB pmrC-lacZ*). wt, wild type.

1×10^{-47}), and *Salmonella enterica* serovar Typhimurium PmrA (58% similarity; E = 7×10^{-43}). The new TCS locus was renamed *preAB*. These two genes form an apparent operon, with the stop codon of *ygiX* overlapping the start codon of *ygiY*, an arrangement suggestive of translational coupling. The high similarity and identity to *E. coli* QseB/QseC, which controls motility in response to AI-2 quorum-sensing signals in *E. coli* strains (37), and the almost-identical map position (Fig. 1) directly upstream of *mdaB*, a putative NADH-dependent oxidoreductase, and *ygiN*, a putative quinol monooxygenase, and downstream of STM3175, an AraC-like regulator, and *ygiW* suggested that these two TCS may be potential orthologs.

Both PreB and PreA affect *pmrCAB* transcription. The regulatory effect of the *preB*::Tn*10*d insertion on *pmrCAB* operon expression was quantified by β-galactosidase enzyme assays upon growth of the bacteria in LB medium to exponential-early stationary phase. Reporter gene activity was measured in a *pmrC*::MudJ background. The results showed an eightfold increase in *pmrCAB* transcription upon introduction of the *preB* polar insertion (strain JSG1039) (Fig. 2). To rule out possible polar effects of the Tn*10*d insertion on the downstream genes *mdaB* and *ygiN* (Fig. 1), we created an unmarked deletion in *preB* (strain JSG2115) using λ-Red mutagenesis. This mutation caused the expected upregulation of *pmrCAB* at levels comparable to those observed in strain JSG1039 (Fig. 2), and its effects were complemented by *preB* in *trans* (Fig. 3). We were also able to complement the regulatory effect of the Δ*preB* mutation with plasmid pQseBC33 (Fig. 3), which carries the homologous *E. coli* operon *qseBC*, implying functional homology between the two regulatory systems. In general, null mutations in the sensor kinase inactivate a two-component system, but cross talk with the response regulator by alternative kinase or by acetyl phosphate may lead to sensor-independent expression of the two-component system regulon. To test whether the effect caused by *preB* was replicated by a *preA* mutation, we introduced a deletion in the response regulator by λ-Red mutagenesis (creating strains JSG1998 and JSG2003). The resulting mutants did not produce the 26-kDa polypeptide after Western blot analysis using a polyclonal antibody against PreA (Fig. 3D, lane 2). To our surprise, a *preA* strain did not show upregulation of *pmrC::*MudJ (Fig. 2). This result was also repeated after reengineering the mutants using independently constructed alleles. Similar regulatory patterns were obtained by using other plasmid-borne and chromosomal gene fusions

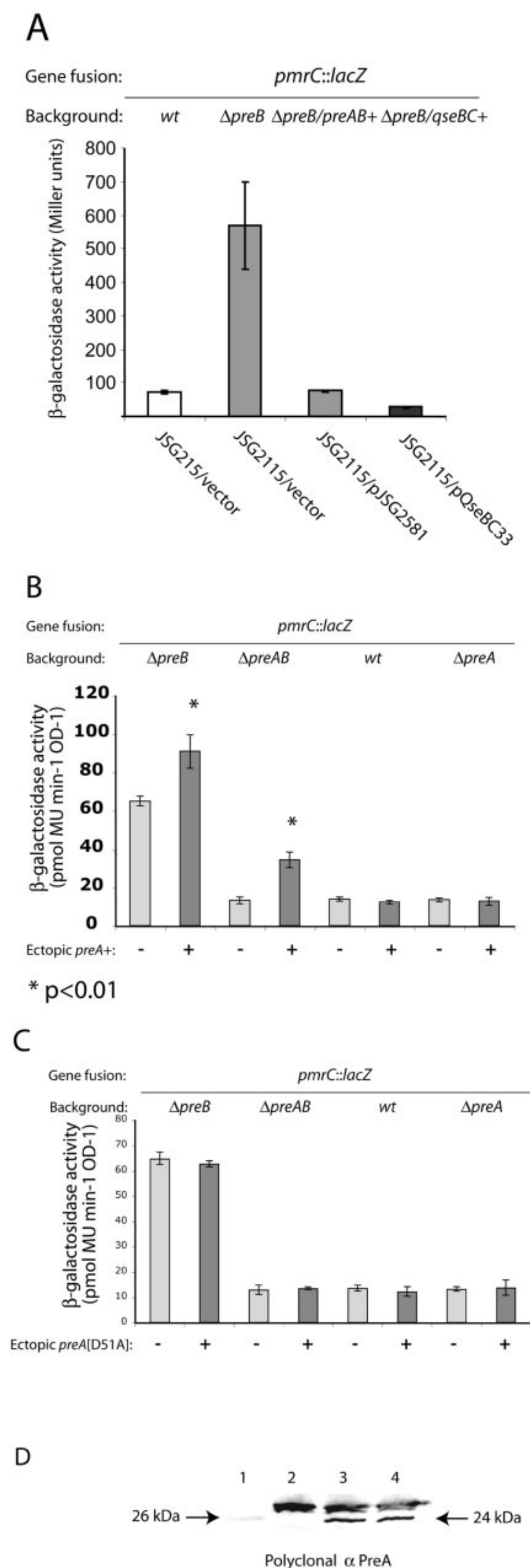

FIG. 3. Ectopic expression of complementing and suppressing regulatory genes. (A) Complementation of *preB* mutant. (B) Ectopic expression of *preA*$^{+}$. (C) Ectopic expression of *preA*[D51A]. (D) Western blot analysis of *Salmonella* whole-cell lysates using polyclonal anti-His$_6$-PreA (α PreA). Lane 1, molecular mass marker; lane 2, JSG1998/pBAD18; lane 3, JSG1998/pJSG2558; lane 4, JSG1998/pJSG2700. *Salmonella* strains were grown in LB medium with L-arabinose (0.2%) to induce expression of the complementing/suppressing genes. β-Galactosidase assays were performed as described in Materials and Methods. Activities are expressed in Miller units (colorimetric protocol) or picomoles of 4-methylumbelliferone (MU) per minute per OD (fluorometric protocol). Error bars indicate the standard deviations. The following strains were used: JSG215 (*pmrC-lacZ*), JSG2115 (Δ*preB pmrC-lacZ*), JSG2624 (Δ*preAB pmrC-lacZ*), JSG2003 (Δ*preA pmrC-lacZ*), JSG2115 (Δ*preB pmrC-lacZ*), and JSG1998 (Δ*preA*). Plasmid pJSG2581 is *preAB* expressed from P_{BAD}, pQseBC is *qseBC* expressed from P_{BAD} (pBAD18 vector), plasmid pJSG2558 is *preA* expressed from pBAD18, and plasmid pJSG2700 expresses *preA*[D51A]. wt, wild type.

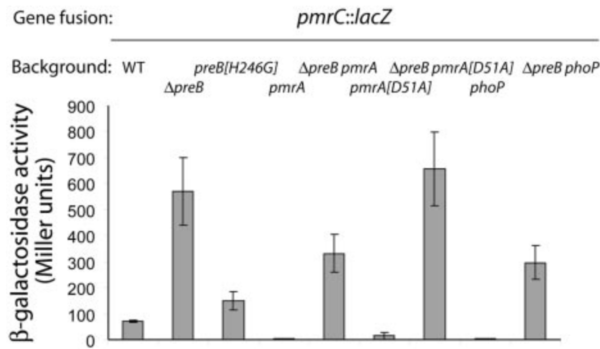

FIG. 4. Effects of *pmrA*, *phoP*, and the PreB putative autophosphorylation site on PreB-mediated regulation of *pmrCAB*. *Salmonella* strains were grown in LB medium, and β-galactosidase assays were performed as described in Materials and Methods. Activities are expressed in Miller units. Error bars indicate the standard deviations. The following strains were used: JSG215 (*pmrC-lacZ*), JSG1039 (*preB pmrC-lacZ*), JSG2422 (*preB*[H246G] *pmrC-lacZ*), JSG420 (*pmrA pmrC-lacZ*), JSG2366 (Δ*preB pmrA*::Tn*10*d *pmrC-lacZ*), JSG2499 (*pmrA*[D51A] *pmrC-lacZ*), JSG2498 (*preB pmrA*[D51A] *pmrC-lacZ*), JSG1060 (*phoP pmrC-lacZ*), and JSG2365 (Δ*preB phoP pmrC-lacZ*). WT, wild type.

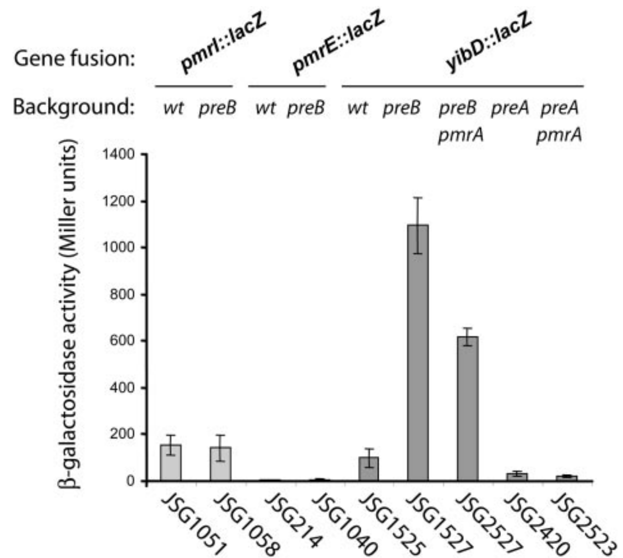

FIG. 5. Effect of *preB* mutations on the expression of *pmrA* regulon promoters. *Salmonella* strains were grown in LB medium, and β-galactosidase assays were performed as described in Materials and Methods. Activities are expressed in Miller units. Error bars indicate the standard deviations. Data are from a representative experiment with two replicates. The following strains were used: JSG1051 (*pmrHFI-lacZ*), JSG1058 (*preB pmrI-lacZ*), JSG214 (*pmrE-lacZ*), JSG1040 (*preB pmrE-lacZ*), JSG1525 (*yibD-lacZ*), JSG1527 (*preB yibD-lacZ*), JSG2527 (Δ*preB pmrA yibD*::*lacZ*), JSG2420 (Δ*preA yibD-lacZ*), and JSG2523 (Δ*preA pmrA yibD-lacZ*).

to the *pmrCAB* promoter (4), ruling out specific effects on the MudJ operon fusion in *pmrC*.

This differential regulatory phenotype implies that the effect on *pmrCAB* was sensor dependent but response regulator independent, possibly due to cross talk of the kinase to unknown regulators. Another interpretation of this result is that under the growth conditions employed, the proper signal activating PreB kinase activity was absent; therefore, a *preA* response regulator mutant would not show differential regulation compared to the wild-type. However, for this to be true, one has to assume that the loss of PreB exposes the PreA protein to nonphysiological kinase cross talk that leads to its constitutive activation. To test these models, a Δ*preAB*::*cat pmrC*::MudJ double-mutant strain was created and *pmrC* transcription was analyzed upon growth in LB medium. The loss of *preA* in this double mutant reversed the phenotype of *pmrC* upregulation observed in the *preB* mutant (Fig. 2), confirming that the effect of deleting *preB* was mediated by PreA and not by PreB cross talk to unknown regulators. In an additional attempt to disprove the cross talk hypothesis, the PreB conserved histidine at residue 246, the putative autophosphorylation site identified by sequence alignments to other transmitter domains of TCS kinases, was mutated to a glycine residue. Expression of *pmrC*::MudJ was measured in wild-type, *preB*, and *preB*[H246G] backgrounds (Fig. 4). Transcriptional activity of the fusion was not significantly increased in the *preB*[H246G] background compared to that of wild type (ca. twofold). This regulatory phenotype is identical to that of a *preA* or a *preAB* mutation. These data suggest a model in which (i) both PreA and PreB affect *pmrCAB* transcription, (ii) the PreB sensor is acting as a phosphatase rather than an activating kinase when bacteria are grown in LB medium, (iii) the PreB[H246G] protein retains its phosphatase activity, and (iv) PreA is phosphorylated by cross talk in the absence of PreB.

As additional confirmation of the involvement of PreA in *pmrC* regulation, we analyzed the Δ*preAB*::*cat pmrC*::MudJ strain with an inducible plasmid-borne *preA*. Ectopic expression of *preA* led to a more-than-twofold increase of β-galactosidase levels, demonstrating that PreA can activate, directly or indirectly, the *pmrCAB* operon (Fig. 3B). The increase of activity by ectopic expression of *preA* was also statistically significant in the Δ*preB* background ($P = 0.008$) but less than twofold in magnitude, likely because the chromosomal copy of *preA*$^+$ was also expressed (Fig. 3B). In support of the general paradigm that PreA requires phosphorylation for activating its target genes, a *preA*[D51A] allele ectopically expressed in pBAD18 failed to activate *pmrCAB* in all the backgrounds tested, in spite of the production of a polypeptide of the expected molecular weight detected using a polyclonal antibody raised against a His$_6$-PreA protein (Fig. 3C). Consistent with the hypothesis that PreB is acting as a phosphatase during growth in LB medium, upregulation of *pmrCAB* by ectopic *preA* was not observed in *preB*$^+$ backgrounds (wild type and the *preA* mutant) (Fig. 3B). Gel shift assays using unphosphorylated or chemically phosphorylated PreA failed to show binding of this protein to the *pmrAB* regulatory region (4), implying indirect regulation of *pmrCAB* by PreA.

Regulation of *pmrCAB* transcription is independent of PmrA and PhoP. PmrA is the only known direct activator of *pmrCAB* transcription. PmrA is directly activated by the PmrB kinase and indirectly activated by PhoP/PhoQ via the small protein PmrD, which stabilizes the phosphorylated form of PmrA (22). A model that explains the apparent repressing effect of PreB on *pmrCAB* transcription might have involved interference of the PreB sensor with PhoP/PhoQ activity. When *phoP* was disrupted, transcription of *pmrCAB* was greatly decreased (Fig.

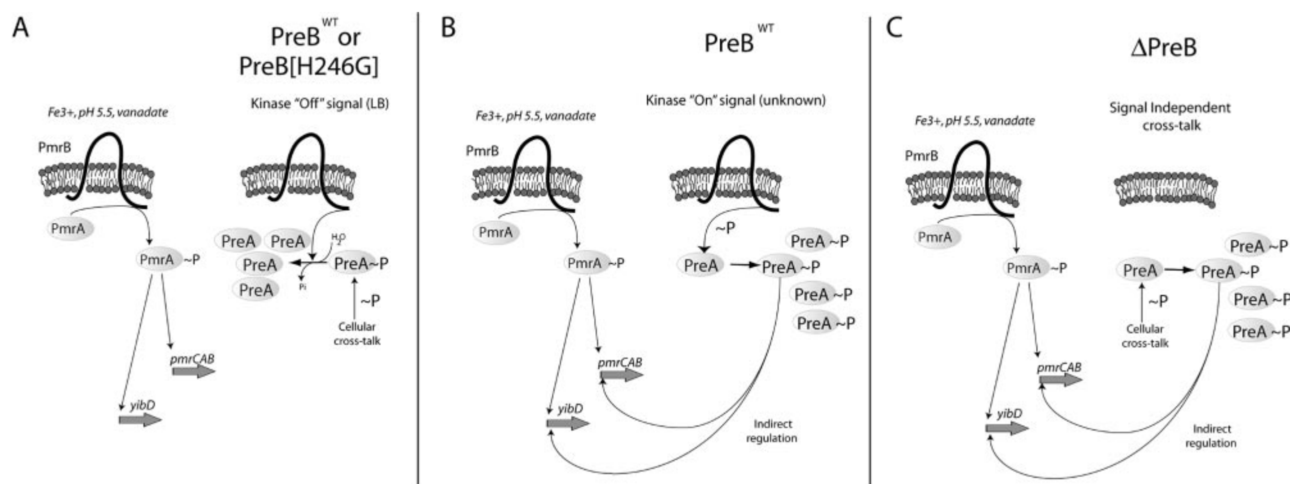

FIG. 6. Working model for PreA/PreB regulation of *pmrCAB*. (A) The genes *pmrCAB* and *yibD* are part of the PmrA regulon. Extracellular signals that affect the PreB sensor are unknown, but growth in LB medium activates its phosphatase activity, maintaining the PreA response regulator in the unphosphorylated conformation. In a PreB[H246G] background, PreA is also inactivated by the phosphatase activity of the mutant protein. (B) Under PreA/PreB-inducing conditions not yet identified, PreA indirectly regulates *pmrCAB* and *yibD*. (C) Ectopic PreA, but not PreA[D51A], can activate *pmrCAB* in a Δ*preB* mutant even when grown in LB medium, presumably because of signal-independent cellular cross talk, which may become unmasked and more important due to the absence of the cognate sensor protein.

4) as expected due to the decreased concentration of cellular PmrD. However, when the *phoP* null mutation was introduced into a *preB* background, the transcriptional activity of *pmrCAB* still increased by 7-fold compared to strain JSG215 *pmrC*::MudJ (Fig. 4) and by ca. 100-fold compared to strain JSG1060 *phoP pmrC*::MudJ. This result indicates that the effect of a PreB deletion on *pmrCAB* transcription was independent of the PhoQ/PhoP/PmrD proteins.

An alternative model of PreB-mediated regulation of *pmrCAB* could involve PmrA/PmrB, known direct regulators of *pmrCAB*. To address this, we introduced a missense substitution to alanine in the putative phosphorylation site of PmrA at residue D51, and the resulting allele was exchanged into a *pmrC*::MudJ reporter strain. Under *pmrCAB*-inducing conditions, the *pmrA*[D51A] allele resulted in decreased *pmrCAB* expression, but the addition of a null mutation in *preB* again strongly upregulated the *pmrC*::MudJ reporter fusion (JSG2499 versus JSG2498) (Fig. 4). A similar result was obtained by using a *pmrA* null mutation (JSG420 versus JSG2366) (Fig. 4). Collectively, these results demonstrate that the PreA/PreB-mediated regulation of *pmrCAB* is independent of PmrA and PhoP.

PreB does not affect PmrA-regulated genes involved in LPS modifications. Increases in the transcription levels of *pmrCAB* could be expected to upregulate PmrA-dependent loci. The PmrA-regulated loci *pmrE* and *pmrHFIJKLM* encode proteins necessary for LPS modifications leading to PMB resistance. To examine if these loci were affected by the loss of *preB*, β-galactosidase activities in JSG1040 (*preB pmrE*::MudJ) and JSG1058 (*preB pmrI*::MudJ) were measured and compared to those of reporter strains with a wild-type regulatory background (JSG214 and JSG1051). The activity of both fusions was unaffected by the loss of *preB* (Fig. 5). Consistent with the transcriptional data, MIC assays for PMB resistance indicated that the *preB* mutant showed equal levels of resistance (2 μg/ml) to the wild-type strain. Interestingly, when we examined the effect of *preB* on another PmrA-regulated gene of unknown function, *yibD* (strain JSG1525 versus strain JSG1527) (Fig. 5), we observed a ninefold upregulation of its expression in a *preB* background, similar to what was seen with *pmrCAB*. Also, as observed with *pmrCAB*, the upregulation of *yibD* in a *preB* mutant was largely independent of PmrA, and the loss of *preA* had little impact on *yibD* expression (Fig. 5). Although not all PmrA-regulated genes have been examined, PreB has only been shown to affect *yibD* and *pmrCAB* transcription thus far.

DISCUSSION

In this study, we describe an uncharacterized TCS of *Salmonella enterica* serovar Typhimurium, termed PreA/PreB, that activates transcription of the *pmrCAB* operon and of another PmrA-regulated gene, *yibD*. The *pmrAB* TCS is critical for the activation of a large regulon involved in polymyxin B resistance. In a Tn*10*d transposon screen for genes having an effect on *pmrC* transcription, we isolated an insertion in the PreB sensor kinase. Based on sequence similarity and map position, the PreA/PreB TCS is homologous to the previously identified *E. coli* QseB/QseC TCS (37). This *E. coli* TCS was shown to be responsive to *luxS*-dependent quorum-sensing signals for regulating flagellar gene expression. PreA/PreB and QseB/QseC behave as potential orthologs based on the successful complementation of *preB* null mutants by the *E. coli* TCS. In spite of functional complementation, in experiments not reported in this paper, we show that their regulons are not overlapping and that the biological role of this TCS in the two organisms may be different. The transcriptional regulation of *preAB*, its genome-wide regulatory effects, and its role in virulence will be described in a separate manuscript.

Initially, the regulation of *pmrCAB* by PreA/PreB appeared to be unusual in that the sensor kinase, rather than the response regulator, seemed responsible for repressing its transcription during growth in LB medium. Typically, upon phosphorylation, the response regulator activates or represses gene

transcription in response to signals perceived by its cognate sensor kinase. Therefore, null mutations in the response regulator usually have the same phenotype of mutations in the sensor kinase. This is not the case for *preA* and *preB*. There are a few examples of TCS pairs cross talking with each other and leading to a bifurcation of regulatory pathways. For instance, the *Salmonella enterica* serovar Typhimurium CpxA sensor was shown to indirectly activate *hilA*, an AraC-like transcription factor regulating SPI-1, at the transcriptional level in a CpxR response regulator-independent manner (32). Another example of cross talk between response regulators and kinases of different cognate pairs is found in *E. coli*, where the CreC sensor kinase regulates the PhoB-PhoR system (47). Subsequent experiments using *preAB* double mutants and ectopic expression of *preA* led us to formulate a different model whereby PreA acts as an activator of *pmrCAB*, likely in an indirect fashion. Furthermore, the observation that neither a *preA* nor a *preAB* mutation affects *pmrCAB* expression, while *preB* mutations do, suggests that under the growth conditions used, the PreA/PreB system is not perceiving a signal able to stimulate the kinase activity of the sensor. Concurrently, we must hypothesize that the absence of the cognate sensor kinase leads to a constitutive activation of PreA by cross talk. Based on the data collected with the ectopic expression of *preA*[D51A], the activation of wild-type PreA occurs by phosphorylation at residue D51, following a common TCS paradigm. Point mutations abolishing only the putative phosphorylation site of PreB showed phenotypes similar to those of *preA* and *preAB* mutations, presumably because its phosphatase activity is unaffected, and/or the PreB[H246G] protein complexes PreA, protecting it from cross talk.

Because the only two known activators of *pmrCAB* are PmrA, by autoregulation (14, 36), and PhoP, via the PmrD small protein (23), we tested both regulatory systems for their genetic interactions with the *preB* mutation. We found that functional null mutations in *pmrA* (both deletions and missense mutations) or *phoP* did not affect the observed upregulation of *pmrC* in the *preB* background. This suggests that PreA/PreB does not act through the two known regulatory pathways controlling *pmrCAB*.

Given that *preB* null mutations lead to the upregulation of *pmrCAB* transcription, one would expect that the concomitant increased levels of PmrA and PmrB proteins may result in the upregulation of genes in the PmrA regulon. This was not the case, because of several genes tested, only *pmrCAB* and *yibD*, an open reading frame of unknown function regulated by PmrA (41), were upregulated in a *preB* background, and the polymyxin resistance phenotype was not altered compared to that of the wild type. Perhaps PreA/PreB potentiates the PmrA/PmrB system by increasing the levels of these proteins to allow a quicker response upon encountering PmrA/PmrB-activating conditions. It is not clear why *yibD* is the only PmrA-regulated gene to also be regulated by PreA/PreB.

Our data imply that the signal activating PreA/PreB, not yet identified, is absent during growth in LB medium to exponential-early stationary phase (Fig. 6). The analysis of the genome-wide regulon controlled by this novel TCS may help to build a new hypothesis concerning its function in vivo and the signals modulating its activation in various niches.

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