

PigS and PigP Regulate Prodigiosin Biosynthesis in *Serratia* via Differential Control of Divergent Operons, Which Include Predicted Transporters of Sulfur-Containing Molecules[∇]

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***Serratia* sp. strain ATCC 39006 produces the red-pigmented antibiotic prodigiosin. Regulation of prodigiosin biosynthesis involves a complex hierarchy, with PigP a master transcriptional regulator of multiple genes involved in prodigiosin production. The focus of this study was a member of the PigP regulon, *pigS*, which encodes an ArsR/SmtB family transcriptional repressor. Mutations in *pigS* reduced production of prodigiosin by decreasing the transcription of the biosynthetic operon. The *pigS* gene is the first in a four-gene operon, which also encodes three membrane proteins (*pmpABC*) of the COG2391 (DUF395; YedE/YeeE) and COG0730 (DUF81; TauE/SafE) families that we propose constitute transport components for sulfur-containing compounds. We provide the first experimental evidence confirming the membrane localization of a COG2391 protein, that of PmpB. Divergently transcribed from *pigS-pmpABC* is a bicistronic operon (*blhA-orfY*), which encodes a metallo- β -lactamase and a coenzyme A-disulfide reductase containing a rhodanese homology domain, both of which may participate in reactions with sulfur-containing compounds. The overproduction of the BlhA and OrfY enzymes and the PmpABC membrane proteins differentially affected pigmentation. We have dissected the contributions of these various proteins and determined their importance in the control of prodigiosin production. PigS-mediated control of prodigiosin occurred via binding directly to a short inverted repeat sequence in the intergenic region overlapping the predicted -10 regions of both *pigS* and *blhA* promoters and repressing transcription. PigP was required for the activation of these promoters, but only in the absence of PigS-mediated repression.**

Serratia sp. strain ATCC 39006 is a Gram-negative enteric bacterium originally isolated from a salt marsh in New Jersey, where it was associated with plant material (25). *Serratia* sp. ATCC 39006 produces several secondary metabolites, including a red antimicrobial pigment, prodigiosin (Pig), and a β -lactam antibiotic, carbapenem (Car) (6, 30). There is considerable interest in Pig and its derivatives due to their anticancer and immunosuppressive activities (34). The biosynthetic genes required for production of Pig and Car in *Serratia* sp. ATCC 39006 have been identified as *pigA* to $-O$ (*pigA-O*) and *carA* to $-H$ (*carA-H*), respectively (17, 35, 37), and several studies have revealed that biosynthesis is controlled via a complex hierarchical network of regulators (10–12, 14, 15, 29–31, 35, 36). Regulation includes a LuxIR-type quorum sensing (QS) system (SmaIR), which allows gene expression to be regulated in response to cell density via the production and detection of low-molecular-weight signal molecules called *N*-acyl homo-

serine lactones (AHLs) (11, 29, 31). The *Serratia* sp. ATCC 39006 QS system modulates secondary metabolism via the transcriptional regulation of four other regulators (CarR, an AHL-independent LuxR regulator; Rap, similar to RovA from *Yersinia*; PigR, an adenylate cyclase; and PigQ, a GacA response regulator) (11). Pig production in *Serratia* spp. can also be modulated by a number of environmental cues, including the availability of inorganic phosphate (15), carbon source (10), salt concentration, temperature, oxygen availability, and multiple metal ion concentrations (reviewed in reference 35).

Previously, PigP was identified as a master regulator of secondary metabolism in *Serratia* sp. ATCC 39006 (11). PigP is the founding member of a novel class of transcriptional regulators present in a restricted number of *Enterobacteriaceae* and has been shown to control secondary metabolism via transcriptional regulation of seven other regulators (CarR; PigQ; PigR; Rap; PigS, an ArsR family regulator; PigV, a homologue of YgfX from *Escherichia coli*; and PigX, a homologue of CsrD from *Escherichia coli* containing a GGDEF/EAL domain) (11). Thus, the PigP regulon overlaps the QS regulon in *Serratia* sp. ATCC 39006.

Fineran et al. (11) identified *pigS* following a transposon mutagenesis screen due to the observed reduction in Pig production following disruption of *pigS*. In addition, expression of *pigS* was activated by the master regulator PigP (11). Sequencing revealed that *pigS* encoded a putative ArsR/SmtB family transcriptional regulator (11). ArsR/SmtB family regulators are metalloregulatory transcriptional repressors which can

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TABLE 1. Bacterial strains and phage used in this study

Strain or plasmid	Genotype/phenotype	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80 Δ dlacZM15 Δ (lacZYA-argF)U169 endA1 recA1 hsdR17 (r _K ⁻ m _K ⁺) deoR thi-1 supE44 λ ⁻ gyrA96 relA1	Gibco/BRL
SM10 λ pir	thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu λ pir Km ^r	7
<i>Serratia</i>		
LacA (parental strain; WT)	Lac ⁻ derivative of <i>Serratia</i> sp. ATCC 39006, made by EMS mutagenesis	31
Mutants derived from LacA		
13S26L	<i>pigP</i> ::mini-Tn5Sm/Sp <i>pigS</i> ::mini-Tn5lacZ1 Sp ^r Km ^r	11
HSPIG26	<i>pigS</i> ::mini-Tn5lacZ1 Km ^r	11
KFAAG6	<i>pigA</i> ::TnuidACm Cm ^r	N. Williamson, unpublished data
PIG13S	<i>pigP</i> ::mini-Tn5Sm/Sp Sp ^r	11
TG70	<i>pigS</i> ::mini-Tn5lacZ1 <i>pigA</i> ::TnuidACm Km ^r Cm ^r	This study
Plasmids		
pQE-80L	Protein expression vector; Ap ^r	Qiagen
pQE-80LoriT	pQE-80L containing <i>oriT</i> from RP4; Ap ^r	J. Ramsay, unpublished data
pMAT29	His ₆ -PmpB expression vector, pQE-80LoriT based; Ap ^r	This study
pTA25	<i>pigS</i> promoter <i>lacZ</i> fusion (-86 to +44), Tc ^r	This study
pTA27	Native PigS expression vector, pQE-80L based; Ap ^r	This study
pTA132	PmpABC expression vector, pQE-80LoriT based; Ap ^r	This study
pTA133	BlhAOrfY expression vector, pQE-80LoriT based; Ap ^r	This study
pTA134	PmpABC, BlhA-OrfY expression vector, pQE-80LoriT based; Ap ^r	This study
pTA154	BlhA expression vector, pQE-80LoriT based; Ap ^r	This study
pTA155	OrfY expression vector, pQE-80LoriT based; Ap ^r	This study
pTA156	PmpBC expression vector, pQE-80LoriT based; Ap ^r	This study
pTA157	PmpC expression vector, pQE-80LoriT based; Ap ^r	This study
pTA159	PmpAC expression vector, pQE-80LoriT based; Ap ^r	This study
pTA160	PmpA expression vector, pQE-80LoriT based; Ap ^r	This study
pTA161	PmpB expression vector, pQE-80LoriT based; Ap ^r	This study
pTA162	PmpAB expression vector, pQE-80LoriT based; Ap ^r	This study
pTG7	His ₆ -PigS expression vector, pQE-80L based; Ap ^r	This study
pTG41	<i>blhA</i> promoter <i>lacZ</i> fusion (-151 to +18); Tc ^r	This study

control the expression of genes linked to stress-inducing concentrations of heavy metals (5). Binding of ArsR/SmtB family regulators to metal ions, including Zn(II), Cd(II), Pb(II), Bi(II), Co(II), Ni(II) and Sb(II), has been observed (5). Upon metal binding, the DNA binding affinity of the protein is reduced, allowing derepression (5).

Here, we investigate the role of PigS in the regulation of Pig production. The *pigS* gene is the first gene in an operon encoding three putative membrane proteins of the COG2391 (clusters of orthologous groups) and COG0730 families, which is divergently transcribed from a second operon encoding BlhA, a metallo- β -lactamase superfamily protein, and OrfY, a coenzyme A-disulfide reductase containing a rhodanese homology domain (CoADR-RHD). Here we show that PigS binds directly to these divergent promoters and represses transcription and that in the absence of Pig, the master regulator, PigP, is required for activation. Currently, 30 to 40% of genes in newly sequenced bacteria have no known function (4, 13), and although challenging, it is clearly important to study these genes to understand bacterial physiology (26). We show that the balance between the *pmpABC* and *blhA-orfY* operons and their specific proteins is important for determining Pig levels and provide bioinformatic data that proteins of these families are functionally linked. In addition, we propose from *in silico* analyses that COG2391 proteins (including PmpAB), constitute transporters for sulfur-containing compounds and dem-

onstrate that PmpB is localized to the cell membrane. To the best of our knowledge, this is the first experimental evidence confirming the membrane localization of any COG2391 protein.

MATERIALS AND METHODS

Bacterial strains, phage, and culture conditions. Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were grown at 37°C and *Serratia* sp. ATCC 39006 strains were grown at 30°C in LB medium (5 g liter⁻¹ yeast extract, 10 g liter⁻¹ Bacto tryptone, 5 g liter⁻¹ NaCl) with shaking or on LB agar supplemented with 1.5% (wt/vol) agar. When required, medium was supplemented with antibiotics at the following concentrations; ampicillin (Ap), 100 mg ml⁻¹; spectinomycin (Sp), 50 mg ml⁻¹; tetracycline (Tc), 35 mg ml⁻¹. The generalized transducing phage ϕ OT8 (8) was used as described previously (31).

DNA manipulations and sequence analyses. All molecular biology techniques, unless stated otherwise, were performed by standard methods (28). Sequence similarity searches were performed using BLAST (2), subcellular localization of proteins was predicted using PSORTb v3.0 (38), transmembrane spanning regions were detected using TMHMM v2.0 (21), and figures were generated using TOPO2 (www.sacs.ucsf.edu/TOPO2/). Functional linkages based on factors such as gene neighborhood, gene fusion, cooccurrence, and coexpression data were determined using STRING (18) set to COG-mode. Domain architectures were viewed using Pfam (http://pfam.sanger.ac.uk/), and some specific comparisons were made using MicrobesOnline (1). To determine the sequence of the *pigS* locus, a *Serratia* sp. ATCC 39006 cosmid library was constructed using the Epicentre Biotechnologies pWEB-TNC cosmid cloning kit, per the manufacturer's directions. A cosmid carrying the *pigS* locus was identified by PCR using primers PF64 (5'-GTACGAATTCAAAGGATCCCTATG-3') and PF65 (5'-CTGACTGCAGCAATATTAATGGTC-3'), and primer walking was used to sequence the complete *pigS* locus.

Prodigiosin, β -galactosidase, and β -glucuronidase assays. Prodigiosin production was assayed as described by Slater et al. (29). β -Galactosidase and β -glucuronidase activities were determined as described by Fineran et al. (11) and Gristwood et al. (15), respectively.

Mapping the transcriptional start site of *pigS* and RT-PCR. The transcriptional start of *pigS* was determined by 5' RACE (rapid amplification of cDNA ends) as described previously (12), except primers PF88 (5'-GAGTAAAACGA GGACTTTCGG-3'), PF87 (5'-CTAAGTCTGCGATAATGTTG-3'), and PF86 (5'-GTACTGCAGACGATCACTATTAGCC-3') were used as specific primers. RNA for reverse transcriptase PCR (RT-PCR) was extracted from an early stationary-phase culture (10 h growth in LB medium) of *Serratia* strain LacA. A total of 250 ng of RNA was used for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers (Amersham). PCR was performed using primers PF64 and JCO24 (5'-TTTGGTACCCGAA TTTGCGAACCCATTAG-3'), PF264 (5'-TTTGAATTCGAAATAGAAGGC GCAGGTC-3') and PF265 (5'-CCACCTAAAGCTTCTGAGGG-3'), and PF266 (5'-TTTGAATTCGCGACGTAAGGAGTTAACC-3') and PF269 (5'-C GGACGCCATCTCGATATC-3'). For quantitative RT-PCR, RNA was extracted and cDNA synthesis was performed as described above using *Serratia* strains HSPIG26, PIG13S, and 13S26L. The quantitative PCRs (qPCRs) were performed using the Applied Biosystems SYBR green PCR mix and an Applied Biosystems Prism 7300. Primers OTG156 (5'-CAGCACCGTCAGTTACGTT-3') and OTG157 (5'-GACCTGATCGCGTAAATAGTG-3') were used to detect *blhA* mRNA, and primers 3916SF and 3916SR (36) were used to detect 16S rRNA. Cycle threshold (C_T) values were calculated using ABI SDS software, and relative gene expression was calculated using 16S rRNA as the internal control and *blhA* mRNA/16S rRNA equal to a value of 1 in strain LacA.

Cellular localization experiments. A construct that enabled expression of N-terminally His-tagged PmpB (pMAT29) was generated using primers MMO75 (5'-GATGGATCCATGAACTTACTTTTT-3') and PF305 (5'-TTTAAGCTTG ATTAATGCCTTTGTCTGG-3'). The PCR fragment was digested with EcoRI/HindIII and cloned into pQE-80LoriT, a derivative of pQE-80L containing the RP4 *oriT*. The plasmid generated (pMAT29) was conjugated into LacA using the *E. coli* donor strain SM10 λ pir. LacA(pMAT29) was grown until an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 was reached, induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and incubated overnight. Cells were pelleted at 10,000 \times g for 20 min, washed twice, and then resuspended in 50 mM potassium phosphate and lysed by several passages through a French press at 16,000 lb/in². Cellular debris was removed by centrifugation at 27,000 \times g for 15 min. The supernatant was subjected to centrifugation at 100,000 \times g for 90 min. The supernatant (containing soluble fraction) was decanted, while the membrane pellet was washed twice in 50 mM potassium phosphate buffer and resuspended in 2 ml of potassium phosphate buffer. Protein quantification was determined using bicinchoninic acid (BCA) assays from Thermo Scientific, and fractions were kept on ice at 4°C before Western blot analysis using mouse monoclonal anti-His antibody (Sigma) and, as a secondary antibody, goat anti-mouse IgG-horseradish peroxidase (Santa Cruz). Bands were visualized on a ChemiDoc imaging system (Bio-Rad) using the SuperSignal West Pico chemiluminescent substrate kit (Pierce).

Plasmids for expression of *pmpABC* and *blhA-orfY*. Vectors were created to allow expression of the *pmpABC* and *blhA-orfY* operons as outlined below. The *pmpABC* and *blhA-orfY* operons were amplified by PCR, using primer pair PF264 and PF265 and primer pair PF266 and PF267 (5'-TTTAAGCTTCTCG AGTCAGGCGGACAACGTC-3'), respectively. PCR fragments were cloned into pQE-80LoriT, giving plasmids pTA132 (*pmpABC*) and pTA133 (*blhA-orfY*). A plasmid (pTA134) for overexpression of both *pmpABC* and *blhA-orfY* operons from divergent IPTG-inducible promoters was made by cloning the *blhA-orfY* region from pTA133 on XhoI sites into pTA132 cut with XhoI. A plasmid expressing *blhA* (pTA154) was constructed by digesting pTA133 with EcoRI and SphI and ligating the fragment containing *blhA* into pQE-80LoriT. An OrfY expression plasmid (pTA155) was generated by PCR using primers PF302 (5'-TTTGAATTCAAAAAGGAGTTTGTCTGTG-3') and PF267 and ligating the EcoRI/HindIII-digested product into pQE-80LoriT. A series of plasmids with different combinations of *pmpABC* were generated. Plasmid pTA132 (*pmpABC*) was digested with BamHI/HindIII, which removed *pmpBC*, blunted with Klenow fragment, and religated, giving plasmid pTA160 (*pmpA*). The *pmpB* and *pmpAB* genes were amplified using primer MMO67 (5'-GATGAATTCAGGAGGACA GGGATGAACCTTACTTTTT-3') or PF264 and PF305 (5'-TTTAAGCTTGT TAATGCCTTTGTCTGG-3'), and the EcoRI/HindIII-digested products were ligated into pQE-80LoriT, giving plasmids pTA161 (*pmpB*) and pTA162 (*pmpAB*), respectively. Next, *pmpBC* and *pmpC* were amplified with primer MMO67 or MMO69 (5'-GATGAATTCAGGAGGACAGGGATGTTTCATTTCATTA-3') and primer PF265, and the products were ligated into pQE-80LoriT, resulting in

plasmids pTA156 and pTA157, respectively. To generate a plasmid expressing PmpAC, pTA132 was digested with BamHI/SphI, blunted with Klenow fragment, and religated to yield plasmid pTA159. Plasmids were conjugated into LacA and HSPIG26 using *E. coli* SM10 λ pir. Where indicated, expression of these plasmids was induced with 0.1 mM IPTG.

Construction of *pigS* and *blhA* promoter *lacZ* fusions and assay conditions. The *pigS* and *blhA* promoters were amplified using primers PF58 (5'-GCATGA ATTCGGTAACTCCTTAC-3') and PF59 (5'-GCACAAGCTTAGGGATCC TTTC-3') and primers OTG173 (5'-ATGTGAATTCGCTAATCGTCGCGTT ATCC-3') and OTG172 (5'-ATGTAAGCTTCTCGATGTGTGTGTCATGG-3'), respectively. The resulting PCR products were cloned into EcoRI/HindIII-digested pRW50 (10, 22), giving plasmids pTA25 and pTG41, and promoter expression was determined as described previously (10).

Native and His-tagged PigS expression plasmids. Constructs that enabled expression of native, untagged PigS, and hexahistidine-tagged PigS (His₆-PigS; PigS protein with the N-terminal extension MRGSHHHHHHGS) were created as outlined below. The *pigS* gene was amplified by PCR, using primers PF64 and PF65 for native PigS or OTG3 (5'-ATAGGCATCGATAACGCGACGATTA GCC-3') and OTG4 (5'-ATAGAAGCTTTAATGGTCATGACCTGCG-3') for His₆-PigS. The resulting PCR fragments were cloned into pQE-80L, yielding plasmids pTA27 and pTG7, respectively. Expression of plasmid pTG7 in both *E. coli* and *Serratia* sp. ATCC 39006 was induced with 1 mM IPTG.

Purification of His₆-PigS. A 500-ml culture of strain HSPIG26 carrying pTG7 was grown to an OD_{600} of 0.6 to 0.8, induced with 1 mM IPTG, and incubated overnight at 16°C. Cells were harvested by centrifugation at 4°C, and His₆-PigS was purified using Ni-nitriloacetic acid (Qiagen) as described previously (14). Purified His₆-PigS was dialyzed overnight at 4°C against 2 liters of storage buffer (250 mM NaCl, 20 mM Tris-HCl [pH 7.9], 1 mM EDTA, 1 mM dithiothreitol [DTT], 50% [wt/vol] glycerol) and stored at -20°C.

EMSA. The *pigS-blhA* intergenic region was PCR amplified from LacA genomic DNA using primers PF58 and OTG42 (5'-AGGGATCCTTTCACAA CTAAG-3'), resulting in a 130-bp DNA fragment (fragment A). Truncated fragments of the *pigS-blhA* intergenic region were obtained by PCR amplification from LacA genomic DNA using forward primers OTG41 (5'-CTATTGCCTTA AACAA-3'), OTG43 (5'-CAAGACTTGAATTAATATAC-3'), OTG44 (5'-A TATTATATTATATAACATTG-3'), and OTG68 (5'-ATAACATTGTAAAGT GTTTTGTG-3'), and the reverse OTG42 to give 105-bp (fragment B), 84-bp (fragment C), 64-bp (fragment D), and 52-bp (fragment E) DNA fragments, respectively. DNA fragments were 3'-end labeled with DIG-11-ddUTP using terminal transferase, and electrophoretic mobility shift assay (EMSA) reactions were carried out according to the DIG gel shift kit protocol (Roche). Each 20- μ l reaction mixture contained the indicated amount of His₆-PigS and 1.5 nM DIG-labeled DNA in binding buffer [20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.02% (wt/vol) Tween, and 30 mM KCl], 5 μ g ml⁻¹ poly-L-lysine, and 50 μ g ml⁻¹ poly[d(I-C)]. Where indicated, reaction mixtures also contained a 50-fold excess (75 nM) of unlabeled competitor DNA. The *pigS-blhA* fragment A was used as a specific competitor, and a promoter DNA fragment from *Pectobacterium carotovorum* was used as a nonspecific competitor (kindly donated by Tom Burr). Polyacrylamide gel electrophoresis, electroblotting, and chemiluminescent detection were carried out according to the DIG gel shift kit protocol (Roche).

RESULTS

PigS is a transcriptional regulator of Pig biosynthesis. It was unknown to what extent the ArsR/SmtB family regulator PigS affected prodigiosin production and whether this was due to transcriptional control of *pigA-O*. In a *pigS* mutant strain, Pig production was reduced by approximately 50% throughout growth compared with Pig production by the WT (throughout this study, WT refers to the LacA parental strain) (Fig. 1A). There was no effect on Car or AHL production in the *pigS* mutant strain compared with the WT (data not shown). Pig was restored to WT levels following complementation of the *pigS* mutation in *trans* (Fig. 1B), confirming that the absence of *pigS* was responsible for the observed phenotype. Expression of a chromosomal *pigA::uidA* transcriptional fusion was reduced 1.5-fold throughout growth in a *pigS* mutant background compared with its expression in the parental strain (Fig. 1C).

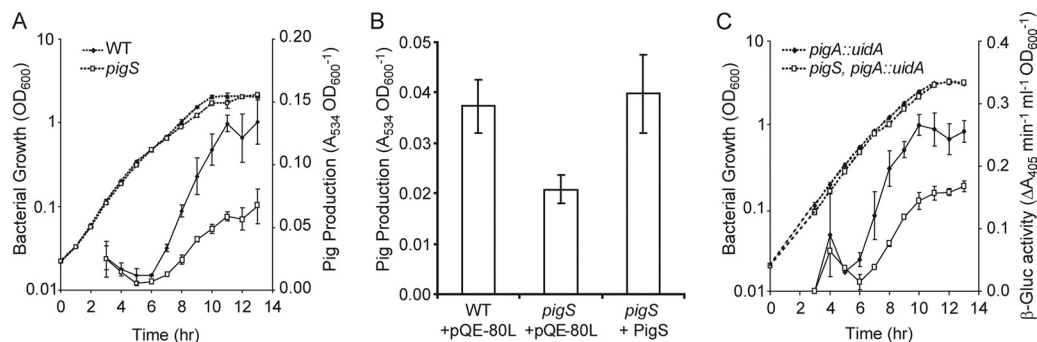


FIG. 1. Pig production and *pigA-O* transcription are reduced in a *pigS* mutant. (A) Pig production by the WT (diamonds) or a *pigS* mutant strain (HSPIG26) (unfilled squares) throughout growth. (B) Pig production by the WT and a *pigS* mutant (HSPIG26) in the presence of empty vector control pQE-80L and Pig production by a *pigS* mutant (HSPIG26) in the presence of pTA27, encoding native PigS at late exponential phase (9 h), are shown. (C) β -Glucuronidase activity was measured from a chromosomal *pigA::uidA* fusion in an otherwise WT background (KFAAG6) (diamonds) or in a *pigS* mutant background (TG70) (unfilled squares) throughout growth. Solid lines represent either Pig or β -glucuronidase assays, and dashed lines represent bacterial growth. Data shown are the means \pm SD of the results of three independent experiments.

Therefore, the phenotypic effects on Pig production following mutation of *pigS* were occurring, at least in part, at the level of transcription of the Pig biosynthetic operon, consistent with PigS being a predicted transcriptional regulator.

Sequence analysis of the *pigS* locus. Sequencing of the region surrounding *pigS* revealed five additional genes predicted to be in two operons (Fig. 2A) that were closely linked to the *pigA-O* biosynthetic cluster; the translational start of *pigA* is located 3.6 kb upstream of the start of *pigS*. The *pmpA* and *pmpB* (putative membrane protein) genes encode putative COG2391 membrane proteins (also known as the YeeE/YedE or DUF395; domain of unknown function). The *pmpC* gene encodes a COG0730 (TauE/Safe or DUF81) (20, 33) putative membrane protein. For consistency, we will refer to COG numbers throughout this article. The *blhA* and *orfY* genes, divergently transcribed from *pigS*, are predicted to encode two cytoplasmic proteins, a metallo- β -lactamase superfamily protein (COG0491) and a coenzyme A-disulfide reductase con-

taining a rhodanese homology domain (CoADR-RHD; COG0446 and COG0607).

***blhA-orfY* and *pigS-pmpABC* are divergently transcribed operons.** The *pigS* transcriptional start site was mapped, a single transcriptional start site (+1) was identified, and located upstream of this are putative -10 and -35 regions, based on the *E. coli* σ^{70} consensus sequences (16). Putative -10 and -35 regions were also predicted for the *blhA* promoter. However, attempts to map the +1 start site were unsuccessful (data not shown). Two inverted repeat sequences were identified within the *pigS-blhA* intergenic region; these were predicted to be regulatory protein binding sites (Fig. 2B). RT-PCR was performed to determine whether the six genes in the *pigS* locus were transcribed as two operons. RT-PCR of regions spanning the gene junctions generated products of the predicted size (Fig. 2C) with no products in the control lacking RT (-RT control) (data not shown). These results indicated that the

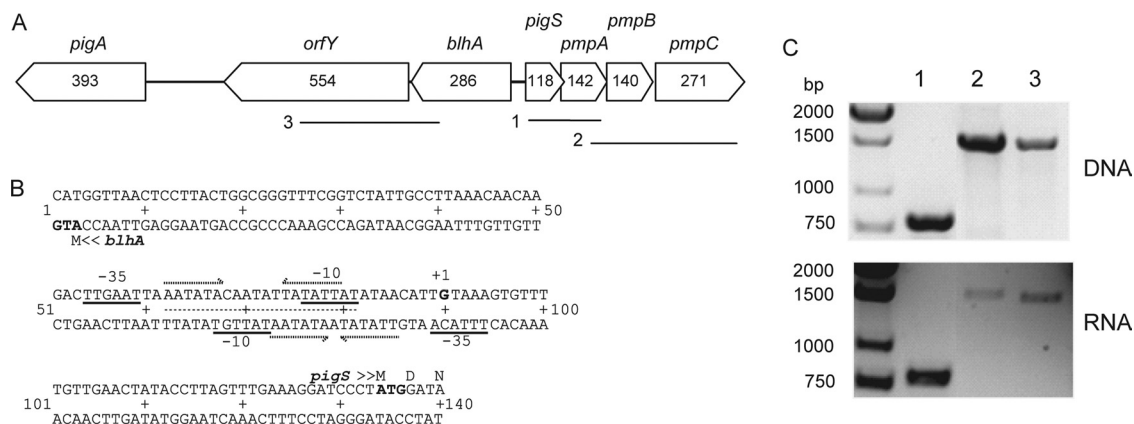


FIG. 2. The *pigS* locus. (A) The *Serratia* sp. ATCC 39006 *pigS* locus consists of two divergent operons, bearing six genes. Numbers shown represent the lengths in amino acids of the putative protein products. Solid lines indicate products generated by RT-PCR, as described in Materials and Methods and shown in panel C. (B) The *pigS-blhA* intergenic region. The *pigS* transcriptional start site is denoted +1, and potential -10 and -35 sites of the *pigS* and *blhA* promoters are underlined. Inverted repeats are indicated by dashed arrows, and a dotted line indicates a conserved BigR box consensus sequence (3). (C) RT-PCRs were performed to determine the operonic nature of *blhA-orfY* and *pigS-pmpABC*. RT-PCR products were generated with the following primer combinations: 1, PF64 and JCO24; 2, PF264 and PF265; and 3, PF266 and PF269. The locations of the PCR products are shown in panel A.

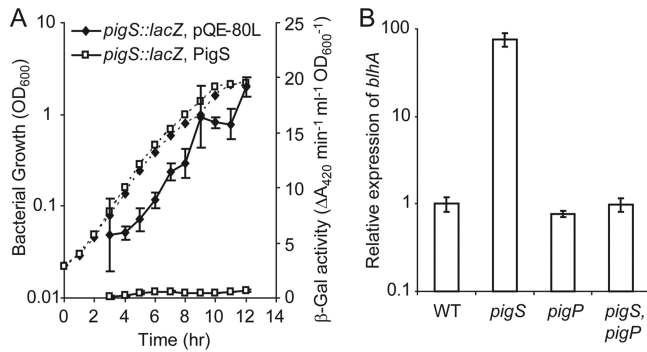


FIG. 3. PigS autoregulates and, along with PigP, controls expression of *blhA*. (A) β -Galactosidase activity was measured from a chromosomal *pigS::lacZ* fusion (HSPIG26) in the presence of an empty vector control, pQE-80L (diamonds), or pTA27, encoding native PigS (unfilled squares), throughout growth. Solid lines represent β -galactosidase assays, and dashed lines represent bacterial growth. (B) Quantitative RT-PCR was used to measure *blhA* transcript levels, relative to the WT (LacA), in a *pigS* mutant (HSPIG26), a *pigP* mutant (PIG13S), and a *pigS pigP* double mutant (13S26L). Data shown are the means \pm SD of the results of three independent experiments.

blhA-orfY genes are bicistronic and that the *pigS-pmpABC* genes comprise a four-gene operon.

Expression of *pigS-pmpABC* and *blhA-orfY* is controlled by PigP and PigS. The transcriptional organization, and the similarity of PigS to ArsR family transcriptional repressors, suggested that PigS might control the expression of the *pigS-pmpABC* and *blhA-orfY* operons. Expression of a chromosomal *pigS::lacZ* transcriptional fusion was measured throughout growth in the presence of plasmid-encoded PigS or an empty vector control. β -Galactosidase activity was reduced by PigS, indicating that PigS is negatively autoregulatory and represses expression of *pigS-pmpABC* (Fig. 3A). In contrast, it was previously shown that the master regulator PigP activates *pigS-pmpABC* transcription (11). Quantitative RT-PCR was performed to investigate whether PigS and PigP also regulate transcription from the divergent *blhA* promoter (Fig. 3B). Expression of *blhA* was increased 75-fold in a *pigS* mutant compared with the WT, indicating that PigS represses transcription of *blhA-orfY* from the *blhA* promoter. Mutation of *pigP* returned *blhA* transcripts to WT levels in the *pigS* mutant background but had no effect in a WT background. These data indicate that *blhA-orfY* expression is activated by PigP but only when there is also derepression in the absence of the repressor, PigS. While the effect of either protein might be direct or indirect based on these data, we will show later that the PigS effects are direct.

PmpA, PmpB, and PmpC are predicted to be inner membrane proteins involved in the transport of sulfur-containing compounds. The PigS- and PigP-mediated control of *pigS-pmpABC* prompted further analysis of PmpABC. PmpA and PmpB are members of the uncharacterized COG2391 family of putative inner membrane proteins with a conserved cysteine and four transmembrane helices (Fig. 4A and B). PmpC is a member of the COG0730 family of proteins, which Weinitschke et al. (33) proposed to be novel permeases for the transport of anions (typically sulfur containing) across cytoplasmic membranes. Proteins of this group have been impli-

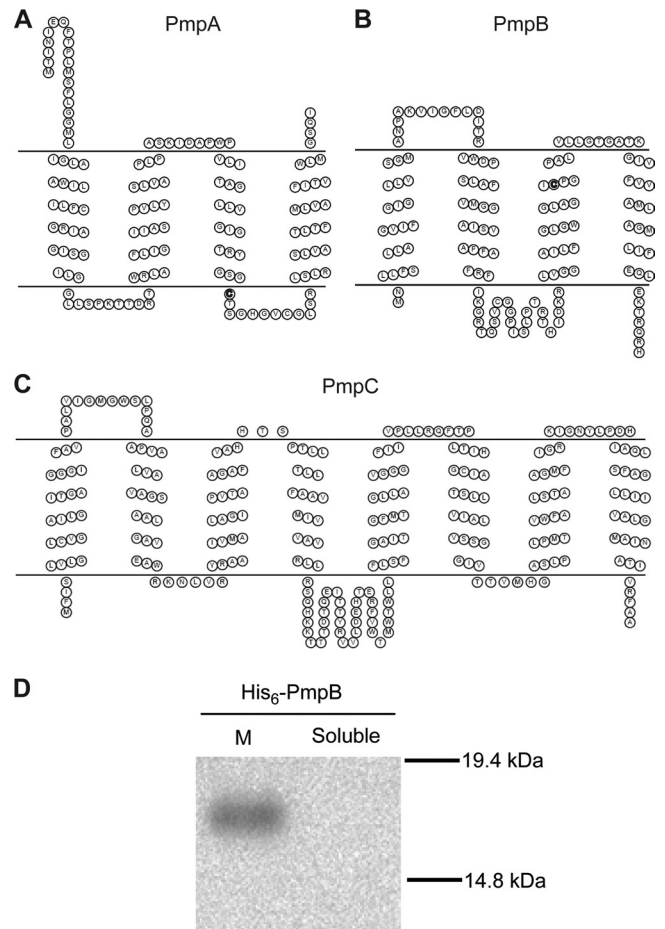


FIG. 4. PmpABC are predicted to localize to the membrane. Topology mapping of PmpA (A), PmpB (B), and PmpC (C) was performed using TMHMM v2.0 (21), and figures were generated using TOPO2. The highly conserved cysteine residues in PmpA and PmpB are shown in bold. (D) Cellular localization of N-terminally His-tagged PmpB (His₆-PmpB) expressed in LacA was performed as described in Materials and Methods. Fifty micrograms of total protein from the membrane fraction (M) or soluble fraction was analyzed by Western blotting to detect the presence of His₆-PmpB.

cated in the uptake of 4-toluenesulfonate (19, 23), sulfate, and sulfite (27) and in the export of sulfite (33) and sulfoacetate (20). PmpC was predicted to contain eight transmembrane domains (Fig. 4C). Since there is no experimental evidence demonstrating the membrane localization of COG2391 or COG0730 proteins, we performed cellular fractionation experiments to localize PmpABC. Unfortunately, despite testing multiple induction conditions, we could not express N-terminally His-tagged or C-terminally FLAG-tagged PmpA or PmpC (data not shown). However, His₆-PmpB was overexpressed in the WT and, following cellular fractionation, was detected by Western blotting in the membrane fraction yet was absent from the soluble fraction (Fig. 4D).

To gain insight into the possible role of COG2391 (PmpAB) proteins, STRING (18) was used in COG-mode. STRING identifies putative functional linkages between proteins by weighting and integrating information about factors such as their gene neighborhood, gene fusion, cooccurrence, and co-

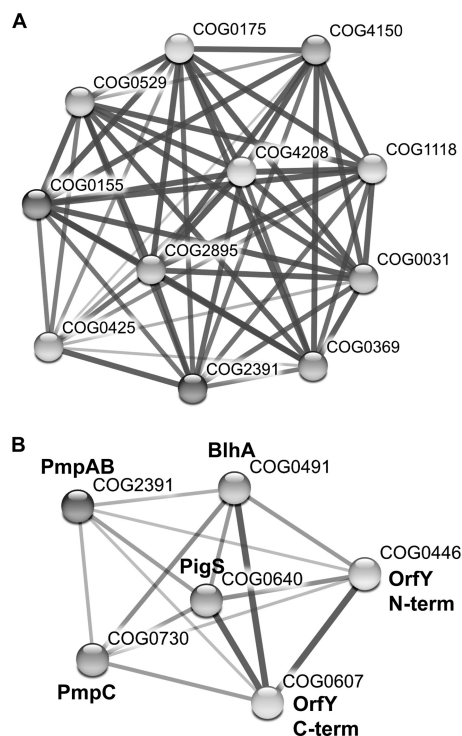


FIG. 5. PmpAB proteins are associated with proteins involved in sulfur metabolism, and members of the *pigS-pmpABC* and *blhA-orfY* system are frequently associated. (A) Phylogenomic profile of functions related to COG2391 (PmpAB; DUF395) shows possible interactions generated in STRING (high confidence, 10 top interactors shown). Shown are COG0425 (SirA), a predicted regulator of disulfide bond formation; COG2895, GTPase-sulfate adenylate transferase subunit 1; COG4208, ABC-type sulfate transport system, permease component; COG0031, cysteine synthase; COG0175, 3-phosphoadenosine 5-phosphosulfate sulfotransferase; COG0529, adenylylsulfate kinase and related kinases; COG1118, ABC-type sulfate/molybdate transport systems, ATPase component; COG0155, sulfite reductase, beta subunit (hemoprotein); COG4150, ABC-type sulfate transport system, periplasmic component; and COG0369, sulfite reductase, alpha subunit (flavoprotein). (B) Putative functional interactions between all six domain members of the *blhA-orfY* and *pigS-pmpABC* operons. In both panels A and B, the thickness of the lines relates to the degree of confidence in an association.

expression. Functional relationships were predicted, and the top 10 are shown in Fig. 5A. Associated functions include SirA, involved in disulfide bond formation, and other proteins involving sulfur-containing compounds (for details, see Fig. 5A). Interestingly, COG2391 (PmpAB) domains are also found fused as a single protein to SirA (COG0425) or COG0607 (C-terminal of OrfY) domains in some species. Therefore, we propose that PmpAB are involved in the transport of sulfur-containing molecules and may be linked to proteins involved in sulfur transfer reactions (see below).

BlhA and OrfY are predicted to function in sulfur transfer reactions with PmpABC. The coregulation of *pmpABC* and *blhA-orfY* indicated that they might be involved in similar processes. Insight into the role of OrfY comes from the recent crystal structure of the dimeric *Bacillus anthracis* protein BaCoADR-RHD (32). Like OrfY, BaCoADR-RHD contains both a coenzyme A disulfide reductase (COG0446) and a rhodanese homology domain (COG0607). The physiological role

of BaCoADR-RHD is unclear but via an interesting mechanism is proposed to reduce polysulfide (S_5^{2-}) to HS^- using flavin adenine dinucleotide (FAD) as a cofactor and reducing equivalents from NADH. We predict that OrfY is involved in disulfide reduction, but the substrate is unknown. BlhA is a member of the metallo- β -lactamase superfamily and is related to the ubiquitous COG0491 family of Zn-dependent hydrolases. COG0491 proteins include β -lactamases, thiolesterases, lactonases, and glyoxalases, but their diverse functions make it difficult to accurately predict the function of BlhA. Gene neighborhood analyses (STRING [18]) using COG0491 (BlhA) demonstrated that four of the eight most commonly linked domains belonged to COG0730 (PmpC), COG0446 and COG0607 (both OrfY), and PigS (COG0640), and similar results were obtained using either domain of OrfY as the query (data not shown). Interestingly, there are examples of gene fusions between COG0491 (BlhA) and COG0446 and/or COG0607 (OrfY) domains, and COG0607 (OrfY C terminus) is also found fused to COG0640 (PigS). To investigate the associations of all domains in the divergent *pigS-pmpABC* and *blhA-orfY* operons, STRING was used (COG queries) to predict functional linkages between these six different domains (Fig. 5B). Our data presented suggest that BlhA and OrfY are acting in similar processes related to sulfur transfer or disulfide reduction reactions that are linked to the PmpABC transport components of sulfur-containing molecules.

Overexpression of *blhA-orfY*, relative to *pmpABC* levels, decreases Pig production. We hypothesized that the decrease in Pig production in the *pigS* mutant was the result of increased expression of *blhA-orfY* in the absence of the PigS repressor. Furthermore, due to the transposon insertion within *pigS*, it was likely that *pmpABC* would no longer be under correct PigS-mediated control. To test this model, and the proposed functional linkage of both divergent operons, we measured the effect of overexpression of *blhA-orfY* (pTA133), *pmpABC* (pTA132), or both the *blhA-orfY* and *pmpABC* operons (pTA134) on Pig production in a WT or *pigS* mutant background. Overexpression of *blhA-orfY* in the WT or *pigS* mutant resulted in reduced Pig production and growth inhibition (Fig. 6A). Overexpression of *pmpABC* in the WT or *pigS* mutant resulted in increased Pig production (Fig. 6A). However, the concomitant overexpression of *blhA-orfY* and *pmpABC* restored Pig production, and growth, to levels comparable to those of the parental strains, indicating that the balance between expression levels of these operons is important for Pig production and for avoiding growth inhibition (Fig. 6A).

To assess the role of BlhA and OrfY further, the effect of overexpressing *blhA* or *orfY* on Pig production was measured in a WT background. As overexpression of *blhA-orfY* in the WT or *pigS* mutant had been shown to result in a growth defect (Fig. 6A), we also assessed the impact of leaky *blhA* and *orfY* expression from the pQE-80L promoter in the absence of IPTG induction. Leaky expression of *blhA-orfY* resulted in reduced Pig production without growth inhibition, but leaky expression of either *blhA* or *orfY* did not reduce Pig levels or growth (Fig. 6B). However, following IPTG induction, overexpression of BlhA or OrfY alone caused reduced Pig production but did not inhibit growth. This demonstrates that both BlhA and OrfY affect Pig levels and that when overexpressed together they are detrimental to growth.

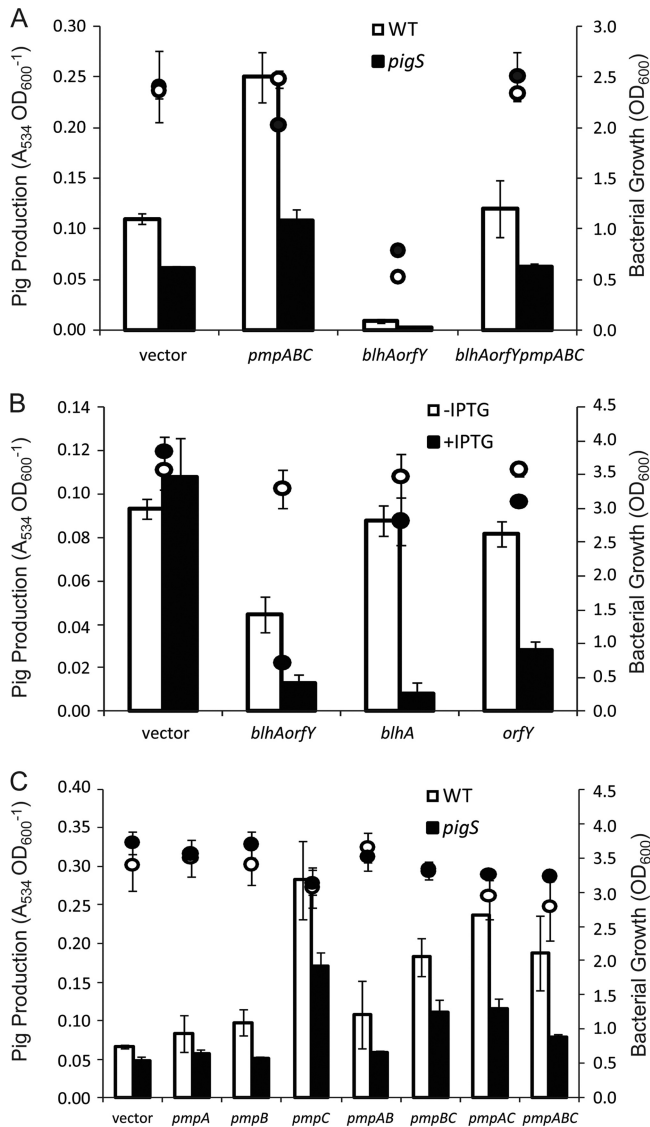


FIG. 6. Overexpression of PmpABC and/or BlhA-OrfY. (A) Pig production by WT (white bars) or *pigS* mutant strains (HSPiG26) (black bars) after 12 h of growth carrying pQE-80LoriT (vector), pTA132 (*pmpABC*), pTA133 (*blhA-orfY*), and pTA134 (*pmpABC blhA-orfY*). Bacterial growth is indicated by white (WT) or black (*pigS* mutant) circles. (B) Pig production by WT carrying pQE-80LoriT (vector), pTA133 (*blhAorfY*), pTA154 (*blhA*), and pTA155 (*orfY*) after 12 h of growth. Cultures were grown in the absence of IPTG induction (white bars) or with 0.1 mM IPTG (black bars). Bacterial growth is indicated by white (-IPTG) or black (+IPTG) circles. (C) Pig production by WT (white bars) or *pigS* mutant strains (HSPiG26) (black bars) carrying pQE-80LoriT (vector), pTA160 (*pmpA*), pTA161 (*pmpB*), pTA157 (*pmpC*), pTA162 (*pmpAB*), pTA156 (*pmpBC*), pTA159 (*pmpAC*), or pTA132 (*pmpABC*) after 12 h of growth. Bacterial growth is indicated by white (WT) or black (*pigS* mutant) circles. Except where indicated, cultures were induced with 0.1 mM IPTG. Data shown are the means \pm SD of the results of three independent experiments.

Similarly, the effect of *pmpABC* on Pig production was investigated in more detail. Overexpression of *pmpA* or *pmpB*, or *pmpAB*, did not significantly affect Pig production in either the WT or *pigS* mutant (Fig. 6C). Overexpression of *pmpC* in the

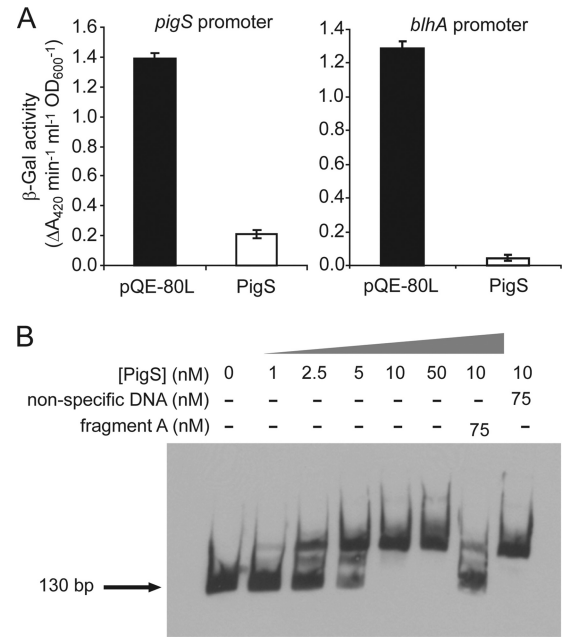


FIG. 7. PigS represses the *pigS* and *blhA* promoters by direct binding to the *pigS-blhA* intergenic region. (A) β -Galactosidase activity was measured in *E. coli* carrying pTA25 or pTG41 (*pigS* or *blhA* promoters upstream of a promoterless *lacZ* gene) and either an empty vector control (pQE-80L) (black bar) or pTA27, encoding native PigS (white bar). Data shown are the means \pm SD of the results of three independent experiments. (B) Binding of His₆-PigS to the *pigS-blhA* intergenic region (fragment A) was assessed using EMSAs. Each lane contains 1.5 nM DIG-labeled fragment A DNA and the indicated amount of His₆-PigS. Competition experiments were carried out in which a 50-fold excess (75 nM) of either unlabeled fragment A or a nonspecific fragment was added.

WT or *pigS* mutant caused an increase in Pig production greater than that caused by overexpression of the entire *pmpABC* operon. Overexpression of *pmpBC* or *pmpAC* partially attenuated the hyperpigmentation observed following overexpression of *pmpC* alone (Fig. 6C). These data suggest that PmpC is responsible for the increased Pig levels following overexpression of the *pmpABC* operon and that PmpA and PmpB partially limit or oppose the phenotypic effect of PmpC.

PigS regulates transcription via direct binding to the *pigS-blhA* intergenic region. The regulation and overexpression data above indicated that PigS was a repressor of the divergent *blhA-orfY* and *pmpABC* operons, the products of which affect Pig production. To characterize PigS repression further in a heterologous host, β -galactosidase production from *E. coli* strains carrying vectors with either the *pigS* or *blhA* promoter fused to a promoterless *lacZ* (pTA25 or pTG41) was measured in the presence or absence of PigS (pTA27 and pQE-80L). In both cases, promoter activity was decreased by PigS (Fig. 7A), indicating that PigS represses transcription, possibly via direct binding to the *pigS-blhA* intergenic region without requiring additional *Serratia* sp. ATCC 39006 proteins (e.g., PigP). Since PigS is an ArsR family transcriptional regulator, this reporter system was used to investigate whether PigS-mediated repression was allosterically inhibited by the presence of metal ions [Fe(III), Cd(III), Zn(II), Ni(II), Mn(II), Mg(II), Cu(II), Li(I), Ag(I), K(I)]. However, derepression was not observed, provid-

coordinately regulated (by PigS and PigP) but that these genes represent a complete functional system.

Despite numerous COG2391 (PmpAB) proteins in bacteria, archaea, and eukaryotes, to the best of our knowledge, there is no experimental evidence suggesting their function. We demonstrated that PmpB was localized to the cell membrane and provide the first experimental data supporting the prediction that COG2391 proteins are integral membrane proteins. Based on functional partnership predictions, we propose a possible role of COG2391 proteins (and PmpAB) as transporters of sulfur-containing compounds. Interestingly, COG2391 proteins contain a highly conserved cysteine (Fig. 4), which might enable disulfide bond formation or the generation of a reactive persulfide that could participate in S transfer reactions (24). In *E. coli*, cobalt stress was recently shown to disrupt Fe/S biogenesis and decrease *yeeE* (COG2391) by ~2.6-fold (9). Furthermore, *yeeE* was activated by IscR, the master regulator of Fe/S biogenesis in *E. coli*.

COG0730 (PmpC) proteins are present in bacteria, archaea, and eukaryotes, and a number of studies indicate that COG0730 proteins may import or export sulfur-containing molecules in bacteria (19, 20, 23, 27, 33). Despite the genetic linkage between PmpAB and PmpC (and their COG groups), and the possibility that they are involved in transport of S compounds, COG2391 and COG0730 are not always found together. This suggests that these proteins can act together in a process in some organisms but perform functionally separable tasks. Indeed, our expression data show that PmpC (COG0730) acts independently but that PmpA and PmpB (COG2391) can temper the effects of PmpC. What is transported via this system awaits identification, but it, or a product of BlhA and/or OrfY, may be the inducer(s) of PigS, enabling expression of these operons under appropriate conditions. Of interest are two highly conserved cysteines present in the PigS/BigR subfamily of ArsR regulators (3), which we predict could be involved in modulating its DNA-binding activity.

Experimental and bioinformatic data demonstrate that OrfY and BlhA are linked to a process similar to that of PmpABC with regard to Pig production. It is likely that OrfY is involved in disulfide reduction reactions and the mechanism is understood (32) but the substrate is unknown. The evidence suggests that BlhA is acting in concert with OrfY, but the details are unclear due to the diverse roles of COG0491 (BlhA) proteins. BlhA is unlikely to act as an AHL lactonase since levels of lactone were unaffected in the BlhA-overexpressing *pigS* mutant (data not shown). Likewise, a β -lactamase function of the related BigR system from *X. fastidiosa* and *A. tumefaciens* could not be demonstrated (3).

Due to the challenges involved in deciphering the function of conserved uncharacterized proteins, there is still much to learn about the role of the *pigS* locus and related genes in other species. Based on the current data, we propose one possible model in which the enzymes OrfY and BlhA generate a S-containing product (z), which is currently unidentified. Product z may indirectly inhibit *pigA-O* transcription and hence Pig levels. PmpC acts as a transporter, exporting z and maintaining appropriate intracellular levels. PmpAB partially inhibit or modulate this PmpC-mediated effect, possibly by also acting as transporters. Mutation of *pigS*, or overexpression of *blhA-orfY*, increases levels of z within the cell, causing reduced Pig pro-

duction. The concomitant overexpression of *pmpABC* allows excess z to be exported, and thus appropriate intracellular z levels, and Pig, are restored. Overexpression of PmpABC results in reduced intracellular z levels, and hence Pig levels are increased.

In conclusion, we have characterized in detail an ArsR family transcriptional regulator which modulates expression of the prodigiosin antibiotic genes via the control of divergent operons that are predicted to constitute a system involved in the transport and modification of a S-containing compound(s). In addition, we have provided experimental and bioinformatic evidence that PmpB (COG2391) is membrane associated and have provided a prediction on the function of this uncharacterized family of proteins in the transport of sulfur-containing molecules. This study represents a step forward in understanding the role of COG2391 and COG0730 proteins and their association with metallo- β -lactamase (COG0491) and CoADR-RHD (COG0446 and COG0607) proteins. These components are important in the control of prodigiosin antibiotic production in *Serratia* sp. ATCC 39006, presumably by functioning in the transport and modification of sulfur-containing molecules. The clear phenotypes, and their newly hypothesized roles, will provide a highly tractable system to further unravel the function of these poorly characterized proteins and their regulation by PigS.

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