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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Received 30 March 2010/Accepted 14 December 2010

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 (SmallR), 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 homoserine 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...
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), and although challenging, it is clearly important to study these...<br><br>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 demonstrate 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, plasmids, 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 φDOS8 (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 cosmI library was constructed using the Epicentre Biotechnologies pWEB-TNC cosmI cloning kit, per the manufacturer's directions. A cosmI carrying the pigS locus was identified by PCR using primers PFM6 (5'–GTACGAATTCAAGGATCCATTG-3') and PF65 (5’–TGACTGCAGCAATATTAGTGTC-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 PF85 (5′-GAGTGAAAGCAGGACGTTTCGG-3′), PF87 (5′-CTAATGCTGCGACTATATGGCTGGT-3′), and PF86 (5′-CTGCTCCACGACTCACTATACC-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 (Amer sham). PCR was performed using primers PF64 and JC24 (5′-TTTGTTAATCCTAATATGCAGAC GACGCTT-3′) and PF265 (5′-CCACCTAAAGGTCTTGAGGAG-3′) and PF266 (5′-TCTTGATCGCTAGTATGCTAT-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 PCR (qPCRs) were performed using the Applied Biosystems SYBR green PCR mix and an Applied Biosystems Prism 7300. Primers OTG156 (5′-GATGAAATGCTGCGACTATATGGCTGGT-3′) and OTG158 (5′-GACC TTGATCGCTAGTATGCTAT-3′) were used to detect pigS mRNA, and primers 3916SF and 3916SR (36) were used to detect 16S rRNA. Cycle threshold (Ct) values were calculated using ABI SDS software, and relative gene expression was calculated using 16S rRNA as the internal control and bglA mRNA as a reference equal to a value of 1 in strain LacA.

Cellular localization experiments. A construct was created that enabled expression of N-terminally His-tagged PigS (pMAT29) was generated using primers MM070 (5′-GATGAAATGCTGCGACTATATGGCTGGT-3′) and PF305 (5′-TTTGAATTCAAAAAAGGAGTT TGCTGTG-3′), and the products were ligated into pQE-80LoriT, giving plasmids pTA132 (pTA131) and pTA160 (pTA159). Plasmids were conjugated into LacA and HSPIG26 using E. coli SM10 Δpir. Where indicated, expression of these plasmids was induced with 0.1 mM IPTG.

Construction of pigS and blhA promoter fusions and assay conditions. The pigS and blhA promoters were amplified using primers PF85 (5′-GATGAAATGCTGCGACTATATGGCTGGT-3′) and PF86 (5′-CTGCTCCACGACTCACTATACC-3′) and primers OTG173 (5′-GATGAAATGCTGCGACTATATGGCTGGT-3′) and OTG175 (5′-GACCTGATCGCTAGTATGCTAT-3′) were used to detect pigS mRNA, and primers 3916SF and 3916SR (36) were used to detect 16S rRNA. Cycle threshold (Ct) values were calculated using ABI SDS software, and relative gene expression was calculated using 16S rRNA as the internal control and bglA mRNA as a reference equal to a value of 1 in strain LacA. PCR was performed using primers PF64 and JCO24 (5′-TTTGTTAATCCTAATATGCAGAC GACGCTT-3′) and PF265 (5′-CCACCTAAAGGTCTTGAGGAG-3′) and PF266 (5′-TCTTGTTAATCCTAATATGCAGACGACGCTT-3′) and primers 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 Δpir. Where indicated, expression of these plasmids was induced with 0.1 mM IPTG.

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Purification of HsPigS. A 500-ml culture of strain HSPIG26 carrying pTF7 was grown to an OD600 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 HsPigS was purified using Ni-NTA agarose (Qiagen) as described previously (14).

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).
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 DU9395; domain of unknown function). The pmpC gene encodes a COG0730 (TauE/SafE or DUF81) 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-/H9252-lactamase superfamily protein (COG0491) and a coenzyme A-disulfide reductase containing 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 σ70 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 (data not shown). These results indicated that the

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 ± SD of the results of three independent experiments.

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-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.
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. /H9252-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.

\[blhA-orfY\] 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 Weintitschke 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 implicated 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 PmpB (data not shown). However, His6-PmpB was overexpressed in the WT and, following cellular fractionation, was detected by Western blotting in the membrane fraction (M) or soluble fraction was analyzed by Western blotting to detect the presence of His6-PmpB.
The coregulation of reactions with PmpABC.

sulfur transfer reactions (see below).

Therefore, we fused as a single protein to SirA (COG0425) or COG0607

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ing 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 PmpABC 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 pro-

cesses. 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 rham-
dean homology domain (COG0607). The physiological role

of BaCoADR-RHD is unclear but via an interesting mecha-
nism is proposed to reduce polysulfide (S\textsubscript{2}\textsuperscript{−}) to HS\textsuperscript{−} 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-\beta-lactamase superfAMILY and is related to the ubiquitous COG0491 family of Zn-dependent hy-
drolases. COG0491 proteins include \beta-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 pre-
dict 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-8L 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.
Similarly, the effect of\textit{pmpABC} on Pig production was investigated in more detail. Overexpression of\textit{pmpA} or\textit{pmpB}, or\textit{pmpAB}, did not significantly affect Pig production in either the WT or\textit{pigS} mutant (Fig. 6C). Overexpression of\textit{pmpC} in the

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

\textit{PigS} regulates transcription via direct binding to the\textit{pigS-blhA} intergenic region. The regulation and overexpression data above indicated that\textit{PigS} was a repressor of the divergent\textit{blhA-orfY} and\textit{pmpABC} operons, the products of which affect Pig production. To characterize\textit{PigS} repression further in a heterologous host,\textit{pQE-80L}-galactosidase production from\textit{E. coli} strains carrying vectors with either the\textit{pigS} or\textit{blhA} promoter fused to a promoterless\textit{lacZ} (pTA25 or pTG41) was measured in the presence or absence of\textit{PigS} (pTA27 and pQE-80L). In both cases, promoter activity was decreased by\textit{PigS} (Fig. 7A), indicating that\textit{PigS} represses transcription, possibly via direct binding to the\textit{pigS-blhA} intergenic region without requiring additional\textit{Serratia} sp. ATCC 39006 proteins (e.g.,\textit{PigP}). Since\textit{PigS} is an ArsR family transcriptional regulator, this reporter system was used to investigate whether\textit{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-
FIG. 8. PigS binds directly to the pigS-blhA intergenic region. (A) The pigS-blhA intergenic region (fragment A) and truncated fragments (fragments B to E) were generated by PCR as described in Materials and Methods. The predicted pigS and blhA −10 and −35 sequences are represented by unfilled and filled boxes, respectively. A dashed line indicates the location of the putative BigR box consensus sequence. (B) Binding of His6-PigS to truncated fragments of the pigS-blhA intergenic region was assessed using EMSAs. Each lane contains a 1.5 mM concentration of the appropriate fragment of DIG-labeled DNA and either 0 or 10 nM His6-PigS, as indicated in the figure. (C) The predicted PigS binding region shows similarity (17/20 nucleotides) to the BigR box consensus sequence, which has 100% conserved bases represented by capital letters (3). The blhA and pigS −10 sequences are underlined. An “x” indicates nucleotides that do not match the BigR consensus; a colon indicate nucleotides that match.

The genomic context of pigS shares some similarity with the BigR locus from the plant pathogen Xylella fastidiosa 9aSc and other members of the Rhizobiaceae (3). Barbosa and Benedetti show that in X. fastidiosa, the ArsR/SmtB family regulator, BigR, controls transcription of a five-gene operon, encoding a metallo-β-lactamase superfamily protein (COG0491-COG3453 fusion), BigR, two COG2391 (PmpAB) proteins, and a COG0730 (PmpC) protein (3). However, the bigR locus does not encode an orthologue of OrfY. This operon was implicated in biofilm growth in X. fastidiosa and in Agrobacterium tumefaciens. BigR bound directly to a 20-bp region spanning the blh −10 sequence, and a BigR box consensus sequence was proposed (3). PigS bound the pigS-blhA intergenic region near a BigR box like sequence (17/20 matches to BigR box consensus), which overlaps the predicted pigS and blhA −10 sequences (Fig. 8C). However, PigS was still able to bind to the 64-bp fragment D, in which 8 nucleotides of the putative BigR-like box had been removed, indicating that either a BigR-like half-site is sufficient to bind PigS or that the BigR box is not required for binding. We suggest that in the absence of a specific inducer molecule, PigS represses transcription of the biosynthetic genes via direct binding to the blhA and pigS promoters. In this study, we set out to determine the role of the ArsR family protein PigS, a member of the PigP regulon, in the control of Pig production in Serratia. PigS repressed the level of Pig at least partly via the transcription of the biosynthetic genes pigA-O. However, PigS did not directly activate, or bind near, the pigA promoter. Despite the lack of direct regulation of pigA-O, PigS was shown to repress transcription from two divergent operons which drive the expression of six genes (blhA, orfY, pigS, pmpA, pmpB, and pmpC) via direct binding to the blhA-pigS intergenic region. Since pigS is within one of these operons, it is autoregulated. In addition, PigP, the founding member of a novel class of transcriptional regulators (11), activates transcription from the blhA and pigS promoters in the absence of repression by PigS. It is currently unknown whether PigP acts directly at the pigS and blhA promoters.

The DISCUSSION

In this study, we set out to determine the role of the ArsR family protein PigS, a member of the PigP regulon, in the control of Pig production in Serratia. PigS repressed the level of Pig at least partly via the transcription of the biosynthetic genes pigA-O. However, PigS did not directly activate, or bind near, the pigA promoter. Despite the lack of direct regulation of pigA-O, PigS was shown to repress transcription from two divergent operons which drive the expression of six genes (blhA, orfY, pigS, pmpA, pmpB, and pmpC) via direct binding to the blhA-pigS intergenic region. Since pigS is within one of these operons, it is autoregulated. In addition, PigP, the founding member of a novel class of transcriptional regulators (11), activates transcription from the blhA and pigS promoters in the absence of repression by PigS. It is currently unknown whether PigP acts directly at the pigS and blhA promoters.

To more precisely identify the binding site(s) of His6-PigS, truncated fragments of the pigS-blhA intergenic region (Fig. 8A) were used in an EMSA (Fig. 8B). His6-PigS bound to the 130-, 105-, 84-, and 64-bp fragments (fragments A, B, C, and D) but did not bind to the 52-bp fragment (fragment E). This implied that the His6-PigS binding site lies within the region overlapping the pigS −10 region sequence. Using EMSAs, it was determined that there was no specific binding by His6-PigS to the pigA promoter nor did PigS affect expression of the pigA promoter cloned in E. coli, indicating that PigS does not directly regulate pigA-O transcription (data not shown).
coordinate transcriptionally (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 exporters 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, coxib 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 numerous COG2391 proteins in bacteria, COG0730 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-oversexpressing 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 production. 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.

ACKNOWLEDGMENTS

We thank Josh Ramsay for plasmid pQE-80LoriT and Neil William-son for strain KFAAG6.

This work was supported by the BBSRC, United Kingdom, the University of Otago, and the Marsden Fund, Royal Society of New Zealand. M.B.M. was supported by a Bright Futures Top Achiever doctoral scholarship from the Tertiary Education Commission of New Zealand.

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