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Virulence and Prodigiosin Antibiotic Biosynthesis in *Serratia* Are Regulated Pleiotropically by the GGDEF/EAL Domain Protein, PigX▽

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Gram-negative bacteria of the genus *Serratia* are opportunistic human, plant, and insect pathogens. *Serratia* sp. strain ATCC 39006 secretes pectinases and cellulases and produces the secondary metabolites carbapenem and prodigiosin. Mutation of a gene (*pigX*) resulted in an extremely pleiotropic phenotype: prodigiosin antibiotic biosynthesis, plant virulence, and pectinase production were all elevated. PigX controlled secondary metabolism by repressing the transcription of the target prodigiosin biosynthetic operon (*pigA-pigO*). The transcriptional start site of *pigX* was determined, and *pigX* expression occurred in parallel with Pig production. Detailed quantitative intracellular proteome analyses enabled the identification of numerous downstream targets of PigX, including OpgG, mutation of which reduced the production of the plant cell wall-degrading enzymes and virulence. The highly pleiotropic PigX regulator contains GGDEF and EAL domains with noncanonical motifs and is predicted to be membrane associated. Genetic evidence suggests that PigX might function as a cyclic dimeric GMP phosphodiesterase. This is the first characterization of a GGDEF and EAL domain protein in *Serratia* and the first example of the regulation of antibiotic production by a GGDEF/EAL domain protein.

Gram-negative bacteria of the genus *Serratia* are members of the family *Enterobacteriaceae* (22). Some species, such as *Serratia marcescens*, are becoming a major cause of nosocomial infections (25) and are often difficult to treat due to the prevalence of resistance to multiple antibiotics (48). *Serratia* sp. strain ATCC 39006 was originally isolated from a salt marsh in Cheesequake, NJ, by E. R. Squibb and Sons, Inc., in an effort to mine producers of novel antibiotics (39). In common with certain *S. marcescens* strains, *Serratia* strain ATCC 39006 produces the red, linear tripyrrole pigment prodigiosin (Pig; 2-methyl-3-pentyl-6-methoxyprodigiosin), a secondary metabolite with antimicrobial, immunosuppressant, and anticancer properties (56). Indeed, Pig is in preclinical anticancer trials, and a derivative is in phase I/II clinical trials as an anticancer agent (56). A simple β-lactam antibiotic, carbapenem (Car; 1-carbapen-2-em-3-carboxylic acid) (14), and plant cell wall-degrading exoenzymes (47) are also produced by *Serratia* strain ATCC 39006. *Serratia* strain ATCC 39006 is also virulent in *Caenorhabditis elegans* (15) and potato tuber-rotting (17) infection models, demonstrating a broad-host-range capacity for pathogenesis in insect and plant hosts.

The Pig biosynthetic pathway was recently elucidated and requires gene products from the *pigA-pigO* operon (24, 57), which is transcribed as a polycistronic mRNA (47). Considerable progress has been made in unraveling the complex regulatory network that governs the production of Pig in *Serratia* strain ATCC 39006. For example, an *N*-acyl homoserine lactone quorum-sensing system (55), encoded by the SmaIR locus, derepresses the transcription of the Pig biosynthetic genes in response to cell density by modulating the expression of at least three pigment regulators (20, 47, 51). In addition, molecular mechanisms involved in altering *pigA-pigO* expression in response to changes in inorganic phosphate and gluconate levels have been characterized (19, 47). The regulatory hierarchy of Pig biosynthesis in *Serratia* strain ATCC 39006 employs at least 15 genetic loci and has been reviewed recently (56).

Recently, we identified a novel master regulator, PigP, that controls the transcription of the *pigA-pigO* biosynthetic operon by modulating the expression of six other regulators (20). Random transposon insertion mutations within one of these members of the PigP regulon resulted in hyperpigmented phenotypes (20). Partial sequence analysis suggested that the gene disrupted in these, designated *pigX*, might encode a GGDEF/EAL domain protein (20). GGDEF/EAL domain proteins are involved in the synthesis and degradation of the bacterial intracellular secondary messenger bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) (32). The aim of the current study was to investigate the phenotypic consequences of the *pigX* mutation with respect to secondary metabolism and virulence in *Serratia* strain ATCC 39006. A combination of genetics and proteomic analyses enabled the dissection of pathways mediated by PigX in the control of virulence and secondary metabolism. Finally, we show that PigX contains GGDEF and EAL domains and provide genetic evidence that PigX might exert phenotypic effects by functioning as a c-di-GMP phosphodiesterase (PDE).

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and culture conditions. Bacterial strains and plasmids are listed in Table 1. *Serratia* sp. strain ATCC 39006 strains were grown at 30°C and *Escherichia coli* strains were grown at 37°C in Luria broth (LB;

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain/plasmid	Genotype/phenotype	Reference or source
Escherichia coli		
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA–argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	Gibco/BRL
ER2566	F$^-$ λ$^-$ *fhuA2* (lon) *ompT lacZ*::T7 *gene1 gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10* R(*mcr-73*::mini-Tn*10*–Tet^s)*2* R(*zgb-210*::Tn*10*)(Tet^s) *endA1* (dcm)	NEB
Serratia		
LacA (WT)	Lac$^-$ derivative of ATCC 39006	51
4SOPG	*pigX*::mini-Tn*5*Sm/Sp *opgG*::Tn-DS1028 Sp^r Cm^r	This study
4SPAL	*pigX*::mini-Tn*5*Sm/Sp *pigA*::mini-Tn*5lacZ1* Sp^r Km^r	This study
HSPIG66	*pigX*pro::mini-Tn*5lacZ1* Km^r (insertion + 16 in *pigX* transcript)	20
MCP2L	*pigA*::mini-Tn*5lacZ1* Km^r	47
NW34	*opgG*::Tn-DS1028; Cm^r	This study
ROP4	*pigX*::mini-Tn*5lacZ1* Km^r (insertion + 207 in *pigX* transcript)	20
ROP4S	*pigX*::mini-Tn*5*Sm/Sp Sp^r (insertion + 259 in *pigX* transcript)	20
Phage		
ϕOT8	*Serratia* generalized transducing phage	Crow et al., unpublished
Plasmids		
pBluescript II SK+	Cloning vector, ColE1 replicon, Ap^r	Stratagene
pNRW94	EAL domain (residues 77–362) of the *E. coli* protein YahA expression vector, pQE-80L based, Ap^r	This study
pNRW98	*opgG*::Tn-DS1028 plasposon clone (digested with NsiI and self-ligated), Cm^r	This study
pNRW104	N-terminal and GGDEF domain of PigX expression vector, pQE-80L based, Ap^r	This study
pNRW105	GGDEF domain of PigX expression vector, pQE-80L based, Ap^r	This study
pNRW106	Extended EAL domain of PigX expression vector, pQE-80L based, Ap^r	This study
pNRW107	EAL domain of PigX expression vector, pQE-80L based, Ap^r	This study
pNRW108	GGDEF and EAL domain of PigX expression vector, pQE-80L based, Ap^r	This study
pQE-80L	Expression vector for native or N-terminal hexahistidine proteins, Ap^r	QIAGEN
pTA40	Native PigX expression vector, pQE-80L based, Ap^r	This study
pTA41	pTA40 derivative with ^{424}ELI to ^{424}ALI point mutation, pQE-80L based, Ap^r	This study
pTA48	pTA40 derivative with ^{424}ELI to ^{424}AAA point mutations, pQE-80L based, Ap^r	This study

5 g/liter yeast extract, 10 g/liter Bacto tryptone, and 5 g/liter NaCl) at 300 rpm or on LB agar supplemented with 1.5% (wt/vol) agar (LBA). Bacterial growth (optical density at 600 nm [OD_{600}]) was measured in a Unicam Heλios spectrophotometer at 600 nm. When required, LB was supplemented with antibiotics at the following concentrations: kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; ampicillin, 50 μg/ml; and chloramphenicol, 25 μg/ml. The generalized transducing phage ϕOT8 was used for the transduction of chromosomal mutations as previously described (51).

DNA manipulations and sequencing. Molecular biology techniques, unless stated otherwise, were performed by standard methods (45). Oligonucleotide primers were obtained from Sigma Genosys and are listed in Table 2. DNA sequencing was performed at the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom. The nucleotide sequence data were analyzed using GCG (Genetics Computer Group, University of Wisconsin, WI) and compared with the GenBank DNA or nonredundant protein sequence databases using BLAST (2).

A partial sequence of *pigX* was determined previously (20) and was completed as described below. Random-primed PCR was performed as described previously (19), using the specific primers PF111 (first-round PCR) and PF112 (second-round PCR), which generated ~300 bp more of the *pigX* sequence. To complete the *pigX* sequence, a degenerate primer (PF117) was designed to the 5′ end of *mreB*, which was predicted based on sequence alignments to be located 3′ of *pigX* in *Serratia* strain ATCC 39006. Primers PF112 and PF117 were used to amplify two independent products that were sequenced on both strands. The completed *pigX* sequence was assembled and is depicted schematically in Fig. 1A.

The insertion site of transposon Tn-DS1028 in the *opgG* mutant was determined using random-primed PCR and the plasposon cloning approach. For random-primed PCR, primers facing out the right-hand side (NW128 and TBOL58) were used by following the method described previously (19). Plasposon cloning was performed as described previously (34). Plasposon pNRW98, containing the sequence flanking *opgG*, was generated by digesting the genomic DNA of the *opgG*::Tn-DS1028 mutant with NsiI followed by self-ligation. The plasposon was sequenced in a primer-walking strategy initially using primers NW127 and NW128.

Plasmid constructions. The different domains of *pigX* were amplified by PCR and cloned into the expression vector pQE-80L. The primers used, and their restriction sites, were PF120 (EcoRI) and PF121 (HindIII) for the full-length PigX (pTA40), PF120 (EcoRI) and NW148 (PstI) for the N-terminal GGDEF domain (pNRW104), NW147 (EcoRI) and NW148 (PstI) for the GGDEF domain (pNRW105), NW132 (BamHI) and PF121 (HindIII) for the extended EAL domain (pNRW106), NW133 (BamHI) and PF121 (HindIII) for the EAL domain (pNRW107), and finally, NW147 (EcoRI) and PF121 (HindIII) for the GGDEF and EAL domain construct (pNRW108). Note that primers PF120 and NW147 introduced consensus ribosome-binding sites (AGGAGGA).

To generate two plasmids with amino acid substitution mutations in the EAL domain of PigX (pTA41, ELI→ALI, and pTA48, ELI→AAA), an overlap PCR strategy was used. The *pigX* gene was amplified in two fragments. Primer pairs PF120 and PF129 (pTA41) or PF120 and PF136 (pTA48) were used to generate the N-terminal *pigX* fragments. Primer pairs PF121 and PF128 (pTA41) or PF121 and PF135 (pTA48) were used to generate the C-terminal *pigX* fragments. Next, the N- and C-terminal fragments were mixed and used as a template in a second PCR, using primers PF120 and PF121. The resulting PCR fragments of the *pigX* mutants were digested with EcoRI and HindIII and cloned into pQE-80L, previously digested with the same enzymes.

A construct that enabled the expression of an EAL domain known to function as a PDE was created as outlined below. The EAL domain (residues 77 to 362) of the *E. coli* protein YahA was PCR amplified from chromosomal DNA of *E. coli* ER2566 with primers NW130 and NW139 and then digested with SphI and cloned into the SphI site of pQE-80L, generating construct pNRW94. The veracity of all plasmids was confirmed by DNA sequencing.

5′ RACE to determine transcriptional start site of *pigX* and primer extension studies. Total RNA was extracted from wild-type (WT) *Serratia* strain ATCC 39006 using a QIAGEN RNeasy mini kit according to the manufacturer's instructions. 5′ RACE (*r*apid *a*mplification of *c*DNA *e*nds) was performed using a Roche 5′/3′ RACE second-generation kit, following the manufacturer's specifi-

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)
HS34	GCTGACTCATAAATATCTGACTG
NW128	GATAATAAGCGGATGAATGGCAG
NW130	CTGCATGCCGCGATATTTTCTTTCAGTAC
NW132	GAAGGATCCACGATTGCTGCTCAACTGGTG
NW133	GGGGGATCCTGGTGTGTGTATGACCGCCAG
NW139	GTTGCATGCAACCACCTGCTTTCATTAC
NW147	GGGGAATTCAGGAGGACAGGGATGATACA TTGATTCGCGCTTTC
NW148	CATACTGCAGTCAGCCATTCCCTCCTTGTA ATAC
PF92	GTATCTAGACCACTCGACAACATTAAG
PF93	GCACGAGTAAAGCCTGG
PF94	GCTTATGCCCGTCACG
PF106	GACCACACGTCGACTAGTGCNNNNNNNNNN AGAG
PF107	GACCACACGTCGACTAGTGCNNNNNNNNNN ACGCC
PF108	GACCACACGTCGACTAGTGCNNNNNNNNNN GATAC
PF109	GACCACACGTCGACTAGTGC
PF111	CAGGTTAATGATAGTGGCGGAC
PF112	CGATCCACTGATTGGTATACCTC
PF117	CRTTGGAAAACATGCCACG
PF120	GATGAATTCAGGAGGACAGGGATGGGATT TACTG
PF121	GTCTAAGCTTCTACGCCAATTCGCAC
PF128	AGGCGAAGTGCATCATCGAGCATTAATCAG CCGTATTTATG
PF129	CATAAATACGGCTGATTAATGCTCGATGAT GCACTTCGCCT
PF135	GCGAAGTGCATCATCGAGCAGCAGCCAGCC GTATTTATGATGGTT
PF136	AACCATCATAAATACGGCTGGCTGCTGCTC GATGATGCACTTCGC
TBOL58	CTAGAGTCGACCTGCAGGCATGCAAGC

cations. The following specific primers were used for mapping the transcriptional start of *pigX*: PF94, PF93, and PF92. The 5′ RACE PCR products were digested with XbaI (on PF92) and SalI (on the anchor primer) and cloned into pBluescript II SK+. To determine the transcriptional start site of *pigX*, a number of clones were sequenced, using primer M13-20, and analyzed. RNA extraction and primer extension analysis for the *pigA* transcript were performed as described previously, using primer HS34 (47). All primer extension reactions were performed with 25 μg of total RNA and 0.2 pmol of the appropriate ^{32}P-labeled primer.

Prodigiosin, Car, exoenzyme, swimming motility, β-galactosidase, and potato virulence assays. The assays for Pig and Car were performed as described previously (47). Pig production was plotted as $(A_{534}/\text{ml}/\text{OD}_{600}) \times 50$. The activities of pectinase (Pel) and cellulase (Cel) were analyzed on agar plates containing the substrates as described previously (4) and plotted as the halo area (cm^2). Swimming and β-galactosidase assays were performed as previously described (20). Potato-rotting assays were performed as described previously (8), except that potatoes were surface sterilized by submersion in 1% Virkon for 10 min and 10^3 CFU of each strain was used as the inocula. Experiments were performed at least seven times, and harvesting was done after 96 h. The mass of rot generated by each strain was compared, and the differences between the strains assessed for statistical significance using a two-tailed paired *t* test.

2D-DiGE proteomic analysis. Cell-associated (intracellular) two-*d*imensional *di*fference in *g*el *e*lectrophoresis (2D-DiGE) proteomics comparing the *pigX* mutant (ROP4S) with the WT strain was performed (biological replicates and reciprocally labeled), initially as described previously but with the detergent ASB-14 instead of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (16) and then in a more detailed manner as described below. LB (25 ml) was inoculated to a starting OD_{600} of 0.02, and four biological replicates of WT and ROP4S cultures were grown for 16 h at 30°C. Protein was harvested from the cultures as previously described (16) and quantified using a Bio-Rad protein quantification assay according to the manufacturer's instructions. The experimental approach was as described previously to allow biological variance analysis of the experimental data set using DeCyder version 5.0 (1, 36). In every gel ($n = 4$), 50 μg of WT and ROP4S protein samples were minimally labeled with either Cy3 or Cy5 and 50 μg of a pooled sample labeled with a third dye, Cy2, was loaded. Proteins were separated on 24-cm, pI 4 to 7, isoelectric-focusing strips and then in the second dimension on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (36), and the images were collected on a Typhoon 9410 (GE Healthcare) (16). To identify any spots that were significantly altered (Student's *t* test; $P < 0.01$), image analysis was performed using the DeCyder biological variance analysis software package (GE Healthcare), and spots of interest were excised and identified by tandem mass spectrometry (MS-MS) as previously described (36).

RESULTS

PigX is a GGDEF/EAL domain protein that represses prodigiosin. Previously, the prodigiosin master regulator, PigP, was shown to negatively affect the expression of the *pigX* gene. Hyperpigmented random transposon mutant strains (ROP4 and ROP4S) and a decreased pigment production mutant strain (HSPIG66) were identified in a screen for regulators of Pig biosynthesis and were mapped either to within *pigX* (ROP4 and ROP4S) or 5′ of *pigX* (HSPIG66) (20). The sequence and genomic context of *pigX* were completed and are depicted schematically in Fig. 1A. The 649-amino-acid (aa) predicted PigX protein is similar to YhdA from *E. coli* (64% similarity/54% identity) and is most closely related to the predicted product of ECA0266 from *Erwinia carotovora* subsp. *atroseptica* SCRI1043 (80% similarity/73% identity). Divergently transcribed from and 5′ of *pigX* is a 984-bp open reading frame, which is predicted to encode a homolog of YhdH from *E. coli* (76% similarity/69% identity), a putative quinone oxidoreductase. PigX is predicted to have two N-terminal transmembrane helices and a central GGDEF domain (aa 222 to 384), as well as a C-terminal EAL domain (aa 398 to 633) (Fig. 1B). Therefore, it was predicted that PigX might be involved in intracellular c-di-GMP metabolism.

PigX acts as a transcriptional repressor of prodigiosin biosynthesis. Pig production by the WT, the *pigX* mutant (ROP4), and the intergenic *pigX* mutant (HSPIG66) strains was measured throughout growth in LB. Pig production by the *pigX* mutant was increased by up to approximately 350% compared

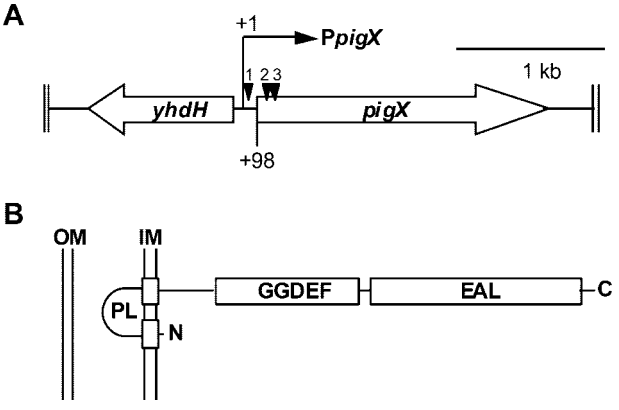

FIG. 1. The *pigX* gene encodes a GGDEF/EAL domain protein. (A) Genomic location of *pigX*, indicating the transcriptional start site (+1) and the insertion sites of transposons (black triangles) in strains HSPIG66 (1), ROP4S (2), and ROP4 (3). (B) Predicted GGDEF/EAL protein domain structure and inner membrane (IM) localization of PigX. OM, outer membrane; PL, periplasmic loop.

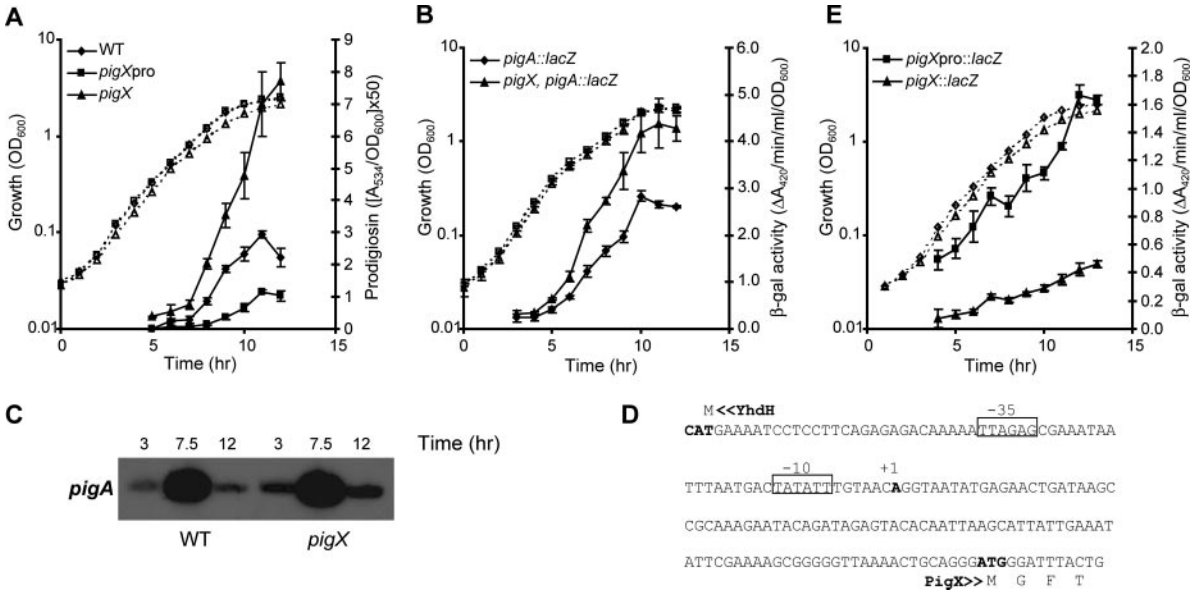

FIG. 2. PigX represses Pig production by controlling transcription of the Pig biosynthetic operon, *pigA-pigO*. (A) Pig levels in WT, HSPIG66 (*pigX*pro), and ROP4 (*pigX*) strains throughout growth in LB. (B) β-Galactosidase activity was measured from a chromosomal *pigA*::*lacZ* fusion in an otherwise WT background (MCP2L) or in a strain containing a mini-Tn*5*Sm/Sp chromosomal insertion in *pigX* (4SPAL). (C) Primer extension analysis of the *pigA-pigO* transcript from the WT and ROP4S (*pigX*) strains throughout the time of growth in LB. (D) *yhdH-pigX* intergenic region showing the transcription start site of *pigX* (+1) and the predicted −10 and −35 elements. (E) β-Galactosidase activity was measured from chromosomal *pigX*::*lacZ* and *pigX*pro::*lacZ* fusion strains throughout growth in LB. Solid symbols and lines represent the results of either Pig or β-galactosidase assays, whereas the open symbols and dashed lines represent the growth curves of the corresponding strains. Data shown are the means ± standard deviations of the results of at least three independent experiments. β-gal, β-galactosidase.

with the level in the WT, whereas the intergenic mutant produced between 30 and 40% of the WT Pig levels (Fig. 2A). In the intergenic mutant, the transcription of *pigX* is probably being driven from an internal mini-Tn*5lacZ1* promoter (18), resulting in deregulated levels of the PigX repressor, causing decreased Pig production. In the *pigX* mutants, the pigment was confirmed as prodigiosin by liquid chromatography-MS analysis and the production of another antibiotic, carbapenem, was shown to be unaffected (data not shown). Next, we assessed the effect of PigX on the transcription of *pigA-pigO*. The expression of a chromosomal *pigA*::*lacZ* fusion was increased by more than 150% in the *pigX* mutant strain compared with its expression in the WT background (Fig. 2B). This result was confirmed by primer extension analysis of the *pigA-pigO* transcript, which demonstrated increased *pigA-pigO* mRNA levels in the *pigX* mutant (Fig. 2C). Finally, the introduction of a plasmid containing the entire *pigX* gene (pTA40) complemented Pig production in a *pigX* mutant strain to the levels observed in a WT strain carrying the vector control (see Fig. 5). Therefore, the GGDEF/EAL domain protein, PigX, represses the biosynthesis of Pig by decreasing the transcription of the *pigA-pigO* operon.

***pigX* is transcribed throughout growth, in parallel with Pig production.** The transcriptional start of *pigX* was determined, with only one transcriptional start (+1) predicted to be located 98 bp 5′ of the predicted translational start (ATG). Promoter −35 and −10 regions were proposed based on the *E. coli* σ^{70} consensus sequence (23) (Fig. 2D). To examine the transcriptional profile of *pigX*, β-galactosidase activities from both the chromosomal *pigX*::*lacZ* fusion and the fusion 5′ of the *pigX* ATG were measured throughout growth in LB (Fig. 2E). The expression of *pigX* increased in parallel with growth and continued to increase in stationary phase (Fig. 2E). Interestingly, β-galactosidase activity from the intergenic fusion was approximately fourfold higher than that from the fusion in *pigX*. Therefore, the transcription of *pigX* increases throughout growth under the culture conditions used in the current study and mirrors the expression of the target *pigA-pigO* operon and Pig production (Fig. 2A and B).

Numerous proteins are altered in abundance in a *pigX* mutant. It was clear that the PigP regulon member PigX was an important secondary metabolite regulator in *Serratia* strain ATCC 39006 that controlled the transcription of the biosynthetic operon, *pigA-pigO*. To define cellular PigX targets, global proteomic analysis was used to determine the proteins that were altered in abundance in the *pigX* mutant in comparison to their abundance in the WT strain. In an initial 2D-DiGE proteomic analysis ("difference in gel analysis"), we detected that the PigA and OpgG (an *o*smoregulated *p*eriplasmic *g*lucan [OPG] biosynthesis protein) proteins, among others, were altered between the *pigX* mutant (ROP4S) and the WT (data not shown). This prompted a more thorough proteome analysis. Total cell-associated proteins were prepared from the *pigX* mutant (ROP4S) and the WT strains and compared using 2D-DiGE. Thirty-three protein spots were identified as being significantly altered (26 were increased and 7 were decreased) in the *pigX* mutant compared with their abundance in the WT. Of the variously abundant proteins, 23 were visible on a Coomassie blue-stained gel and were excised for MS analysis. A Cy2 image of the pooled sample is shown in Fig. 3, and the identities of the protein spots which gave single hits are given in Table 3. Several spots gave either no significant hit

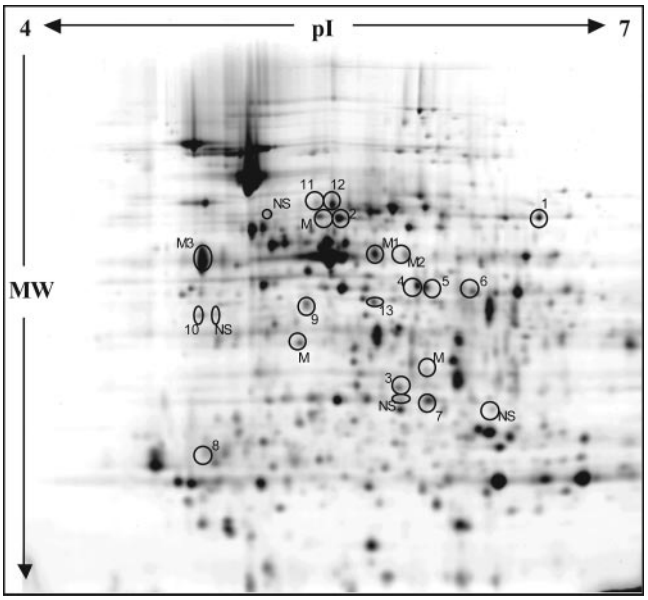

FIG. 3. The 2D-DiGE intracellular protein profile in the *pigX* mutant strain is altered in comparison to that in WT *Serratia* strain ATCC 39006. Cy2 image of the pooled sample of the 2D gel (24 cm, pI 4 to 7, 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel), with proteins which were picked for identification by MS circled. NS means that no significant hits were obtained by MS analysis, and M identifies the protein spot as a mixed hit. M1, M2, and M3 contained FabB (M1 and M2) and FliC (M3) mixed with elongation factor Tu. A list of proteins that were altered in abundance is provided in Table 3. MW, molecular weight.

or produced a mixed hit. The mixed hits are not included in Table 3, as it is not possible to determine which protein was actually altered in the mutant. These proteomic analyses demonstrated that PigX modulates the levels of multiple proteins, the significance of which will be covered in Discussion.

PigX represses exoenzyme production and virulence in planta via OpgG. Examination of the intracellular proteome of the *pigX* mutant strain indicated elevated levels of a protein from *Pseudomonas syringae* pv. tomato strain DC3000 with similarity to OpgG from *Erwinia chrysanthemi* (Table 3). In *Erwinia chrysanthemi*, the *opgGH* operon is involved in the synthesis of OPGs (6), and mutation of *opgG* resulted in dramatic reductions in the plant cell wall-degrading enzymes and virulence (38). We predicted that the upregulated levels of OpgG in the *pigX* mutant might result in an increase in exoenzyme production and a hypervirulent phenotype. Therefore, phenotypes associated with plant virulence, including Cel and Pel production and potato tuber rotting, were assessed. Pel activity was increased in the *pigX* mutant compared with its level in the WT strain (Fig. 4A), whereas Cel production was not detectably altered (Fig. 4B). Interestingly, overexpression of PigX in *trans* in the *pigX* mutant repressed the production of Pel (Fig. 4C) but had no detectable effect on Cel (data not shown). Finally, the *pigX* mutant displayed a statistically significant increase in virulence in planta based on potato tuber-rotting assays (Fig. 4D). Serendipitously, in an independent study we isolated a *Serratia* strain ATCC 39006 *opgG* transposon mutant with decreased swimming motility (Fig. 4E) and no effect on growth in LB or prodigiosin production (data not

TABLE 3. Intracellular proteins significantly altered in abundance in the *pigX* mutant compared with their levels in *Serratia* strain ATCC 39006

Relative level of *pigX* mutant	Spot no.[a]	Protein identity	Name[b]	Change in abundance (fold)	Bacterium[b]	Accession no.[b]
Increased	1	Glycerol kinase	GlpK	1.10	*Escherichia coli*	gi 1134966
	2	Aldehyde dehydrogenase	PutA	1.31	*Shewanella baltica* OS155	gi 68541744
	3	GTP cyclohydrolase I	Sden_1513	1.28	*Shewanella denitrificans* OS217	gi 91792870
	4	L-Prolyl-PCP dehydrogenase	PigA	2.22	*Serratia* strain ATCC 39006	gi 55581716
	5	Phosphoserine aminotransferase	SerC	1.17	*Yersinia enterocolitica*	gi 134441
	6	ABC-type branched-chain amino acid transport systems, periplasmic component	LivK	1.27	*Yersinia mollaretii* ATCC 43969	gi 77961773
	7	Acyl-carrier protein, enoyl reductase (NADH)	FabI	1.41	*Erwinia carotovora* subsp. *atroseptica*	gi 49611426
	8	Aminopeptidase N	PepN	1.69	*Rubrivivax gelatinosus* PM1	gi 47573617
	9	Predicted periplasmic lipoprotein involved in iron transport	YP_1538	1.13	*Yersinia pestis* biovar microtus strain 91001	gi 45436217
	10	HBC *O*-methyl transferase	PigF	2.07	*Serratia* strain ATCC 39006	gi 55581721
		Periplasmic glucan biosynthesis protein[c]	OpgG	1.78	*Pseudomonas syringae* pv. tomato strain DC3000	gi 28855528
Decreased	11 and 12	Aspartate ammonia-lyase 2	AspA	−1.34 −1.25	*Erwinia carotovora* subsp. *atroseptica*	gi 49610097
	13	Glycerol dehydrogenase	GldA	−1.26	*Erwinia carotovora* subsp. *atroseptica*	gi 49610315

[a] The spot number is the spot number in the 2D-DiGE image in Fig. 3.
[b] Protein name, bacterium name, and accession number refer to the top hits for the *Serratia* strain ATCC 39006 peptides identified by MS using MASCOT.
[c] The spot described here was picked from the original "difference in gel analysis" 2D-DiGE analysis (not shown) and identified by MS.

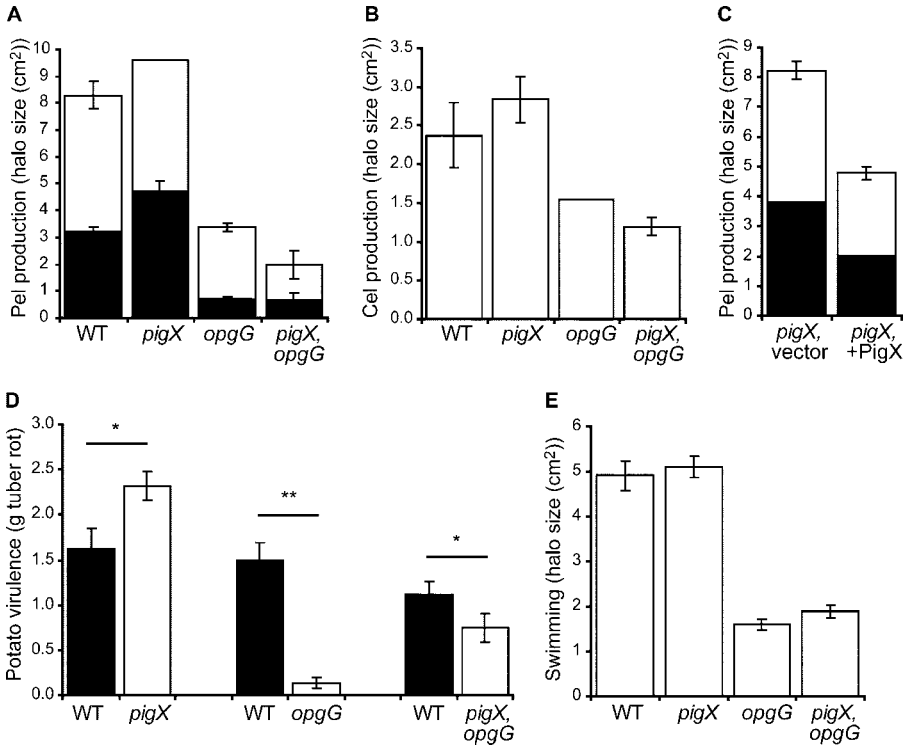

FIG. 4. PigX represses Pel activity and virulence in a potato tuber-rotting virulence assay. (A) Pel activities of the WT (LacA), a *pigX* mutant (ROP4S), an *opgG* mutant (NW34), and a *pigX opgG* double mutant (4SOPG) strain. Two halos of enzyme activity were observed and are shown as black (inner halo) and white (outer halo). (B) Cel activity of the WT (LacA), a *pigX* mutant (ROP4S), an *opgG* mutant (NW34), and a *pigX opgG* double mutant (4SOPG) strain. (C) Pel activity of the *pigX* mutant strain plus vector (ROP4 and pQE-80L) and the *pigX* mutant strain plus PigX (ROP4 and pTA40). (D) Results of potato tuber-rotting assays comparing the amount of rot caused by the WT with the amount caused by the *pigX* mutant (ROP4S), the *opgG* mutant (NW34), and the *pigX opgG* double mutant (4SOPG) strain, using 10^3 CFU as inocula. The difference between the WT and each mutant was statistically significant using a two-tailed paired *t* test with a *P* value of either <0.05 (*) or <0.001 (**), and the data shown are the means ± standard errors of the means of the results of at least seven independent experiments. (E) Swimming motility of the WT (LacA), a *pigX* mutant (ROP4S), an *opgG* mutant (NW34), and a *pigX opgG* double mutant (4SOPG) strain. Data shown are the means ± standard deviations of the results of at least three independent experiments (unless otherwise stated).

shown). Sequence analysis of the *opgG* region in *Serratia* strain ATCC 39006 (Materials and Methods) demonstrated that the predicted product, OpgG, was 88% similar and 85% identical (of 450 aa aligned) to OpgG from *Erwinia chrysanthemi*. The *Serratia opgG* gene is also predicted to be in an operon with *opgH*, as in *Erwinia* and *E. coli* (data not shown). Pel and Cel production levels in the *opgG* mutant were reduced compared with their levels in the WT (Fig. 4A and B), and the *opgG* mutant's virulence was dramatically reduced in planta (Fig. 4D). A *pigX opgG* double mutant displayed Pel and Cel production phenotypes slightly lower than those of the *opgG* single mutant (Fig. 4A and B). The *pigX opgG* double mutant strain had reduced virulence compared to that of the WT strain in potato tuber-rotting assays but was more virulent than the *opgG* single mutant strain (Fig. 4D). Therefore, the proteomic, exoenzyme, and virulence data suggest that PigX represses the level of OpgG, which is required for full exoenzyme production and plant pathogenicity in *Serratia* strain ATCC 39006.

PigX is predicted to function as a c-di-GMP PDE. It was of interest to examine whether PigX could be affecting virulence and secondary metabolism by modulating the levels of the intracellular secondary messenger c-di-GMP. Recently, biochemical data have provided unequivocal evidence that GGDEF domains possess diguanylate cyclase activity responsible for the synthesis of c-di-GMP from two molecules of GTP (40, 44). Conversely, EAL domains are c-di-GMP-specific PDEs required for the turnover of this secondary messenger (12, 46, 50). PigX contains a GGDEF domain with the noncanonical active-site sequence YHSDF instead of the conserved GGDEF motif, which is necessary for the synthesis of c-di-GMP (40, 44) (Fig. 5A). Therefore, PigX is unlikely to catalyze the production of c-di-GMP. In addition, PigX does not contain the conserved sequence RXXD (X is any amino acid) of the inhibitory c-di-GMP-binding site situated adjacent to the GGDEF motif (10, 32). Analysis of the EAL domain of PigX revealed that PigX contains the amino acids ELI (Fig. 5A). Multiple functional studies have demonstrated the importance of the EAL motif in the c-di-GMP PDE activity (29, 50, 53). Our bioinformatic analyses led to the hypothesis that PigX might function (via its EAL domain) as a c-di-GMP-specific PDE.

Cultures of the WT, the *pigX* mutant (ROP4), and the *pigX*-pro mutant (HSPIG66) strains were grown in the presence of $^{32}P_i$, and nucleotides were extracted and analyzed by 2D thin-layer chromatography to examine total intracellular c-di-GMP levels as previously described (5, 26, 52). Unfortunately, despite good extractions and separation of nucleotides, c-di-GMP was not detected in any of the cultures (data not shown).

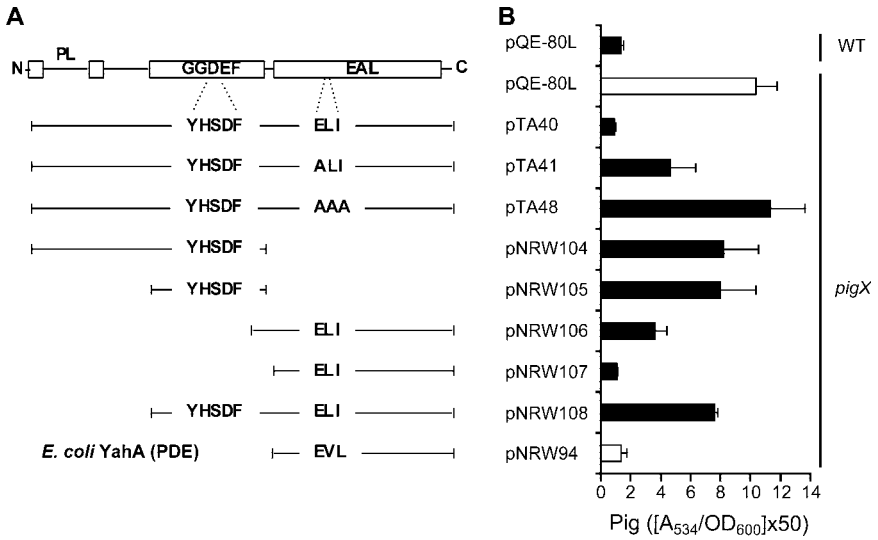

FIG. 5. PigX is predicted to function as a c-di-GMP PDE. (A) Schematic of domain organization of PigX and regions cloned into pQE-80L. The sequences of the divergent motifs in the GGDEF and EAL domains are shown (YHSDF and ELI, respectively). Also shown (bottom) is the domain of the *E. coli* PDE YahA that was cloned into pQE-80L. PL, periplasmic loop. (B) Pig assays of WT and *pigX* mutant (ROP4) strains in the presence of the PigX plasmids and YahA plasmid shown in panel A. Data shown are the means ± standard deviations of the results of at least three independent experiments.

Therefore, a genetic strategy was used to determine the possible function of PigX.

Alanine substitution mutants were constructed in the ELI motif, resulting in two plasmids with altered forms of PigX. A plasmid with an E424A (ELI→ALI) substitution mutation could partially repress Pig production in a *pigX* mutant strain, demonstrating that the function of PigX was impaired (Fig. 5A and B). An E424A L425A I426A (ELI→AAA) triple amino acid substitution mutant plasmid could not complement the *pigX* mutant for the biosynthesis of Pig (Fig. 5A and B). These complementation experiments suggested that the ELI motif was essential for the activity of PigX. To further examine the roles of the different PigX domains, a series of plasmids was constructed encoding different domains of PigX (Fig. 5A). Strikingly, the EAL domain alone fully complemented Pig production in the *pigX* mutant strain back to WT levels (Fig. 5A and B). Furthermore, plasmids expressing the GGDEF domain alone or in combination with the potential membrane-spanning region had no significant effect on Pig production in the *pigX* mutant. In addition, the expression of a construct possessing both GGDEF and EAL domains, but not the membrane spanning regions, failed to complement the *pigX* mutant.

To test if the PDE activity of a biochemically characterized EAL protein could function analogously to PigX, the *pigX* mutant strain was transformed with a plasmid expressing the EAL domain of YahA from *E. coli* (46) and examined for Pig production. The EAL domain of YahA complemented Pig production in the *pigX* mutant to WT levels (Fig. 5A and B). These heterologous expression experiments suggest that artificial modulation of c-di-GMP levels can affect secondary metabolism in *Serratia* strain ATCC 39006 and support the proposed assignment of PigX as a PDE. A definitive designation awaits biochemical characterization.

DISCUSSION

The current study has investigated the role of the pleiotropic regulator, PigX, in *Serratia* strain ATCC 39006. PigX controlled diverse phenotypes, including virulence and the biosynthesis of prodigiosin, presumably by functioning as a PDE modulating levels of the intracellular secondary messenger, c-di-GMP. This is the first experimental report examining the physiological role of a GGDEF/EAL domain protein in a *Serratia* species. Furthermore, we have recently demonstrated that PigX has a key role in the regulation of a conditional biosurfactant production and swarming phenotype in *Serratia* strain ATCC 39006 (N. R. Williamson, P. C. Fineran, W. Ogawa, L. R. Woodley, and G. P. C. Salmond, submitted for publication).

The master secondary metabolite regulator, PigP, was shown to repress the expression of *pigX* (20), the predicted product of which contained GGDEF and EAL domains (Fig. 1). PigX repressed the transcription of the biosynthetic operon *pigA-pigO* and the production of the red-pigmented secondary metabolite Pig (Fig. 2). Furthermore, levels of the PigA and PigF pigment biosynthetic proteins were elevated in the *pigX* mutant strain in a proteomic analysis (Fig. 3 and Table 3). Therefore, bioassay, gene fusion, primer extension, and proteomic experiments showed that PigX repressed (presumably indirectly) the transcription of the Pig biosynthetic gene cluster to control secondary metabolism.

A proteomic strategy was devised to identify cellular changes in the absence of a functional PigX (Fig. 3 and Table 3). As mentioned above, the levels of proteins implicated in the biosynthesis of Pig were elevated in the hyperpigmented mutant strain. Furthermore, proteins involved in amino acid biosynthesis and uptake were altered in the *pigX* mutant strain. Increased levels of SerC, involved in serine biosynthesis; PutA, a

*p*roline *ut*ilization membrane protein; and LivK, a periplasmic component of an ABC-type branched-chain amino acid transporter, were detected in the *pigX* mutant. In addition, the *pigX* mutant displayed decreased levels of AspA, an enzyme required for L-aspartate metabolism (L-aspartate is linked to serine and glycine biosynthesis) (37). Serine and proline are precursors in the biosynthetic pathway of prodigiosin (54). It is possible that the elevated level of SerC, which catalyzes the second step of serine biosynthesis (37), provides increased serine for the overproduction of pigment in the *pigX* background. The multifunctional PutA flavoenzyme converts proline into glutamate in a two-step reaction and is also a DNA binding protein that represses its own expression and that of *putP*, a proline transporter (37). Therefore, the increased levels of PutA in the *pigX* mutant strain might suggest a drop in intracellular proline. However, it has been observed that proline transport mutants of *Streptomyces coelicolor* A3(2) can also lose the ability to degrade proline while still retaining proline biosynthesis. The result was an increase in the production of undecylprodigiosin, which was suggested to be acting as a "metabolic sink" for excess proline (30). Finally, PepN, an intracellular aminopeptidase, was increased in the *pigX* mutant background. In *E. coli*, PepN has been implicated as the major aminopeptidase involved in the degradation of cytosolic proteins and may have a role in some cellular stress responses (9). Therefore, although we have speculated about links between specific altered proteins involved in amino acid metabolism and enhanced Pig production in the *pigX* mutant, it is possible that PigX plays a more general role in the regulation of amino acid metabolism.

Glycerol can be phosphorylated in a GlpK-catalyzed, ATP-dependent mechanism, yielding glycerol-3-phosphate (37). Alternatively, GldA (glycerol dehydrogenase) can convert glycerol to glycerone in an NAD^+-dependent reaction (37). The decreased GldA and increased GlpK levels in the *pigX* mutant suggest that the level of glycerol-3-phosphate, which may be funneled into either glycolysis or phospholipid biosynthesis, will be elevated.

The abundance of FabI, an enoyl-ACP reductase involved in the elongation cycle of fatty acid biosynthesis, was increased in the *pigX* background, and FabB (β-ketoacyl-ACP synthase I protein) was present in an elevated mixed hit in the *pigX* mutant strain. FabI can provide the acyl-ACP precursors of the *N*-AHL quorum-sensing signals (28). However, mutation of *pigX* had no effect on *N*-AHL production or SmaI expression throughout the time of growth (data not shown). It is clear that proteins involved in fatty acid metabolism are altered in the *pigX* mutant, and it is possible that these pathways might be linked to the biosurfactant production observed in the *pigX* mutant (Williamson et al., submitted).

Vitamin B_6 and vitamin B_9 metabolism may also be increased in the *pigX* strain due to elevated levels of the SerC and GTP cyclohydrolase I enzymes, respectively (37). Furthermore, a predicted periplasmic lipoprotein involved in iron transport was also increased in the *pigX* mutant. It is interesting to note that a transcriptomic study of a diguanylate cyclase-overexpressing strain of *E. coli* displayed repression of iron uptake genes (35).

Finally, increased levels of OpgG were detected in the *pigX* mutant. In *Erwinia chrysanthemi*, a number of genes, including OpgG, are involved in the synthesis of OPGs, branched glucans consisting of a β-1,2-linked glucose backbone with branched β-1,6 linkages (6, 13, 38). The exact role of OPGs is unknown, but they are important for osmoprotection, membrane integrity, and virulence in proteobacteria (6). The *opgGH* genes in *Serratia* strain ATCC 39006 were organized in a predicted operon, similar to the arrangement in *Erwinia chrysanthemi* (38). OpgG is a periplasmic protein, but its role in OPG synthesis is still unclear (6). However, mutation of *opgG* abolished the production of OPGs (38). We predicted that PigX (via *opgG*) may regulate OPG production, membrane integrity, exoenzyme production, and virulence. Indeed, Pel production was shown to be repressed by PigX and the *pigX* mutant was hypervirulent in a potato tuber-rotting model (Fig. 4). Mutation of *opgG* resulted in reduced exoenzyme production and decreased virulence in planta, even in the hyperproducing *pigX* mutant background (Fig. 4). Therefore, PigX represses levels of OpgG, which positively influences exoenzyme production and virulence in planta. A recent proteomic study was performed on an *opgG* mutant of *Erwinia chrysanthemi* (7). However, there is little overlap between those results and the ones reported here for the *pigX* mutant. To our knowledge, this is the first example of a proteomic study on a GGDEF/EAL mutant. These experiments revealed that, in addition to the alteration in the secondary metabolite production phenotype of a *pigX* mutant strain, proteins involved in primary metabolism and virulence are also affected, indicating a far more pleiotropic role of PigX than initially thought.

GGDEF and EAL domain proteins can modulate levels of the intracellular secondary messenger c-di-GMP and control multiple phenotypes, including biofilm formation, motility, and virulence (21, 32, 41). A number of lines of genetic evidence suggest that PigX functions as a PDE (via its EAL domain) and that levels of c-di-GMP might influence secondary metabolism, exoenzyme production, and virulence in *Serratia* strain ATCC 39006. First, overexpression of a biochemically characterized PDE protein from *E. coli* affected the PigX-controlled pigment and swarming (data not shown) phenotypes analogously to PigX, implicating altered levels of c-di-GMP in the modulation of Pig biosynthesis and swarming. Second, the EAL domain of PigX alone could fully complement Pig production and swarming (data not shown) in the *pigX* mutant strain. Finally, site-directed mutagenesis of the EAL motif of PigX abolished the function of PigX. Interestingly, during the preparation of the manuscript it was reported that the PigX homologue YhdA from *E. coli* (now designated CsrD) was not involved in c-di-GMP signaling and instead may be involved in binding RNA (49). In contrast to our results, the authors were unable to complement the *yhdA* mutant strain with the EAL domain alone, suggesting that YhdA and PigX might not have identical functions (49). It is worth noting that sequence deviation in EAL motifs can be tolerated and still allow PDE activity (42). For example, FimX from *P. aeruginosa* and CdgR from *Salmonella enterica* serovar Typhimurium possess EVL and EII motifs, respectively, yet retain PDE activity (27, 33). There is still no three-dimensional structural data of an EAL domain protein, which is likely to function as a monomer. Therefore, questions remain in our understanding of the details of c-di-GMP PDE activities. Finally, it is interesting that PDE mutations (and increased c-di-GMP) commonly result in decreased

virulence, whereas in this study, mutation of *pigX* caused an increase in virulence.

Despite the recent advances in the biochemistry of c-di-GMP signaling, it is still unclear how this secondary messenger regulates cellular phenotypes at the molecular level. However, recent bioinformatic and biochemical analyses have led to the identification of the PilZ domain that can bind c-di-GMP specifically and therefore may be the "missing link" in these signal transduction systems (3, 11, 43). It has also been proposed that certain nonconsensus GGDEF and EAL domain proteins may bind c-di-GMP and fulfill the role of downstream c-di-GMP receptor proteins (32). No PilZ domain proteins were identified in the known Pig regulators (data not shown). Therefore, any c-di-GMP binding proteins in *Serratia* strain ATCC 39006 await identification.

The pleiotropic regulator PigX may be regulated at the transcriptional and/or posttranscriptional level, which would influence its effects on secondary metabolism and virulence. The transcriptional start site of *pigX* was determined, and its expression followed a pattern similar to that of Pig production, increasing throughout the time of growth in LB, with maximal levels present in stationary phase (Fig. 2). The pleiotropic master regulator PigP was shown to strongly repress the transcription of *pigX* (20). In addition to transcriptional control by PigP, the activity of the PigX protein may be modulated. PigX is predicted to be anchored in the inner membrane via two transmembrane-spanning helices (Fig. 1), which might enable signal perception leading to an alteration in the activity of PigX. A number of proteins involved in c-di-GMP signaling, such as PleD from *Caulobacter crescentus* and FimX from *Pseudomonas aeruginosa*, can have a spatially constrained membrane localization, potentially resulting in fluctuations in the local subcellular c-di-GMP pool (31, 33, 40).

In conclusion, we have shown that PigX, a GGDEF/EAL domain protein, has phenotypic impacts on virulence and secondary metabolism in *Serratia* strain ATCC 39006. Proteomic analyses enabled the identification of a protein (OpgG), the levels of which were modulated by PigX. Mutation of *opgG* had impacts on exoenzyme production and virulence in planta. Despite sequence divergence in the EAL domain, genetic evidence implicated PigX as a PDE, involved in the breakdown of the intracellular secondary messenger c-di-GMP. From this study, it is clear that GGDEF/EAL domain proteins can also regulate the transcription of genes involved in the production of secondary metabolites, such as antibiotics, in addition to the regulation of motility, biofilm formation, and virulence. Our study also demonstrates the power of proteomics in the generation of hypotheses, which can then be validated by more-traditional genetic approaches.

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