A GntR family transcriptional regulator (PigT) controls gluconate-mediated repression and defines a new, independent pathway for regulation of the tripyrrole antibiotic, prodigiosin, in *Serratia*

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Biosynthesis of the red, tripyrrole antibiotic prodigiosin (Pig) by *Serratia* sp. ATCC 39006 (39006) is controlled by a complex regulatory network involving an *N*-acyl homoserine lactone (*N*-AHL) quorum-sensing system, at least two separate two-component signal transduction systems and a multitude of other regulators. In this study, a new transcriptional activator, PigT, and a physiological cue (gluconate), which are involved in an independent pathway controlling Pig biosynthesis, have been characterized. PigT, a GntR homologue, activates transcription of the *pigA–O* biosynthetic operon in the absence of gluconate. However, addition of gluconate to the growth medium of 39006 repressed transcription of *pigA–O*, via a PigT-dependent mechanism, resulting in a decrease in Pig production. Finally, expression of the *pigT* transcript was shown to be maximal in exponential phase, preceding the onset of Pig production. This work expands our understanding of both the physiological and genetic factors that impinge on the biosynthesis of the secondary metabolite Pig in 39006.

INTRODUCTION

Serratia sp. ATCC 39006 (39006) is a taxonomically ill-defined member of the Enterobacteriaceae that produces a number of interesting secondary metabolites. Like many strains of *Serratia marcescens*, 39006 displays swimming motility and produces the red, tripyrrole antibiotic prodigiosin (Pig; 2-methyl-3-pentyl-6-methoxyprodigiosin), a molecule which also displays anticancer and immunosuppressant activities (Manderville, 2001; Perez-Tomas *et al.*, 2003). The biosynthetic pathway of Pig has recently been elucidated in this laboratory (Harris *et al.*, 2004; Williamson *et al.*, 2005). In addition to Pig, 39006 also synthesizes a broad-spectrum β -lactam antibiotic, carbapenem (Car; 1-carbapen-2-em-3-carboxylic acid), which is also synthesized by some strains of *Erwinia carotovora* ssp. *carotovora* and *Photobacterium luminescens* ssp. *laumondii* TT01 (reviewed by Coulthurst *et al.*, 2005). 39006 also shares with *Erwinia* species the ability to macerate plant cell walls via production of secreted pectate lyases and cellulases (Crow, 2001; Slater *et al.*, 2003). Furthermore, recent studies have demonstrated

that 39006 is virulent in a *Caenorhabditis elegans* infection model (Coulthurst *et al.*, 2004).

Previous research in this laboratory has demonstrated numerous regulatory mechanisms, both genetic and environmental, involved in the control of secondary metabolite and (in some cases) exoenzyme production. Briefly, an *N*-acyl homoserine lactone (*N*-AHL) quorum-sensing (QS) system (SmaIR) functions, via a de-repression mechanism, to control Pig, Car and exoenzymes in response to increasing concentrations of *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (HHL) synthesized by SmaI (Fineran *et al.*, 2005; Slater *et al.*, 2003; Thomson *et al.*, 2000). QS controls the transcription of the Pig and Car biosynthetic genes by affecting expression of at least four other regulatory genes (*carR*, *pigR*, *pigQ* and *rap*) (Fineran *et al.*, 2005; Slater *et al.*, 2003).

Another key regulator is PigP, a novel putative DNA-binding protein, which controls expression of Pig and Car via modulation of at least seven other regulatory genes (*carR*, *pigQ*, *pigR*, *pigS*, *pigV*, *pigX* and *rap*) (Fineran *et al.*, 2005). In addition, a GacAS family two-component system (PigQW) regulates Pig production via activation of the biosynthetic operon *pigA–O* (Fineran *et al.*, 2005). We have also shown that Pig and Car levels are altered in response to phosphate concentration, sensed by the *pstSCAB/phoBR* system (Slater *et al.*, 2003). Our current model of secondary metabolite production involves an intricate hierarchical

Abbreviations: 39006, *Serratia* sp. ATCC 39006; BHL, *N*-butanoyl-L-homoserine lactone; Car, carbapenem; CRP, cAMP receptor protein; HHL, *N*-hexanoyl-L-homoserine lactone; HTH, helix–turn–helix; *N*-AHL, *N*-acyl homoserine lactone; Pig, prodigiosin; QS, quorum sensing; RBS, ribosome-binding site; WT, wild-type.

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network of transcriptional regulation that integrates numerous cues, including cell density, phosphate and unknown signal(s), presumably detected by the sensor kinase PigW (Fineran *et al.*, 2005).

In the current study, we characterize a new physiological cue (gluconate) and a predicted sensor/effector (PigT) that regulate Pig production, in 39006, independently of the known regulators mentioned above. PigT is homologous to the *Escherichia coli* GntR protein, which is a repressor of the GntI group of genes required for gluconate utilization (Izu *et al.*, 1997; Tong *et al.*, 1996). We demonstrate that PigT activates transcription of the Pig biosynthetic operon (*pigA*–*O*), whereas addition of gluconate causes a reduction in transcription of *pigA*–*O*. Furthermore, a putative PigT binding site was identified in the promoter of *pigA*, based on sequence similarity to the *gnt* operator site (Porco *et al.*, 1997). Therefore, PigT is predicted to activate *pigA*–*O* transcription directly. We demonstrate that PigT increases expression of a *pigA* promoter cloned in *E. coli* and that this activation is inhibited by gluconate. The transcription profile of a chromosomal *pigT::lacZ* fusion and primer extension of *pigT* demonstrated that *pigT* expression was maximal in exponential phase and decreased in stationary phase. The gluconate/PigT system represents a new, independent pathway involved in regulation of Pig production.

METHODS

Bacterial strains, plasmids, phage and culture conditions.

Bacterial strains and plasmids are listed in Table 1. *Serratia* sp. ATCC 39006 derivative strains were grown at 30 °C and *E. coli* strains were grown at 37 °C in Luria broth (LB; 5 g yeast extract l⁻¹, 10 g Bacto tryptone l⁻¹ and 5 g NaCl l⁻¹) or minimal medium [0.1%, w/v, (NH₄)₂SO₄, 0.41 mM MgSO₄, 0.2%, w/v, glucose, 40 mM K₂HPO₄, 14.7 mM KH₂PO₄, pH 6.9–7.1] in shake flasks at 300 r.p.m., or on minimal or LB agar supplemented with 1.5% (w/v) agar (LBA) (Miller, 1972). Bacterial growth (OD₆₀₀) was measured in a Unicam HeZios spectrophotometer. When required, LB and minimal medium were supplemented with antibiotics at the following final concentrations: kanamycin (Km) 50 µg ml⁻¹, spectinomycin (Sp) 50 µg ml⁻¹, streptomycin (Sm) 50 µg ml⁻¹, ampicillin (Ap) 100 µg ml⁻¹ and tetracycline (Tc) 35 µg ml⁻¹. The generalized transducing phage φOT8 was used for transduction of chromosomal mutations, as described previously (Thomson *et al.*, 2000).

DNA manipulations and sequencing of *pigT*. All molecular biology techniques, unless stated otherwise, were performed by standard methods (Sambrook *et al.*, 1989). 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 on an Applied Biosystems 3730xl DNA Analyser. Nucleotide sequence data were analysed using GCG (Genetics Computer Group, University of Wisconsin) and compared with GenBank DNA or non-redundant protein sequence databases using BLAST (Altschul *et al.*, 1997).

Random transposon mutagenesis of *Serratia* sp. ATCC 39006 strain LacA was performed by conjugation with either *E. coli* S17-1 λ pir harbouring plasmid pUTmini-Tn5lacZ1 or *E. coli* SM10 λ pir harbouring plasmid pUTmini-Tn5Sm/Sp, as described previously (Thomson *et al.*, 2000). Mutations were moved by φOT8-mediated transduction into 'clean' LacA backgrounds to ensure that the

phenotypes were associated with single transposon insertions. Genomic DNA from the *pigT* mutant (HSPIG36) was prepared and subjected to Southern blot and hybridization analysis using the *pig* cluster as a probe [pTON245 labelled with DIG using the Random Primed DNA Labelling Kit (Roche)]. The precise site of the transposon insertion in strain HSPIG36 was determined by 'single primer site PCR', followed by DNA sequence analysis across the transposon/chromosomal junction, as described previously (Fineran *et al.*, 2005). Additionally, random primed PCR was used to complete the approximately 2 kb sequence containing *pigT* in a method similar to that described previously (Jacobs *et al.*, 2003). Briefly, PCR was performed using a random primer mix (PF106, PF107 and PF108) and the *pigT*-specific primers HS3 (5' sequence) or *gntR* seq R (3' sequence) on LacA chromosomal DNA. A second PCR was performed using an aliquot of DNA from the first PCR with primers PF109 and either PF110 (5' sequence) or PF113 (3' sequence). The resulting mix of PCR fragments was purified and sequenced with PF110 or PF113, as appropriate. Sequencing was completed on the opposite strands using primers PF115 and PF116.

Construction of a plasmid (pTA38) that expresses native PigT.

A construct that enabled expression of native, untagged PigT was created as outlined below. The *pigT* gene was amplified by PCR, using primers PF103 and PF69, which contain *EcoRI* and *PstI* restriction sites, respectively. Additionally, primer PF103 contains a consensus ribosome-binding site (RBS, AGGAGGA). The PCR fragment of *pigT* was cloned into pQE-80L, previously digested with the enzymes *EcoRI* and *PstI*. The resulting plasmid, pTA38, was confirmed by DNA sequencing. Expression of plasmid pTA38 in both *E. coli* and 39006 hosts was induced with 1 mM IPTG.

Generation of a *pigT::mini-Tn5Sm/Sp* mutant by transposon exchange mutagenesis.

Previously, we reported an efficient *in vivo* method based on phage transduction that allows exchange of a chromosomal transposon insertion for an alternative transposon (Fineran *et al.*, 2005). Using this exchange method, strain HSPIG36S (*pigT::mini-Tn5Sm/Sp*) was constructed from the parental strain HSPIG36 (*pigT::mini-Tn5lacZ1*). The location of the new transposon was determined by PCR amplification across the insertion site using primers facing out of the transposon (LER1 and SP2 for mini-Tn5Sm/Sp) and the *pigT*-specific primers HS3 and HS4. PCR products in the expected size range were analysed further by sequencing and the point of insertion was determined. The *pigT::mini-Tn5Sm/Sp* mutation was transduced into a 'clean' LacA background using φOT8, and the nature of the transductants was confirmed by phenotype and PCR analysis.

Marker exchange mutagenesis of *pigW*. Strain PIG62L (*pigW::lacZKm*) was generated by a marker exchange strategy. Firstly, primers PF95 and PF96, containing *XbaI* and *Sall* restriction sites, respectively, were used to PCR-amplify a fragment of the *pigW* gene. This fragment was ligated into pBluescript II KS+ digested with *XbaI* and *Sall*, and the resulting construct (pTA35) was confirmed by DNA sequencing. Secondly, a promoterless *lacZ* gene and a Km resistance cassette were amplified from plasmid pUTmini-Tn5lacZ1 using primers PF97 and PF98, which contain *NcoI* and *NheI* restriction sites, respectively. The *lacZKm* fragment was ligated to specific *NcoI* and *NheI* sites internal to the *pigW* gene in pTA35, generating plasmid pTA36. Next, the entire *pigW::lacZKm* fragment was excised from plasmid pTA36 on the unique *XbaI* and *Sall* restriction sites and ligated into the marker exchange vector pKNG101 digested with *XbaI* and *Sall*, generating plasmid pTA37. Marker exchange with plasmid pTA37 was performed using a sucrose selection protocol similar to that described elsewhere (Kaniga *et al.*, 1991). The *pigW* mutant was confirmed by PCR, sequencing and phenotypic analysis.

Construction of *pigA* promoter::*lacZ* fusions and assay conditions. The *pigA* promoter was delineated into four different

Table 1. Bacterial strains, bacteriophage and plasmids used in this study

Strain/Plasmid	Genotype/phenotype	Reference
<i>Escherichia coli</i>		
CC118 λ pir	<i>araD</i> , Δ (<i>ara</i> , <i>leu</i>), Δ <i>lacZ</i> 74, <i>phoA</i> 20, <i>galK</i> , <i>thi</i> -1, <i>rspE</i> , <i>rpoB</i> , <i>argE</i> , <i>recA</i> 1, λ pir	Herrero <i>et al.</i> (1990)
DH5 α	F ⁻ , ϕ 80 Δ <i>lacZ</i> M15, Δ (<i>lacZYA</i> - <i>argF</i>)U169, <i>endA</i> 1, <i>recA</i> 1, <i>hsdR</i> 17 (<i>r</i> _K ⁻ <i>m</i> _K ⁺), <i>deoR</i> , <i>thi</i> -1, <i>supE</i> 44, λ ⁻ , <i>gyrA</i> 96, <i>relA</i> 1	Gibco-BRL
ESS	β -Lactam super sensitive indicator strain	Bainton <i>et al.</i> (1992)
HH26	Marker exchange mobilization strain for conjugal transfer	Grinter (1983)
SM10 λ pir	<i>thi</i> -1, <i>thr</i> , <i>leu</i> , <i>tonA</i> , <i>lacY</i> , <i>supE</i> , <i>recA</i> ::RP4-2-Tc::Mu, λ pir, Km ^R	de Lorenzo <i>et al.</i> (1990)
S17-1 λ pir	<i>recA</i> , <i>pro</i> , <i>hsdR</i> , <i>recA</i> ::RP4-2-Tc::Mu, λ pir, Tmp ^R , Sp ^R , Sm ^R	de Lorenzo <i>et al.</i> (1990)
YU563	Δ <i>lacX</i> 74, <i>galOP</i> 308, <i>rpsL</i> , <i>gntR</i> :: <i>Cm</i>	Tsunedomi <i>et al.</i> (2003)
<i>Serratia</i>		
ATCC 39006	Wild-type (Car ⁺ , Pig ⁺)	Bycroft <i>et al.</i> (1987)
LacA (WT)	Lac ⁻ derivative of ATCC 39006, made by EMS mutagenesis	Thomson <i>et al.</i> (2000)
Mutants derived from LacA		
13S36L	<i>pigP</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain PIG13S	This study
17S36L	<i>pigQ</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain PIG17S	This study
36S62L	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigW</i> :: <i>lacZ</i> Km, Sp ^R , Km ^R ; derivative of PIG62L following transduction using ϕ OT8 grown on strain HSPIG36S	This study
36SPAL	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigA</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of MCP2L following transduction using ϕ OT8 grown on strain HSPIG36S	This study
36SRAPL	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>rap</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of RAPL following transduction using ϕ OT8 grown on strain HSPIG36S	This study
36SSIL	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>smal</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of LC13 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
62S36L	<i>pigW</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain PIG62S	This study
HSROP4::36	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigX</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of ROP4 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
HSPIG17	<i>pigQ</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
HSPIG17::36	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigQ</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG17 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
HSPIG23	<i>pigR</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
HSPIG23::36	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigR</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG23 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
HSPIG26	<i>pigS</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
HSPIG26::36	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigS</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG26 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
HSPIG36	<i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
HSPIG36::R2	<i>pstS</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain ROP2	This study
HSPIG36::R4	<i>pigX</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain ROP4S	This study
HSPIG36S	<i>pigT</i> ::mini-Tn5Sm/Sp, Sp ^R ; derivative of HSPIG36, constructed by transposon exchange	This study
HSPIG46	<i>pigV</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
HSPIG46::36	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG46 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
HSPIG67	<i>pigP</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
HSPIG67::36	<i>pigT</i> ::mini-Tn5Sm/Sp, <i>pigV</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG67 following transduction using ϕ OT8 grown on strain HSPIG36S	This study
IS36L	<i>smal</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain LIS	This study
LC13	<i>smal</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Thomson <i>et al.</i> (2000)

Table 1. cont.

Strain/Plasmid	Genotype/phenotype	Reference
LIS	<i>smal</i> ::mini-Tn5Sm/Sp, Sp ^R	Thomson <i>et al.</i> (2000)
MCP2L	<i>pigA</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Slater <i>et al.</i> (2003)
PIG13S	<i>pigP</i> ::mini-Tn5Sm/Sp, Sp ^R	Fineran <i>et al.</i> (2005)
PIG17S	<i>pigQ</i> ::mini-Tn5Sm/Sp, Sp ^R	Fineran <i>et al.</i> (2005)
PIG62L	<i>pigW</i> :: <i>lacZ</i> Km, Km ^R ; derivative of <i>LacA</i> , constructed by marker exchange	This study
PIG62S	<i>pigW</i> ::mini-Tn5Sm/Sp, Sp ^R	Fineran <i>et al.</i> (2005)
RAP36L	<i>rap</i> ::mini-Tn5Sm/Sp, <i>pigT</i> ::mini-Tn5 <i>lacZ</i> 1, Sp ^R , Km ^R ; derivative of HSPIG36 following transduction using ϕ OT8 grown on strain RAPS	This study
RAPL	<i>rap</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
RAPS	<i>rap</i> ::mini-Tn5Sm/Sp, Sp ^R	Fineran <i>et al.</i> (2005)
ROP2	<i>pstS</i> ::mini-Tn5Sm/Sp, Sp ^R	Slater <i>et al.</i> (2003)
ROP4	<i>pigX</i> ::mini-Tn5 <i>lacZ</i> 1, Km ^R	Fineran <i>et al.</i> (2005)
ROP4S	<i>pigX</i> ::mini-Tn5Sm/Sp, Sp ^R	Fineran <i>et al.</i> (2005)
Phage		
ϕ OT8	<i>Serratia</i> generalized transducing phage	M. A. Crow and others, unpublished results
Plasmids		
pBluescript II KS+	Cloning vector, ColE1 replicon, Ap ^R	Stratagene
pKNG101	Marker exchange suicide vector, <i>sacBR</i> , <i>mobRK2</i> , <i>oriR6K</i> , Sm ^R	Kaniga <i>et al.</i> (1991)
pNJ5000	Mobilizing plasmid used in marker exchange, Tc ^R	Grinter (1983)
pQE-80L	Expression vector for native or N-terminal hexahistidine proteins, Ap ^R	QIAGEN
pRW50	Promoterless <i>lacZ</i> fusion plasmid, RK2 replicon, Tc ^R	Lodge <i>et al.</i> (1992)
pTA15	<i>pigA</i> promoter <i>lacZ</i> fusion (−367 to +49 of <i>pigA</i> promoter in pRW50), Tc ^R	This study
pTA30	<i>pigA</i> promoter <i>lacZ</i> fusion (−319 to +49 of <i>pigA</i> promoter in pRW50), Tc ^R	This study
pTA31	<i>pigA</i> promoter <i>lacZ</i> fusion (−193 to +49 of <i>pigA</i> promoter in pRW50), Tc ^R	This study
pTA32	<i>pigA</i> promoter <i>lacZ</i> fusion (−43 to +49 of <i>pigA</i> promoter in pRW50), Tc ^R	This study
pTA35	<i>pigW</i> region for <i>lacZ</i> Km marker exchange in pBluescript II KS+	This study
pTA36	<i>pigW</i> :: <i>lacZ</i> Km in pBluescript II KS+	This study
pTA37	<i>pigW</i> :: <i>lacZ</i> Km in pKNG101	This study
pTA38	Native <i>PigT</i> expression vector, derivative of pQE-80L, Ap ^R	This study
pTON245	pHCP19 vector containing 24 kb including the 39006 <i>pigA</i> –O cluster	Harris <i>et al.</i> (2004)
pUTmini-Tn5 <i>lacZ</i> 1	Delivery plasmid for mini-Tn5 <i>lacZ</i> 1, Ap ^R , Km ^R	de Lorenzo <i>et al.</i> (1990)
pUTmini-Tn5Sm/Sp	Delivery plasmid for mini-Tn5Sm/Sp, Ap ^R , Sp ^R	de Lorenzo <i>et al.</i> (1990)

size fragments and cloned into the promoterless *lacZ* plasmid pRW50 (Lodge *et al.*, 1992). The plasmids, named pTA15, pTA30, pTA31 and pTA32 in order of decreasing size, were created by cloning *Bam*HI/*Hind*III-digested PCR products [generated with forward primers HS78, PF70, PF71 and PF72, respectively, and the reverse primer HS79 (Table 2, Fig. 3)] into *Bam*HI/*Hind*III-digested pRW50. The nature of all plasmids was confirmed by DNA sequencing.

Promoter activity assays were performed in an *E. coli gntR* mutant (YU563) to avoid interference by native *GntR* in *E. coli*. YU563 was transformed with the pRW50-derived construct (pTA15, 30, 31 or 32) and either the vector control (pQE-80L) or the *PigT* expression plasmid (pTA38). Additionally, *E. coli* strains containing pRW50 had no detectable β -galactosidase activity. Overnight cultures of strains tested were grown with Ap and Tc selection for approximately 16 h and subcultured into 25 ml LB containing Ap, Tc and IPTG in 250 ml conical flasks to a starting OD₆₀₀ of 0.04. The bacterial cultures were incubated at 300 r.p.m. at 37 °C for approximately 3 h, or until the OD₆₀₀ reached between 0.4 and 0.6. Aliquots (1 ml) of culture were removed, pelleted by centrifugation and subjected to β -galactosidase assays, as outlined below.

β -Galactosidase assays. β -Galactosidase activity in bacterial cultures grown in liquid media was determined using ONPG as

substrate, as described elsewhere (Miller, 1972). Enzyme activity was expressed as the initial reaction rate per ml of sample per OD₆₀₀ of the bacterial culture tested ($\Delta A_{420} \text{ min}^{-1} \text{ ml}^{-1} \text{ OD}_{600}^{-1}$). Results presented are the mean $\pm$ standard deviation (SD) of three independent experiments, unless stated otherwise.

Bioassays of prodigiosin, carbapenem, N-AHLs, exoenzymes and motility. The assays for *Pig* and *Car* were performed as described previously (Slater *et al.*, 2003). *Pig* production was plotted as ($A_{534} \text{ ml}^{-1} \text{ OD}_{600}^{-1}$) $\times$ 50. Detection of N-AHLs was performed using the *Serratia* LIS bioassay described in Thomson *et al.* (2000). Activity of pectate lyase and cellulase was analysed on agar plates containing the corresponding substrates, as described elsewhere (Andro *et al.*, 1984). Motility was assessed on tryptone swarm agar (TSA) plates (10 g Bacto tryptone l^{−1}, 5 g NaCl l^{−1} and 3 g agar l^{−1}). Overnight bacterial cultures were adjusted to an OD₆₀₀ of 0.2, 3 μ l was spotted onto the plates, and halo size was examined after growth for 16 h at 30 °C.

Primer extension and RNA studies. A hot-acidic phenol method (Aiba *et al.*, 1981) was used to extract total RNA from 39006. Primer extension analysis for the *pigA* transcript was performed as described previously (Slater *et al.*, 2003) using primer HS34. All primer extension reactions were performed with 25 μ g total RNA

Table 2. Oligonucleotide primers used in this study

Name	Sequence (5'–3')	Notes	Restriction site
<i>gntR</i> seq R	CGCCAGCGGTGGAAGATGTTGG	<i>pigT</i> forward primer for random primed PCR	
<i>gntR</i> ss(2)	GCCCAGTTCATCAAGCACTATGGCG	<i>pigT</i> primer extension primer	
HS3	CAGTATGTGTACGCTCGGCC	<i>pigT</i> reverse primer (and for random primed PCR)	
HS4	GACCGGTACTCCAGGATGTC	<i>pigT</i> internal forward primer	
HS78	GACGGATCCTAGCTGATGACCTTCG	Forward primer for pTA15 <i>pigA</i> promoter construct	<i>Bam</i> HI
HS79	GACAAGCTTCCATAAAACACTCCATTGC	Reverse primer for pTA15, 30, 31 and 32 <i>pigA</i> promoter constructs	<i>Hind</i> III
LER1	GCGTGCATAATAAGCCCTACAC	mini-Tn5Sm/Sp out right hand side	
LL-68	TGCATCAATCGCTGCTTTTACCTG	mini-Tn5 <i>lacZ1</i> out left hand side	
LR-68	CAGCAACACCTTCTTCACGAGG	mini-Tn5 <i>lacZ1</i> out right hand side	
PF69	CTGACTGCAGACGTCTGACTGATG	<i>pigT</i> reverse primer for construction of pTA38	<i>Pst</i> I
PF70	GACGGATCCAATGGCCTCTATTTAC	Forward primer for pTA30 <i>pigA</i> promoter construct	<i>Bam</i> HI
PF71	GACGGATCCGTTTGTATTATAAAAGTG	Forward primer for pTA31 <i>pigA</i> promoter construct	<i>Bam</i> HI
PF72	GACGGATCCAATGTTGCATTTTG	Forward primer for pTA32 <i>pigA</i> promoter construct	<i>Bam</i> HI
PF95	GTATCTAGACTCGGCCTGCTCTCC	<i>pigW</i> forward primer for construction of pTA35	<i>Xba</i> I
PF96	CTAGTCGACGGCTTGGTGGCTG	<i>pigW</i> reverse primer for construction of pTA35	<i>Sal</i> I
PF97	GTACCATGGTTCCGCTGGCG	<i>lacZKm</i> forward primer for pUTmini-Tn5 <i>lacZ1</i>	<i>Nco</i> I
PF98	CATGCTAGCTGCCAGTGTTACAACC	<i>lacZKm</i> reverse primer for pUTmini-Tn5 <i>lacZ1</i>	<i>Nhe</i> I
PF103	GATGAATTCAGGAGGACACGGATGATAA-AGAAGAAAAG	<i>pigT</i> forward primer for construction of pTA38, contains consensus RBS	<i>Eco</i> RI
PF106	GACCACACGTCGACTAGTGCNNNNNNN-NNNAGAG	Random primed PCR primer 1	
PF107	GACCACACGTCGACTAGTGCNNNNNNN-NNNACGCC	Random primed PCR primer 2	
PF108	GACCACACGTCGACTAGTGCNNNNNNN-NNNGATAC	Random primed PCR primer 3	
PF109	GACCACACGTCGACTAGTGC	Random primed PCR adapter primer	
PF110	CCTGATTGGTCAACGAGGG	<i>pigT</i> reverse primer for random primed PCR	
PF113	CGCTGTTGGCAGGGG	<i>pigT</i> forward primer for random primed PCR	
PF115	GACGCCAGATCTGGATTTCAG	<i>pigT</i> forward primer for sequencing 5' region	
PF116	GCAGCAGTAAAGCAACGGC	<i>pigT</i> reverse primer for sequencing 3' region	
SP2	GCCTTGATGTTACCCGAGAGC	mini-Tn5Sm/Sp out left hand side	
T3	TTAATTGGGAGTGATTTCCC	pBluescript II KS+ sequencing primer	
T7	GTAATACGACTCACTATAGGGC	pBluescript II KS+ sequencing primer	

and 0.2 pmol of the appropriate ³²P-labelled primer. Primer extension analysis of *pigT* was performed using primer *gntR* SS(2), which anneals 122 bp downstream of the predicted *pigT* translational start site. mRNA transcripts were quantified using ImageJ, a densitometry program available at <http://rsb.info.nih.gov/nih-image/Default.html>.

RESULTS

A GntR homologue, PigT, regulates Pig production and swimming motility

From a mutagenesis programme aimed at identifying regulators of secondary metabolite production, one mutant (HSPIG36) was analysed in more detail because it showed a strong defect in Pig production (compare Fig. 1a with Fig. 1b). The impact of the mutation in HSPIG36 on other phenotypes (swimming motility, Pel, Cel, Car and *N*-AHL production) was assessed in plate assays from stationary-phase overnight cultures. Mutant HSPIG36 showed reduced

swimming motility compared to the 'wild-type' (WT refers to the parental precursor strain LacA throughout this paper) (compare Fig. 1c with Fig. 1d). There was no detectable difference between the WT and mutant HSPIG36 in the exoenzyme assays or in *N*-AHL production (data not shown).

The site of the mini-Tn5*lacZ1* insertion in HSPIG36 was determined as described in Methods. The transposon interrupted a 999 bp ORF with homology to *gntR* from *E. coli*. We designated this gene in 39006 as *pigT*. The predicted PigT protein is similar (77 % identity/81 % similarity) to GntR from *E. coli* K-12 (Tong *et al.*, 1996), a member of the GalR/LacI family of transcriptional regulators (Fukami-Kobayashi *et al.*, 2003; Peekhaus & Conway, 1998). In *E. coli*, in the absence of gluconate, GntR binds DNA via an N-terminal helix–turn–helix (HTH) domain and represses the GntI group of genes involved in gluconate utilization (Peekhaus & Conway, 1998). The location and sequence of

Fig. 1. Mutation of *pigT* or addition of gluconate to 39006 culture media affects Pig production in 39006. Pig production by strains (a) LacA and (b) HSPiG36 (*pigT*) grown on LB agar supplemented with 1.5% (w/v) agar (LBA). Swimming motility of strains (c) LacA and (d) HSPiG36 (*pigT*) grown on tryptone swarm agar (TSA). Complementation of Pig production in (e) HSPiG36 (*pigT*) carrying plasmid pTA38 (PigT) compared to (f) HSPiG36 (*pigT*) with the vector control (pQE-80L). Pig production by strain LacA on minimal medium with either 0.2% (w/v) glucose (g) or 0.2% (w/v) gluconate (h) as the sole carbon source. Pig production phenotype of strain LacA grown on 0.2% glucose minimal medium (i) or on 0.2% glucose minimal medium supplemented with 0.02% gluconate (j). Production of Pig by strain LacA on (k) LBA or (l) LBA containing 0.02% gluconate.

the gene 5' of *pigT* is similar (67% identity/73% similarity of 45 amino acids aligned) to YhhW from *E. coli* K-12. Furthermore, an ORF 3' of *pigT* is similar (57% identity/67% similarity) to GntK from *E. coli* K-12. These results indicate that *pigT* shares a similar, although not identical, genomic context to *gntR* of *E. coli* K-12.

To demonstrate that the transposon disruption in *pigT* was responsible for the Pig phenotype, complementation was performed. Plasmid pTA38, which expresses native PigT, was able to complement the Pig phenotype in HSPiG36, whereas a pQE-80L control could not (compare Fig. 1e with Fig. 1f). Therefore, PigT affects both swimming motility and Pig production in 39006 and shares sequence similarity with GntR from *E. coli*.

PigT is a transcriptional regulator of Pig

To examine the impact of PigT on Pig production in more detail, Pig was assessed throughout growth in LB in the *pigT* mutant and compared to that of the WT (Fig. 2a). Pig production was approximately 10% of the levels observed in the WT. To enable construction of double chromosomal mutants, strain HSPiG36S (*pigT*::mini-Tn5Sm/Sp) was constructed (see Methods). HSPiG36S was shown to have the same swimming motility and Pig production defects as HSPiG36. Expression of a chromosomal *pigA*::*lacZ* fusion was studied throughout growth in the WT and *pigT* mutant backgrounds (Fig. 2b), and was found to be reduced to approximately 30% of WT levels. In addition, primer extension studies demonstrated that the *pigA*-O transcript in the *pigT* mutant was similarly reduced compared to that of the WT (Fig. 2c). The *pigT* mutant was unaffected for Car and BHL/HHL production when examined throughout growth in LB (data not shown). These results were consistent with a

model in which PigT regulates Pig production by controlling the transcription of the biosynthetic genes (*pigA*-O).

PigT does not operate via known regulators

In recent reports, we elucidated a complex circuit controlling Pig production that involved numerous transcriptional regulators, including the SmaIR QS system (Fineran *et al.*, 2005; Slater *et al.*, 2003; Thomson *et al.*, 1997, 2000). Given this plethora of known regulatory inputs, it was possible that PigT was controlling *pigA* expression indirectly via one or more of these other known secondary metabolite regulators. Therefore, the effect of a chromosomal mini-Tn5Sm/Sp mutation in *pigT* on the expression of *lacZ* transcriptional fusions in *pigP*, *pigQ*, *pigR*, *pigS*, *pigV*, *pigW*, *pigX*, *rap* and *smaI* was examined in early-exponential, mid-exponential, late-exponential/transition and stationary phase. However, *pigT* did not affect β -galactosidase activity from any of these fusions (data not shown). Furthermore, chromosomal mini-Tn5Sm/Sp mutations in *smaI*, *pigP*, *pigQ*, *pigW*, *pigX*, *pstS* and *rap* did not alter expression of the *pigT*::mini-Tn5*lacZ1* fusion in early-exponential, mid-exponential, late-exponential/transition and stationary phase (data not shown). Therefore, PigT defines a new prodigiosin regulator operating via a novel route, independent of the members of the regulatory network examined in this study. PigT could be activating *pigA*-O transcription directly, or could be repressing expression of an, as yet, unidentified repressor of Pig.

PigT is predicted to act directly at the *pigA* promoter

The transcriptional hierarchy data described above indicate that *pigT* does not control the transcription of the other regulators examined, consistent with the notion that PigT

Fig. 2. PigT regulates Pig production by controlling transcription of the Pig biosynthetic operon *pigA*-O. (a) Pig levels in WT (squares) and HSPIG36 (*pigT*) (triangles) throughout growth in LB. (b) β -Galactosidase activity was measured from a chromosomal *pigA::lacZ* fusion in an otherwise WT background (squares) or in a strain containing a mini-Tn5Sm/Sp chromosomal insertion in *pigT* (triangles). (c) Primer extension analysis of the *pigA*-O transcript from WT and HSPIG36 (*pigT*) throughout growth in LB. The *pigA* transcript was quantified and the *pigT*/WT ratios are given below the lanes. In (a) and (b), solid symbols and lines represent Pig and β -galactosidase activity, respectively, whereas the open symbols and dashed lines represent the growth curves of the corresponding strains. Data shown are the mean $\pm$ standard deviation (SD) of at least three independent experiments.

directly activates *pigA*-O transcription. Examination of the sequence 5' of *pigA*-O revealed a potential PigT binding site (ATGTTACTGGTAACTG) centred at -78.5 relative to the *pigA* transcriptional start site (Fig. 3a) (Slater *et al.*, 2003). The bold type represents the two conserved half sites of the predicted PigT/GntR binding sites. This predicted binding site is based on the *E. coli* GntR palindromic consensus binding sequence (ATGTTA-(N₄, GC-rich)-TAACAT) (Porco *et al.*, 1997) present in the promoter regions of GntR-regulated genes. The position of this binding site suggests that PigT may be binding directly to the *pigA*-O promoter region and functioning as a Class I activator to regulate transcription (Busby & Ebright, 1999).

To test if PigT directly activates transcription of the Pig biosynthetic operon, the *pigA* promoter was cloned into the low-copy, promoterless *lacZ* expression vector pRW50 (Lodge *et al.*, 1992). Promoter delineation studies were performed on the *pigA* promoter to determine regions required for PigT activation and promoter activity (see Methods). The results showed that, in an *E. coli* *gntR* mutant (YU563), the presence of PigT caused an increase in transcription from the *pigA* promoter (Fig. 3b). Furthermore, the shortest construct (pTA32), which still possessed the -35 and -10 elements, but not the predicted PigT binding site, was not activated by PigT. These results suggest that PigT directly activates transcription of *pigA*-O and this activation requires a 150 bp region 5' of the *pigA* promoter. Within this region there is an inverted repeat sequence similar to the *E. coli* GntR box, which we predict is a PigT box.

We had reported previously that transposon insertions within the ROP1 (repressor of pigment) region of the *pigA* promoter in 39006 resulted in a hyper-pigmented phenotype and an increase in *pigA::lacZ* expression (Slater *et al.*, 2003). It was suggested that the ROP1 region might (1) be a repressor binding site, (2) be important for DNA conformation or (3) encode a small RNA that acts negatively on Pig production. Deletion of the ROP1 region had little effect on promoter activity in *E. coli* (Fig. 3b). Furthermore, PigT was still able to activate *pigA* promoter constructs that lacked the ROP1 region, indicating that ROP1 is not required for the PigT-mediated activation of *pigA*. Thus, the DNA region between -193 and -44 inclusive is required for PigT-mediated activation of *pigA* transcription.

Expression of *pigT* is maximal in exponential phase

To assess the expression of *pigT*, β -galactosidase activity was measured from a chromosomal *pigT::lacZ* fusion throughout growth (Fig. 4a). In addition, primer extension analysis of the *pigT* transcript was performed (Fig. 4b). These experiments showed that levels of the *pigT* transcript peak in exponential phase (6 h) and then decline as the cultures reach stationary phase (12 h).

Fig. 3. In *E. coli*, the *pigA* promoter is activated by PigT and contains a predicted palindromic PigT binding site. (a) Partial sequence of the *pigA* promoter that was cloned into constructs pTA15, pTA30, pTA31 and pTA32 (indicated). Features of the promoter include the ROP1 (repressor of pigment) region (bold italics), the transcriptional start site (+1), the predicted -10 and -35 elements (boxed) (Slater *et al.*, 2003), and the proposed PigT binding site (bold boxed, centred at -78.5) based on the GntR binding site (Porco *et al.*, 1997). A putative cAMP receptor protein (CRP) binding site centred at -64.5 relative to the +1 overlaps the PigT box and is shown underlined with the two conserved half sites in italics. Primer binding sites are shown underlined and the translational initiation codon of *pigA* is shown in bold (ATG). (b) Schematic representation of the *orfY*-*pigA* intergenic region and the pRW50-derived *pigA* promoter fusion constructs (pTA15, pTA30, pTA31 and pTA32). The scale of the promoter region is indicated, but genes are not drawn to scale. β -Galactosidase activity of the *pigA* promoter fusions in *E. coli* YU563 in the absence (pQE-80L, -) or presence (pTA38, +) of PigT is indicated. Data shown are the mean $\pm$ SD of at least three independent experiments.

Gluconate regulates Pig production transcriptionally

The identification of a putative GntR homologue (PigT) that controls Pig encouraged us to examine whether gluconate affects pigmentation. 39006 grown on minimal medium with 0.2 % (w/v) gluconate as sole carbon source was non-pigmented when compared with the 0.2 % (w/v) glucose-grown controls (compare Fig. 1g with Fig. 1h). Addition of 0.02 % gluconate to minimal medium containing 0.2 % glucose caused a reduction in Pig (compare Fig. 1i with Fig. 1j). Furthermore, when LBA was supplemented with 0.02 % gluconate, Pig production was decreased (compare Fig. 1k with Fig. 1l). To examine further the gluconate-mediated repression of Pig, 0.02 % gluconate was added to glucose minimal medium, either at the start of

growth (t_0) or after 8 h (t_8), and Pig was examined throughout growth (Fig. 5a). Gluconate repressed Pig to approximately 35 % of WT levels when added at t_0 . Gluconate added just prior to the onset of pigmentation (t_8) decreased Pig to approximately 50 % of WT levels, after it had initially been produced (Fig. 5a). The level of *pigA*-O transcription was measured using a strain with a chromosomal *pigA*::*lacZ* reporter (MCP2L) under identical conditions to those described above. Gluconate added at t_0 and t_8 resulted in approximately 12 and 35 %, respectively, of *pigA*-O transcription when measured by β -galactosidase activity (Fig. 5b). Car and BHL/HHL levels were unaffected throughout growth in LB by the addition of 0.02 % gluconate (data not shown). These experiments showed that gluconate, even in the presence of glucose, could repress Pig biosynthesis, and that this physiological

Fig. 4. Expression of *pigT* is maximal in the exponential growth phase and decreases in stationary phase. (a) β -Galactosidase activity was measured from *pigT::lacZ* (HSPIG36) throughout growth in LB. (b) Primer extension analysis of the *pigT* transcript from a WT culture throughout growth in LB. In (a), bars represent β -galactosidase activity, whereas the open symbols and dashed lines represent the growth curves of the corresponding strain. Data shown are the mean $\pm$ SD of at least three independent experiments.

repression operates, at least in part, at the level of transcription of the *pigA*-O operon.

Gluconate-mediated repression of Pig requires PigT

In *E. coli*, the GntR protein responds to gluconate and alters gene expression accordingly. Since the phenotypic consequences of a *pigT* mutation and addition of gluconate were similar, it was predicted that the gluconate response required functional PigT. Therefore, an epistasis test was performed by growing the WT and *pigT* mutant in LB with or without gluconate (0.02%), and Pig was measured in stationary phase. The presence of gluconate resulted in a 50% reduction in Pig in the WT strain (Fig. 6a). No additional decrease in Pig was observed in the *pigT* mutant in the presence of gluconate, implying that PigT was required for the gluconate-mediated repression (Fig. 6b). Furthermore, PigT-mediated activation of a *pigA* promoter *lacZ* fusion in an *E. coli gntR* mutant was inhibited by 0.02% gluconate (Fig. 6c). Addition of gluconate, in the absence of PigT, had no effect on the expression of the *pigA* promoter in this *E. coli* background (Fig. 6c). The available data suggested that gluconate, by inhibiting the activity of PigT, was repressing Pig production.

Fig. 5. Addition of gluconate inhibits Pig production and transcription of the Pig biosynthetic genes, *pigA*-O. (a) Pig production throughout growth in glucose minimal medium by the WT after addition of 0.02% (w/v) gluconate at the start of growth (*t*₀, triangles), after 8 h (*t*₈, diamonds) or with no added gluconate (squares). (b) β -Galactosidase activity was measured from a chromosomal *pigA::lacZ* fusion throughout growth in glucose minimal medium after addition of 0.02% gluconate at the start of growth (*t*₀, triangles), after 8 h (*t*₈, diamonds) or with no added gluconate (squares). Solid symbols and lines represent either Pig or β -galactosidase activity, whereas the open symbols and dashed lines represent the growth curves of the corresponding strains. Data shown are the mean $\pm$ SD of at least three independent experiments.

Fig. 6. *PigT* is required for the gluconate-mediated repression of *Pig* production. *Pig* production by (a) WT and (b) *pigT* mutant (HSPIG36) in stationary phase (12 h). Strains were grown in LB in the presence (open bars) or absence (black bars) of 0.02% (w/v) gluconate added at the start of growth. Note that the scales of (a) and (b) are different. (c) β -Galactosidase activity was measured from a *pigA* promoter *lacZ* fusion (plasmid pTA30) in an *E. coli gntR* mutant (YU563) containing either vector-only control (No *PigT*; pQE-80L) or a *PigT* expression plasmid (*PigT*; pTA38) in LB in the presence (open bars) or absence (black bars) of 0.02% gluconate added at the start of growth. Data shown are the mean $\pm$ SD of at least three independent experiments.

DISCUSSION

We have shown previously that in *Serratia* sp. ATCC 39006 (39006), production of prodigiosin (*Pig*) is regulated by a complex regulatory network involving an *N*-AHL QS system, *PigP*, *Rap*, *GacAS* and *PhoBR* two-component systems, and several other regulators (Fineran *et al.*, 2005; Slater *et al.*, 2003; Thomson *et al.*, 1997, 2000) (Fig. 7). Furthermore, numerous studies have implicated a spectrum of physiological factors in the production of *Pig* in strains of *S. marcescens* (Cang *et al.*, 2000; Giri *et al.*, 2004; Rjasantseva *et al.*, 1994; Silverman & Munoz, 1973; Sole *et al.*, 1997; Williams *et al.*, 1971a, b; Witney *et al.*, 1977). Therefore, it is apparent that multiple environmental, physiological and genetic cues influence *Pig* production in *Serratia* species. In this paper, we have characterized the mechanism underlying gluconate-mediated repression of the biosynthesis of *Pig*: yet another regulatory input in the control of this red antibiotic in 39006.

Firstly, we demonstrated that mutation of a gene predicted to encode a *GntR* homologue (*pigT*) resulted in lower *Pig* production and a reduction in swimming motility (Figs 1 and 2). The reduction in *Pig* was the result of decreased transcription of the biosynthetic operon (*pigA*–*O*) observed both by chromosomal *pigA*::*lacZ* fusion analysis and by primer extension of *pigA*–*O* transcript levels in the *pigT* mutant (Fig. 2). Furthermore, complementation of the *pigT* mutation with a plasmid encoding the *pigT* ORF restored *Pig* production (Fig. 1).

In *E. coli*, *GntR* negatively regulates the *GntI* group of genes involved in gluconate utilization. However, when gluconate is present in the medium, *GntR* is thought to bind gluconate (or one of its metabolic intermediates), and repression of the

GntI genes is alleviated (Izu *et al.*, 1997; Tong *et al.*, 1996). The gluconate-sensing role of *GntR*, and the sequence similarity to *PigT*, prompted us to examine the effects of gluconate on secondary-metabolite biosynthesis in 39006.

Gluconate, even in the presence of glucose or in complex media (LB), could decrease *Pig* production in 39006 (Figs 1 and 5a). The gluconate-mediated decrease in *Pig* production was accompanied by a reduction in *pigA* transcription, even if gluconate was added after the onset of pigmentation (Fig. 5b).

These data suggested a model whereby *PigT* mediates gluconate repression of *Pig* production. Based on genetic and biochemical data from *E. coli GntR*, it is clear that *GntR* represses gene expression by binding to DNA in the promoter regions of *GntI* genes in the absence of gluconate (Peekhaus & Conway, 1998). However, in 39006, *PigT*-activated *Pig* production is hypothesized to be 'deactivated' by the addition of gluconate. It was possible that *PigT* was controlling transcription of *pigA*–*O* indirectly by repressing a repressor of *Pig*. Therefore, to try and identify a potential regulatory intermediate, the effects of a *pigT* mutation on the transcription of multiple known secondary metabolite regulators were examined. However, the effect of *PigT* on *Pig* production was independent of all the regulators investigated. *PigT* did not control transcription of any of those tested and, likewise, no regulator examined controlled expression of *pigT*. This result suggested that *PigT* might directly activate transcription of the *pigA*–*O* operon.

Interestingly, a putative *PigT* binding site, based on that demonstrated for *GntR* (Porco *et al.*, 1997), was identified upstream of the -35 element in the *pigA* promoter (Fig. 3a). Furthermore, promoter delineation experiments in *E. coli*

Fig. 7. Schematic model of physiological and genetic factors that affect Pig production in 39006. Gluconate ‘deactivates’ PigT, which, in the absence of gluconate, acts via an independent pathway to activate transcription of *pigA*–O. Low phosphate results in activation of transcription of both *smal* and *pigA*–O via the PstSCAB/PhoBR system (Slater *et al.*, 2003). Pig production is also controlled by cell density via the SmalR quorum-sensing (QS) system (Slater *et al.*, 2003; Thomson *et al.*, 2000). SmalR also regulates transcription of *pigQ* of the PigQW (GacAS family) two-component system. Presumably, PigQ is activated in response to signals sensed by the histidine kinase PigW (Fineran *et al.*, 2005). Transcription of *rap* (regulator of antibiotic and pigment), which is a member of the SlyA/MarR family, is also controlled by QS (Fineran *et al.*, 2005). Both *rap* and *pigQ* are also activated by a master Pig regulator (PigP), which controls expression of numerous other Pig and Car regulators (not shown). A deeper discussion of all these regulatory inputs is described in Fineran *et al.* (2005).

implied that PigT only activated *pigA* promoter constructs that contained a 150 bp region (from –193 to –44) which includes this putative PigT binding site (Fig. 3b). Based on this genetic evidence, our model, that PigT directly activates expression of *pigA*–O in the absence of gluconate, contradicts the conventional role of GntR, which functions as a repressor of gluconate metabolism genes in *E. coli*. However, in our model, the binding of PigT (like GntR) to DNA is dependent on the absence of gluconate. We suggest that the binding of gluconate to PigT results in PigT being unable to bind DNA, and therefore activation of transcription is prevented. Interestingly, we have evidence that, in *E. coli*, GntR can activate the cloned *pigA* promoter, and this activation can be inhibited by the presence of gluconate (P. Fineran, unpublished results). This suggests that, *E. coli* GntR can also be an activator of gene expression if the placement of the GntR binding site is permitting. It remains to be determined whether PigT functions analogously to GntR by regulating genes involved in uptake and utilization

of gluconate. However, both the sequence context of *pigT*, with respect to genes potentially involved in gluconate utilization, and the presence of a putative PigT binding site in the promoter of the putative gluconokinase (data not shown), indicate that PigT may have a role in regulating gluconate metabolism.

The *pigA* promoter studies raised a few interesting points about the regulation of the *pigA*–O operon. Firstly, the transcriptional start site of *pigA* has been mapped by primer extension, and putative –10 and –35 elements identified (Slater *et al.*, 2003). A TAAAGA→TAAAGG substitution in the predicted –10 element resulted in a greater than 50 % reduction in *pigA* promoter activity (data not shown), supporting this assignment. Secondly, interrogation of the sequence 5′ of *pigA* has revealed a predicted CRP binding site centred at –64.5, which overlaps one half of the predicted PigT binding site (Fig. 3a). Preliminary data demonstrated an increase in *pigA* promoter expression in an *E. coli* *crp* mutant (P. Fineran, unpublished results). However, the role of CRP requires further investigation. Interestingly, the *gntT* promoter in *E. coli* is also regulated by both GntR and CRP (Peekhaus & Conway, 1998). Furthermore, it is interesting that, in a previous mutagenesis screen, we isolated a predicted novel adenylate cyclase mutant (*pigR*) with decreased Pig (Fineran *et al.*, 2005).

Primer extension and expression of a chromosomal *pigT*::*lacZ* fusion was used to assess the transcription profile of *pigT* throughout growth. *pigT* was maximally expressed during mid–late exponential phase, but was attenuated during the transition into stationary phase (Fig. 4a, b). This transcript profile is consistent with a role for PigT as a major activator of *pigA*, and hence Pig, levels of which increase dramatically in mid–late exponential phase (Fig. 2a, b).

Our model of PigT directly activating the *pigA*–O operon (and hence Pig production) and inhibition of this interaction in the presence of gluconate is based on the following lines of evidence: (1) PigT shares significant sequence similarity with *E. coli* GntR (77 % identity/81 % similarity), which has been shown biochemically to respond to gluconate and alter its DNA binding affinity accordingly (Peekhaus & Conway, 1998); (2) addition of gluconate or mutation of *pigT* had similar effects on Pig production and transcription of *pigA*–O (Figs 2 and 5), but no effect on Car and BHL/HHL synthesis; (3) epistasis experiments demonstrated that gluconate could not further repress pigmentation in a *pigT* mutant (Fig. 6); (4) finally, in an *E. coli* *gntR* mutant, PigT-mediated activation of the *pigA* promoter was inhibited by gluconate (Fig. 6).

The physiological benefit of 39006 repressing Pig in the presence of gluconate is unknown. Little is known of the true habitat(s) of 39006, but it is possible that gluconate is present in a niche where Pig is not required. GntR homologues have been associated with the regulation of various phenotypes in bacteria. For example, a GntR homologue

regulates production of antibiotics and the onset of aerial mycelia and spore formation in *Streptomyces coelicolor* (Sprusansky *et al.*, 2003).

The current investigation has increased our understanding of the physiological and genetic regulation of the biosynthesis of a tripyrrole antibiotic pigment, prodigiosin (Pig). We have identified a new physiological cue (gluconate) that represses Pig production and characterized the potential sensor of this signal (PigT). The gluconate/PigT pathway represents a system for control of pigmentation that is apparently completely independent of the regulatory network of secondary metabolite production that we have recently characterized in some detail (Fineran *et al.*, 2005; Slater *et al.*, 2003; Thomson *et al.*, 1997, 2000). Fig. 7 highlights some of the important physiological and genetic factors that modulate Pig biosynthesis in 39006, including the PigT/gluconate system. Both the physiological rationale and molecular mechanism driving this gluconate-mediated regulation of the red, tripyrrole antibiotic warrant further investigation.

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