

# The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation

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The prodigiosin biosynthesis gene cluster (*pig* cluster) from two strains of *Serratia* (*S. marcescens* ATCC 274 and *Serratia* sp. ATCC 39006) has been cloned, sequenced and expressed in heterologous hosts. Sequence analysis of the respective *pig* clusters revealed 14 ORFs in *S. marcescens* ATCC 274 and 15 ORFs in *Serratia* sp. ATCC 39006. In each *Serratia* species, predicted gene products showed similarity to polyketide synthases (PKSs), non-ribosomal peptide synthases (NRPSs) and the Red proteins of *Streptomyces coelicolor* A3(2). Comparisons between the two *Serratia* *pig* clusters and the *red* cluster from *Str. coelicolor* A3(2) revealed some important differences. A modified scheme for the biosynthesis of prodigiosin, based on the pathway recently suggested for the synthesis of undecylprodigiosin, is proposed. The distribution of the *pig* cluster within several *Serratia* sp. isolates is demonstrated and the presence of cryptic clusters in some strains shown. The *pig* cluster of *Serratia marcescens* ATCC 274 is flanked by *cueR* and *copA* homologues and this configuration is demonstrated in several *S. marcescens* strains, whilst these genes are contiguous in strains lacking the *pig* cluster.

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## INTRODUCTION

Gram-negative bacteria of the genus *Serratia* are opportunistic human, plant and insect pathogens and are members of the Enterobacteriaceae. Some strains of *Serratia marcescens* cause nosocomial infections that are clinically problematic because multidrug resistance is widespread within the species (Stock *et al.*, 2003; Traub, 2000). *S. marcescens* has been isolated from soil, water, plants and air (Grimont & Grimont, 1978). The ability of some *S. marcescens* strains to colonize a wide range of ecological niches has been linked to the production of a spectrum of extracellular products including chitinases, proteases, lipases, nucleases, bacteriocins, surfactants and wetting agents (Braun & Schmitz, 1980; Clegg & Allen, 1985; Hines *et al.*, 1988; Matsuyama *et al.*, 1995; Yanagida *et al.*, 1986).

**Abbreviations:** ACP, acyl carrier protein; Ecc, *Erwinia carotovora* subsp. *carotovora*; MAP, 2-methyl-3-n-amy-l-pyrrole; MBC, 4-methoxy-2,2-bipyrrole-5-carboxyaldehyde; OHHL, *N*-(3-oxohexanoyl)-L-homoserine lactone; PCP, peptidyl carrier protein; PLP, pyridoxal phosphate; Sma 274, *Serratia marcescens* strain Atcc 274.

The GenBank/EMBL/DBJ accession number for the *Serratia* sp. ATCC 39006 prodigiosin biosynthesis gene cluster is AJ833001, and for the *Serratia marcescens* ATCC 274 prodigiosin biosynthesis gene cluster, AJ833002.

The red pigment, prodigiosin (Pig), is a member of the prodiginines produced by some *Serratia* species, actinomycetes and a few other bacteria (Grimont & Grimont, 1978). Prodigiosin is a linear tripyrrole and a typical secondary metabolite, appearing only in the later stages of bacterial growth (Williams *et al.*, 1971). The actinomycete *Streptomyces coelicolor* A3(2) produces a closely related linear tripyrrole, undecylprodigiosin, and a cyclic derivative, butyl-*meta*-cycloheptylprodiginine in a 2:1 ratio (Tsao *et al.*, 1985).

A bifurcated pathway has been proposed for the synthesis of prodigiosin culminating in the enzymic condensation of the terminal products of the two pathways, 4-methoxy-2,2-bipyrrole-5-carboxyaldehyde (MBC) and the monopyrrole, 2-methyl-3-n-amy-l-pyrrole (MAP). The precursors for prodigiosin were shown to be acetate, serine, alanine, methionine and proline (Williams, 1973). Recently, the mechanism of proline incorporation into a pyrrole moiety has been shown biochemically and a pathway for synthesis of undecylprodigiosin proposed (Cerdeño *et al.*, 2001; Thomas *et al.*, 2002).

Prodiginosins have no defined role in the physiology of producing strains, but have been reported to have antifungal, antibacterial and antiprotozoal/antimalarial activities

(Demain, 1995; Williams & Quadri, 1980). Consequently, prodigiosins are of interest because they might have potential clinical utility. Prodigiosins and synthetic derivatives have been shown to have potent and specific immunosuppressive activity, with novel targets clearly distinct from other drugs (D'Alessio *et al.*, 2000; Mortellaro *et al.*, 1999; Tsuji *et al.*, 1990, 1992). Montaner *et al.* (2000) showed that prodigiosin extracted from *S. marcescens* could induce apoptosis in haematopoietic cancer cell lines including acute T-cell leukaemia, myeloma and Burkitt's lymphoma, with little effect on non-malignant cell lines. Prodigiosin has also been shown to induce apoptosis in human primary cancer cells in the case of B and T cells in B-cell chronic lymphocytic leukaemia samples (Cámpas *et al.*, 2003).

Previous attempts to reconstitute prodigiosin production in *Escherichia coli* have been unsuccessful. Dauenhauer *et al.* (1984) isolated a *S. marcescens* genomic clone capable of condensing the two prodigiosin precursors, MAP and MBC, to form prodigiosin. However, no sequence data were reported for this clone. We were able to demonstrate expression of prodigiosin in an heterologous host (Thomson *et al.*, 2000). The *pig* gene cluster from *Serratia* sp. ATCC 39006 (*Serratia* 39006) was expressed in *Erwinia carotovora* subsp. *carotovora* (Ecc; 25 out of 36 strains tested), though it was not expressed in several other members of the Enterobacteriaceae, including *E. coli* (Thomson *et al.*, 2000). In *Serratia* 39006 the production of prodigiosin is regulated by multiple factors, including a quorum-sensing system, via the LuxIR homologues, *SmaI* and *SmaR* (Slater *et al.*, 2003; Thomson *et al.*, 2000). Interestingly, prodigiosin production became *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) dependent when expressed in Ecc, despite the latter producing a different signalling molecule from that made by *Serratia* 39006 (Thomson *et al.*, 2000).

Initially it was thought that *Serratia* 39006 was an *S. marcescens* strain (Thomson *et al.*, 1997). It is now clear that this *Serratia* strain is atypical and does not fall into the *marcescens* classification. We now refer to this strain by the ATCC nomenclature, strain 39006. In this paper we report the organization of the prodigiosin biosynthetic gene (*pig*) clusters in *Serratia* 39006 and in an *S. marcescens* strain, ATCC 274 (*Sma* 274) and we define the genomic context of the *pig* cluster in several *S. marcescens* strains. We discuss a pathway for prodigiosin biosynthesis based on predicted protein functions of the genes of the *pig* clusters and comparison with the recently proposed pathway for undecylprodigiosin (Cerdeño *et al.*, 2001).

## METHODS

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains were cultured at 37 °C and *Erwinia* and *Serratia* strains were cultured at 30 °C in Luria-Bertani medium. *Serratia* strains were also cultured on peptone glycerol media (PGM, 1% glycerol, 0.5% peptone, 1.6% agar) to enhance pigmentation. Antibiotic

final concentrations were as follows, unless otherwise stated: 100 µg ml<sup>-1</sup> ampicillin (Ap), 50 µg ml<sup>-1</sup> kanamycin (Km), 50 µg ml<sup>-1</sup> spectinomycin (Sp), 100 µg ml<sup>-1</sup> streptomycin (Sm) and 10 µg ml<sup>-1</sup> tetracycline (Tc).

**DNA manipulations.** All DNA manipulations were performed as described in Sambrook *et al.* (1989). Genomic DNA from *Serratia* strains was extracted with the DNAeasy Tissue kit (Qiagen). DNA fragments were isolated from agarose gels using a gel extraction kit (Qiagen). Isolation of cosmids and plasmids was performed with the Qiagen Miniprep kit. Oligonucleotide primers were purchased from Sigma-Genosys. Double-stranded sequencing was performed at the DNA Sequencing facility, Department of Biochemistry, University of Cambridge, using an ABI 3700 DNA analyser.

**Isolation and sequencing of the biosynthetic cluster of *Serratia* sp. ATCC 39006 and *S. marcescens* ATCC 274.** The cosmid pNRT104 carrying the *pig* genes of *Serratia* 39006 was identified previously (Thomson *et al.*, 2000), and this was then subcloned to the smallest insert size (pTON245) that would still produce pigment in an Ecc host. The insert was further subcloned into pDAH330 (McGowan *et al.*, 1995) and primer walking was used to sequence the resultant plasmids.

Chromosomal DNA of *Sma* 274 was partially digested with *Sau*3AI, dephosphorylated and then ligated into the *Bam*HI sites of SuperCos-1. The ligation products were packaged with a Gigapack III XL λ packaging kit (Stratagene) and used to transduce *E. coli* XL-1 Blue as per the manufacturer's instructions. A prodigiosin-producing clone (pPIG4) was identified.

A transposon-based approach using the EZ::TN <TET-1> system (Epicentre) was employed to sequence pPIG4. Transposition *in vitro* was performed on pPIG4 and the transposition mix was used to transform *E. coli* XL-1 Blue by electroporation. Transformants were selected on LBA Tc plates and non-pigmented colonies selected for further analysis. Transposon-specific primers were used to obtain the sequence of the flanking DNA (forward primer 5'-CTCTAGAGTTCGACCTGCAGGCATGCAAGC-3' and reverse primer 5'-TAAATTGCACTGAAATCTAGAAA-3'). The sequences were assembled using the Staden package (Staden, 1996). Primer walking was used to join contigs and cover problematic areas. In total, 22 794 bp covering the *pig* cluster were determined with double-stranded coverage of each base in the consensus. ORFs were determined using GeneMark.hmm ([http://opal.biology.gatech.edu/GeneMark/whmm\\_info.html](http://opal.biology.gatech.edu/GeneMark/whmm_info.html)) and Glimmer 2.0 (Delcher *et al.*, 1999) and annotation was done using Artemis (Rutherford *et al.*, 2000).

### Southern blotting using *S. marcescens* ATCC 274 and *Serratia* sp. ATCC 39006 *pig* clusters as probes.

Genomic DNA from *Serratia* strains was digested overnight with *Acl*I (New England Biolabs). The digested DNA was transferred to a nylon membrane (Hybond-N, Amersham). The DNA probe was generated through a *Bfal*-*Ascl* digest of pPIG4 from which three fragments (9.5, 5.6, 5.3 kb) containing the *pig* genes were gel extracted (Qiagen) and DIG labelled by random prime (Roche). The DNA probe for the *Serratia* 39006 cluster was generated by DIG labelling pTON245 using random prime and this was used to probe *Mlu*I-digested genomic DNA of *S. marcescens* strains.

**PCR amplification of *cueR-pigA* and *pigN-copA*.** The regions between *cueR* and *pigA* and *pigN* and *copA* were amplified by PCR using the primers *cueR* (5'-TCGTA AAAACGAATCGTC-3'), PE1 (5'-GCAAACTCTGAGCGGATTTCGC-3'), ab77 (5'-GAAACACTTAACCTGACG-3') and PE2 (5'-CGCAGTTCATGCAGGACAGC-3').

**Table 1.** Bacterial strains and plasmids used in this study

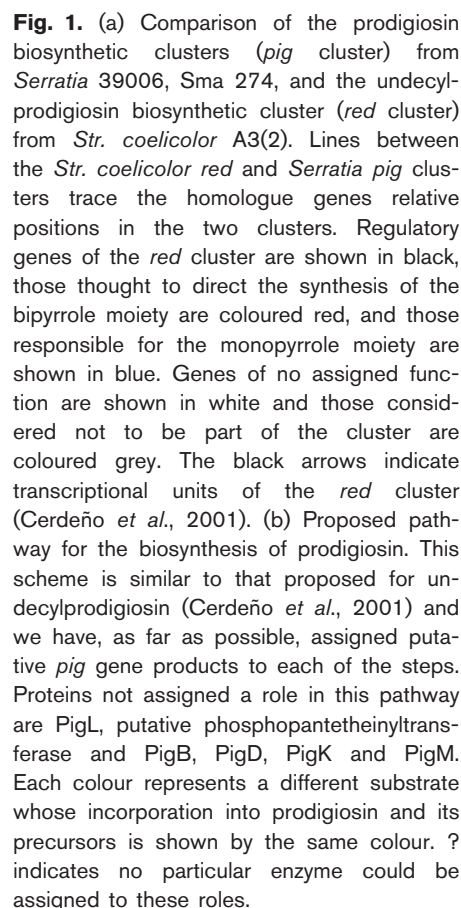
The strains shown in bold are pigmented and the strains in normal font are non-pigmented. The *Serratia marcescens* strains 1–19 were kindly donated by Hazel Aucken of the Public Health Laboratory Service (PHLS), UK.

Bacterial strains	Strain	Note	Source/reference
<b><i>Serratia</i></b>			
1	Sma 1695	Clinical	Blood
2	Sma 0006	Environmental	Food
3	Sma 1047	Clinical	Blood
4	Sma 0026	Environmental	Insect
5	Sma 4028	Clinical	Urine
<b>6</b>	<b>Sma 3078</b>	Clinical	Stool
<b>7</b>	<b>Sma 0038</b>	Environmental	Reservoir
8	Sma 1024	Clinical	Urine
9	Sma 0022	Environmental	Insect
10	Sma 3127	Clinical	Wound drain
<b>11</b>	<b>Sma 0035</b>	Environmental	Fig
12	Sma 3888	Clinical	Blood
13	Sma 0028	Environment	Insect
<b>14</b>	<b>Sma 3255</b>	Clinical	Urine
<b>15</b>	<b>Sma 0040</b>	Environmental	Reservoir
16	Sma 2595	Clinical	Blood
17	Sma 0030	Environmental	Plant
<b>18</b>	<b>Sma 3127</b>	Clinical	Wound drain
19	Sma 3078*	Clinical	Stool
<b>20</b>	<b>Sma ATCC 274</b>	Environmental	Lab stock
21	Sma S6	Clinical	Yang <i>et al.</i> (1990)
22	Sma Db11	Environmental	Insect (Flyg <i>et al.</i> , 1980)
<b>23</b>	<b><i>Serratia</i> sp. ATCC 39006</b>	Environmental	Salt marsh (Bycroft <i>et al.</i> , 1987)
	<i>Serratia</i> sp. ATCC 39006 HSPiG28 ( <i>pigB</i> ::miniTn5 <i>lacZ</i> 1, Km <sup>r</sup> )	Biosynthetic mutant	H. Slater & G. P. C. Salmond (unpublished)
	Sma ATCC 274 MUT12 ( <i>pigI</i> ::miniTn5 Sm/Sp, Sp <sup>r</sup> )	Biosynthetic mutant	Harris (2003)
<b><i>Erwinia</i></b>			
	<i>Erwinia carotovora</i> subsp. <i>atroseptica</i> , strain SCRI 1043		Lab stock
	<i>Erwinia carotovora</i> subsp. <i>carotovora</i> , strain ATTn10	Wild-type, restrictionless, carbapenem-producing strain	Lab stock
	<i>E. coli</i> DH5α		Lab stock
	<i>E. coli</i> XL-1 Blue MR		Stratagene
<b>Plasmids</b>			
	SuperCos	ColE1 replicon, Ap <sup>r</sup> , Kn <sup>r</sup>	Stratagene
	pPIG4	SuperCos vector with ~41 kb insert containing the <i>Sma</i> 274 <i>pig</i> genes	This study
	pNRT104	pSF6 vector with ~35 kb insert containing the <i>Serratia</i> 39006 <i>pig</i> genes	Thomson <i>et al.</i> (2000)
	pTON245	pHCP19 vector, containing the Pig cluster (24 kb) from <i>Serratia</i> 39006	This study

\*Strain Sma 19 is a spontaneous non-pigmented variant of Sma 6.

We previously identified a cosmid (pNRT104) containing *Serratia* 39006 genomic DNA which encoded synthesis of prodigiosin in the heterologous host *Ecc* (Thomson *et al.*, 2000). This cosmid was further subcloned and sequenced by primer walking to identify a cluster of 21 634 bp containing 15 candidate prodigiosin biosynthetic genes. To identify the *pig* genes in Sma 274, a cosmid library was constructed and used to transform *E. coli* XL-1 Blue. Approximately 0.1 % of the Ap<sup>r</sup> colonies recovered after transduction produced a red pigment, presumed to be prodigiosin. A prodigiosin-producing clone (pPIG4) was

The *Serratia pig* clusters contain 14 candidate genes common to both strains and are arranged *pigA* through to *pigN* (Fig. 1a). As described below, we have putatively assigned the products of five genes to the biosynthesis of MBC (red) and four to the MAP (blue) pathway. Four are unassigned, with the remaining protein (PigL) proposed to be involved in the post-translational modification of some of the proteins in the cluster (discussed later). The order of the genes is conserved between the two different *Serratia* species and the corresponding 14 predicted proteins are similar in size and share significant amino acid





identities between the species. However, the relative genomic contexts of the clusters and their regulation are very different.

The *Serratia* 39006 *pig* gene cluster contains an additional gene, tentatively designated *pigO*. RT-PCR and primer extension has confirmed that there is transcriptional read-through between *pigN* and *pigO* (Slater *et al.*, 2003), consistent with *pigO* being part of the *pig* operon in that strain. However, the *pigO* gene has been inactivated and the pigment produced by the *pigO* mutant has been shown to be prodigiosin, the same as wild-type *Serratia* 39006 and Sma 274, by LC-MS analysis (unpublished). These data suggest that *pigO* is not involved in the biosynthesis of prodigiosin, but could have a regulatory role. The *Serratia* 39006 *pig* cluster (*pigA–O*) is transcribed as a polycistronic message from a promoter upstream of *pigA* (Slater *et al.*, 2003), and so we presume that the *pig* cluster (*pigA–pigN*) of Sma 274 is also transcribed as a polycistronic message.

The only substantial gap in both *pig* clusters is between *pigC* and *pigD*. There is a 184 bp gap between *pigC* and *pigD* in *Serratia* 39006, suggesting a possibility of two transcriptional units. However, transcription levels of the

genes either side of this gap were shown to be similar in studies using *lacZ* reporter fusions to *pigA* and *pigH* (Crow, 2001). The Sma 274 *pig* cluster has a 64 bp gap between *pigC* and *pigD*, smaller than that in *Serratia* 39006, with no other significant intergenic gaps.

Table 2 shows the relatedness of each *Serratia* 39006 Pig protein to its homologue in Sma 274. Although the 39006 and Sma 274 clusters contain the same genes and are orientated identically, they are only 68% identical, on average, at the DNA level. There is also wide variation in the level of identity between proteins encoded by genes in the *pig* clusters. For example, the *Serratia* PigE homologues are 85.2% identical (90.4% similar) whilst the PigL homologues are only 52.7% identical (56.1% similar). This implies different functional constraints altering the rates of molecular evolution in different genes within the same cluster. Another striking difference between the two *pig* clusters is the G+C content of the genes. Each *Serratia* 39006 *pig* gene has a lower G+C content than its Sma 274 counterpart. The *Serratia* 39006 *pig* genes have a mean G+C content of 51.4% whilst the mean G+C content of the Sma 274 *pig* genes is 59.8% per gene. This compares to a mean of 58.4% across the whole genus (Grimont &

**Table 2.** Deduced functions and homologues of the gene products from the *pig* clusters

Putative functions were assigned based on the results of BLASTP searches and comparison with the *red* cluster. The relatedness of each Sma 274 Pig protein to its homologue in *Serratia* 39006 is shown in the comparison of *Serratia* Pig proteins percentage sequence identity column. Sma 274 has no homologue of PigO. The number of amino acid residues (aa) in each of the Pig proteins is listed. In the Red homologue percentage sequence identity column, values on the left refer to *Serratia* 39006 and values on the right refer to the percentage sequence identity for the corresponding Sma 274 protein. PigL contains motifs which are conserved within the phosphopantetheinyl transferases (Lambalot *et al.*, 1996). *pigD* and *pigE* are the only genes from the *Serratia* *pig* clusters not to contain a homologue in the *red* cluster.

Protein	Comparison of <i>Serratia</i> Pig proteins			Putative function	Red homologue and highest protein sequence similarity	
	<i>Serratia</i> 39006 (aa)	Sma 274 (aa)	Identity (%)		Protein	Identity (%)
PigA	386	385	79	L-Prolyl-PCP dehydrogenase	RedW, <i>Str. coelicolor</i>	44/43
PigB	670	671	71.3	No assigned function	RedS (N-terminal 146 aa), <i>Str. coelicolor</i>	39/41
PigC	890	888	75.6	Phosphotransferase	RedH, <i>Str. coelicolor</i>	41/39
PigD	866	904	83.4	No assigned function	PdorFL, <i>P. stutzeri</i>	38/38
PigE	853	853	85.2	Aminotransferase	PdorFM, <i>P. stutzeri</i>	50/48
PigF	348	338	82.8	O-Methyltransferase	RedI, <i>Str. coelicolor</i>	37/25
PigG	87	87	78.2	Peptidyl carrier protein (PCP)	RedO, <i>Str. coelicolor</i>	44/63
PigH	653	648	79.7	Aminotransferase	RedN, <i>Str. coelicolor</i>	49/47
PigI	491	490	68.1	L-Prolyl-AMP ligase	RedM, <i>Str. coelicolor</i>	37/38
PigJ	770	762	68.8	$\beta$ -Ketomyristol-ACP synthase	RedX, <i>Str. coelicolor</i>	46/52
PigK	104	104	71.2	No assigned function	RedY, <i>Str. coelicolor</i>	44/44
PigL	234	215	52.7	4'-Phosphopantetheinyltransferase	RedU, <i>Str. coelicolor</i>	
PigM	359	352	59.4	No assigned function	RedV, <i>Str. coelicolor</i>	29/35
PigN	242	242	76.6	Oxidoreductase	RedF, <i>Str. coelicolor</i>	24/34
PigO	157	—	—	No assigned function	PAB0452, VirR-related protein, <i>Pyrococcus abyssi</i>	41/34

Grimont, 1984) and 59 % for the whole genome of *S. marcescens* Db11 ([http://www.sanger.ac.uk/Projects/S\\_marcescens/](http://www.sanger.ac.uk/Projects/S_marcescens/)). The significance of this difference in G+C content is unclear, although it may reflect the fact that *Serratia* 39006 is an atypical strain.

Biochemical, syntrophic and genetic analyses have also been done using the undecylprodigiosin-producing strain, *Str. coelicolor* A3(2) (Cerdeño *et al.*, 2001; Coco *et al.*, 1991; Feitelson *et al.*, 1985; Rudd & Hopwood, 1980). Undecylprodigiosin synthesis is encoded by the *red* gene cluster, as shown in Fig. 1(a) (White & Bibb, 1997). A biosynthetic pathway for undecylprodigiosin synthesis has been proposed recently, based on the predicted functions of the proteins in the *red* cluster (Cerdeño *et al.*, 2001).

Table 2 shows the predicted functions of *pig* gene products based on BLAST searches (<http://www.ncbi.nlm.nih.gov/blast/>) and the Red homologues of each of the Pig proteins. Comparison of the three prodigiosin gene clusters proved extremely interesting. The *red* cluster is larger (23 genes) than the *pig* clusters (14–15 genes), possibly reflecting the greater complexity of the undecylprodigiosin products made by the former (Tsao *et al.*, 1985). Twelve genes are conserved between all three clusters, as might be expected of pathways making such similar products. However, their orientation and regulation seem to have undergone pronounced variation. The *Serratia* *pig* clusters are orientated and transcribed unidirectionally (Slater *et al.*, 2003). In contrast, the Red cluster is arranged in four transcriptional units and is regulated by RedZ and RedD (Fig. 1a). The RedD-dependent promoter region lies between RedQ and RedP and contains two divergent promoters directing transcription in opposite directions. RedZ, a response regulator, is thought to bind upstream of the RedD gene (White & Bibb, 1997). The dissimilar arrangement of these homologous genes, in clusters that produce chemically similar products, raises questions about the origins and evolution of prodigiosin biosynthetic gene clusters in both Gram-positive and Gram-negative bacteria. It is unclear whether there is a species-related functional significance to the dissimilar arrangement of the genes or whether the differences have arisen by random events.

Differences between the other genes (see Fig. 1a) present in these operons might explain differences in the final molecules produced, or the biochemical routes to their production. *pigD* and *pigE* are the only *pig* genes that do not have homologues in the *red* cluster. Their respective homologues, *pdtorfL* and *pdtorfM*, are part of a gene cluster encoding the synthesis of the carbon tetrachloride dechlorination agent pyridine-2,6-bis(thiocarboxylic acid) by *Pseudomonas stutzeri* (Lewis *et al.*, 2000). *pdtorfL* and *pdtorfM* are contiguous, as are *pigD* and *pigE* in the *pig* cluster. PigE is also similar to a putative aminotransferase (SC6A5.18, 461 aa) from *Str. coelicolor* A3(2), encoded by a gene unlinked to the *red* gene cluster (38 % identical to PigE from *Serratia* 39006 and 39 % identical to PigE from Sma 274; both are 50 % similar over 460 aa). Indeed, it is

possible that genes encoding proteins that take part in the synthesis of prodigiosin and undecylprodigiosin may not be associated with the gene clusters. RedR, RedQ and RedP do not have homologues encoded by the *pig* cluster, yet are thought to direct the condensation of acetate to form three of the carbon atoms of the pyrrole ring and the undecyl side chain of undecylprodigiosin (Cerdeño *et al.*, 2001). The genes encoding the corresponding homologues could be present at another location on the *Serratia* chromosome, or other enzymes may perform the necessary catalytic roles in prodigiosin biosynthesis in *Serratia*. However, as we have shown that prodigiosin can be made in recombinant *E. coli* and *Erwinia*, this would necessitate functional activity of the corresponding *E. coli* and *Erwinia* enzymes to supplement the activity of the enzymes encoded by the *pig* cluster.

The twelve Red proteins with homologues encoded in the *pig* cluster (the 'core' prodigiosin enzymes) are similar in size to their Pig counterparts. An exception to this is PigB (670 aa), whose homologue RedS (Sc3f7.05c) is a much smaller protein (146 aa) and is similar to only the N-terminal region of PigB. The rest of PigB shows sequence identity with amine oxidases. The PigI and PigJ proteins are also significantly smaller than their respective RedM and RedX homologues.

### Biosynthesis of prodigiosin

Cerdeño *et al.* (2001) have suggested a likely sequence of steps for the biosynthesis of the closely related undecylprodigiosin in *Str. coelicolor* A3(2) and have assigned putative Red proteins to most of the steps. Undecylprodigiosin is thought to be formed from combination of the identical bipyrrole unit, MBC, with a slightly different monopyrrole (2-undecylpyrrole). We now suggest a scheme for the biosynthesis of prodigiosin, shown in Fig. 1(b), which is similar to that proposed for undecylprodigiosin and we have, as far as possible, assigned putative *pig* gene products to each of the steps.

### Biosynthesis of MBC

We propose that the biosynthesis of MBC follows the same general chemical pathway in *Serratia* as in *Str. coelicolor* A3(2). Recent studies have shown that RedM, RedO and RedW from the *red* cluster, which have homologues in both *pig* clusters, are sufficient for the *in vitro* synthesis of a pyrrole group from L-proline (Thomas *et al.*, 2002). RedM from *Str. coelicolor*, which is homologous to adenylation domains in non-ribosomal peptide synthases (NRPSs), has been shown to activate the carboxyl group of proline *in vitro* and transfer the prolyl group to RedO, a homologue of peptidyl carrier protein (PCP) domains of NRPSs (Cerdeño *et al.*, 2001). The prolyl group is then oxidized by RedW, which shares sequence identity with flavin-dependent acyl-CoA dehydrogenases. Homologues of these three proteins have been shown to be required for the incorporation of L-proline into the pyrrole moieties of other pyrrole-containing

compounds (Thomas *et al.*, 2002; Xu *et al.*, 2002). Thus PigI, which is homologous to RedM, is similarly presumed to adenylate proline and transfer the prolyl group to PigG, a homologue of RedO (Table 2). The prolyl-PigG is then presumably oxidized by PigA, which is a homologue of RedW. It is not known whether PigA performs both oxidations required to convert the prolyl group to a pyrrole, whether a second enzyme is also involved, or whether the second oxidation is non-enzymic (Thomas *et al.*, 2002). PigA has good sequence identity with a wide range of acyl-CoA dehydrogenases. Sequence alignment with human isovaleryl-CoA dehydrogenase, the crystal structure of which has been determined, showed that the majority of amino acids around the flavin and the phosphopantetheinyl moiety of CoA are conserved but the amino acids involved in binding the adenosyl group of CoA are not conserved (Tiffany *et al.*, 1997). This supports the suggestion that the substrate for PigA is a prolyl PCP rather than prolyl-CoA.

In the case of *Str. coelicolor*, it was proposed that the next enzyme in the biosynthesis of MBC is RedL, which appears to be a hybrid NRPS/polyketide synthase (PKS), consisting of the following six domains: an adenylation domain, an acyl carrier protein (ACP), a keto-synthase, an acyltransferase, another ACP, and a pyridoxal phosphate-dependent domain thought to be a serine transferase (Cerdeño *et al.*, 2001). There is no similar multifunctional enzyme among the Pig gene products and so we assume that the various reactions are all catalysed by smaller monofunctional enzymes or by distinct mechanisms.

The next step in MBC biosynthesis in *Serratia* should, by analogy with that proposed for *Str. coelicolor*, be condensation of prolyl-PigG with malonyl-CoA or a malonyl ACP. As there is no apparent ACP in the *pig* cluster, we have shown malonyl-CoA as the substrate in Fig. 1(b), but it is quite possible that an ACP from outside the *pig* cluster is used, such as the fatty acid synthase ACP. The keto-synthase which catalyses this condensation is also unknown. There is one putative keto-synthase in the cluster (PigJ), which could catalyse this reaction, but PigJ has highest homology with RedX, which has been assigned to the biosynthesis of the monopyrrole moiety of undecylprodigiosin. Accordingly, we have assigned PigJ to the biosynthesis of MAP in Fig. 1(b) and not assigned any of the Pig proteins to the keto-synthase reaction in the biosynthesis of MBC.

In *Str. coelicolor*, it was proposed that formation of pyrrole ring B of undecylprodigiosin is catalysed by the terminal pyridoxal phosphate (PLP)-dependent domain of RedL. In the *pig* cluster there are two gene products with homology to aminotransferases, PigE and PigH. PigH is homologous to RedN, which is proposed to be involved in the biosynthesis of the monopyrrole moiety, so here we suggest that PigE is the one involved in MBC biosynthesis in *Serratia*, although it only has low homology (35% identity) with the putative PLP-dependent domain of RedL (Cerdeño *et al.*, 2001).

The remaining two steps required for the formation of MBC are (i) oxidation of the hydroxymethyl group to an aldehyde and (ii) methylation of the hydroxyl group on ring B. PigF shows sequence identity to S-adenosylmethionine-dependent methyltransferases, including RedI, and is assumed to be responsible for the methylation (Cerdeño *et al.*, 2001; Summers *et al.*, 1992). In *Str. coelicolor* the gene product proposed for the oxidation step was RedK, which shows sequence identity to NAD(P)-dependent dehydrogenases. No homologue of RedK was found in the *pig* cluster and so the enzyme that catalyses this reaction is uncertain. One possibility is PigB, the majority of which shows sequence identity to FAD-dependent amine oxidases. Another possibility is that PigB catalyses one of the two oxidations required for the conversion of the prolyl-PigG to the corresponding pyrrole.

### Biosynthesis of MAP

Four of the Pig proteins share homology with Red proteins proposed to be involved in the biosynthesis of 2-undecylpyrrole. The first of these, PigJ, is homologous to RedX. Both of these proteins appear to have two keto-synthase-like domains, but in each case only the C-terminal domain can be active as the N-terminal domain has the active-site cysteine replaced by an aspartate residue. In PigJ, unlike RedX, the amino acids flanking this aspartate are poorly conserved when compared with the active site of a true keto-synthase. It is proposed that the apparently active C-terminal domain of PigJ catalyses the condensation of a malonyl thioester with a hexanoyl thioester to give a 3-keto-octanoyl thioester. The hexanoyl group is probably derived from fatty acid biosynthesis or degradation and may be introduced as either hexanoyl-CoA or a hexanoyl ACP. PigH, thought to be the next enzyme in the pathway, appears to have two ACP-like domains at its N-terminus, as does its homologue RedN. One possibility is that one or other of these ACP domains is malonylated (by an unknown acyltransferase) and this malonyl group then condenses with the hexanoyl thioester in the active site of PigJ to give a 3-keto-octanoyl thioester of PigH. In this pathway we assign PigE and PigH to their respective reactions in MBC and MAP biosynthesis; however, both proteins are putative aminotransferases and therefore their roles could be exchanged.

The remainder of PigH (other than the two N-terminal ACP domains) shows sequence similarity with PLP-dependent enzymes, in particular 8-amino-7-oxononanoate synthase (AONS), one of the enzymes of biotin biosynthesis. In the AONS-catalysed reaction, glycine is decarboxylated and the remaining aminomethyl group attacks a CoA thioester, releasing free CoA (Alexeev *et al.*, 1998; Webster *et al.*, 2000). The reaction proposed for PigH is similar, except that the amino acid decarboxylated is alanine and the thioester has a  $\beta$ -keto group. It is this ketone group which is attacked by the  $\alpha$ -carbon of the decarboxylated alanine while the thioester is attacked by the amino group, giving the five-membered pyrrolinone ring. Red N, the

homologue of PigH in *Str. coelicolor*, was proposed to catalyse a similar reaction, except that the amino acid involved is glycine and the  $\alpha$ -carbon attacks the thioester while the amine attacks the ketone. Sequence alignment of PigH with AONS from *E. coli*, for which a crystal structure is known, shows complete conservation of the amino acids surrounding the PLP in the active site (Webster *et al.*, 2000). The lysine which forms the Schiff's base with the PLP is predicted to be Lys-485 in PigH, and the aspartate which is hydrogen bonded to N-1 of PLP is predicted to be Asp-451.

The final step required for the formation of MAP is reduction of the amide group. The equivalent step in *Str. coelicolor* was proposed to be catalysed by RedH and RedF, and the homologous proteins in *Serratia* are PigC and PigN. PigC (like RedH) appears to have three domains. The N-terminal and C-terminal domains share sequence identity with the first two domains of pyruvate phosphate dikinase, which are the ATP-binding domain and a swivelling phosphoryl transfer domain, respectively. By homology, the phosphorylation site on the phosphoryl transfer domain of PigC is His-840 (Cerdeño *et al.*, 2001; Herzberg *et al.*, 1996). In PigC these two domains are separated by a domain that has no significant sequence identity to any protein of known function. This is presumably the binding domain for the substrate that is to be phosphorylated. From a chemical point of view, it makes sense that phosphorylation of the amide occurs, because without some form of activation, amides are generally too stable to be reduced by a NAD(P)H-dependent reductase. PigN is homologous to RedF and has low homology to known NAD(P)-dependent oxidoreductases (Prosite entry PS50299). It is therefore proposed to be the reductase that generates MAP.

### Biosynthesis of prodigiosin and other Pig proteins

The remaining *pig* gene products that have not been included in Fig. 1(b) are PigB, PigD, PigK, PigL and PigM. Of these, PigL has sequence identity (and contains conserved motifs) with phosphopantetheinyltransferases (PPTases) (Lambalot *et al.*, 1996; Reuter *et al.*, 1999). PigL is therefore presumed to transfer the 4'-phosphopantetheinyl moiety from CoA to the hydroxyl of the conserved serine residues in PigG and/or the ACP domains of PigH, converting these enzymes from the inactive apo- to the active holo- form. As mentioned above, PigB is a putative amine oxidase and is possibly involved in one or other of the proposed oxidation steps. Thus the remaining candidates for the enzyme which couples MBC and MAP to give prodigiosin are PigD, PigK and PigM. PigD is not likely to be the enzyme for this step as it has no homologue in the Red cluster. There are currently no significant sequence identities of PigK or PigM with enzymes of known function, so it is not possible to predict which is more likely to catalyse the final coupling reaction.

**Table 3.** Heterologous expression of the *pig* genes from Sma 274 (pPIG4) and *Serratia* 39006 (pTON245)

+, Pigment; –, no pigment; ND, not determined. Transformants were incubated at 25 and 30 °C at a range of densities. Strains listed in the table are described in Table 1.

Host	pPIG4	pTON245
<i>E. coli</i> XL-1 Blue	+	–
<i>E. coli</i> DH5 $\alpha$	+	–
<i>Serratia</i> 39006 HSPIG28	+	+
Sma 274 MUT 12	+	ND
Ecc	–	+

\*Expression of pigment in *Serratia* 39006 HSPIG28 biosynthetic mutant complemented with pPIG4 is not to wild-type levels.

### Heterologous expression of prodigiosin genes in *Serratia*, *Erwinia* and *E. coli* strains and mutants

The two Pig<sup>+</sup> cosmids (pPIG4 and pTON245) were used to transform electrocompetent cells of various *Erwinia*, *E. coli* and *Serratia* strains and the results are shown in Table 3. Surprisingly, pPIG4 from Sma 274 was not capable of heterologous expression in Ecc, despite encoding pigment production in *E. coli* XL-1 Blue and *E. coli* DH5 $\alpha$ . The reverse was true for the *Serratia* 39006-derived cosmid, pTON245: it encoded prodigiosin synthesis in Ecc, but not in *E. coli*. The Sma 274 pPIG4 cosmid could complement biosynthetic mutants in both clusters, although complementation was not to wild-type levels in the *Serratia* 39006 biosynthetic mutant. The reasons for the genus/species-dependent expression of the two recombinant cosmids are not known.

### Differences in *pig* cluster regulation

Although the global arrangement of the *pig* clusters is very similar in *Serratia* 39006 and Sma 274, the regulation of the two *Serratia* *pig* clusters is markedly different. In *Serratia* 39006, transcription of the *pig* cluster is under quorum-sensing control via the *smal/R* genes and the *N*-acyl homoserine lactones (AHLs) *N*-butanoyl-L-homoserine lactone (BHL) and *N*-(hexanoyl)-L-homoserine lactone (HHL) (Thomson *et al.*, 2000). Sma 274 has been tested for production of AHLs and the presence of *smal/R* homologues (data not shown), and no AHL-dependent quorum-sensing system has been detected (data not shown). Recently, *luxS*, which is responsible for the production of Autoinducer-2 (AI-2), has been shown to regulate prodigiosin production via extracellular signalling in Sma 274 (Coulthurst *et al.*, 2004). No evidence of an AHL-dependent quorum-sensing system controlling pigment biosynthesis has been found in any of the other 22 *S. marcescens* strains tested, of which seven are pigmented (data not shown), implying that quorum-sensing control of prodigiosin production is both highly strain

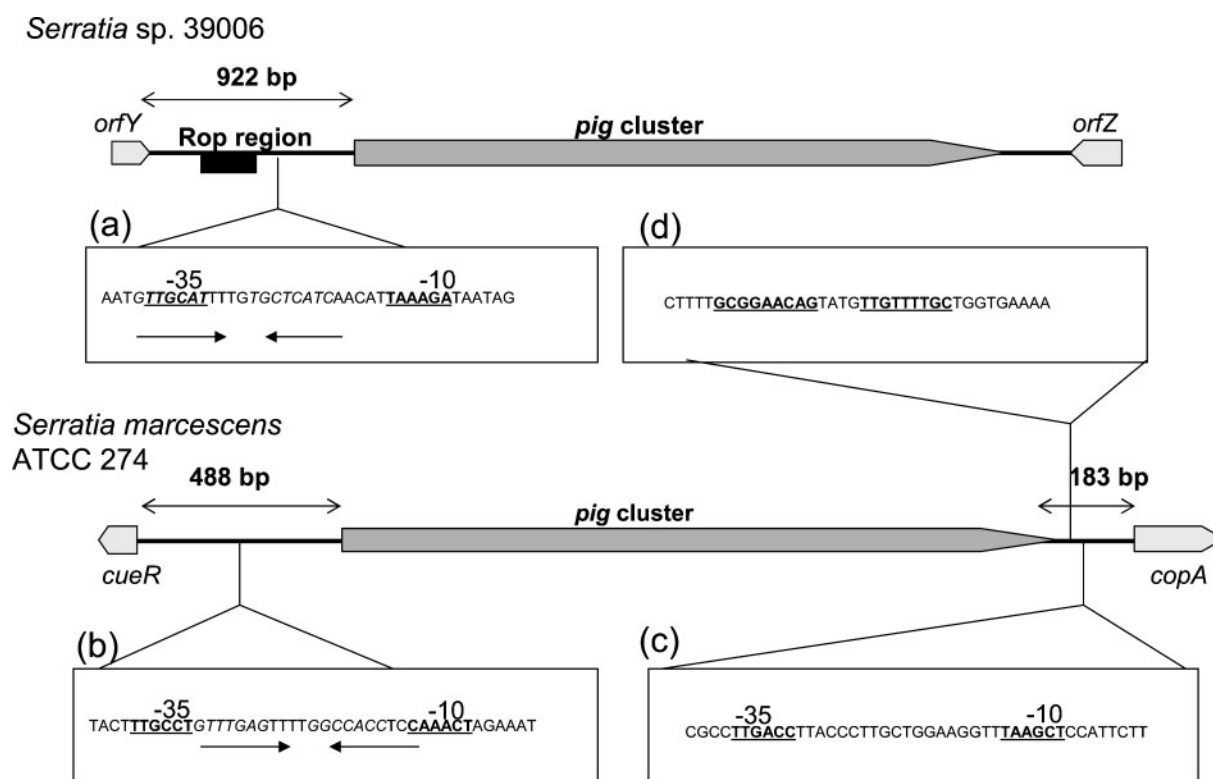


dependent and comparatively rare. Interestingly, a recent report has described a strain of *S. marcescens* in which pigment is affected by production of OHHL, another AHL (Hörng *et al.*, 2002). Where AHLs are made by *S. marcescens* strains, BHL is usually the molecule produced. Therefore, there is considerable strain-dependent variation in both the ability to make AHLs and in the chemical nature of the molecule made.

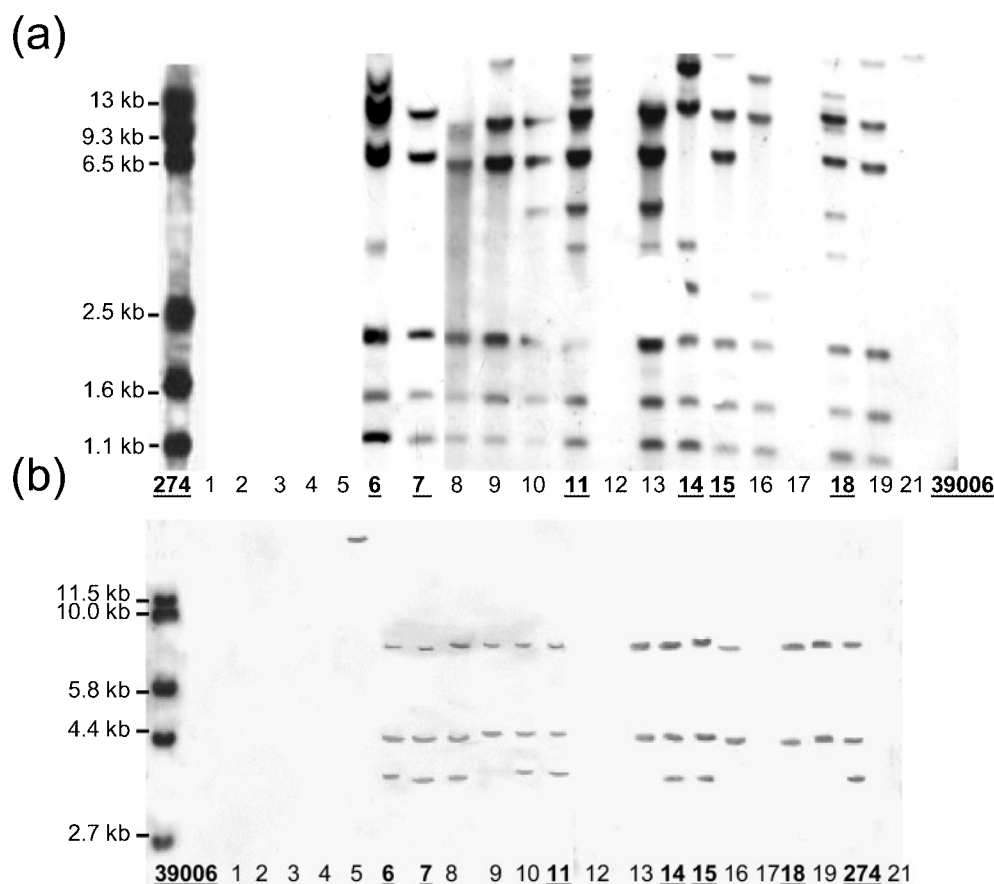
Another conspicuous difference between the *pig* clusters is the intergenic region upstream of *pigA*. In Sma 274 this region is 488 bp compared to 922 bp in *Serratia* 39006. In *Serratia* 39006, transposon insertions in the region 200–320 bp upstream of the *pigA* transcriptional start site cause hyperpigmentation. This has been termed the ‘Rop1’ class of mutation and has been hypothesized to be due to disruption of a binding site for a repressor(s) (Slater *et al.*, 2003). The *Serratia* 39006 promoter has been mapped (Slater *et al.*, 2003). Based on this, corresponding –35 and –10 regions are proposed for Sma 274 in the region upstream of *pigA* (Fig. 2).

A long-documented phenomenon is hyperpigmentation

of *S. marcescens* strains when grown on PGM (Morrison, 1966). Recent work in *Serratia* 39006 has confirmed that phosphate ( $P_i$ ) limitation is a cause of prodigiosin operon upregulation (Slater *et al.*, 2003). In *Serratia* 39006, a mutation in the Pst transport system, the major route for inorganic phosphate uptake under phosphate-limiting conditions, physiologically mimics the phenotype of  $P_i$  limitation. This notion is consistent with the identification of the Pho box covering the –35 and –10 regions of the *pig* cluster promoter of *Serratia* 39006 (Fig. 2) (Slater *et al.*, 2003). Hyperpigmentation due to growth on PGM is widely reported in various *S. marcescens* strains (Grimont & Grimont, 1978) and so the Pho box may be present in the 5′ regulatory region of all *S. marcescens* *pig* clusters. Indeed, a predicted Pho box has been identified 5′ of *pigA* in Sma 274 (Fig. 2). The Pho box consensus CTGTCAT A(AT)A(TA) CTGT(CA)A(CT) is composed of two 7 bp direct repeats separated by a 4 bp segment that is part of the –35 region (VanBogelen *et al.*, 1996). The two predicted Pho boxes in *Serratia* 39006 and Sma 274 are 4/7 identical to each other in the 7 bp repeated region and 3/4 in the middle region, but the 4 bp segment is not part of the –35 region in either.



**Fig. 2.** Promoter regions of the prodigiosin biosynthetic clusters (Pig clusters) from *Serratia* 39006 (top) and *S. marcescens* ATCC 274 (bottom). (a) Promoter region of the 39006 *pig* cluster with the –10 and –35 regions underlined. The arrows show the predicted Pho box. (b) Predicted promoter region of the Sma 274 *pig* cluster with the –10 and –35 regions underlined and a predicted Pho box italicized and denoted with arrows. (c) Predicted *copA* promoter. (d) Putative transcriptional terminator sequence. The ‘Rop’ region is 200 to 320 bp upstream of the translational start of *pigA* in *Serratia* 39006.



**Fig. 3.** Distribution of the *pig* cluster within pigmented and non-pigmented *S. marcescens* strains. Southern hybridization of restriction enzyme digested chromosomal DNAs of *S. marcescens* strains isolated from different sources using (a) *AclI*-digested genomic DNA and probed with a *Sma* 274 *pig* cluster-specific probe and (b) *MluI*-digested genomic DNA and probed with a *Serratia* 39006 *pig* cluster-specific probe. Numbers are as in Table 1, with the pigmented strains underlined.

### Southern blotting using *S. marcescens* ATCC 274 and *Serratia* sp. ATCC 39006 *pig* clusters as probes

The *Sma* 274 *pig* cluster cosmid (pPIG4) was digested, DIG labelled and used to probe genomic DNA of several pigmented and non-pigmented *Serratia*. DNA from 12 of the 23 *S. marcescens* strains tested hybridized to the 274 *pig* cluster probe (Fig. 3). All of the pigmented strains have bands, many corresponding to the restriction pattern of *Sma* 274 from which the probe is generated, suggesting that restriction sites are conserved between many of the *pig* clusters. Interestingly, the prodigiosin biosynthetic operon is also present in some non-pigmented *S. marcescens* strains. These 'cryptic' *pig* clusters are found in six non-pigmented *S. marcescens* isolates (*Sma* 8, 9, 13, 10, 16 and 21). These strains were tested on LBA and PGM at 25, 30 and 37 °C to see if pigment production could be induced. They were also tested to see if cross-feeding from other pigmented strains could provide any intermediates or signal molecules that may be required for pigment synthesis, but

with the exception of *Sma* 10, which appeared slightly pink on PGM, no pigment was induced in any of the experiments (data not shown).

The *Serratia* 39006 *pig* genes were DIG labelled using random prime (Roche) and used to probe genomic DNA from the same *S. marcescens* strains. The restriction pattern of all of the strains that hybridized was more similar to the *Sma* 274 restriction pattern than the *Serratia* 39006 restriction pattern. This is consistent with the observation that *Serratia* 39006 is not in the species *marcescens* and is quite different from the other *S. marcescens* strains. Five strains (8, 9, 10, 13 and 16) were shown to harbour cryptic *pig* clusters when probed with both cluster-specific probes. Of these strains, three are clinical isolates and two are environmental. It has long been observed that non-pigmented antibiotic-resistant strains of *S. marcescens* are much more common in the clinical setting than in the natural environment (Aucken & Pitt, 1998; Carbonell *et al.*, 2000; Grimont & Grimont, 1978). There is no obvious reason for the prevalence of non-pigmented clinical strains,

though multiple theories have been presented (Blazevic, 1970; Cooksey *et al.*, 1976; Ding & Williams, 1983; Grimont & Grimont, 1978; Holland & Dale, 1978). Presumably, there is some pressure counter-selecting expression of prodigiosin in a clinical environment where fitness of *Serratia* strains may be improved by loss of prodigiosin production due to biosynthetic or regulatory mutations.

### Relative genomic contexts of the *pig* clusters

Flanking the *Serratia* 39006 *pig* cluster are genes transcribed in the same direction as the *pig* genes. The product of the 5' gene (*orfY*) shows sequence similarity to NADH oxidases and contains the pyridine nucleotide-disulphide oxidoreductase dimerization domain. The 3' gene encodes a hypothetical protein with no known significant homologues. In contrast to *Serratia* 39006, the genes flanking the *pig* gene cluster in Sma 274 (Fig. 1a) encode proteins similar to CueR (68 % identity and 76 % similarity to YbbI, accession number AAK06655) and CopA (69 % identity and 80 % similarity to CopA, accession number Q59385) of *E. coli* (Rensing *et al.*, 2000; Stoyanov *et al.*, 2001). CueR is the transcriptional regulator of CopA. CueR is a member of the MerR family of transcriptional regulators, which occur in a range of bacterial genera and respond to a wide variety of external stimuli (Amabile-Cuevas & Demple, 1991; Brocklehurst *et al.*, 1999; Chiu *et al.*, 1999; Stoyanov *et al.*, 2001; Summers, 1992). CopA is a copper-transporting P-type ATPase efflux pump and a central component of copper homeostasis in *E. coli* (Rensing *et al.*, 2000). As shown in Fig. 4, the *copA* and *cueR* genes and their homologues are typically contiguous and divergently transcribed. It is therefore interesting that the complete *pig* gene cluster interrupts these genes in strain Sma 274.

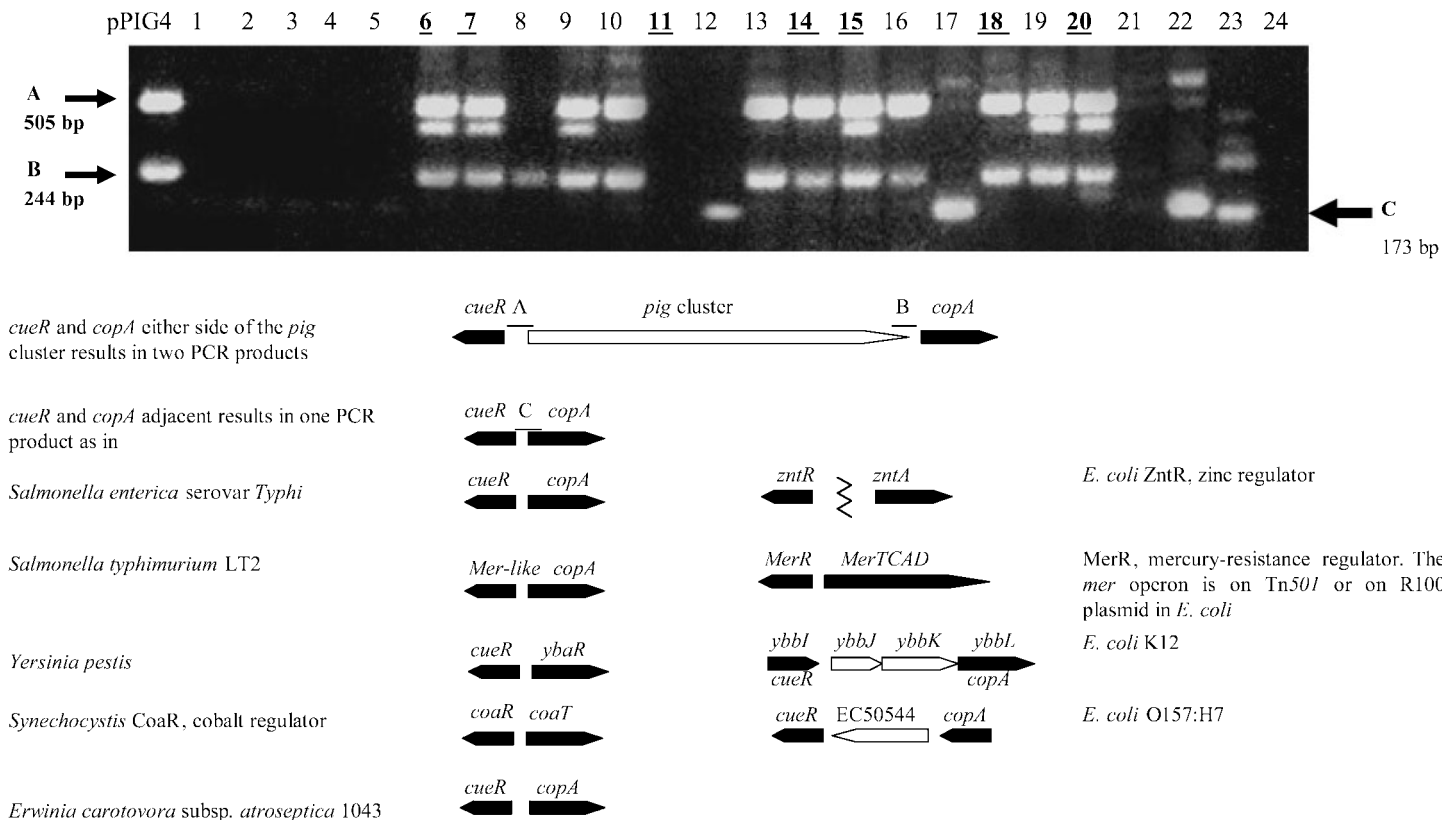
We examined 22 *S. marcescens* isolates to see whether the *cueR/copA* genes always flank the *pig* cluster, or are adjacent – as more typically found in other bacteria. The regions between *cueR* and *pigA* and *pigN* and *copA* were amplified by PCR using the primers *cueR*, PE1, ab77 and PE2. As shown in Fig. 4, this PCR can generate a *cueR–copA* product if these homologues are adjacent to each other, or can generate two products (*cueR–pigA* and *pigN–copA*) if *cueR* and *copA* flank the *pig* cluster. The pigmented strains had the *cueR–copA* homologues arranged at either end of the *pig* cluster, as in Sma 274, with the exception of strain Sma 11 which generated no PCR products (Fig. 4). The *cueR* and *copA* homologues also flanked the *pig* cluster in four *S. marcescens* strains containing a cryptic *pig* locus. In Sma 17, 12 and Db11 the 173 bp PCR product was sequenced and found to be similar to the intergenic region between *cueR* and *copA*, showing that the *cueR–copA* genes are adjacent and divergent in these *S. marcescens* strains, as more commonly found in other bacteria. This may suggest an ancestral state of adjacent *cueR–copA* genes, with the *pig* cluster having recombined into this intergenic locus comparatively recently in some strain lineages. These observations could suggest that the *pig* cluster may be under CueR-mediated control and/or that one or more of

the *Pig* enzymes requires copper, or a related metal, as a co-factor. The CueR/CopA system is involved in copper efflux (Changela *et al.*, 2003; Petersen & Møller, 2000; Stoyanov *et al.*, 2001). It is therefore intriguing that prodigiosin has been shown to effect Cu-dependent DNA cleavage (Melvin *et al.*, 2001; Park *et al.*, 2003) and so it is conceivable that intracellular copper availability must be regulated in concert with prodigiosin biosynthesis to avoid DNA damage.

In *E. coli* K-12 the *cueR–copA* intergenic region contains the *copA* promoter where CueR binds and promotes transcription of *copA* (Stoyanov *et al.*, 2001). This entire intergenic region between *cueR* and *copA* is very similar to the sequence found in the intergenic region between *pigN* and *copA* in Sma 274 (Fig. 2). The promoter is highly conserved between *E. coli* K-12 and Sma 274, with 32/40 bases identical over the whole region. Sequence analysis of the PCR products from strains Db11, Sma 12 and 17, in which the *cueR–copA* genes are adjacent to each other rather than either side of the *pig* cluster, had an identical predicted *copA* promoter region to Sma 274 (data not shown). Database searches have shown this promoter to be highly conserved in many bacteria that contain *cueR* and *copA* homologues, such as *E. coli* O157:H7, *Yersinia pestis*, *Salmonella typhi* and *Sal. typhimurium* (data not shown). Despite the location of the *pig* cluster, the presence of the *copA* promoter, the putative transcriptional terminator (Fig. 2) and the 183 bp gap between *pigN* and *copA* might suggest that the *copA* gene is regulated independently of the *pig* genes in Sma 274.

### Summary

Our results show that the genes for prodigiosin biosynthesis in *Serratia* lie in a large operon, at least for strains from two different *Serratia* species. Although some of the encoded 'core' enzymes are similar to those found in the *red* cluster of *Str. coelicolor*, there are some clear differences between the biosynthetic components from the Gram-negative and Gram-positive clusters. We have assigned a role for the protein of each gene where a putative function can be predicted based on sequence identity with proteins of known function and comparison with the proposed pathway for undecylprodigiosin biosynthesis. We are currently investigating the biosynthesis of prodigiosin further to confirm the function of each protein in the biosynthetic pathway. The transcriptional organization is retained in the two *Serratia* clusters, but is completely different in *Streptomyces*. Furthermore, the genomic context is quite different in the two species of *Serratia* investigated in this study. Among *S. marcescens* strains there is considerable heterogeneity. Some strains have no *pig* genes or have cryptic *pig* genes. Where a *pig* cluster was present in the *S. marcescens* strains, the *pig* genes were flanked by *cueR* and *copA*, genes involved in metal (copper)-dependent regulation and efflux. This observation begs the question of whether or not there is a physiological connection between copper (or other metal) homeostasis and prodigiosin biosynthesis.



**Fig. 4.** PCR amplification of the *cueR* to *pigA* intergenic region and the *pigN* to *copA* intergenic region from *S. marcescens* strains 1–21. Two PCR products are amplified (B, *cueR*–*pigA* and C, *pigN*–*copA*) if *cueR* and *copA* flank the *pig* cluster as is the case for Sma 274 (lane 20). PCR product C is amplified if *cueR*–*copA* are contiguous. The cosmid pPIG4 contains the *pig* genes and flanking DNA (50 kb total) from Sma 274. Numbers are as in Table 1 with pigmented strains underlined, except number 23 which is *Erwinia carotovora* subsp. *atroseptica* strain SCRI 1043 ([http://www.sanger.ac.uk/Projects/E\\_carotovora/](http://www.sanger.ac.uk/Projects/E_carotovora/)). Lane 24 is a negative control.



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