Metabolic and regulatory engineering of *Serratia marcescens*: mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities

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*Serratia marcescens* is an important cause of opportunistic human infections. Many, but not all, strains produce prodigiosin, a secondary metabolic, red-pigment antibiotic, the biosynthesis of which is directed by the *pig* gene cluster. Quorum sensing (QS) involves the production and detection of chemical signal molecules as a means to regulate gene expression in response to population cell density. Several strains of *S. marcescens* have previously been shown to possess an *N*-acyl-**L**-homoserine lactone (aHSL) QS system. This study aimed to determine the impact of introducing, by phage-mediated horizontal gene transfer, a biosynthetic gene cluster (*pig*) and a regulatory locus (aHSL QS) into strains lacking the respective trait. The *pig* cluster from *S. marcescens* ATCC 274 (*Sma* 274) was transferred to the non-pigmented strain, *S. marcescens* strain 12 (*Sma* 12). In the engineered strain, pigment was expressed and brought under the control of the recipient’s native regulatory systems (aHSL QS and *luxS*). Moreover, transfer of the aHSL locus from *Sma* 12 to the non-QS *Sma* 274 resulted in the imposition of aHSL control onto a variety of native traits, including pigment production. In addition, during this study, the QS regulon of the clinical strain, *Sma* 12, was characterized, and some novel QS-regulated traits in *S. marcescens* were identified. The results have implications for the evolution and dissemination of biosynthetic and QS loci, illustrating the genetic modularity and ease of acquisition of these traits and the capacity of phages to act as vectors for horizontal gene transfer.

INTRODUCTION

*Serratia marcescens* is a Gram-negative, enteric bacterium that is able to inhabit a wide variety of ecological niches and cause disease in plant, vertebrate and invertebrate hosts (Grimont & Grimont, 1978). It is an opportunistic human pathogen and is responsible for an increasing number of serious nosocomial infections, a problem exacerbated by the resistance of many strains to multiple antibiotics (Hejazi & Falkiner, 1997). A characteristic feature of many *S. marcescens* strains, particularly those of environmental origin, is production of the red tripyrrole antibiotic prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin). Prodigiosins are currently of great interest because they have been shown to possess antimicrobial, antiprotozoal, immuno-suppressive and anti-oncogenic properties (Williamson et al., 2005 and references therein). However, the physiological role of prodigiosin in the producing organism remains elusive. In this study we used two strains of *S. marcescens*: *S. marcescens* ATCC 274 (*Sma* 274) is a pigmented, environmental strain, whereas *S. marcescens* strain 12 (*Sma* 12) is a non-pigmented, clinical isolate.

Previously, we have cloned and sequenced the prodigiosin biosynthetic (*pig*) gene clusters from two different *Serratia* species: *Sma* 274 and *Serratia* ATCC 39006 (*S 39006*), which contain 14 and 15 genes, respectively (Harris et al., 2004). In strains of *S. marcescens*, we have shown that the pigment cluster, if present, is always flanked by the *cueR* and *copA* genes, thought to be involved in controlling copper efflux.

**Abbreviations:** aHSL, *N*-acyl-**L**-homoserine lactone; BHL, *N*-butanoyl-**L**-homoserine lactone; HHL, *N*-hexanoyl-**L**-homoserine lactone; QS, quorum sensing.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the *smaIR* locus from *Sma* 12 is AM236917.
and homeostasis. Strains lacking the pigment genes have contiguous cueR-copA genes, implying that, in some strains, the prodigiosin gene cluster may have been acquired horizontally by insertion into the cueR-copA intergenic region (Harris et al., 2004). In addition, we have recently defined the biosynthetic pathway of prodigiosin from primary metabolites in Serratia (Williamson et al., 2005). Regulation of prodigiosin production has been extensively studied in S 39006, where it has been shown to involve a complex regulatory network, integrating information from a variety of environmental cues, including quorum sensing (Slater et al., 2003; Fineran et al., 2005).

Quorum sensing (QS) is a process of intercellular communication by which bacteria detect their population cell density and regulate gene expression accordingly. Bacterial cells produce signal molecules which accumulate in their surroundings as the population increases. When the concentration of the signal exceeds a threshold value, signalling pathways are activated and a concerted physiological response is mounted throughout the population. A wide spectrum of important processes, in diverse bacterial species, is regulated by QS, e.g. virulence, production of secondary metabolites, symbiosis, sporulation and biofilm formation (Whitehead et al., 2001). The most extensively studied QS systems in Gram-negative bacteria are those utilizing N-acyl-L-homoserine lactone (aHSL) signal molecules, in which LuxI homologues synthesize various aHSL signals and LuxR-type transcriptional regulatory proteins bind their cognate signal at high cell densities and alter gene expression (as reviewed by Lazdunski et al., 2004; Pappas et al., 2004; Whitehead et al., 2001). In S 39006, production of prodigiosin and carbapenem antibiotic is regulated by Smal [which produces N-butanoxy-1-L-homoserine lactone (BHL) and N-hexanoyl-1-L-homoserine lactone (HHL)] and the cognate receptor, SmaR (Slater et al., 2003). In S. marcescens SS-1 (Sma SS-1), prodigiosin, secreted nuclease and sliding motility are under the control of SmaR (Horn et al., 2002). In the non-pigmented strain S. marcescens MG1 (Sma MG1; formerly S. liquefaciens MG1), swarming motility, production of secreted protease and biofilm formation are under aHSL QS control, via the BHL/HHL synthase, Smal (Labbate et al., 2004; Riedel et al., 2001).

Another type of QS system has also been described in Gram-negative bacteria. As reviewed (Vendeville et al., 2005), autoinducer-2 (AI-2), whose synthesis depends on LuxS, was first described as a QS signalling molecule regulating bioluminescence in Vibrio harveyi, but occurrence of luxS/AI-2 production now appears to be widespread. LuxS/AI-2 have been implicated in ‘QS’ in diverse bacterial species on the basis of the multiple phenotypes that have been reported for luxS mutants of different bacteria, including many affecting virulence determinants. However, since LuxS has a metabolic role in the activated methyl cycle, some of the phenotypes reported for luxS mutants could also be due to metabolic defects (Winzer et al., 2002; Vendeville et al., 2005). The luxS mutant of Sma 274 exhibits reduced prodigiosin production, haemolysis and virulence, and luxS regulation of pigment production does indeed occur via an extracellular signal, most likely AI-2 (Coulthurst et al., 2004).

Prior to this study, we noted that some strains of S. marcescens possess pig clusters, whilst others do not, and some possess aHSL QS systems, whereas others do not (Harris et al., 2004: unpublished results). Moreover, several Serratia QS loci appear to be flanked by remnants of transposable elements, namely smallIR in S 39006 and spinIR in Sma SS-1 (Thomson et al., 2000; Horn et al., 2002). We therefore speculated that the pig cluster and/or QS loci might be mobile between Serratia strains (Thomson et al., 2000). We wondered whether we could engineer a Pig+ strain from a Pig− strain, not by the use of physiologically artefactual multicopy cloning vectors, but by in vivo stable transfer of the biosynthetic locus into the chromosome. Similarly, we aimed to determine the physiological consequences of stable genetic transfer of an aHSL QS locus into the chromosome of a strain lacking any such detectable locus. Such interchromosomal movement should mimic the ‘horizontal transfer’ of gene sets. Phages are thought to play an important evolutionary role in the horizontal transfer of genes, in particular for the evolution of pathogenicity islands and other gene clusters encoding secondary metabolites. Hence we decided to use a transducing phage to move specific gene sets between strains, to mimic as closely as possible the natural situation.

In this work, in addition to identifying multiple QS-regulated phenotypes in the clinical strain, Sma 12, we used a generalized transducing phage to effect the metabolic engineering of strains of S. marcescens. A regulatory locus (QS) and a large secondary metabolite biosynthetic locus (Pig) were reciprocally transferred between strains lacking these traits to yield chromosomally engineered derivatives that had newly acquired metabolic and regulatory capabilities. Our results have implications for the remarkable evolutionary plasticity of bacteria and the capacity of phages to act as vectors for horizontal gene transfer.

**METHODS**

**Bacterial strains, media and culture conditions.** The bacterial strains, plasmids and phage used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37°C and *S. marcescens* strains were grown at 30°C in Luria broth (LB; 10 g tryptone l−1, 5 g yeast extract l−1, 5 g NaCl l−1). *V. harveyi* BB170 was grown at 30°C in AB medium (Greenberg et al., 1979). Cultures of *S. marcescens* strains were grown in 25 ml LB inoculated to a starting OD600 of 0·02 and incubated at 30°C with shaking (300 r.p.m.) in a 250 ml conical flask. Growth was measured as OD600 using a Unicam Hēξios spectrophotometer and 1 cm pathlength cuvettes. Where necessary, antibiotics were added at the following final concentrations: ampicillin (Ap), 100 μg ml−1; chloramphenicol (Cm), 50 μg ml−1; kanamycin (Km), 50 μg ml−1; streptomycin (Sm), 100 μg ml−1; and tetracycline (Tc), 10 μg ml−1.

**Generalized transduction.** An overnight culture of the *S. marcescens* recipient strain was grown in 5 ml LB. Bacterial cells were
pelleted by centrifugation and the pellet resuspended in 1 ml LB. Then 100 μl of a high-titre φ3M phage lysate grown on the donor strain was added to the cell suspension, mixed and incubated at 30°C for 1 h. Aliquots (100 μl) of the phage-infected cells were plated onto LB containing the appropriate antibiotic and incubated at 30°C overnight. Transductants were purified by streaking twice onto fresh plates to eliminate any phage carry-over.

**Molecular biological techniques.** All DNA manipulations were performed as described by Sambrook et al. (1989). Genomic DNA was extracted with the DNeasy Tissue kit (Qiagen) and 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 DNA sequencing was performed by the DNA Sequencing facility, Department of Biochemistry, University of Cambridge. PCR reactions were performed using either Expand High Fidelity Taq polymerase (Roche) or BioTaq (Bioline), depending on the application.

**Sequencing of the smallR locus.** In order to identify a luxI homologue in Sma 12, the oligonucleotide primers Small-5’ (S’-TWAGAAAAAAGGTIT3’-3”) and Small-3’ (5’-CAATGCCAAAACATGCCCAT3’) were designed to conserved regions between Small from S 39006 and swrl from Sma MG1. PCR amplification of Sma 12 genomic DNA using Small-5’ and Small-3’ (with the following PCR parameters: 1 cycle of 94°C for 5 min, 45°C for 30 s and 68°C for 50 s, followed by 30 cycles of 94°C for 20 s, 45°C for 30 s, 68°C for 50 s, and a final step of 72°C for 7 min) resulted in a 600 bp product, which was sequenced and found to encode part of a LuxI homologue. The sequence of this internal fragment was then used to design primers for use in determining the flanking sequences by

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

<table>
<thead>
<tr>
<th>Strain/plasmid/phage</th>
<th>Genotype/phenotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>DH5α</td>
<td>F′ φ80lacZM15 Δ(lacZYA-argF)U169 endA1 recA1 hisR17 (r6K mrr) deoR thi-1 supE44 Δλ- gyrA96 relA1</td>
<td>GibcoBRL, Life Technologies</td>
</tr>
<tr>
<td>S17-1 λpir</td>
<td>recA pro hsdR recA::RP4-2-Tc::Mu λpir TeR SmR SpR</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
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<td><strong>Serratia sp.</strong></td>
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<td>LIS</td>
<td>Serratia ATCC 39006 smal::miniTnSm/Sp, SmR</td>
<td>Thomson et al. (2000)</td>
</tr>
<tr>
<td>S. marcescens ATCC 274 (Sma 274)</td>
<td>Wild-type (Pig−), non-motile</td>
<td>Lab stock</td>
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<tr>
<td>S. marcescens strain 12 (Sma 12)</td>
<td>Wild-type (Pig−), clinical isolate</td>
<td>Harris et al. (2004)</td>
</tr>
<tr>
<td>S. marcescens ATCC 274 motile isolate (Sma M274)</td>
<td>Wild-type (Pig−), motile</td>
<td>Matsuyama et al. (1995)</td>
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<tr>
<td>NW7</td>
<td>Sma 274, copA::cat, CmR</td>
<td>Williamson et al. (2006)</td>
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<tr>
<td>SCC4</td>
<td>Sma 274, luxS::KmR, KmR</td>
<td>Coulthurst et al. (2004)</td>
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<tr>
<td>Sma 12I</td>
<td>smal::miniTn5Km1, KmR</td>
<td>This study</td>
</tr>
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<td>Sma 12P</td>
<td>pig−, copA::cat, CmR derivative of Sma 12; generated by transduction using φ3M grown on strain NW7</td>
<td>This study</td>
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<tr>
<td>Sma 12IP</td>
<td>pig−, smal::miniTn5Km1, CmR KmR derivative of Sma 12P; generated by transduction using φ3M grown on strain Sma 121</td>
<td>This study</td>
</tr>
<tr>
<td>Sma 12S</td>
<td>luxS::KmR, KmR derivative of Sma 12; generated by transduction using φ3M grown on strain SCC4</td>
<td>This study</td>
</tr>
<tr>
<td>Sma 12SP</td>
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<td>This study</td>
</tr>
<tr>
<td>Sma 274I</td>
<td>smal::miniTn5Km1, SmalR derivative of Sma 274</td>
<td>This study</td>
</tr>
<tr>
<td>Sma M274I</td>
<td>following transduction using φ3M grown on strain Sma 121</td>
<td>This study</td>
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<td><strong>Other bacterial strains</strong></td>
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<td>Chromobacterium violaceum CV026</td>
<td>evI::Tn5, aHSL bioassay strain</td>
<td>McClean et al. (1997)</td>
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<tr>
<td>Vibrio harveyi BB170</td>
<td>luxN::Tn5, AI-2 bioassay strain</td>
<td>Surette et al. (1999)</td>
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<td><strong>Phage</strong></td>
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<td>φ3M</td>
<td>S. marcescens generalized transducing phage</td>
<td>Williamson et al. (2006)</td>
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<td><strong>Plasmids</strong></td>
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<td>pACYC184</td>
<td>Low-copy cloning vector, CmR, TeR</td>
<td>Chang &amp; Cohen (1978)</td>
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<td>pNMRW75</td>
<td>675 bp PCR-amplified fragment containing smal from Sma 12 in the EcoRI site of pACYC184</td>
<td>This study</td>
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<tr>
<td>pNMRW85</td>
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<td>pUTminiTn5Km1</td>
<td>Delivery plasmid for miniTn5Km1, ApR, KmR</td>
<td>de Lorenzo et al. (1990)</td>
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single-specific-primer PCR (Shyamala & Ames, 1989) and primer walking. An average 3-4-fold coverage was achieved and the sequence was assembled using the Staden package (Staden, 1996). Sequence analysis was performed using the BLAST suite of programs (Altschul et al., 1990) at GenBank (http://www.ncbi.nlm.nih.gov/blast/).

**Generation of an aHSL mutant.** A random transposon mutagenesis of Smal 12 was performed. E. coli S17-1Ap carrying pUTminiTn5Km1 was used to introduce the transposon into Smal 12 by conjugation as described previously (Williamson et al., 2006). A total of 2700 KmR transconjugants were picked onto lawns of the aHSL bioassay strain, Chromobacterium violaceum CV026. Five aHSL-deficient mutants were identified visually (by the lack of a purple halo). One aHSL-deficient mutant was selected for further study and was transduced into a clean genetic background to generate Smal 12I. To identify the location of the transposon insertion in Smal 12I, PCR amplification was performed using Smal 12-specific primers (NW110 and NW111, see below) in combination with specific primers (NW136 (5'-GATCGTGGCCAGGGCGTTATTGATCC-3') and the resulting PCR products were sequenced.

**Construction of plasmids containing smal and smaR from Smal 12.** The smal gene was PCR-amplified from Smal 12 genomic DNA using primers NW110 (5'-CTGAAATCCGCGGATGCGGACCAGGCTG-3') and NW111 (5'-GCGGATCTTGGTTGGAAGCGGCGGAC-3') and the resulting product cloned into the EcoRI site of pACYC184, generating plasmid pNRW75. The smaR gene was PCR-amplified from Smal 12 genomic DNA using primers NW135 (5'-CGCTGACGATTTAAGCGCTTCTATG-3') and NW136 (5'-GATCGTGGCTTGATGTTGGAAGCGGCGGAC-3') and the resulting product cloned into the PstI site of pACYC177, generating plasmid pNRW85. Smal strains were transformed with the appropriate plasmids by electroporation.

**Measurement of prodigiosin.** Cells were harvested from 1 ml samples of liquid culture by centrifugation and the pellet was resuspended in 1 ml acidified ethanol (4 % 1 M HCl) to extract prodigiosin from the cells. Following a second centrifugation step, the A534 of the supernatant was measured (Slater et al., 2003).

**Measurement of aHSL and AI-2 production.** aHSLs were detected using the CV026 and LIS biosensor strains (McClean et al., 1997; Thomson et al., 2000). Briefly, top agar lawns were prepared by diluting the sensor strain 1/50 in 0-7 % LB agar (LBA) and pouring onto normal (1 %) LBA plates. Either samples of overnight cultures of the strains to be tested were spotted onto the lawn, or wells were cut in the agar plate and filled with cell-free supernatant. The bioassays were incubated at 30 °C overnight until coloured haloes, indicating aHSL production, became visible. For measurement of aHSL production throughout growth, uniformly sized cut-wells in a LIS bioassay plate were filled with cell-free supernatant samples harvested throughout growth and aHSL production expressed as halo area (mm²).

The V. harveyi BB170 bioassay was used to detect AI-2 activity (Surette & Bassler, 1998). Samples (10 μl) of cell-free supernatant were added to the wells of a black microtitre plate. A 16 h overnight culture of BB170, grown at 30 °C in AB medium to an OD₅₆₀ of 1-20, was diluted 1 in 5000 in fresh AB medium and 90 μl added to each sample. The bioassay was incubated for 4-5 h at 30 °C and light production measured using an Anthos LUCY1 luminometer. Positive control samples were obtained from 5 ml AB overnight cultures of BB152, grown for 16 h to an OD₅₆₀ of 1-25.

Cell-free supernatant samples were prepared by centrifugation at 13 400 g for 5 min, followed by passage of the supernatant through a 0-22 μm filter (Millipore), and were stored at 90 °C.

**Other phenotypic assays**

**Swarming, haemolysis and secreted inhibitory activity.** Swarming agar contained 5 g peptone 1⁻¹, 1 % (v/v) glycerol and 0-75 % agar. Blood agar, for detecting secreted haemolytic activity, was prepared by adding 5 % washed erythrocytes to LBA. Defibrinated horse blood was obtained from TCS Biosciences and the erythrocytes were washed in cold phosphate-buffered saline. Production of secreted inhibitory activity was detected using top agar lawns containing 5 μl of an overnight culture of LIS in 4 ml 0-7 % LBA.

**Secreted caseinase activity.** Culture samples were centrifuged to pellet the cells, supernatant samples were removed and kept on ice and protease activity in the supernatant was measured using azocasein (Braun & Schmitz, 1980). A 150 μl sample of supernatant was mixed with 500 μl 2 % azocasein, 100 μl 1 M Tris/HCl (pH 8-0) and 650 μl H₂O; a negative control sample contained H₂O instead of supernatant. After 2 h incubation at 30 °C, 375 μl 14 % perchloric acid was added to stop each reaction. The samples were then centrifuged (13000 g, 5 min), 0-75 ml of the supernatant mixed with 75 μl 10 M NaOH and the A₄₅₀ measured. Activity was calculated as ΔA₄₅₀ h⁻¹ ml⁻¹, the background activity of the negative control was subtracted, and activity was reported as ΔA₄₅₀ h⁻¹ ml⁻¹per OD₆₀₀ unit.

**Secreted chitinase activity.** Cultures of each strain to be tested were grown in LB supplemented with 1-5 % (w/v) crab shell chitin (BDH) for 16 h. Culture samples were centrifuged to pellet the cells, and the supernatant was removed and stored on ice. A 150 μl sample of the culture supernatant was mixed with 450 μl of the substrate mixture and the enzymatic reaction was incubated at 37 °C for 6 h. The substrate mixture consisted of 1 vol. 0-2 % (w/v) chitin azure (Sigma) and 2 vols succinate/NaOH buffer (100 mM, pH 6-0). After 6 h, the samples were centrifuged at 13 400 g for 5 min to remove any unreacted chitin azure and the A₅₅₀ of the supernatant measured. Chitinase activity was reported as ΔA₅₅₀ h⁻¹ ml⁻¹ per OD₆₀₀ unit.

**Biofilm assay.** Overnight cultures of the strains to be tested were diluted to an OD₆₀₀ of 0-2 in LB, 200 μl of each diluted culture was placed in eight wells of a 96-well microtitre plate (Nunclon, catalogue no. 167008), and the plate was incubated for 5 days at 30 °C. To develop the assay, culture medium was removed from the wells by aspiration and the wells were washed with water and then filled with 240 μl 0-1 % crystal violet and allowed to stain for 1 h. The crystal violet was then removed and the wells washed with 3 × 400 μl water to remove non-attached material. Next, 250 μl 50 % ethanol was added to each well and gently agitated for several hours to allow the crystal violet to dissolve. Finally, the contents of each well derived from the same original culture were pooled and the A₅₂₀ was measured.

**Synthetic BHL and preparation of conditioned medium (CM).** For use in complementation assays, BHL (N-butanoyl-L-homoserine lactone) was chemically synthesized as described by Glansdorp et al. (2004). BHL was dissolved in DMSO and used at a final concentration of 1 μM. An equivalent volume of DMSO alone was added to control cultures. CM was obtained from Smal 12 and Smal 12S as follows: cultures were grown for 4 h in LB, cells were removed by centrifugation and the supernatant passed through a 0-22 μm sterile filter (Millipore). CM was added to cultures at a final concentration of 10 %.
RESULTS

Introduction of a functional prodigiosin biosynthetic gene cluster from Sma 274 into Sma 12

We have reported previously that pigmented strains of S. marcescens carry the pig prodigiosin biosynthetic gene cluster inserted between copA and cueR, whereas non-pigmented strains have contiguous cueR-copA genes (Harris et al., 2004). A copA mutant, with a chloramphenicol resistance (Cm) cassette inserted into the copA gene, was constructed in Sma 274 and showed normal prodigiosin production in unsupplemented LB medium (Williamson et al., 2006). Since copA is adjacent to the pig cluster, this mutation provided a selectable marker (Cm) closely linked with the pig genes.

A φ3M lysate was prepared from the copA mutant of Sma 274 and used to transduce the non-pigmented strain, Sma 12, selecting for Cm-resistant transductants. Several hundred pigmented, Cm-resistant transductants were obtained. One of these, Sma 12P, was selected for further study. Sma 12P produced a red pigment with a strong absorbance at 534 nm (data not shown; see also Fig. 2), showing that this strain is able to express the heterologous pig genes and to produce the complex secondary metabolite prodigiosin. Growth of Sma 12P in LB was indistinguishable from that of wild-type Sma 12 (data not shown).

Sma 12 has an aHSL QS system

Several phenotypes, including prodigiosin production, have been reported to be under QS control in other strains of Serratia (see Introduction). It was therefore of interest to determine whether Sma 12 possessed an aHSL QS system and/or produced AI-2, and whether these systems might affect pigment production by Sma 12P. Sma 12 was tested for aHSL production using the aHSL biosensor strains CV026 and LIS, which produce the purple pigment, violacein, and prodigiosin, respectively, in response to exogenous aHSL (McClean et al., 1997; Thomson et al., 2000). Sma 12 gave a positive result with both biosensors (Fig. 1, Fig. 2; data not shown), indicating that it produces a short-chain aHSL molecule(s). Thin-layer chromatography was used to separate ethyl acetate-extracted cell free supernatant from Sma 12 alongside aHSL standards, and aHSL components were detected using LIS/CV026 overlays. Sma 12 was found to produce two aHSL molecules with Rf values corresponding to those of BHL and HHl (data not shown). Further evidence for a central role for BHL in Sma 12 QS was subsequently provided by the use of chemically synthesized BHL in complementation experiments (see below). Production of BHL by Sma 12 was measured throughout growth and was found to be growth-phase-dependent, peaking at the transition to stationary phase (Fig. 1a).

In order to identify a luxl homologue encoding an aHSL synthase in Sma 12, oligonucleotide primers were designed to regions conserved between two known Serratia luxl homologues: smal from S 39006 (smal39006) and swrI from Sma MG1. This approach identified a luxl homologue, named smal, and smal and flanking regions were sequenced by PCR-based methods (see Methods). The complete smal gene encoded a predicted protein of 224 aa, homologous to other LuxI-family aHSL-synthases. Database searches revealed that smal from Sma 12 was most similar to SwrI from Sma MG1 (97% identity over 198 aa) and Smal39006 (63% identity over 222 aa). Downstream of smal, conveniently transcribed, was a gene, smar, encoding a predicted protein of 248 aa, homologous to other LuxR-family transcriptional regulators. Smar from Sma 12 was most similar to SwrR from Sma MG1 (96% identity over 248 aa) and Smar39006 (70% identity over 247 aa). The smal and smar genes overlap by 54 bp (Fig. 1c). The regions sequenced up- and downstream of smal and smar are similar to the corresponding regions flanking swrIR, but do not include any predicted protein-coding sequences.

An aHSL non-producing mutant of Sma 12 was isolated by miniTn5 transposon mutagenesis followed by screening on CV026 bioassay plates (see Methods). Strain Sma 12I does not produce any detectable aHSL activity throughout growth (Fig. 1a). The transposon insertion was mapped to the smal gene, inserted after base pair 236. Therefore Sma 12 production of aHSL activity is dependent on a functional smal gene.

Sma 12 produces luxS-dependent AI-2 activity

The V. harveyi BB170 AI-2 bioassay was used to show that Sma 12 produces extracellular AI-2 activity, with production peaking sharply in late-exponential phase (Fig. 1b). We have previously reported the construction of a defined luxS mutant (Km insertion) in Sma 274 (strain SCC4; Coulthurst et al., 2004). Generalized transduction using φ3M was used to introduce the luxS mutation from Sma SCC4 into Sma 12, generating strain Sma 12S. As expected, Sma 12S was unable to produce detectable AI-2 activity (Fig. 1b). Therefore Sma 12 does produce luxS-dependent AI-2 activity. Production of AI-2 was unaffected in the smal mutant, Sma 12I, and production of aHSL was unaffected in the luxS mutant, Sma 12S, demonstrating that the two potential QS signalling systems are independent, at least at the level of signal production (data not shown).

The heterologous prodigiosin cluster in Sma 12P is brought under the control of the native Sma 12 QS systems

It was of interest to determine whether the pig cluster in Sma 12P, originally from Sma 274, was brought under the control of the native Sma 12 QS systems, namely aHSL QS (smalR) and luxS. Sma 12P was transduced with the smal mutation from Sma 12I to generate Sma 12IP (smal pig+). Production of prodigiosin was severely reduced in Sma 12IP compared with Sma 12P. Pigment production by Sma 12IP could be restored to wild-type levels by exogenous BHL,
cross-feeding from wild-type *Sma* 12 and overexpression of *smaI* in trans (Fig. 2). Interestingly, addition of exogenous BHL to *Sma* 12P caused an increase in prodigiosin production. Overexpression of *smaI* in trans was achieved by the introduction of plasmid pNRW75, which also restored aHSL production (Fig. 2c), providing further confirmation that *smaI* is responsible for aHSL production in *Sma* 12. Therefore the heterologous pig cluster has been brought under the control of the endogenous *Sma* 12 aHSL QS system in *Sma* 12P.

Similarly, *Sma* 12S was transduced with the pig cluster from *Sma* 274 *copA* to generate *Sma* 12SP (*luxS pig*'). Pigment production by *Sma* 12SP was significantly reduced compared with *Sma* 12P (Fig. 2d) and could be partially complemented by the addition of 10% conditioned medium from wild-type *Sma* 12 (AI-2') (data not shown). Therefore the incoming pig cluster has also been brought under the control of *luxS* in *Sma* 12, just as it is under *luxS* control in *Sma* 274 (Coulthurst et al., 2004).

**Determination of other QS-controlled phenotypes in *Sma* 12**

Following the discovery that pigment production in *Sma* 12P was under the control of the native QS systems of *Sma* 12, it was of interest to determine which other phenotypes were normally regulated by QS in wild-type *Sma* 12. *Sma* 12 exhibits swarming motility on 0–75% agar. Swarming motility was eliminated in the aHSL QS mutant, *Sma* 12I, and could be restored by the addition of synthetic BHL (Fig. 3a). Production of secreted haemolytic activity was reduced in *Sma* 12I compared with the wild-type and, again, this phenotype could be complemented by exogenous BHL (Fig. 3b).

Wild-type *Sma* 12, when grown on a lawn of LIS (a *smaI* mutant of S 39006), produced a zone of inhibition in the lawn. Production of this inhibitory activity was dependent on the aHSL QS system, being reduced in the *smaI* mutant, *Sma* 12I (Fig. 3c). Production of this inhibitory activity by...
Sma 12I was comparable to that of wild-type Sma 12 when grown on a lawn of wild-type S 39006, implying that BHL/HHL produced by S 39006 was able to complement the reduced inhibition by the smal mutant of Sma 12 (data not shown). Note that this inhibitory activity was not observed in the LIS aHSL bioassay (e.g. Fig. 1), where the LIS biosensor was exposed to cell-free supernatant samples harvested throughout growth, rather than to actively growing cells.

Production of both secreted caseinase activity and secreted chitinase activity was reduced in the smal mutant, Sma 12I, compared with the wild-type. In each case, wild-type levels of enzyme production were restored by the addition of synthetic BHL (Fig. 3d, e). Finally, a crude assay to assess biofilm forming potential, by measuring the extent of attachment to the walls of a plastic microtitre plate, was used to compare Sma 12I with the wild-type. As shown in Fig. 3(e), 'biofilm formation' by Sma 12I was reduced by ~50 % compared with the wild-type. Addition of synthetic BHL to Sma 12I restored biofilm production and even increased it above wild-type levels. In contrast, no difference in the production of secreted nuclease, gelatinase or lipase activity, or in swimming motility in 0-3 % agar, was detected in Sma 12I compared with the wild-type (data not shown).

The luxS mutant, Sma 12S, was found to exhibit slightly increased 'biofilm formation' compared with the wild-type (Fig. 3f), as well as increased mucoidy on certain media (data not shown). However no reproducible difference was detected between Sma 12S and the wild-type in any of the other phenotypes tested (data not shown).

**Imposition of aHSL-QS control onto prodigiosin production in Sma 274 by introduction of the Sma 12 QS locus**

We also looked for an aHSL QS system in Sma 274, but were unable to detect any such system in this strain. No aHSL signalling molecules were detected using the LIS and CV026 bioassays and no luxIR-like genes were detected using the PCR-based approach used for Sma 12 or by Southern blotting using a smalR probe (data not shown). Since the pig genes of Sma 274 were brought under the Sma 12 aHSL QS system in Sma 12P, we decided to see what the impact, if any, would be of introducing the Sma 12 aHSL QS system into Sma 274. The smalR locus from Sma 12I, with a KmR cassette disrupting smal, was introduced into Sma 274 by generalized transduction and selection on Km. In the resulting strain, Sma 274I (smal smalR), considered equivalent to Sma 12I, any phenotypes becoming QS-dependent should be responsive to exogenous BHL.

Prodigiosin production was greatly reduced in Sma 274I compared with the wild-type and was fully restored by
addition of synthetic BHL, whereas BHL did not increase pigment production in the wild-type strain (Fig. 4a). (The growth rate of Sma274I was indistinguishable from that of the wild-type; data not shown.) This result implied that the incoming QS system was controlling pigment production in Sma274I. We confirmed that the observed reduction in pigment in Sma274I was indeed due to the introduction of smaR by introducing plasmid pNRW85, carrying smaR from Sma12, into Sma274. As expected, pigment production by Sma274(pNRW85) was significantly reduced compared with Sma274(pACYC177) and this decrease was alleviated by the addition of BHL (Fig. 4b).

Next we determined whether several of the other phenotypes found to be QS-dependent in Sma12 had also become QS-dependent in Sma274I. Production of secreted haemolytic activity was reduced in Sma274I compared with the wild-type and could be restored to wild-type levels by the addition of synthetic BHL (Fig. 4c) (The haemolysis produced by wild-type Sma274 was unaffected by the addition of BHL; data not shown.) Production of secreted caseinase activity was modestly, but significantly, reduced in Sma274I compared with the wild-type. Addition of BHL to Sma274I restored caseinase production to wild-type levels, but addition of BHL to wild-type Sma274 had no effect on caseinase production (data not shown). Hence the foreign Sma12 QS system appears to exert a regulatory effect on native gene expression in Sma274I.

Our laboratory isolate of Sma274 is non-motile (data not shown). Since swarming motility is a characteristic phenotype of QS mutants of S. marcescens, we obtained a motile

Fig. 3. aHSL QS-dependent phenotypes of Sma12. (a) Swarming motility on 0·75 % agar by Sma12 (wild-type, left), Sma12I (smaI, right) and Sma12I + 1 μM BHL (bottom). (b) Production of secreted haemolytic activity by Sma12 (left), Sma12I (right) and Sma12I + 1 μM BHL (bottom). (c) Production of inhibitory activity by Sma12 (left) and Sma12I (right) on a lawn of LIS. (d) Production of secreted caseinase activity by Sma12, Sma12I and Sma12I + 1 μM BHL after 16 h growth in LB. (e) Production of secreted chitinase activity by Sma12, Sma12I and Sma12I + 1 μM BHL after 16 h growth in LB + 1·5 % chitin. (f) Biofilm formation by Sma12, Sma12I, Sma12I + 1 μM BHL and Sma12S (luxS) after incubation for 5 days. For (a)–(c), assay plates, prepared as described in Methods, were inoculated with 5 ml overnight culture, diluted to an OD 600 of 0·2, and incubated for 16 h (swarming), 72 h (haemolysis) or 48 h (inhibition). The plates shown are representative of three independent experiments. For (d)–(f), caseinase, chitinase and biofilm assays were performed as described in Methods and bars show mean ± SD (n ≥ 3).
isolate of Sma 274, here called Sma M274 to distinguish it from our own isolate, in order to assess the impact of the Sma 12 QS system on swarming in a normally non-QS background. The smaIR locus from Sma 12I, with a KmR cassette disrupting smaI, was therefore introduced into Sma M274 by generalized transduction to generate strain Sma M274I (smaI smaR+). As expected, Sma M274I showed reduced pigmentation compared with the wild-type (data not shown). As for the smal mutant of Sma 12, Sma M274I was impaired in swarming motility compared with the wild-type. As shown in Fig. 4(d), wild-type Sma M274 and Sma M274I + BHL exhibited characteristic swarming motility on 0-75 % agar, whereas Sma M274I did not. This result again demonstrates that a heterologous Sma 12 QS system is able to exert QS control on native gene expression in Sma 274.

**DISCUSSION**

Prior to this work, we hypothesized that contiguous cueR-copA genes represent the ancestral state of this genetic locus in S. marcescens and that, in pigmented strains, the pig cluster has been recently acquired, between cueR and copA, by horizontal gene transfer. In this study, we exploited the tight linkage to copA in order to introduce a complete functional prodigiosin biosynthetic cluster into a non-pigmented clinical strain, Sma 12, thus mimicking natural horizontal gene transfer. This result not only validates our original hypothesis, but also demonstrates the capability of phages to act as vectors for the horizontal gene transfer of large secondary metabolite gene clusters. We have thus engineered a new strain capable of expressing the complete biosynthetic pathway of prodigiosin from a single
chromosomal copy of the pig cluster in a physiologically relevant manner.

During this study we identified and characterized the QS system of a clinical S. marcescens isolate, Sma 12. Sma 12 was shown to produce aHSL molecules which appear to be BHL and HHL, the aHSL molecules produced by Sma MG1 and S 39006, and to possess convergently transcribed smalR genes similar to those of Sma MG1 and S 39006 (Eberl et al., 1996; Thomson et al., 2000). An aHSL non-producing smal mutant of Sma 12 was identified and characterized, identifying multiple smal-dependent phenotypes, all of which could be fully complemented by exogenous BHL, thereby confirming their aHSL-dependence.

Several previously described S. marcescens aHSL QS-dependent phenotypes were observed in Sma 12: Sma 12I was unable to swarm, had reduced ability to form biofilms and produced reduced levels of secreted caseinase activity, compared with the wild-type. In Sma MG1, swimming motility has been shown to be dependent on the production of the extracellular lipopeptide biosurfactant serrawettin, and expression of swrA, which encodes the serrawettin synthetase, is aHSL QS-dependent (Matsuyama et al., 1995; Lindum et al., 1998). Therefore the inability of Sma 12I to swarm is likely to be because biosurfactant production is aHSL-dependent. In Sma MG1, biofilm formation, differentiation and sloughing have been shown to be QS dependent (Labbate et al., 2004; Rice et al., 2005). The regulatory effect of aHSL QS on secreted protease activity in Sma 12 is likely to be mediated via aHSL QS control of the lipB Type I secretion system, as has been shown in Sma MG1 and Serratia proteamaculans (Christensen et al., 2003; Riedel et al., 2001), although secreted lipase activity was not affected in Sma 12I.

Several novel QS-controlled phenotypes in S. marcescens were also observed. Production of secreted chitinase and secreted haemolytic activities was reduced in the smal mutant of Sma 12. To the best of our knowledge, this is the first report of aHSL QS controlling chitinase or haemolytic activity in S. marcescens, although chitinase activity has been shown to be affected in QS mutants of C. violaceum and S. proteamaculans (Chernin et al., 1998; Christensen et al., 2003). Sma 12 was found to secrete an as-yet-uncharacterized, QS-dependent compound with apparent antibiotic activity. This compound may be a bacteriocin, since production of bacteriocins is known to be widespread in strains of S. marcescens (Guasch et al., 1995).

These results demonstrate that several important virulence factors, including production of secreted enzymes, swarm-ing motility and biofilm formation (the latter two important in surface colonization and adherence), are under aHSL QS control in a clinical strain of S. marcescens. Hence aHSL QS may have an important role in the virulence of Sma 12 and other clinical S. marcescens strains. In addition, the luxS-dependent production of AI-2 activity by Sma 12 has been described. Analysis of the luxS mutant of Sma 12 revealed very little impact of luxS inactivation on the native phenotypes of this strain. We have previously reported that the impact of luxS inactivation appears to be strain-dependent in both Serratia spp. and Erwinia carotovora (Coulthurst et al., 2004, 2006). However the luxS mutant did show a modest increase in biofilm formation compared with the wild-type. Biofilm formation has been reported to be affected in the luxS mutants of several other species of pathogenic bacteria, e.g Helicobacter pylori and Streptococcus mutans (Cole et al., 2004; Merritt et al., 2003).

We next asked whether the engineered pig cluster in Sma 12P had been brought under the control of native regulatory systems of Sma 12 and found that the foreign pig cluster had indeed been brought under the control of both aHSL QS and luxS. As in the native pig host, Sma 274 (Coulthurst et al., 2004), pigment production was reduced in the luxS mutant of Sma 12P, a phenotype at least partially due to lack of extracellular signal. More surprisingly, we found that the incoming pig cluster, from a strain lacking any detectable aHSL QS system, was brought under the tight control of the native Sma 12 aHSL QS system. It is not yet clear how this control operates, although both direct and indirect scenarios can be envisaged. Direct control would involve binding of SmaR to pig promoter sequences, whereas indirect control would involve smaIR regulating the expression of one or more other regulatory proteins, perhaps conserved between QS and non-QS strains of S. marcescens, which in turn regulate expression of the pig cluster. We cannot currently distinguish between these possibilities (e.g. there is no obvious lux-box upstream of the pig cluster; data not shown), although the indirect scenario has preceded in S 39006, where regulation of pigment production by QS appears to be indirect (Fineran et al., 2005).

We next used phage-mediated transduction to engineer a new level of regulation, aHSL QS, onto the Pig biosynthetic cluster in its native background, Sma 274, causing pigment production to become aHSL-dependent in a strain without any detectable native aHSL QS system. Moreover, other phenotypes expressed at a high level in a QS-independent manner in Sma 274 (swarming, secreted haemolysis and protease production), were also subsumed into the engineered QS regulon in Sma 274I. To the best of our knowledge, this is the first example of ‘engineered’ horizontal acquisition of a QS system, followed by the immediate imposition of QS control onto multiple native traits. The introduction of SmaR into Sma 274, whether by the introduction of smaR, smal:: : KmR (in Sma 274I) or by its introduction on a plasmid, caused repression of various phenotypes, including pigment production and swarming motility, with this repression being relieved by the addition of BHL. These observations are consistent with SmaR being a repressor, either directly or indirectly, of gene expression in the absence, but not the presence, of aHSL.

Typically, aHSL QS involves LuxR-family proteins acting as transcriptional activators in the presence of threshold levels of their cognate aHSL (Pappas et al., 2004). However several
examples have been reported of LuxR-family proteins acting as repressors of gene expression in the absence of aHSL, with this repression being relieved by the cognate aHSL. SmaR39006 has been shown genetically to be a repressor of pigment and carbapenem production in the absence of aHSL and has also been shown to bind to the carA promoter in the absence, but not the presence, of BHL (Fineran et al., 2005). In Pantoea stewartii, EsaR, in the absence of aHSL, represses production of extracellular polysaccharide by direct repression of the transcriptional coactivator rcsA, and also directly represses its own expression (Minogue et al., 2002, 2005). SpnR of Sma SS-1 and VirR of Er. carotovora have also been shown genetically to act as repressors (Burr et al., 2006; Horng et al., 2002). Interestingly, to date, the phenomenon of LuxR homologues acting as repressors, with aHSL-dependent derepression, appears to be confined to the Enterobacteriaceae.

As already noted for the pig genes in Sma 12P, the level at which SmaR of Sma 12 acts, on native or foreign target genes, remains to be determined. It is formally possible that SmaR can bind to the promoters of all the genes, native and foreign, that it regulates. However, we consider a more likely scenario to be that SmaR acts via the repression/aHSL-dependent derepression of a yet-to-be-identified, conserved regulator(s) common to all Sma strains. Such a regulatory protein(s) would have conserved promoter element(s) that SmaR is able to bind in the absence of aHSL, preventing transcription. Having a pleiotropic regulator(s) under QS control has been seen elsewhere, e.g. Rap is QS regulated and itself regulates pigment and carbapenem production in S 39006 (Fineran et al., 2005). In Fig. 5, we present a simple model for how the smallR QS locus may exert QS control on a variety of phenotypes, both native and foreign, both in its original background and immediately upon its introduction into a new, QS-naïve, genetic background.

However mediated, this work suggests that smallR-like loci are self-contained ‘exchangeable’ units that can be readily moved by horizontal gene transfer and ‘plugged into’ a novel host strain’s existing regulatory systems. They can then immediately impose QS control on pre-existing traits, when such control becomes advantageous. We would like to propose that this may be the reason for having a LuxR-homologue, like SmaR, that functions as a repressor, rather than an activator, of gene expression. Given an existing, highly expressed QS-independent trait (e.g. prodigiosin production or swarming motility), horizontal acquisition of a genetic unit consisting of a repressor plus a synthase of the relieving QS molecule allows the trait to be instantly repressed at low cell densities/expressed at high cell densities without the need for any further adaptation. When QS control is no longer advantageous, loss of the repressive smallR-like gene, or the entire locus, immediately results in

![Fig. 5. A simple model for the modular control of multiple phenotypes of Sma by aHSL QS. Left: in strains without a QS locus, a pleiotropic regulator (represented by ‘?’) activates, directly or indirectly, the expression of genes required for pigment production (where present), swarming motility, production of secreted protease and haemolytic activity and other phenotypes (e.g. biofilm formation). Middle: in strains with a QS locus, at low cell density/low aHSL concentrations, SmaR represses expression of the pleiotropic regulator ‘?’. In the absence of ‘?’; swarming motility is eliminated and expression of other phenotypes, such as pigment production (where present) and secreted protease and haemolysin production, is reduced. This low cell density/low BHL situation is mimicked by strains in which functional SmaR, but not functional Small, is present, namely Sma 12I and Sma 274I. Right: in strains with a QS locus, at high cell density/high aHSL concentrations, SmaR binds aHSL, presumably altering its DNA-binding properties, and does not repress the expression of the pleiotropic regulator ‘?’.

Expression of ‘?’ allows high-level expression of pigment, swarming and other downstream phenotypes. This high cell density/high BHL situation is mimicked by the addition of exogenous BHL to strains in which functional SmaR, but not Small, is present, namely Sma 12I and Sma 274I. Dark circles represent the aHSL signal molecules BHL/HHL; solid arrows with ‘+’ signs indicate positive activation and the flattened arrowhead indicates repression; ‘?’ may represent more than one regulatory protein.](http://mic.sgmjournals.org)
QS-independent expression of the trait, without further adaptation to restore high levels of expression. Such modularity may be facilitated by the QS locus exerting control via widely conserved regulator(s), as illustrated in Fig. 5. Of course, as we have shown here, this apparently inherent flexibility and modularity also extends to the traits regulated by QS, since the pig biosynthetic cluster, when horizontally transferred by phage-mediated transduction, is properly expressed and regulated by the recipient strain.

In conclusion, we have successfully demonstrated that the complete, functional biosynthetic system of a complex secondary metabolite can be easily acquired by phage-mediated horizontal gene transfer and integrated into the recipient’s gene regulatory systems. We have shown that QS regulation is also easily acquired by horizontal gene transfer and suggested that LuxR-family repressors may facilitate immediate and reversible imposition of QS control. These results have implications for the evolution and dissemination of biosynthetic clusters and QS regulatory systems, illustrating their modular nature and facile acquisition by horizontal transfer. This work highlights both the remarkable evolutionary plasticity of bacteria and the capacity of phages to act as vectors for horizontal gene transfer, indicating the potential power of using transducing phage to perform such metabolic and regulatory engineering.

ACKNOWLEDGEMENTS

The authors would like to thank Katrina Evetts for help in initially identifying a smaI mutant of Sma 12. This work was funded by the BBSRC.

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