

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.

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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; Aucken & Pitt, 1998). *S. marcescens* strains produce a range of secreted products, including proteases, nuclease, lipase, chitinases and haemolysin, many of which are likely to represent virulence factors in human infection (Aucken & Pitt, 1998; Braun *et al.*, 1993; Hines *et al.*, 1988;

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, immunosuppressive 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

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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 *smalR* 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 SmaI [which produces *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-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 SpnIR (Hornig *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, SmaI (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 *smaIR* in *S* 39006 and *spnIR* in *Sma* SS-1 (Thomson *et al.*, 2000; Hornig *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 inter-chromosomal 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 cultured 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 Helios 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

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

Strain/plasmid/phage	Genotype/phenotype	Source or reference
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80 Δ dlacZM15 Δ (lacZYA-argF)U169 <i>endA1 recA1 hsdR17</i> (r _k ⁻ m _k ⁻) <i>deoR thi-1 supE44 λ⁻ gyrA96 relA1</i>	GibcoBRL, Life Technologies
S17-1 λ pir	<i>recA pro hsdR recA::RP4-2-Tc::Mu λpir Tc^R Sp^R Sm^R</i>	de Lorenzo <i>et al.</i> (1990)
<i>Serratia</i> sp.		
LIS	<i>Serratia</i> ATCC 39006 <i>smaI::miniTn5Sm/Sp, Sp^R</i>	Thomson <i>et al.</i> (2000)
<i>S. marcescens</i> ATCC 274 (<i>Sma</i> 274)	Wild-type (Pig ⁺), non-motile	Lab stock
<i>S. marcescens</i> strain 12 (<i>Sma</i> 12)	Wild-type (Pig ⁻), clinical isolate	Harris <i>et al.</i> (2004)
<i>S. marcescens</i> ATCC 274 motile isolate (<i>Sma</i> M274)	Wild-type (Pig ⁺), motile	Matsuyama <i>et al.</i> (1995)
NW7	<i>Sma</i> 274, <i>copA::cat, Cm^R</i>	Williamson <i>et al.</i> (2006)
SCC4	<i>Sma</i> 274, <i>luxS::Km^R, Km^R</i>	Coulthurst <i>et al.</i> (2004)
<i>Sma</i> 12I	<i>smaI::miniTn5km1, Km^R</i>	This study
<i>Sma</i> 12P	<i>pig⁺, copA::cat, Cm^R</i> derivative of <i>Sma</i> 12; generated by transduction using ϕ 3M grown on strain NW7	This study
<i>Sma</i> 12IP	<i>pig⁺, smaI::miniTn5km1, Cm^R Km^R</i> derivative of <i>Sma</i> 12P; generated by transduction using ϕ 3M grown on strain <i>Sma</i> 12I	This study
<i>Sma</i> 12S	<i>luxS::Km^R, Km^R</i> derivative of <i>Sma</i> 12; generated by transduction using ϕ 3M grown on strain SCC4	This study
<i>Sma</i> 12SP	<i>luxS::Km^R, pig⁺, copA::cat, Cm^R Km^R</i> derivative of <i>Sma</i> 12S; generated by transduction using ϕ 3M grown on strain NW7	This study
<i>Sma</i> 274I	<i>smaI::miniTn5km1, smaR⁺</i> derivative of <i>Sma</i> 274 following transduction using ϕ 3M grown on strain <i>Sma</i> 12I	This study
<i>Sma</i> M274I	<i>smaI::miniTn5km1, smaR⁺</i> derivative of <i>Sma</i> M274 following transduction using ϕ 3M grown on strain <i>Sma</i> 12I	This study
Other bacterial strains		
<i>Chromobacterium violaceum</i> CV026	<i>cviI::Tn5</i> , aHSL bioassay strain	McClellan <i>et al.</i> (1997)
<i>Vibrio harveyi</i> BB170	<i>luxN::Tn5</i> , AI-2 bioassay strain	Surette <i>et al.</i> (1999)
Phage		
ϕ 3M	<i>S. marcescens</i> generalized transducing phage	Williamson <i>et al.</i> (2006)
Plasmids		
pACYC184	Low-copy cloning vector, Cm ^R , Tc ^R	Chang & Cohen (1978)
pACYC177	Low-copy cloning vector, Km ^R , Ap ^R	Chang & Cohen (1978)
pNRW75	675 bp PCR-amplified fragment containing <i>smaI</i> from <i>Sma</i> 12 in the <i>EcoRI</i> site of pACYC184	This study
pNRW85	889 bp PCR-amplified fragment containing <i>smaR</i> from <i>Sma</i> 12 in the <i>PstI</i> site of pACYC177	This study
pUTminiTn5Km1	Delivery plasmid for miniTn5Km1, Ap ^R , Km ^R	de Lorenzo <i>et al.</i> (1990)

pelleted by centrifugation and the pellet resuspended in 1 ml LB. Then 100 μ l of a high-titre ϕ 3M phage lysate grown on the donor strain was added to the cell suspension, mixed and incubated at 30 °C for 1 h. Aliquots (100 μ l) 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 *smaIR* locus. In order to identify a *luxI* homologue in *Sma* 12, the oligonucleotide primers *SmaI*-5' (5'-TWAGAAAAAACGTTT-3') and *SmaI*-3' (5'-CAATGCCAAAC-ATGCCAT-3') were designed to conserved regions between *smaI* from S 39006 and *swrI* from *Sma* MG1. PCR amplification of *Sma* 12 genomic DNA using *SmaI*-5' and *SmaI*-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 30s, 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

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 *Sma* 12 was performed. *E. coli* S17-1 λ pir carrying pUTminiTn5Km1 was used to introduce the transposon into *Sma* 12 by conjugation as described previously (Williamson *et al.*, 2006). A total of 2700 Km^R 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 *Sma* 12I. To identify the location of the transposon insertion in *Sma* 12I, PCR amplification was performed using *Sma* 12 *smal*-specific primers (NW110 and NW111, see below) in combination with a primer which binds to both ends of the miniTn5Km1 transposon (3-Tn5km1a; 5'-CGCTGCGCAGGGCTTTATTGATTC-3') and the resulting PCR products were sequenced.

Construction of plasmids containing *smal* and *smarR* from *Sma* 12. The *smal* gene was PCR-amplified from *Sma* 12 genomic DNA using primers NW110 (5'-CTTGAATTCGCCGATGCCGC-ACCGCCTG-3') and NW111 (5'-GCCGAATTCCTAGGTGTGGA-GCTGCAAC-3') and the resulting product cloned into the *EcoRI* site of pACYC184, generating plasmid pNRW75. The *smarR* gene was PCR-amplified from *Sma* 12 genomic DNA using primers NW135 (5'-CGCTGCAGCATTATTAACGCATCCTCTATG-3') and NW136 (5'-GATCTGCAGTTGGATTCATGGCCATTATG-3') and the resulting product cloned into the *PstI* site of pACYC177, generating plasmid pNRW85. *Sma* 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 A_{534} 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 (McClellan *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 (1·6%) 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 μ l) 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 OD₆₀₀ of 1·20, was diluted 1 in 5000 in fresh AB medium and 90 μ l 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 OD₆₀₀ 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 μ m filter (Millipore), and were stored at -80 °C.

Other phenotypic assays

Swarming, haemolysis and secreted inhibitory activity. Swarm agar contained 5 g peptone l⁻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 μ l 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 μ l sample of supernatant was mixed with 500 μ l 2% azocasein, 100 μ l 1 M Tris/HCl (pH 8·0) and 650 μ l H₂O; a negative control sample contained H₂O instead of supernatant. After 2 h incubation at 30 °C, 375 μ l 14% perchloric acid was added to stop each reaction. The samples were then centrifuged (13 000 g, 5 min), 0·75 ml of the supernatant mixed with 75 μ l 10 M NaOH and the A_{436} measured. Activity was calculated as $\Delta A_{436} \text{ h}^{-1} \text{ ml}^{-1}$, the background activity of the negative control was subtracted, and activity was reported as $\Delta A_{436} \text{ h}^{-1} \text{ ml}^{-1}$ per 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 μ l sample of the culture supernatant was mixed with 450 μ l of the substrate mixture and the enzymic 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 A_{560} of the supernatant measured. Chitinase activity was reported as $\Delta A_{560} \text{ h}^{-1} \text{ ml}^{-1}$ per OD₆₀₀ unit.

Biofilm assay. Overnight cultures of the strains to be tested were diluted to an OD₆₀₀ of 0·2 in LB, 200 μ l 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 μ l 0·1% crystal violet and allowed to stain for 1 h. The crystal violet was then removed and the wells washed with 3 \times 400 μ l water to remove non-attached material. Next, 250 μ l 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 A_{550} 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 μ M. An equivalent volume of DMSO alone was added to control cultures. CM was obtained from *Sma* 12 and *Sma* 12S as follows: cultures were grown for 4 h in LB, cells were removed by centrifugation and the supernatant passed through a 0·22 μ m 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^R) 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^R) closely linked with the *pig* genes.

A $\phi 3\text{M}$ 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 (McClellan *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 R_F 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 aHSL 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 *luxI* homologue encoding an aHSL synthase in *Sma* 12, oligonucleotide primers were designed

to regions conserved between two known *Serratia luxI* homologues: *smaI* from S 39006 (*smaI*₃₉₀₀₆) and *swrI* from *Sma* MG1. This approach identified a *luxI* homologue, named *smaI*, and *smaI* and flanking regions were sequenced by PCR-based methods (see Methods). The complete *smaI* gene encoded a predicted protein of 224 aa, homologous to other LuxI-family aHSL-synthases. Database searches revealed that *SmaI* from *Sma* 12 was most similar to *SwrI* from *Sma* MG1 (97% identity over 198 aa) and *SmaI*₃₉₀₀₆ (63% identity over 222 aa). Downstream of *smaI*, convergently 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 *SmaR*₃₉₀₀₆ (70% identity over 247 aa). The *smaI* and *smaR* genes overlap by 54 bp (Fig. 1c). The regions sequenced up- and downstream of *smaI* 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 *smaI* gene, inserted after base pair 236. Therefore *Sma* 12 production of aHSL activity is dependent on a functional *smaI* 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^R insertion) in *Sma* 274 (strain SCC4; Coulthurst *et al.*, 2004). Generalized transduction using $\phi 3\text{M}$ 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 *smaI* 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 (*smaIR*) and *luxS*. *Sma* 12P was transduced with the *smaI* mutation from *Sma* 12I to generate *Sma* 12IP (*smaI 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,

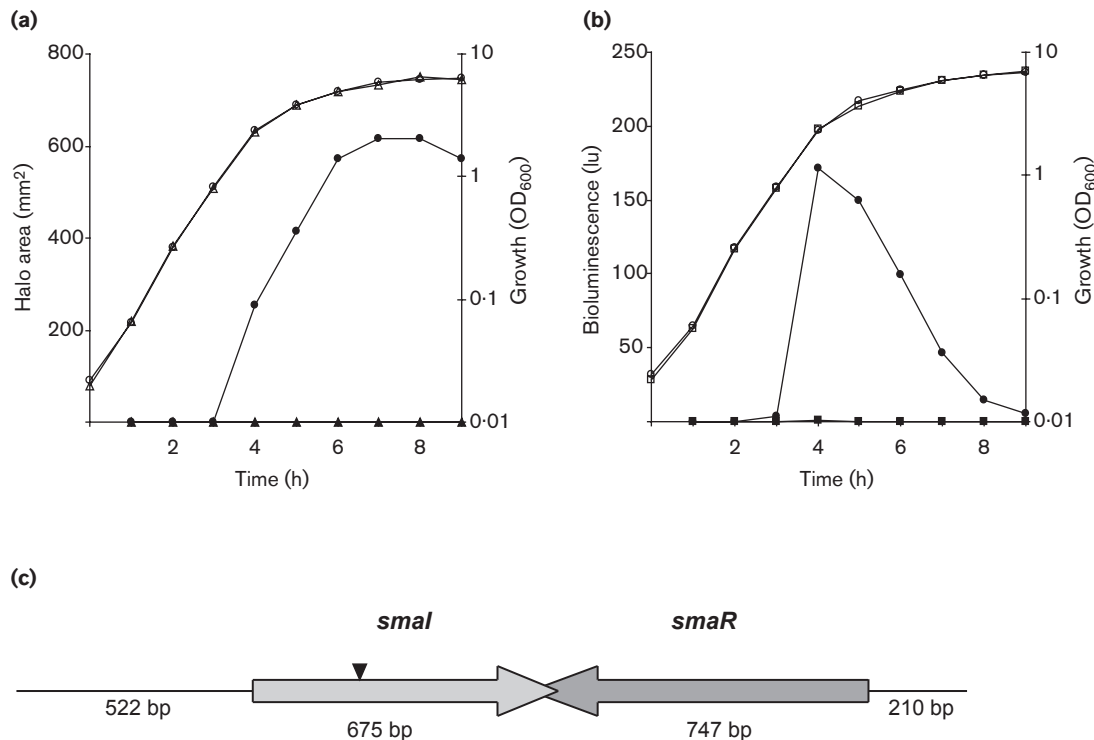


Fig. 1. Production of aHSL and AI-2 activity by *Sma 12*. (a) Production of aHSL by *Sma 12* (wild-type, ●) and *Sma 12I* (*smal*, ▲) was measured during growth in LB. Growth of strains, measured by OD₆₀₀, is represented by open symbols (○, △). Supernatant samples were harvested at intervals and their aHSL activity measured using the LIS bioassay. aHSL activity induces pigment production in the sensor strain, reported as the area of the pigmented halo induced. (b) Production of AI-2 by *Sma 12* (wild-type, ●) and *Sma 12S* (*luxS*, ■) was measured during growth in LB (OD₆₀₀ values are plotted as ○, □). Supernatant samples were harvested at hourly intervals and their AI-2 activity was measured using the *V. harveyi* BB170 bioassay. AI-2 activity induces bioluminescence in the sensor strain, reported as light units (lu). For comparison, a positive control sample from *V. harveyi* BB152 produced 20 lu and media alone produced <0.05 lu. Each experiment was performed at least in duplicate with essentially identical results. (c) Schematic representation of the sequenced *smalR* locus of *Sma 12*. The position of the transposon insertion in *Sma 12I* (after base pair 236) is shown with an arrowhead.

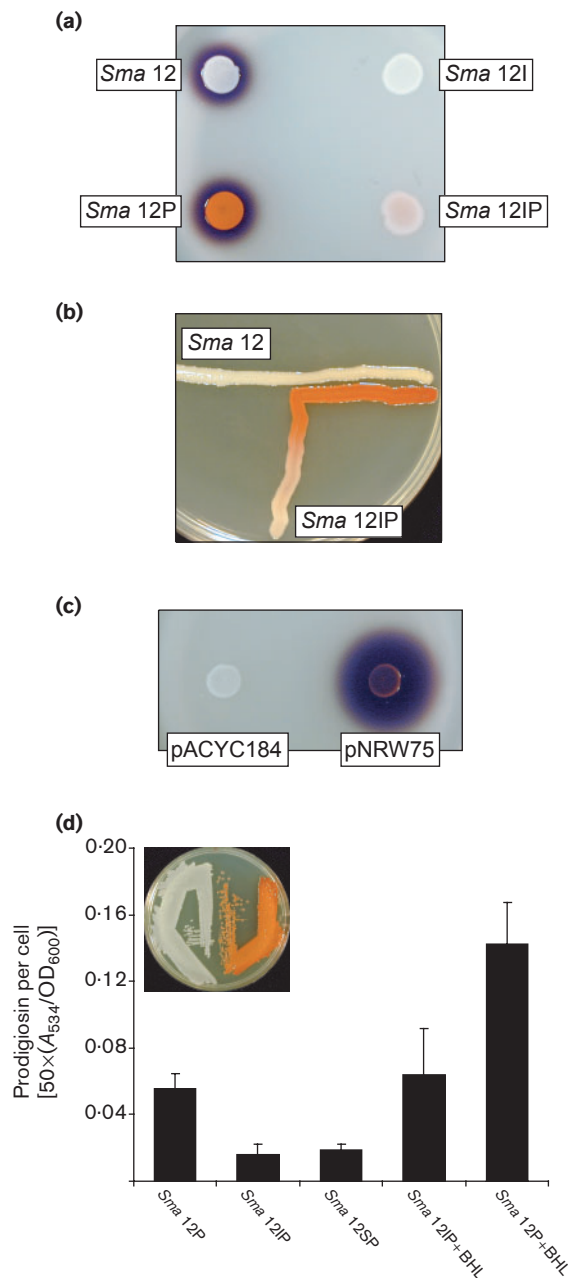
cross-feeding from wild-type *Sma 12* and overexpression of *smal* *in trans* (Fig. 2). Interestingly, addition of exogenous BHL to *Sma 12P* caused an increase in prodigiosin production. Overexpression of *smal* *in trans* was achieved by the introduction of plasmid pNRW75, which also restored aHSL production (Fig. 2c), providing further confirmation that *smal* 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 *smal* 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 *smal* 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 *smaI* 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 *smaI* mutant, *Sma* 12I, compared with the wild-type. In each case, wild-type levels of enzyme production were restored by the addition of

Fig. 2. QS-regulated production of prodigiosin in *Sma* 12P. (a) Pigmentation and aHSL production by *Sma* 12 (wild-type, top left), *Sma* 12P (*pig*⁺, bottom left), *Sma* 12I (*smaI*, top right) and *Sma* 12IP (*smaI*, *pig*⁺, bottom right). A 5 μl drop of each strain was spotted onto a top lawn of CV026 as shown. aHSL production is indicated by purple halos around the *Sma* colonies. (b) *Sma* 12 (wild-type, top line) is able to restore pigment production to *Sma* 12IP (*smaI* *pig*⁺, bottom L-shape) by cross-feeding. (c) Prodigiosin and aHSL production can be restored to *Sma* 12IP by the introduction of the *smaI* gene *in trans*; left, *Sma* 12IP(pACYC184) [vector control]; right, *Sma* 12IP(pNRW75) [*smaI* *in trans*]. (d) Prodigiosin production by *Sma* 12P, *Sma* 12IP, *Sma* 12SP (*luxS* *pig*⁺), *Sma* 12IP+1 μM BHL and *Sma* 12P+1 μM BHL was measured, as described in Methods, after 16 h growth in LB. Bars show mean ± SD (*n*=4). Inset: the red pigment, prodigiosin, is produced by *Sma* 12P (right) but not wild-type *Sma* 12 (left).

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 *smaIR* 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 *smaIR* locus from *Sma* 12I, with a Km^R cassette disrupting *smaI*, was introduced into *Sma* 274 by generalized transduction and selection on Km. In the resulting strain, *Sma* 274I (*smaI* *smaR*⁺), 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

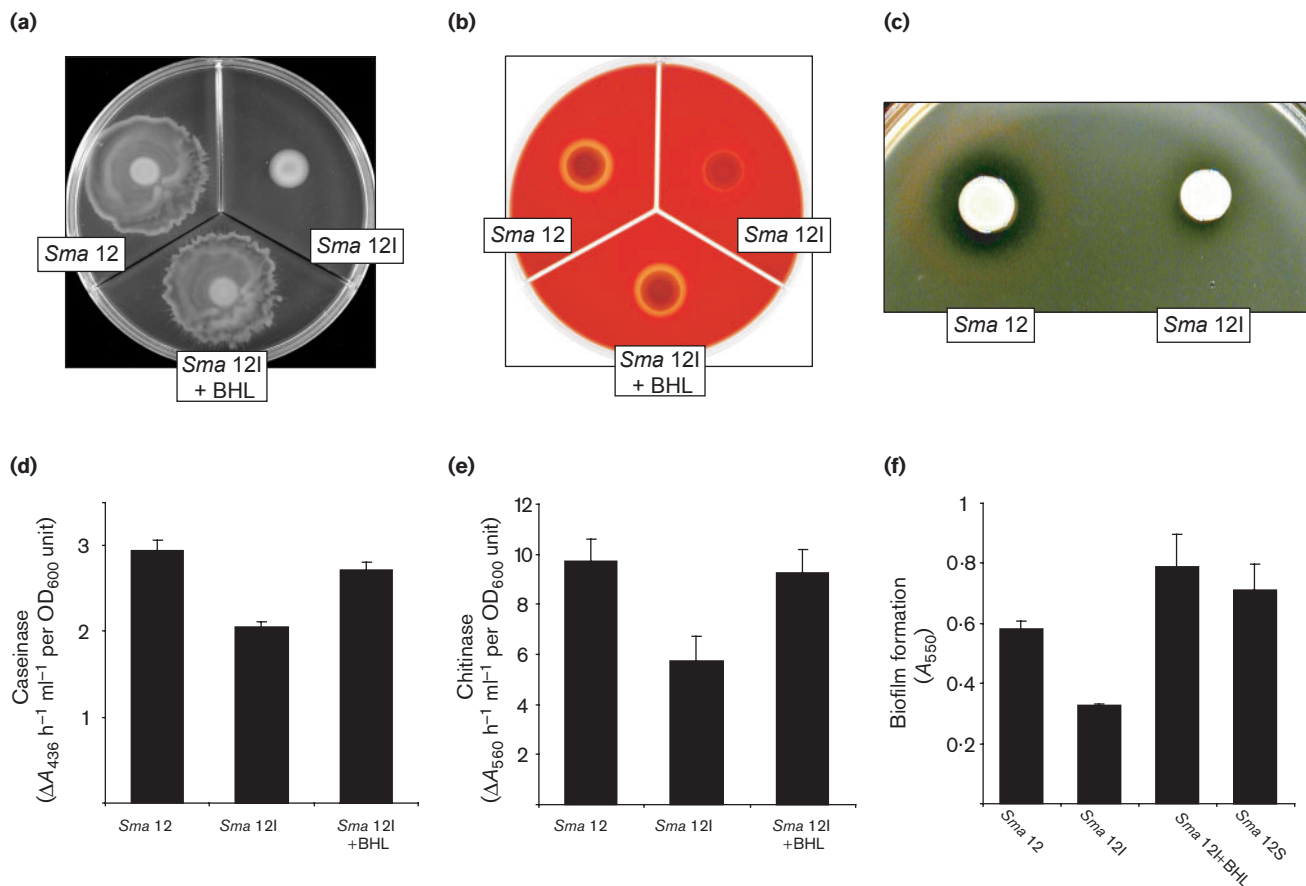


Fig. 3. aHSL QS-dependent phenotypes of *Sma* 12. (a) Swarming motility on 0.75% agar by *Sma* 12 (wild-type, left), *Sma* 12I (*smal*, right) and *Sma* 12I + 1 μM BHL (bottom). (b) Production of secreted haemolytic activity by *Sma* 12 (left), *Sma* 12I (right) and *Sma* 12I + 1 μM BHL (bottom). (c) Production of inhibitory activity by *Sma* 12 (left) and *Sma* 12I (right) on a lawn of LIS. (d) Production of secreted caseinase activity by *Sma* 12, *Sma* 12I and *Sma* 12I + 1 μM BHL after 16 h growth in LB. (e) Production of secreted chitinase activity by *Sma* 12, *Sma* 12I and *Sma* 12I + 1 μM BHL after 16 h growth in LB + 1.5% chitin. (f) Biofilm formation by *Sma* 12, *Sma* 12I, *Sma* 12I + 1 μM BHL and *Sma* 12S (*luxS*) after incubation for 5 days. For (a)–(c), assay plates, prepared as described in Methods, were inoculated with 5 μl 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 \pm SD ($n \geq 3$).

addition of synthetic BHL, whereas BHL did not increase pigment production in the wild-type strain (Fig. 4a). (The growth rate of *Sma* 274I was indistinguishable from that of the wild-type; data not shown.) This result implied that the incoming QS system was controlling pigment production in *Sma* 274I. We confirmed that the observed reduction in pigment in *Sma* 274I was indeed due to the introduction of *smar* by introducing plasmid pNRW85, carrying *smar* from *Sma* 12, into *Sma* 274. As expected, pigment production by *Sma* 274(pNRW85) was significantly reduced compared with *Sma* 274(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 *Sma* 12 had also become QS-dependent in *Sma* 274I. Production of secreted haemolytic

activity was reduced in *Sma* 274I 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 *Sma* 274 was unaffected by the addition of BHL; data not shown.) Production of secreted caseinase activity was modestly, but significantly, reduced in *Sma* 274I compared with the wild-type. Addition of BHL to *Sma* 274I restored caseinase production to wild-type levels, but addition of BHL to wild-type *Sma* 274 had no effect on caseinase production (data not shown). Hence the foreign *Sma* 12 QS system appears to exert a regulatory effect on native gene expression in *Sma* 274I.

Our laboratory isolate of *Sma* 274 is non-motile (data not shown). Since swarming motility is a characteristic phenotype of QS mutants of *S. marcescens*, we obtained a motile

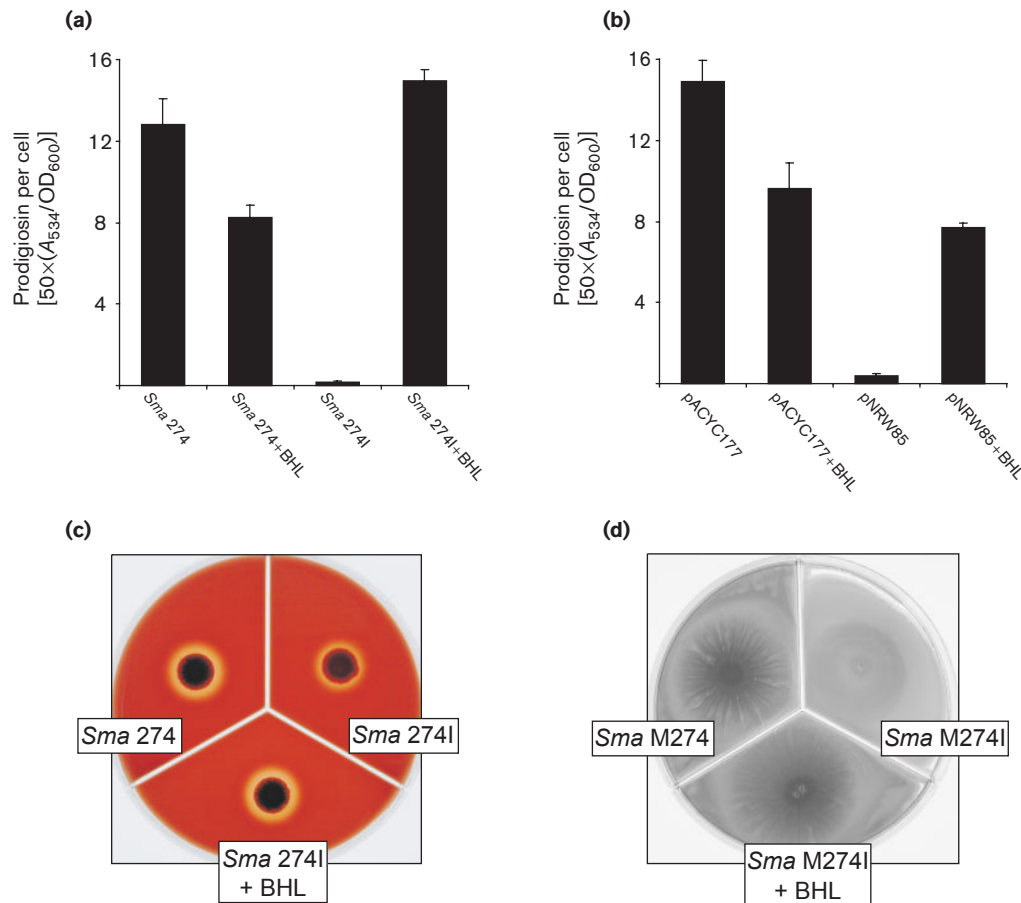


Fig. 4. Phenotypes of *Sma 274* subject to superimposition of aHSL QS control. Production of prodigiosin by (a) *Sma 274* (wild-type) and *Sma 274I* (*smaI smaR*⁺), and (b) *Sma 274*(pACYC177) [vector control] and *Sma 274*(pNRW85) [*smaR in trans*], with and without 1 μM BHL, was measured after 16 h growth in LB. Measurement of prodigiosin is described in Methods and bars show mean ± SD (*n* = 4). (c) Production of secreted haemolytic activity by *Sma 274* (left), *Sma 274I* (right) and *Sma 274I*+1 μM BHL (bottom). Blood agar plates were inoculated with 5 μl overnight culture, diluted to an OD₆₀₀ of 0.2, and incubated for 72 h. (d) Swarming motility of *Sma M274* (left), *Sma M274I* (right) and *Sma M274I*+1 μM BHL (bottom). Swarm assay plates were inoculated with 3 μl overnight culture, diluted to an OD₆₀₀ of ~0.2, and incubated overnight. Note that the characteristic swarming morphology of the wild-type is missing in *Sma M274I* but restored by BHL; the plate is shown in black and white to aid clarity. The plates shown are representative of three independent experiments.

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 *smaI* locus from *Sma 12I*, with a Km^R 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 *smaI* 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 *smaIR* genes similar to those of *Sma* MG1 and S 39006 (Eberl *et al.*, 1996; Thomson *et al.*, 2000). An aHSL non-producing *smaI* mutant of *Sma* 12 was identified and characterized, identifying multiple *smaI*-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, swarming 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 *smaI* 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, swarming 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 precedent 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*, *smaI*::Km^R (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 *smaIR* 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 *smaIR*-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 *smaIR*-like gene, or the entire locus, immediately results in

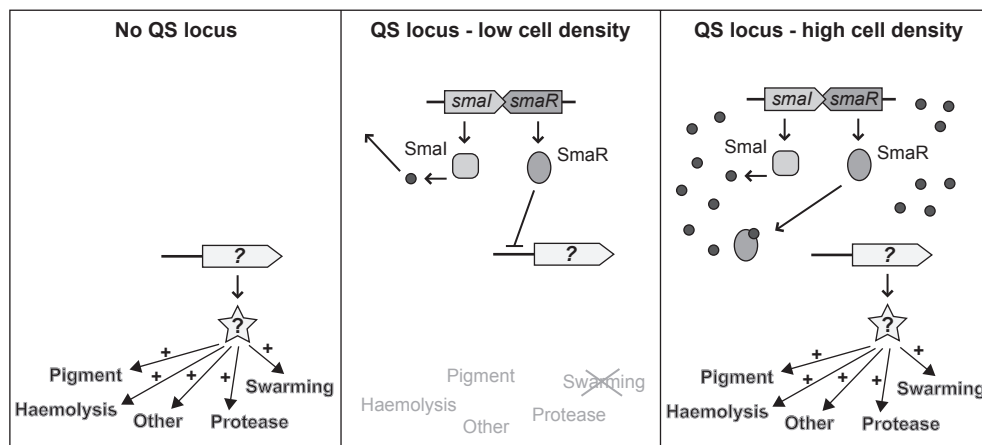


Fig. 5. A simple model for the modular control of multiple phenotypes of *Sma* by aHSL QS. Left: in strains without a QS locus, a pleiotropic regulator (represented by ‘?’) activates, directly or indirectly, the expression of genes required for pigment production (where present), swarming motility, production 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 *SmaI*, 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 *SmaI*, 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.

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.

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