Role of the phosphopantetheinyltransferase enzyme, PswP, in the biosynthesis of antimicrobial secondary metabolites by *Serratia marcescens* Db10

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INTRODUCTION

Phosphopantetheinyltransferase (PPTase) enzymes fulfil essential roles in primary and secondary metabolism in prokaryotes, archaea and eukaryotes. PPTase enzymes catalyse the essential modification of the carrier protein domain of fatty acid synthases, polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs). In bacteria and fungi, NRPS and PKS enzymes are often responsible for the biosynthesis of secondary metabolites with clinically relevant properties; these secondary metabolites include a variety of antimicrobial peptides. We have previously shown that in the Gram-negative bacterium *Serratia marcescens* Db10, the PPTase enzyme PswP is essential for the biosynthesis of an NRPS-PKS dependent antibiotic called althiomycin. In this work we utilize bioinformatic analyses to classify PswP as belonging to the F/KES subfamily of Sfp type PPTases and to putatively identify additional NRPS substrates of PswP, in addition to the althiomycin NRPS-PKS, in *Ser. marcescens* Db10. We show that PswP is required for the production of three diffusible metabolites by this organism, each possessing antimicrobial activity against *Staphylococcus aureus*. Genetic analyses identify the three metabolites as althiomycin, serrawettin W2 and an as-yet-uncharacterized siderophore, which may be related to enterobactin. Our results highlight the use of an individual PPTase enzyme in multiple biosynthetic pathways, each contributing to the ability of *Ser. marcescens* to inhibit competitor bacteria by the production of antimicrobial secondary metabolites.

Phosphopantetheinyltransferase (PPTase) enzymes have essential roles in the biosynthetic pathways of primary and secondary metabolism in prokaryotes, archaea and eukaryotes. PPTase enzymes catalyse the essential modification of the carrier protein domain of fatty acid synthases, polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are all dependent upon the catalytic action of PPTase enzymes to function (Beld et al., 2014). FASs, PKSs and NRPSs are generally large, multimodular proteins. Each module within the protein incorporates one acyl or amino acid unit into the growing metabolite and comprises a minimum of three essential domains, including the carrier protein domain (Fischbach & Walsh, 2006). PPTase enzymes catalyse the transfer of a 4'-phosphopantetheine (PPT) prosthetic group from coenzyme A to a conserved serine residue in the carrier protein domain of FASs, PKSs and NRPSs (Beld et al., 2014; Elovson & Vagelos, 1968; Lambalot et al., 1996). The 4'-phosphopantetheine prosthetic group of the carrier protein domain fulfils two main functions: the thiol-terminated 4'-phosphopantetheine arm serves as the point of attachment for the FAS, PKS or NRPS enzyme to the biosynthetic machinery and the flexibility of this arm allows the biosynthetic intermediate access to the catalytic reaction centres within the FAS, PKS or NRPS module (Beld et al., 2014).

PPTase enzymes can be divided into three families: the Sfp family whose prototype is Sfp of *Bacillus subtilis* (Lambalot et al., 1996; Reuter et al., 1999); the AcpS family whose prototype is AcpS of *Escherichia coli* (Elovson & Vagelos, 1968; Lambalot & Walsh, 1995); and a third family whose members are integrated as domains within the FAS or PKS enzyme that they modify (Beld et al., 2014; Copp & Neilan, 2006; Lambalot et al., 1996; Walsh et al., 1997). The Sfp family of PPTases have broad substrate specificity and are generally required for the activation of PKS and NRPS enzymes of secondary metabolism (Mootz et al., 2001; Quadri et al., 1998); these enzymes are approximately 230 residues in length and exist as monomers (Reuter et al., 1999). Based on sequence comparisons, members of the Sfp family were further divided into the F/KES and W/KEA subfamilies (Copp & Neilan, 2006; Lambalot et al., 1996). F/KES subfamily...
members were shown to largely be associated with NRPS enzymes whereas W/KEA subfamily members were more often associated with PKS enzymes (Copp & Neilan, 2006). Members of the AcpS family of PPTases are generally around 120 residues in length and are thought to exist as trimers (Chirgadze et al., 2000; Parris et al., 2000). The AcpS family of PPTases show a higher degree of specificity towards the carrier protein domain that they modify and are generally required for the modification of FAS enzymes from primary metabolism (Lambalot et al., 1996; Mootz et al., 2001; Parris et al., 2000). Despite sharing limited overall sequence similarity, the AcpS and Sfp family members share homology at the structural level; an AcpS dimer resembles one Sfp monomer (Lambalot et al., 1996; Reuter et al., 1999). As previously described, there exists a third family of PPTases that are translationally fused to the FAS or PKS enzyme that they modify (Copp & Neilan, 2006; Fichtlscherer et al., 2000; Lambalot et al., 1996). Examples of these integrated PPTases are few and, to date, only two bacterial integrated PPTases have been experimentally validated (Murugan & Liang, 2008; Weissman et al., 2004).

Serratia marcescens is a Gram-negative bacterium belonging to the family Enterobacteriaceae. Members of this genus are known to produce a variety of secondary metabolites (Fineran et al., 2005; Kadouri & Shanks, 2013; Thomson et al., 2000). Many strains, including Ser. marcescens ATCC 274, produce the secondary metabolite prodigiosin that gives the bacterium a distinctive red colour (Williamson et al., 2006). Ser. marcescens Db10 is a model insect pathogen but lacks the genes to produce prodigiosin and so is an example of a non-pigmented strain of Serratia (Flyg et al., 1980). However, we recently described the production of the antibiotic secondary metabolite, althiomycin, by Ser. marcescens Db10 (Gerc et al., 2012). Althiomycin is the product of the six-gene alb operon, which encodes the following: a hybrid NRPS-PKS enzyme, appropriate tailoring enzymes and an export/resistance protein. The PPTase encoded by SMA2452, which is not genetically linked with the alb operon, was shown to be required for althiomycin biosynthesis in this organism (Gerc et al., 2012). PswP, in Ser. marcescens ATCC 274, is the PPTase required for the production of prodigiosin and the surfactant serrawettin W1 (Sunaga et al., 2004). Ser. marcescens Db10 does not produce serrawettin W1 dependent on the NRPS SwrW (Li et al., 2005), instead, it produces serrawettin W2 dependent on the NRPS SwrA (Matsuyama et al., 1992). Serrawettins W1 and W2 are both cyclic lipopeptides but have quite distinct chemical structures (Matsuyama et al., 1992; Wasserman et al., 1962). The PPTase SMA2452 and PswP from Ser. marcescens ATCC 274 show 96% identity at the protein level; therefore, SMA2452 will henceforth be referred to as PswP.

In this study, we provide evidence that PswP is required for the biosynthesis of two further secondary metabolites in Ser. marcescens Db10 and, in addition to althiomycin, these metabolites have an antimicrobial effect against Staphylococcus aureus. The production of one of these secondary metabolites, serrawettin W2, is known to be dependent on the action of PswP (Pradel et al., 2007). We further show that the second PswP-dependent metabolite is a siderophore, likely enterobactin or a related molecule. Together, althiomycin, serrawettin W2 and the siderophore comprise a repertoire of secondary metabolites that have antimicrobial activity and are dependent on PswP for their biosynthesis.

## METHODS

### Strains and culture media.

Strains used in this study are detailed in Table 1. E. coli was routinely cultured in Luria–Bertani (LB) medium (1 %, w/v, Bacto tryptone, 0.5 %, w/v, Bacto yeast extract, 1 %, w/v, NaCl). Ser. marcescens in low salt Luria–Bertani (LB) medium (1 %, w/v, Bacto tryptone, 0.5 %, w/v, Bacto yeast extract, 0.5 %, w/v, NaCl) and Sta. aureus in tryptic soya broth (TSB) (0.5 %, w/v, NaCl, 0.5 %, w/v, soytone, 1.5 %, w/v, tryptone). Chroma azulo S (CAS) agar plates were prepared as described by Swynn & Neillands (1987). Growth media were solidified through addition of agar to 1.5 % (w/v). When required, media were supplemented with FeCl3, to a final concentration of 50 μM, or with antibiotics: 100 μg ampicillin ml⁻¹; 1000 μg kanamycin ml⁻¹ and 100 μg streptomycin ml⁻¹.

### Construction of strains and plasmids.

A Ser. marcescens chromosomal mutant with an in-frame deletion in entB was constructed by marker (allelic) exchange using the suicide vector pKNG101 (Kaniga et al., 1991), as described previously (Coulthurst et al., 2006). Briefly, the upstream and downstream flanking regions of the gene SMA4415 (entB) were cloned into pBluescript KS(+) so as to generate a non-polar, in-frame deletion of the gene. This deletion allele was then cloned into pKNG101 and the resulting marker exchange plasmid introduced into Ser. marcescens by conjugation. Selection on streptomycin-containing agar and then on high sucrose agar allowed isolation of mutants in which the deletion allele had replaced the wild-type copy. The plasmids used in this study are detailed in Table 1. The upstream and downstream regions of entB were amplified using the primers AG157 (TATAAGCTTCTTTTGGAAAGGCTGCATGCA) and AG158 (TATAAGCTGCACTCTCATCGACAGGCAGAGGGATCCTTAATGCTGAG) (upstream), and AG159 (TATAAAAGCTTCTAAGGAGGAAAGGGCTGATTTTAAAAATGCTGAG) (downstream). The resulting product was cloned into pBluescript KS(+) using restriction sites engineered into the primers (underlined in the primer sequences). Following marker exchange, the integrity of the disrupted region was confirmed by DNA sequencing. The ΔalbB-4-5ΔentB double mutant (SAN180) was constructed by introduction of the entB deletion into SAN5 (ΔalbB-5), as described above. The swrA::Tn5 mutation was introduced into the single ΔalbB-4-5 mutant and the double ΔalbB-4-5 ΔentB mutant by phage λIF3-mediated transduction as described previously (Petty et al., 2006), generating strains SAN124 and SAN181, respectively. Growth curve measurement was also performed on each mutant strain, this showed that the growth rate was unaffected compared with wild-type Ser. marcescens Db10 (data not shown).

### Antimicrobial activity assays.

Sta. aureus was grown in liquid culture in a volume of 5 ml in a universal tube at 37 °C with rotation and 100 μl culture was spread onto an agar plate. Strains of Ser. marcescens being tested for antimicrobial production were grown in liquid culture in a volume of 5 ml in a universal tube at 30 °C with rotation and 10 μl culture was then spotted on the Sta. aureus lawn. The plates were incubated (15 h) at 30 °C prior to photography.

### CAS assay.

Ser. marcescens strains were grown to stationary phase at 30 °C and 10 μl of culture was spotted on to the CAS agar plate. The plates were incubated (15 h) at 30 °C prior to photography. For complementation analysis, plasmids were maintained by the addition of kanamycin to both the culture medium and to the CAS agar plates.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Db10</td>
<td>Serratia marcescens wild-type</td>
<td>Flyg et al. (1980)</td>
</tr>
<tr>
<td>Staphylococcus aureus 113</td>
<td>ATCC 35556</td>
<td>Professor T. Palmer (University of Dundee)</td>
</tr>
<tr>
<td>JESM267</td>
<td>Newman spa::tet sbi::kan</td>
<td>Sibbald et al. (2010)</td>
</tr>
<tr>
<td>SAN112</td>
<td>Db10 (Δarb5-Tn5) in-frame</td>
<td>Pradel et al. (2007)</td>
</tr>
<tr>
<td>SAN124</td>
<td>Db10 (ΔpswP::cml) in-frame</td>
<td>Gerc et al. (2012)</td>
</tr>
<tr>
<td>SAN176</td>
<td>Db10 (ΔentB) in-frame</td>
<td>Gerc et al. (2012)</td>
</tr>
<tr>
<td>SAN180</td>
<td>Db10 (Δarb5-ΔentB)</td>
<td>This study</td>
</tr>
<tr>
<td>SAN181</td>
<td>Db10 (Δarb5, swrA::Tn5, ΔentB)</td>
<td>This study</td>
</tr>
<tr>
<td>Escherichia coli MC1061</td>
<td>F' lacIQ lacZM15 Tn10 (TetR), cloning host</td>
<td>Perego &amp; Hoch (1988)</td>
</tr>
<tr>
<td>E. coli CC118/pir</td>
<td>Cloning host and donor strain for pKNG101-derived marker exchange plasmids (pir)</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>E. coli HH26 pNJ5000</td>
<td>Mobilizing strain for conjugal transfer</td>
<td>Grinter (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript KS(+)</td>
<td>High copy cloning vector (AmpR)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pKNG101</td>
<td>Suicide vector for marker exchange (SmR, sacBR, mobRK2, ori R6K)</td>
<td>Kaniga et al. (1991)</td>
</tr>
<tr>
<td>pSUPROM</td>
<td>Vector for constitutive expression of cloned genes under the control of the E. coli tat promoter (kan)</td>
<td>Jack et al. (2004)</td>
</tr>
<tr>
<td>pNW572</td>
<td>pKNG101-derived marker exchange plasmid for the generation of chromosomal Δarb5-5</td>
<td>Gerc et al. (2012)</td>
</tr>
<tr>
<td>pSAN69</td>
<td>pKNG101-derived marker exchange plasmid for the generation of chromosomal ΔentB</td>
<td>This study</td>
</tr>
<tr>
<td>pSAN46</td>
<td>pKNG101 coding sequence in pSUPROM</td>
<td>Gerc et al. (2012)</td>
</tr>
</tbody>
</table>

AmpR, ampicillin resistance; KanR, kanamycin resistance; SmR, streptomycin; TetR, tetracycline resistance.

Bioinformatic analysis. The genome of *S. marcescens* strain Db11 was sequenced by the Pathogen Sequencing Unit, the Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK. *S. marcescens* strain Db11 is a spontaneous Sm-resistant mutant of *S. marcescens* strain Db10 and hence the Db11 genome information can be used for Db10. To identify NRPS or PKS secondary metabolite gene clusters encoded within the *S. marcescens* Db11 genome, the bioinformatics tool antiSMASH was employed (Blin et al., 2011). The genome was uploaded as a nucleotide sequence and the bacterial, archaeal and plant plastid translation code, with a minimal gene length of 50, was used for gene finding by Glimmer. Within antiSMASH, the following parameters were selected: smCOG analysis for functional prediction and phylogenetic analysis of genes, gene cluster BLAST analysis, subcluster BLAST analysis, whole genome PFAM analysis and secondary metabolite detection on all possible ORFs. To investigate the likely function of NRPS or PKS products identified by antiSMASH, further manual analysis using BLASTP (Altschul et al., 1997) was performed.

RESULTS AND DISCUSSION

The PswP PPTase of *S. marcescens* Db10 is required for the biosynthesis of more than one metabolite with antimicrobial activity

During a prior study investigating the mechanism of althiomycin biosynthesis in *S. marcescens* Db10, it was noted that while *Sta. aureus* was sensitive to althiomycin, disruption of althiomycin biosynthesis did not completely abolish the antimicrobial effect of *S. marcescens* Db10 against *Sta. aureus* (Gerc et al., 2012). Aiming to investigate this further, we discovered that deletion of the gene encoding the PPTase protein PswP (which is required for althiomycin biosynthesis) was sufficient to negate the antimicrobial activity of *S. marcescens* Db10 against *Sta. aureus* (Fig. 1a). To confirm that the loss of antimicrobial activity associated with deletion of *pswP* was specific to deletion of this gene, the PswP mutant phenotype was complemented by expression of *pswP* in trans. Expression of *pswP* in trans restored the antimicrobial activity of *S. marcescens* Db10 against *Sta. aureus* (Fig. 1b). PPTase enzymes are known to modify the carrier protein domain of FAS, PKS and NRPS enzymes (Lambalot et al., 1996). We hypothesized that, in addition to being required for althiomycin biosynthesis, PswP was required for the biosynthesis of one or more FAS, PKS or NRPS product(s) with antimicrobial activity against *Sta. aureus*. Following this initial observation, we aimed to determine the identity of this metabolite(s).

Bioinformatic analysis of PswP and potential target proteins

PswP belongs to the Sfp class of PPTases and is one of three PPTases encoded by *S. marcescens* Db10 (Gerc et al.,...
There exists a second Sfp-type PPTase, SMA4147, which was previously identified during a search for the PPTase required for the biosynthesis of althiomycin; however, no biological function for SMA4147 has been ascribed (Gerc et al., 2012). SMA3052 represents a third PPTase encoded by *Ser. marcescens* Db10 that shows homology to AcpS family PPTases (data not shown) and is therefore likely involved in primary metabolism (Lambalot et al., 1996). Therefore, further bioinformatic analysis of the PPTases encoded by *Ser. marcescens* Db10 focused solely on PswP. Based on the presence of the conserved residues within motifs 1A, 1, 2 and 3, we assigned PswP to the F/KES subfamily of Sfp-type PPTases (Fig. 2).

In *Ser. marcescens* Db10, the *pswP* gene is not embedded within or adjacent to an NRPS or PKS biosynthetic gene cluster. We were therefore unable to predict additional PKS or NRPS biosynthetic pathways that might require the activity of PswP based on its genomic context. The bioinformatics tool, antiSMASH, allows the identification and the preliminary analysis of secondary metabolite gene clusters in bacterial and fungal genomes to be performed in an unbiased manner (Blin et al., 2013). Therefore, antiSMASH was employed to identify all genomic regions containing putative PKS, NRPS and NRPS-PKS gene clusters in the *Ser. marcescens* Db10 genome, on the basis that one or more of these might direct the biosynthesis of the secondary metabolite(s) active against *Sta. aureus*. The results of this search are summarized in Table 2. Five genomic regions containing NRPS or NRPS-PKS gene clusters, whose products could potentially be modified by the action of PswP, were identified. Upon closer inspection of the NRPS and NRPS-PKS gene clusters, the althiomycin biosynthetic machinery and the machinery required for the biosynthesis of the surfactant, serrawettin W2, were identified in clusters three and four (Gerc et al., 2012; Db10(a); Db10+VC; Db10+Δalb4–5 Δalb4–5 ΔentB ΔpswP ΔpswP ΔpswP + VC ΔpswP + pswP + VC ΔpswP ΔpswP ΔpswP ΔpswP ΔpswP + pswP (SAN112 pSAN46).

**Fig. 1.** The PPTase PswP is required for the biosynthesis of three secondary metabolites with antimicrobial activity against *Sta. aureus*. (a) Antimicrobial activity assays using *Sta. aureus* 113 as the indicator lawn. The producer strains are indicated above: Db10 (wild-type *Ser. marcescens* Db10), Alb4-5 (SAN5), ΔpswP (SAN112), Alb4-5 swrA::Tn5 (JESM267), Alb4-5 swrA::Tn5 (SAN124) and Alb4-5 swrA::Tn5 ΔentB (SAN181). (b) Antimicrobial activity assays show complementation of the *pswP* deletion by expression of the *pswP* gene in trans, using kanamycin-resistant *Sta. aureus* Newman spa::tet sbi::kan as the indicator lawn. The producer strains are indicated above: Db10+VC (*Ser. marcescens* Db10 pSUPROM, vector control), Db10+pswP (*Ser. marcescens* Db10 pSAN46), ΔpswP+VC (SAN112 pSUPROM) and ΔpswP+pswP (SAN112 pSAN46).

**Fig. 2.** Identification of conserved motifs of the F/KES subfamily of Sfp-type PPTases within PswP of *Ser. marcescens* Db10. Conserved PPT motifs 1A, 1, 2 and 3 are denoted with a dashed boxed. Residues previously identified as being highly conserved across the F/KES subfamily are indicated below the amino acid sequence; residues of PswP that match the consensus shown are shaded black. Analysis is based on the motif alignment analysis performed by Copp & Neilan (2006).
against *S. aureus* mutant showed a slight reduction in antimicrobial activity. DB05 showed a larger reduction in antimicrobial activity than DB10, with a small antibiosis halo observed with the double mutant (DB05::Tn5 vs. DB10). The reduction in size of the antimicrobial halo observed with either single mutant; however, a small antibiosis halo could still be observed (Fig. 1a). Therefore, we concluded that *S. aureus* W2 and althiomycin, which is dependent on the presence of PswP for biosynthesis.

The PswP-dependent surfactant serrawettin W2 has antimicrobial activity against *S. aureus*

Compared with *S. marcescens* Db10, the serrawettin W2 produced by *S. marcescens* ATCC 274 has been shown to have antimicrobial properties against meticillin-resistant *S. aureus* (Kadouri & Shanks, 2013). In *S. marcescens* Db10, PswP was previously shown to be required for the biosynthesis of serrawettin W2 (Pradel et al., 2007). Therefore, to determine whether serrawettin W2 also possesses antimicrobial activity, we tested both mutant strains for antimicrobial activity against *S. aureus* (Fig. 1a). Therefore, we concluded that serrawettin W2 does indeed possess antimicrobial activity against *S. aureus* (Fig. 1a). Therefore, we concluded that serrawettin W2 does indeed possess antimicrobial activity against *S. aureus*.

Table 2. Characterization of NRPS- or PKS-encoding genes identified by antiSMASH (Blin et al., 2013)

<table>
<thead>
<tr>
<th>Gene cluster</th>
<th>Genomic region predicted by antiSMASH</th>
<th>Enzyme predicted by antiSMASH</th>
<th>Gene(s) encoding NRPS (or NRPS-PKS)</th>
<th>Previously reported product</th>
<th>Putative product</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SMA1552–1589</td>
<td>NRPS</td>
<td>SMA1571-1572</td>
<td>–</td>
<td>Unknown</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>SMA1705–1744</td>
<td>NRPS</td>
<td>SMA1729</td>
<td>–</td>
<td>Siderophore</td>
<td>†</td>
</tr>
<tr>
<td>3</td>
<td>SMA2274-1309</td>
<td>NRPS-PKS</td>
<td>SMA2289-2290</td>
<td>Althiomycin</td>
<td>NA</td>
<td>†</td>
</tr>
<tr>
<td>4</td>
<td>SMA3659–3702</td>
<td>NRPS</td>
<td>SMA3680</td>
<td>NA</td>
<td>Siderophore</td>
<td>‡</td>
</tr>
<tr>
<td>5</td>
<td>SMA4386–4438</td>
<td>NRPS</td>
<td>SMA4411</td>
<td>–</td>
<td>Unknown</td>
<td>‡</td>
</tr>
</tbody>
</table>

NA, not applicable.

*Predicted NRPS encoded within a cluster of genes (SMA1575–1568) showing similarity with genes involved in the biosynthesis and secretion of microcins; may synthesize a modifying group added to the microcin.

†Predicted NRPS similar to chrysobactin synthetase (64% identity over the whole length) and flanked by genes encoding proteins with similarity to proteins mediating siderophore export, uptake and iron release.

‡Althiomycin is a broad-spectrum antibiotic. NRPS-PKS is encoded within the six-gene *alb* operon (SMA2288–2293), which also encodes tailoring and export functions (Gerc et al., 2012).

§Serrawettin W2 is a biosurfactant (Pradel et al., 2007).

‖Predicted NRPS is similar to enterobactin synthetase *EntF* and within cluster of genes (SMA4408–4415) similar to the enterobactin biosynthesis, export and uptake cluster of *E. coli* (Fig. 3).

¶Encoded NRPS proteins share similarity with siderophore synthetase enzymes from other organisms but number and nature of product(s) unclear.
The **SMA4408-4415** gene cluster encodes a siderophore that is dependent on PswP for biosynthesis and has antimicrobial activity against *Sta. aureus*

In light of the results presented above, we reasoned that one or more of the three remaining NRPS gene clusters identified by antiSMASH should be responsible for the antimicrobial effect against *Sta. aureus* observed in the absence of althiomycin and serrawettin W2. Two of the three remaining NRPS gene clusters, identified by antiSMASH, encoded proteins with similarity to siderophore biosynthetic proteins (Table 2, Fig. 3). We therefore hypothesized that either a siderophore produced by *Ser. marcescens* Db10 was directly toxic toward *Sta. aureus*, or that siderophore-dependent removal of iron from the environment prevented growth of *Sta. aureus*. In order to investigate these possibilities, FeCl₃ was added to the growth media and the antimicrobial bioassay repeated. The small antibiosis halo observed in the absence of additional iron with the double Δαlbd₄-₅ swrA_::Tn₅ mutant disappeared upon the addition of FeCl₃ (Fig. 4). From these results two scenarios are possible: 1) addition of excess iron negates the iron depletion effect of a siderophore(s), or 2) the addition of iron negatively affects the production of siderophores by *Ser. marcescens* Db10, as observed in *E. coli* and many other Gram-negative bacteria (Crosa & Walsh, 2002). In either case, these findings indicated that siderophore production by *Ser. marcescens* Db10 was likely to inhibit *Sta. aureus* growth.

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**Fig. 3.** Comparison of the SMA2450–2452 and SMA4408–4415 gene clusters of *Ser. marcescens* Db10 with the enterobactin biosynthetic gene cluster of *E. coli* CFT073 (c0668–c0683). Genes are drawn approximately to scale and protein length is indicated below the encoding gene (as the number of amino acids). Homologous genes are indicated by the same colour, the level of identity between homologous proteins is shown below the corresponding genes as an absolute value and as a percentage.

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**Fig. 4.** An althiomycin and serrawettin W2 mutant of *Ser. marcescens* Db10 is unable to kill *Sta. aureus* in the presence of additional iron. Antimicrobial activity assays in the absence (a) or presence (b) of 50 μM FeCl₃, using *Sta. aureus* as the indicator lawn, with a schematic of the location of each producer strain shown (c). The producing strains were: 1, *Ser. marcescens* Db10 (wild-type); 2, SAN5 (Δαlbd₄-₅); 3, JESM267 (swrA_::Tn₅); 4, SAN124 (Δαlbd₄-₅ swrA_::Tn₅); 5, SAN112 (ΔpswP). The insets show a magnified image of producer strain 4 SAN124 in both (a) and (b).
As mentioned above, a set of genes (SMA4408–4415) within cluster five show homology and synten with the enterobactin gene cluster of *E. coli* (Fig. 3). It therefore seemed likely that these genes are involved in the synthesis of enterobactin or a closely related molecule. However, it should be noted that the only close homologue of *entA*, which is located immediately upstream of *entB* in *E. coli*, was identified as SMA2450 in *Ser. marcescens* Db11. In contrast with *E. coli*, the SMA2450 gene is located at a distant locus from the other enterobactin-like genes; however, it is closely genetically linked with *pswP* (SMA2452) in *Ser. marcescens*. Given both the linkage between *entA* and *pswP* in *Ser. marcescens* and that the SMA4408–4415 genomic region showed the most convincing similarity with a well-characterized cluster of genes involved in siderophore biosynthesis and utilization, we tested whether the product of these genes was responsible for the *pswP*- and iron-dependent growth inhibition observed in the Δ*alb4-5 swrA::Tn5* double mutant. The product of SMA4415 shares significant homology with *entB* of the enterobactin biosynthetic gene cluster (Fig. 3). Since EntB is essential for enterobactin biosynthesis in *E. coli* (Crosa & Walsh, 2002), we reasoned that SMA4415 would be essential for biosynthesis of this siderophore in *Ser. marcescens* Db10. An in-frame deletion in *entB* was therefore constructed in *Ser. marcescens* Db10, and also in the althiomycin and serrawettin W2 mutant background to give a triple althiomycin, serrawettin W2 and *EntB* mutant (Δ*alb4-5 swrA::Tn5 ΔentB*). To compare siderophore production by wild-type *Ser. marcescens* Db10 with that of the single *entB* mutant, the triple Δ*alb4-5 swrA::Tn5 ΔentB* mutant and the Δ*pswP* mutant, the relevant strains were grown on CAS indicator plates. CAS/hexadecyltrimethylammonium bromide complex with ferric iron present in these plates acts as an indicator of siderophore biosynthesis; in the presence of an iron chelator this indicator turns from a blue to an orange colour (Schwyn & Neillands, 1987). In wild-type *Ser. marcescens* Db10, production of a diffusible siderophore was clearly observed (Fig. 5a). In contrast, no siderophore production was observed in either the *ΔentB* or *pswP* PPTase (*ΔpswP*) single mutants (Fig. 5a). Furthermore, no siderophore production was observed in the althiomycin, serrawettin W2 and *EntB* triple mutant (Δ*alb4-5 swrA::Tn5 ΔentB*) (Fig. 5a). The loss of siderophore production was specific to the deletion of *entB* or *pswP*, with siderophore production being unaffected in strains unable to produce althiomycin and/or serrawettin (Fig. 5a). These results clearly show that both *entB* and *pswP* are essential for the biosynthesis of a siderophore in *Ser. marcescens* Db10. In addition, this assay confirmed that construction of the *pswP* (SMA2452) PPTase mutant did not affect siderophore biosynthesis through downstream polar effects on the expression of SMA2450, which is predicted to encode an EntA homologue (Fig. 3), since the loss of siderophore biosynthesis observed with the *pswP* mutant could be complemented by the expression of *pswP* in trans (Fig. 5b). Having shown that *entB* was required for biosynthesis of a siderophore in *Ser. marcescens* Db10, the triple althiomycin, serrawettin W2 and siderophore mutant was tested for antimicrobial activity against *Sta. aureus*. No antibiotic halo was observed with the Δ*alb4-5 swrA::Tn5 ΔentB* triple mutant (Fig. 1a), thus providing a phenocopy of the *pswP* PPTase mutant and identifying the *entB*-dependent siderophore as the third *pswP*-dependent antimicrobial secondary metabolite produced by *Ser. marcescens* Db10.

**CONCLUDING REMARKS**

In this study, we have shown that *Ser. marcescens* Db10 produces three diffusible molecules with antimicrobial activity against the clinically relevant pathogen *Sta. aureus*. Production of all three requires *pswP*, consistent with their biosynthesis being dependent on NRPS enzymes. Genetic evidence has revealed these three metabolites to be: the antibiotic althiomycin, the product of Alb1-6 (Gerc et al., 2012); the biosurfactant serrawettin W2, the product of SwrA (Pradel et al., 2007); and a siderophore whose production is associated with NRPS-containing cluster of.

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**Fig. 5.** The PPTase *pswP* and SMA4415 (*EntB*) are required for the biosynthesis of a siderophore in *Ser. marcescens* Db10. CAS assay for the detection of siderophore biosynthesis, as indicated by the presence of an orange halo. (a) The producer strains are indicated above: Db10 (wild-type *Ser. marcescens* Db10), Δ*alb4-5* (SAN5), swrA::Tn5 (JESM267), Δ*alb4-5 swrA::Tn5* (SAN124), Δ*entB* (SAN176), Δ*pswP* (SAN112) and Δ*alb4-5 swrA::Tn5* Δ*entB* (SAN181). (b) Complementation of the *pswP* deletion by expression of the *pswP* gene in trans. The producer strains are indicated above: Db10 + VC (Ser. marcescens Db10 pSUPROM, vector control), Db10 + *pswP* (Ser. marcescens Db10 pSAN46), Δ*pswP* + VC (SAN112 pSUPROM) and Δ*pswP* + *pswP* (SAN112 pSAN46).
genes, *SMA4408–4415*. Importantly, this is the first time, to our knowledge, that the latter two molecules have been shown to act against *Sta. aureus*. Furthermore, production of this siderophore and its association with PswP and the *SMA4408–4415* gene cluster has not been reported previously in *Ser. marcescens*. Whilst we speculate that this molecule is likely to be closely related, or even identical, to enterobactin produced by *E. coli*, its chemical structure remains to be confirmed. Whilst we speculate that the ability to use this siderophore to scavenge Fe$^{3+}$ is likely to be important for *Ser. marcescens* to survive within the iron-limited environment of the host during infection (Miethke & Marahiel, 2007), this remains to be investigated. In addition, our data highlight how an individual bacterial PPTase enzyme can play an essential role in the biosynthesis of multiple secondary metabolites with disparate physiological roles. PswP provides another example, in addition to others reported previously (Beld et al., 2014), illustrating that PPTase enzymes are not necessarily pathway-specific or genetically linked with their substrates but may act on multiple NRPS enzymes. Finally, our data also illustrate how several distinct secondary metabolites can contribute to the inhibition of growth of competitor bacteria, a scenario that is likely to occur at polymicrobial infection sites and in varied environmental niches. In particular, we note the potential utility of iron restriction to inhibit the growth of clinically significant pathogens such as *Sta. aureus*.

**Note added in proof**

The siderophore product dependent on the enterobactin-like gene cluster *SMA4408–4415* is likely to be identical or related to the serratiochelin molecules recently reported to be produced by *Serratia* sp. V5 using a ‘shuffled’ combination of enterobactin-like and vibriobactin-like genes (Seyedsayamdost et al., 2012).

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