The plant pathogen *Streptomyces scabies* 87-22 has a functional pyochelin biosynthetic pathway that is regulated by TetR- and AfsR-family proteins

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Siderophores are high-affinity iron-chelating compounds produced by bacteria for iron uptake that can act as important virulence determinants for both plant and animal pathogens. Genome sequencing of the plant pathogen *Streptomyces scabies* 87-22 revealed the presence of a putative pyochelin biosynthetic gene cluster (PBGC). Liquid chromatography (LC)-MS analyses of culture supernatants of *S. scabies* mutants, in which expression of the cluster is upregulated and which lack a key biosynthetic gene from the cluster, indicated that pyochelin is a product of the PBGC. LC-MS comparisons with authentic standards on a homochiral stationary phase confirmed that pyochelin and not enantio-pyochelin (*ent*-pyochelin) is produced by *S. scabies*. Transcription of the *S. scabies* PBGC occurs via ~19 kb and ~3 kb operons and transcription of the ~19 kb operon is regulated by TetR- and AfsR-family proteins encoded by the cluster. This is the first report, to our knowledge, of pyochelin production by a Gram-positive bacterium; interestingly regulation of pyochelin production is distinct from characterized PBGCs in Gram-negative bacteria. Though pyochelin-mediated iron acquisition by *Pseudomonas aeruginosa* is important for virulence, *in planta* bioassays failed to demonstrate that pyochelin production by *S. scabies* is required for development of disease symptoms on excised potato tuber tissue or radish seedlings.

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INTRODUCTION

The genus *Streptomyces* is comprised of hundreds of species, most of which are soil-dwelling and saprophytic. The ability of these filamentous actinobacteria to produce biologically active secondary metabolites is well known; streptomycetes produce nearly two-thirds of the world's naturally occurring antibiotics (Bentley *et al.*, 2002). Since soil is poor in nutrients but rich in microbial competitors, most secondary metabolites are thought to serve as antimicrobial agents. However, secondary metabolites have other roles, one of which is iron acquisition. Iron is relatively unavailable to soil bacteria due to the low solubility of the Fe³⁺ ion under aerobic conditions at

neutral pH. The most common way that bacteria cope with low bioavailability of iron is the production of iron-chelating compounds called siderophores (Guerinot, 1994). Production of siderophores by saprophytic *Streptomyces* species has been known since the 1960s; desferrioxamine siderophores are produced by multiple *Streptomyces* species (Barona-Gómez *et al.*, 2004, 2006; Bickel *et al.*, 1960; Imbert *et al.*, 1995; Schupp *et al.*, 1988). Recently, streptomycetes have been found to produce siderophores other than desferrioxamines. *Streptomyces* sp. Tü 6125 produces enterobactin, a catecholate-type siderophore typically produced by members of the family *Enterobacteriaceae* (Fiedler *et al.*, 2001). *Streptomyces* sp. ATCC 700974 and *Streptomyces griseus* produce griseobactin, a siderophore containing catechol, threonine and arginine that has only been partially characterized, and *Streptomyces coelicolor* produces coelichelin, a novel tris-hydroxamate siderophore (Lautru *et al.*, 2005; Patzer & Braun, 2010).

Interestingly, the genus *Streptomyces* also contains more than a dozen pathogens of agricultural crops, some of which

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Abbreviations: *ent*-pyochelin, enantio-pyochelin; LC, liquid chromatography; NRPS, non-ribosomal peptide synthetase; PBGC, putative pyochelin biosynthetic gene cluster.

A supplementary table of primer sequences and two supplementary figures are available with the online version of this paper.

cause scab diseases of potato and tap root crops (Loria *et al.*, 2006). Scab-causing streptomycetes are general necrotic pathogens that aggressively colonize rapidly expanding root and tuber tissue. These pathogens possess multiple virulence genes, some of which are conserved among pathogenic species while others are species- or even strain-specific (Bignell *et al.*, 2010a; Kers *et al.*, 2005; Loria *et al.*, 2003, 2006, 2008). Among plant pathogenic streptomycetes, *Streptomyces scabies* 87-22 has emerged as a model system for studying plant-microbe interactions for Gram-positive plant pathogens, and a genome sequence is now available for this strain (GenBank Accession no. FN554889).

It is well established that siderophore-mediated iron acquisition is critical for the successful infection of mammalian hosts by bacterial pathogens (Miethke & Marahiel, 2007), but less is known about the role of siderophores in infection of plants by bacteria. To the best of our knowledge, studies of iron acquisition and its importance in plant pathogenicity have only been conducted with Gram-negative bacteria. In *Pseudomonas syringae* pv. tabaci 6605, which causes wildfire disease, the siderophore pyoverdine is required for infection of tobacco (Taguchi *et al.*, 2010). Transcription of virulence genes in the bacterial speck pathogen, *P. syringae* pv. tomato DC3000 is enhanced in the presence of iron (Kim *et al.*, 2009). Similarly, siderophore-mediated iron acquisition by the bacterial soft rot pathogen, *Erwinia chrysanthemi*, and production of pectate lyase virulence proteins are coupled (Enard *et al.*, 1988; Franza *et al.*, 2002). Iron acquisition is also important for systemic pathogenicity of apple flowers by the fire blight pathogen, *E. amylovora* (Dellagi *et al.*, 1998).

Iron acquisition by Gram-positive plant pathogens remains entirely unexplored. Analysis of the *S. scabies* 87-22 genome (<http://strepdb.streptomyces.org.uk/>) revealed gene clusters predicted to direct the production of desferrioxamine, pyochelin and other potential siderophores. The putative pyochelin biosynthetic gene cluster (PBGC) was of particular interest because pyochelin is produced by the mammalian pathogens *P. aeruginosa* (Cox *et al.*, 1981) and some members of the *Burkholderia cepacia* complex (Thomas, 2007), as well as plant-associated *P. fluorescens* (Castignetti, 1997). Pyochelin-mediated iron acquisition by *P. aeruginosa* during infection of mice is important for pathogenesis (Cox, 1982; Takase *et al.*, 2000), because pyochelin is capable of removing iron from transferrin and lactoferrin from human serum to facilitate bacterial growth (Ankenbauer *et al.*, 1985; Sriyosachati & Cox, 1986).

The biosynthesis of pyochelin in *P. aeruginosa* is well characterized and requires the activities of seven proteins (PchABCDEFG). PchAB utilize chorismate to biosynthesize salicylate (Serino *et al.*, 1995), which is adenylated by PchD and subsequently loaded onto a thiolation domain of the PchE non-ribosomal peptide synthetase (NRPS) multi-enzyme. PchE catalyses adenylation of cysteine, which

subsequently autoacylates a second thiolation domain within the multienzyme. Condensation of the cysteinyl thioester with the salicyl thioester, catalysed by a heterocyclization domain of PchE, produces a 2-hydroxyphenyl-thiazolanyl thioester intermediate, which is condensed with a second molecule of cysteine via a similar mechanism by the NRPS PchF (Quadri, *et al.* 1999). This results in a 2-hydroxyphenyl-bis-thiazolanyl thioester intermediate attached to the thiolation domain of PchF (Quadri *et al.*, 1999). An external thiazoline reductase, PchG, reduces one of the thiazolines in the intermediate to a thiazolidine, which undergoes concomitant *N*-methylation catalysed by the methyltransferase domain of PchF. Mature pyochelin is released from PchF by thioester hydrolysis catalysed by the C-terminal thioesterase domain (Patel & Walsh, 2001; Reimmann *et al.*, 2001). PchC serves as a proofreading enzyme that removes aberrantly charged molecules from the peptidyl carrier protein domains of PchE and PchF (Reimmann *et al.*, 2004).

Transcription of the pyochelin biosynthetic genes in *P. aeruginosa* is regulated by the ferric uptake regulator, Fur, and is repressed during growth in iron-rich conditions (Prince *et al.*, 1993). A pathway-specific AraC-family regulator, PchR, requires ferripyochelin as a ligand to activate transcription and, interestingly, PchR represses its own transcription during times of low iron availability (Heinrichs & Poole, 1993, 1996; Michel *et al.*, 2005).

Here, we describe the only known PBGC in Gram-positive bacteria. This is also the first report, to our knowledge, of pyochelin production by a plant pathogen. We also demonstrate that, although the *S. scabies* and *P. aeruginosa* PBGCs are similar, TetR- and AfsR-family proteins, rather than AraC-family proteins, regulate transcription of the pyochelin biosynthetic genes in *S. scabies* 87-22.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains were cultured as described by Sambrook *et al.* (1989). *Streptomyces* strains were cultured at 28 °C using international *Streptomyces* project 4 agar (ISP4) medium or liquid minimal medium (MM) containing 2 g NH₄SO₄ l⁻¹, 0.6 g MgSO₄ l⁻¹, 2.6 g K₂HPO₄ l⁻¹, 0.1 g CaCl₂ l⁻¹, 2.5% (v/v) glycerol, 2.5 g glucose l⁻¹, 0.05 g yeast extract l⁻¹ and trace element solution as described previously (Kieser *et al.*, 2000) without FeCl₃. All liquid cultures were shaken at ~250 r.p.m. Media were supplemented with antibiotics at the following concentrations: 100 µg hygromycin B ml⁻¹, 100 µg apramycin ml⁻¹, 50 µg kanamycin ml⁻¹, 50 µg nalidixic acid ml⁻¹, 10 µg thiostrepton ml⁻¹. All *Streptomyces* strains generated in this study were created by cross-genera conjugation from the non-methylating *E. coli* strain ET12567/pUZ8002 (MacNeil *et al.*, 1992) as described previously (Kieser *et al.*, 2000). Cloning and plasmid construction were performed as described previously (Seipke & Loria, 2008). Strains and plasmids are described in Table 1.

Generation of mutant strains. Null mutants of *S. scabies* 87-22 were created using the PCR-targeting REDIRECT technology (Gust *et al.*, 2003a). A disruption cassette consisting of an *oriT* and either the apramycin resistance gene, *aac(3)IV* from pIJ773 (Gust *et al.*, 2003a), or the hygromycin B resistance gene from pIJ10700 (Gust *et al.*,

Table 1. Bacterial strains and plasmids used in this study

Amp, ampicillin; Apr, apramycin; Hyg, hygromycin B; Kan, kanamycin; Tsp, thiostrepton.

Strain or plasmid	Genotype or characteristic	Source or reference
<i>S. scabies</i>		
87-22	Wild-type	This study
Δ SCAB1371	87-22 SCAB1371 null mutant	This study
Δ SCAB1401	87-22 SCAB1401 null mutant	This study
87-22 attB ϕ BT1::pIJ10257	87-22 bearing the empty overexpression vector	This study
87-22 attB ϕ BT1::pRFSRL38	87-22 bearing the SCAB1371 overexpression plasmid	This study
Δ SCAB1401- Δ SCAB1471	87-22 SCAB1401-SCAB1471 double mutant	This study
Δ SCAB1401 attB Φ C31::pAU3-45	SCAB1401 null mutant bearing empty complementation vector	This study
Δ SCAB1401 attB Φ C31::pRFSRL34	Complemented SCAB1401 null mutant	This study
<i>E. coli</i>		
BW25113	Host for REDIRECT PCR targeting system	Gust <i>et al.</i> (2003a)
ET12567	Non-methylating host for transfer of DNA into <i>Streptomyces</i> strains; <i>dam dcm hsdM</i>	MacNeil <i>et al.</i> (1992)
DH5 α	F ⁻ general cloning host	GibcoBRL
TOP10	Host for pCR2.1TOPO cloning system	Invitrogen
Plasmids		
Supercos1	Cosmid backbone for <i>S. scabies</i> cosmids; Amp ^R Kan ^R	Stratagene
Cosmid 632	Supercos1 derivative containing the <i>S. scabies</i> 87-22 PBGC; Kan ^R Amp ^R	This study
pCR2.1TOPO	Cloning vector for PCR products; Kan ^R Amp ^R	Invitrogen
pIJ773	PCR template for <i>aac(3)IV</i> + <i>oriT</i> cassette used in REDIRECT PCR targeting system	Gust <i>et al.</i> (2003a/b)
pIJ10700	PCR template for <i>hygR</i> cassette used in REDIRECT PCR targeting system	Gust <i>et al.</i> (2003b)
pUZ8002	Encodes conjugation machinery for mobilization of plasmids from <i>E. coli</i> to <i>Streptomyces</i> ; Kan ^R	MacNeil <i>et al.</i> (1992)
pKD46	Encodes lambda RED recombination machinery induced by arabinose; Amp ^R	Datsenko & Wanner (2000)
pAU3-45	pSET152 derivative, integrates into the Φ C31 attB site in <i>Streptomyces</i> ; Apr ^R Tsp ^R	Bignell <i>et al.</i> (2005)
pIJ10257	pMS81 derivative containing <i>ermEp*</i> , integrates into the Φ BT1 attB site in <i>Streptomyces</i> ; Hyg ^R	Hong <i>et al.</i> (2005)
pRFSRL34	pAU3-45 derivative containing the SCAB1401 locus cloned into the <i>EcoRI</i> site	This study
pRFSRL38	pIJ10257 derivative containing the SCAB1371 coding sequence cloned into the <i>NdeI</i> - <i>HindIII</i> sites	This study

2003b) was generated by PCR amplification with primers that contained 39 nt of homology. The cassette included the start or stop codons and 36 nt downstream or upstream of the coding sequence for SCAB1401 and SCAB1471, while the 39 nt of homology extended 39 nt internal to the coding sequence of SCAB1371 and did not contain the start or stop codons (Supplementary Table S1, available with the online version of this paper). The resulting PCR products were gel-purified and transferred by electroporation into *E. coli* BW25113 containing pKD46 (which contains the λ RED genes) (Datsenko & Wanner, 2000) and *S. scabies* 87-22 cosmid 632, which contains the entire PBGC and 269 bp of sequence upstream of SCAB1481 and 18 944 bp of sequence downstream of SCAB1371. Transformants were screened for the presence of the mutagenized cosmid by colony PCR and mutagenesis was verified by restriction digestion. Mutagenized cosmid DNA was transferred to *S. scabies* 87-22 via conjugation. Transconjugants resulting from double crossover homologous recombination were selected on the basis of apramycin resistance and kanamycin sensitivity (Δ SCAB1401), resistance to hygromycin B and kanamycin sensitivity (Δ SCAB1471 and Δ SCAB1371), or resistance to apramycin and hygromycin B, and sensitivity to kanamycin

(Δ SCAB1401- Δ SCAB1471). Correct construction of mutant strains was confirmed by Southern blot hybridization.

Overexpression of the SCAB1371 regulatory gene.

Overexpression of SCAB1371 was achieved by cloning the SCAB1371 coding sequence amplified by PCR into the *NdeI* and *HindIII* restriction sites of pIJ10257 (Hong *et al.*, 2005), which integrates into the ϕ BT1 phage site present in streptomycete chromosomes (Gregory *et al.*, 2003). The resulting plasmid, pRFSRL38, was moved to the wild-type strain by conjugation. Transconjugants were selected for using hygromycin B. *S. scabies* strains containing pRFSRL38 were merodiploid, with one copy of SCAB1371 under the control of the native promoter, and the second copy under the control of *ermEp** provided by pIJ10257 (Hong *et al.*, 2005).

Complementation of the Δ SCAB1401 mutant strain.

To complement the Δ SCAB1401 mutant, a ~1.7 kb fragment containing the SCAB1401 coding sequence and 1 kb of upstream DNA was amplified by PCR and cloned into the *EcoRI* site of the ϕ C31 integrative plasmid, pAU3-45 (Bignell *et al.*, 2005). The resulting

plasmid, pRFSRL34, was moved to the Δ SCAB1401 strain by conjugation. Transconjugants were selected for thioestrepton resistance.

Southern blot hybridization. Southern analysis was performed as described previously (Seipke & Loria, 2008). To confirm proper construction of the Δ SCAB1401 strain, *Kpn*I-digested DNA was probed with a gene-specific fragment internal to SCAB1391 that was labelled with dioxigenin-11-dUTP (DIG) (Roche). Correct construction of the Δ SCAB1401- Δ SCAB1471 and Δ SCAB1471 mutant strains was confirmed by probing *Pst*I/*Kpn*I-digested DNA with a DIG-labelled fragment internal to SCAB1451. Correct construction of the Δ SCAB1371 mutant strain was confirmed by probing *Not*I/*Eco*RI-digested DNA with a DIG-labelled fragment internal to SCAB1381. Hybridizations were performed in a rotary oven overnight at 55 °C. Washing conditions for all membranes used in this study were as follows: 2 × SSC (1 × SSC: 0.15 M NaCl plus 0.015 M sodium citrate) at 55 °C for 20 min, 1 × SSC at 55 °C for 20 min and 0.2 × SSC at 55 °C for 20 min. Processing of each membrane from this point forward was performed according to the manufacturer's instructions (Roche). Mutant genotypes were confirmed by the presence of the expected polymorphic shift when compared with the DNA from the wild-type strain probed with the same probes.

RNA analysis. For co-transcription and transcriptional regulation experiments, *S. scabies* strains were grown for 3 days in liquid MM with or without FeCl₃. RNA was extracted as described previously (Joshi *et al.*, 2007). DNase-treated RNA (1 µg) was reverse transcribed using the Superscript III first strand synthesis system and 250 ng random hexamer primers (Invitrogen). Control reactions in which the enzyme was omitted were also performed. For co-transcription analysis, PCR with nine primer sets (Supplementary Table S1) was performed. For transcriptional regulation studies, PCR with two primer sets targeting the SCAB1411 and SCAB1451 genes present within the large biosynthetic operon as well as primer sets targeting SCAB1381, SCAB1371, SCAB1391 and SCAB1401 was performed (Supplementary Table S1). All products resulting from PCR of cDNA were cloned into pCR2.1TOPO (Invitrogen) and sequenced (Biotechnology Resource Center, Cornell University) using oligonucleotide primer M13r (Invitrogen).

Liquid chromatography (LC)-MS analyses. *S. scabies* 87-22 and the Δ SCAB1401 and Δ SCAB1401- Δ SCAB1471 mutants were grown in a previously described iron-deficient medium (Barona-Gómez *et al.*, 2004) for 4 days. The cultures were centrifuged for 10 min at 4000 r.p.m. and 4 °C. The supernatants were lyophilized and resuspended in 5 ml methanol for LC-MS analysis on a Dionex Ultimate 3000RS instrument equipped with Supelco Acentis Express C18 column (150 × 2.1 mm, 2.7 µm) coupled to a Bruker MaXis mass spectrometer [ESI in positive mode; full scan 50–1500 *m/z*; End plate offset, −500 V; Capillary, −4500 V; Nebulizer gas (N₂), 1.6 bar; Dry gas (N₂), 8 l min^{−1}; Dry Temperature, 180 °C]. The solvents used for elution of the column were as follows. Solvent A, water/0.1 % formic acid; Solvent B, methanol/0.1 % formic acid. The elution profile was as follows: 0–5 min, 100 % A; 5–30 min, linear gradient from 100 % A to 100 % B; 30–35 min, 100 % B; 35–40 min, linear gradient from 100 % B to 100 % A; 40–55 min, 100 % A. The flow rate was 0.2 ml min^{−1}. Absorbance was monitored at 210 and 280 nm. The mass spectrometer was calibrated with 10 mM sodium formate at the beginning of each run. An Agilent 1100 HPLC coupled to a Bruker HCTplus mass spectrometer [ESI in positive mode; full scan 50–1500 *m/z*; End plate offset, −500 V; Capillary, −4500 V; Nebulizer gas (N₂), 40 p.s.i.; Dry gas (N₂), 10 l min^{−1}; Dry Temperature, 300 °C] was used for analysis of pyochelin samples on a homochiral stationary phase. Pyochelin and enantio-pyochelin (*ent*-pyochelin) standard solutions (both 10 µl, 10 µg ml^{−1} in methanol) and concentrated culture supernatant from the Δ SCAB1401 mutant were analysed on a

ChiralPAK IA analytical column (250 × 4.6 mm, 5 µm) in reverse phase mode using the eluents and elution conditions described above, except that the flow rate was 1 ml min^{−1}.

In planta bioassays. Excised potato tuber tissue assays (cv. Russett Burbank) were performed as described previously (Loria *et al.*, 1995). Agar plugs from iron-deficient solid minimal medium containing mycelia from either the wild-type or SCAB1471 mutant strain were used as inoculum. Inoculated potato tuber slices (four slices per strain) were incubated for ~1 week in a Parafilm-sealed Petri plate containing moist filter paper to prevent drying. Virulence assays with radish (cv. Burpee White) seedlings were performed as described previously (Bignell *et al.*, 2010b). Five germinated seeds were placed in wells carved in nanopure water agar (8 g ultrapure agarose l^{−1}) by using a cork borer. Following placement in the agar wells, germinated seeds were inoculated with 75 µl wild-type or mutant spore suspensions. Plants were incubated at 21 ± 2 °C under a 12 h photoperiod and analysed ~4 days after inoculation.

RESULTS

Sequence analysis of the *S. scabies* PBGC

Analysis of the recently sequenced genome of *S. scabies* 87-22 (GenBank Accession no. FN554889) revealed the presence of a cluster of twelve genes, six of which encode proteins (SCAB1381, SCAB1411, SCAB1421, SCAB1461, SCAB1471 and SCAB1481) that appear to be functionally analogous to known pyochelin biosynthetic proteins PchABCDEFGF, from the opportunistic mammalian pathogen *P. aeruginosa* (Patel & Walsh, 2001; Quadri *et al.*, 1999; Serino *et al.*, 1997) (Fig. 1, Table 2).

SCAB1381 contains a chorismate-utilizing anthranilate synthase domain (InterPro ID, IPPR005801) and all 10 active site residues of Irp9, the salicylate synthase for yersiniabactin in *Yersinia enterocolitica* (Kerbarh *et al.*, 2006). The predicted AMP-ligase SCAB1411 probably adenylates salicylate, because sequence comparisons identified the putative active site residues ALPSQGVLNK, which are 80 % identical to the active site residues (PLPAQGVLNK) of MbtA, an AMP-ligase from *Mycobacterium tuberculosis* that adenylates salicylate for mycobactin production (Challis *et al.*, 2000; Quadri *et al.*, 1998; Rausch *et al.*, 2005).

The organization and predicted functions of NRPS domains in SCAB1481 and SCAB1471 are identical to those of PchE and PchF, respectively. The adenylation domains of SCAB1481 and SCAB1471 are predicted to contain the substrate-binding pocket residues DLWNLSLIWK and DLYNLSLIWK, which are 90 and 100 % identical to characterized cysteine-selective adenylation domains, respectively (Challis *et al.*, 2000; Quadri *et al.*, 1999; Rausch *et al.*, 2005). Based on the similarity between SCAB1481 and PchE and SCAB1471 and PchF, it is likely that SCAB1481 catalyses assembly of a 2-hydroxyphenyl-thiazolanyl thioester from the salicyl adenylate generated by SCAB1411, and SCAB1471 elongates this thioester with a second molecule of cysteine to create a 2-hydroxyphenyl-bis-thiazolanyl thioester tethered to the T domain of

Fig. 1. Organization of the PBGCs in *S. scabies* 87-22 and *P. aeruginosa* PAO1. DNA sequences for these PBGCs can be accessed via GenBank accession nos FN554889 and NC_002516, respectively. Pyochelin biosynthetic gene orthologues are colour-coded based on conserved function. Genes presumably involved in pyochelin export are grey, while non-orthologous genes are white.

SCAB1471. SCAB1461 encodes a predicted thiazoline reductase (InterPro ID, IPP010091) and probably catalyses reduction of one of the thiazoline rings in the *bis*-thiazolinyl thioester intermediate prior to *N*-methylation of the resulting thiazolidine catalysed by SCAB1471 (Patel & Walsh, 2001). After *N*-methylation, a terminal thioesterase domain in SCAB1471 is expected to release mature pyochelin. SCAB1421 encodes a putative thioesterase that is likely to be functionally analogous to PchC, which is a proofreading enzyme that removes aberrantly charged molecules from the thiolation domains of PchE and PchF (Reimann *et al.*, 2004). The above analyses indicate that the substrate specificities and catalytic properties of SCAB1381, SCAB1411, SCAB1421, SCAB1461, SCAB1471 and SCAB1481 are identical to the pyochelin biosynthetic proteins PchABCDEFGH from *P. aeruginosa* and suggest that *S. scabies* produces pyochelin. A proposed pathway for pyochelin biosynthesis in *S. scabies* is shown in Fig. 2.

Pyochelin production by *S. scabies*

In order to evaluate our prediction that *S. scabies* produces pyochelin, we analysed culture supernatants from *S. scabies* wild-type grown for 4 days in an iron-deficient medium using LC-MS. While we were able to detect a metabolite with the expected *m/z* for the $[M+H]^+$ ion of pyochelin, this result was poorly reproducible. We postulated that this was because the gene cluster was not always sufficiently induced under the growth conditions used to allow detection of the metabolite. Since pathway-specific regulators are typically encoded by biosynthetic gene clusters in *Streptomyces* species, it appeared likely that the putative TetR-family regulatory gene (SCAB1401) in the *S. scabies* PBGC (Fig. 1) might repress transcription of the gene cluster. In order to assess this possibility, we deleted SCAB1401 using the λ RED-based PCR-targeting gene replacement technology (Gust *et al.*, 2003a). The genotype of the Δ SCAB1401 mutant strain was confirmed by Southern blot hybridization (data not shown). LC-MS

Table 2. Proposed functions of the proteins encoded by the *S. scabies* 87-22 pyochelin biosynthetic gene cluster

NRPS, Nonribosomal peptide synthetase.

<i>S. scabies</i> protein	Proposed function	Closest homologue		
		Name	Organism	Identity (%)
SCAB1371	AfsR-family regulator	AfsR	<i>S. coelicolor</i>	40
SCAB1381	Salicylate synthase	TrpE	<i>Saccharopolyspora erythraea</i>	51
SCAB1391	Unknown	Sav760	<i>S. avermitilis</i>	72
SCAB1401	TetR-family regulator	OrfH2	<i>S. griseoruber</i>	55
SCAB1411	Salicyl-AMP-ligase	DhbE	<i>Bacillus subtilis</i>	50
SCAB1421	Thioesterase	PchC	<i>P. fluorescens</i>	44
SCAB1431	ABC transporter permease/ATPase	Nfa28020	<i>Nocardia farcinica</i>	66
SCAB1441	ABC transporter permease/ATPase	Nfa28010	<i>N. farcinica</i>	65
SCAB1451	Major facilitator superfamily protein	Fraal6304	<i>Frankia alni</i>	46
SCAB1461	Thiazoline reductase	Strop2818	<i>Salinispora tropica</i>	49
SCAB1471	Pyochelin NRPS	PchF	<i>P. fluorescens</i>	45
SCAB1481	Pyochelin NRPS	Psto2602	<i>P. syringae</i>	40

Fig. 2. Model of pyochelin biosynthesis in *S. scabies* 87-22 based on biochemical analysis of orthologous proteins from *P. aeruginosa*. (a) The SCAB1411 A domain catalyses *trans* acylation of the N-terminal T domain of SCAB1481 with salicylate and the internal A domain of SCAB1481 catalyses *cis* acylation of the C-terminal T domain of SCAB1481 with L-Cys. Both reactions are ATP-dependent and involve adenylate intermediates. The Cy domain catalyses condensation of the L-Cys thioester with the salicyl thioester and subsequent cyclodehydration to form the thiazoline ring. The MT-like domain catalyses epimerization of the Cys-derived stereocenter during or after heterocycle formation. (b) The SCAB1471 A domain catalyses ATP-dependent acylation of the downstream T domain with L-Cys. The Cy domain of SCAB1471 catalyses condensation of the thioesters bound to SCAB1481 and SCAB1471 to generate a *bis*-thiazolyl thioester. SCAB1461 catalyses reduction of one of the thiazolines to a thiazolidine and the MT domain of SCAB1471 catalyses *N*-methylation, and thioesterase domain-mediated thioester hydrolysis yields pyochelin. The biosynthetic scheme was adapted from the paper by Kadi & Challis (2009).

analyses of culture supernatants from the Δ SCAB1401 mutant reproducibly detected a compound with a retention time of approximately 23.5 min that afforded an $m/z=325.0669$ ion, which exactly matches the m/z for the $[M+H]^+$ ion derived from pyochelin and corresponds closely to the $[M+H]^+$ ion (m/z 325.0676) derived from *ent*-pyochelin (Fig. 3). Comparison of the measured mass spectrum for the *S. scabies* metabolite with the simulated mass spectrum for a compound with the molecular formula of $C_{14}H_{17}N_2O_3S_2^+$ demonstrated that there is good agreement between the measured and predicted m/z values for each isotopomer as well as the predicted relative abundances (Supplementary Fig. S1, available with the online version of this paper). The recent report of the isolation of *ent*-pyochelin from *P. fluorescens* prompted us to analyse the stereochemistry of pyochelin produced by *S. scabies* (Youard *et al.*, 2007). Culture supernatants of the Δ SCAB1401 mutant were compared by LC-MS on a homochiral stationary phase with synthetic standards of pyochelin and *ent*-pyochelin (Youard *et al.*, 2007). These analyses showed conclusively that *S. scabies* produces pyochelin and not *ent*-pyochelin (Fig. 3).

In order to confirm that pyochelin was indeed the product of the PBGC and not the product of another biosynthetic gene cluster in *S. scabies*, we replaced the NRPS gene, SCAB1471, in the Δ SCAB1401 mutant strain with a gene conferring resistance to hygromycin B. The integrity of the resulting Δ SCAB1401– Δ SCAB1471 mutant strain was verified by Southern blot hybridization (data not shown). As predicted, pyochelin was reproducibly not detected in the supernatant of the Δ SCAB1401– Δ SCAB1471 mutant by LC-MS analysis (Fig. 3). Taken together, these data strongly suggest that pyochelin is the product of the *S. scabies* PBGC and further suggest that SCAB1401 is a transcriptional repressor that modulates transcription of the pyochelin biosynthetic genes.

***S. scabies* PBGC is transcribed as two mRNA transcripts**

The genetic organization of the *S. scabies* PBGC (Fig. 1) suggests that several genes in the PBGC are co-transcribed. In order to analyse the transcriptional organization of the PBGC, we isolated RNA from *S. scabies* 87-22 grown in iron-deficient liquid minimal medium and performed RT-PCR analysis using nine pairs of oligonucleotide primers (Supplementary Table S1). The oligonucleotide primers were designed to span the intergenic regions of the *S. scabies* PBGC and to capture the DNA sequence at least 500 bp upstream of the putative start codons, in order to account for independent promoters driving transcription from multiple sites within the PBGC. A total of eight products were obtained by RT-PCR analysis; no products were obtained when reverse transcriptase was omitted from the reaction (Fig. 4). These results indicate that the SCAB1481–SCAB1411 genes are co-transcribed as a large ~19 kb mRNA transcript and that the putative salicylate

synthase, SCAB1381, and the AfsR-family regulator, SCAB1371, are co-transcribed in a ~3 kb operon (Fig. 4). RT-PCR did not yield any PCR products for the large (~350 bp) SCAB1391–SCAB1401 intergenic region.

SCAB1401 represses transcription of the PBGC

The proximity of the gene, encoding a TetR-family protein SCAB1401, to the PBGC (Fig. 1) and analysis of pyochelin production in wild-type *S. scabies* and the Δ SCAB1401 mutant indicated that SCAB1401 is a transcriptional repressor of the *S. scabies* PBGC. Before we could test this hypothesis, we first needed to identify conditions under which transcription of the PBGC was repressed. Transcription of siderophore gene clusters in other taxa is typically repressed by high levels of iron (Miethke & Marahiel, 2007); therefore, we used RT-PCR to analyse transcription of the PBGC in wild-type *S. scabies* in the presence of 100 μ M FeCl₃. As expected, transcription of the PBGC was repressed in the presence of high levels of FeCl₃ (Supplementary Fig. S2, available with the online version of this paper). Interestingly, transcription of SCAB1371 and SCAB1391 was not repressed by 100 μ M FeCl₃ (Supplementary Fig. S2). In order to determine if SCAB1401 is a transcriptional repressor of the *S. scabies* PBGC, we analysed transcription in the Δ SCAB1401 mutant strain using RT-PCR. RNA was isolated from 3-day-old cultures of *S. scabies* wild-type and Δ SCAB1401 grown in liquid minimal medium supplemented with 100 μ M FeCl₃. RT-PCR was used to analyse the transcription of two genes, SCAB1411 and SCAB1451, which served as markers for the large mRNA transcript of the PBGC. Transcription of SCAB1411 and SCAB1451 was elevated in the Δ SCAB1401 mutant compared with the wild-type strain (Fig. 5). Deletion of SCAB1401 did not affect transcription of SCAB1371, SCAB1381 and SCAB1391 (data not shown). To ensure that the observed difference in transcription between the *S. scabies* wild-type and the Δ SCAB1401 mutant was due solely to the deletion of SCAB1401, we created pRFSRL34, which contained a copy of the SCAB1401 gene under the control of its native promoter. When pRFSRL34 was introduced into the Δ SCAB1401 mutant, transcription of SCAB1411 and SCAB1451 was indistinguishable from the wild-type strain (Fig. 5). These data demonstrate that SCAB1401 represses transcription of the 19 kb operon encoding part of the pyochelin biosynthetic machinery, but not the 3 kb operon encoding the AfsR-family regulatory gene and putative salicylate synthase.

SCAB1371 activates transcription of the PBGC

The SCAB1371 gene is predicted to encode an AfsR-family protein and, as it is co-transcribed with the predicted salicylate synthase gene, SCAB1381, we hypothesized that SCAB1371 is a positive transcriptional regulator of the PBGC. To test this hypothesis, we created a null mutant strain, Δ SCAB1371, using PCR targeting-based gene replacement technology (Gust *et al.*, 2003a) and confirmed

Fig. 3. LC-MS analyses of culture supernatants from the $\Delta SCAB1401$ and $\Delta SCAB1401$ - $\Delta SCAB1471$ mutant strains. (a) Extracted ion chromatograms (EICs) at $m/z=325$ from analyses of supernatants from the $\Delta SCAB1401$ mutant (top trace) and the

$\Delta SCAB1401$ - $\Delta SCAB1471$ mutant (bottom trace). (b) Measured mass spectrum of the compound with retention time of ~23.5 min in the analysis of the $\Delta SCAB1401$ mutant (top panel), pyochelin (middle panel) and *ent*-pyochelin (bottom panel) standards. (c) EICs at $m/z=325$ from analyses on a homochiral stationary phase of authentic *ent*-pyochelin (top trace), authentic pyochelin (middle trace) and pyochelin produced by *S. scabies* (bottom trace). I, *ent*-pyochelin I; II, *ent*-pyochelin II; III, pyochelin I; IV, pyochelin II. (d) Stereochemical configurations of pyochelin and *ent*-pyochelin adapted from Youard *et al.* (2007). The occurrence of two isomers for both pyochelin and *ent*-pyochelin is a result of a metal-induced epimerisation at C-2'' (Ino & Murabayashi, 2001; Schlegel *et al.*, 2004).

the deletion by Southern blot hybridization (data not shown). We isolated RNA from 3-day-old cultures of *S. scabies* wild-type and the $\Delta SCAB1371$ strain grown in liquid minimal medium. RT-PCR analysis demonstrated that transcription of *SCAB1411* and *SCAB1451* was significantly reduced in the $\Delta SCAB1371$ mutant compared with in the *S. scabies* wild-type (Fig. 6). However, deletion of *SCAB1371* had no effect on the transcription of *SCAB1381*, *SCAB1391* and *SCAB1401* (data not shown). To further characterize *SCAB1371* regulation of the PBGC, we created a *SCAB1371* overexpressing strain. The plasmid pRFSRL38 was engineered to contain the *SCAB1371* gene under the control of the strong constitutive streptomycete promoter *ermEp** (Bibb *et al.*, 1994). If *SCAB1371* is an activator of PBGC transcription, then introduction of pRFSRL38 into *S. scabies* wild-type should increase transcription of *SCAB1411* and *SCAB1451*. In order to test this hypothesis, we isolated RNA from 3-day-old cultures of *S. scabies* wild-type harbouring pRFSRL38 grown in iron-deficient minimal medium. *SCAB1411* and *SCAB1451* mRNA was not elevated in *S. scabies* harbouring pRFSRL38 compared with the vector control strain (data not shown). Interestingly, when *S. scabies* harbouring pRFSRL38 was grown in the presence of 100 μM FeCl₃, transcription of *SCAB1411* and *SCAB1451* was elevated compared with the vector control strain (Fig. 6), suggesting that the DNA sequence to which *SCAB1371* binds is saturated with *SCAB1371* during growth in iron-deficient liquid minimal medium. Taken together, these data suggest that *SCAB1371* activates transcription of the large operon in the *S. scabies* PBGC.

Pyochelin is not required for pathogenicity on excised potato tissue or radish seedlings

To analyse the importance of pyochelin in plant pathogenicity we generated a *SCAB1471* null mutant ($\Delta SCAB1471$) using PCR targeting-based gene replacement technology (Gust *et al.*, 2003a). The integrity of the $\Delta SCAB1471$ strain was confirmed by Southern blot hybridization (data not shown). We first inoculated excised potato tuber tissue with plugs of agar containing mycelia of *S. scabies* wild-type or $\Delta SCAB1471$. Potato tuber tissue was heavily necrotized by both wild-type and $\Delta SCAB1471$ strains 7 days post-inoculation, suggesting that pyochelin

is not required for pathogenicity of excised potato tissue (data not shown). Next, we performed a radish (cv. Burpee White) seedling assay, in which seedlings were grown in Petri dishes containing water agar and inoculated with *S. scabies* wild-type or Δ SCAB1471 mutant spores. After ~4 days of incubation, both wild-type- and Δ SCAB1471-inoculated plants were heavily necrotized and roots were stunted compared with the mock-inoculated control (data not shown), suggesting that pyochelin is not an important siderophore for pathogenesis of radish.

DISCUSSION

This is the first report, to our knowledge, of pyochelin production by a Gram-positive bacterium and a plant pathogen. Mining of the recently completed *S. scabies* 87-22 genome sequence allowed us to identify a putative PBGC. SCAB1411, SCAB1421, SCAB1461, SCAB1471 and SCAB1481 appear to be orthologues of the characterized *P. aeruginosa* pyochelin biosynthetic proteins PchCDEFG. The only significant difference in the biosynthetic proteins is that in *P. aeruginosa*, PchA and PchB catalyse the formation of salicylate from chorismate (Serino *et al.*, 1995), while in *S. scabies* 87-22, production of salicylate from chorismate is predicted to be catalysed by a single protein, SCAB1381. Interestingly, SCAB1381 is 40 and 41 % identical to Irp9 from *Y. enterocolitica* and MbtI from *Mycobacterium tuberculosis*, respectively, both of which catalyse the conversion of chorismate to salicylate, an intermediate in the biosynthesis of the siderophores yersiniabactin and mycobactin (Harrison *et al.*, 2006; Kerbarh *et al.*, 2006). The *S. scabies* and *P. aeruginosa* PBGCs differ from one another in three ways: gene organization, transcriptional regulation and the predicted

biosynthetic mechanism for salicylate. These differences suggest that *S. scabies* may have independently evolved the ability to produce pyochelin and that the PBGC was neither acquired from a common ancestor of *S. scabies* and *P. aeruginosa* nor horizontally transferred from a *Pseudomonas* species to *S. scabies*. It is also interesting to note that the *S. scabies* PBGC does not contain an orthologue of the inner membrane pyochelin permease FptX and there is not an FptX orthologue located elsewhere in the genome, suggesting that import of ferric-pyochelin in *S. scabies* occurs via one of the putative transport proteins (SCAB1431, SCAB1441 or SCAB1451) encoded within the cluster.

LC-MS analysis of culture supernatants from the Δ SCAB1401 mutant grown in iron-deficient liquid medium identified a metabolite with the same molecular mass as pyochelin. Curiously, we were unable to consistently detect production of this metabolite in supernatants of *S. scabies* wild-type grown in iron-deficient liquid medium. Inconsistent detection of this metabolite by the wild-type strain was unexpected given that we detected transcripts for pyochelin biosynthetic genes under these growth conditions. Deletion of the SCAB1471 gene in the Δ SCAB1401 mutant abrogated production of the metabolite, confirming that it is a product of the PBGC. LC-MS analyses on a homochiral stationary phase confirmed that *S. scabies* produces pyochelin and not *ent*-pyochelin, consistent with the presence of a *pchG* orthologue (SCAB1461) and the absence of a *pchK* orthologue in the PBGC, as well as the presence of a putative methyl transferase-like epimerization domain in the PchE orthologue SCAB1481 (Youard *et al.*, 2007).

The *S. scabies* PBGC is transcribed as two operons, one large ~19 kb operon consisting of the pyochelin biosynthetic genes, and a smaller operon (~3 kb) composed of a

Fig. 4. Demonstration of co-transcription of the *S. scabies* 87-22 PBGC by RT-PCR. (a) Schematic representation of the *S. scabies* PBGC and the locations of the eight RT-PCR products (1–8) obtained. Black arrows represent the large (~19 kb) and small (~3 kb) operons identified. Genes immediately adjacent to the PBGC, SCAB1351, SCAB1361, SCAB1491 and SCAB1501, are predicted to encode a transposase and three hypothetical proteins, respectively, and are presumed not to play a significant role in the biosynthesis of pyochelin. There is a predicted stem-loop 20 nt downstream of the stop codon for SCAB1491. (b) Agarose gel electrophoresis of RT-PCR products. Lanes: i, cDNA from *S. scabies* wild-type grown in iron-deficient liquid minimal medium; ii, reverse transcription reaction mix with enzyme omitted. PCR products were sequenced to verify their identity.

Fig. 5. RT-PCR analysis of Δ SCAB1401 mutant strain demonstrating that SCAB1401 is a repressor of *S. scabies* 87-22 pyochelin biosynthetic genes. cDNA from *S. scabies* wild-type (lane 1), Δ SCAB1401 (2), Δ SCAB1401 harbouring pAU3-45 (3) or pRFSRL34 (4) grown in liquid minimal medium + 100 μ M FeCl₃ was used as template for PCRs. The *murX* gene served as a loading control.

salicylate synthase gene and a positive transcriptional regulator, while two genes (*SCAB1391* and *SCAB1401*) are transcriptionally isolated within the cluster. Mutational studies indicate that two regulatory proteins control transcription of the large biosynthetic operon and do not act on either the small operon or the two isolated genes in the cluster. Transcription of the large biosynthetic operon is repressed during growth in high iron conditions by SCAB1401, a TetR-family repressor protein, while transcription is activated by an AfsR-family regulator, SCAB1371, during times of low iron availability. Pyochelin production also appears to be regulated at the post-transcriptional level, because the salicylate synthase gene, *SCAB1381*, possesses a UUA Leu codon that is only recognized by the *blaD*-encoded tRNA (Leskiw *et al.*, 1991). Regulation of the *S. scabies* pyochelin biosynthetic

Fig. 6. RT-PCR analysis of the Δ SCAB1371 mutant and SCAB1371 overexpression strain demonstrating that SCAB1371 is an activator of *S. scabies* 87-22 pyochelin biosynthetic genes. cDNA from *S. scabies* wild-type (lane 1) and Δ SCAB1371 (2) grown in iron-deficient liquid minimal medium or *S. scabies* wild-type harbouring pIJ10257 (3) or pRFSRL38 (4) grown in liquid minimal medium + 100 μ M FeCl₃ was used as template for PCRs. The *murX* gene served as a loading control.

genes contrasts with regulation of the pyochelin cluster in *P. aeruginosa*, which is controlled by the AraC-family regulator PchR and the ferric uptake regulator, Fur (Heinrichs & Poole, 1993, 1996; Michel *et al.*, 2005).

Transcription of the large operon in the *S. scabies* PBGC and *SCAB1381* is repressed by 100 μ M FeCl₃; however, the regulatory protein(s) that mediates iron repression is unknown. Iron metabolism in Gram-negative organisms and firmicutes is controlled by Fur (Hantke, 2001). Streptomyces possess four Fur-family proteins, all of which have been characterized in the model streptomyces *S. coelicolor*, including CatR (SCO5206), Nur (SCO4180), Zur (SCO2508) and FurA (SCO0561); FurA has also been characterized in *Streptomyces reticuli* and is called FurS (Ahn *et al.*, 2006; Hahn *et al.*, 2000a, b; Ortiz de Oru Lucana & Schrempf, 2000; Shin *et al.*, 2007; Zou *et al.*, 1999). *S. scabies* contains four genes (*SCAB30481*, *SCAB49681*, *SCAB61951* and *SCAB9521*) encoding Fur proteins orthologous to those of *S. coelicolor* and *S. reticuli*. Of these Fur-family proteins, only FurA/FurS are involved in iron metabolism. In the presence of iron, FurA/FurS repress a two-gene operon consisting of itself and a catalase-peroxidase gene, *SCO0560* or *cpeB* (orthologous to *SCAB9531*). The *S. scabies* FurA orthologue (*SCAB9521*) is most closely related to FurS from *S. reticuli*; analysis of the genome sequence revealed a 26mer (5'-AACTTGGGTGGTTCGTTTTCTTGAAC-3') upstream of *SCAB9521* that is 80% identical to the binding site (5'-AAATTGCACCGTTCGTTTTCTTGAAC-3') bound by FurS (Zou *et al.*, 1999). Global iron metabolism in actinobacteria is regulated by DxtR-family proteins (e.g. DmdRs in *Streptomyces* species) (Hantke, 2001). DmdR was initially identified as a repressor of the *des* cluster in *S. pilosus*, which directs production of desferroxamine siderophores. DmdR1 and DmdR2 were later characterized in *S. coelicolor* as repressors of not only the *des* cluster but also multiple loci (Flores *et al.*, 2003, 2005; Flores & Martn, 2004). *S. pilosus* and *S. coelicolor* DmdR proteins bind a 19mer (5'-TTAGGTTAGGCTCACCTAA-3') located upstream of the desferroxamine biosynthetic gene *desA* (Flores *et al.*, 2003; Flores & Martn, 2004). *S. scabies* contains a single DmdR homologue encoded by *SCAB51401* as well as a putative DmdR-binding sequence (5'-TTAGGTTAGCCTAACC-TAA-3') upstream of a *desA* orthologue (*SCAB57951*) that is nearly identical to that of the DmdR-binding consensus (5'-ttaggttAGGCTcaCCTaa-3') for streptomyces (Flores & Martn, 2004), suggesting that SCAB51401 may function as a global regulator of iron homeostasis in *S. scabies* as it does in other streptomyces. However, BLASTN analysis of the *S. scabies* genome did not reveal the presence of putative FurA or DmdR binding sequences within the PBGC, suggesting that transcription of the PBGC is not directly controlled by *S. scabies* FurA or DmdR orthologues, but this hypothesis remains to be tested. It seems unlikely that the TetR-family regulator, SCAB1401 would directly sense and respond to iron, as TetR-family repressor proteins typically release DNA upon binding an organic

ligand. *In silico* promoter prediction (http://bioinformatics.biol.rug.nl/websoftware/ppp/ppp_start.php) was unable to predict suitable promoters for *SCAB1401* and the ~19 kb and ~3 kb operons of the PBGC, and a comparison of the DNA sequence upstream of *SCAB1401* and the ~19 kb operon (the genes of which are repressed by 100 μ M FeCl₃) did not reveal significant homology between the two promoter regions. Future studies will employ electrophoretic mobility shift assays with purified SCAB1401 and SCAB1371 proteins to characterize their DNA- and ligand-binding activities and address whether these regulator proteins directly or indirectly control transcription of the pyochelin biosynthetic genes.

When interpreting the lack of an *in planta* disease phenotype for the Δ SCAB1471 pyochelin non-producing mutant it is worth noting that we cannot eliminate the possibility that iron may not have been a limiting growth factor in our assays. However, on the other hand, *S. scabies* possesses the potential to produce other siderophores in addition to pyochelin. Putative genes for the biosynthesis of desferrioxamines (SCAB57921–SCAB57951), which have been implicated in virulence of *Erwinia amylovora* on apple flowers (Dellagi *et al.*, 1998), and other structurally uncharacterized siderophores have been identified in the *S. scabies* 87-22 genome (<http://strepdb.streptomyces.org.uk/>). Thus, elucidating the iron-acquisition systems required for pathogenesis is a difficult task. *In planta* transcriptional analysis of the pyochelin, desferrioxamine and other putative siderophore biosynthetic gene clusters will provide clues to the importance of iron acquisition in *S. scabies* pathogenicity, and mutational analysis of the putative desferrioxamine and other siderophore biosynthetic gene clusters will allow us to determine which siderophores are critical for pathogenicity.

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