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.

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 Fe$^{3+}$ 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...
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 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).

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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) multienzyme. 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-thiazolinyl 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*-thiazolinyl 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 thiazoldine, 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 ferricrocin 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 agar (ISP4) medium or liquid minimal medium (MM) containing 2 g NH₄SO₄ l⁻¹, 0.6 g MgSO₄ 1⁻¹, 2.6 g KH₂PO₄ 1⁻¹, 0.1 g CaCl₂ 1⁻¹, 2.5 % (v/v) glycerol, 2.5 g glucose 1⁻¹, 0.05 g yeast extract 1⁻¹ 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 thiodestromin ml⁻¹. All *Streptomyces* strains generated in this study were created by cross-genera conjugation from the non-methylating *E. coli* strain ET15267/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 pJJ773 (Gust et al., 2003a), or the hygromycin B resistance gene from pJJ10700 (Gust et al.,
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristic</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>S. scabies</strong></td>
<td></td>
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<tr>
<td>87-22</td>
<td>Wild-type</td>
<td>This study</td>
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<tr>
<td>ΔSCAB1371</td>
<td>87-22 SCAB1371 null mutant</td>
<td>This study</td>
</tr>
<tr>
<td>ΔSCAB1401</td>
<td>87-22 SCAB1401 null mutant</td>
<td>This study</td>
</tr>
<tr>
<td>87-22 attB φBT1::pRFSRL38</td>
<td>87-22 bearing the empty overexpression vector</td>
<td>This study</td>
</tr>
<tr>
<td>87-22 attB φBT1::pRFSRL38</td>
<td>87-22 bearing the SCAB1371 overexpression plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>ΔSCAB1401-ΔSCAB1471</td>
<td>87-22 SCAB1401–SCAB1471 double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>ΔSCAB1401 attB ΔC31::pAU3-45</td>
<td>SCAB1401 null mutant bearing empty complementation vector</td>
<td>This study</td>
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<tr>
<td>ΔSCAB1401 attB ΔC31::pRFSRL34</td>
<td>Complemented SCAB1401 null mutant</td>
<td>This study</td>
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<td><strong>E. coli</strong></td>
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<tr>
<td>BW25113</td>
<td>Host for REDIRECT PCR targeting system</td>
<td>Gust et al. (2003a)</td>
</tr>
<tr>
<td>ET12567</td>
<td>Non-methylating host for transfer of DNA into Streptomyces strains; dam dcm hsdM</td>
<td>MacNeil et al. (1992)</td>
</tr>
<tr>
<td>DH5α</td>
<td>F’ general cloning host</td>
<td>GibroBRL</td>
</tr>
<tr>
<td>TOP10</td>
<td>Host for pCR2.1TOPO cloning system</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>Superco1</td>
<td>Cosmid backbone for <em>S. scabies</em> cosmids; AmpR KanR</td>
<td>Stratagene</td>
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<tr>
<td>Cosmid 632</td>
<td>Superco1 derivative containing the <em>S. scabies</em> 87-22 PBGC; KanR AmpR</td>
<td>This study</td>
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<td>pCR2.1TOPO</td>
<td>Cloning vector for PCR products; KanR AmpR</td>
<td>Invitrogen</td>
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<td>pJ1773</td>
<td>PCR template for acl(3)IV+oriT cassette used in REDIRECT PCR targeting system</td>
<td>Gust et al. (2003a/b)</td>
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<td>pJ10700</td>
<td>PCR template for hygR cassette used in REDIRECT PCR targeting system</td>
<td>Gust et al. (2003b)</td>
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<td>pUZ8002</td>
<td>Encodes conjugation machinery for mobilization of plasmids from <em>E. coli</em> to <em>Streptomyces</em>; KanR</td>
<td>MacNeil et al. (1992)</td>
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<tr>
<td>pKD46</td>
<td>Encodes lambda RED recombination machinery induced by arabinose; AmpR</td>
<td>Datsenko &amp; Wanner (2000)</td>
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<td>pAU3-45</td>
<td>pSET152 derivative, integrates into the ΔC31 attB site in <em>Streptomyces</em>; AprR TspR</td>
<td>Bignell et al. (2005)</td>
</tr>
<tr>
<td>pJJ10257</td>
<td>pMS81 derivative containing ermEp*, integrates into the ΔBT1 attB site in <em>Streptomyces</em>; HygR</td>
<td>Hong et al. (2005)</td>
</tr>
<tr>
<td>pRFSRL34</td>
<td>pAU3-45 derivative containing the SCAB1401 locus cloned into the EcoRI site</td>
<td>This study</td>
</tr>
<tr>
<td>pRFSRL38</td>
<td>pJJ10257 derivative containing the SCAB1371 coding sequence cloned into the Ndel–HindIII sites</td>
<td>This study</td>
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</table>

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 18944 bp of sequence downstream of *SCAB1371*. Transformants were screened for the presence of the mutated 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 (*ΔSCAB1401*), resistance to hygromycin B and kanamycin sensitivity (*ΔSCAB1471* and *ΔSCAB1371*), or resistance to apramycin and hygromycin B, and sensitivity to kanamycin (*ΔSCAB1401-ΔSCAB1471*). 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 Ndel and HindIII restriction sites of pJJ10257 (Hong et al., 2005), which integrates into the ΔBT1 phage site present in streptomyces 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 promotor, and the second copy under the control of ermEp* provided by pJJ10257 (Hong et al., 2005).

**Complementation of the ΔSCAB1401 mutant strain.** To complement the ΔSCAB1401 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 ΔC31 integrative plasmid, pAU3-45 (Bignell et al., 2005). The resulting

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

Southern blot hybridization. Southern analysis was performed as described previously (Seipke & Loria, 2008). To confirm proper construction of the SCAB1401 strain, KpnI-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 SCAB1401–SCAB1471 and SCAB1471 mutant strains was confirmed by probing PstI/KpnI-digested DNA with a DIG-labelled fragment internal to SCAB1451. Correct construction of the SCAB1371 mutant strain was confirmed by probing NotI/ EcoRI-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 SCAB1401 and SCAB1401–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 reconstituted 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), 81 m/min; 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. 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 psi; Dry gas (N₂), 10 l/min; Dry Temperature, 300 °C] was used for analysis of pyochelin samples on a homochiral stationary phase. Pyochelin and enantiopyochelin (est-pyochelin) standard solutions (both 10 μl, 10 μg ml⁻¹ in methanol) and concentrated culture supernatants from the 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 mycobactin 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. FN54889) 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 PchABCDEFG, 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 ALPSQGVNLK, which are 80 % identical to the active site residues (PLPAQGVLN) 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 DLWNLSLWIK and DLYNLSLWIK, 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-thiazolinyl 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-thiazolinyl thioester tethered to the T domain of...
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 (Reimmann 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 PchABCDEFG from *P. aeruginosa* and suggest that *S. scabies* produces pyochelin. A proposed pathway for pyochelin biosynthesis in *S. scabies* is shown in Fig. 2.

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

<table>
<thead>
<tr>
<th><em>S. scabies</em> protein</th>
<th>Proposed function</th>
<th>Closest homologue</th>
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<tbody>
<tr>
<td>SCAB1371</td>
<td>AfsR-family regulator</td>
<td>AfsR, <em>S. coelicolor</em></td>
</tr>
<tr>
<td>SCAB1381</td>
<td>Salicylate synthase</td>
<td>TrpE, <em>Saccharopolyspora erythraea</em></td>
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<td>SCAB1391</td>
<td>Unknown</td>
<td>Sav760, <em>S. avermitilis</em></td>
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<td>SCAB1401</td>
<td>TetR-family regulator</td>
<td>OrfH2, <em>S. griseoruber</em></td>
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<td>SCAB1411</td>
<td>Salicyl-AMP-ligase</td>
<td>DhbE, <em>Bacillus subtilis</em></td>
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<td>SCAB1421</td>
<td>Thioesterase</td>
<td>PchC, <em>P. fluorescens</em></td>
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<td>SCAB1431</td>
<td>ABC transporter permease/ATPase</td>
<td>Nfa28020, <em>Nocardia farcinica</em></td>
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<td>SCAB1441</td>
<td>ABC transporter permease/ATPase</td>
<td>Nfa28010, <em>N. farcinica</em></td>
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<td>SCAB1451</td>
<td>Major facilitator superfamily protein</td>
<td>Fral6304, <em>Frankia alni</em></td>
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<td>SCAB1461</td>
<td>Thiazoline reductase</td>
<td>Strop2818, <em>Salinispora tropica</em></td>
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<td>SCAB1471</td>
<td>Pyochelin NRPS</td>
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<td>SCAB1481</td>
<td>Pyochelin NRPS</td>
<td>Psto2602, <em>P. syringae</em></td>
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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
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-thiazolinyl thioester. SCAB1461 catalyses reduction of one of the thiazolines to a thiazoline 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 ΔSCAB1401 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-pyrochelin (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 C₁₄H₁₃NO₃S₅+ 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-pyrochelin from P. fluorescens prompted us to analyse the stereochemistry of pyochelin produced by S. scabies (Youard et al., 2007). Culture supernatants of the ΔSCAB1401 mutant were compared by LC-MS on a homochiral stationary phase with synthetic standards of pyochelin and ent-pyrochelin (Youard et al., 2007). These analyses showed conclusively that S. scabies produces pyochelin and not ent-pyrochelin (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, SCABI471, in the ΔSCAB1401 mutant strain with a gene conferring resistance to hygromycin B. The integrity of the resulting ΔSCAB1401–ΔSCAB1471 mutant strain was verified by Southern blot hybridization (data not shown). As predicted, pyochelin was reproducibly not detected in the supernatant of the ΔSCAB1401–ΔSCAB1471 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 SCABI481–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 SCABI401, to the PBGC (Fig. 1) and analysis of pyochelin production in wild-type S. scabies and the ΔSCAB1401 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 μM 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 μM FeCl₃ (Supplementary Fig. S2). In order to determine if SCAB1401 is a transcriptional repressor of the S. scabies PBGC, we analysed transcription in the ΔSCAB1401 mutant strain using RT-PCR. RNA was isolated from 3-day-old cultures of S. scabies wild-type and ΔSCAB1401 grown in liquid minimal medium supplemented with 100 μM FeCl₃. RT-PCR was used to analyse the transcription of two genes, SCABI411 and SCAB1451, which served as markers for the large mRNA transcript of the PBGC. Transcription of SCABI411 and SCAB1451 was elevated in the ΔSCAB1401 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 ΔSCAB1401 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 ΔSCAB1401 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, ΔSCAB1371, using PCR targeting-based gene replacement technology (Gust et al., 2003a) and confirmed
the deletion by Southern blot hybridization (data not shown). We isolated RNA from 3-day-old cultures of S. scabies wild-type and the ΔSCAB1371 strain grown in liquid minimal medium. RT-PCR analysis demonstrated that transcription of SCAB1411 and SCAB1451 was significantly reduced in the ΔSCAB1371 mutant compared with 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 (ΔSCAB1471) using PCR targeting-based gene replacement technology (Gust et al., 2003a). The integrity of the ΔSCAB1471 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 ΔSCAB1471. Potato tuber tissue was heavily necrotized by both wild-type and ΔSCAB1471 strains 7 days post-inoculation, suggesting that pyochelin

ΔSCAB1401–ΔSCAB1471 mutant (bottom trace). (b) Measured mass spectrum of the compound with retention time of ~23.5 min in the analysis of the ΔSCAB1401 mutant (top trace), 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).

Fig. 3. LC-MS analyses of culture supernatants from the ΔSCAB1401 and ΔSCAB1401–ΔSCAB1471 mutant strains. (a) Extracted ion chromatograms (EICs) at m/z=325 from analyses of supernatants from the ΔSCAB1401 mutant (top trace) and the

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Pyochelin production and regulation in S. scabies

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 Mbt1 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 ΔSCAB1401 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 ΔSCAB1401 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 that 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

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**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.
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 AsfR-family regulator, SCAB1361, 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 bldA-encoded tRNA (Leskiw et al., 1991). Regulation of the S. scabies pyochelin biosynthetic 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 μM FeCl₃, however, the regulatory protein(s) that mediates iron repression is unknown. Iron metabolism in Gram-negative organisms and firmicutes is controlled by Fur (Hanke, 2001). Streptomyces possess four Fur-family proteins, all of which have been characterized in the model streptomycete S. coelicolor, including CatR (SCO5206), Nur (SCO4180), Zur (SCO2508) and FurA (SCO0561); FurA has also been characterized in Streptomyces reticuli and is called FurR (Ahn et al., 2006; Hahn et al., 2000a, b; Ortiz de Orué Lucana & Schremppf, 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/FurR are involved in iron metabolism. In the presence of iron, FurA/FurR 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 FurR from S. reticuli; analysis of the genome sequence revealed a 26mer (5′-AACATTGGGTGG- TTCGTTTTCTTGAAC-3′) upstream of SCAB9521 that is 80% identical to the binding site (5′-AAATTGACCCG- TTCGTTTTCTTGAAC-3′) bound by FurR (Zou et al., 1999). Global iron metabolism in actinobacteria is regulated by Dxr-family proteins (e.g. DmdRs in Streptomyces species) (Hanke, 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 & Martin, 2004). S. pilosus and S. coelicolor DmdR proteins bind a 19mer (5′-TTAGTTAGGCTACCTA-3′) located upstream of the desferroxamine biosynthetic gene desA (Flores et al., 2003; Flores & Martin, 2004). S. scabies contains a single DmdR homologue encoded by SCAB51401 as well as a putative DmdR-binding sequence (5′-TTAGTTAGGCTACCTA-3′) upstream of a desA orthologue (SCAB57951) that is nearly identical to that of the DmdR-binding consensus (5′-ttagttAGGCTcaCTTA-3′) for streptomycetes (Flores & Martin, 2004), suggesting that SCAB51401 may function as a global regulator of iron homeostasis in S. scabies as it does in other streptomycetes. 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 μM 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 ASCAB1471 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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