Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in Streptomyces coelicolor A3(2) and Streptomyces ambofaciens ATCC 23877

Francisco Barona-Gómez,† Sylvie Lautru,† François-Xavier Francou, Pierre Leblond, Jean-Luc Pernodet and Gregory L. Challis

Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK
CNRS, Institut de Génétique et Microbiologie, UMR 8621, Université Paris-Sud 11, 91405 Orsay Cedex, France
Laboratoire de Génétique et Microbiologie, UMR INRA 1128 IFR 110, Faculté des Sciences et Techniques, Université Henri Poincaré Nancy 1, Boulevard des Aiguillettes, BP239, 54506 Vandoeuvre-les-Nancy Cedex, France

Correspondence
Gregory L. Challis
G.L.Challis@warwick.ac.uk

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INTRODUCTION

Iron is an essential nutrient for virtually all micro-organisms because it is required for vital life processes such as aerobic and anaerobic adenosine triphosphate (ATP) biosynthesis. However, the bioavailability of iron, which exists predominantly in its ferric form in aerobic environments such as soil, is very low, despite the fact that it is the fourth most abundant element in the Earth’s crust. This is because, at neutral and alkaline pH, ferric iron forms insoluble, polymeric oxyhydroxide complexes, which cannot be assimilated by micro-organisms (Chipperfield & Ratledge, 2000). Consequently, iron acquisition in aerobic environments poses a significant challenge to saprophytic micro-organisms. Similar problems are encountered by pathogens of eukaryotes, where ferric iron is tightly bound to...
solubilizing transport and storage glycoproteins. Thus, iron assimilation by invading pathogens, which is often important for establishing infection (reviewed by Wandersman & Delepelaire, 2004; Crosa et al., 2004), also poses a significant challenge.

A common strategy used by many pathogenic and saprophytic micro-organisms to tackle the problem of low iron bioavailability is the biosynthesis and excretion of high affinity iron chelators known as siderophores (Wandersman & Delepelaire, 2004). The structural diversity of these metabolites is remarkable (Winkelmann & Drechsel, 1997), given that they all perform the same function – iron chelation. Many siderophores are polypeptides that are biosynthesized by members of the non-ribosomal peptide synthetase (NRPS) multienzyme family (Crosa & Walsh, 2002), which is also responsible for the biosynthesis of the majority of microbial peptide antibiotics. The enzymology of NRPS-catalysed peptide biosynthesis has been intensively studied over the last decade and the biosynthetic mechanisms for several types of structurally diverse peptides are now well understood (Challis & Naismith, 2004). On the other hand, many hydroxamate and \( \alpha \)-hydroxyacid-containing siderophores are not polypeptides, but are assembled instead from alternating dicarboxylic acid and diamine or amino alcohol building blocks (which are nevertheless derived from amino acids) linked by amide or ester bonds. Such siderophores are assembled by the much less well studied NRPS-independent siderophore (NIS) pathway (Challis, 2005), which is widely utilized in bacteria. Once an excreted siderophore has scavenged ferric iron from the environment, the resulting iron–siderophore complex is taken up by bacterial cells via membrane-associated transport systems containing an ATP-binding cassette (ABC) importer and a receptor protein. In Gram-negative bacteria several such transport systems have been extensively characterized at the genetic, biochemical and structural levels (Wandersman & Delepelaire, 2004; Crosa et al., 2004). In contrast, only the ABC importer utilizing the cell surface-associated ferric hydroxamate uptake receptor lipoprotein FhuD has been studied in detail in Gram-positive bacteria, in particular in the low-G+C content organisms Bacillus subtilis (Schneider & Hantke, 1993) and Staphylococcus aureus (Sebulskey & Heinrichs, 2001).

Actinomycetes belonging to the high-G+C content Gram-positive Streptomyces genus are well known as important producers of antibiotics and for their complex life cycle (Kieser et al., 2000). Streptomyces are ubiquitous in soil and also colonize the rhizosphere and marine habitats. Little is known about siderophore-mediated iron acquisition in streptomycetes. Desferroxamines are \( \alpha \)-hydroxamate ferric-iron-chelating metabolites produced by many Streptomyces species (Bickel et al., 1960). Streptomyces pilosus can take up ferrioxamines B, D₁, D₂ and E (Muller & Raymond, 1984), while Streptomyces viridosporis has been shown to take up ferrioxamines B, E and G₁, and Streptomyces lividans has been shown to take up ferrioxamines B and G₁ (Imbert et al., 1995). The uptake of different ferrioxamines in S. pilosus is believed to be mediated by the same importer system (Muller & Raymond, 1984). Desferroxamines have also been reported to cause interspecies stimulation of Streptomyces growth and development (Yamanaka et al., 2005). Recently, four putative iron–siderophore-binding lipoprotein receptors and four putative ATPase components of predicted ABC siderophore importer systems have been identified in the membrane-associated proteome of Streptomyces coelicolor (Kim et al., 2005). Desferroxamine B biosynthesis in S. pilosus is regulated by a DtxR-like ferric-iron-dependent repressor (Günter et al., 1993; Günter-Seethoth & Schupp, 1995). Similar repressor proteins (DmdR1 and DmdR2) have been identified in S. coelicolor (Flores & Martin, 2004).

Early steps of desferroxamine B biosynthesis in S. pilosus involve decarboxylation of L-lysine and hydroxylation of the resulting cadaverine to give \( N \)-hydroxycadaverine (Schupp et al., 1987, 1988). Very recently, two gene clusters that direct the biosynthesis of the \( \alpha \)-hydroxamate iron chelators desferroxamine E and coelichelin (Fig. 1), which could potentially function as siderophores, have been discovered in S. coelicolor A3(2) by genome mining (Barona-Gómez et al., 2004; Lautru et al., 2005). The des cluster encodes a NIS-like pathway proposed to use four enzymes, DesA, DesB, DesC and DesD, in the assembly of desferroxamine E, and a previously unidentified \( \alpha \)-hydroxamate from lysine, succinyl CoA and molecular oxygen (Fig. 2; Barona-Gómez et al., 2004). The cch cluster encodes an unusual NRPS-dependent pathway, which utilizes a trimodular NRPS and a separately encoded thioesterase to assemble the novel tetrapeptide coelichelin from L-Thr and the non-proteinogenic amino acids L-Nupyter, L-N5-formyl-N5-hydroxyornithine and L-N5-hydroxyornithine (Fig. 2; Lautru et al., 2005).

Here we report further investigation of the \( \alpha \)-hydroxamate metabolites produced by S. coelicolor and the first investigation of the production of such metabolites by Streptomyces ambifaciens. The biological function of these metabolites as siderophores is examined and the selectivity of two putative siderophore uptake systems towards cognate and non-cognate siderophores is investigated.

**METHODS**

**Growth conditions and growth promotion assays.** Standard Streptomyces growth conditions, including media and antibiotic concentrations were as described by Kieser et al. (2000). An iron-deficient liquid medium was used for analysis of \( \alpha \)-hydroxamate production (Muller & Raymond, 1984).

Growth promotion assays with purified siderophores were carried out on a silica medium described by Hood et al. (1992). Colloidal silica or Ludox (Grace Davison) was prepared by dialysis using two changes of 1 litre 1 mM phosphate buffer (pH 6.8) after 24 h, and two changes of 1 litre distilled water after 4 h. For preparing one plate, 13 · 2 ml dialysed Ludox was mixed with 2 · 5 ml of a salts solution containing 28 · 7 mM \( K_2HPO_4 \) (5 g l\(^{-1}\)), 8 · 11 mM MgSO\(_4\) · 7H\(_2\)O (2 g l\(^{-1}\)) and 75 · 68 mM
(NH$_4$)$_2$SO$_4$ (10 g l$^{-1}$) in 5-5.5 ml distilled water, and autoclaved. This solution was supplemented, per plate, with 3-7.5 ml 100 mM phosphate buffer (pH 6-7) and 0-7 ml 50% glycerol, previously autoclaved. When iron was required, a 100 mM FeCl$_3$ sterile solution was supplemented, per plate, with 3 mol of each compound to be tested and the diameter of the growth halo around the disc was measured for at least 2 h in a laminar-flow hood. Plates were carefully inoculated with 3.5 ml medium per plate) and allowing the silica to settle without lids for at least 2 h. The plugs were placed on these plates and incubated for 48-60 h at 30°C and the halo of growth around the plug was recorded.

**Construction and complementation of S. coelicolor and S. ambofaciens mutants.** Details of the mutants constructed in this study are included in Table 3 and Figs 3 and 4. The S. coelicolor and S. ambofaciens mutants were constructed using the M145 (Bentley et al., 2002) and OSC2 strains (Raynal et al., 2006), respectively. The Escherichia coli DH5α was used for cloning experiments.

For the gene replacements in S. coelicolor, a PCR-based method, commercially registered as REDIRECT, was used (Gust et al., 2003). The protocol, plasmids and strains were provided by Plant Bioscience Ltd. The oligonucleotides used for the REDIRECT replacements are as follows: S. coelicolor sequence underlined, all 5'-3'; desE–desD (des cluster), CCGATGCTGATGACGCGCCGGGTGGCGCACCGGCCCGGGCGAGCTG; cchA–K (cch cluster), CCGGCCCGTCCGCGGGGCCGGTTGGTGTAGGCTGGAGCTG; cchH (cch cluster), CCGGCCCGTCCGCGGGGCCGGTTGGTGTAGGCTGGAGCTG; cchH (cch cluster), CCGGCCCGTCCGCGGGGCCGGTTGGTGTAGGCTGGAGCTG.

The purified siderophores were supplied by diffusion from filter paper discs impregnated with 1 μmol of each compound to be tested and the diameter of the growth halo around the disc was measured after 5 days' incubation at 30°C.

**Fig. 1.** Structures of tris-hydroxamate metabolites produced by S. coelicolor. 200 μM 2,2'-dipyridyl. The strains tested were streaked out onto plates containing 25 ml of these media. After 24 h incubation at 30°C, plugs (0.6 cm diam.) were obtained from confluent regions. At this point, plates containing the same medium were evenly spread with approximately 10$^8$ spores of S. coelicolor W13 suspended in 1 ml sterile water and allowed to dry. The plugs were placed on these plates and incubated for 48-60 h at 30°C and the halo of growth around the plug was recorded.

The plasmids used for gene inactivation in S. ambofaciens were derivatives of pBC SK+ (Stratagene) in which the chromosomally expressed resistance gene had been inactivated by insertion of a cassette containing the RK2 oriT and the ωaac or ωhyg interposons. The construct bearing the internal fragment of desC (pOSID2) conferred resistance to hygromycin only when the ωhyg cassette, whereas the plasmid containing the ωcchH fragment (pOSID4) contains the ωaac cassette, conferring resistance to apramycin (Blondelet-Rouault et al., 1997). Fragments internal to the coding sequences of these genes were cloned using primers 5'-TGACACACCCCGAAAGGCGCGGG-3' and 5'-GCCTCTCACTGCGTTGCCGAGAC-3' for desC.

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and 5'-CCCTCCACCGAACCTGGCCGCACGG-3' and 5'-GGGCGCAGGGGATGGTGTGATGACAGTCCCCCAT-3' for cchH. After RP4-based conjugation between S. ambofaciens and E. coli strain S17-1 (Simon et al., 1983) previously transformed with pOSID2 and pOSID4, S. ambofaciens transconjugants resistant to hygromycin and apramycin, respectively, were isolated. The double desC and cchH mutant OSID2/4 was obtained by mating the single mutants OSID2 and OSID4 and selecting for both antibiotic markers.

Siderophores and chemicals. Purified siderophores were obtained from EMC microcollections, other than coelichelin, which was purified as described previously (Lautru et al., 2005). Desferriere siderophores were added to the sterile filter paper discs (0.6 cm diam.) using the appropriate amounts of a 0.2 mM siderophore aqueous solution, except desferrioxamine E which was dissolved in 50% dimethyl sulfoxide. 2,2'-Dipyridyl and antibiotics were purchased from Sigma.

**LC-MS analysis of tris-hydroxamates in culture supernatants.**

Cultures of wild-type and mutants of S. coelicolor and S. ambofaciens were centrifuged and the supernatants concentrated using a rotary evaporator. Dry extracts were redissolved in the minimum amount of water and siderophores were converted to their ferric complexes by addition of FeCl₃. Prior to HPLC injection, the concentrated supernatants were filtered using a Vivaspin 0.5 ml concentrator (10,000 molecular mass cut-off). An Agilent 1100 HPLC instrument equipped with a binary pump and a diode array detector was used for HPLC analysis. Samples were analysed on a Supelco Discovery HSF5 column (150×4.6 mm, 5 μm i.d., column temp. 20 °C) and eluted with 10 mM ammonium carbonate, pH 7.0 (solvent A)/MeOH (solvent B) (10:90) at 1 ml min⁻¹ for 10 min, followed by a gradient to 100:0 A/B over 8 min, 10 min isocratic conditions at 100:0 A/B, a gradient to 10:90 A/B over 8 min and isocratic conditions at 10:90 A/B for 4 min. Ferric-tris-hydroxamate complexes were detected by monitoring A₄35. The identities of compounds with retention times of approximately 2.8, 16.6 and 36.1 min were confirmed as ferricoelichelin, ferrioxamine E and ferrioxamine B, respectively, by either LC-MS or direct injection MS analysis on the collected fractions. For LC-MS analysis, the HPLC outflow was connected via a splitter (10% flow to MS, 90% flow to waste) to a Bruker HCT+ mass spectrometer equipped with an electrospray...
RESULTS

Sequence analysis of the S. coelicolor des and cch clusters

Upstream of the desABCD putative operon, previously implicated in desferrioxamine E biosynthesis (Barona-Gómez et al., 2004), there are two genes in the same orientation, desE (Sco2780) and desF (Sco2781) (Fig. 3). DesE is similar to ferric-siderophore lipoprotein receptors and DesF is similar to ViuB, which is proposed to be a hydrolase involved in the release of iron from ferrivibriobactin (Table 1; Butterton & Calderwood, 1994). DesE contains the N-terminal sequence ALGLGAVLAAC which matches the Prosite prokaryotic membrane lipoprotein lipid attachment site and contains a cysteine residue which is proposed to be modified by lipidation. It has also been localized in the membrane-associated proteome of S. coelicolor (Kim et al., 2005). A putative DmdR1/DmdR2 binding site lies upstream of desE (Flores & Martín, 2004).

The gene upstream of desE is acdH, which has previously been shown to encode an acyl-CoA dehydrogenase required for leucine, isoleucine and valine catabolism in Streptomyces spp. (Zhang et al., 1999), and the hexA gene downstream of desD encodes a protein with 93% similarity to a β-N-acetylhexosaminidase of Streptomyces plicatus (Mark et al., 1998). No rational role for either of these proteins in desferrioxamine biosynthesis or excretion, or ferrioxamine uptake or utilization can be envisaged. Thus, the first and last genes of the des cluster are proposed to be desE and desD, respectively. This proposal is supported by the finding that both the organization and chromosomal location of the desEFABCD cluster are highly conserved in the genomes of S. ambofaciens (see below), Streptomyces avermitilis (Ikeda...
Table 1. Proposed functions of proteins encoded by the des cluster

<table>
<thead>
<tr>
<th>Protein</th>
<th>Homologue (percentage identity)</th>
<th>Proposed function</th>
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</thead>
<tbody>
<tr>
<td>DesA</td>
<td>Ddc Acinetobacter baumannii (37)</td>
<td>1-L-Lysine decarboxylase</td>
</tr>
<tr>
<td>DesB</td>
<td>AlcA Bordetella bronchiseptica (47)</td>
<td>1.5-Diaminopentane mono-oxygenase</td>
</tr>
<tr>
<td>DesC</td>
<td>AlcB Bordetella bronchiseptica (46)</td>
<td>Acyl CoA acyl transferase</td>
</tr>
<tr>
<td>DesD</td>
<td>AlcC Bordetella bronchiseptica (51)</td>
<td>Type C siderophore synthetase</td>
</tr>
<tr>
<td>DesE</td>
<td>FluD Bacillus subtilis (24)</td>
<td>Ferric-siderophore lipoprotein receptor</td>
</tr>
<tr>
<td>DesF</td>
<td>ViuB V. cholerae (30)</td>
<td>Ferric-siderophore hydrolase</td>
</tr>
</tbody>
</table>

We recently isolated coelichelin from S. coelicolor, elucidated its structure and identified a cluster of genes required for coelichelin biosynthesis (Lautru et al., 2005). The first and last genes of the cluster are defined as cchA (Sco0499) and cchK (Sco0489), respectively (Fig. 4). These assignments are consistent with the location of putative DmdR1 and DmdR2 iron-dependent repressor (IdeR) protein binding sites in the intergenic regions between cchA and the cchBCDEFGHI putative operon, and cchJ and cchK (Fig. 4; Flores & Martin, 2004). The genes flanking cchA and cchK are divergently transcribed and appear not to be under the control of DmdR1 and DmdR2. The proposed cluster boundaries are also consistent with the recently reported heterologous expression of the cch cluster in Streptomyces fungicidicus (Lautru et al., 2005).

In addition to the cchJ and cchH genes previously implicated in coelichelin biosynthesis (Lautru et al., 2005), cchA and cchB encode a monooxygenase and an acyl transferase, respectively, believed to be required for conversion of ornithine to the non-proteinogenic amino acids L-5-formyl-5-hydroxynornithine and 1-5-N5-hydroxyornithine and 1-5-N5-formyl-N5-hydroxyornithine incorporated into coelichelin (Table 2). Two genes (cchG and cchH) are proposed to encode ABC exporters of coelichelin containing both ATPase and permease domains as found in other ABC exporters (Table 2, Fig. 2; Fath & Kolter, 1993). Four genes (cchCDEF) encode a ferric-siderophore uptake system similar to those found in other Gram-positive bacteria, consisting of a lipoprotein receptor (ChhF), an ATPase (ChhE) and two permeases (ChhC and ChhD) (Table 2, Fig. 2). ChhF contains the sequence AALGVGLLAGC in its N terminus, which matches the Prosite prokaryotic membrane lipoprotein lipid attachment site well and contains a cysteine residue which is proposed to be the site of post-translational modification. Recently, it has also been shown that ChhF is localized in the membrane-associated proteome of S. coelicolor (Kim et al., 2005). The remaining gene in the cluster (cchK) encodes a protein similar to MbtH-like proteins of unknown function encoded within many NRPS gene clusters (Yeats et al., 2003).

Conservation of the cch and des clusters in S. ambofaciens

The sequence of about 1-4 Mb of each chromosome arm of S. ambofaciens ATCC 23877 has been determined (Choulet et al., 2006). In addition, the insert extremities of about 5000 clones from a BAC library of the S. ambofaciens chromosome have been sequenced, leading to 40 % coverage of the complete chromosome and providing some information on the genes present in the central part of the chromosome. A complete des gene cluster is most probably present in the core of the S. ambofaciens chromosome because partial sequence data, obtained from insert extremities of BACs, indicate the presence of desBCD homologues (accession no. AM287205). Moreover, as the synteny is strong in the central region of the S. coelicolor and S. ambofaciens

Table 2. Proposed functions of proteins encoded by the cch cluster

<table>
<thead>
<tr>
<th>Protein</th>
<th>Homologue (percentage identity)</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CchA</td>
<td>FxBa Mycobacterium smegmatis (49)</td>
<td>Formyl transferase</td>
</tr>
<tr>
<td>CchB</td>
<td>PsBa Pseudomonas sp. B10 (39)</td>
<td>1-O Ornithine-N5-mono-oxygenase</td>
</tr>
<tr>
<td>CchC</td>
<td>FpD Yersinia spp. (39)</td>
<td>Permease component of ABC importer</td>
</tr>
<tr>
<td>CchD</td>
<td>FpG Yersinia spp. (35)</td>
<td>Permease component of ABC importer</td>
</tr>
<tr>
<td>CchE</td>
<td>FpC Yersinia enterocolitica (54)</td>
<td>ATPase component of ABC importer</td>
</tr>
<tr>
<td>CchF</td>
<td>FluD Bacillus subtilis (26)</td>
<td>Ferric-siderophore lipoprotein receptor</td>
</tr>
<tr>
<td>CchG</td>
<td>ExT Mycobacterium smegmatis (50)</td>
<td>ATPase/permease of exporter</td>
</tr>
<tr>
<td>CchH</td>
<td>FxbC Mycobacterium smegmatis (42)</td>
<td>Coelichelin NRPS</td>
</tr>
<tr>
<td>CchI</td>
<td>StrV Streptomyces glaucescens (33)</td>
<td>ATPase/permease of exporter</td>
</tr>
<tr>
<td>CchJ</td>
<td>Fes Escherichia coli (30)</td>
<td>Thioesterase</td>
</tr>
<tr>
<td>CchK</td>
<td>MbtH Mycobacterium tuberculosis (70)</td>
<td>Unknown</td>
</tr>
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</table>
Table 3. Production of desferrioxamines and coelichelin by wild-type S. coelicolor, wild-type S. ambofaciens and des/cch mutants

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Siderophore</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Desferrioxamine E</td>
</tr>
<tr>
<td><strong>S. coelicolor</strong></td>
<td></td>
</tr>
<tr>
<td>W1 [desEEABCD::aac(3)IV]</td>
<td>–</td>
</tr>
<tr>
<td>W2 [desD::aac(3)IV]</td>
<td>–</td>
</tr>
<tr>
<td>W3 [cchABCDDEFGHIJK::vph]</td>
<td>+</td>
</tr>
<tr>
<td>W4 [cchH::vph]</td>
<td>+</td>
</tr>
<tr>
<td><strong>S. ambofaciens</strong></td>
<td></td>
</tr>
<tr>
<td>OSID2 (desC::pOSID2)</td>
<td>–</td>
</tr>
<tr>
<td>OSID4 (cchH::pOSID4)</td>
<td>+</td>
</tr>
</tbody>
</table>

chromosomes (Choulet et al., 2006), the other des genes are likely to be conserved as well and present in the same chromosomal region. Analysis and annotation of the chromosome arm sequences identified a cluster in the right arm virtually identical (80–94% identity at the protein level) to the cch cluster found in S. coelicolor (coding sequences SAMR0548 to SAMR0559, accession no. AM238664). The only difference between the two gene clusters is an insertion of a gene (SAMR0550) encoding a possible integral membrane protein of unknown function between cchl and cchf in the S. ambofaciens cluster (Fig. 4). It should be noted that the cch cluster is located in the terminal variable parts of the S. ambofaciens chromosome and that the genes flanking the cch clusters in S. coelicolor and S. ambofaciens are not homologues, except Sco0500, Sco0502 and Sco0503 and the corresponding S. ambofaciens homologues, which are known not to be required for coelichelin biosynthesis (Lautru et al., 2005).

Mutagenesis of the des and cch gene clusters in S. coelicolor and S. ambofaciens and analysis of tris-hydroxamate production in the mutants

To examine the requirement of coelichelin and desferrioxamines for growth of S. coelicolor and as a first step towards examining the role of the putative ferric-siderophore uptake proteins encoded within the des and cch clusters, seven new mutants of S. coelicolor lacking just biosynthetic or both biosynthetic and uptake genes were constructed as described in Methods. desEFABCD::aac(3)IV (W1), cchABCDDEFGHIJK::vph (W3) and cchH::vph (W4) mutants of S. coelicolor were constructed (Figs 3 and 4; Table 3). The W1 and W3 mutants were complemented in cis by the introduction of cosmids SCF34 and SCC105, respectively, from the S. coelicolor ordered cosmid library (Redenbach et al., 1996) and selected for double homologous recombination to restore the wild-type alleles. These mutants, together with the previously reported desD::aac(3)IV mutant (W2; Barona-Gómez et al., 2004) were used to create desEFABCD::aac(3)IV/cchABCDDEFGHIJK::vph (W13), desEFABCD::aac(3)IV/cchH::vph (W14), desD::aac(3)IV/cchABCDDEFGHIJK::vph (W23) and desD::aac(3)IV/cchH::vph (W24) double mutants as described in Methods (Figs 3 and 4; Table 3). While no difficulty was encountered in obtaining any of the single mutants, when preparing the double mutants the initially obtained single-crossover transconjugants had to be subcultured several times to obtain the desired double-crossover siderophore non-producing mutants.

We previously reported independent and mutually incompatible HPLC methods for analysis of ferrioxamine and ferricoelichelin (formed by addition of ferric iron to culture supernatants), respectively, in S. coelicolor culture supernatants (Barona-Gómez et al., 2004; Lautru et al., 2005). Here the HPLC method for ferricoelichelin analysis was modified to allow LC-MS analysis of ferricoelichelin and ferrioxamines using the same method (Fig. 5). It has been reported that desferrioxamine E and desferrioxamine G1 are produced by S. coelicolor (Imbert et al., 1995). However, analysis of ferric-ferrooxidative mutants showed the presence of ferrioxamine B rather than ferrioxamine G1 along with ferrioxamine E (Fig. 5). Coelichelin, desferrioxamine E and desferrioxamine B production by the mutants grown in iron-deficient medium was analysed by LC-MS, confirming the expected metabolite pattern for each mutant (Table 3, data not shown). Restoration of metabolite production in the complemented W1 and W3 mutants, containing cosmids SCC105 and SCF34 inserted in cis, respectively, was also confirmed by LC-MS (Table 3, data not shown).
To determine whether the *S. ambofaciens* des and cch clusters direct desferrioxamine and coelichelin biosynthesis, single desC::pOSID2 (OSID2) and cchH::pOSID4 (OSID4) mutants, and a double desC::pOSID2/cchH::pOSID4 (OSID24) mutant of *S. ambofaciens* were constructed. Ferricoelichelin, ferrioxamine E and ferrioxamine B were identified by LC-MS in ferrated culture supernatants of *S. ambofaciens* grown in iron-deficient medium (Fig. 5). Neither ferrioxamine could be detected in ferrated supernatants of the OSID2 mutant, nor could ferricoelichelin be detected in ferrated culture supernatants of the OSID4 mutant, as expected (Table 3, data not shown). Production of all three tris-hydroxamate metabolites was abolished in the OSID24 mutant (Table 3, data not shown).

**Growth of des and cch mutants is impaired under iron-deficient and iron-sufficient conditions**

We used medium employing colloidal silica as the solidifying agent, originally developed to avoid organic impurities, including agar itself (Hood *et al.*, 1992; see also Methods), to examine the ability of wild-type *S. coelicolor* and the mutants to grow under iron-deficient and iron-sufficient conditions. This medium does not contain any xenosiderophores, which are likely to be present in other standard *Streptomyces* growth media and might complicate interpretation of the results of such an analysis. Neither the wild-type, nor any of the single mutants W1–W4 were able to grow on this medium unless 1 µM FeCl₃ was added. Growth of the single mutants was comparable to the wild-type in the presence of iron. In contrast none of the double mutants W13, W14, W23 and W24 grew on this medium even in the presence of high concentrations (up to 1 M) of FeCl₃.

**Growth restoration of double mutants with exogenously added siderophores**

Small filter paper discs impregnated with coelichelin, desferrioxamine E or desferrioxamine B placed onto the colloidal silica medium restored the ability of the W13, W14, W23 and W24 mutants to grow in the presence of ferric iron, but not in its absence (Table 4). However, the growth halo for the W13 and W23 mutants around a disc containing coelichelin and the growth halo for the W13 and W14 mutants around a disc containing desferrioxamine E was significantly smaller compared with the growth haloes for other mutants around discs containing any of the three *S. coelicolor* tris-hydroxamate metabolites. We also tested the ability of the four double mutants to utilize xenosiderophores representative of different chemical classes (Table 4). We found similar growth haloes for the four double mutants (albeit to different absolute extents) with the ferric complexes of the hydroxamate siderophores desferrioxamine G₁ and coprogen, as well as with the mixed hydroxamate/α-hydroxyacid siderophores aerobactin and schizokinen. The hydroxamate siderophore ferrichrome also stimulated growth of all four double mutants, but W13 and W14 grew less extensively than W23 and W24, in parallel with the results obtained with desferrioxamine E. In contrast, neither ornibactin (another mixed hydroxamate/α-hydroxyacid siderophore, but significantly bigger) nor catecholate-containing siderophores such as enterobactin and pyoverdin A stimulated growth of any of the mutants.

**Growth promotion of *S. coelicolor* W13 by other mutants**

The ability of the *S. coelicolor* single and double mutants to promote growth of the W13 mutant (presumably by cross-feeding of ferric siderophore complexes) was examined by placing plugs from plates of each of the mutants on a lawn of *S. coelicolor* W13 grown under iron-deficient conditions as described in Methods (Fig. 6). While plugs of the W13 and W14 mutants did not stimulate growth of W13, plugs containing the W23 and W24 mutants did stimulate growth of W13 to a small extent. Plugs containing the W1, W2, W3 and W4 mutants all caused significant growth of the W13 mutant, although to varying extents, presumably as a result of different ability to produce and deliver siderophores.
of uptake of the ferric complexes of siderophores excreted by the single mutant by the W13 mutant.

DISCUSSION

In both *S. coelicolor* and *S. ambofaciens* the *des* cluster has been shown to be required for biosynthesis of both desferrioxamine E and B. In light of our previously proposed pathway for desferrioxamine biosynthesis (Barona-Gómez et al., 2004) this strongly suggests that DesC possesses relaxed substrate specificity and is capable of catalysing acylation of N-hydroxy-1,5-diaminopentane (hDAP) with acetyl-CoA or succinyl-CoA to give the corresponding monohydroxamic acids (haDAP and hsDAP, respectively; Fig. 2). DesD is proposed to catalyse either condensation and cyclization of 3 units of hsDAP to give desferrioxamine E or condensation of 2 units of hsDAP and 1 unit of haDAP to give desferrioxamine B (Fig. 2).

Despite circumstantial evidence for the biological function of desferrioxamines and coelichelin as *S. coelicolor* siderophores, no direct evidence for this role has been available before now. The results of the experiments examining the ability of the various single and double biosynthetic mutants to grow in the presence and absence of iron on the xenosiderophore-free medium strongly suggest that coelichelin and desferrioxamines E/B all function as siderophores in *S. coelicolor* and that excretion of at least one of these metabolites is required for growth in a xenosiderophore-free environment. These conclusions are supported by the results of the experiments examining growth promotion of *S. coelicolor* W13 by the other mutants and the results of the growth promotion experiments with exogenously added cognate siderophores using the double biosynthetic mutants on the xenosiderophore-free medium. The difficulty in isolating the *S. coelicolor* double mutants lacking the ability to produce desferrioxamines and coelichelin compared with the single mutants lacking the ability to produce only one of these siderophores further supports the conclusion that these tris-hydroxamates are important for growth of *S. coelicolor*. The double mutants were derived from single-crossover integration of the appropriate mutagenized cosmid into the chromosome of the single mutants followed by screening for a second crossover resulting from loss of the cosmid containing the wild-type allele. Cells in which the second

**Table 4.** Growth restoration of *S. coelicolor* double des/cch mutants by supplementation of desferrioxamines, coelichelin and xenosiderophores

The genotypes of the mutants are given in Table 3. Levels of growth promotion were recorded as: good, similar size halo of growth around filter paper disc to W13 + desferrioxamine B (typically ≥ 17 mm diam); poor, significantly smaller size halo of growth around filter paper disc relative to W13 + desferrioxamine B (typically ≤ 10 mm diam.); none, no halo of growth around filter paper disc. One microlitre of each siderophore, except for coelichelin, the concentration of which was unknown, was used as described in Methods. Growth restoration was good for all mutants with desferrioxamine B, desferrioxamine G1, coprogen and aerobactin. Poor growth restoration was observed in all mutants with schizokinen, and no growth restoration was observed in any of the mutants with ornibactin, enterobactin and pyoverdin A. Similar results to those encountered with desferrioxamine B and mutant W24 were obtained using the *S. ambofaciens* OSID24 mutant.

<table>
<thead>
<tr>
<th>Siderophore</th>
<th>Mutant</th>
<th>W13</th>
<th>W14</th>
<th>W23</th>
<th>W24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desferrioxamine E</td>
<td>Poor</td>
<td>Poor</td>
<td>Good</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Coelichelin</td>
<td>Poor</td>
<td>Good</td>
<td>Poor</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Ferrichrome</td>
<td>Poor</td>
<td>Poor</td>
<td>Good</td>
<td>Good</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 6.** *S. coelicolor* W13 growth is promoted by other *S. coelicolor* des and cch mutants. Haloes of growth promotion are indicated by the production of the blue-pigmented polyketide actinorhodin. All four double mutants were included in a single plate (top left), whereas the single mutants were analysed independently because of larger growth promotion haloes. See text for further details.
crossover event occurs are probably counter-selected due to the complete loss of siderophore biosynthetic systems.

On the basis of the results of the growth promotion experiments with the various double mutants and exogenously added cognate siderophores, it is tempting to speculate that the Chf putative lipoprotein receptor exhibits significant selectivity for ferriochrome over ferrioxamine E and that the DesE putative lipoprotein receptor appears to exhibit significant selectivity for ferriochrome E over ferrichrome (Fig. 6). Interestingly, the ATPase and permease partners of DesE are not encoded within the des cluster and remain to be identified. The fact that desferrioxamine B can stimulate significant growth in a mutant lacking the entirety of both the cch and des clusters (i.e. W13) demonstrates that a third uptake system capable of efficiently transporting ferrioxamine B (and with significantly lower efficiency ferrioxamine E and ferrichrome) must be present in S. coelicolor. It is tempting to speculate that a potential operon consisting of Sco7400, Sco7399 and Sco7398, encoding a putative ATPase, a putative lipoprotein showing significant similarity to ferric-siderophore-binding lipoprotein receptors and a protein containing two putative permease domains, respectively, with a putative DmdR1/DmdR2 binding site upstream of Sco7400, is likely to encode this third uptake system (Fig. 2). Interestingly, Sco7400 and Sco7399 along with DesE and Chf have recently been identified in the membrane-associated proteome of S. coelicolor (Kim et al., 2005). Orthologues of Sco7400, Sco7399 and Sco7398 are present in the left arm of the S. ambofaciens chromosome (SAML0724, 90 % aa identity with Sco7398; SAML0723, 91 % aa identity with Sco7399; and SAML0722, 92 % aa identity with Sco7400). The proposed role of Sco7398–7400 as a third siderophore uptake system is supported by a recent report examining the comparative sensitivity to the siderophore-antibiotic conjugate salmycin of S. coelicolor M145 and a mutant containing the Sco7400, Sco7399 and Sco7398 genes replaced with an oriT-aac(3)IV resistance cassette, together with the ability of desferrioxamine B to reduce salmycin sensitivity in the M145 strain (But et al., 2006). The analysis of the ability of S. coelicolor double mutants to grow on the colloidal silica-based medium in the presence of ferric iron and a range of exogenously added siderophores other than desferrioxamines E/B and coelichelin indicates that this system is able to transport several xenosiderophores, but still exhibits significant selectivity for hydroxamate-containing iron–siderophore complexes. Interestingly, this analysis also suggests that only the DesE putative lipoprotein receptor is able to efficiently transport the cyclic tris-hydroxamate fungal xenosiderophore ferrichrome, although the DesF protein may play a role in efficient utilization of iron from cyclic tris-hydroxamate iron–siderophore complexes.

Multiple siderophore biosynthetic and uptake systems have been reported for other bacteria, including Bacillus anthracis (anthrachelin and anthractin; Cendrowski et al., 2004), Erwinia chrysanthemi (achromobactin and chrysoactin; Franza et al., 2003) and Pseudomonas aeruginosa (pyochelin and pyoverdin; Poole & McKay, 2003). These reports hint towards functional duplication conferring an advantage for the bacterium as it colonizes different ecological niches. They also suggest that in these pathogenic strains only one siderophore is important during certain stages of infection (Cendrowski et al., 2004; Franza et al., 2005). Desferrioxamine production seems to be conserved among Streptomyces spp., yet several soil-dwelling non-actinomycetes can utilize the ferric complexes of these hydroxamate metabolites (Meyer & Abdallah, 1980; Berner et al., 1988; Kachadourian et al., 1996). It is tempting to speculate that uptake and utilization of ferrioxamines as xenosiderophores by microbial competitors in the environment of S. coelicolor and S. ambofaciens have driven acquisition of the cch cluster by these organisms as a ‘contingency plan’ to overcome such biological competition for iron (Challis & Hopwood, 2003). Whereas a des cluster identical to the one described in this paper is present in S. avermitilis (Ikeda et al., 2003) and S. scabies (www.sanger.ac.uk/Projects/S_scabies/) these organisms lack the cch cluster. It would be interesting, therefore, to ascertain whether desferrioxamines are the only siderophores produced by S. avermitilis and S. scabies or whether they also contain other gene clusters directing the production of structurally distinct siderophores.

In conclusion, we have shown that ferric iron acquisition during vegetative growth of S. coelicolor and S. ambofaciens involves a complex interplay of three different tris-hydroxamate siderophores (coelichelin, desferrioxamine E and desferrioxamine B), which are biosynthesized by two independent, but apparently co-regulated pathways, and at least three uptake systems, which appear to possess different selectivity towards their cognate siderophores as well as several xenosiderophores. This work sets the stage for unravelling the molecular basis and functional significance of such a complex ferric iron acquisition system, which should further our understanding of how streptomycetes have adapted to survive in their complex and highly competitive soil environment.

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