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

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Siderophore-mediated iron acquisition has been well studied in many bacterial pathogens because it contributes to virulence. In contrast, siderophore-mediated iron acquisition by saprophytic bacteria has received relatively little attention. The independent identification of the *des* and *cch* gene clusters that direct production of the *tris*-hydroxamate ferric iron-chelators desferrioxamine E and coelichelin, respectively, which could potentially act as siderophores in the saprophyte *Streptomyces coelicolor* A3(2), has recently been reported. Here it is shown that the *des* cluster also directs production of desferrioxamine B in *S. coelicolor* and that very similar *des* and *cch* clusters direct production of desferrioxamines E and B, and coelichelin, respectively, in *Streptomyces ambofaciens* ATCC 23877. Sequence analyses of the *des* and *cch* clusters suggest that components of ferric-siderophore uptake systems are also encoded within each cluster. The construction and analysis of a series of mutants of *S. coelicolor* lacking just biosynthetic genes or both the biosynthetic and siderophore uptake genes from the *des* and *cch* clusters demonstrated that coelichelin and desferrioxamines E and B all function as siderophores in this organism and that at least one of these metabolites is required for growth under defined conditions even in the presence of significant quantities of ferric iron. These experiments also demonstrated that a third siderophore uptake system must be present in *S. coelicolor*, in addition to the two encoded within the *cch* and *des* clusters, which show selectivity for coelichelin and desferrioxamine E, respectively. The ability of the *S. coelicolor* mutants to utilize a range of exogenous xenosiderophores for iron acquisition was also examined, showing that the third siderophore-iron transport system has broad specificity for *tris*-hydroxamate-containing siderophores. Together, these results define a complex system of multiple biosynthetic and uptake pathways for siderophore-mediated iron acquisition in *S. coelicolor* and *S. ambofaciens*.

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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

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Abbreviations: ABC, ATP-binding cassette; NIS, NRPS-independent siderophore; NRPS, non-ribosomal peptide synthetase.

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 α-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* (Sebulsky & 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). Streptomycetes are ubiquitous in soil and also colonize the rhizosphere and marine habitats. Little is known about siderophore-mediated iron acquisition in streptomycetes. Desferrioxamines are *tris*-hydroxamate ferric-iron-chelating metabolites produced by many *Streptomyces* species (Bickel *et al.*, 1960). *Streptomyces pilosus* can take up ferrioxamines B, D_1, D_2 and E (Muller & Raymond, 1984), while *Streptomyces viridosporus* has been shown to take up ferrioxamines B, E and G_1, and *Streptomyces lividans* has been shown to take up ferrioxamines B and G_1 (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). Desferrioxamines 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). Desferrioxamine B biosynthesis in *S. pilosus* is regulated by a DtxR-like ferric-iron-dependent repressor (Günter *et al.*, 1993; Günter-Seeboth & Schupp, 1995). Similar repressor proteins (DmdR1 and DmdR2) have been identified in *S. coelicolor* (Flores & Martin, 2004).

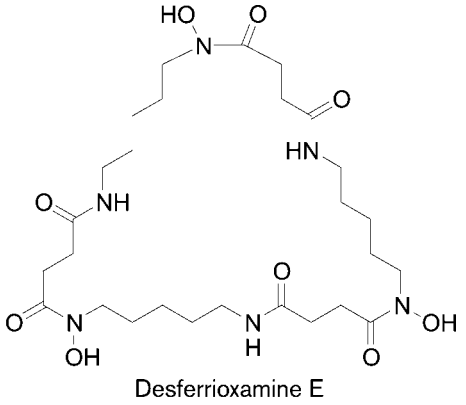
Early steps of desferrioxamine B biosynthesis in *S. pilosus* involve decarboxylation of L-lysine and hydroxylation of the resulting cadaverine to give *N*-hydroxycadaverine (Schupp *et al.*, 1987, 1988). Very recently, two gene clusters that direct the biosynthesis of the *tris*-hydroxamate iron chelators desferrioxamine 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 desferrioxamine E, and a previously unidentified *tris*-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-*N*5-formyl-*N*5-hydroxyornithine and L-*N*5-hydroxyornithine (Fig. 2; Lautru *et al.*, 2005).

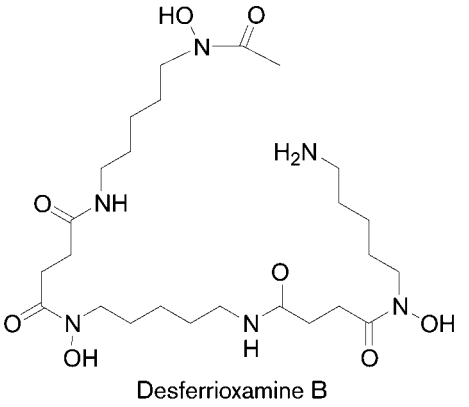
Here we report further investigation of the *tris*-hydroxamate metabolites produced by *S. coelicolor* and the first investigation of the production of such metabolites by *Streptomyces ambofaciens*. The biological function of these metabolites as siderophores is examined and the selectivity of two putative 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 *tris*-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 $MgSO_4.H_2O$ (2 g l^{-1}) and 75·68 mM





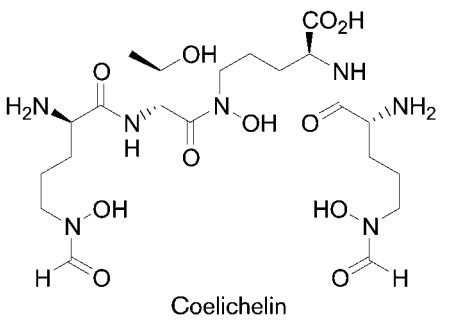


Fig. 1. Structures of *tris*-hydroxamate metabolites produced by *S. coelicolor*.

$(NH_4)_2SO_4$ (10 g l^{-1}) in 5·5 ml distilled water, and autoclaved. This solution was supplemented, per plate, with 3·75 ml 100 mM phosphate buffer (pH 6·8) and 0·7 ml 50 % glycerol, previously autoclaved. When iron was required, a 100 mM $FeCl_3$ sterile solution was added at this point. Solidification of the medium was achieved by adding 10 ml sterile 2 M NaCl per plate (leading to approximately 35 ml medium per plate) and allowing the silica to settle without lids for at least 2 h in a laminar-flow hood. Plates were carefully inoculated by adding spores in solution and allowing drying (silica becomes brittle). 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.

Growth promotion of *S. coelicolor* W13 by the other *S. coelicolor* mutants was examined using either R2 or R2YE supplemented with 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 10^6 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.

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* strain 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), CGGATGCTGATCGCACGGGAGTTGGGGCTGGTGGGCTGAATTCCGGGGATCCGTCGACC and CCGTCTCCGGGGTGCCCGCCGTCCGCGGGGCCGGTTGGTGTAGGCTGGAGCTGCTTC; *cchA–K* (*cch* cluster), CGGGCCCTGCCCGTCATGGGTGTCCGGTCGCGGCGCTCAATTCCGGGGATCCGTCGACC and TGGGAGTTCACGGGCGACGCTTGACGGGGCTCGGCCTCATGTAGGCTGGAGCTGCTTC; and *cchH*, ATGATGGAACCGACCGCTTCTCTCGTACGGCTTTCTCCCATTCCGGGGATCCGTCGACC and AGGTCATGGTGGAGCCGTGGGCGACCAGCGCCGTCCGGTTGTAGGCTGGAGCTGCTTC.

desD:: *aac(3)IV* was as described by Barona-Gómez *et al.* (2004). The apramycin and viomycin cassettes used for the replacements, containing the antibiotic resistance markers *aac(3)IV* and *vph*, were obtained from pIJ773 and pIJ780, respectively (Gust *et al.*, 2003), after excision with *Hin*dIII and *Eco*RI. PCR amplification using Expand high-fidelity DNA polymerase (Roche) and the conditions recommended in the REDIRECT manual (John Innes Centre, Norwich) was carried out. After RP4-based conjugation between *E. coli* ET12576(pUZ8002) and *S. coelicolor* M145, the double cross-over recombination events were confirmed by PCR using the following screening primers: 5′-GAGCCGTTCAAGAAGGAC-3′ and 5′-GACTGGGACACCTACAAG-3′ for the *des* allele; 5′-GCCAGCGGTCGTTCCGGCGC-3′ and 5′-CGACGCGGGGTGGCGCACCT-3′ for the *cch* allele; and 5′-GCCTGCCTTCATTCCTTG-3′ and 5′-CCTGGTAGAGACCCATGAG-3′ for *cchH*. *Streptomyces* chromosomal DNA was extracted using a FastDNA Spin Kit (for soil) (Q-BIOgene) from biomass obtained from a 1 cm^2 patch grown on MS agar. For complementation of the *S. coelicolor* W1 and W3 mutants, the SuperCos1 backbone of cosmids SCC105 and SCF34 from the *S. coelicolor* ordered genomic library (Redenbach *et al.*, 1996) was re-engineered, targeting the *neo* gene with the pIJ780 (*vph*) and pIJ773 [*aac(3)IV*] cassettes, respectively, as described previously (Barona-Gómez *et al.*, 2004).

The plasmids used for gene inactivation in *S. ambofaciens* were derivatives of pBC SK+ (Stratagene) in which the chloramphenicol resistance gene had been inactivated by insertion of a cassette containing the RK2 *oriT* and the Ω*aac* or Ω*hyg* interposons. The construct bearing the internal fragment of *desC* (pOSID2) conferred resistance to hygromycin as it contains the Ω*hyg* cassette, whereas the plasmid containing the *cchH* fragment (pOSID4) contains the Ω*aac* cassette, conferring resistance to apramycin (Blondelet-Rouault *et al.*, 1997). Fragments internal to the coding sequences of these genes were cloned using primers 5′-TGACCACCCCCACGAAGGCCGCCGG-3′ and 5′-GCCCTCTCGAACTGCTCGCGGGTGCAGAAAC-3′ for *desC*;

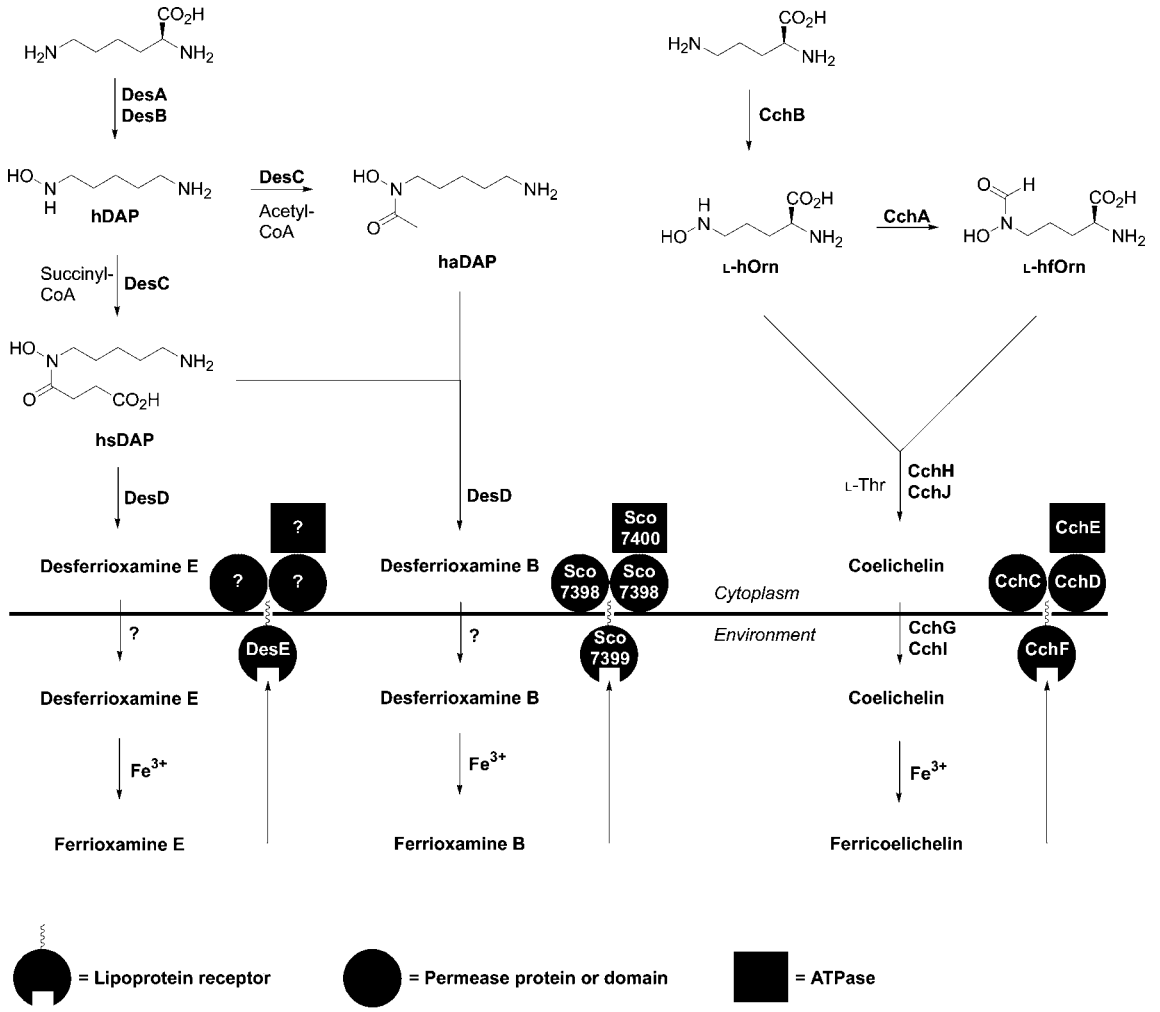


Fig. 2. Model for the biosynthesis, export and uptake of coelichelin, desferrioxamine E and desferrioxamine B by *S. coelicolor* M145. Abbreviations for intermediates in coelichelin and desferrioxamine biosynthesis are as follows: hDAP, *N*-hydroxy-1,5-diaminopentane; haDAP: *N*-hydroxy-*N*-acetyl-1,5-diaminopentane; hsDAP: *N*-hydroxy-*N*-succinyl-1,5-diaminopentane; L-hOrn; L-*N*5-hydroxyornithine; L-*N*5-hydroxy-*N*5-formylornithine.

and 5′-CCCTCCACCCGAACCTGGCCGCACGG-3′ and 5′-GCGCGCAGGGGGATCGTGTTGATGAACAGTCCCACCAT-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). Desferri-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 $FeCl_3$. 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 A_{435}. 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

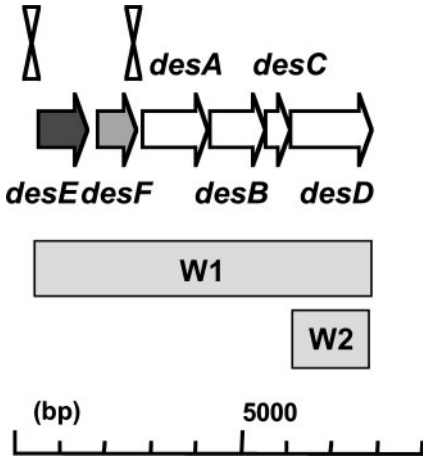


Fig. 3. Organization of the conserved *Streptomyces des* cluster found in *S. coelicolor*, *S. avermitilis*, *S. scabies* and *S. ambofaciens* (sequence data for the latter are only partially available for *desBCD* – accession no. AM287205). Regions replaced with the *oriT-aac(3)IV* cassette derived from pIJ773 in the *S. coelicolor* mutants W1 and W2 are indicated by the pale grey boxes. The *desC* gene in *S. ambofaciens* was mutated by insertional inactivation. White arrows, siderophore biosynthetic genes; mid-grey arrows, putative siderophore utilization genes; dark grey arrows, siderophore uptake genes. The double arrowhead symbol indicates the location of iron boxes for DmdR or IdeR.

source with parameters as follows: nebulizer flow 40 p.s.i., dry gas flow 10 l min^{-1}, dry temperature 300 °C, capillary −4 kV, skimmer 40 V, capillary exit 121 V, ion charge control target (ICC) 100 000, spectra averages, 3. For direct injection MS, the sample was introduced via a syringe pump at 4 µl min^{-1} and the parameters were as for LC-MS analysis except as follows: nebulizer flow 10 p.s.i., dry gas flow 4 l min^{-1}.

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 ferric-vibriobactin (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

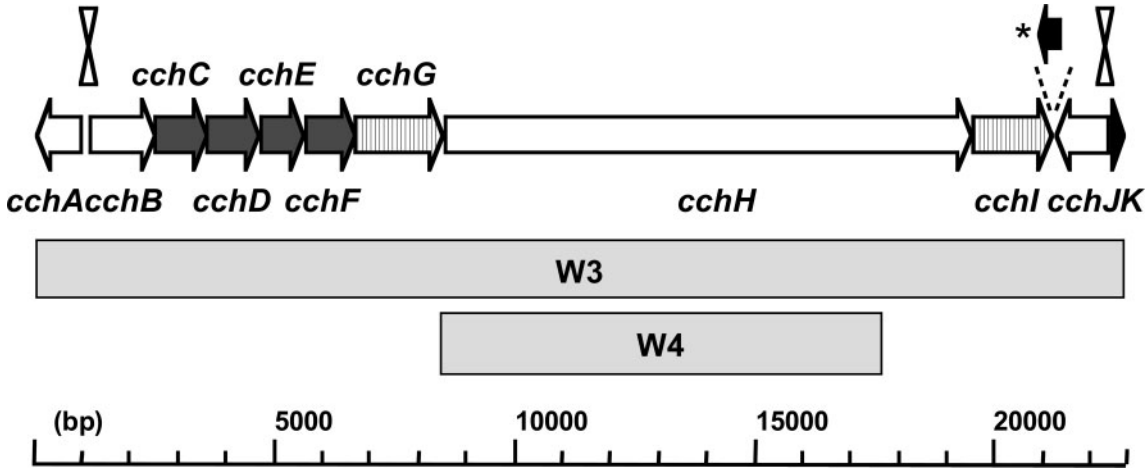


Fig. 4. Organization of the *cch* gene clusters in *S. coelicolor* and *S. ambofaciens*. The only difference between the two clusters is the extra gene marked with * present in *S. ambofaciens*. The regions replaced with the *oriT-vph* cassette derived from pIJ780 in the *S. coelicolor* mutants W3 and W4 are indicated by the pale grey boxes. The *cchH* gene in *S. ambofaciens* was mutated by insertional inactivation. In addition to the symbols used in Fig. 3, black arrows indicate genes of unknown function, and striped arrows indicate putative siderophore export genes.

Table 1. Proposed functions of proteins encoded by the *des* cluster

Protein	Homologue (percentage identity)	Proposed function
DesA	Ddc *Acinetobacter baumannii* (37)	L-Lysine decarboxylase
DesB	AlcA *Bordetella bronchiseptica* (47)	1,5-Diaminopentane monooxygenase
DesC	AlcB *Bordetella bronchiseptica* (46)	Acyl CoA acyl transferase
DesD	AlcC *Bordetella bronchiseptica* (51)	Type C siderophore synthetase
DesE	FhuD *Bacillus subtiltis* (24)	Ferric-siderophore lipoprotein receptor
DesF	ViuB *V. cholerae* (30)	Ferric-siderophore hydrolase

et al., 2003) and *Streptomyces scabies* (www.sanger.ac.uk/Projects/S_scabies/).

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 & Martín, 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-*N*5-hydroxyornithine and L-*N*5-formyl-*N*5-hydroxyornithine incorporated into coelichelin (Table 2). Two genes (*cchG* and *cchI*) 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 (CchF), an ATPase (CchE) and two permeases (CchC and CchD) (Table 2, Fig. 2). CchF 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-tanslational modification. Recently, it has also been shown that CchF 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

Protein	Homologue (percentage identity)	Proposed function
CchA	FxbA *Mycobacterium smegmatis* (49)	Formyl transferase
CchB	PsbA *Pseudomonas* sp. B10 (39)	L-Ornithine-N5-monooxygenase
CchC	FepD *Yersinia* spp. (39)	Permease component of ABC importer
CchD	FepG *Yersinia* spp. (35)	Permease component of ABC importer
CchE	FepC *Yersinia enterocolitica* (54)	ATPase component of ABC importer
CchF	FhuD *Bacillus subtilis* (26)	Ferric-siderophore lipoprotein receptor
CchG	ExiT *Mycobacterium smegmatis* (50)	ATPase/permease of exporter
CchH	FxbC *Mycobacterium smegmatis* (42)	Coelichelin NRPS
CchI	StrV *Streptomyces glaucescens* (33)	ATPase/permease of exporter
CchJ	Fes *Escherichia coli* (30)	Thioesterase
CchK	MbtH *Mycobacterium tuberculosis* (70)	Unknown

Table 3. Production of desferrioxamines and coelichelin by wild-type *S. coelicolor*, wild-type *S. ambofaciens* and *des*/*cch* mutants

Data for strains *S. coelicolor* W13 (W1 and W3 genotypes), W14 (W1 and W4 genotypes), W23 (W2 and W3 genotypes), W24 (W2 and W4 genotypes) and *S. ambofaciens* OSID24 (OSID2/OSID4 genotypes) were all negative. Data for strains *S. coelicolor* M145, W1 + SCC105 (*des*$^+$), W3 + SCF34 (*cch*$^+$) and *S. ambofaciens* OSC2 were all positive.

Strain (genotype)	Siderophore		
	Desferrioxamine E	Desferrioxamine B	Coelichelin
S. coelicolor			
W1 [*desEFABCD*::*aac(3)IV*]	−	−	+
W2 [*desD*::*aac(3)IV*]	−	−	+
W3 (*cchABCDEFGHIJK*::*vph*)	+	+	−
W4 (*cchH*::*vph*)	+	+	−
S. ambofaciens			
OSID2 (*desC*::*pOSID2*)	−	−	+
OSID4 (*cchH*::*pOSID4*)	+	+	−

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 *cchI* and *cchJ* 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), *cchABCDEFGHIJK*::*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*/*cchABCDEFGHIJK*::*vph* (W13), *desEFABCD*::*aac(3)IV*/*cchH*::*vph* (W14), *desD*::*aac(3)IV*/*cchABCDEFGHIJK*::*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 G_1 are produced by *S. coelicolor* (Imbert *et al.*, 1995). However, analysis of ferrated culture supernatants of wild-type *S. coelicolor* grown in iron-deficient medium using our LC-MS method showed the presence of ferrioxamine B rather than ferrioxamine G_1 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).

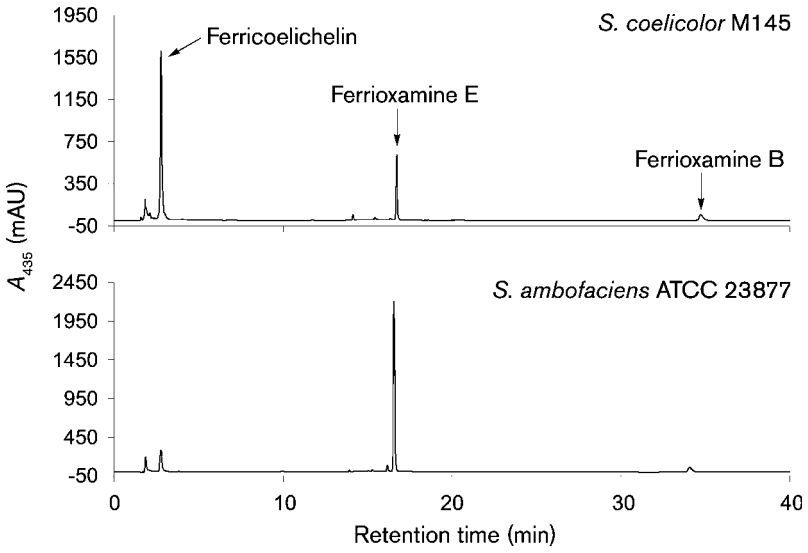


Fig. 5. HPLC analysis of coelichelin and desferrioxamines produced by *S. coelicolor* and *S. ambofaciens*. Ferric *tris*-hydroxamate complexes are selectively detected by monitoring A_{435} following addition of ferric iron to the culture supernatants. The identity of the ferric complexes was confirmed by ESI-MS.

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 $FeCl_3$ 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 $FeCl_3$.

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 G_1 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

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 $\geqslant$17 mm diam); poor, significantly smaller size halo of growth around filter paper disc relative to W13 + desferrioxamine B (typically $\leqslant$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 G_1, 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.

Siderophore	Mutant			
	W13	W14	W23	W24
Desferrioxamine E	Poor	Poor	Good	Good
Coelichelin	Poor	Good	Poor	Good
Ferrichrome	Poor	Poor	Good	Good

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

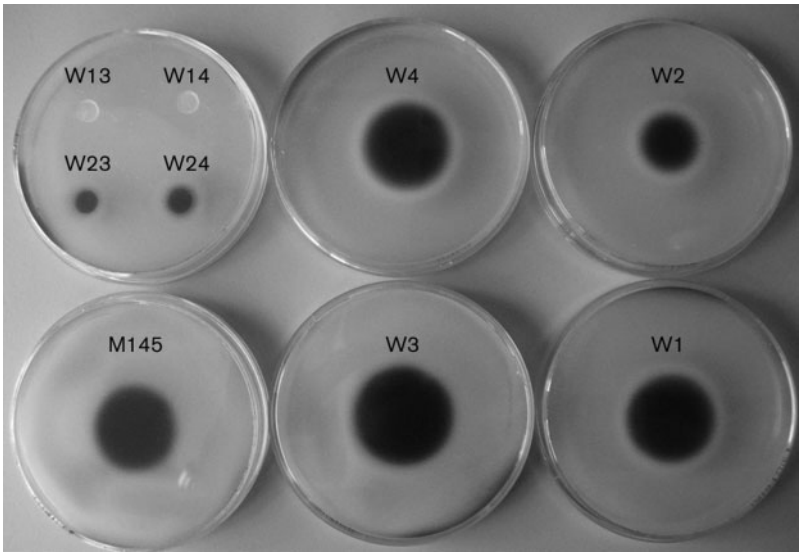


Fig. 6. *S. coelicolor* W13 growth is promoted by other *S. coelicolor des* and *cch* mutants. Haloes of growth promotion are indicated by the production of the blue-pigmented polyketide actinorhodin. All four double mutants were included in a single plate (top left), whereas the single mutants were 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 CchF putative lipoprotein receptor exhibits significant selectivity for ferricoelichelin over ferrioxamine E and that the DesE putative lipoprotein receptor appears to exhibit significant selectivity for ferrioxamine E over ferricoelichelin (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 ferricoelichelin) 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 CchF 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 (Bunet *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 anthrabactin; Cendrowski *et al.*, 2004), *Erwinia chrysanthemi* (achromobactin and chrysobactin; Franza *et al.*, 2005) 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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