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Identification of genes involved in siderophore transport in *Streptomyces coelicolor* A3(2)

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Abstract

The potential iron siderophore transporter genes have been determined from the genome sequence of *Streptomyces coelicolor* A3(2). One of these gene clusters, cdtABC, was disrupted and characterized to determine its role in the uptake of the siderophores produced by *S. coelicolor*. Resistance to the siderophore-like antibiotics, salmycin and albomycin, was tested in the parent and cdtABC mutant, showing that the parent, but not the mutant, was sensitive to salmycin, while both were resistant to albomycin. Ferrioxamine competition assays against salmycin suggest that the uptake of salmycin is via a ferrioxamine transport system. However, Fe-55 ferrioxamine B uptake experiments did not reveal any difference between the parent and mutant. This suggests that CdtABC specifically transports salmycin, while ferrioxamine uptake maybe substituted by another transport system.

Introduction

Most microorganisms require iron for growth and survival. However, the availability of iron in the environment is often low and acquisition of iron thus becomes a challenge. Siderophores are selective and strong Fe(III)-binding low-molecular-weight molecules secreted by most microorganisms under low iron concentration to ensure iron availability. The siderophores bind iron with high affinity and are subsequently transported back into the cells. They are essential for growth of bacteria and are also required for virulence of many pathogens (for a review, see Schalk et al., 2004).

The most prominent siderophore is the enterobactin first isolated from *Salmonella enterica* (Pollack & Neilands, 1970) and the enterochelin from *Escherichia coli* (O’Brien & Gibson, 1970), which have identical structures (Fig. 1).

*Streptomyces* are Gram-positive soil bacteria and are commercially important for their ability to produce antibiotics. They also produce the only commercially important siderophore, desferrioxamine B, called Desferal, to treat excess iron in humans (Tam et al., 2003). In *Streptomyces coelicolor*, the model streptomycete, desferrioxamine E and G1 (Imbert et al., 1995) (Fig. 1) and coelichelin (Lautru et al., 2005) are known to be produced (Fig. 1). The biosynthesis of desferrioxamine is encoded by the four genes desA, B, C and D (SCO2782–2785) (Barona-Gomez et al., 2004), while biosynthesis of coelichelin involves a cluster of genes including ones homologous to transport genes...
A recent report suggests that siderophores may be involved in growth and development in streptomycetes (Yamanaka et al., 2005). The uptake of iron siderophores in Gram-negative bacteria requires two steps (Raymond et al., 2003). Firstly, to cross the outer membrane, it associates with a specific outer membrane transporter and is transported into the periplasm. Secondly, it binds to a cognate periplasmic binding protein and is actively transported across the inner membrane by an ATP-binding cassette (ABC) transporter. In the case of enterobactin, the iron-bound enterobactin binds to FepB, the periplasmic binding protein, and is carried across to the cytoplasmic transmembrane proteins FepD and FepG. Transport across the cytoplasmic membrane is driven by hydrolysis of ATP by the ATP-binding FepC, which is attached to the cytoplasmic membrane. In Bacillus subtilis, a similar uptake system for ferrichrome has been identified. FhuD has similarity to FepB; FhuBG to FepDG; and FhuC to FepC (Schneider & Hantke, 1993).

Some antibiotics are actively transported using transport systems of siderophores that have similar chemical structures (see Braun, 1999, for review). For example, albomycin, which has a structure related to ferrichrome (Fig. 1), is actively transported through the outer membrane of E. coli using FhuA, which is normally known to transport iron-bound ferrichrome and through the cytoplasmic membrane using FhuBCD. Salmycin, a hydroxamate antibiotic, which has an Fe$^{3+}$-siderophore of the ferrioxamine group with an antibiotically active aminodisaccharide (Fig. 1) is also thought to be actively transported by use of an iron transporter (see Vertesy et al., 1995; Braun, 1999, for review). By looking for resistance to these antibiotics, it is

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**Fig. 1.** Chemical structures of siderophores and antibiotics. The names of the siderophores and antibiotics are noted underneath. The bacteria from which it has been isolated are in brackets.
possible to suggest the structure of the siderophore that is transported by an unknown transporter.

Although the siderophores produced by *S. coelicolor* have been reported, the transport mechanisms have yet to be identified. Using the genome sequence of *S. coelicolor*, we have identified homologues of the iron siderophore transporters FepGD, FepB and FepC, which correspond to SCO7398, 7399 and 7400, respectively, and have demonstrated, for the first time, their involvement in iron uptake.

### Materials and methods

#### Bacterial strains, plasmids and culture conditions

*Streptomyces coelicolor* A3(2) strains M145 (Kieser et al., 2000) and LW17 (this work) were manipulated as described previously (Kieser et al., 2000). *Escherichia coli* K-12 strains JM101 (Sambrook et al., 1989), ET12567 (MacNeil et al., 1992) and BW25113 (Datsenko & Wanner, 2000) were grown and transformed according to Sambrook et al. (1989). The vectors used were SC10G8 (Redenbach et al., 1996), pTE135 (this work), pUZ8002 (Flett et al., 1997), plJ773 and plJ790 (Gust et al., 2003). SMM was as reported by Takano et al. (2001). SFM, DNAgar, HT, LB, R2YE, R2, R5, MM, YEME and TSB media were as described by Kieser et al. (2000).

#### DNA sequence analysis

Homology searches were performed using the Sanger Centre *S. coelicolor* blast search (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_coelicolor).

#### DNA manipulations

Cosmid DNA was extracted from *E. coli* by the alkaline method (Sambrook et al., 1989). *Streptomyces* genomic DNA was isolated as described (Leblond et al., 1996).

#### PCR conditions

For amplification of the disruption cassette oriT-aac(3)IV with primers forfep/revfep, the PCR conditions were as described (Gust et al., 2003). For confirmation of the deletion of SCO7398, SCO7399 and SCO7400 by PCR with the flanking primers upfep and downfep, the amplification reaction contained 5 U of Taq polymerase (Qiagen), 1 × PCR buffer (Qiagen), 2.5% (v/v) dimethyl sulfoxide, 10 pM of each primer and ~50 ng of *Streptomyces* genomic DNA in 50 µL. [5 min at 95 °C, 30 cycles of (45 s at 95 °C, 45 s at 56 °C, 90 s at 72 °C), 5 min at 72 °C].

### Deletion of cdtdABC

The strategy to replace the three genes was based on the PCR-targeting system described in Gust et al. (2003). A selectable cassette containing the apramycin resistance marker aac(3)IV and the origin of transfer oriT<sub>RK</sub>, was amplified by PCR with primers forfep (5′-ctacgcagcctttatgtttgccc taaccccactgtcctaatccgggtatcgggtggctac-3′; the 3′ of the primer allowing the amplification of the disruption cassette is in italics, while the 5′ part with homology to the upstream sequence of *cdtC* is in bold, and the first two nucleotides of the GTG start codon of *cdtC* are underlined) and revfep (5′-atctcgagcaccggttttctggccggagatacctaagttgcgagcttctg cttc-3′; the 3′ of the primer allowing the amplification of the disruption cassette is in italics, while the 5′ part with homology to the downstream sequence of *cdtA* is in bold, and the stop codon of *cdtA* is underlined) using the oriT-<br>aac(3)IV gel-purified disruption cassette from pIJ773 as a template (Gust et al., 2003). The replacement of the three ORFs was first created on the cosmid SC10G8 (Redenbach et al., 1996) according to the PCR-targeting protocol (Gust et al., 2003), generating pTE135. The targeted cosmid pTE135 was mobilized into *S. coelicolor* M145 by conjugation after passing through the methylation-deficient *E. coli* ET12567 (MacNeil et al., 1992) containing the nontransmissible plasmid pUZ8002, which carries the transfer functions (Kieser et al., 2000). The allelic exchange was confirmed by Southern and PCR analysis with downfep (5′-tccggctgcgta ggctac-3′; 208 bp downstream from the stop codon of *cdtA*) and upfep (5′-cgcggcagaggtttgagtc-3′; 120 bp upstream from the start codon of *cdtC*), generating a fragment of 1602 bp in the mutant instead of 4278 bp in the wild type. The disruption mutant of SCO7398, SCO7399 and SCO7400 was designated LW17.

### Southern hybridization analysis

Cosmid and genomic DNA were digested with BamHI (New England Biolabs) and run on a 1% (w/v) agarose gel. DNA was transferred onto a Hybond-N nylon membrane (Amerham) by vacuum blotting. Cosmids SC10G8 and pTE135 were digoxigenin-labelled according to the manufacturer’s protocol (Roche) and used together as probes. Hybridization, washings and detection were performed according to the recommendations of the manufacturer (Roche). Signals were recorded on an X-ray film.

### Antibiotic resistance assay

The parent M145 and LW17 spores or mycelia, which had been grown in 5 mL 50%YEME/50%TSB for 2 days at 30 °C, were confluently plated on DNAgar, and MM with or without Fe. Paper discs with 0.01–5.0 µg of salmycin or albomycin (a gift from Volkmar Braun) were placed on both
the parent and mutant plates. The plates were incubated at 30 °C for 2 days for DNAgar and 3 days for MM.

**Radiolabelled Fe-bound desferrioxamine uptake assay**

The parent M145 and LW17 were grown in SMM with or without Fe at 30 °C until the exponential phase (0.5 OD$_{450}$ nm) or the early stationary phase (1.0 OD$_{450}$ nm). The cells were harvested and then washed three times with SMM with 5 mM glucose, no casamino acids and Fe, and then resuspended into the same medium to a final concentration of 20–40 mg mL$^{-1}$ of cells. Seven millilitres cell suspension was preincubated for 5 min at 27 °C with shaking in a 50 mL glass beaker. Fe-55 ferrioxamine B was added to a final concentration of 0.5 or 0.2 μM. One millilitre samples were taken at 2, 5, 8, 12, 15 and 20 min, filtered onto membranes (GN-6 Metricel Membrane filters, PALL), and washed twice with 2 mL 0.1 M LiCl. The dry filters were counted for radioactivity with a scintillation counter. Fe-55 ferrioxamine B was made from desferal by mixing it with equimolar amounts of 55-FeCl$_3$/FeCl$_3$ (Amersham). The transport assay contained 0.5 nMole 55-FeCl$_3$-ferrioxamineB with 2.48 kBq mL$^{-1}$.

**Ferrioxamine competition assay**

The parent M145 and LW17 spores were inoculated into 10 mL 50%YEME/50%TSB for 1 day at 30 °C (in the case of LW17, a final concentration of 5 μg mL$^{-1}$ of apramycin was added). The mycelia were collected, resuspended in 20% glycerol and sonicated for 2 s, with 5 s intervals on ice, three times to disperse the mycelia. One hundred microlitres of cells was then confluenly plated on DNAgar. Paper strips with 0.5 μg of salmycin, albomycin and ferrioxamine B were placed on the parent and mutant plates, so that the salmycin and albomycin strips were parallel and the ferrioxamine strip crossed both strips. The plates were incubated at 30 °C for 1 day.

**Results and discussion**

**Identification of iron transport gene homologues**

*In silico* analysis of the genome sequence of *S. coelicolor* (Bentley et al., 2002) revealed three gene clusters homologous to iron uptake genes. Four genes (SCO0494–0497) homologous to *E. coli* FepB (24% AA identity), FepC (52% AA identity), FepG (38% AA identity) and FepD (44% AA identity), respectively, were found in the coelichelin biosynthesis gene cluster. Another cluster was found at nine o’clock of the chromosome (SCO1785–1787), which had similarity to *E. coli* FepC (61% AA identity), FepG (44% AA identity) and FepD (46% AA identity), respectively, were found in the coelichelin biosynthesis gene cluster. Another cluster was found at nine o’clock of the chromosome (SCO1785–1787), which had similarity to *E. coli* FepC (61% AA identity), FepG (44% AA identity) and FepD (46% AA identity), respectively. The third gene cluster was found at the right-hand end of the chromosome as SCO7398, with homology to FepG/D (33% AA identity, 33% AA identity), respectively. Interestingly, FepG and FepD were fused into one protein in the case of SCO7398, while the other homologues were translated into two separate proteins.

Fig. 2. Disruption of cdtABC by allelic exchange with a resistance cassette [oriT-aac(3)IV]. (a) BamHI restriction map of the Streptomyces coelicolor M145 insert of the cosmid SC10G8. BamHI sites are indicated by black triangles. The sizes of BamHI fragments are given in basepairs. (b) Schematic representation of the replacement of cdtABC by the oriT-aac(3)IV cassette. The positions of primers used for PCR-targeting mutagenesis (forfep/revfep) and for verification of the mutant (downfep/upfep) are indicated by arrows. The black triangles denote BamHI sites, and the sizes of the two newly created BamHI fragments (1407 and 781 bp) after replacement with the resistance cassette are indicated by dotted brackets.
Incomplete gene clusters were also found. SCO2272–2274 have homology to FepB (22% AA identity), FepD (35% AA identity) and FepC (37% AA identity), respectively. SCO0997 (frtD) and 0998 (frtE) have homology to FepD (37% AA identity), and FepC (38% AA identity), respectively. SCO7216 and 7217 have homology to FepD (34% AA identity) and is annotated as an FecCD homologue, and FepC (40% AA identity), which also has homology to FecE, respectively. SCO0997/0998 and SCO7216/7217 are both adjacent to a gene (SCO0996 and SCO7218) that has homology to a lipoprotein that is found in the daptomycin antibiotic biosynthesis cluster (GenBank accession no. AY787762.1) (Miao et al., 2005). Thus, it is likely that these two clusters, SCO0996–998 and SCO7216–7218, are not involved in iron transport.

**Construction of S. coelicolor LW17, a deletion mutant of SCO7398, SCO7399 and SCO7400**

To analyse the function of the third gene cluster, which we have designated cdtA (coelicolor iron-desferal transport), cdtB, cdtC, for SCO7398, SCO7399 and SCO7400, respectively, we constructed a deletion mutant of all three genes in *S. coelicolor* M145 using the PCR targeting method developed by Gust et al. (2003). The chromosomal region encompassing the three ORFs was replaced by the selectable oriT-aac(3)IV resistance cassette, deleting the three coding sequences from the start codon of cdtC to the stop codon of cdtA (Fig. 2). The disruption was confirmed by PCR (data not shown) and Southern analysis of the LW17 genomic DNA (Fig. 3).

These genes potentially form a transcriptional unit, as suggested by their arrangement on the chromosome. The coding sequence of cdtC overlaps that of cdtB by 4 bp, while the intergenic region between cdtB and cdtA does not exceed 7 bp. The intergenic region separating cdtC from the upstream ORF, SCO7401, which is orientated in the same direction, is potentially large enough (104 bp) to allow the presence of a promoter region for a coupled transcription of cdtA, cdtB and cdtC.

**LW17 is resistant to the antibiotic albomycin**

The mutant *S. coelicolor* strain LW17 showed growth, morphological characteristics and antibiotic production identical to those of the *S. coelicolor* wild-type strain M145 when grown on rich solid media (SFM, DNAagar, HT, LB, R2YE, R2, R5) or minimal media as MM with glycerol or glucose and SMM as well as in 50%YEME/50%TSB and SMM liquid media (data not shown).

The parent M145 and LW17 were tested for resistance to salmycin and albomycin to determine the effect on iron transport in the mutant. Spores of parent and LW17 were spread confluentl onto DNAagar and MM with or without Fe. Paper discs with 0.01–5.0 μg of salmycin or albomycin were placed on these plates. The plates were incubated at 30 °C. A faint growth inhibition halo was observed in the parent with salmycin but the inhibition was not very clear (data not shown). The transport proteins may not be expressed in spores, which may be the cause of a very high background of growth in the parent M145. To avoid this high background growth, parent and LW17 were precultured in YEME/TSB for 2 days at 30 °C. The mycelia were then collected, homogenized and plated confluentl as described for the spores. The plates were incubated for 2 days for DNAagar and 3 days for MM. In all media tested, the growth of the parent M145 was clearly inhibited.

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**Fig. 3.** Confirmation of the allelic replacement of cdtABC by the oriT-aac(3)IV cassette in Streptomyces coelicolor LW17. A mixture of digoxgenin-labelled SC10G8 and pTE135 cosmids was used as a probe in Southern analysis of BamHI-digested SC10G8 (lane 1), pTE135 (lane 2), S. coelicolor M145 (parent) genomic DNA (lane 3) and LW17 (mutant lacking cdtABC) genomic DNA (lane 4). The replacement of cdtA, cdtB and cdtC by the resistance cassette results in the loss of two BamHI restriction fragments (1021 and 3740 bp), which are replaced by 1407 and 781 bp fragments (see Fig. 2). Both wild-type cosmids SC10G8 (lane 1) and M145 (parent) genomic DNA (lane 3) possess 1021 and 3740 bp fragments denoting the presence of cdtABC, whereas in both the targeted cosmids pTE135 (lane 2) and the LW17 genomic DNA (lane 4), the 3740 bp fragment disappeared, to yield the 1407 bp fragment, which denotes the replacement of cdtABC by the resistance cassette. The loss of the 1021 bp fragment in pTE135 (lane 2) and LW17 DNA (lane 4) was barely detectable as another fragment of 1021 bp is present in this BamHI restricted region (see Fig. 2a). Similarly, the new 781 bp fragment in pTE135 (lane 2) and LW17 (Fig. 3, lane 4) was not clearly visible due to the presence of a 774 bp BamHI restriction fragment (see Fig. 2a), which is unchanged in both wild type and mutant. All the other BamHI restriction fragments remained unchanged, which excludes additional integrations of pTE135 in LW17. The arrows indicate fragments of interest. Supercos-1 denotes the BamHI fragment embedding the vector part. M denotes the digoxgenin-labelled molecular weight marker.
with salmycin while LW17 was not (Fig. 4). However, with albomycin, no difference was observed between the parent and the mutant (Fig. 4). This suggests that LW17 cannot take up salmycin due to the disruption of \textit{cdtA}, \textit{cdtB} and \textit{cdtC}, and thus the mutant is resistant to salmycin. The parent M145, on the other hand, can transport salmycin and cannot grow. This also suggests that in spores and in germinating spores, these genes are not efficiently expressed. The necessity of iron in spores or germinating spores is not known.

LW17 has an apramycin resistance cassette in the chromosome. Apramycin inhibits protein synthesis, which is the same mode of action as salmycin (Perzynski et al., 1979; Braun, 1999). To confirm that the resistance to salmycin is not due to the apramycin resistance cassette, LW6, a mutant in \textit{kasO} that also has an apramycin resistance cassette in the chromosome (Takano et al., 2005), was also tested for resistance to salmycin. Mycelia of LW6 were confluent plated onto DNAgar and salmycin paper discs were placed onto the plates and incubated. LW6 was sensitive to salmycin and showed inhibition of growth (data not shown). This suggests that the apramycin resistance cassette is not responsible for the resistance to salmycin in LW17 and that mutation of the transport genes is the most likely cause of the resistance.

To further determine whether salmycin is transported using the ferrioxamine uptake system, ferrioxamine competition assays were conducted by placing paper strips with 0.5 µg of salmycin, albomycin and ferrioxamine B onto the parent and LW17 lawns. The strip containing ferrioxamine B was placed at right angles to the salmycin and albomycin strips to see whether the inhibition zone made by salmycin in the parent would be inhibited only where ferrioxamine B is present. As predicted, ferrioxamine B antagonized the sensitivity of the parent to salmycin (Fig. 5). This indicates that salmycin is taken up by a ferrioxamine B transporter.

From the structural similarity of salmycin to ferrioxamines (Fig. 1), it is likely that the \textit{cdtA}, \textit{cdtB} and \textit{cdtC} are responsible for the uptake of ferrioxamines produced in \textit{S. coelicolor}. As albomycin did not show any effect on the resistance of the mutant, it is likely that these genes cannot transport albomycin. This is also suggested by the fact that albomycin is taken up by the FhuABCD transporter for ferrichrome in Gram-negative bacteria (Braun & Braun, 2002) and albomycin has a chemical structure different from that of ferrioxamines (Fig. 1).

To further clarify this point, radioactive Fe-55 ferrioxamine uptake assays were conducted. The parent M145 and LW17 were grown in SMM with and without Fe until the exponential phase or the early stationary phase. The cells were harvested, washed and resuspended in SMM without Fe. Then, 0.1–0.5 µM radioactive Fe-55 ferrioxamine B was added to the culture. After 2, 5, 8, 12, 15 and 20 min, the radioactivity of Fe-55 taken up by the cells was measured with a scintillation counter. In all cases, good uptake was...
observed and there was no difference between the parent and the mutant (data not shown).

CdtABC are most likely to function as a transport system for ferrioxamines from the results obtained from the antibiotic resistance tests. However, it is interesting to note that it must not be the sole transporter of ferrioxamines as the radiolabelled ferrioxamine uptake experiments failed to show any difference between the parent and the mutant. This suggests that the antibiotic salmycin is specifically transported by CdtABC. However, ferrioxamine uptake may be substituted by another transport system.

Two proteins with low similarity to a siderophore-binding protein, FepB, are SCO2780 (26% AA identity) and SCO2272 (22% AA identity). Interestingly, SCO2780 is two genes away from the recently identified desferrioxamine gene cluster (SCO2782–2785) (Barona-Gomez et al., 2004) and may therefore be involved in ferrioxamine uptake. However, in the vicinity of SCO2780, there is no ABC transporter encoded. SCO1785–1787 are also homologues of FepC, G and D, respectively, and may function as an iron transporter. As there is no periplasmic-binding protein homologue of FepB encoded nearby, SCO2780, together with SCO1785–1787, may play a role in ferrioxamine transport. Similarly, in B. subtilis, a periplasmic protein, YxeB, which is located away from the fluBCGD operon, seems to function together with these proteins to take up ferrichrome (Moore & Helmann, 2005).

Participation of the other potential iron transport gene clusters encoded by SCO2272–2274 or SCO0494–0497 (coelichelin) cannot be excluded. However, the specificity of these transporters remains to be clarified. It is also possible that there are other iron transport systems apart from those mentioned in this manuscript within the genome. The redundancy of the ferrioxamine transport systems may be an indication of the importance of ferrioxamine uptake for Streptomyces.

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**Fig. 5.** Effect of ferrioxamine B on salmycin activity in the parent M145 and LW17. Paper strips of 0.5 µg salmycin and albomycin were placed parallel onto DNAagar plates with M145 or LW17 as a lawn. A paper strip of 0.5 µg ferrioxamine B was placed at right angles. The plates were incubated for 1 day at 30 °C. The samples contained in the paper strips are indicated and shown with an arrow. The dark area shows growth inhibition.


