Disruption of the siderophore-binding desE receptor gene in *Streptomyces coelicolor* A3(2) results in impaired growth in spite of multiple iron–siderophore transport systems

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Summary

Ferrioxamines-mediated iron acquisition by *Streptomyces* *coelicolor* A3(2) has recently received increased attention. In addition to the biological role of desferrioxamines (dFOs) as hydroxamate siderophores, and the pharmaceutical application of dFO-B as an iron-chelator, the ferrioxamines have been shown to mediate microbial interactions. In *S. coelicolor* the siderophore-binding receptors DesE (Sco2780) and CdtB (Sco7399) have been postulated to specifically recognize and uptake FO-E (cyclic) and FO-B (linear) respectively. Here, disruption of the desE gene in *S. coelicolor*, and subsequent phenotypic analysis, is used to demonstrate a link between iron metabolism and physiological and morphological development. *Streptomyces coelicolor desE* mutants, isolated in both wild-type (M145) and a coelichelin biosynthesis and transport minus background (mutant W3), a second hydroxamate siderophore system only found in *S. coelicolor* and related species, resulted in impaired growth and lack of sporulation. This phenotype could only be partially rescued by expression in trans of either desE and cdtB genes, which contrasted with the ability of FO-E, and to a lesser extent of FO-B, to fully restore growth at μM concentrations, with a concomitant induction of a marked phenotypic response involving precocious synthesis of actinorhodin and sporulation. Moreover, growth restoration of the desE mutant by complementation with desE and cdtB showed that DesE, which is universally conserved in *Streptomyces*, and CdtB, only present in certain streptomycetes, have partial equivalent functional roles under laboratory conditions, implying overlapping ferrioxamine specificities. The biotechnological and ecological implications of these observations are discussed.

Introduction

Siderophore-mediated iron acquisition by *Streptomyces* species, including siderophore biosynthesis and its regulation, and aspects of iron–siderophore uptake have recently received increased attention (Barona-Gómez et al., 2004; Flores and Martín, 2004; Barona-Gómez et al., 2006; Bunet et al., 2006; Kadi et al., 2007; Tunca et al., 2007; 2009). These studies have paved the way for developing the main siderophores produced by these organisms, i.e. the desferrioxamines (dFOs, Fig. 1), into many novel biotechnological processes, from agriculture and bioremediation (Dimkpa et al., 2008a,b; 2009; Fialho de Oliveira et al., 2010; Rajkumar et al., 2010) to medicine (Dayani et al., 2004; Warshawsky et al., 2005; Banin et al., 2008). Despite the isolation of several siderophores produced by members of this genus, including the novel hydroxamate siderophore coelichelin (Cch, Fig. 1; Lautru et al., 2005) and catechol siderophores previously isolated from other organisms (Fiedler et al., 2001; Patzer and Braun, 2009), only the dFOs have found biotechnological applications. Before the many applications that have been recently postulated for the dFOs, one member of this chemical complex, i.e. desferrioxamine B (dFO-B, Fig. 1), has been commercialized, under the name of desferal®, for treating iron overload in blood for almost four decades (http://www.desferal.com; Gaeumann et al., 1964; Keri et al., 2005). Efforts aimed at replacing this iron-chelator, mainly due to problems associated with its subcutaneous application, have been reported in the literature (Chaston and Richardson, 2003); unfortunately, these efforts have not been clinically successful, and as a consequence, dFO-B...
is currently the main treatment for iron overload caused by multiple transfusions, including thalassaemia. An interest from the biotechnology industry on dFO-B has recently been reinforced by publications of novel methods aimed at its efficient purification (Keri et al., 2005; Braich and Codd, 2008). The aim of these developments is to obtain a highly pure preparation of dFO-B, which is co-produced with several other chemically related dFOs, mainly desferrioxamine E (dFO-E, Fig. 1), by large-scale Streptomyces industrial fermentations.

From a less anthropocentric view, of the many siderophores produced by Streptomyces species, only the dFOs seem to be conserved throughout this and other related genera. For instance, the desABCD genes, which direct the synthesis of these hydroxamate siderophores (Barona-Gómez et al., 2004; Kadi et al., 2007), have been found in all Streptomyces genomes sequenced to date, as well as in other related genera, such as Salinispora (Nett et al., 2009; our unpublished observations). Moreover, these metabolites have been isolated from culture supernatants of virtually all Streptomyces strains that have been screened for this purpose (Imbert et al., 1995; our unpublished observations), with both the cyclic dFO-E and the linear dFO-B, being the two main dFOs produced by this genus. These observations emphasize the important biological role of these metabolites in Streptomyces.

Different ferrioxamines have been shown to promote growth of many microorganisms, including the only known dFO non-producing streptomycete, i.e. Streptomyces tanashiensis IAM0016 (Yamanaka et al., 2005), as well as non-actinomycetes, such as the enteric bacterial pathogen Salmonella enterica (Kingsley et al., 1999). Furthermore, it has recently been reported that other siderophores of the dFOs class can promote growth of uncultured distantly related microorganisms (D’Onofrio et al., 2010). These results suggest that, beyond their important physiological role as siderophores, dFOs have the potential to mediate ecological relationships of related and unrelated bacteria, through uptake of iron–(xeno)siderophore complexes by means of siderophore transport systems with broad specificities (Challis and Hopwood, 2003; Barona-Gómez et al., 2006). Along
these lines, it is also interesting to note that the ecological roles of dFOs may expand into plant–bacterial interactions, as it has been shown that dFOs promote plant growth (Dimkpa et al., 2008a,b; 2009), probably by means of dissolving and mobilizing iron and other metals (Rajkumar et al., 2010).

Two different loci involved in transport and biosynthesis of FOs and dFOs, respectively, have been previously identified in Streptomyces coelicolor A3(2) (Barona-Gómez et al., 2006; Bunet et al., 2006). These studies showed that the desEFABCD locus (Sco2780 to Sco2785) directs the synthesis of dFOs (desABCD), plus FO recognition (desE) and assimilation (desF), with a particular specificity towards FO-E, whereas the cdt locus (Sco7400 to Sco7398 or cdtABC) encodes for a complete iron–siderophore importer with an apparent specificity towards FO-B and related linear hydroxamate (xeno)siderophores, but not coelichelin. However, many of the components needed for proper export and import of dFOs and FOs, respectively, remain to be identified. Of particular relevance are the genes encoding for the transporters that allow translocation of dFOs and FOs through the bacterial membrane, which, in the case of FOs import, must interact with the presumed siderophore-binding DesE receptor (Fig. 2).

Despite DesE representing both the front line of interaction with the environment, and being an obvious target for generating dFOs accumulating industrial strains, this protein has not been studied in any detail. Here, we report the construction and analysis of S. coelicolor desE mutants, in both wild-type (M145) and a coelichelin biosynthetic and transport minus mutant (W3) backgrounds (Table 1). This strain was used as background since it lacks all coelichelin biosynthesis and transport genes, ruling out any possible interference of this second siderophore system. Our experiments reveal the importance of DesE for proper growth and morphological development of S. coelicolor. Moreover, it is shown that CdtB can complement in trans, at least partially but to a similar extent than desE, the nutritional functional role of DesE, suggesting that these siderophore-binding receptors have overlapping specificities for FOs. Interestingly, addition of

![Fig. 2. Current model for siderophore-mediated iron acquisition in S. coelicolor. The proposed model is based in our previous report (Barona-Gómez et al., 2006). Of particular relevance for this study are the specificities of the siderophore-binding proteins DesE and CdtB, as well as the yet-to-be-identified ABC and permease genes, encoding for the FOs import and dFOs export systems (marked as empty boxes).](image-url)
purified FO-E into the media induced a marked phenotypic response of the desE mutant, involving precocious actinorhodin biosynthesis and sporulation, which was less notorious when FO-B was used.

Results

Disruption of desE (Sco2780) affects cell growth and development of S. coelicolor

The desE gene, predicted to encode for an iron–siderophore-binding receptor protein, hypothesized to be FO-specific due to its proximity and co-regulation with the desABCD dFO biosynthetic genes (Flores and Martín, 2004; Tunca et al., 2007, 2009), was mutated using the λ-RED-mediated mutagenesis protocol adapted for Streptomyces (Gust et al., 2003). Replacement of the desE gene by the apramycin antibiotic marker aac(3)IV was carried out in two different backgrounds, M145 and W3, resulting in the strains V1 and V3 respectively (Table 1). The genotypes of both V1 and V3 were confirmed by PCR amplification of the mutated allele and sequencing, and polar effects upon desABCD, affecting dFOs biosynthesis, were ruled out by detection of dFOs by high-performance liquid chromatography (HPLC) and mass spectrometry (data not shown). Both mutants were characterized in parallel leading to identical results. Therefore, to avoid repetition only experiments with V3 are reported, since in this strain coelicchin biosynthesis and its cognate transport systems are absent, ruling out any possible interference of this second siderophore system with our results.

Disruption of desE dramatically affects growth of V3 and development in R2YE medium supplemented with the iron-chelator 2,2′-dipyridyl (Fig. 3). We have previously used this compound to reproduce an iron-limited condition, even in rich media, with the concomitant induction of dFOs synthesis (Barona-Gómez et al., 2006). Interestingly, impaired growth of V3 was recorded even without addition of the iron-chelator, although the phenotype became more apparent as the concentration of 2,2′-dipyridyl increased. An excess concentration of 200 μM was used for subsequent characterization of the mutant strains, both in liquid and in solid media, since this concentration ensures a phenotypic response related to iron limitation, even in M145 and W3.

Cross-feeding of FOs, from W3 into V3, which could be expected to occur and revealed by a gradual growth promotion at the edges of the patches, could not be detected (Fig. 3). Furthermore, the impaired growth of V3, with regards to W3, which was accompanied by precocious production of actinorhodin, represents a phenotype reminiscent of the mutant strain S. coelicolor W1, which lacks the entire des locus including desE (Barona-Gómez et al., 2006). However, the observation that the phenotype of V3 is less severe than that recorded for W1 (data not shown) suggests that both import of FOs and synthesis of dFOs are essential under iron-limited conditions.

Complementation of V3 with desE and cdtB restores growth but not morphological development

Impaired growth of the desE mutant V3 was also recorded in YEME liquid medium supplemented with 200 μM 2,2′-dipyridyl. At this concentration, as can be seen in Fig. 4, the mutant V3 transformed with the chromosomally integrating PtipA empty expression vector pJ6902, i.e. strain V3plJ6902, has an extended lag phase of around 50 h. This situation is reversed when V3 is transformed with pJ6902 constructs containing either desE and cdtB genes, under the control of the PtipA promoter, even without induction of expression with thiostrepton (Fig. 4). However, the phenotypes of the complemented strains,
called V3desE and V3cdtB, were only partially rescued on solid R2YE medium supplemented with the iron-chelator at the same concentration. Sporulation was not fully recovered (Fig. 4) and unexpectedly, the increased restoration of sporulation was obtained with pIJ6902cdtB, rather than with pIJ6902desE. Indeed, in both solid and liquid iron-limited media V3cdtB grows better than V3desE, and produces more actinorhodin, as judged by visual inspection.

**Differential growth restoration of the desE mutant V3 by addition of FO-B and FO-E**

The lack of dFOs cross-feeding, from W3 into V3 (Fig. 3) and discussed above, is curious, since in V3 the cdT locus remains intact. These genes have been previously shown to support uptake of FOs (Bunet et al., 2006) and growth of V3 promoted by dFOs produced by W3 would be expected. A similar situation was found with the complemented strains, V3desE and V3cdtB, which overproduce DesE and CdtB (data not shown). However, the excess of 2,2′-dipyridyl used in these experiments is sufficient to limit iron availability, given that growth of W3 is impaired under these conditions (Fig. 3). Therefore, the ability of pure FO-B and FO-E to promote growth of the desE mutant V3 in media without the iron-chelator was tested.

These experiments showed that both FO-B and FO-E could promote growth and sporulation of the desE mutant V3, albeit with some phenotypic differences (Fig. 5); 1 μM FO-B induced sporulation without precocious production of actinorhodin. In contrast, the phenotype associated with overproduction of actinorhodin was increased with
addition of FO-E and sporulation was only partially rescued (Fig. 5). Nevertheless, all together, these observations suggest that, in disagreement with Barona-Gómez and colleagues (2006), CdtB can also uptake cyclic hydroxamate siderophores. Moreover, it is very interesting to note that addition of very low concentrations of both FO-B and FO-E promoted phenotypic responses. Disruption of \textit{desE} does not lead to accumulation of dFOs

Using the semi-synthetic liquid medium GG1, which was developed for reproducible production of \textit{Streptomyces} secondary metabolites (Avignone-Rossa et al., 2002), the different \textit{desE} mutants constructed in this study, including their pIJ6902-derived exconjugants, were analysed by HPLC, and in selected cases the presence of FO-E and FO-B on their culture supernatants was confirmed by mass spectrometry (Fig. 6). Again, in order to reproduce an iron-limited condition, 200 \(\mu\)M 2,2'’-dipyridyl was used. In contrast with the previously \textit{Streptomyces} iron-limited liquid medium used to investigate dFOs synthesis as a response to iron deficiency (Barona-Gómez \textit{et al.}, 2006), this medium reflects more closely industrial conditions, yet due to its defined nature, subsequent analysis of dFOs by HPLC and mass spectrometry is feasible.

Systematic HPLC analysis of the \textit{desE} mutants and exconjugants, including \textit{cdtB} and \textit{desE} overexpressing M145 and W3 strains, failed to identify two expected dFOs production profiles. Although V3 accumulates dFO-E over dFO-B, which is in agreement with the hypothesized DesE specificity towards cyclic hydroxamates, none of the strains analysed accumulated dFOs on their culture supernatant. A typical chromatogram of dFOs produced by V3 and M145 (Fig. 6), showing two peaks (a and b) eluting at approximately 16 and 17 min that both correspond to FO-E (as confirmed by mass spectrometry, inset), demonstrates that M145 produces as much or even more dFO-E and dFO-B than the \textit{desE} mutant V3.
Moreover, analysis of the overexpressing strains failed to identify conditions in which the accumulation of either dFO could be reproducibly obtained.

Discussion
Reports dealing with FOs-specific uptake systems involving DesE homologues in different microorganisms have been previously reported (e.g. *S. enterica*; Kingsley et al., 1999 and references therein). However, to our knowledge, none of these studies dealt with ferrioxamines-binding proteins in dFO-producing actinomycetes, which are the main source of these hydroxamate metabolites in nature. Thus, the mutational analysis of *desE* in *S. coelicolor* reported herein is the first step towards investigating the physiological and ecological roles of FOs, within the producing organism and in the environment respectively. Moreover, these studies, together with the very recent confirmation that DesE is a Tat-dependent lipoprotein (Thompson et al., 2010), release of its 3D X-ray crystallographic structure (PDB: 2X4L; Oke et al., 2010), and its recent in vitro biochemical characterization (Patel et al., 2010), could inform on novel biotechnological developments with the aim of selectively accumulate dFO-B over dFO-E.

The fact that the physiology of *S. coelicolor desE* disruption mutant leads to such a dramatic phenotype, even in R2YE standard rich medium conditions, emphasizes the importance of iron as an essential nutrient. The inability to fully rescue growth and normal development by *desE* complementation in trans suggests that any minor perturbation, such as chromosomal gene positioning or expression levels (important for maintaining protein stoichiometry), can have very dramatic and negative consequences on whole-cell physiology. A similar scenario was found when complementation of the *desD* biosynthetic gene was attempted (Barona-Gómez et al., 2004). In this case, single-gene complementation in trans could not be achieved, and dFOs biosynthesis of the *desD* W2 mutant had to be rescued by complementation in cis using the entire *des* locus. These observations hint towards protein–protein interactions, of both transport and biosynthetic enzymes, as a key feature of *S. coelicolor* FO-mediated iron acquisition.

The current model for *S. coelicolor* FO-mediated iron acquisition (Fig. 2) takes into account two FO-binding receptors, i.e. DesE and CdtB. However, this apparent functional redundancy is not conserved throughout the *Streptomyces* genus. Whereas DesE is universally conserved and the *des* locus shows synteny, clear orthologues of CdtB can only be found in a few streptomycetes whose genomes are currently been sequenced (e.g. *Streptomyces ambofaciens* ATCC 23877, *S. ghanaensis* ATCC 14672, *S. griseoflavus* Tü 4000 and *S. flavogriseus* ATCC 33331). This observation becomes relevant when the potential identity of the ABC and permease proteins interacting with these FO-binding receptors is taken into account, as discussed below.

On one hand, the question of why certain streptomycetes have two different FO-binding receptors, with
somehow differing but also overlapping specificities, as shown by our results and confirmed in vitro while this article was under revision (Patel et al., 2010), may be raised. The idea of molecular contingency in siderophore biosynthesis and uptake of iron–siderophore complexes has been previously postulated (Challis and Hopwood, 2003; Barona-Gómez et al., 2006), and is gaining support in the literature. Non-specific uptake of (xeno)siderophores by related and unrelated organisms has been extensively documented in recent years (Yamanaka et al., 2005; D’Onofrio et al., 2010 and references therein).

Within this context, having multiple means of acquiring iron–siderophore complexes seems likely to contribute into Streptomyces fitness.

On the other hand, cdtB (Sco7399) appears to be part of an operon, which also encodes for its cognate permeases and ABC protein, needed for import of FOs bound by CdtB. Could it be possible that in the organisms encoding for orthologous cdtABC operons the membrane proteins CdtA and CdtC interact with both CdtB and DesE? A precedent to this scenario is the pioneering studies by Nikaido and Ames (1999) on regarding the histidine and arginine ABC and permease transporter of Salmonella typhimurium, which shows a different subunit arrangement (i.e. different receptor) depending upon which substrate needs to be imported. However, given the fact that the entire des locus is universally conserved in Streptomyces, it is reasonable to expect that DesE-specific membrane proteins involved in import of FOs will also be conserved in the Streptomyces genome.

A reasonable expectation in terms of dFOs accumulation after disruption of desE could not be confirmed. Despite several attempts to characterize by HPLC the dFOs production profiles in the different strains constructed in this study, even when a favourable ratio of dFO-B over dFO-E was found, this could not be reproduced. Indeed, it is interesting to realize that to our knowledge production kinetics of dFOs have never been reported. In our hands, this represented an unfulfilled serious technical challenge that either requires rigorous

Fig. 6. HPLC and MS analysis of dFOs accumulated by the desE mutant V3. The figure shows a representative chromatogram for M145 and V3, and a mass spectrum for FO-E (inset). All cultures were grown and treated under identical conditions, i.e. GG1 medium supplemented with 200 μM 2,2′-dipyridyl and incubation at 30°C for 5 days with vigorous agitation (250 r.p.m.). Two associated peaks, eluting at approximately 16 and 17 min, were identified by MS analysis as FO-E, whereas FO-B elutes at 33.6 min, as confirmed with the standard.

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synchronization of cultures, or a more detailed understanding of Streptomyces iron metabolism. The recent report by Tunca and colleagues (2009), showing that the biosynthesis of both dFOs and the pigmented antibiotics produced by S. coelicolor may be regulated coordinately, by means of two overlapping antiparallel regulatory genes (i.e. dmdR1 and adm), may have an impact on this observation. It would be interesting to test the idea of selective accumulation of dFOs, after controlled expression of desE and cdtB, within a dFO biosynthetic de-repressed DmdR1 minus background (Tunca et al., 2009).

Along these lines, the ability of both FO-B and FO-E, when exogenously added even at μM concentrations, to induce very dramatic phenotypic responses (Fig. 5), including precocious synthesis of actinorhodin and restoration of sporulation, is a puzzling observation. This observation implicates iron metabolism with the developmental programme of S. coelicolor. In addition to the regulatory roles of DmdR1 and Amp discussed above, it has been shown that the developmental protein WhiD, involved in the late stages of sporulation, binds a [4Fe–4S] cluster (Jakimowicz et al., 2005). Thus, given that FOs biosynthesis seems conserved throughout the Streptomyces genus, which implies a sustained environmental pool of these metabolites, it is tempting to speculate that FOs/dFOs may have a regulatory or signalling role, as it has been shown for bona fide secondary metabolites, including actinorhodin in S. coelicolor and the phenazines in Pseudomonas (Dietrich et al., 2008).

Experimental procedures

Bacterial strains, plasmids and culture conditions

Streptomyces coelicolor M145 (Bentley et al., 2002), W1 and W3 (Barona-Gómez et al., 2006), V1 and V3, including their plJ6902-related exconjugants (this work, Table 1), were grown and manipulated as described previously (Kieser et al., 2000). GG1 medium has been previously reported (Avignone-Rossa et al., 2002). Escherichia coli strains and plasmids used for the λ-RED-mediated mutagenesis protocol adapted for Streptomyces have been previously described (Gust et al., 2003). The FOs were added as droplets of varying concentrations (from 0 to 10 mM), at the centre of the R2YE agar plate and allowed to dry before 10^8 spores of V3 were plated as a lawn.

Streptomyces molecular genetics

Streptomyces coelicolor genomic DNA, suitable for screening by PCR, was extracted using the FastDNA® SPIN Kit for Soil (Qbiogene). For this extraction, biomass from 1 cm² of patches grown on Soya Flour Mannitol (SFM) agar plates, were used. The correct replacement of desE (Sco2780) was confirmed by standard PCR and sequencing protocols, using the following flanking oligonucleotides: desE_For (5′-atgtgatgcagcgggagttg), which is 165 bp upstream from the start codon of desE; and desE_Rev (5′-acagggagctgagaagac), which is 73 bp downstream from the stop codon of desE. PCR amplification of the mutated allele yields a product of 1626 bp, instead of 1288 bp of the wild-type allele. For replacement of the desE gene by an ortT-aac(3)IV cassette, obtained from plJ773 (Gust et al., 2003), primers were carefully designed such that both the stop and start codons (underlined below) of desE were left behind, avoiding a frameshift. The primers used for targeting of desE gene (bold) were as follows: Mut_desE_For (5′-ccggagctcgcggagctgagaagacagccacccagcatggccgggaatgctgagctgagcagcgggagttgaggtgaattc), and Mut_desE_Rev (5′-ccggtctcaaggggtcgggggagggggagggggggctgagctgagcagcgggagttgaggtgaattc). Replacement of desE in M145 yielded the mutant strain V1, which was subsequently mated with W3 (Barona-Gómez et al., 2006), using standard protocols (Kieser et al., 2000). The double desE::aac(3)IV and chhAB-cdtB cassette, obtained from pIJ773 (Gust et al., 2008), was inserted into the chromosome of S. coelicolor M145, W3, V1 and V3, following standard protocols (Kieser et al., 2000). The resulting strains were verified by PCR using the following screening primers: Sc_Com_For (5′-cccgagctgctgagcagcgggagttg) and Sc_Com_Rev (5′-ttctcactccgctgaaactg).

HPLC and mass spectrometry analysis

Samples were prepared and analysed as previously reported (Barona-Gómez et al., 2004; 2006), with minor modifications. HPLC was carried out using a binary pump (Agilent 1200) equipped with a diode array detector with a fraction collector, and fitted with a Discovery HSFF column, Supelco (150 × 6.6 mm, 5 μm i.d., column temperature 20°C). The mobile phase comprised a binary system of: eluent A, 10 mM ammonium carbonate, pH 7, and eluent B: 100% methanol. The run consisted of an isotropic elution of 90% A : 10% B in 10 min, followed by a gradient of 0:100 over 8 min, 10 min isotonic conditions 0:100, a gradient to 90:10 over 8 min, and 10 min to isotonic conditions at 90:10 (A:B). The tris-hydroxamylate–Fe²⁺ complexes were detected by monitoring absorbance at a wavelength of 435 nm. The identities of compounds with retention times of approximately 16.5 and 33.6 min were confirmed as FO-E and FO-B, respectively, by injection into a Waters Q-TOF MicroMass spectrometer in positive ion mode.

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The mesylate salt of dFO-B (BD9533) was purchased from Sigma, and iron-chelator 2,2′-dipyridyl (14454) was purchased from Fluka. FO-E was purified by HPLC from S. coelicolor M145 grown under iron-limited conditions. The exact concentration of FO-E was calculated from the molar extinction coefficient of this iron complex, as previously reported (Fernández and Winkelmans, 2005).

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