Desferrioxamine E produced by *Streptomyces griseus* stimulates growth and development of *Streptomyces tanashiensis*

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The authors previously reported that interspecific stimulatory events between *Streptomyces* species for antibiotic production and/or morphological differentiation mediated by putative diffusible metabolites take place at a high frequency. This paper reports the isolation and characterization of a substance produced by *Streptomyces griseus* that stimulates the growth and development of *Streptomyces tanashiensis*. The substance was purified from the culture supernatant of *S. griseus* by using anion-exchange chromatography, gel filtration chromatography and reverse-phase HPLC. FAB-MS and NMR analyses of the purified preparation indicated the substance to be desferrioxamine E (synonym: nocardamine), a siderophore that is widely produced by *Streptomyces* species and related organisms. Similar stimulatory effects on the growth and development of *S. tanashiensis* were exerted by desferrioxamine E produced by another actinomycete strain, but not by other siderophores tested, including ferrichrome and nocobactin and free ferric ion. An exogenous supply of desferrioxamine E stimulated secondary metabolite formation and/or morphological differentiation in various actinomycete strains. Disruption of the desferrioxamine biosynthesis gene cluster in *Streptomyces coelicolor* A3(2) abolished the production of desferrioxamine E and the activity to stimulate the growth and differentiation of *S. tanashiensis*. The *S. coelicolor* mutant showed impaired growth and development on Bennett’s/glucose agar medium, but it was rescued by the exogenous supply of desferrioxamine E. These results indicate that desferrioxamines play an important role in streptomycete physiology. Similar to several pathogenic bacteria and fungi, *S. tanashiensis* may be defective in the production of siderophores; however, it can utilize the siderophores excreted by other organisms.

**INTRODUCTION**

The Gram-positive bacterial genus *Streptomyces* is characterized by the ability to perform complex morphological differentiation resembling that of filamentous fungi. In the initial stages of the life cycle, the organism grows as a branching multinucleoid substrate hypha on solid media (Chater, 1993). The substrate hypha then produces aerial mycelium, which culminates in a long spore chain by forming septa at regular intervals. Streptomycetes are also characterized by the ability to produce a wide variety of secondary metabolites, which have many applications in the pharmaceutical, chemical and agricultural industries (Miyadoh, 1993).

Evidence is accumulating that the onset of morphological differentiation and secondary metabolite formation in streptomycetes is induced by autoregulatory substances which have a γ-butyrolactone structure. A-factor (2-isocapryloyl-3R-hydroxyxymethyl-γ-butyrolactone) of *Streptomyces griseus*, the best-studied γ-butyrolactone autoregulator, induces the onset of both aerial growth and secondary metabolism in this organism (Horinouchi et al., 1984). The studies of Horinouchi, Beppu and coworkers have revealed the details of the A-factor signalling cascade, which involves multiple regulatory gene functions (Horinouchi, 1999, 2002; Horinouchi & Beppu, 1992). On the other hand, Nihira, Yamada and colleagues have characterized the role and function of virginiae butanolides in *Streptomyces virginiae*...
(Yamada, 1999). They have also identified the plethora of γ-butyrolactones in a variety of Streptomyces species, including Streptomyces coelicolor A3(2), the model organism whose genome has been completely sequenced (Bentley et al., 2002; Takano et al., 2000). It is generally understood that the action of γ-butyrolactone autoregulators is specific to the producer organism because of the specificity of the cognate receptor proteins.

In contrast to the idea of autoregulation, we have previously shown that interspecific stimulation of morphogenesis and/or secondary metabolism takes place among various Streptomyces species and related organisms (Ueda et al., 2000). Cross-feeding experiments on solid media demonstrated the response of an array of colonies of one strain to a concentration gradient of a substance diffusing from a colony of another strain. Comprehensive cross-feeding tests among 76 Streptomyces strains showed that more than 20% of the strains showed response(s) to putative metabolite(s) excreted by another strain and exhibited precocious colony development and/or enhanced secondary metabolism formation. These stimulatory events between different species may include those involving the function of a specific metabolite. In this study, we focused on the stimulation of the growth and development of Streptomyces tanashiensis; this stimulation is caused by a substance, revealed to be a type of siderophore, secreted by S. griseus.

METHODS

Bacterial strains, plasmids and culture conditions. S. griseus st-21-2 and S. tanashiensis IAM0016 have been described previously (Ueda et al., 2000). S. coelicolor A3(2) M145 used for the gene disruption experiment and cosmid C105 containing the des operon were obtained from the John Innes Centre (Norwich, UK). Escherichia coli DH5α was used as a host for general DNA manipulation. Plasmids and other conditions for genetic manipulation in E. coli and Streptomyces were as described by Maniatis et al. (1982) and Kieser et al. (2000) respectively. E. coli was grown at 37 °C in Luria–Bertani medium containing the following (in g l⁻¹): Bactotryptone (Difco), 10; Bactoyeast extract (Difco), 5; NaCl (Kokusan), 5; and agar (Kokusan), 10 (for solid medium). Streptomyces strains were cultivated at 28 °C on Bennett's sugar medium containing the following (in g l⁻¹): Bactoyeast extract (Difco), 1; meat extract (Kyokuto), 1; NZ amine (Wako Pure Chemical Industries), 2; and an appropriate sugar (Kokusan), pH 7.2. Agar (Kokusan) was supplied at a concentration of 1.5% for solid media. For the selection of transformants, ampicillin (Wako) and kanamycin (Wako) at a final concentration of 50 μg ml⁻¹ were used for E. coli. For S. coelicolor, thiostrepton (Sigma) and kanamycin (Wako) were added at a final concentration of 20 μg ml⁻¹. The enzymes used for genetic manipulation were purchased from Takara-shuzo. Siderophores other than desferrioxamine E were purchased from Sigma, with the exception of nocardactin, which was a kind gift from J. Ishikawa (National Institute of Health, Japan). Nocardactin was isolated from the culture supernatant of Nocardia farcinica as a mixture of several analogues. Desferrioxamine E was previously isolated from the culture broth of an unidentified actinomycete strain (Matsubara et al., 1998).

Isolation of the stimulant produced by S. griseus. In order to isolate the substance that stimulates the development of S. tanashiensis, S. griseus st-21-2 was cultured in Bennett’s glucose liquid medium. The seed culture was grown at 28 °C for 5 days; shaking was carried out at 135 r.p.m. in a 500 ml baffled Erlenmeyer flask in a working volume of 100 ml. After removing the mycelia by centrifugation and filtration, 100 ml of the culture supernatant was first extracted three times with 100 ml ethyl acetate, and 20 g cation-exchange resin (Amberlite IRC50; Organo) was added to the resultant water phase. Following incubation for 1 h at room temperature with gentle stirring, the resin was removed by filtration. The unab sorbed filtrate thus obtained was then applied onto a Dowex 1×2 anion-exchange column (2.5×7.5 cm; Amersham Biosciences). The column was developed with DW (0.6 ml min⁻¹) and fractions of 10 ml were collected. The activity, which was recovered in fractions 13–15, was concentrated by evaporation and lyophilization to dryness. The sample was then dissolved in 3 ml DW and applied to a reverse-phase HPLC column (ODS-7515-12A, 2.5×14 cm; Amersham). After washing with water, the column was developed with a stepwise gradient of methanol in water (water : methanol 10:0–10:10). The activity was recovered by elution with water : methanol (6:4:4:6). The fractions were combined and lyophilized to dryness, dissolved in 1 ml water and applied onto a reverse-phase HPLC column (RESOURCES RPC, 5×0.2 cm; Amersham). Following washing with water, the column was developed with a gradient of 10–90% acetonitrile in 0.05% formic acid at 1 ml min⁻¹ and fractions of 1 ml were collected, monitoring UV absorption spectra at 218 nm. The active fractions were combined, lyophilized, dissolved in 1 ml water and applied onto a reverse-phase HPLC column operated under the same conditions as above, except that the elution was performed at 0.8 ml min⁻¹. The HPLC performed previously afforded the stimulatory activity as a single peak at a concentration of 25% acetonitrile. The activity of the purified substance as a siderophore was examined by the CAS assay according to Schwyn & Neilands (1987), which visualizes ferric-sequestering activity by the formation of a clear halo on the CAS assay plate.

Structural analysis. The 1H NMR spectrum was recorded on a JEOL JMN-500 spectrometer at 500 MHz. The FAB-MS spectrum was obtained on a JEOL JMS-700T spectrometer using xenon as the fast atom. Spectral data of the active substance were as follows: FAB-MS (positive, glycerol matrix) m/z 601 (M+H)+, 623 (M+Na)+; 1H NMR δ (500 MHz, DMSO-dj): 1.15–1.24 (m, 6H), 1.33–1.40 (m, 6H), 1.45–1.52 (m, 6H), 2.27 (t, J=7 Hz, 6H), 2.58 (t, J=7 Hz, 6H), 2.93–3.02 (m, 6H), 3.46 (t, J=7 Hz, 6H), 7.77 (br. s, 3H, N-H), 9.60 (br. s, 3H, N-OH).

Gene disruption. The desABC mutant of S. coelicolor A3(2) was generated by the standard homologous recombination technique, which replaced the wild-type allele with a mutated construct on a disruption plasmid. The disruption plasmid was constructed as follows. The two DNA fragments, a and b (see Fig. 4a), which correspond to the internal region of desA and desD respectively, were amplified from the chromosomal DNA of S. coelicolor A3(2) by standard PCR. The PCR primers that were used were as follows: 5’-CGA-CAGGCTTGAGGACGCTCTA-3’ (corresponding to 3035832–3035852 of SCO2782) (http://www.sanger.ac.uk/Projects/S_coelicolor) (Bentley et al., 2002) and 5’-AAGGATCTCCAGGACTCTC-3’ (3037049–3037068 of SCO2782) for fragment a and 5’-CTTCGAGAGTCTCAGGCTCTA-3’ (3039416–3039436 of SCO2782) and 5’-CAGGGATCTCCAGGGTAC-3’ (3040650–3040670) for fragment b. Fragments a and b were then digested with the restriction endonucleases HindIII/BglII and BglII/EcoRI, respectively, and cloned onto pUC18, which was digested with HindIII/EcoRI by three-fragment ligation. In order to generate the disruption plasmid, the plasmid thus
formed was cleaved with BglII and ligated to an aphII (kanamycin resistance) cassette (Beck et al., 1982), which was recovered as a BamHI-digested fragment. The disruption plasmid was introduced into S. coelicolor A3(2) M145 cells by standard transformation. As a result, two kanamycin-resistant colonies were obtained; one colony was confirmed for true recombination by the standard Southern hybridization technique using appropriate probes and described as a desABCD mutant (KY1 strain). The KY1 strain lacks the region containing the C-terminal part of desA, the entire region for desB and desC and the N-terminal portion of desD. The insertion of aphII does not affect the gene expression of the flanking regions, because aphII oriented in the opposite direction to the des operon.

For the genetic complementation of the mutant, an 8.6 kb BamHI–PstI fragment that contains the desABCD operon and the flanking coding sequences (Fig. 4a) was excised from cosmids C105 and cloned between the BamHI–PstI sites of pUC19. In order to generate pDES, the DNA fragment containing the des operon was recovered from the resultant plasmid as a BamHI–HindIII fragment and cloned between the BamHI–HindIII sites of pX4, a Streptomyces plasmid carrying a gene for thiostrepton resistance (Kieser et al., 2000). In order to confirm its genetic complementation activity against the phenotype of KY1, pDES was introduced into the KY1 strain by standard transformation.

RESULTS

Identification of the substance produced by S. griseus that stimulates the growth and differentiation of S. tanashiensis

We performed screening for interspecific stimulatory events in Streptomyces species, as described previously (Ueda et al., 2000), and found that co-culture with S. griseus stimulates the growth, morphological differentiation and antibiotic production of S. tanashiensis (Fig. 1a). The result suggested that a diffusible substance produced by S. griseus exhibits an activity that induces colonial development and secondary metabolism of S. tanashiensis.

In order to determine the chemical structure of the stimulatory substance produced by S. griseus, the active principle was isolated and purified from culture supernatant (see Methods). The activity was assessed by a filter disc assay; a filter disc containing the sample was placed on a Bennett’s/glucose agar plate in order to observe their response to the concentration gradient of the substance(s) diffusing from a colony of S. griseus (a) and a filter disc containing desferrioxamine E or B (b). For the experiment shown in (b), the responses of three S. tanashiensis strains (IAM0016, JCM4086 and JCM4671) were examined. Each filter disc contained approximately 3 μg desferrioxamine E isolated from the S. griseus culture supernatant (left panels) or 25 μg commercial desferrioxamine B (right panels). The colonies that exhibit aerial growth appear white, while those exhibiting only vegetative growth appear yellow or brown. The colonies of S. tanashiensis IAM0016 were overlaid with Bacillus subtilis cells, which allowed the visualization of the amount of antibiotic produced by this organism. The patches were photographed after 7 days cultivation at 28°C for streptomycete strains and overnight incubation at 37°C for B. subtilis.

Stimulatory effect of desferrioxamines on Streptomyces growth and development

As shown in Fig. 1(b), approximately 3 μg of the purified desferrioxamine E applied onto a filter discstimulated the growth, cell differentiation and antibiotic production of S. tanashiensis. The same effect was exerted by purified desferrioxamine E, previously isolated from the culture supernatant of an unidentified actinomycete strain (Matsubara et al., 1998) (data not shown). While the stimulation was clearly observed under this condition, we failed to determine...
the exact minimum effective concentration of desferrioxamine E, largely due to the ambiguous response of the colony appearances to low concentrations of the substance.

Desferrioxamines have several analogous forms, including desferrioxamine B (Fig. 2), which is commercially available. In comparison with desferrioxamine E, the growth-stimulation activity of commercial desferrioxamine B against *S. tanashiensis* was weak (Fig. 1b), although it showed distinct stimulation when it was mixed with 0.1 µg FeCl₂ prior to application onto the filter disc. The exogenous supply of other siderophores, including 2,3-dihydroxybenzoic acid, ferrozine, ferrichrome, 8-quinolinol, transferrin and nocobactin and free ferric ion, did not affect the phenotype of *S. tanashiensis* (data not shown).

Fig. 3 shows the effect of exogenous desferrioxamine E on the phenotypes of various actinomycete strains. The supply of the substance induced precocious aerial growth in both *S. griseus* st-21-2, the strain used as a producer of desferrioxamine E in this study, and *S. coelicolor* A3(2). In addition, various effects were observed with the strains that were freshly isolated from soil (Fig. 3): the supply of desferrioxamine E stimulated yellow pigment and antibiotic production (strain no. 17), aerial mycelium formation (no. 70 and no. 507), melanin-like pigment production (no. 82) and growth (no. 326).

**Disruption of the desferrioxamine biosynthesis gene cluster in *S. coelicolor* A3(2)**

The above results indicated that the model organism *S. coelicolor* A3(2) is also stimulated into colony development by desferrioxamine E. Recently, Barona-Gomez *et al.* (2004) reported the identification of the gene cluster of this organism that is responsible for desferrioxamine biosynthesis. The cluster has an operon structure, which consists of four coding sequences, namely, desABCD (SCO2782–2785; http://www.sanger.ac.uk/Projects/S_coelicolor) (Bentley *et al.*, 2002) (Fig. 4a). In order to assess the role of desferrioxamines in *S. coelicolor* development, a null mutant for the operon (KY1 strain) was generated by the standard homologous recombination technique. In KY1, the wild-type des allele was replaced by a mutational construct that carries the kanamycin-resistance gene cassette (Fig. 4a) (see Methods).

KY1 showed impaired growth, morphological development and pigment production on Bennett’s/glucose solid medium (Fig. 4b). The deficiency was partially rescued by an exogenous supply of desferrioxamine E isolated from *S. griseus* culture broth (Fig. 4b) and was fully complemented by the introduction of a plasmid that carries the intact des operon (pDES) (Fig. 4c). The cross-feeding assay showed that *S. coelicolor* A3(2) wild-type exhibits an activity that
stimulates the growth and development of \emph{S. tanashiensis}, while the KY1 strain was defective in the stimulation activity (Fig. 4c). The stimulation activity was restored by the introduction of pDES. Purification of desferrioxamine E by the same method as employed for \emph{S. griseus} showed the presence of the compound in the culture broth of \emph{S. coelicolor} A3(2) wild-type and its absence in that of KY1 (data not shown).

**Fig. 4.** Disruption of the des operon of \emph{S. coelicolor} A3(2). (a) Schematic representation of desferrioxamine biosynthesis gene cluster of \emph{S. coelicolor} A3(2). The mutant construction used to generate the des mutant (KY1) is also shown; \textit{aphII}, kanamycin resistance gene cassette. (b) Colonies of \emph{S. coelicolor} A3(2) wild-type and KY1 grown on Bennett’s/glucose solid medium supplied with (left) or without (right) exogenous desferrioxamine E isolated from the \emph{S. griseus} culture supernatant. Each filter disc for the former condition contained approximately 3 µg of the purified desferrioxamine E. The blue pigment diffusing from the \emph{S. coelicolor} colonies is the antibiotic actinorhodin. The exogenous supply of desferrioxamine E stimulated colonial growth, development and antibiotic production in both the strains. KY1 without desferrioxamine E showed impaired growth (rightmost panel). The patches were photographed after 5 days cultivation at 28°C. (c) Cross-feeding experiment between \emph{S. coelicolor} strains and \emph{S. tanashiensis} IAM0016. In each assay, one colony of the former strain (left end) and two colonies of the latter strain were grown on a single Bennett’s/glucose agar plate. While the \emph{S. coelicolor} wild-type and KY1 harbouring pDES stimulated the growth and aerial mycelium formation of \emph{S. tanashiensis} grown in close proximity, KY1 did not affect the phenotype of \emph{S. tanashiensis}. The patches were photographed after 7 days cultivation at 28°C. WT, \emph{S. coelicolor} A3(2) M145 (wild-type); KY1, KY1 strain (a mutant for the des operon); KY1/pDES1, KY1 strain harbouring pDES1, a plasmid which carries an intact des operon.

**DISCUSSION**

This study revealed that the substance produced by \emph{S. griseus} that stimulates the growth and development of \emph{S. tanashiensis} is the siderophore desferrioxamine E. Desferrioxamines have been isolated from the culture broth of various actinomycetes, including \emph{Streptomyces}, \emph{Nocardia} and \emph{Micromonospora} species (Zahner et al., 1962). They are also isolated from Gram-negative bacteria such as species of \emph{Pseudomonas} (Meyer & Abdallah, 1980) and \emph{Erwinia} (Berner et al., 1988). While it is well known that desferrioxamines act as siderophores and have important clinical applications (Tam et al., 2003), the physiological role of the compounds in the producer and related organisms has not been extensively characterized. The results obtained in this study suggest that in \emph{Streptomyces} species, siderophores have a role in development as well as growth.

Ferric uptake in \emph{Streptomyces} species has been poorly characterized, except in \emph{Streptomyces pilosus}, the organism used for the fermentation production of desferrioxamine B (Müller & Raymond, 1984; Müller et al., 1984). This organism produces various desferrioxamine analogues, among which desferrioxamine B is the major constituent. Müller et al. (1984) demonstrated that \emph{S. pilosus} can take up not only desferrioxamines but also ferrichrome, another major microbial siderophore, although the organism cannot produce the latter type of siderophore. On the other hand, we found that the growth and development of \emph{S. tanashiensis} are not affected by the exogenous supply of ferrichrome. Evidence suggests that there is diversity in the utilization of siderophores in streptomycetes; however, the major siderophores in the group of bacteria may be desferrioxamines.

The stimulation of \emph{S. tanashiensis} growth and development by \emph{S. griseus} was not detected in our previous study (Ueda et al., 2000). This may be attributed to the difference in the length of the incubation period. While the stimulation was assessed after a 4 day culture in the previous screening study, the marked stimulation in \emph{S. tanashiensis} was observed after prolonged incubation for more than 1 week. The wide occurrence of desferrioxamine in \emph{Streptomyces} species and its stimulatory effect on the growth and/or development of various streptomycetes and related organisms indicate that the stimulatory event is not specific to the two strains mentioned above but common to the constituents of the bacterial group.

The studies on \emph{S. pilosus} have shown that desferrioxamines are commonly synthesized from lysine (Müller & Raymond, 1984; Schupp et al., 1987). Although we were successful in isolating only desferrioxamine E from the \emph{S. griseus} culture broth in this study, during the purification we also observed the presence of several minor fractions with stimulatory activities for \emph{S. tanashiensis} development (unpublished result). These fractions may contain desferrioxamine analogues, which are also produced by the \emph{S. griseus} strain. The lower effect of desferrioxamine B on the growth and
development of *S. tanashiensis* in comparison with that of desferrioxamine E (Fig. 1b) suggests that the siderophore uptake mechanism in *S. tanashiensis* has higher affinity for the latter compound than for the former. Müller & Raymond (1984) previously described that *S. pilosus* takes up desferrioxamine B more efficiently than desferrioxamine E, together with information on the conformation of ferric-desferrioxamine complexes. The diversity in uptake specificity may help to prevent competition for the acquisition of the environmental ferric ion in this group of bacteria. The precise characterization of the activity spectrum of each desferrioxamine will reveal the various roles of specific siderophores in streptomycete physiology.

The *S. coelicolor* mutant defective in desferrioxamine biosynthesis (KY1) showed impaired growth and development on Bennett’s/glucose solid medium. Since ferric ion is essential for the viability of nearly all life forms, the growth impairment is probably due to iron deficiency. The partial but marked restoration activity of desferrioxamine E (Fig. 4b) indicates that the substance transports iron into the cell of this organism. However, the partial effect implies that another type of desferrioxamine(s) also plays an essential role in the viability of *S. coelicolor* A3(2). Additionally, the fact that KY1 is still viable suggests that the organism has another ferric uptake system(s). Challis & Ravel (2000) suggested the occurrence of a peptidic siderophore termed coelichelin in *S. coelicolor* A3(2), although this has not been biochemically confirmed. Both KY1 and *S. tanashiensis* grow and develop well on Bennett’s medium supplied with maltose (our unpublished observation). Thus, we currently speculate that the siderophore production in *Streptomyces* species is under complex regulation that links not only to ferric limitation but also to carbohydrate metabolism.

Desferrioxamines have long been known as a fungal growth factor (Prelog, 1963). The uptake of desferrioxamines takes place in the budding yeast *Saccharomyces cerevisiae* (Lesuisse et al., 2001; Yun et al., 2000) as well as pathogenic bacteria such as *Salmonella* (Kingsley et al., 1999) and *Neisseria* species (Schryvers & Stojilkovic, 1999), which do not have the ability to produce their own ferric-chelating agent. *Salmonella* strains have been shown to have receptors for siderophores that they do not synthesize (Kingsley et al., 1999). Since our attempts to amplify des genes from *S. tanashiensis* genomic DNA by PCR have failed, we assume that the *S. tanashiensis* strain does not carry the desferrioxamine biosynthesis gene cluster (unpublished observation). Microbes defective in siderophore production may survive in the natural environment by utilizing the siderophores produced by other organisms.

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**References**


