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Pleiotropic role of the Sco1/SenC family copper chaperone in the physiology of *Streptomyces*

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Summary

Antibiotic production and cell differentiation in *Streptomyces* is stimulated by micromolar levels of Cu^{2+}. Here, we knocked out the Sco1/SenC family copper chaperone (ScoC) encoded in the conserved gene cluster 'sco' (the *Streptomyces* copper utilization) in *Streptomyces coelicolor* A3(2) and *S. griseus*. It is known that the Sco1/SenC family incorporates Cu^{2+} into the active centre of cytochrome oxidase (cox). The knockout caused a marked delay in antibiotic production and aerial mycelium formation on solid medium, temporal pH decline in glucose-containing liquid medium, and significant reduction of cox activity in *S. coelicolor*. The *scoC* mutant produced two- to threefold higher cellular mass of the wild type exhibiting a marked cox activity in liquid medium supplied with 10 μM $CuSO_4$, suggesting that ScoC is involved in not only the construction but also the deactivation of cox. The *scoC* mutant was defective in the monoamine oxidase activity responsible for cell aggregation and sedimentation. These features were similarly observed with regard to the *scoC* mutant of *S. griseus*. The *scoC* mutant of *S. griseus* was also defective in the extracellular activity oxidizing *N*,*N*′-dimethyl-*p*-phenylenediamine sulfate. Addition of 10 μM $CuSO_4$ repressed the activity of the conserved promoter preceding *scoA* and caused phenylalanine auxotrophy in some *Streptomyces* spp. probably because of the repression of *pheA*; *pheA* encodes prephenate dehydratase, which is located at the 3′ terminus of the putative operon structure. Overall, the evidence indicates that Sco is crucial for the utilization of copper under a low-copper condition and for the activation of the multiple Cu^{2+}-containing oxidases that play divergent roles in the complex physiology of *Streptomyces*.

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Introduction

The Gram-positive soil bacteria belonging to *Streptomyces* have a complex developmental life cycle, resembling that of the filamentous fungi. First, this organism forms branched, multinucleoid vegetative hyphae that grow into the substrate. These substrate hyphae then produce aerial mycelia that create a long spore chain by forming septa at regular intervals (Chater, 2006; Chater *et al*., 2010). *Streptomyces* is also characterized by the ability to produce a wide variety of secondary metabolites, which include antibiotics, pigments, and other biologically active substances that have wide industrial applications (Miyadoh, 1993; Hopwood, 2007). Accumulating evidence has indicated that the genetic control mechanisms for morphological differentiation and secondary metabolite formation are linked to each other by the pleiotropic role of multiple regulatory proteins.

It has long been observed that the morphological development and antibiotic production in *Streptomyces* are stimulated by supplying copper to the culture media. Originally, T. Kieser described that the addition of copper sulfate promoted pronounced aerial mycelium formation in *Streptomyces lividans* (Kieser and Hopwood, 1991). Our previous study regarding the deficiency of morphological differentiation and antibiotic production in a mutant strain of *Streptomyces tanashiensis* showed that the addition of 10 μM $CuSO_4$ restored the parental phenotype (Ueda *et al*., 1997). Keijser and colleagues (2000) reported that elevated copper concentration rescued aerial mycelium and spore formation in the mutant for *ram*, encoding membrane translocators and transcriptional regulators involved in the aerial mycelium formation in *S. lividans*.

Copper is essential for life. To date, more than 30 types of copper-containing proteins are known (Messerschmidt *et al*., 2001). The major class includes oxidases such as cytochrome oxidase (cox) complex, which is involved in terminal oxidation in the respiratory chain; lysyl oxidase, which is involved in cross-linkage formation in collagen; and tyrosinase, which is involved in melanin production. Another class includes electron carriers such as plastocyanins and azurines, in which copper alternates between

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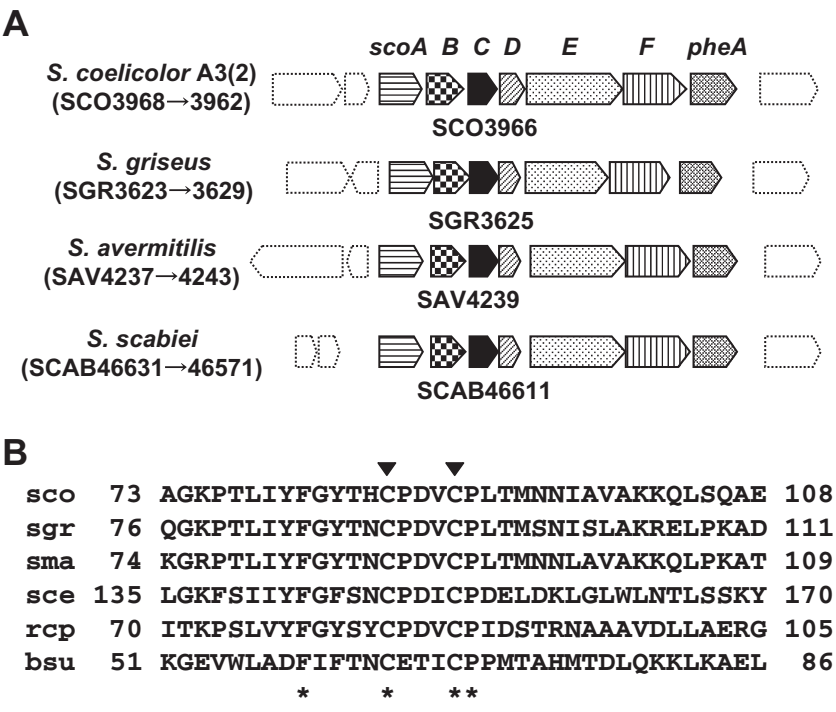

Fig. 1. The *sco* operon of *Streptomyces*.
A. Schematic representation of the *sco* operon distributed in the four genome-sequenced *Streptomyces* spp. (*Streptomyces coelicolor*, *S. griseus*, *S. avermitilis* and *S. scabiei*). Coding sequence numbers are those assigned in each genome sequence database.
B. The amino acid sequence alignment of the active site of ScoC orthologues and the SCO1/SenC family proteins from *S. cerevisiae* (Sco1), *R. capsulatus* (SenC) and *B. subtilis* (YpmQ). The regions containing the two cysteine residues essential for the incorporation of Cu^{2+} into cox (indicated by arrowheads) are compared. Asterisks indicate identical amino acids. sco, *S. coelicolor* A3(2); sgr, *S. griseus*; sma, *S. avermitilis*; sce, *S. cerevisiae*; rcp, *R. capsulatus*; bsu, *B. subtilis*.

the redox states Cu(I) and Cu(II) to serve as an electron acceptor and donor (Messerschmidt *et al*., 2001). The stimulatory effect of copper in *Streptomyces* development suggests that some of the copper-dependent function significantly correlates with the biochemical basis of this bacterial group's complex life cycle.

This article deals with the Sco1/SenC protein family distributed in *Streptomyces coelicolor* A3(2) and *S. griseus*. Sco1 (synthesis of cytochrome *c* oxidase) of *Saccharomyces cerevisiae* (Rigby *et al*., 2008) and SenC of *Rhodobacter capsulatus* (Swem *et al*., 2005) and related proteins have been studied extensively for their role in the construction of cytochrome oxidase. Typically, this protein family serves as copper chaperones delivering copper to the active site of cox. This well-characterized family of protein was encoded in a unique conserved gene cluster consisting of genes for the putative copper-utilizing function in *Streptomyces*. The gene clustering made us think of the possibility that a complex system is involved in the utilization of copper in this group of bacteria and that the detailed characterization of the role of this gene cluster will provide an insight into the copper-dependent developmental physiology. The evidence indicates that the Sco1/SenC homologue is involved in the utilization of copper under a low-copper condition and the incorporation of copper into multiple copper-dependent enzymes including cox that play crucial roles in the initiation of development.

Results

Gene organization of the copper-utilization gene cluster

Figure 1A schematically represents the gene organization of the *sco* (*Streptomyces* copper utilization) locus. The results of sequence similarity (BLAST) and motif (MOTIF) searches as well as the annotation supplied in the genomic database (http://www.genome.jp/) indicated that the gene cluster consists of seven unidirectional coding sequences encoding the following proteins (the amino acid numbers in parentheses are those for *S. coelicolor* proteins): ScoA (284 aa), an integral membrane protein; ScoB (253 aa), a hypothetical protein containing the eukaryotic lysine-oxoglutarate reductase/saccharopine dehydrogenase (LOR/SDH) bifunctional enzyme conserved region; ScoC (216 aa), an Sco1/SenC-like protein containing a 1–16 aa N-terminal prokaryotic membrane lipoprotein lipid attachment site; ScoD (178 aa), a hypothetical protein; ScoE (680 aa), a membrane-associating protein containing an N-terminal prokaryotic membrane lipoprotein lipid attachment site (1–21 aa) and CopC- (13–128 aa) and CopD-like (341–420, 498–540 aa) domains; ScoF (445 aa), a secreted protein containing the Tat (twin arginine translocation) signal peptide (1–64 aa) and the 87–433-aa Dyp (dye-decolorizing peroxidase)-type peroxidase domain; and PheA (310 aa), prephenate dehydratase involved in phenylalanine biosynthesis. It appears likely that most Sco proteins are membrane-associated. The tandem localization of these seven coding sequences

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is conserved in all the genome-sequenced *Streptomyces* spp. (Fig. 1A).

Our database search of each Sco protein indicated that all except for ScoC and ScoE do not show end-to-end similarity to other proteins of known function. ScoB exhibits a similarity with Gle1, a eucaryotic protein involved in mRNA export and translational termination (Alcázar-Román *et al*., 2010; and references cited therein), but its functional similarity with this eukaryotic protein is not known. Genes encoding ScoB homologues are widely distributed in Actinobacteria, by frequently clustering with genes encoding CopC- and/or CopD-like proteins. In *Bacillus subtilis*, an ScoB homologue, YcnI, is encoded in an operon-like structure (*ycnK–ycnJ–ycnI*) whose transcription is induced by copper-limited condition (Chillappagari *et al*., 2009). It is assumed that YcnK and YcnJ encoded in this operon serve as a copper-dependent transcriptional regulator responsible for the copper-dependent repression of this operon and a membrane protein involved in copper uptake respectively (Chillappagari *et al*., 2009).

ScoE shows partial similarity with CopC and CopD, the proteins encoded in the copper resistance operon of *Pseudomonas*. These proteins are assumed to be involved in copper uptake (Cha and Cooksey, 1993; Cooksey, 1994). The copper-binding modes of CopC and homologues have been characterized precisely (Boal and Rosenzweig, 2009). The aforementioned putative copper transporter YcnJ of *B. subtilis* exhibits end-to-end similarity with ScoE. Truncated forms of ScoE lacking CopC- or CopD-like domain are widely distributed in the genome of Actinobacteria. They frequently constitute a cluster with genes encoding homologues of ScoD and ScoF.

The limited information do not provide a clear view with respect to the exact function of Sco proteins in *Streptomyces*, but it appears likely that they are associated with membrane-extracytoplasmic fraction and involved in copper uptake and utilization.

Phenotype of an scoC *mutant*

To study the role of the Sco1/SenC family copper chaperone protein, a marker-less knockout mutant for the corresponding coding sequence (*scoC*) was generated with respect to *S. coelicolor* A3(2) (Fig. 2). As shown in Fig. 2A (upper panels), aerial mycelium formation and pigment antibiotic production in the *scoC* mutant of *S. coelicolor* was delayed significantly. The wild type formed aerial mycelia and produced pigment antibiotics on day 2 on Bennett's medium supplied with 1% glucose, whereas the mutant was pale brown in colour and formed only vegetative hyphae. Scanning electron microscope observation (Fig. 2B) showed the presence of abundant aerial mycelia and spores in the wild type but only substrate hyphae in the *scoC* mutant. However, on day 5, the mutant formed aerial mycelia and pigment to the same extent as did the wild type (Fig. 2A). The delay in development of the *scoC* mutant was recovered by supplying 10 μM $CuSO_4$ to the culture medium (Fig. 2A, lower panels) or by introducing an intact *scoC* using an integration vector (Fig. 2C). The delay of development was also observed when the mutant was cultured on the medium supplied with maltose (Fig. 2A), indicating that the phenotype is not specific to glucose. Similar delay of development was observed with respect to the *scoC* mutant of *S. griseus* (data not shown). These results indicate that the copper utilization by ScoC and subsequent activation of some copper-dependent function(s) is crucial for the initiation of developmental growth.

Previously, Mattatall and colleagues (2000) reported that the two conserved cysteine residues (see Fig. 1B) of YpmQ, the Sco1/SenC homologue of *B. subtilis*, were crucial for the cox activity of this organism. The authors showed that a C64S/C68S mutant did not restore cytochrome oxidase activity in the *ypmQ* mutant. Based on this knowledge, the corresponding mutant (C86S/C90S) was generated for the *scoC* of *S. coelicolor* and introduced into the *scoC* mutant (Fig. 2C). The introduction of this mutated *scoC* did not restore the wild-type phenotype in the *scoC* mutant, indicating that the two cysteine residues are essential for the function of ScoC.

Growth profile and cox activity

Figure 3 shows the growth profiles of the *scoC* mutant of *S. coelicolor* A3(2) cultured in Bennett's liquid medium containing glucose or maltose at 1%. Overall, the *scoC* mutant grew effectively; the growth yield was even higher than that of the wild type. This suggests that ScoC is involved in primary metabolism and affects some energy yielding process. A notable feature observed with respect to the *scoC* mutant was the remarkable pH decline during its early growth in glucose medium. The acidic pH of the culture was then neutralized and alkalified up to 8.6 (Fig. 3). A similar pH profile was also exhibited by the wild type when it was cultured in glucose medium supplemented with 400 μM of bathocuproinedisulfonic acid (BCDA), a copper-specific chelating agent (data not shown). However, such a marked pH shift was not observed when strains were cultured in the maltose medium (Fig. 3). This raises the possibility that the ScoC-dependent function is related to the efficiency in glucose metabolism.

Another notable feature regarding the growth of the *scoC* mutant was the high cellular yield in glucose medium supplied with 10 μM $CuSO_4$ (Fig. 3). In this condition, the mutant grew rapidly and yielded a cellular mass threefold (50 mg ml^{-1}) higher than that of the wild type at

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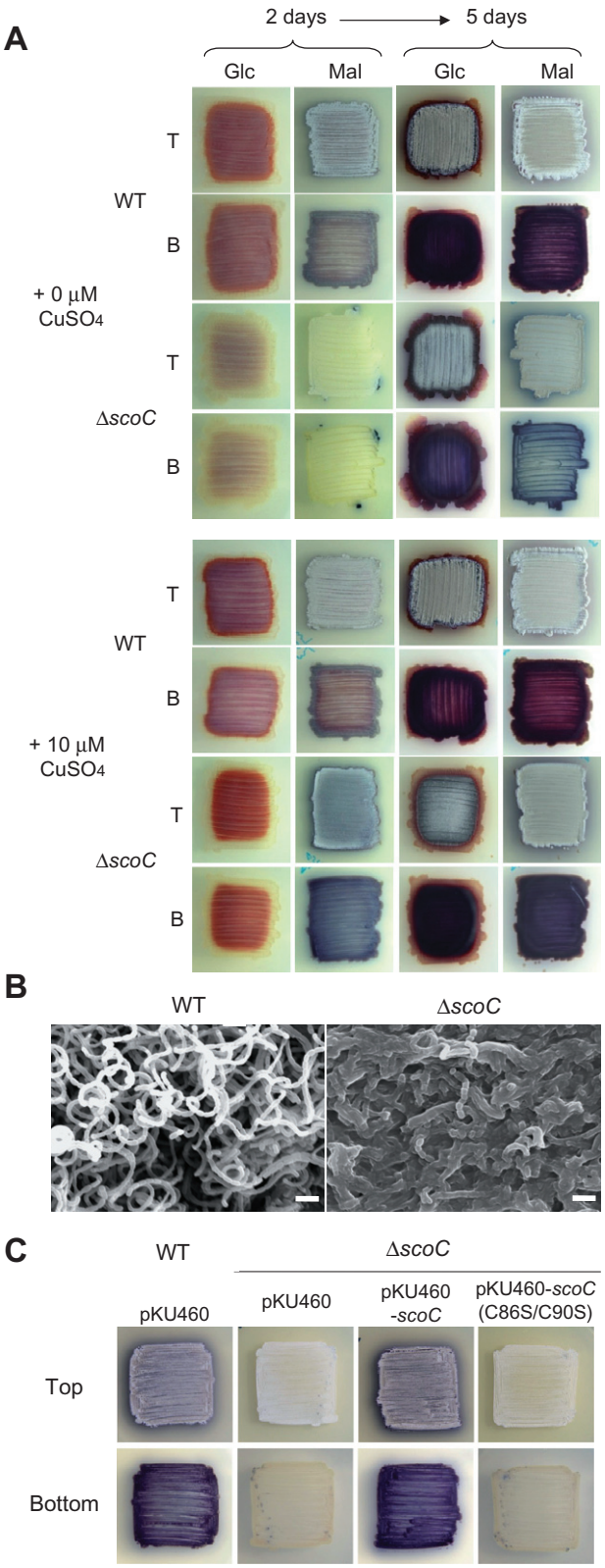

Fig. 2. Phenotype of the *scoC* mutant of *S. coelicolor* A3(2).
A. Macroscopic observation of the wild-type strain (WT) and the *scoC* mutant (Δ*scoC*). Colonies were photographed at days 2 and 5 from the top (T) and bottom (B) to show the occurrence of aerial mycelium and the pigment antibiotic actinorhodin respectively. Strains were grown at 28°C on Bennett's solid medium containing 1% glucose (Glc) and maltose (Mal) without and with 10 μM $CuSO_4$. Colonies forming aerial mycelia appear white, whereas those forming only substrate mycelium appear yellow or light brown. The purple or blue colour is due to the production of the pigmented antibiotic, actinorhodin. *Streptomyces coelicolor* also produces an intracellular red pigment undecylprodigiosin.
B. Scanning electron microscopy observation of the wild type and the *scoC* mutant grown for 3 days on Bennett's/glucose solid medium. Bar, 2 μm.
C. Macroscopic observation of the genetically complemented *scoC* mutant. The *scoC* mutants harbouring the empty integration vector (pKU460), and pKU460 containing the intact (pKU460-*scoC*) and mutated [pKU460-*scoC* (C86S/C90S)] *scoC*-coding sequence, were grown on Bennett's/maltose medium for 2 days. The wild type harbouring pKU460 is also shown.

128 h. This growth promotion by $CuSO_4$ was abolished when 400 μM BCDA was supplied to the medium (data not shown).

Because Sco1/SenC proteins are involved in the incorporation of copper into cox, its activity was measured using *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD; see *Experimental procedures*) with respect to the cells grown in the aforementioned conditions. As shown in Fig. 4A (upper), the wild type of *S. coelicolor* exhibited high cox activities during early growth phases in Bennett's/glucose medium. In contrast, the *scoC* mutant exhibited remarkably low activities. The activity in the *scoC* mutant, however, was restored by the addition of 10 μM $CuSO_4$, to a level higher than that of the wild type, and this restoration by $CuSO_4$ was abolished by the addition of 400 μM BCDA. A similar cox activity profile was obtained with respect to *scoC* mutant of *S. griseus* (Fig. 4A, lower).

To study the correlation of other *sco* genes with cox, apramycin-resistant knockout mutants were generated, and their cox activity in the early growth phase (24 h) was studied by using Bennett's/glucose and maltose media (Fig. 4B). With the exception of *scoC* and *scoF*, cox activity was largely unaffected by the knockout. The activity in all mutants was remarkably reduced by the addition of BCDA. The *scoF* mutant showed extremely low cox activities in both glucose and maltose media; the activity in maltose medium was below the detectable level. The activity in the *scoF* mutant was restored to the same level as in the *scoC* mutant (i.e. twofold higher than the wild-type activity) by the addition of 10 μM $CuSO_4$ (data not shown). This result indicated that the putative secreted protein containing a peroxidase-like domain encoded by *scoF* also plays an important role in the activity of the terminal oxidase.

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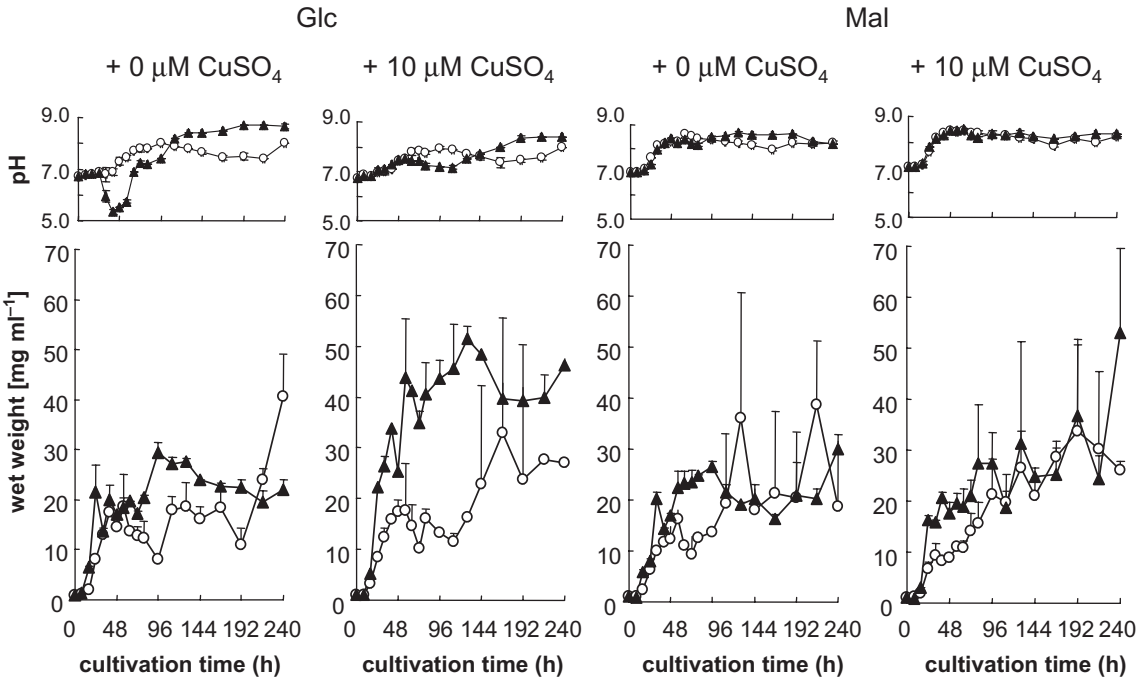

Fig. 3. Transition of pH and growth yield in a 10-day culture of *S. coelicolor* A3(2) in liquid medium. The wild-type strain (open circle) and the *scoC* mutant (closed triangle) were grown in Bennett's liquid medium containing 1% glucose (Glc) and maltose (Mal) without and with 10 μM $CuSO_4$. Wet cellular weight and pH of culture broth were measured every 12 h.

Cell aggregation and monoamine oxidase activity

The *scoC* mutant also differed from the wild type in terms of the cell aggregation phenotype. The wild-type cells of *S. coelicolor* A3(2) and *S. griseus* in the early growth phase (24 h) rapidly precipitated when collected from the shaking flask into a standing test tube (Fig. 5A). Cell in this growth phase aggregated effectively and formed large pellets (micrographs of *S. coelicolor* cells are shown in Fig. 5B). In contrast, the *scoC* mutants at the corresponding growth phase neither precipitated (Fig. 5A) nor formed pellets (Fig. 5B). Aggregation was restored through genetic complementation or supplementation of 10 μM $CuSO_4$ to the mutants.

Recently, Koebsch and colleagues (2009) reported that the cell aggregation of *S. lividans*, the close relative of *S. coelicolor* A3(2), is based on the activity of HyaS, a secreted copper-containing monoamine oxidase that catalyses the oxidation of monoamine to form aldehyde, NH_3 and H_2O_2. To evaluate the possible linkage of the aforementioned aggregation phenotype with HyaS activity, we used the 3,3′-diaminobenzidine method to detect *in situ* enzyme activity (Koebsch *et al*., 2009). Wild-type cells of *S. griseus* were stained dark because of the high level of H_2O_2, whereas *scoC* mutant cells appeared yellowish (background; Fig. 5C). The enzyme activity was restored by genetic complementation or supplementation with 10 μM $CuSO_4$.

To verify the involvement of HyaS in cell aggregation, a knockout mutant was generated in *S. griseus*. The mutant of the corresponding coding sequence (SGR 3840) was defective in cell precipitation and aggregation and in *in situ* monoamine oxidase activity (Fig. 5C). This suggests that the enzyme encoded by this coding sequence is involved in cell aggregation in *S. griseus*.

Phenol oxidase activity

We previously reported that *S. griseus* retains a phenol-oxidizing activity and identified a laccase-like copper-containing extracytoplasmic oxidase EpoA (Endo *et al*., 2002; 2003). Based on this knowledge, the *scoC* mutant of *S. griseus* was assessed for extracellular phenol-oxidizing activity by using *N,N′*-dimethyl-*p*-phenylenediamine sulfate (DMP) as a substrate (*Experimental procedures*). The DMP-oxidizing activity in the crude extract of the *scoC* mutant cells was lower than that in the wild-type cells (Fig. 6). A marked DMP-oxidizing activity was restored in this mutant by supplying 10 μM $CuSO_4$ to the culture medium.

Transcription of sco

To study the transcription level of the *sco* genes, S1 nuclease mapping was performed in *S. griseus* using the

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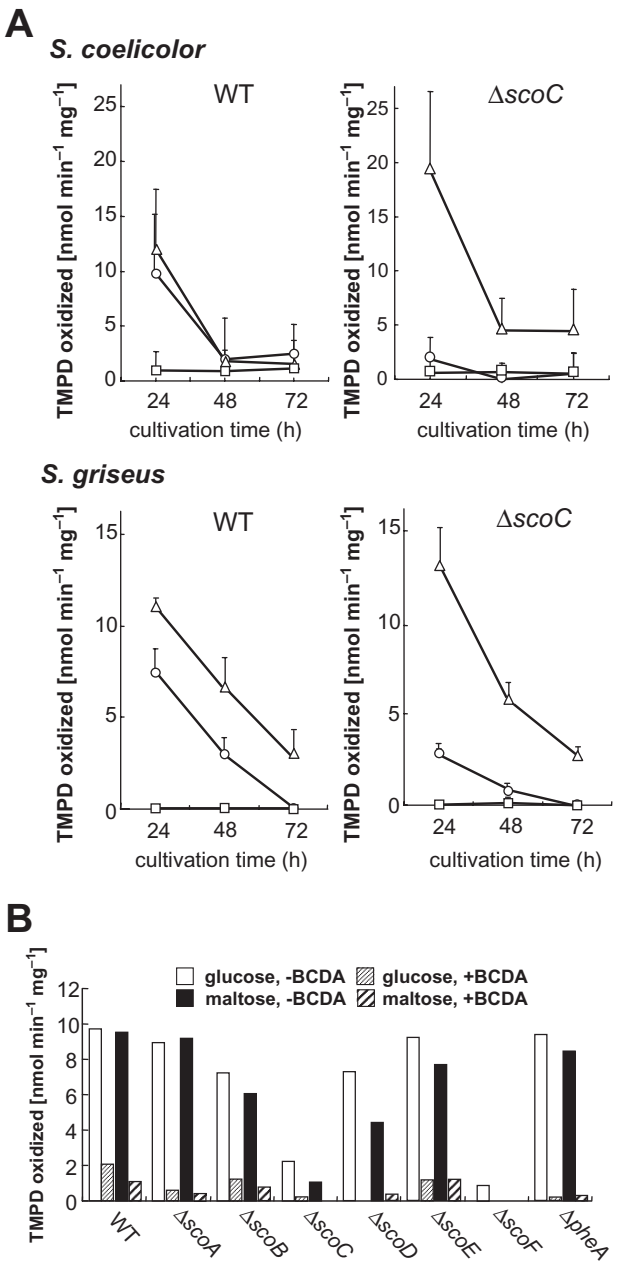

Fig. 4. Cox activity of the *sco* mutants.
A. Transition of cox activity in the wild-type strain and the *scoC* mutant of *S. coelicolor* A3(2) (upper) and *S. griseus* (lower). TMPD-oxidizing activity was measured with respect to the cells grown for 24, 48 and 72 h in Bennett's/glucose (*S. coelicolor*) or YMP/glucose (*S. griseus*) liquid medium supplied with neither $CuSO_4$ nor BCDA (open circle), 10 μM $CuSO_4$ (open triangle), or 10 μM $CuSO_4$ and 400 μM (*S. coelicolor*) or 200 μM (*S. griseus*) BCDA (open square). Data are means of duplicated measurements.
B. Cox activity of the other *sco* mutants of *S. coelicolor* A3(2). Each mutant was grown in Bennett's/glucose and maltose liquid medium supplied without and with 400 μM BCDA for 24 h, and studied for TMPD-oxidizing activity. Data for the wild type and *scoC* mutant are also shown.

region preceding *scoA* as a probe (Fig. 7A and B). The high-resolution analysis (Fig. 7A) assigned a single transcriptional start site at the G residue 4 bp upstream from the A residue of the translational start codon (ATG) of ScoA (Fig. 7C). The nucleotide sequences of the potential −35 and −10 regions were conserved in *S. griseus*, *S. coelicolor* and *S. avermitilis* (Fig. 7C). The low-resolution analysis (Fig. 7B) showed that the promoter of the wild type was activated throughout the 3-day culture on YMP glucose medium, whereas it was notably repressed by the addition of 10 μM $CuSO_4$.

The conserved tandem localization of the *sco* genes suggested that these coding sequences constitute a polycistron and that their expression is repressed simultaneously by exogenous copper. This raised the possibility that phenylalanine biosynthesis is repressed by copper because *pheA* is a constituent of the proposed operon structure. Hence, we studied growth on minimal medium, and discovered that exogenous $CuSO_4$ caused phenylalanine auxotrophy in *S. coelicolor* A3(2) and *S. avermitilis*; these organisms did not grow on minimal agar media supplied with 10 μM $CuSO_4$ but did grow on media supplemented with phenylalanine (Fig. 8). This is probably due to the dependence of *pheA* expression on the transcription from the copper-repressive promoter preceding *scoA* in these two species. On the other hand, the growth of *S. griseus* on the medium supplied with $CuSO_4$ indicates that *pheA* of this organism is transcribed by another promoter. The long intergenic region (285 bp) between *scoF* and *pheA* of *S. griseus* may contain a promoter region. We also assessed the growth of four environmental isolates and found that the two strains exhibited copper-dependent phenylalanine auxotrophy (Fig. 8).

Discussion

This study revealed that the Sco1/SenC family protein ScoC is crucial for the activity of copper-dependent enzymes in *Streptomyces*. The occurrence of *scoC* within the probable operon structure suggests the functional correlation of ScoC with other Sco proteins. Although we do not yet know the exact function of each Sco protein, the wide distribution of the *sco* operon in *Streptomyces* makes us assume that the possible coordinated function of Sco is crucial for copper utilization by this group of soil bacteria. The similarity of phenotypes caused by the knockout of *scoC* between the two divergent species, *S. coelicolor* A3(2) and *S. griseus*, reinforce the view that the physiological background for copper requirement is common to *Streptomyces*. The transcriptional repression observed with exposure to high concentrations of copper ions (10 μM $CuSO_4$ = *c.* 0.6 ppm Cu^{2+}) indicates that the *sco* operon is activated under low-copper conditions. Normally, soil solution contains copper at 0.01–0.06 ppm (Bowen, 1966).

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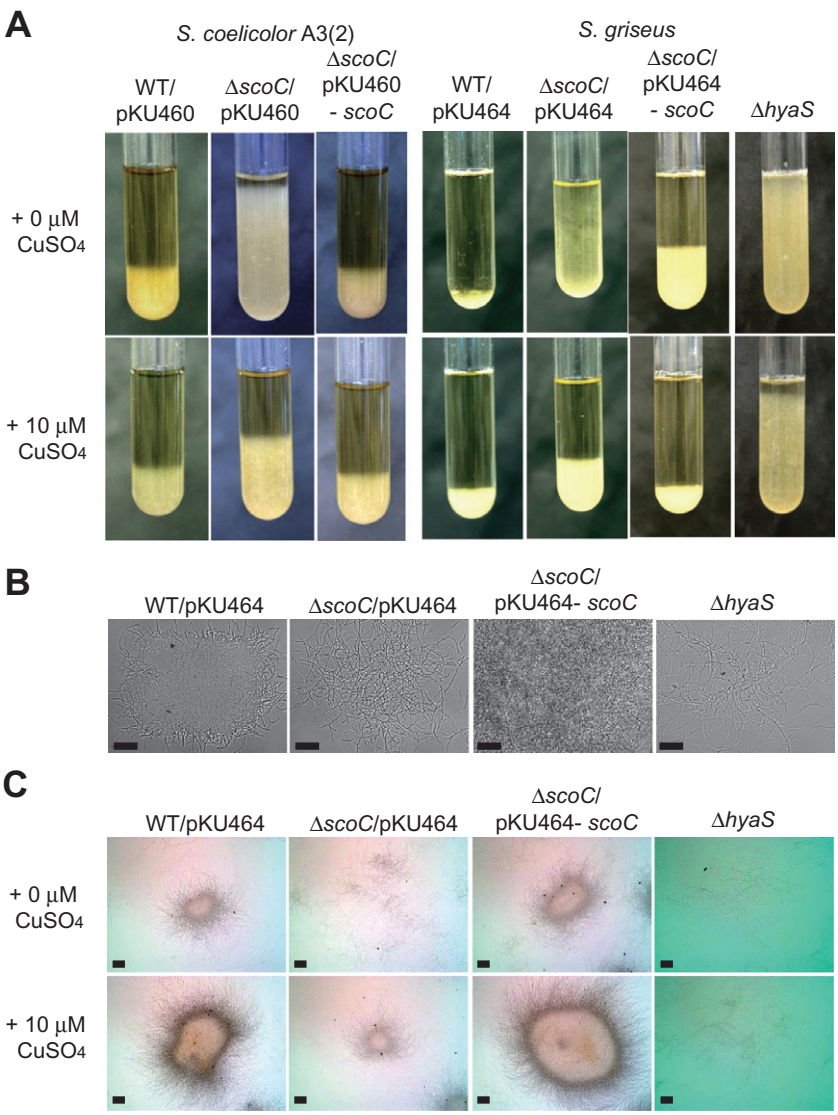

Fig. 5. Cell aggregation and precipitation phenomenon in *S. coelicolor* A3(2) and *S. griseus*.
A. Precipitation in the early growth phase. Culture broth containing the cells grown for 24 h in Bennett's/glucose (*S. coelicolor*) or YMP/glucose (*S. griseus*) liquid medium (without/with 10 μM $CuSO_4$) was collected from the shaking flask into a test tube, left to stand for 3 min and photographed.
B and C. (B) Optical micrograph and (C) *in situ* activity stain for monoamine oxidase of the *S. griseus* cells grown for 24 h in YMP/glucose liquid medium. Activity stain was performed by the method described by Koebsch and colleagues (2009). Strain designations for *S. coelicolor* A3(2) are as in Fig. 2. The *S. griseus* strains shown are: the wild type (WT) and *scoC* mutant (Δ*scoC*) harbouring the empty plasmid (pKU464) and pKU464 carrying the intact *scoC* (pKU464-*scoC*). A *hyaS* mutant (Δ*hyaS*) is also shown. Bars, 20 μm (B) and 50 μm (C).

Hence, it is predicted that *Streptomyces* cells in normal environments are expressing *sco* to utilize copper.

Sco1/SenC family proteins are known for their involvement in the production of cox in several microorganisms including *S. cerevisiae*, *Rhodobacter* spp., *Pseudomonas aeruginosa* and *B. subtilis* (Frangipani and Haas, 2009). Biochemical and structural evidence has revealed that this family of proteins is involved in the incorporation of the Cu^{2+} ion into the dinuclear Cu_A site of cox complex. The marked amino acid sequence similarity as well as the significance of the two conserved cysteine residues (Fig. 1B) indicates that ScoC of *Streptomyces* has a similar function. The remarkably low cox activity in the *scoC* mutant (Fig. 4A) may be due to a defect in its ability to incorporate Cu^{2+}. The restoration of cox activity in the *scoC* mutant by the addition of $CuSO_4$ indicates that the incorporation of Cu^{2+} into cox is independent of ScoC under high Cu^{2+} conditions.

The involvement of ScoC in the synthesis of cox suggests that the phenotype of the *scoC* mutant is based on a deficiency in the terminal respiratory chain. We have successfully generated knockout mutants for the genes encoding cox domains and observed that they have defective developmental growth (our unpublished results). Recently, Worrall and Vijgenboom (2010) published a review article on the copper-dependent function of *Streptomyces* in which they refer to an unpublished observation regarding the *scoC* homologue of *S. lividans* and suggest that cox activity is crucial for development in this organism. The delayed developmental growth of the *scoC* mutants of *S. coelicolor* and *S. griseus* may also reflect a correlation between terminal oxidation efficiency and developmental regulation. How energy metabolism is related to developmental fate is an important issue in terms of both basic physiology and industrial application of *Streptomyces*.

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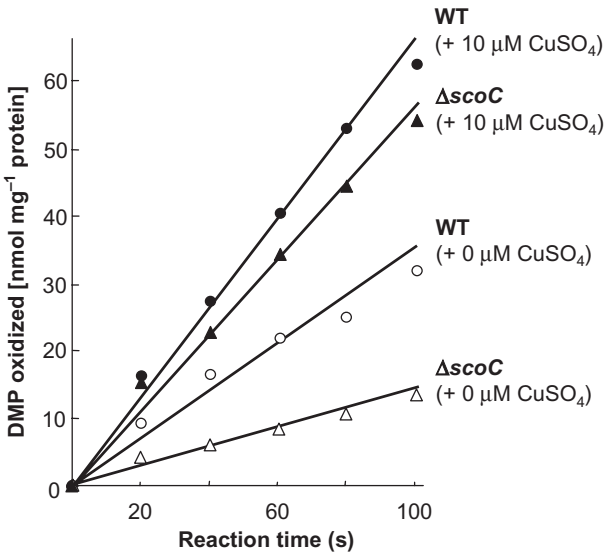

Fig. 6. DMP-oxidizing activity of *S. griseus*. The wild-type strain and *scoC* mutant of *S. griseus* grown for 5 days in Bennett's/maltose solid medium (without/with 10 μM $CuSO_4$) were studied for DMP-oxidizing activity by measuring the increment of absorbance at 550 nm (Endo *et al*., 2003).

Cultivation of the *scoC* mutant in glucose medium caused a temporal pH decline (Fig. 3). Similar glucose medium acidification was observed with respect to the wild-type culture in the presence of BCDA (data not shown). This phenomenon may also be the result of low cox activity. *Streptomyces* retains the copper-independent cytochrome *bd* quinol oxidase. It is known that this type of terminal oxidase is activated under microaerophilic condition (Junemann, 1997). This alternative terminal oxidase may compensate for the low cox activity in the *scoC* mutant, but its energy production efficiency would be relatively low; this would significantly affect the metabolic balance during glucose assimilation, thus producing the unusual acidification that was observed.

A noteworthy observation was that the supply of $CuSO_4$ to the glucose medium markedly promoted the growth of the *scoC* mutant, yielding a cellular mass that was two to three times higher than that of the wild type (Fig. 3). This surprising evidence suggests that *scoC* may negatively regulate some copper-dependent energy-yielding process, and that its deregulation causes outgrowth if a high level of Cu^{2+} is available. A simple explanation is that ScoC regulates cox activity both in a positive and in a negative manner; it may activate and deactivate cox by

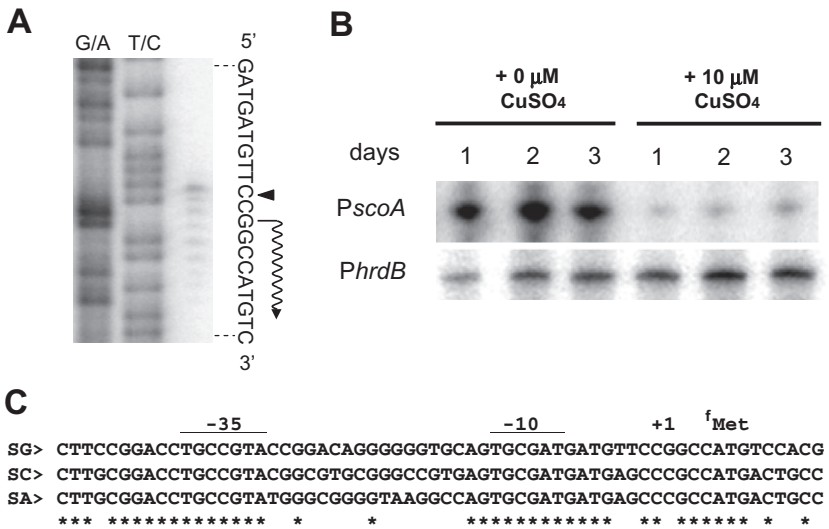

Fig. 7. S1 protection analyses of the promoter preceding *scoA* of *S. griseus*.
A. High-resolution analysis for the determination of transcriptional start site. Maxam-Gilbert sequencing ladders (G+A and T+C reactions) were generated using the same ^{32}P-labelled fragment as the probe DNA. The position of the S1-protected fragment is denoted by an arrowhead and the transcriptional start site assigned to the residue is denoted by a wavy arrow. The fragments generated by the chemical-sequencing reactions migrate 1.5 nt further than the corresponding fragments generated by the S1 nuclease digestion of the DNA–RNA hybrids (half a residue from the presence of the 3′-terminal phosphate group and 1 residue from the elimination of the 3′-terminal nucleotide; Sollner-Webb and Reeder, 1979). The RNA prepared from the wild-type cells grown for 2 days on YMP/glucose solid medium was used for hybridization.
B. Low-resolution analysis. Activities of the promoters preceding *scoA* and *hrdB* (housekeeping sigma factor gene; control) were estimated by the signal intensities of protected fragments. RNA was isolated from cells grown for 1–3 days on YMP/glucose solid medium without and with 10 μM $CuSO_4$.
C. Nucleotide sequence of the promoter region preceding *scoA*. Sequence alignment of the corresponding regions of *S. griseus* (SG), *S. coelicolor* A3(2) (SC) and *S. avermitilis* (SA) is shown. The transcriptional start position determined by the high-resolution analysis in *S. griseus* is designated as +1, and the potential –35 and –10 regions are indicated. The position of the translational initiation codon (ATG) is also indicated.

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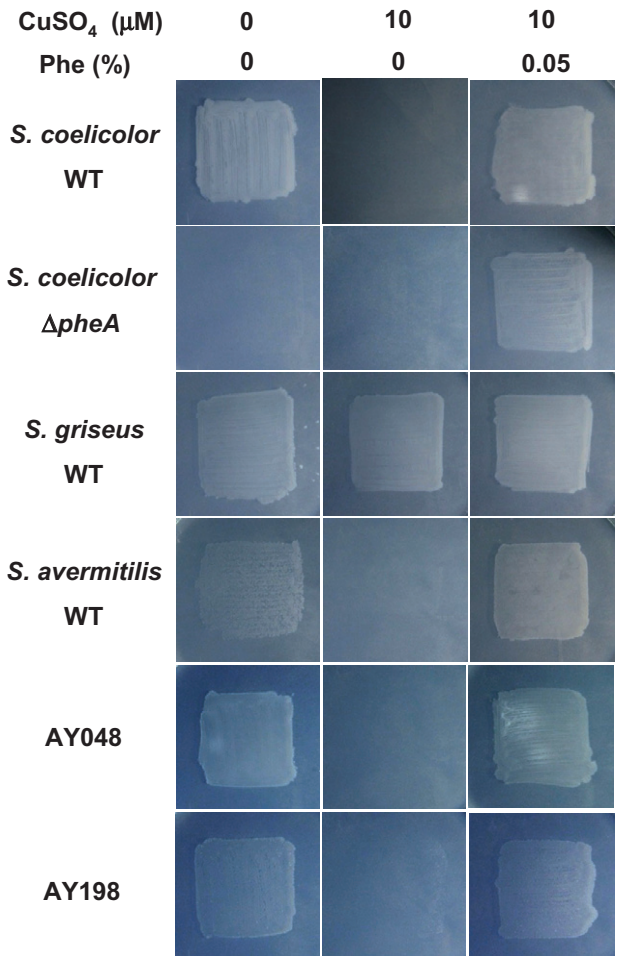

Fig. 8. Copper-dependent phenylalanine auxotrophy in *Streptomyces* spp. Strains were cultured on minimal agar medium without/with 10 μM $CuSO_4$ and 0.05% phenylalanine. Colonies of the wild type and *pheA* mutant of *S. coelicolor* A3(2), wild-type strains of *S. griseus* and *S. avermitilis*, and two environmental isolates are shown. Patches were photographed at 2 days.

controlling the efficiency of Cu^{2+} incorporation. This view is supported by the remarkably high cox activity in the *scoC* mutant under the Cu^{2+}-plus condition (Fig. 4A). The constitutive hyperactivation of cox may enable a high degree of energy recovery and effective propagation of vegetative cells. However, the marked reduction of cox activity observed in the later growth phase (48 and 72 h) in both the wild type and the *scoC* mutant (Fig. 4A) suggests the presence of an *scoC*-independent repression mechanism controlling the cox activity in the transition phase.

The *scoC* knockout also affected the activity of secreted copper-containing enzymes including monoamine oxidase and phenol oxidase. This indicates that copper utilization by ScoC is crucial for the creation of not only cox but also other copper-containing enzymes. The putative lysylamine oxidase HyaS has been shown to be involved in cell aggregation and pellet formation in *S. lividans* (Koebsch *et al*., 2009). This study confirmed that HyaS plays a similar role in *S. griseus*. Data from eukaryotic lysyl oxidases suggest that this enzyme has adhesion properties and that amine oxidase activity induces cross-linkage formation between hyphae-associated proteins and related substances (Koebsch *et al*., 2009). Similar surface cross-linkage can be formed due to the activity of phenol oxidases, and this cross-linkage induces polymerization of phenolics. In fungi, it is suggested that polymerization of oxidized phenolic substrates contributes to the rigidity of differentiating cells (Burke and Cairney, 2002). We previously showed that phenol oxidases including tyrosinase and laccase generate a substance that stimulates aerial mycelium formation in *S. griseus* (Endo *et al*., 2002). Thus, it appears that the activities of the copper-dependent oxidases may be significantly correlated with the complex cell structure in *Streptomyces*, and that copper utilization by Sco is fundamental to these functions.

The transcriptional analysis revealed that the promoter preceding *scoA* is repressed by exogenous supply of copper. This indicates that the promoter is controlled by a copper-dependent transcriptional regulatory mechanism. The identity of the promoter sequence (Fig. 7C) implies that the copper-dependent repression widely occurs in *Streptomyces*. Transcriptional regulation regarding metal homeostasis is well characterized with respect to the acquisition of ferric, in which ferric-bound form of Fur (ferric uptake regulator) protein represses the transcription of genes involved in the ferric uptake (Cornelis *et al*., 2009). Involvement of Fur family regulators in metal homeostasis is also known with respect to zinc, manganese and nickel (Shin *et al*., 2007, and references cited therein). In *S. coelicolor* A3(2), Zur (zinc uptake regulator) controls zinc homeostasis via the activation of the redox-regulated sigma factor σ^R (Owen *et al*., 2007).

Knowledge about copper homeostasis in prokaryotes has been mostly obtained with respect to the response to the excessive level of copper. Multiple types of transcriptional repressors have been identified to be the regulator responsible for the copper-dependent transcriptional activation of copper homeostasis genes (Solioz *et al*., 2010, and references cited therein). Meanwhile, response to copper limitation has been known only for the aforementioned *ycnK–ycnJ–ycnI* operon of *B. subtilis* (Chillappagari *et al*., 2009). In this system, it is predicted that YcnK containing domains for DNA and Cu(I) binding is involved in the copper-dependent transcriptional repression of this operon. Currently, we speculate that the copper-dependent transcriptional repression in *Streptomyces* is based on a different regulatory mechanism since we cannot find YcnK homologue in *Streptomyces* genome (our unpublished observation).

We discovered that the copper-dependent phenylalanine auxotrophy occurs in certain *Streptomyces* spp. This

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means that these bacteria depend their ability to synthesize proteins on exogenous supply of phenylalanine under high copper conditions. Currently, we cannot explain why such auxotrophy occurs, but speculate that it improves the survival of these organisms in the natural environment. The details about the role of each copper-dependent function require further investigation, but the evidence indicates that the utilization of copper is a crucial factor for *Streptomyces* physiology.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The wild-type strain of *S. coelicolor* A3(2) M145 was obtained from John Innes Centre, UK, and that of *S. griseus* IFO13350 from the Institute for Fermentation, Osaka, Japan. *Streptomyces avermitilis* MA-4680 was provided by H. Ikeda at Kitasato University. The *Escherichia coli* DH5α strain (Takara Shuzo; Kyoto, Japan) was used as a host for conventional DNA manipulation, and the GM2163 strain, a methylation-deficient strain, was used for generating disruption cosmids. pUC19 (Takara Shuzo) was used for general DNA manipulation. TA cloning of polymerase chain reaction (PCR)-generated DNA fragments was done with the help of pMD19 (Takara Shuzo). pUWLFLP carrying an Flp recombinase gene (Fedoryshyn *et al*., 2008) was used for the construction of a marker-less mutant. The integration plasmids pKU460 and pKU464 carrying the phiC38 and phiBT1 integrase gene, respectively, pKU474 carrying a kanamycin-resistance gene cassette flanked by the *loxP* sequences, and pKU250*cre* carrying the Cre recombinase gene at the PstI site of pKU250 (Komatsu *et al*., 2010) were obtained from H. Ikeda at Kitasato University. pIJ773 and pIJ774 were obtained from John Innes Centre. The enzymes used for DNA manipulation were purchased from Takara Shuzo. Chemicals were purchased from Kokusan (Tokyo, Japan) if not indicated otherwise. The standard experimental conditions and materials used for the genetic manipulation of *E. coli* and *Streptomyces* strains were adopted from those described by Maniatis and colleagues (1982) and Kieser and colleagues (2000) respectively. *Streptomyces* strains were cultured at 28°C in Bennett's/sugar medium [composition: 1 g l^{-1} yeast extract (Difco Laboratories, Detroit, Michigan), 1 g l^{-1} meat extract (Kyokuto, Tokyo, Japan), 2 g l^{-1} NZ amine (Wako Pure Chemical Industries, Osaka, Japan) and 10 g l^{-1} of the appropriate sugar (pH 7.2)], R2YE medium (Kieser *et al*., 2000), YMP/sugar medium (Komatsu *et al*., 2006), tryptic soy broth (TSB; Nissui, Tokyo, Japan) and minimal medium [composition: 0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4$ $7H_2O$, 0.01 g l^{-1} $FeSO_4$ $7H_2O$, 1.0 g l^{-1} $(NH_4)_2SO_4$ and 10 g l^{-1} of the appropriate sugar (pH 7.2)]. $CuSO_4$ (usually at 10 μM) and phenylalanine (0.05%) were supplied when required. BCDA (Sigma-Aldrich, Tokyo, Japan), a Cu^{2+}-specific chelating agent, was added at 400 μM. Solid media were prepared by adding 1.5% agar to the above mixtures. *Escherichia coli* transformants were selected in media containing a final concentration of 50 μg ml^{-1} ampicillin (Wako), neomycin (Wako) or apramycin (Sigma-Aldrich Japan, Tokyo, Japan). *Streptomyces* transformants were selected in media containing a final concentration of 20 μg ml^{-1} kanamycin or 5 μg ml^{-1} apramycin.

Gene disruption

Disruption of *sco* genes was performed using a homologous recombination technique based on Redirect technology (Gust *et al*., 2003). The cosmid clone (SCO1E4) used for disruption construction in *S. coelicolor* was obtained in this study. The apramycin-resistance gene cassettes used for each disruption construction were prepared by PCR using the primer sets DisAc-F/DisAc-R (*scoA*), DisCc-F/DisCc-R (*scoC*), DisDc-F/DisDc-R (*scoD*), DisEc-F/DisEc-R (*scoE*), DisFc-F/DisFc-R (*scoF*) and Dis*pheA*-F/Dis*pheA*-R (*pheA*) using pIJ733 (obtained from John Innes Centre) as a template (the oligonucleotide primer sequences are summarized in Table S1). The cassettes were then substituted for the corresponding coding sequence by *in vivo* recombination using λ RED. The resulting apramycin-resistant cosmids purified from *E. coli* GM2163 were introduced into the wild-type strain of *S. coelicolor*. Apramycin-resistant recombinants were then screened and checked for true recombination by PCR using appropriate primer sets.

A marker-less mutant for *scoC* of *S. coelicolor* was constructed using pUWLFLP to direct the expression of Flp recombinase (Fedoryshyn *et al*., 2008). The introduction of this plasmid into the aforementioned apramycin-resistant *scoC* mutant eliminated the apramycin resistance cassette flanked by Flippase recognition target (FRT) sites. The pKU460-*scoC* used for genetic complementation was constructed as follows: DNA fragments containing the coding sequence for *scoC* and the promoter region preceding *scoA* were amplified by PCR using the P*scoA*c-F/P*scoA*c-R and *scoC*c-F/*scoC*c-R primers respectively. The ligation mixture of these two fragments was used as a template for PCR using primers P*scoC*c-F/*scoC*c-R to amplify the correctly fused DNA fragment. The resultant fragment was then cloned onto pMD19 by TA-cloning, recovered as a HindIII-digested fragment and re-cloned onto pKU460 to generate pKU460-*scoC*. pKU460-*scoC* was then introduced into the *scoC* marker-less mutant by transformation to generate a kanamycin-resistant transformant carrying the plasmid integrated at the phiC38 site. The mutant *scoC* (C86S/C90S) was generated as follows: two DNA fragments amplified from pKU460-*scoC* by PCR using the P*scoA*c-F/*scoC*cmt-MR and *scoC*cmt-MF/*scoC*c-R primers were mixed and used as a template for the second PCR using the *scoA*c-F/*scoC*c-R primers. The resulting mutated *scoC* cassette was treated as described above to construct pKU460-*scoC* (C86S/C90S).

To disrupt *scoC* in *S. griseus*, the flanking regions were amplified by PCR using primers DisCg-F/DisCg-MR and DisCg-MF/DisCg-R. These fragments were digested with BamHI and BglII and ligated to a kanamycin-resistance cassette flanked by *loxP* sequences. The kanamycin-resistance cassette was prepared by PCR with primers aphII-F/aphII-R using pKU474 as a template. The ligation mixture was used as a template for the second PCR using primers DisCg-F/DisCg-R to amplify the correctly fused fragment. The amplicon was cloned onto pMD19 by TA cloning, recovered as an EcoRI/HindIII-digested fragment, and cloned onto pUC19. The resulting disruption plasmid was isolated from *E. coli* GM2163 and introduced into the wild-type strain of *S. griseus* to obtain kanamycin-resistant recombinants. The mutants were checked for true recombination by PCR using appropriate primer sets. The marker-less *scoC* mutant was

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constructed by introducing pKU250*cre* into the kanamycin-resistant *scoC* mutant. The introduction of this plasmid directed the xylose-inducible expression of Cre recombinase, which catalysed recombination between the two *loxP* sequences, eliminating the kanamycin-resistance cassette (Komatsu *et al*., 2010). pKU250*cre* lacking the SCP2 stability region (Komatsu *et al*., 2010) was cured from the cell by cultivating the transformant in a thiostrepton-free medium. To construct the complementation plasmid pKU464-*scoC*, the promoter and coding regions of *scoC* of *S. griseus* were amplified using primers P*scoA*g-F/P*scoA*g-R and *scoC*g-F/*scoC*g-R, respectively, recovered as SphI/BamHI-digested fragments, and ligated with BamHI-digested pKU464 by three-fragment ligation. Disruption of *hyaS* in *S. griseus* was based on Redirect technology (see above). The cosmid clone (SGR2G5) used for disruption was obtained in this study. The apramycin-resistance gene cassette was prepared by PCR using the primer sets DishyaSg-F/DishyaSg-R (Table S1) and processed similarly as described above.

Scanning electron microscopy

Cells were fixed with 2% osmium tetroxide for 30 h and then dehydrated by freeze-drying. Each specimen was sputter-coated with palladium/gold using an E-1010 ion sputter (Hitachi, Tokyo, Japan) and scanned on a VE8800 scanning electron microscope (Keyence, Tokyo, Japan).

Enzyme activity measurements

The activity of cox was measured by using TMPD as an electron donor (Frangipani and Haas, 2009), as this makes it possible to quantify whole-cell cox activity as an increment in absorbance at $\lambda = 520$ nm. *Streptomyces coelicolor* and *S. griseus* strains grown in Bennett's and YMP liquid media, respectively, were collected every 24 h and washed twice in 0.9% (w/v) NaCl. Approximately 5 mg of cells were added to 1.4 ml of 33 mM potassium phosphate buffer (pH 7.0) in a cuvette. The reaction was started by adding 5 μl of 0.54 M TMPD; cox activity was expressed as nanomolar TMPD oxidized per minute per milligram of cells, using 6.1 as the millimolar extinction coefficient for TMPD (Matsushita *et al*., 1982).

HyaS activity was detected according to the description by Koebsch and colleagues (2009). Cells were grown at 28°C by shaking (300 r.p.m.) in a test tube (ϕ18 mm) containing 10 ml of TSB liquid medium. Cells pre-cultured for 3 days were harvested by centrifugation at 1630 *g* for 5 min, washed twice with saline and inoculated into the main culture at 1 mg (wet weight) ml^{-1}. Then, 0.1 mg ml^{-1} 3,3′-diaminobenzidine was added to this culture at 8 h. Cells were collected at 24 h and observed by using a BZ-9000 optical microscope (Keyence, Tokyo).

Laccase-like phenoloxidase activity was measured using DMP as a substrate (Endo *et al*., 2003). *Streptomyces griseus* cells (*c.* 0.3 g) grown on cellophane-covered Bennett's/maltose agar medium were collected and suspended in 1 ml of 100 mM NaH_2PO_4 containing 10% glycerol (pH 6.5), disrupted by sonication and centrifuged at 18 000 *g* for 10 min at 4°C. Then, 0.1 ml of the resultant supernatant containing 4.0 mg ml^{-1} protein was added to 0.1 ml of DMP solution (20 mg ml^{-1}) and 2.5 ml of citrate buffer (prepared by mixing 37 mM citric acid with 180 mM Na_2HPO_4 to adjust pH to 6.5). The initial increase of absorbance at 550 nm was scanned during a 100 s incubation at 40°C using a U-2800A spectrophotometer (Hitachi, Tokyo, Japan). The oxidizing activity was expressed as micromolar DMP oxidized per microgram cellular protein, using 0.2 as the micromolar extinction coefficient for DMP (Clutterbuck, 1972).

S1 nuclease mapping

Methods and conditions for RNA preparation and S1 nuclease mapping were described previously (Kieser *et al*., 2000; Kelemen *et al*., 2001; Takano *et al*., 2007). The probes were prepared by PCR using primer sets SS1-F/SS1-R* (P_{scoA}) and HS1-F/HS1-R* (P_{hrdB}; Table S2; primers with an asterisk were labelled at the 5′-end with [γ-^{32}P]-ATP using T4-polynucleotide kinase).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotide primers used in disruption and complementation construction.

Table S2. Oligonucleotide primers used in S1 protection analysis.

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