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

Masahiro Fujimoto, Akio Yamada, Junpei Kurosawa, Akihiro Kawata, Teruhiko Beppu, Hideaki Takano and Kenji Ueda*

Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa 252-0880, Japan.

Summary

Antibiotic production and cell differentiation in *Streptomyces* is stimulated by micromolar levels of Cu²⁺. Here, we knocked out the Sco1/SenC family copper chaperone (ScoC) encoded in the conserved gene cluster ‘sco’ (the *ScoC* copper utilization) in *Streptomyces coelicolor* A3(2) and *S. griseus*. It is known that the Sco1/SenC family incorporates Cu²⁺ 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 CuSO₄, suggesting that ScoC is involved not only in the construction but also the deactivation of cox. The scoC mutant was defective in the formation of 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 CuSO₄ restored the parental phenotype (Keijser 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...
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 identical amino acids, sco, S. coelicolor A3(2); sgr, S. griseus; sma, S. avermitilis; sce, S. cerevisiae; rcp, R. capsulatus; bsu, B. subtilis).

| Protein  | Number of Amino Acids | Sequence Numbers |
|----------|----------------------|-----------------|
| Sco      | 284                  | SCO3968→3962    |
| S. griseus  | 629                  | SGR3623→3629   |
| S. avermitilis | 4243              | SAV4237→4243   |
| S. scabiei  | 46571                | SCAB46631→46571|

Figure 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 Cu²⁺ 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.

© 2011 The Authors
Microbial Biotechnology © 2011 Society for Applied Microbiology and Blackwell Publishing Ltd, Microbial Biotechnology, 5, 477–488
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 eukaryotic 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–ycnL) whose transcription is induced by copper-limited condition (Chilappagarai 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 (Chilappagarai 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 CuSO4 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, Mattattall 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 alkalfied 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 CuSO4 (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...
This growth promotion by CuSO₄ 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 CuSO₄, to a level higher than that of the wild type, and this restoration by CuSO₄ 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 CuSO₄ (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.
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 CuSO₄ 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, NH₃ and H₂O₂. 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 H₂O₂, whereas scoC mutant cells appeared yellowish (background; Fig. 5C). The enzyme activity was restored by genetic complementation or supplementation with 10 μM CuSO₄.

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 CuSO₄ 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
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 CuSO4.

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 CuSO4 caused phenylalanine auxotrophy in S. coelicolor A3(2) and S. avermitilis; these organisms did not grow on minimal agar media supplemented with 10 μM CuSO4 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 CuSO4 indicates that pheA of this organism is transcribed by another promoter. The long intergenic region (285 bp) between the two divergent species, S. griseus and S. coelicolor, reinforces 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 CuSO4 = c. 0.6 ppm Cu2+) indicates that the sco operon is activated under low-copper conditions. Normally, soil solution contains copper at 0.01–0.06 ppm (Bowen, 1966).

**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 CuSO4 = c. 0.6 ppm Cu2+) indicates that the sco operon is activated under low-copper conditions. Normally, soil solution contains copper at 0.01–0.06 ppm (Bowen, 1966).
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*.

**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 \(\mu\)M 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 Koebisch 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 (\(\Delta\)scoC) harboring the empty plasmid (pKU464) and pKU464 carrying the intact scoC (pKU464-scoC). A hyaS mutant (\(\Delta\)hyaS) is also shown. Bars, 20 \(\mu\)m (B) and 50 \(\mu\)m (C).
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 CuSO₄ 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 Cu²⁺ 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...
controlling the efficiency of Cu²⁺ incorporation. This view is supported by the remarkably high cox activity in the scoC mutant under the Cu²⁺-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 σR (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–ycnl* operon of *B. subtilis* (Chillagargari 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...
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 Kita-sato University. The *Escherichia coli* DH5α strain (Takara Shuzo; Kyoto, Japan) was used as a host for conventional DNA manipulation, and the GM2163 strain, a methylion-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. Integration plasmids pKU460 and pKU464 carrying the phiC38 and phiBT integrase gene, respectively, pKU474 carrying a kanamycin-resistance gene cassette flanked by the loxP sequences, and pKU250 carrying the Cre recombinase gene at the PstI site of pKU250 (Komatsu et al., 2010) were obtained from H. Ikeda at Kita-sato University. pUJ773 and pUJ774 were obtained from John Innes Centre. The enzymes used for DNA manipulation were purchased from Takara Shuzo. Chemicals were purchased from Kohsusan (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), YMPl/sugar medium (Komatsu et al., 2006), tryptic soy broth (TSB; Nissui, Tokyo, Japan) and minimal medium [composition: 0.5 g l−1 KH2PO4, 0.2 g l−1 MgSO4, 7H2O, 0.01 g l−1 FeSO4, 7H2O, 1.0 g l−1 (NH4)2SO4, and 10 g l−1 of the appropriate sugar (pH 7.2)]. CuSO4 (usually at 10 μM) and phenylalanine (0.05%) were supplied when required. BCDA (Sigma-Aldrich, Tokyo, Japan), a Cu²⁺-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), DisFf-F/DisFf-R (scoF) and Disphe-A-F/Disphe-A-R (pheA) using pUJ733 (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 *PscoAc*-F/*PscoAc-R and *scoCc*-F/*scoCc-R primers respectively. The ligation mixture of these two fragments was used as a template for PCR using primers *PscoCc*-F/*scoCc-R to amplify the correctly fused DNA fragment. The resulting 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* (C68S/C90S) was generated as follows: two DNA fragments amplified from pKU460-*scoC* by PCR using the *PscoAc*-F/*scoCcMt-MR and *scoCcMt-MF/*scoCc-R primers were mixed and used as a template for the second PCR using the *PscoAc*-F/*scoCc-R primers. The resulting mutated *scoC* cassette was treated as described above to construct pKU460-*scoC* (C68S/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 aphil-F/aphil-R using pUK474 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 ampli-con 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* strain 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
constructed by introducing pKU250cre 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). pKU250cre 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 scoG{F}/scoG{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 \( \mu l \) 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 Koebusch and colleagues (2009). Cells were grown at 28°C by shaking (300 r.p.m.) in a test tube (18 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-8000 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 cellobiose-covered Bennett's/maltose agar medium were collected and suspended in 1 ml of 100 mM NaH\(_2\)PO\(_4\) 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 NaH\(_2\)PO\(_4\), 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\(_{scoA}l\) and HS1-F/HS1-R* (P\(_{nab}l\); Table S2; primers with an asterisk were labelled at the 5'-end with \([\gamma\rceil^32P\]-ATP using T4-polynucleotide kinase).

**Acknowledgements**

We thank Haruo Ikeda for providing pKU series of plasmids and Andyi Lzhetzsyk for providing pWULFLP. This study was supported by the High-tech Research Center Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan; and a grant from the Institute of Fermentation, Osaka, Japan. M.F. was supported by the Nakato fellowship.

**References**

Alcázar-Román, A.R., Bolger, T.A., and Wente, S.R. (2010) Control of mRNA export and translation termination by inositol hexakisphosphate requires specific interaction with Gie1. J Biol Chem 285: 16683–16692.

Boal, A.K., and Rosenzweig, A.C. (2009) Structural biology of copper trafficking. Chem Rev 109: 4760–4779.

Bower, H.J.M. (1966) Trace Elements in Biochemistry. London and New York: Academic Press.

Burke, R.M., and Cairney, J.W. (2002) Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. Mycorrhiza 12: 105–116.

Cha, J.S., and Cooksey, D.A. (1993) Copper hypersensitivity and uptake in *Pseudomonas syringae* containing cloned components of the copper resistance operon. Appl Environ Microbiol 59: 1671–1674.

Chater, K.F. (2006) *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. Philos Trans R Soc Lond B Biol Sci 361: 761–768.

Chater, K.F., Biro, S., Lee, K.J., Palmer, T., and Schrempf, H. (2010) The complex extracellular biology of *Streptomyces*. FEMS Microbiol Rev 34: 171–198.

Chillappagari, S., Mietheke, M., Trip, H., Kuipers, O.P., and Marahiel, M.A. (2009) Copper acquisition is mediated by YcnJ and regulated by YcnK and CsoR in *Bacillus subtilis*. J Bacteriol 191: 2362–2370.

Clutterbuck, A.J. (1972) Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J Gen Microbiol 70: 423–435.

Cooksey, D.A. (1994) Molecular mechanisms of copper resistance and accumulation in bacteria. FEMS Microbiol Rev 14: 381–386.

© 2011 The Authors Microbial Biotechnology © 2011 Society for Applied Microbiology and Blackwell Publishing Ltd, Microbial Biotechnology, 5, 477–488.
Cornelis, P., Matthys, S., and Van Oeckelen, L. (2009) Iron uptake regulation in Pseudomonas aeruginosa. Biometals 22: 15–22.

Endo, K., Hosono, K., Beppu, T., and Ueda, K. (2002) A novel extracytoplasmic phenol oxidase of Streptomyces: its possible involvement in the onset of morphogenesis. Microbiology 148: 1767–1776.

Endo, K., Hayashi, Y., Hibi, T., Hosono, K., Beppu, T., and Ueda, K. (2003) Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by Streptomyces griseus. J Biochem 133: 671–677.

Fedoryshyn, M., Petzke, L., Welle, E., Bechthold, A., and Luzhetskyy, A. (2008) Marker removal from actinomycetes genome using Flp recombinesin. Gene 419: 43–47.

Frangipani, E., and Haas, D. (2009) Copper acquisition by the SenC protein regulates aerobic respiration in Pseudomonas aeruginosa PA01. FEMS Microb Lett 298: 234–240.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003) PCR-targeted Streptomyces gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100: 1541–1546.

Hopwood, D.A. (2007) Streptomyces in Nature and Medicine: The Antibiotic Makers. New York, NY: Oxford University Press.

Junemann, S. (1997) Cytochrome bd terminal oxidase. Biochim Biophys Acta 1321: 107–127.

Keijser, B.J., van Wezel, G.P., Canters, G.W., Kieser, T., and Vijnboomen, E. (2000) The ram-dependence of Streptomyces lividans differentiation is bypassed by copper. J Mol Microbiol Biotechnol 2: 565–574.

Kelemen, G.H., Viollier, P.H., Tenor, J., Marli, L., Buttnr, M.J., and Thompson, C.J. (2001) A connection between stress and development in the multicellular prokaryote Streptomyces coelicolor A3(2). Mol Microbiol 40: 804–814.

Kieser, T., and Hopwood, D.A. (1991) Genetic manipulation of Streptomyces: integrating vectors and gene replacement. Methods Enzymol 204: 430–458.

Kieser, T., Bibb, M.J., Buttnr, M.J., Chater, K.F., and Hopwood, D.A. (2000) Practical Streptomyces Genetics. Norwich, UK: The John Innes Foundation.

Koebsch, I., Overbeck, J., Pliemeyer, S., Meschke, H., and Schrempf, H. (2009) A molecular key for building hyphae aggregates: the role of the newly identified Streptomyces protein HyaS. Microb Biotechnol 2: 343–360.

Komatsu, M., Takano, H., Hiratsuka, T., Ishigaki, Y., Shimada, K., Beppu, T., and Ueda, K. (2006) Proteins encoded by the conservon of Streptomyces coelicolor A3(2) comprise a membrane-associated heterocomplex that resembles eukaryotic G protein-coupled regulatory system. Mol Microbiol 62: 1534–1546.

Komatsu, M., Uchiyama, T., Omura, S., Cane, D.E., and Ikeda, H. (2010) Genome-minimized Streptomyces host for the heterologous expression of secondary metabolism. Proc Natl Acad Sci USA 107: 2646–2651.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Matsushita, K., Shinagawa, E., Adachi, O., and Ameyama, M. (1982) o-Type cytochrome oxidase in the membrane of aerobically grown Pseudomonas aeruginosa. FEBS Lett 139: 255–258.

Mattattall, N.R., Jazairi, J., and Hill, B.C. (2000) Characterization of YpmQ, an accessory protein required for the expression of cytochrome c oxidase in Bacillus subtilis. J Biol Chem 275: 28802–28809.

Messerschmidt, A., Huber, R., Pouls, T., and Wieghardt, K. (eds) (2001) Handbook of Metalloproteins, Vol. 1. New York: John Wiley & Sons, pp. 1149–1414.

Miyadoh, S. (1993) Research on antibiotic screening in Japan over the last decade: a producing microorganisms approach. Actinomycetologica 7: 100–106.

Owen, G.A., Pascoe, B., Kalilifadas, D., and Paget, M. (2007) Zinc-responsive regulation of alternative ribosomal protein genes in Streptomyces coelicolor involves Zur and α5. J Bacteriol 189: 4078–4086.

Rigby, K., Cobine, P.A., Khalimonchuk, O., and Winge, D.R. (2008) Mapping the functional interaction of Sco1 and Cox2 in cytochrome oxidase biogenesis. J Biol Chem 283: 15015–15022.

Shin, J.H., Oh, S.Y., Kim, S.J., and Roe, J.H. (2007) The zinc-responsive regulator Zur controls a zinc uptake system and some ribosomal proteins in Streptomyces coelicolor A3(2). J Bacteriol 189: 4070–4077.

Solioz, M., Abicht, H.K., Mermod, M., and Mancini, S. (2010) Response of gram-positive bacteria to copper stress. J Biol Inorg Chem 15: 3–14.

Sollner-Webb, B., and Reeder, R.H. (1979) The nucleotide sequence of the initiation and termination sites for ribosomal RNA transcription in X. laevis. Cell 18: 485–499.

Swem, D.L., Swem, L.R., Setterdahl, A., and Bauer, C.E. (2005) Involvement of SenC in assembly of cytochrome c oxidase in Rhodobacter capsulatus. J Bacteriol 187: 8081–8087.

Takano, H., Asano, K., Beppu, T., and Ueda, K. (2007) Role of α5 paralogs in intracellular melanin formation and spore development in Streptomyces griseus. Gene 393: 43–52.

Ueda, K., Tomaru, Y., Endoh, K., and Beppu, T. (1997) Stimulatory effect of copper on antibiotic production and morphological differentiation in Streptomyces tanashiensis. J Antibiot (Tokyo) 50: 693–695.

Worrall, J.A., and Vigenboom, E. (2010) Copper mining in Streptomyces: enzymes, natural products and development. Nat Prod Rep 27: 742–756.

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.