Iron competition triggers antibiotic biosynthesis in *Streptomyces coelicolor* during coculture with *Myxococcus xanthus*

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Abstract
Microbial coculture to mimic the ecological habitat has been suggested as an approach to elucidate the effect of microbial interaction on secondary metabolite biosynthesis of *Streptomyces*. However, because of chemical complexity during coculture, underlying mechanisms are largely unknown. Here, we found that iron competition triggered antibiotic biosynthesis in *Streptomyces coelicolor* during coculture with *Myxococcus xanthus*. During coculture, *M. xanthus* enhanced the production of a siderophore, myxochelin, leading *M. xanthus* to dominate iron scavenging and *S. coelicolor* to experience iron-restricted conditions. This chemical competition, but not physical contact, activated the actinorhodin biosynthetic gene cluster and the branched-chain amino acid degradation pathway which imply the potential to produce precursors, along with activation of a novel actinorhodin export system. Furthermore, we found that iron restriction increased the expression of 21 secondary metabolite biosynthetic gene clusters (smBGCs) in other *Streptomyces* species. These findings suggested that the availability for key ions stimulates specific smBGCs, which had the potential to enhance secondary metabolite biosynthesis in *Streptomyces*.

Introduction

*Streptomyces*, ubiquitous soil gram-positive bacteria, are well-known for their ability to produce diverse secondary metabolites, including many compounds that are currently in clinical use [1]. Functions of secondary metabolites are not directly involved in the natural growth of *Streptomyces* in their native habitats, but they serve important ecological roles in interspecies interaction and communication [2]. Biosynthesis of each secondary metabolite is tightly controlled by the complex regulatory networks in response to various biotic stresses (e.g., nutrient competition, quorum sensing, and physical interaction) and abiotic stresses (e.g., pressure, temperature, and salinity) in natural environments [3]. Under the axenic laboratory culture condition, however, those environmental stimuli are lacking; thus, most of secondary metabolite biosynthetic gene clusters (smBGCs) in *Streptomyces* are not activated [4].

Elucidation of signals that trigger the biosynthesis of the secondary metabolites enables to understand the ecological role of secondary metabolites in the microbial community and increase the industrial production of secondary metabolites. Thus, tremendous efforts have focused on awakening the silent *Streptomyces* smBGCs, including media
optimization, epigenetic modifier treatment, mutagenesis, and genetic engineering [5–7]. In addition to these approaches, although accurately mimicking natural habitats is challenging, microbial coculture of two or more different microorganisms in which bacterial populations cohabitate with complex communities, is an effective approach to understand the ecological roles of the chemical diversity of Streptomyces and to turn on their cryptic pathways [8, 9]. However, because of the chemical and molecular complexity of microbial communication, success with revealing the interaction mechanisms has been limited. For example, Myxococcus xanthus is a mobile predator that is able to lyse a wide variety of bacteria, including Streptomyces [10]. Specifically, the interaction between M. xanthus and S. coelicolor stimulates aerial mycelium formation and actinorhodin overproduction by S. coelicolor at the contact area between the two species, but still, the underlying mechanism is ambiguous [11]. In this study, we aimed to elucidate the mechanisms of interaction of these two bacteria during coculture in order to provide insights into actinorhodin biosynthesis and its ecological role. Our findings supported the hypothesis that chemical competition with nearby microbes plays a critical role in stimulating secondary metabolite biosynthesis in Streptomyces. An iron-restricted condition triggered the expression of 21 smBGCs in eight Streptomyces species, indicating that secondary metabolites involved in different types of microbial communications and interactions have common environmental factors triggering their expression. Precise characterization of the microbial interaction governed by secondary metabolites will uncover the unexplored ecological systems and provide accurate selection pressure for improving the industrial production of secondary metabolites.

Materials and methods

Bacterial strains and media

S. coelicolor A3(2) M145 (ATCC BAA-471) and seven Streptomyces species (S. subrubilus, S. kanamyceticus, S. coeruleorubidus, S. cinereoruber, S. roseosporus, S. rimosus, and S. venezuelae) were cultured in 50 mL liquid CTT media (1% Difco casitone, 10 mM Tris-HCl (pH 7.6), 8 mM MgSO4, and 1 mM KHPO4) with 0.16 g/mL glass beads (3 mm ± 0.3 mm diameter) for 24 h at 30 °C using a 200 rpm orbital shaker. In parallel, M. xanthus DK1622 was cultured under the identical conditions used for Streptomyces species without the glass beads. Cells were harvested at exponential growth phase (OD600nm between 2 and 3) and resuspended in 1 mL of 20% glycerol for inoculation on solid CTT media. For additional iron treatment, 100 mM FeCl2 stock was added to the media at the desired concentration. For iron-restricted conditions, media were supplemented with 10 mM of 2,2′-bipyridyl stock to the desired concentration (350 μM for S. coelicolor and 250 μM for seven other Streptomyces species to avoid growth retardation under the same conditions as for S. coelicolor).

Solid cocultures

Cocultures of S. coelicolor as a prey with M. xanthus as a predator were carried out on solid CTT medium plates. Twenty microliters S. coelicolor stock was spread on half (width = 3 cm, length = 6 cm) of the solid CTT medium plate. M. xanthus was then spread on the other half, maintaining 3 mm distance from the section of S. coelicolor, whereas identical species was spread for pure-culture condition (Fig. 1a). The culture plates were then incubated at 30 °C until the desired morphology appeared. Morphology observation of S. coelicolor and M. xanthus during coculture using scanning electron microscopy described in Supplementary Methods.

Genome sequencing for six Streptomyces species

Among the eight Streptomyces species used in this study, the complete genome sequences of only two species (S. coelicolor [NC_003888] and S. venezuelae [NC_018750]) were reported. Briefly, genomic DNA (gDNA) from each species was prepared from cultured cells harvested at the mid-exponential phase using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. For long-read (PacBio) genome sequencing, a library was prepared using 5 μg gDNA with SMRTbell, according to the manufacturer’s protocol. The prepared SMRTbell library (20 kb size) was sequenced using P6-C4-chemistry (DNA Sequencing Reagent 4.0) on a PacBio RS II sequencing platform (Pacific Biosciences, Menlo Park, CA, USA). For generation of a short-read (Illumina Inc., San Diego, CA, USA) genome sequencing library, gDNA was fragmented to ~350 bp by using a Covaris instrument (Covaris Inc., Woburn, MA) with the following conditions: power, 175; duty factor, 20%; C. burst, 200; time, 23 s. The library was then constructed using a TruSeq DNA PCR-Free LT kit (Illumina) according to the manufacturer’s protocol. Finally, prepared short-read genome sequencing libraries were sequenced on a Hiseq 2500 instrument with a 100-bp single-read running scale.

Obtained long-read and short-read genome sequencing read were de novo assembled into contigs and finally linked into chromosome (Supplementary Table 1). Additional details regarding de novo assembly are described in detail in Supplementary Methods. The six completed genomes were annotated using NCBI’s Prokaryotic Genome Annotation...
Pipeline version 4.5. Genome sequences are available at NCBI under BioProject ID PRJNA412292 (accession numbers: CP023701 [S. subrutilus], CP023699 [S. kana-mycticus], CP023694 [S. coeruleorubidus], CP023693 [S. cinereoruber], PDCL00000000 [S. roseosporus], and CP023688 [S. rimosus]). smBGCs were predicted using each Genbank file by antiSMASH 4.0 (Supplementary Data 3) [12].
RNA sequencing (RNA-Seq)

For Streptomyces species, RNA was extracted from the cells which were harvested from the culture plates and resuspended in 3 mL lysis buffer (20 mM Tris-HCl [pH 7.4], 140 mM NaCl, 5 mM MgCl₂, and 1% Triton X-100). For M. xanthus, RNA was extracted from the cells obtained from the culture plate and resuspended in 3 mL liquid CTT medium. Detailed RNA extraction method is described in Supplementary Methods. RNA-Seq libraries were then constructed using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) according to the manufacturer’s instructions. The RNA-Seq library concentration was measured using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the size of the libraries was determined with an Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). Quantified libraries were then sequenced on a HiSeq2000 Rapid-Run platform (Illumina) using a 1 × 100 cycle V4 kit.

Data processing

Sequencing reads were mapped to the corresponding reference genome sequences (Genome accession numbers described above for Streptomyces species and NC_008095 for M. xanthus) using CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark) (Supplementary Tables 2 and 3). Mapped reads were then counted and normalized using the DESeq2 algorithm in R (Supplementary Data 1–2 and 4–11) [13]. Mapped read information was exported as a BAM file format, which was further converted to a GFF file format containing read counts for each genomic position. The GFF file was visualized on SignalMap (v2.0.0.5; Roche NimbleGen, Basel, Switzerland). The sequencing data were deposited in the European Nucleotide Archive (accession number: PRJEB25075).

Measurement of iron concentration

Extracellular and intracellular iron level of S. coelicolor and M. xanthus were measured using an Iron Bio kit for Cedex Bio (Roche Diagnostics, Indianapolis, IN, USA) [14]. Methods for sample preparation is fully described in Supplementary Methods.

Measurement of actinorhodin concentrations

Two volumes of methanol were added to the cocultured S. coelicolor and solid agar medium (width = 1.5 cm, length = 6 cm) obtained from the culture plates. After overnight incubation at 25 °C, the supernatant was then concentrated in 500 μL methanol by air drying, and 166 μL of 4 N KOH was added to the sample. The concentration of actinorhodin was then measured by UV absorbance at 640 nm with a Tecan Infinite F200 Pro (Tecan Group Lt., Männedorf, Switzerland).

LC-electrospray ionization (ESI)-MS/MS analysis of myxochelin A

For myxochelin A extraction, two volumes of methanol were added to the cocultured M. xanthus and solid agar media obtained from the contact area (width = 1.5 cm, length = 6 cm) in the culture plates. Samples were incubated at 25 °C overnight and supernatant was air-dried and resuspended in 1 mL methanol, which was then analyzed using a Triple Quad 3500 (SCIEX, Framingham, MA, USA) equipped with an ESI source and a Nexera X2 UHPLC system (Shimadzu, Japan). LC-ESI-MS/MS analysis method is described in detail in Supplementary Methods.

Disruption and overexpression of SCO6666 in S. coelicolor

Disruption of SCO6666 gene was performed using CRISPR/Cas9 system based on pCRISPomyces-2 plasmid following the established protocol [15]. Gene disruption was confirmed by PCR amplification of the genomic region containing SCO6666 gene. Since the size of SCO6666 gene is 2.2 kb, the amplified DNA fragment sizes of WT and the deleted strain were 4.8 and 2.6 kb, respectively. Primer sequences used in this validation experiment are listed in Supplementary Table 4. Overexpression of SCO6666 gene under ermE promoter and two endogenous promoters of S. coelicolor was conducted by using pIBR25 plasmid. Vector construction and transformation method are fully described in Supplementary Methods.

Results

Interspecies interactions between S. coelicolor and M. xanthus

To compare the effect of interspecies and intraspecies interaction on actinorhodin production of S. coelicolor, we formed cocultures by spreading S. coelicolor and M. xanthus on solid CTT media with a distance of 3 mm between them. For pure-cultures, each species was spread near itself. However, in the contact region with S. coelicolor, we observed a glossy morphology. The contact region then widened with growth, and actinorhodin production was observed (Fig. 1b). Only the cocultured S.
Iron competition triggers antibiotic biosynthesis in *Streptomyces coelicolor* during coculture with...
actinorhodin biosynthesis, but the actinorhodin production appears to be stimulated only during the chemical interaction between *M. xanthus* and *S. coelicolor*. Based on these results, we speculated that sequential chemical interaction between the two species, including myxochelin-mediated iron uptake by *M. xanthus*, triggered the actinorhodin biosynthesis of *S. coelicolor* during coculture. Indeed, myxochelin is a catecholate-type siderophore that is a stronger iron-chelating compound than the hydroxamate-type siderophore of *S. coelicolor*, desferrioxamine. Accordingly, *M. xanthus* is capable of fully utilizing iron available in the culture medium, whereas *S. coelicolor* senses reduced iron level followed by the initiation of actinorhodin biosynthesis [24, 25].

![Fig. 2 Effects of iron competition between *S. coelicolor* and *M. xanthus* on actinorhodin production.](image)
Iron competition triggers antibiotic biosynthesis in *Streptomyces coelicolor* during coculture with...  

To address the iron competition between the two species, we measured extracellular and intracellular iron levels. Even at the T1 time point of *S. coelicolor* under both pure-culture and coculture conditions, the extracellular iron levels, i.e., the amount of iron remaining in the media, decreased lower than the detection limit (<1 μM; Fig. 2d). Owing to the active iron acquisition of *S. coelicolor*, the extracellular iron level of cocultured *M. xanthus* decreased about 6.4-fold more rapidly than that of the pure-cultured *M. xanthus*, indicating the difference in iron requirements of two microbes (Fig. 2d). To respond to the iron-reduced environment caused by *S. coelicolor*, *M. xanthus* overproduced myxochelin. Myxochelin biosynthetic genes (MXAN_3646-3640) possesses a ferric uptake regulator (Fur) binding motif in the upstream region (Supplementary Fig. 6c). Similar to Fur-mediated regulation of the biosynthesis of enterobactin, a siderophore of *Escherichia coli*, myxochelin biosynthesis is activated when Fur is unbound from the promoter region under iron-depletion conditions [26]. Indeed, myxochelin production was increased under iron-restricted pure-culture conditions but decreased under iron-added coculture conditions (Fig. 2e, f).

We then examined changes in intracellular iron levels during the coculture. The intracellular iron concentration of cocultured *M. xanthus* at T3 was about two times higher than that of pure-cultured *M. xanthus*, supporting that the overproduction of myxochelin during coculture occurred in intracellular iron levels via iron transportation (Fig. 2g). *M. xanthus* apparently sequesters more iron than it needs for growth during the coculture condition, to prevent *S. coelicolor* from utilizing the iron. Considering the low extracellular iron levels under the coculture conditions, *M. xanthus* seemed to absorb iron at the expense of *S. coelicolor* ability to maintain intracellular iron to high levels. Indeed, in contrast to *M. xanthus*, intracellular iron levels in cocultured *S. coelicolor* were 1.7 times lower than under pure-culture conditions at T3 (Fig. 2h). These results demonstrate that iron competition occurred between the two species during the coculture, when *S. coelicolor* was in a state of the reduced intracellular iron level and stimulated actinorhodin overproduction.

Next, we sought to determine the relationships between iron availability and actinorhodin production. As the amount of iron in the media increased, the actinorhodin production of *S. coelicolor* was decreased during coculture without growth inhibition (Fig. 2i and Supplementary Fig. 7). In addition, we tested whether actinorhodin overproduction was affected by other metal ions, such as divalent cations, including Ca\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), and Co\(^{2+}\). In most cases, high concentrations of metal ions inhibited cell growth of *S. coelicolor* or *M. xanthus*. After accounting the cytotoxicity, iron was the only metal that decreases actinorhodin production with increased concentrations (Supplementary Figs. 7, 8) [27]. The supplementation of excess iron seems to reduce the interspecies iron competition between *M. xanthus* and *S. coelicolor* and decrease actinorhodin production. Nevertheless, the addition of iron during coculture may have a variety of effects on microbial interaction, so these results are insufficient to explain the direct relationship between actinorhodin production and iron availability. Therefore, we next examined whether actinorhodin production in *S. coelicolor* was triggered in iron-restricted pure-culture conditions by the addition of the iron chelator 2,2′-bipyridyl. *S. coelicolor* produced actinorhodin when the 2,2′-bipyridyl concentration was 300 μM or higher (Fig. 2j). When we added more iron after the 2,2′-bipyridyl treatment, actinorhodin production was no longer induced. These results confirm that the iron-restricted environment induces actinorhodin production in *S. coelicolor*. However, actinorhodin production under the iron-restricted condition was less active than the coculture condition, indicating the existence of another interspecies interaction during coculture, which intensifies the actinorhodin production. Taken together, these results suggest that during coculture, *S. coelicolor* actively took up extracellular iron, resulting in sensing of decreased extracellular iron levels by *M. xanthus* and upregulation of myxochelin biosynthesis. Through the myxochelin-mediated iron acquisition, *M. xanthus* restored intracellular iron levels, which caused *S. coelicolor* to experience iron-restricted conditions and induce actinorhodin production. Also, actinorhodin production seems to be further activated by another interspecies interaction during coculture.

**Effects of iron depletion on the expression of smBGCs in Streptomyces**

We then examined whether iron-restricted conditions altered the expression of smBGCs in other *Streptomyces*. Seven species (i.e., *S. subtililus*, *S. kanamyceticus*, *S. coeruleorubidus*, *S. cinereoruber*, *S. roseosporus*, *S. rimosus*, and *S. venezuelae*) were selected based on morphological changes, such as colony shape and color, under iron-restricted conditions (Supplementary Fig. 9). We completed the genome sequences of six species in a single scaffold (7.6–10.1 Mbp in length) for five species and two scaffolds (5.7 and 2.1 Mbp in length) for *S. roseosporus* using both long-read (PacBio) and short-read (Illumina) genome sequencing methods (Supplementary Fig. 10 and Supplementary Table 1). The completed genomes were then annotated by the NCBI Prokaryotic Genome Annotation Pipeline and smBGCs were predicted using antiSMASH [12]. Finally, 260 smBGCs were predicted from the eight *Streptomyces* genomes (including *S. coelicolor*; ~30 smBGCs genome; Supplementary Data 3).

To determine which smBGCs were activated under iron-restricted conditions, we then performed RNA-Seq under...
iron-restricted (2,2′-bipyridyl-treated CTT media) and normal (CTT media) conditions (Supplementary Table 3). RNA-Seq reads were mapped to the corresponding completed genomes, and the expression value of each gene was normalized using the DESeq2 package in R (Supplementary Data 4–11) [13]. Calculation of Euclidean distances between samples demonstrated high reproducibility between biological replicates (Supplementary Fig. 11). Among 260 smBGCs predicted in the genomes of the eight species, 76 smBGCs were expressed in normal or iron-restricted conditions, and the expression of 21 smBGCs was specifically upregulated in response to iron-restricted conditions (Fig. 3a). Among them, 11 smBGCs were siderophore BGCs, including desferrioxamine, coelichelin, bacillibactin, and scabichelin BGCs (Fig. 3a). Biosynthesis of desferrioxamine and coelichelin in S. coelicolor is regulated by the iron-dependent transcriptional regulator DmdR1 in the same way that Fur regulates myxochelin biosynthesis in M. xanthus [28, 29]. Consistent with this, the remaining nine siderophore BGCs also contained DmdR1-binding sites, indicating that expression of these siderophore BGCs was regulated by DmdR1 (Table 1).

In contrast, the remaining ten smBGCs, activated by iron restriction, corresponded to the synthesis of various secondary metabolites, including ectoine, melanin, actinorhodin, cosmobycin D, ikarugamycin, chloramphenicol, and lanthipeptide. Among these, ectoine and melanin BGCs were conserved in all eight species, but only upregulated in three and one species, respectively, indicating that these BGCs were differently regulated by iron restriction in each species. The remaining six BGCs were species specific, except cosmobycin D BGC, which was conserved in S. coeruleorubidus and S. cinereoruber (Fig. 3a). Unlike siderophore BGCs, putative binding sites of Fur or DmdR1 were not detected in these six BGCs. However, a repeat of the TCGAG sequence at constant intervals (6 nt) was found in actinorhodin and cosmobycin D [30]. The motif was likely to be the binding site for the cluster-situated SARP family regulator, which was located in both BGCs (Supplementary Fig. 12). Overall, through the genome sequencing and transcriptome analyses, our findings suggested that siderophore BGCs of Streptomyces were activated via DmdR1-associated regulation under iron-restricted conditions. In contrast, nonsiderophore BGCs, including actinorhodin BGC, appeared to be upregulated by other mechanisms that may be associated with iron limiting culture conditions.

Among the secondary metabolites expected to be produced from the six nonsiderophore BGCs, actinorhodin and cosmomycin D were aromatic polyketide-type secondary metabolites with a similar backbone structure (Fig. 3b). Interestingly, this backbone structure (close ketone and hydroxyl groups) is highly similar to the structure of another polyketide, tetracycline which is well-known for chelating Fe$^{2+}$/Fe$^{3+}$ ions [31]. In addition, chloramphenicol, a nonribosomal peptide-type secondary metabolite, has three potential sites for interactions with metal ions, and these sites have been reported to form a complex with Fe ion (Fig. 3b) [32]. Taken together, nonsiderophore secondary metabolites, of which BGCs were upregulated under iron-restricted conditions, have the potential to interact with iron. Although further analyses are required, if these secondary metabolites have both antibiotic and iron-chelating functions, production of these secondary metabolites during the iron competition with other microbes would be highly advantageous to Streptomyces by blocking the accessibility of other microbes and increasing the iron-chelating ability.

Identification of novel genes affecting actinorhodin production in S. coelicolor

Although iron-restricted culture conditions triggered actinorhodin production, the amount synthesized was markedly lower, and the produced actinorhodin was rarely excreted compared with that under coculture conditions (Fig. 2j). Because changes in the transcriptome of S. coelicolor during coculture were related to the sophisticated interaction with M. xanthus, iron-restricted conditions were insufficient to mimic the phenotype of coculture. Thus, in order to elucidate the cellular mechanisms that supported actinorhodin production or transport during coculture, we compared the DEGs between coculture and pure-culture conditions with the DEGs between iron-restricted conditions and normal (CTT media) culture condition. Among the 30 upregulated genes during coculture conditions, seven genes were commonly upregulated genes during the iron-restricted condition, simultaneously, four out of eight downregulated genes during coculture conditions were commonly downregulated during the iron-restricted condition (Supplementary Fig. 13). Three of the seven commonly upregulated genes were minimal polyketide synthase-coding genes (SCO5087–5089), which are essential for actinorhodin biosynthesis [19]. However, the branched-chain amino acid degradation pathway, which was upregulated during coculture and potentially supplies precursors for actinorhodin biosynthesis, was not upregulated under iron-restricted conditions. This was thought to be one of the reasons why less actinorhodin was produced under iron-restricted conditions compared with coculture conditions.

Among genes specifically upregulated during coculture, SCO6666, which had the highest fold change in expression between pure-culture and coculture conditions at all four time points, rarely expressed under iron-restricted conditions (Fig. 4a, b). SCO6666 encodes a hypothetical protein but has a protein sequence similar to that of the actinorhodin
BGC-encoded gene SCO5084 (e-value < 3 × 10^-5 and identity = 34%), which has been predicted as a transporter or dehydrogenase to convert actinorhodin to γ-actinorhodin [33]. SCO6666 possesses two mycobacterial membrane proteins large domains and a putative NAD-binding domain. Therefore, we speculated that SCO6666 may play critical roles in actinorhodin production during coculture. Interestingly, SCO5083 and SCO5084 in the actinorhodin BGC were rarely expressed under CTT pure-culture, coculture, and iron-restricted conditions (Fig. 4c).
Table 1 DmdR1-binding sites in 11 siderophore BGCs upregulated under iron-restricted conditions.

| BGC            | BGC number | Species                      | Gene                | DmdR1-binding sites (5′-3′ sequence) |
|----------------|------------|------------------------------|---------------------|--------------------------------------|
| Desferrioxamine B | 1          | *S. kanamyceticus*           | CP970_27940         | TTAGGTTAGGTTCGCTACCTAA (63 nt) ATG    |
|                | 4          | *S. venezuelae*              | SVEN2570            | TTAGGTTAGGTGCTACCTAA (50 nt) ATG      |
|                | 6          | *S. roseosporus*             | CP979_13235         | TTAGGTTAGGTGCTACCTAA (54 nt) ATG      |
|                | 9          | *S. rimosus*                 | CP984_32060         | TTAGGTTAGGTGCTACCTAA (115 nt) ATG     |
|                | 12         | *S. cinereoruber*            | CP977_12440         | TTAGGTTAGGTGCTACCTAA (68 nt) ATG      |
|                | 17         | *S. coelicolor*              | SCO2782             | TTAGGTTAGGTGCTACCTAA (63 nt) ATG      |
|                | 19         | *S. coeruleorubidus*         | CP976_16210         | TTAGGTTAGGTGCTACCTAA (62 nt) ATG      |
| Coelichelin    | 15         | *S. coelicolor*              | SCO0498             | TTAGGTTAGGTGCTACCTAA (25 nt) ATG      |
|                | 21         | *S. coeruleorubidus*         | CP976_37645         | TgAGcTTAGGTAGCCTACCTAA (104 nt) ATG    |
|                |            |                              | CP976_37695         | TTAGGTTAGGTGCTACCTAA (50 nt) ATG      |
| Bacillibactin  | 7          | *S. rimosus*                 | CP984_01935         | TaAGGTaAGGCTAgCCTTA (49 nt) ATG       |
|                |            |                              | CP984_01965         | TaAGGTAGTCGCTACCTAA (124 nt) ATG      |
| Scabichelin    | 11         | *S. cinereoruber*            | CP977_03980         | TTAGGTTAGGTGCTACCTAA (83 nt) ATG      |
|                |            |                              | CP977_04015         | TTAGGTTAGGTGCTACCTAA (101 nt) ATG     |
|                |            |                              | CP977_04025         | TTAGGTTAGGTGCTACCTAA (40 nt) ATG      |

Sequences in lower case indicate nonconserved nucleotides compared with the known DmdR1-binding site in the desferrioxamine BGC of *S. coelicolor*.

Normally, the two genes are transcriptionally repressed by a repressor encoded by SCO5082 (actII-ORF1) and activated when actinorhodin or intermediates bind to the repressor and release it from the promoter region [34]. Because actinorhodin was not produced under the pure-culture conditions, the expression of the two genes may be repressed; in contrast, under actinorhodin-producing conditions, such as coculture and iron-restricted conditions, these two genes were expected to be strongly activated. Therefore, we suspected that there was an additional regulatory mechanism acting on the SCO5083 and SCO5084 genes under CTT media culture conditions. Accordingly, we performed additional RNA-Seq on *S. coelicolor* cultured in R5(−) solid media, the most common culture conditions for *S. coelicolor*. The results revealed that actinorhodin was actively produced and that SCO5083–5084 were highly expressed, as expected (Supplementary Data 4 and Fig. 4e) [35]. The low expression of SCO5083–5084 may be why low actinorhodin production was observed under iron-restricted CTT culture conditions. In contrast, under coculture condition, actinorhodin was still actively produced and exported to the extracellular region, even in the absence of SCO5083–5084 expression. These results suggested that genes, including SCO6666, activated only during coculture had the potential to act as alternative genes to replace SCO5083–SCO5084.

To verify the functional roles of SCO6666 in actinorhodin production or export, a deletion strain was constructed using a CRISPR/Cas9-mediated knockout system (Fig. 4d). The SCO6666 deletion strain did not produce actinorhodin under pure-culture conditions, similar to the wild-type strain. Moreover, the deletion strain produced approximately three times less actinorhodin during coculture compared with the wild-type strain (Fig. 4e). To further elucidate the functional role of this gene, we examined the correlations between SCO6666 expression and actinorhodin production under pure-culture conditions. SCO6666 is a membrane protein that can be toxic to the host if overexpressed [36]. Accordingly, based on the RNA-Seq results, we selected two weak promoters from SCO1797 and SCO1855, which showed constant expression during cell growth (Supplementary Fig. 14a). The constructed SCO6666 overexpression strain with the SCO1797 promoter actively produced actinorhodin under pure-culture conditions and iron-restricted conditions (Fig. 4f). In contrast, actinorhodin production in the SCO6666 overexpressing strain with the SCO1855 promoter, showing tenfold less RNA expression than that with the SCO1797 promoter, was similar to that of the wild-type strain, indicating that SCO6666 expression was insufficient to activate actinorhodin production. Consistent with this, SCO6666 expression levels of the strain with the SCO1797 promoter were 5.73- to 2.66-fold higher than that in the wild-type and the strain with the SCO1855 promoter, respectively (Supplementary Fig. 14b). In addition, the complemented strain constructed by introducing SCO6666 overexpression vector into SCO6666 deletion strain showed the same phenotype as the SCO6666 overexpression strain in coculture and pure-culture conditions (Fig. 4e, f). The fact that actinorhodin export by the SCO6666 deletion strain did not completely disappear under coculture conditions suggested that actinorhodin export during coculture...
Iron competition triggers antibiotic biosynthesis in *Streptomyces coelicolor* during coculture with...
Because actinorhodin acts as a repellent to *M. xanthus* and has the structural potential to chelate iron, actinorhodin production appears to support *S. coelicolor* to be in a favorable position in iron competition during coculture [45]. Considering that actinorhodin production is only triggered by the interspecies iron competition, not intraspecies iron competition, it seems to have a selective advantage to tolerate conspecifics but be more aggressive against xenospecies. In the same context, actinorhodin production during the iron-restricted condition is less intense than the coculture condition, indicating that another interspecies interaction between *S. coelicolor* and *M. xanthus* exists which intensifies the actinorhodin production.

Indeed, by comparing the transcriptome data for *S. coelicolor* under coculture and iron-restricted conditions, we found that actinorhodin cluster-related genes as well as expression of other genes affected actinorhodin biosynthesis. Actinorhodin and its intermediates induce the expression of SCO5083–5084 expression during the actinorhodin production. In addition, a knockout and overexpression study of SCO6666 suggested its functional role to replace SCO5083–5084 for actinorhodin biosynthesis. Although further studies are required, assembly of a membrane-associated multiprotein complex, such as SCO5083–5084 and SCO6666, is required for high-level secondary metabolite production [46]. Taken together, actinorhodin production seems to be not only determined by genes present in the actinorhodin cluster but also determined by a more complex network involving other unknown genes (Fig. 5).

In addition, in order to identify the effect of iron depletion on other secondary metabolites production, we performed genome sequencing and transcriptome analyses on seven other *Streptomyces* species, determining that iron-restricted conditions activated 11 siderophore BGCs and 10 nonsiderophore BGCs from eight *Streptomyces* species, including *S. coelicolor*. The 11 siderophore BGCs contained regulatory sites controlled by DmdR1, which was well-conserved in all eight *Streptomyces* species, suggesting that siderophore BGC expression may be regulated by DmdR1 in response to iron levels. In contrast, nonsiderophore BGCs seem to be activated by mechanisms other than Fur- or DmdR1-mediated regulation under iron-restricted conditions. In the environment, siderophores produced by *Streptomyces* are sometimes stolen and utilized by other nearby microbes, such as fungi and other *Streptomyces* species [47]. Therefore, *Streptomyces* may activate defense mechanisms against neighboring species to survive under conditions of iron competition, as in the example of *S. coelicolor* and *M. xanthus*. In the same context, pathogenic bacteria use iron-depletion conditions as a marker for host recognition and virulence expression to take nutrients, including iron, from the host [21]. Accordingly, when *Streptomyces* species reside with various microbes and undergo iron competition, they increase the production of nonsiderophore secondary metabolites, which have antimicrobial activity and potentially iron-chelating function to obtain advantages during competition.

In order to increase the chemical diversity of known secondary metabolites and to search new drug candidates from *Streptomyces*, activating the silent biosynthetic pathways of *Streptomyces* is important, as is improving our understanding the awakening mechanism. Overall, our findings provided insights into the mechanisms through which chemical competition in the microbial community activates the silent secondary biosynthetic pathways in unexpected ways and thus will elevate the potential of *Streptomyces* as a production host for a diverse set of secondary metabolites.

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**Author contributions** B-KC conceived and supervised the study. NL, SC, and B-KC designed the experiments. NL, WK, JC, YL, K-SJ, and SC performed the experiments. NL, K-SJ, SC, SCK, BP, and B-KC analyzed the data. NL, SC, BP, and B-KC wrote the manuscript.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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