Full Paper

Activation of cryptic milbemycin A₄ production in *Streptomyces* sp. BB47 by the introduction of a functional *bldA* gene

(Received March 19, 2021; Accepted April 14, 2021; J-STAGE Advance publication date: September 11, 2021)

Nana Matsui¹, Shizuka Kawakami¹, Dai Hamamoto¹, Sayuri Nohara¹, Reina Sunada¹, Watanalai Panbangred², Yasuhiro Igarashi³, Takuya Nihira¹,⁴,* and Shigeru Kitani¹,⁵

¹ International Center for Biotechnology, Osaka University, Osaka, Japan
² Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand.
³ Biotechnology Research Center, Toyama Prefectural University, Toyama, Japan
⁴ MU-OU Collaborative Research Center for Bioscience and Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand
⁵ Industrial Biotechnology Initiative Division, Institute for Open and Transdisciplinary Research Initiatives, Osaka University

* Deceased 17 September 2018

Running title: Activating cryptic milbemycin production

Correspondence: Dr. S. Kitani, International Center for Biotechnology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan.

E-mail: kitani@icb.osaka-u.ac.jp
Summary

Streptomyces are characterized by their ability to produce structurally diverse compounds as secondary metabolites and by their complex developmental life cycle, which includes aerial mycelium formation and sporulation. The production of secondary metabolites is growth-stage dependent, and generally coincides with morphological development on a solid culture. *Streptomyces* sp. BB47 produces several types of bioactive compounds and displays a bald phenotype that is devoid of an aerial mycelium and spores. Here, we demonstrated by genome analysis and gene complementation experiments that the bald phenotype arises from the *bldA* gene, which is predicted to encode the Leu-tRNA<sub>UUA</sub> molecule. Unlike the wild-type strain producing jomthonic acid A (1) and antarlide A (2), the strain complemented with a functional *bldA* gene newly produced milbemycin (3). The chemical structure of compound 3 was elucidated on the basis of various spectroscopic analyses, and was identified as milbemycin A4, which is an insecticidal/acaricidal antibiotic. These results indicate that genetic manipulation of genes involved in morphological development in streptomycetes is a valuable way to activate cryptic biosynthetic pathways.

Keywords:

*bldA*; cryptic compound; milbemycin; morphological development; *Streptomyces*
Introduction

Natural products are a rich source of bioactive compounds, and have a long history of use in agriculture and human and veterinary medicine (Koehn and Carter, 2005). Microbial metabolites have contributed to the expansion of the structural diversity of bioactive compounds (Newman and Cragg, 2016; Omura, 2011). In the last few years, genomic technologies have revolutionized microbial natural product research. For example, microbial genomic information has revealed various gene clusters encoding enzymes involved in the production of undetected and unknown secondary metabolites, as well as known bioactive compounds (Baltz, 2017; Nett et al., 2009). Interestingly, most of the gene clusters not associated with any of the reported compounds are presumed to be “cryptic” biosynthetic pathways that are either not expressed under standard laboratory cultivation conditions or are expressed at a very low titer (Hoskisson and Seipke, 2020). Strategies to activate these cryptic gene clusters have therefore been developed to gain access to novel/useful bioactive compounds and intriguing biosynthetic pathways (Baltz, 2018; Liu et al., 2013; Zarins-Tutt et al., 2016).

Filamentous bacteria of the genus *Streptomyces* are the most prolific of the microorganisms producing bioactive compounds as secondary metabolites; and have a complex developmental cycle, including aerial mycelial formation and sporulation. The large genome of *Streptomyces* is rich in biosynthetic capacity, with about 20–40 biosynthetic gene clusters of secondary metabolites per strain (Baltz, 2017). Genetic manipulation in streptomycetes has awakened the expression of cryptic biosynthetic pathways, resulting in the finding of new bioactive compounds. The production of secondary metabolites (so-called secondary metabolism) and morphological development are closely linked (McCormick and Flardh, 2012). The *bld* mutants, which lack aerial mycelium formation and thus have a bald appearance, have pleiotropic effects which often cause defects in carbon catabolite repression and in intercellular signaling, and change the profile
of secondary metabolites including antibiotics (Bibb et al., 2000). The bldA mutation of Streptomyces coelicolor A3(2) and other Streptomyces strains has been well-studied, and, in most cases, affects both morphological development and secondary metabolism (Leskiw et al., 1991). The bldA gene is a unique gene that encodes a tRNA molecule required for the translation of the mRNA UUA codons specifying leucine (Leu-tRNAUUA) and is well-conserved in streptomycetes (Rebets et al., 2006; Tercero et al., 1998; Wang et al., 2009). At the DNA level, the TTA codon is the rarest codon in the GC-rich genome of Streptomyces spp. The TTA codons are frequently present in regulatory genes, potentially providing a way to restrict the expression of genes involved in morphological development and/or biosynthesis of secondary metabolites (Chater and Chandra, 2008; Chater, 2013; Chandra and Chater, 2014). Thus, the bldA gene is one of the most important factors in understanding these two striking features in streptomycetes.

Streptomyces sp. BB47 produces jomthonic acids A–C, which induce an activity for adipocyte differentiation (Igarashi et al., 2012; Yu et al., 2014), and antarlides A–H, which act as androgen-receptor antagonists (Saito et al., 2016, 2017) (Fig. 1). In addition, an HPLC/UV analysis of a culture-broth extract using our in-house metabolite database also suggested the presence of clethramycin-type hexaenes and piericidins, both of which are antifungal agents (Cai et al., 2007; Hayakawa et al., 2007; Igarashi et al., 2003). Thus, we conjectured that Streptomyces sp. BB47 may have the ability to synthesize a structurally diverse range of compounds as secondary metabolites. Despite its excellent production capacity for bioactive compounds, this strain shows a bald phenotype with insufficient sporulation and aerial mycelium formation on the ISP medium 2 used routinely in studies of Streptomyces (Fig. 2). These findings led us to hypothesize that restoration of the bald phenotype to normal morphological development could activate the expression of cryptic biosynthetic pathways, leading to the finding of additional useful compounds. Here, we search for and identify the genes responsible for the bald phenotype.
of *Streptomyces* sp. BB47, and demonstrate that the bald phenotype is caused by a loss of function of the *bldA* gene, and that *Streptomyces* sp. BB47 harboring a functional *bldA* copy produces cryptic milbemycin A₄, which is not detected in the culture broth of the wild-type strain.
Materials and Methods

Bacterial strains, plasmids, and growth conditions

*Streptomyces coelicolor* A3(2) M145 was used to PCR-amplify the *bldA* gene (SCOt24; *trn24*), and *Streptomyces* sp. BB47 was used as the host for heterologous expression of the *bldA* gene. *Escherichia coli* DH5α was used for general DNA manipulation, and the DNA methylation-deficient *E. coli* strain ET12567 containing the RP4 derivative pUZ8002 (Paget et al., 1999) was used for *E. coli*/*Streptomyces* sp. BB47 conjugation. The plasmids used were pBluescript II KS for general cloning and pLT129 (Daduang et al., 2015), a derivative of pSET152 containing a strong and constitutive promoter (*ermEp*) and a *tfd* terminator with the hygromycin-resistance gene *hyg*, for overexpression of the *bldA* gene. The medium conditions and general *E. coli* and *Streptomyces* manipulations were as described previously (Kieser et al., 2000). *Streptomyces* sp. BB47 was grown on a Bn-2 medium (soluble starch 0.5%, glucose 0.5%, meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, CaCO₃ 0.1% and agar 1.5%). The glycerol stock prepared from the agar medium was inoculated into 70 mL TSB+YEME medium (including TSB medium and YEME medium in a one-to-one ratio) in a 500-mL baffled flask, and the culture broth was collected after 42 h of cultivation at 28°C on a rotary shaker (160 rpm). The culture broth (1 mL) was inoculated into 100 mL V-22 medium (Igarashi et al., 2010) in a 500-mL baffled flask, and incubated at 160 rpm and 28°C for 4 days. The mycelia were washed, resuspended in fresh V-22 medium and stored at -80°C until use as a seed culture.

Genome sequencing and bioinformatic analyses

Genomic DNA sequencing was performed using an Illumina HiSeq system. The sequencing data were assembled using Velvet assembly software to give 417 contigs with a mean length of 19,211 bp. The assembled contig sequences and the Genome Matcher software (Ohtsubo et al.,
Matsui et al. (2008) were used to screen for a putative \textit{bldA} gene in the genome. The \textit{bldA} sequence was analyzed with the GENETYX software package (GENETYX Corp., Japan). The nucleotide sequence of the \textit{bldA} gene from \textit{Streptomyces} sp. BB47 has been deposited in the DDBJ data bank under accession number LC616657. The secondary structure of \textit{Streptomyces} Leu tRNAs\textsubscript{UUA} was predicted using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) with minimum free energy and partition function in fold algorithms. The expected entropy is visualized as a heat map.

\textbf{Construction of a \textit{Streptomyces} sp. BB47 harboring a \textit{S. coelicolor} \textit{bldA} gene}

The \textit{bldA} gene from \textit{S. coelicolor} A3(2) M145 was amplified by PCR with the primer pair oxbldA-Fw (5'-CGCGGATCCGACGAAAGCCCATACC-3\textquoteright) and oxbldA-Re (5'-CGCGGATCCGACGAAAGCCCATACC-3\textquoteright), both of which include a \textit{Bam}HI site. The resultant fragment was digested with \textit{Bam}HI and then inserted into the \textit{Bam}HI site of pBluescript II KS. The resultant plasmid was digested with \textit{Bam}HI, and the 0.4-kb fragment was recovered and cloned into the \textit{Bam}HI site of pLT129, resulting in pLT176, which was verified by sequencing. \textit{E. coli} ET12567(pUZ8002) harboring pLT176 was conjugated with \textit{Streptomyces} sp. BB47, and then the \textit{bldA} gene from \textit{S. coelicolor} A3(2) was introduced into the genome of \textit{Streptomyces} sp. BB47 as an extra copy. Integration of the plasmid pLT176 was confirmed by hygromycin resistance and PCR analysis, and the \textit{Streptomyces} sp. BB47 harboring the \textit{S. coelicolor} \textit{bldA} gene was designated as the oxbldA strain.

\textbf{Morphological assessment}

Spores of the \textit{Streptomyces} sp. BB47 wild-type strain, the oxbldA strain, and a \textit{Streptomyces} sp. BB47 carrying pLT129 were streaked onto ISP medium 2 (ISP2), ISP medium 4 (ISP4), and
soya flour-mannitol (SFM) medium. These plates were cultivated at 28°C for 3 days before they were analyzed for morphological differences.

**HPLC analysis of metabolite profiles from *Streptomyces* sp. BB47 strains**

The seed culture (500 µL) was inoculated on 2 mL YMS medium (Ueda et al., 2019), ISP2 medium, ISP4 medium, and SFM medium, respectively, followed by incubation at 28°C for 7 days. The agar culture was diced and extracted with an equal volume of methanol, and the methanol extract was evaporated and dissolved in dimethyl sulfoxide (150 µL) as an analytical sample. For liquid cultivation, the seed culture (1 mL) was inoculated into 100 mL liquid YMS medium in a 500-mL baffled flask, and incubated at 28°C and 160 rpm for 6 days. The culture broth (2 mL) was extracted with a half volume of *n*-butanol, and the *n*-butanol layer was evaporated and dissolved in dimethyl sulfoxide (150 µL). These extracts were analyzed by reversed-phase HPLC on a Cadenza CD-C18 column (4.6 i.d. x 75 mm; Imtakt, Japan) developed with a gradient system of acetonitrile (15% for 0–3 min; 15–85% for 3–25 min; 85% for 25–29 min) containing 0.1% formic acid (flow rate, 1.2 mL/min; UV detection, 254 nm).

**Isolation and structural elucidation of compound 3 produced by the oxbldA strain**

The mycelia were harvested from the 4 L culture broth of the oxbldA strain, which was incubated at 28°C and 160 rpm for 8 days by using liquid YMS medium. The mycelia were extracted with an equal volume of methanol and filtrated, after which the methanol extract was evaporated to dryness. The crude extract (3.7 g) was fractionated by silica gel column chromatography eluted with a stepwise gradient of hexane/ethyl acetate (1:0, 2:1, 1:1, and 1:2 v/v). Fractions 2 and 3 were concentrated to give 380 mg, which was further purified by preparative reversed-phase HPLC using a Capcell-Pak C₁₈ column (UG80; 5 µm; 10 i.d. × 250
mm; Osaka Soda, Japan) developed with 80% acetonitrile containing 0.1% formic acid at 3 mL/min followed by detection at 254 nm, yielding 3.1 mg of purified compound 3. HR-ESI-MS was recorded on a JMS-T100LP (JEOL, Japan), and the NMR spectra (¹H, 600 MHz; ¹³C, 150 MHz) were measured on an UltraShield 600 Plus spectrometer (Bruker).
Results

Identification of a putative bldA gene from *Streptomyces* sp. BB47

When the mycelia of *Streptomyces* sp. BB47 are grown on ISP2 medium (including glucose as a carbon source), the strain shows a poor ability to sporulate as a bald phenotype (Fig. 2). The behavior of *bld* mutants from *S. coelicolor* A3(2) depends on the medium composition (Bibb et al., 2000). Thus, we assessed the morphological development of *Streptomyces* sp. BB47 on solid media that included a different carbon source. As shown in Fig. 2, there was no significant difference in color or morphology among the cultivation with ISP2 medium, ISP4 medium (including soluble starch), or SFM medium (including mannitol), suggesting that the bald phenotype is probably not affected by carbon sources. Next, to find potential *bld* mutations of *Streptomyces* sp. BB47, the genome was sequenced using the Illumina sequencing technology. The draft DNA sequences of the genome contained 8.01 Mbp and were dispersed across 417 contigs. Among the *bld* genes, *bldA* plays an important role in morphological development, as described above. By analyzing the draft DNA sequence based on sequence homology of the *S. coelicolor bldA* gene, we found the only potential *bldA* gene in *Streptomyces* sp. BB47. Sequence comparison with *bldA* genes in other streptomycetes, the morphological development of which is normal, demonstrated that the nucleotide sequence of the 45–58 bp region in the *Streptomyces* sp. BB47 *bldA* gene is slightly different from those in the other *bldA* genes (Fig. 3A). To determine whether the slight difference of nucleotide sequences affects the *bldA* function, we predicted the secondary structure of these *bldA* genes, revealing that the 45–58 bp region forms a variable arm in the typical clover-leaf structure of tRNA (Fig. 3B). Furthermore, the entropy of the D arm and ΨC arm is expected to be higher than that of the *bldA* genes in *S. coelicolor* A3(2), *Streptomyces griseus* and *Streptomyces avermitilis*, suggesting that the secondary structure of the putative *bldA* gene from *Streptomyces* sp. BB47 might be unstable, leading to the loss of the
function of Leu-tRNA^{UUA} and to the poor sporulation.

**Introduction of a functional bldA gene induces morphological development**

Based on the sequence and secondary-structure comparison of the bldA genes, we hypothesized that the bald phenotype of *Streptomyces* sp. BB47 comes from the nucleotide sequence of the putative bldA gene. To confirm this hypothesis, we introduced one copy of the bldA gene from *S. coelicolor* A3(2) into the genome of *Streptomyces* sp. BB47 by using a plasmid pLT176, and evaluated the morphological development. The plasmid is an integrative plasmid based on φC31 integrase, which helps to integrate an attB site in the chromosome (Combes et al., 2002). It contains the bldA gene under the control of the constitutive and strong *ermE* promoter. *Streptomyces* sp. BB47 carrying pLT176, designated as an oxbldA strain, formed aerial mycelia and spores normally on ISP2 medium as is typically observed for *Streptomyces* strains (Fig. 2). Moreover, when the oxbldA strain was cultivated on ISP4 medium and SFM medium, the morphological development was also similar to that cultivated on ISP2 medium, indicating that complementation of the functional bldA gene restores the formation of the aerial mycelium with sporulation regardless of the medium composition. On the other hand, *Streptomyces* sp. BB47 carrying the empty pLT129 vector still showed insufficient sporulation and poor aerial mycelium formation as in the parental strain. These findings indicated that the bald phenotype in *Streptomyces* sp. BB47 is attributed to the putative bldA gene.

**Effect of introduction of an additional bldA gene on the metabolite profiling**

To investigate whether an additional introduction of the functional bldA gene would regulate an unexpected production of secondary metabolites, we performed a detailed analysis of the metabolite profiles of the oxbldA strain by HPLC using a diode array detector. Because the
oxbldA strain showed a dramatic change of morphological development on a solid culture, the oxbldA strain was first grown on the solid medium, and metabolites in the crude extract were analyzed by reversed-phase HPLC. In cultures using ISP4 and SFM media, no differences in metabolite profiles were observed between the wild-type carrying pLT129 and the oxbldA strains (data not shown). However, a few peaks (5.7 min and 25.6 min) were detected only in the methanol extract of the oxbldA strain grown on ISP2 and YMS media (Fig. S1). For structural analysis, it is desirable that the target compounds are produced in a liquid culture, where they can be easily prepared in large quantities. Thus, we cultivated the oxbldA strain by using liquid ISP2 and YMS media, and investigated the metabolite profiles. When the oxbldA strain was grown in liquid ISP2 medium, the HPLC chromatogram of the methanol extract was almost identical to that of the wild-type carrying pLT129. However, in a culture using liquid YMS medium, the oxbldA strain produced several compounds eluting from 23 to 27 min, whereas the corresponding peaks were not detected in the HPLC chromatogram of the wild-type strain and the wild-type strain carrying pLT129 (Fig. 4). The UV absorption maximum (244 nm) of one remarkably large peak (25.6 min) was identical to that of the peak (25.6 min) detected from the oxbldA strain grown on solid ISP2 and YMS media. These findings indicated that the production of an unidentified compound 3 (25.6 min) both on a solid culture and in a liquid culture is manifested by the additional introduction of the functional bldA gene. We have previously demonstrated that Streptomyces sp. BB47 has the ability to produce the antifungal agents known as piericidins under a cultivation condition using liquid A-3M medium (Igarashi et al., 2012). The HPLC/UV analysis with our in-house metabolite database also suggested that the small peaks (eluted from 23.4 to 27.1 min) around the peak of compound 3 were piericidins. These results clearly indicated that the additional introduction of the functional bldA gene into the bald-phenotype strain Streptomyces sp. BB47 leads not only to the restoration of morphological development, but also
to the activation of the production of cryptic metabolites in response to cultivation conditions.

Isolation and structural elucidation of compound 3 from the oxbldA strain

To elucidate its structure, we purified the newly appeared compound 3 from 4 L of the liquid YMS culture of the oxbldA strain. Because compound 3 was found to be accumulated in the mycelia, it was successively purified from the mycelia by silica gel column chromatography and preparative reversed-phase HPLC, yielding 3.1 mg of the pure compound 3 (Fig. 4). The molecular formula of compound 3 was deduced to be C_{32}H_{46}O_7 by the HRESITOFMS analysis (positive ion mode), which showed a molecular ion peak at $m/z$ 565.3111 [M + Na]^+ (calculated for C_{32}H_{46}O_7Na^+, 565.3136), indicating the presence of 10 sites of unsaturation in the structure. The UV spectrum of compound 3 showed an absorption maximum at 244 nm with two shoulders (238 nm and 254 nm).

The structure of compound 3 was established by 1D NMR ($^1$H and $^{13}$C NMR) and 2D NMR (HSQC, HMBC, and $^1$H-$^1$H COSY) analysis (Table 1). The $^1$H and $^{13}$C NMR spectra of 3 in combination with the HSQC spectrum indicated the presence of one triplet aliphatic methyl proton ($\delta_H$ 0.92), two doublet aliphatic methyl protons ($\delta_H$ 0.76 and 0.93), and two olefinic or aromatic methyl protons ($\delta_H$ 1.46 and 1.81). The $^{13}$C-NMR spectrum displayed 32 carbon signals, including 5 methyls ($\delta_C$ 10.1, 15.5, 17.8, 20.0, and 22.3), 8 methylenes, 13 methines (3 $sp^2$ methine carbons, 5 $sp^3$ methine centers, and 5 oxymethines), 1 acetal carbon ($\delta_C$ 97.4), and 1 ester carbonyl carbon ($\delta_C$ 173.6). The $^1$H-$^1$H COSY correlations revealed 6 partial structures: H-2 to H-3, H-5 to H-6, H-10 to H-28, H-15 to H-19, H-25 to H-32, and H-24 to H-30 (Fig. 5A). A detailed comparison of the NMR data of 3 with those of milbemycins revealed that the $^1$H and $^{13}$C NMR data of compound 3 were significantly similar to those of milbemycin A$_4$ (Ono et al., 1983; Takiguchi et al., 1980;). HMBC correlations of 3 from methyl protons (H$_3$-26) to C-3 and
C-5, and oxymethine proton (H-6) to C-2 and C-4 (Fig. 5B), combined with the \( ^1H-^1H \) correlations of H-2/H-3 and H-5/H-6, demonstrated that C-2 to C-7 (a hydroxy methine carbon) formed a six-membered ring. The observed long-range correlations of methyl protons (H\(_3\)-28, H\(_3\)-29, H\(_3\)-30, and H\(_3\)-32) and methylene protons (H\(_2\)-31) indicated that three methyl groups (C-28, C-29, and C-30) were attached to C-12, C-14, and C-24, respectively, whereas one ethyl group (C-31 to C-32) was connected with a oxymethine carbon C-25. The geometry of the double bond between C-10 and C-11 was assigned as 10\( E \), on the basis of the vicinal coupling constant (\( J_{10,11} = 14.7 \) Hz on average) (Table 1). Thus, the planar structure of 3 was established (Fig. 1), and was identical to that of milbemycin A\(_4\), which is an antibiotic with acaricidal activity (Takiguchi et al., 1980). Based on all of these results, we concluded that overexpression of the functional \( bldA \) activates the production of cryptic milbemycin A\(_4\) (3) in \textit{Streptomyces} sp. BB47.
Discussion

Through a growing number of new genome sequencing projects, the genome of actinomycetes has been revealed to contain a number of genes involved in the biosynthesis of novel natural products with potentially intriguing biological properties. However, it has become apparent that a vast number of these biosynthetic pathways are poorly expressed under standard laboratory cultivation conditions, and thus they have been referred to as cryptic biosynthetic pathways. To date, considerable research has focused on developing approaches for the discovery of cryptic bioactive compounds. Positive and negative pathway-specific regulators in the biosynthetic gene clusters of individual compounds have been used as target factors as one approach to activate the expression of the cryptic gene clusters. Overexpression of a *Streptomyces* antibiotic regulatory protein (SARP)-family gene adjacent to silent gene clusters has led to the discovery of new bioactive compounds in several *Streptomyces* strains (Du et al., 2016; Koomsiri et al., 2019). On the other hand, global regulators located outside the gene clusters have also been recognized as attractive targets for the activation of cryptic pathways. We previously demonstrated that gene deletion of a *Streptomyces*-hormone receptor homologue not only regulates the production of the major compounds, but also activates the production of cryptic β-carboline alkaloid compounds in *Kitasatospora setae* and cryptic phthoxazolin A in *Streptomyces avermitilis* (Aroonsri et al., 2012 (a, b); Suroto et al., 2017, 2018). In the present study, we have shown that the overexpression of the functional *bldA* gene (encoding one of the global regulators) in *Streptomyces* sp. BB47 showing a bald phenotype restores the aerial mycelial formation with sporulation, and activates the production of cryptic milbemycin A₄ and piericidins, allowing the identification of its structure. Furthermore, the *bldA*-signaling cascade controlling both morphological development and secondary metabolism is also found to be conserved in *Streptomyces* sp. BB47.
On solid cultivation of streptomycetes, the biosynthesis of many secondary metabolites is induced after the onset of morphological development. This means that morphological development and secondary metabolism are closely regulated and may be genetically linked through common regulatory factors. Kalan et al. (2013) showed that complementation of *Streptomyces calvus*, which impairs aerial mycelial formation and sporulation, with the correct copy of the *bldA* gene promotes morphological development, and allows the production of cryptic polyene compounds. A similar approach has been used to change the biosynthetic profile of *Streptomyces* strains classified as poorly sporulating strains, and the production of cryptic compounds, which include new/known compounds, is stimulated by expression of the *bldA* gene (Gessner et al., 2015). However, the morphological development of the strains investigated is not affected by the introduction of the *bldA* gene, and it is not known whether the original *bldA* gene in each *Streptomyces* strain is functional. On the other hand, we proved that the bald phenotype of *Streptomyces* sp. BB47 is due to its own *bldA* gene, because the functional *bldA* gene from *S. coelicolor* A3(2) confers on *Streptomyces* sp. BB47 the ability to form an aerial mycelium with sporulation. The nucleotide sequences of the *bldA* genes differ only slightly between streptomycetes (Fig. 3A). However, the prediction of the secondary structure of the *bldA* gene suggested that the positional entropy of the D arm and ΨC arm of the *Streptomyces* sp. BB47 *bldA* is different from that of other functional *bldA* genes (Fig. 3B). This energy difference is thought to contribute to an instability of the higher-order structure of the *bldA* gene.

Milbemycins are a group of 16-membered macrolides with potent insecticidal and anthelminthic activity, and are produced by several *Streptomyces* species (Ono et al., 1983; Takiguchi et al., 1980; Wang et al., 2009). In particular, milbemycin A₄ is one of the main components of the biosynthetic pathway, and the derivatives of milbemycin A₄ have been commercially used in the fields of agriculture and veterinary medicine. The complete
biosynthetic gene cluster (*mil* cluster) of milbemycins has been identified in the genome of *S. bingchenggensis*, although the enzymes involved in some post-modification steps remain unclear (Wang et al., 2010). Analysis of the draft DNA sequences of *Streptomyces* sp. BB47 using AntiSMASH predicted the presence of several potential biosynthesis genes for milbemycin A₄ in the fragmented contigs. The genes encode homologues of MilC (polyketide modifying enzyme), MilE (cytochrome P450 hydroxylase), MilF (C-5-ketoreductase), and MilR (LuxR family transcriptional regulator), each of which plays an important role in the biosynthesis of milbemycins. However, these putative biosynthetic genes have no TTA codon in the nucleotide sequence, suggesting that cryptic milbemycin A₄ production is not activated through the expression of these genes. On the other hand, certain milbemycin biosynthetic genes, such as the polyketide synthase (PKS) gene, have not yet been found, because the fragmented contigs do not have sufficient information to assemble the PKS genes. Thus, there is a possibility that the remaining biosynthetic genes or global regulatory genes (widely controlling secondary metabolism) might contain the TTA codon and be under the control of Leu-tRNA<sup>UUA</sup>. The overexpression of the functional *bldA* gene also stimulated cryptic production of piericidins. A cluster of putative biosynthetic genes for piericidins is found in the genome of *Streptomyces* sp. BB47, and in its proximal region there is a gene encoding a SARP-family regulator that may be involved as an activator of piericidins production (Li et al., 2019). The putative regulatory gene has a TTA codon in its nucleotide sequence, suggesting that ectopic expression of a functional *bldA* gene might prompt expression of the putative SARP-family regulator, resulting in stimulation of cryptic piericidins production.

Morphological development of streptomycetes requires not only *bldA* but also several other factors, such as other *bld* genes and *whi* genes (Bibb et al., 2000). These factors are involved in aerial mycelial formation and sporulation at various levels of the signaling cascade, and their
function is sometimes dependent on the composition of the medium. The oxbldA strain derived from *Streptomyces* sp. BB47 differentiated its morphology normally in all solid media with different carbon sources. However, the metabolite profiles were different in each solid medium, suggesting that the nutrient condition affects secondary metabolisms beyond the function of *bldA*. In some cases, the peaks observed only in the oxbldA strain disappeared when this strain was cultured in a liquid medium of the same composition as the solid medium. These findings implied that the culture environment probably affects the expression of regulatory genes or biosynthetic enzyme genes, which have the TTA codon in their nucleotide sequences. Gessner et al. (2015) conjectured that increasing the cellular concentration of Leu-tRNA\textsuperscript{UUA} encoded by *bldA* facilitates the translation of UUA codons, leading to regulation of the production of secondary metabolites. Taken together with our results, these findings suggest that, even if morphological development is not rescued by complementation of a functional *bldA* gene into *bldA* mutant strains, careful consideration of the culture conditions could reveal the presence of cryptic compounds. Interestingly, some *bldA* homologues showed a similar entropy distribution of the D arm and TΨC arm as *bldA* from *Streptomyces* sp. BB47. Thus, constitutive expression of the functional *bldA* gene in streptomycetes would be one of the effective approaches for the discovery of new natural bioactive compounds.

**Acknowledgements**

We would like to thank Dr. Tetsuhiro Ogawa (The University of Tokyo) for helpful discussion on the tRNA molecules. This work was supported by a Grant-in-Aid for Scientific Research (C) (grant number 18K05390) from the Japan Society for the Promotion of Science (JSPS) to S.K. and by a Grant-in-Aid for Scientific Research on Innovative Areas (grant number 20H04775) from JSPS to S.K. This work was also supported in part by funding from the Institute
for Fermentation, Osaka (IFO) to S.K. by funding from the Asahi Glass Foundation to S.K., and by a New Chemical Technology Research Encouragement Award (Step-up Award) from the Japan Association for Chemical Innovation to S.K.
Figure legends

Figure 1
Chemical structures of jomthonic acid A (1), antarlide A (2) and milbemycin A₄ (3) produced by *Streptomyces* sp. BB47.

Figure 2
Morphological development of the *Streptomyces* sp. BB47 *oxbldA* strain. WT, *Streptomyces* sp. BB47; *oxbldA*, *Streptomyces* sp. BB47 carrying pLT176 [including a *bldA* gene from *S. coelicolor* A3(2)]; pLT129, *Streptomyces* sp. BB47 carrying the empty pLT129 vector (control).

Each strain was streaked on ISP medium 2 (ISP2), ISP medium 4 (ISP4), and soya flour-mannitol (SFM) medium, respectively, and incubated at 28°C. The plates were photographed from above at 3 days of cultivation.

Figure 3
Sequence analysis of the Leu tRNA⁹⁰ for *Streptomyces* sp. BB47 and other streptomycetes (A), and the predicted secondary structures of the Leu tRNAs⁹⁰ (B). (A) Numbers indicate nucleotide positions within each sequence, and black boxes indicate positions in the alignment at which the same nucleotide is found in at least four of the six sequences.

Figure 4
HPLC chromatograms of *n*-butanol extracts from the *Streptomyces* sp. BB47 wild-type strain (WT), the wild-type strain carrying pLT129 (pLT129) and the wild-type strain carrying pLT176 (*oxbldA*), and purified compound 3. mAU, milliabsorbance units at 254 nm. The peak of compound 3 is indicated by an filled inverted triangle. The peaks of jomthonic acid A and
piericidins are represented by an open diamond and open inverted triangles, respectively.

**Figure 5**

$^1$H-$^1$H COSY correlations of compound 3 (A) and key HMBC correlations of 3 (B). (A) $^1$H-$^1$H COSY correlations are shown as a bold line. (B) HMBC correlations are from proton(s) stated to the indicated carbon.
References

Aroonsri, A., Kitani, S., Ikeda, H. and Nihira, T. (2012-a) Kitasetaline, a novel β-carboline alkaloid from Kitasatospora setae NBRC 14216T. J. Biosci. Bioeng., 114, 56-58.

Aroonsri, A., Kitani, S., Hashimoto, J., Kosone, I., Izumikawa, M. et al. (2012-b) Pleiotropic control of secondary metabolism and morphological development by KsbC, a butyrolactone autoregulator receptor homologue in Kitasatospora setae. Appl. Environ. Microbiol., 78, 8015-8024.

Baltz, R.H. (2017) Gifted microbes for genome mining and natural product discovery. J. Ind. Microbiol. Biotechnol., 44, 573-588.

Baltz, R.H. (2018) Synthetic biology, genome mining, and combinatorial biosynthesis of NRPS-derived antibiotics: a perspective. J. Ind. Microbiol. Biotechnol., 45, 635-649.

Bibb, M.J., Molle, V. and Buttner, M.J. (2000) σBldN, an extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in Streptomyces coelicolor A3(2). J. Bacteriol., 182, 4606-4616.

Cai, P., Kong, F., Fink, P., Ruppen, M.E., Williamson, R.T. et al. (2007) Polyene antibiotics from Streptomyces mediocidicus. J. Nat. Prod., 70, 215-219.

Chandra, G. and Chater, K.F. (2014) Developmental biology of Streptomyces from the perspective of 100 actinobacterial genome sequences. FEMS. Microbiol. Rev., 38, 345-379.

Chater, K.F. and Chandra, G. (2008) The use of the rare UUA codon to define "expression space" for genes involved in secondary metabolism, development and environmental adaptation in Streptomyces. J. Microbiol., 46, 1-11.

Chater, K.F. (2013) Curing baldness activates antibiotic production. Chem. Biol., 20, 1199-1200.

Combes, P., Till, R., Bee, S. and Smith, M.C. (2002) The Streptomyces genome contains multiple pseudo-attB sites for the φC31-encoded site-specific recombination system. J. Bacteriol., 184, 5746-5752.

Daduang, R., Kitani, S., Sudoh, Y., Grace, Umadhay Pait I., Thamchaipenet, A. et al. (2015) 29-Deoxymaklamicin, a new maklamicin analogue produced by a genetically engineered strain of Micromonospora sp. NBRC 110955. J. Biosci. Bioeng., 120, 608-613.

Du, D., Katsuyama, Y., Onaka, H., Fujie, M., Satoh, N. et al. (2016) Production of a novel amide-containing polyene by activating a cryptic biosynthetic gene cluster in Streptomyces sp. MSC090213JE08. Chembiochem., 17, 1464-1471.
Gessner, A., Heitzler, T., Zhang, S., Klaus, C., Murillo, R. et al. (2015) Changing biosynthetic profiles by expressing bldA in Streptomyces strains. Chembiochem., 16, 2244-2252.

Hayakawa, Y., Shirasaki, S., Kawasaki, T., Matsuo, Y., Adachi, K. et al. (2007) Structures of new cytotoxic antibiotics, piericidins C7 and C8. J. Antibiota. (Tokyo.), 60, 201-203.

Hoskisson, P.A. and Seipke, R.F. (2020) Cryptic or silent? The known unknowns, unknown knowns, and unknown unknowns of secondary metabolism. mBio. 11, e02642-20.

Igarashi, Y., Iwashita, T., Fujita, T., Naoki, H., Yamakawa, T. et al. (2003) Clethramycin, a new inhibitor of pollen tube growth with antifungal activity from Streptomyces hygroscopicus TP-A0623. II. Physico-chemical properties and structure determination. J. Antibiota. (Tokyo)., 56, 705-708.

Igarashi, Y., Shimasaki, R., Miyanaga, S., Oku, N., Onaka, H. et al. (2010) Rakicidin D, an inhibitor of tumor cell invasion from marine-derived Streptomyces sp. J. Antibiota. (Tokyo)., 63, 563-565.

Igarashi, Y., Yu, L., Ikeda, M., Oikawa, T., Kitani, S. et al. (2012) Jomthonic acid, a modified amino acid from a soil-derived Streptomyces. J. Nat. Prod., 75, 986-990.

Kalan, L., Gessner, A., Thaker, M.N., Waglechner, N., Zhu, X. et al. (2013) A cryptic polyene biosynthetic gene cluster in Streptomyces calvus is expressed upon complementation with a functional bldA gene. Chem. Biol., 20, 1214-1224.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000) Practical Streptomyces genetics. John Innes Foundation. Norwich.

Koehn, F.E. and Carter, G.T. (2005) The evolving role of natural products in drug discovery. Nat. Rev. Drug. Discov., 4, 206-220.

Koomsiri, W., Inahashi, Y., Leetanasaksakul, K., Shiomi, K., Takahashi, Y.K. et al. (2019) Sarpeptins A and B, lipopeptides produced by Streptomyces sp. KO-7888 overexpressing a specific SARP regulator. J. Nat. Prod., 82, 2144-2151.

Leskiw, B.K., Lawlor, E.J., Fernandez-Abalos, J.M. and Chater, K.F. (1991) TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, Streptomyces mutants. Proc. Natl. Acad. Sci. U. S. A., 88, 2461-2465.

Li, Y., Kong, L., Shen, J., Wang, Q., Liu, Q. et al. (2019) Characterization of the positive SARP family regulator PieR for improving piericidin A1 production in Streptomyces piomogeues var. Hangzhouwanensis. Synth. Syst. Biotechnol., 4, 16-24.
Liu, G., Chater, K.F., Chandra, G., Niu, G. and Tan, H. (2013) Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiol. Mol. Biol. Rev.*, **77**, 112-143.

McCormick, J.R. and Flardh, K. (2012) Signals and regulators that govern *Streptomyces* development. *FEMS Microbiol. Rev.*, **36**, 206-231.

Nett, M., Ikeda, H. and Moore, B.S. (2009) Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat. Prod. Rep.*, **26**, 1362-1384.

Newman, D.J. and Cragg, G.M. (2016) Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod.*, **79**, 629-661.

Ohtsubo, Y., Ikeda-Ohtsubo, W., Nagata, Y. and Tsuda, M. (2008) GenomeMatcher: a graphical user interface for DNA sequence comparison. *BMC Bioinformatics.*, **9**, 376.

Omura, S. (2011) Microbial metabolites: 45 years of wandering, wondering and discovering *Tetrahedron.*, **67**, 6420-6459.

Ono, M., Mishima, H., Takiguchi, Y., Terao, M., Kobayashi, H. et al. (1983) Milbemycins, a new family of macrolide antibiotics. Studies on the biosynthesis of milbemycins a2, a4 and D using 13C labeled precursors. *J. Antibiot. (Tokyo).*, **36**, 991-1000.

Paget, M.S., Chamberlin, L., Atrih, A., Foster, S.J. and Buttner, M.J. (1999) Evidence that the extracytoplasmic function sigma factor σE is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J. Bacteriol.*, **181**, 204-211.

Rebets, Y.V., Ostash, B.O., Fukuhara, M., Nakamura, T. and Fedorenko, V.O. (2006) Expression of the regulatory protein LndI for landomycin E production in *Streptomyces globisporus* 1912 is controlled by the availability of tRNA for the rare UUA codon. *FEMS. Microbiol. Lett.*, **256**, 30-37.

Saito, S., Fujimaki, T., Panbangred, W., Igarashi, Y. and Imoto, M. (2016) Antarlides: a new type of androgen receptor (AR) antagonist that overcomes resistance to AR-targeted therapy. *Angew. Chem. Int. Ed. Engl.*, **55**, 2728-2732.

Saito, S., Fujimaki, T., Panbangred, W., Sawa, R., Igarashi, Y. et al. (2017) Antarlides F-H, new members of the antarlide family produced by *Streptomyces* sp. BB47. *J. Antibiot. (Tokyo).*, **70**, 595-600.

Suroto, D.A., Kitani, S., Miyamoto, K.T., Sakihama, Y., Arai, M. et al. (2017) Activation of cryptic phthoxazolin A production in *Streptomyces avermitilis* by the disruption of autoregulator-receptor homologue AvaR3. *J. Biosci. Bioeng.*, **124**, 611-617.
Suroto, D.A., Kitani, S., Arai, M., Ikeda, H. and Nihira, T. (2018) Characterization of the biosynthetic gene cluster for cryptic phthoxazolin A in *Streptomyces avermitilis*. PLoS. One., 13, e0190973.

Takiguchi, Y., Mishima, H., Okuda, M., Terao, M., Aoki, A. et al. (1980) Milbemycins, a new family of macrolide antibiotics: fermentation, isolation and physico-chemical properties. *J. Antibiot. (Tokyo)*, 33, 1120-1127.

Tercero, J.A., Espinosa, J.C. and Jimenez, A. (1998) Expression of the *Streptomyces alboniger pur* cluster in *Streptomyces lividans* is dependent on the *bldA*-encoded tRNALeu. *FEBS. Lett.*, 421, 221-223.

Ueda, S., Ikeda, H., Namba, T., Ikejiri, Y., Nishimoto, Y. et al. (2019) Identification of biosynthetic genes for the β-carboline alkaloid kitasetaline and production of the fluorinated derivatives by heterologous expression. *J. Ind. Microbiol. Biotechnol.*, 46, .

Wang, J., Schully, K.L. and Pettis, G.S. (2009) Growth-regulated expression of a bacteriocin, produced by the sweet potato pathogen *Streptomyces ipomoeae*, that exhibits interstrain inhibition. *Appl. Environ. Microbiol.*, 75, 1236-1242.

Wang, M., Yang, X.H., Wang, J.D., Wang, X.J., Chen, Z.J. et al. (2009) New β-class milbemycin compound from *Streptomyces avermitilis* NEAU1069: fermentation, isolation and structure elucidation. *J. Antibiot. (Tokyo)*, 62, 587-591.

Wang, X.J., Yan, Y.J., Zhang, B., An, J., Wang, J.J. et al. (2010) Genome sequence of the milbemycin-producing bacterium *Streptomyces bingchenggensis*. *J. Bacteriol.*, 192, .

Yu, L., Oikawa, T., Kitani, S., Nihira, T., Bayanmunkh, B. et al. (2014) Jomthonic acids B and C, two new modified amino acids from *Streptomyces* sp. *J. Antibiot. (Tokyo)*, 67, 345-347.

Zarins-Tutt, J.S., Barberi, T.T., Gao, H., Mearns-Spragg, A., Zhang, L. et al. (2016) Prospecting for new bacterial metabolites: a glossary of approaches for inducing, activating and upregulating the biosynthesis of bacterial cryptic or silent natural products. *Nat. Prod. Rep.*, 33, 54-72.
Table 1. NMR spectroscopic data for compound 3 in chloroform-$d$

| Position | $\delta_C$ | $\delta_H$ mult (J in Hz) |
|----------|------------|--------------------------|
| 1        | 173.6, C   | -                        |
| 2        | 45.7, CH   | 3.20 d (2.3)             |
| 3        | 118.2, CH  | 5.33 m                   |
| 4        | 137.8, qC  | -                        |
| 5        | 67.7, CH   | 4.22 t (6.5)             |
| 6        | 79.2, CH   | 3.90 d (6.2)             |
| 7        | 80.4, qC   | -                        |
| 8        | 139.5, qC  | -                        |
| 9        | 120.3, CH  | 5.77 dt (11.3, 2.3)      |
| 10       | 123.4, CH  | 5.67 dd (14.5, 11.3)     |
| 11       | 142.9, CH  | 5.318 dd (14.8, 11.0)    |
| 12       | 36.0, CH   | 2.35 m                   |
| 13       | 48.5, CH$_2$ | 1.80 m                   |
|          |            | 2.14 m                   |
| 14       | 137.0, qC  | -                        |
| 15       | 120.9, CH  | 4.90 t (7.7)             |
| 16       | 34.7, CH$_2$ | 2.17 m                   |
| 17       | 67.5, CH   | 3.50 m                   |
| 18       | 36.7, CH$_2$ | 0.80 m                   |
|          |            | 1.74 m                   |
| 19       | 68.6, CH   | 5.315 m                  |
| 20       | 41.3, CH$_2$ | 1.29 m                   |
|          |            | 1.93 ddd (1.7, 4.8, 12.1) |
| 21       | 97.4, qC   | -                        |
| 22       | 35.6, CH$_2$ | 1.45 m                   |
|          |            | 1.60 m                   |
| 23       | 27.9, CH$_2$ | 1.45 m                   |
| 24       | 34.2, CH   | 1.26 m                   |
| 25       | 76.0, CH   | 3.06 td (2.6, 10.0)      |
| 26       | 20.0, CH$_3$ | 1.81 br s               |
| 27       | 68.5, CH$_2$ | 4.61 ddd (2.1, 14.6, 25.0) |
| 28       | 22.3, CH$_3$ | 0.93 d (6.3)            |
| 29       | 15.5, CH$_3$ | 1.46 br s               |
| 30       | 17.8, CH$_3$ | 0.76 d (6.2)            |
| 31       | 25.7, CH$_2$ | 1.28 m                 |
|          |            | 1.62 m                   |
| 32       | 10.1, CH$_3$ | 0.92 t (7.5)            |
| C(7)OH   | -          | 4.03 s                   |
Figure 1

Matsui et al.

Jomthonic acid A (1)

Antarlide A (2)

Milbemycin A₄ (3)
Figure 4

Matsui et al.

WT

pLT129

oxbldA

Purified 3

Retention time (min)

Intensity (mAU)

0 5 10 15 20 25 30

0 500 1000 1500

0 500 1000 1500

0 500 1000 1500

0 3000 2000 1000 0

I

II

III

IV

3



