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Characterization of LnmO as a pathway-specific Crp/Fnr-type positive regulator for leinamycin biosynthesis in *Streptomyces atroolivaceus* and its application for titer improvement

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Abstract

The cAMP receptor protein/fumarate and nitrate reductase regulatory protein (Crp/Fnr) family of transcriptional regulators are pleiotropic transcriptional regulators that control a broad range of cellular functions. Leinamycin (LNM) is a potent antitumor antibiotic produced by *Streptomyces atroolivaceus* S-140. We previously cloned and characterized the *Inm* biosynthetic gene cluster from *S. atroolivaceus* S-140. We here report inactivation of *InmO* in *S. atroolivaceus* S-140 and overexpression of *InmO* in the *S. atroolivaceus* S-140 wild-type and *InmE* mutant SB3033 to investigate its role in LNM biosynthesis. Bioinformatics analysis revealed LnmO as the only regulator within the *Inm* gene cluster, exhibiting high sequence similarity to known Crp/Fnr family regulators. Inactivation of *InmO* in *S. atroolivaceus* S-140 completely abolished LNM production but caused no apparent morphological changes, supporting that LnmO is indispensable and specific to LNM biosynthesis. Overexpression of *InmO* in *S. atroolivaceus* S-140 and SB3033 resulted in 3- and 4-fold increase in LNM and LNM E1 production, respectively, supporting that LnmO acts as a positive regulator. While all of the Crp/Fnr family regulators studied to date appeared to be pleiotropic, our results support LnmO as the first Crp/Fnr family regulator that is pathway-specific. LnmO joins the growing list of regulators that could be exploited to improve secondary metabolite production in *Streptomyces*. Engineered strains overproducing LNM and

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Compliance with ethical standards

Animal and human subjects

This article does not contain any studies with human participation or animals performed by any of the authors.

Conflict of interests

The authors declare that they have no competing interests.

LNM E1 will facilitate further mechanistic studies and clinical evaluation of LNM and LNM E1 as novel anticancer drugs.

Keywords

Biosynthesis; regulation; leinamycin; Crp/Fnr regulator; titer improvement

Introduction

Streptomycetes, Gram-positive filamentous soil bacteria, are important sources of bioactive natural products including many clinically important antibiotics, anticancer agents, and other medicines. The genes encoding the biosynthesis of these natural products, also known as secondary metabolites, are usually clustered, the expression of which is typically under the control of pathway-specific and/or pleiotropic transcriptional regulators (Bibb 2005; Liu et al. 2013). Manipulation of pathway regulation, particularly through the pathway-specific regulators, has been widely exploited to improve the production of secondary metabolites in *Streptomyces* (Chen et al. 2010; Liu et al. 2013; Liu et al. 2014; Zhang et al. 2016).

The cyclic adenosine monophosphate (cAMP) receptor protein/ fumarate and nitrate reductase regulatory protein (Crp/Fnr) family transcriptional regulators are widely spread in both Gram-positive and Gram-negative bacteria, and they predominantly function as positive transcriptional regulators (Körner et al. 2003; Matsui et al. 2013). The Crp/Fnr regulators feature an N-terminal signal molecule binding domain and a C-terminal helix-turn-helix (HTH) motif-containing DNA binding domain (Körner et al. 2003; Lawson et al. 2004; McKay and Steitz 1981). They respond to a broad spectrum of intracellular and exogenous signals, such as cAMP, nitric oxide, carbon monoxide, and 2-oxoglutarate, and regulate diverse cellular activities, including metabolism, stress resistance, pathogenesis, and morphology, thereby making them pleiotropic/global transcriptional regulators (Green et al. 2001; Körner et al. 2003; Matsui et al. 2013). The Crp/Fnr regulators could be functionally classified into different types, such as Crp, Fnr, FixK, and Flp (Körner et al. 2003; Matsui et al. 2013). The Crp-type regulators are well studied, especially in *Escherichia coli*, where it mediates carbon catabolite repression upon binding to the effector molecule cAMP (Görke and Stülke 2008; Lawson et al. 2004). Specifically, the binding of cAMP to the N-terminal domain of the *E. coli* Crp resulted in a substantial conformation change of Crp and enabled it to bind to target DNA through the C-terminal DNA-binding domain, which led to activation of more than 100 target genes (Busby and Ebright 1999; McKay and Setiez 1981; Schultz et al. 1991). In streptomycetes, the *S. coelicolor* Crp SCO3571 is the only one that has been functionally studied. Inactivation of Crp in *S. coelicolor* led to not only morphological development defects, e.g., delayed germination, but also changes in secondary metabolite biosynthesis, such as lowering the titers of actinorhodin, undecylprodigiosin, and calcium-dependent antibiotic production, indicative of Crp as an important pleiotropic regulator (Derouaux et al. 2004a; Gao et al. 2012; Piette et al. 2005). In vitro assay also confirmed the ability of *S. coelicolor* Crp to bind cAMP (Derouaux et al. 2004b). Overexpression of Crp in *S. coelicolor* and several *Streptomyces* species stimulated antibiotic production (Gao et al. 2012), supporting the wisdom of exploiting Crp-type

regulators to improve the production of secondary metabolites in *Streptomyces*. Strikingly, all the characterized members of Crp-type regulators appeared to be pleiotropic or global, and no pathway-specific Crp regulator has been reported to date (Körner et al. 2003; Matsui et al. 2013).

Leinamycin (LNM, Fig. 1b) is a potent antitumor antibiotic produced by *Streptomyces atroolivaceus* S-140 (Hara et al. 1989). It features an unusual 1,3-dioxo-1,2-dithiolane moiety that is spiro-fused to a thiazole-containing 18-membered lactam ring, a molecular architecture that has not been found in any other natural products to date. LNM shows potent antitumor activity in vitro and in vivo and is active against tumors that are resistant to clinically important anticancer drugs, such as cisplatin, doxorubicin, mitomycin, and cyclophosphamide (Ashizawa et al. 1999; Gates 2000; Hara et al. 1990). Upon reductive activation in the presence of cellular thiols, LNM exerts its anticancer activity by an episulfonium ion-mediated DNA alkylation, a mode of action (MOA) that is unprecedented for thio-dependent DNA cleavage (Asai et al. 1996; Fekry et al. 2011; Gates 2000). LNM E1 (Fig. 1b), recently isolated and characterized from the *S. atroolivaceus* *InmE* mutant strain SB3033, has also been shown, upon oxidative activation, to be a potent DNA alkylating agent, via a similar episulfonium intermediate as LNM (Huang et al. 2015; Ma et al. 2015). LNM E1 therefore complements the MOA of LNM and could be exploited as a prodrug, activated by reactive oxygen species, to target cancer cells under high cellular oxidative stress. A facile synthesis has also been developed, converting LNM E1 into an LNM analogue that shed new insights into the structure-activity relationship (SAR) of LNM (Liu et al. 2015). However, the low titers of LNM and LNM E1 production in the *S. atroolivaceus* wild-type and the *InmE* mutant SB3033, respectively, impede their further mechanistic and SAR studies, as well as preclinical evaluation, thereby motivating the current effort to develop overproducers.

We have previously cloned the *Inm* biosynthetic gene cluster from *S. atroolivaceus* S-140 (Fig. 1a) and characterized many aspects of the LNM biosynthetic pathway (Fig. 1b) (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004; Tang et al. 2006). Among the 27 genes within the *Inm* cluster, *InmO*, which encodes a putative transcriptional regulator, was identified by sequence analysis (Tang et al. 2004). Here, we report the identification of *InmO* as a pathway-specific Crp/Fnr family regulator, which positively regulates the biosynthesis of LNM. Overexpression of *InmO* in *S. atroolivaceus* S-140 and SB3033 resulted in titer improvement of LNM and LNM E1, respectively, demonstrating the feasibility to improve antibiotics production in *Streptomyces* by manipulating the Crp/Fnr family pathway-specific regulators.

Materials and methods

Bacterial strains and plasmids

E. coli DH5 α , used for the propagation of plasmids (Sambrook et al. 1989), and *E. coli* S17-1, used for intergeneric conjugation to introduce plasmids into *Streptomyces* strains (Kieser et al. 2000), were cultured in Luria-Bertani medium (Sambrook et al. 1989). *S. atroolivaceus* S-140 wild-type, kindly provided by Kyowa Hokko Kogyo Co. Ltd. (Tokyo, Japan) (Cheng et al. 2002), *InmE* mutant SB3033 (Huang et al. 2015), and recombinant

strains derived thereof were grown on ISP-4 agar plate at 28 °C. pSP72 (Promega) and LITMUS 28 (NEB) were from commercial sources. pBS3006 and pBS3007 (Cheng et al. 2002), pBS8004 (Tao et al. 2007), pSET151 and pSET 152 (Bierman et al. 1992), pUO9090 (Prado et al. 1999), and pWHM79 (Shen and Hutchinson 1996) were described previously.

DNA manipulations

Plasmids preparation was carried out using commercial kits (Qiagen). Restriction enzymes and other molecular biology reagents were from standard commercial sources. DNA digestion and ligation followed standard methods (Sambrook et al. 1989). Conditions for growth, intergeneric conjugation, Southern analysis, and general DNA manipulation of *S. atroolivaceus* wild-type and recombinant strains were carried out as previously reported (Cheng et al. 2002; Cheng et al. 2003; Huang et al. 2015; Kieser et al. 2000).

Construction of the *InmO* mutant strain *S. atroolivaceus* SB3048

A pSET151 based plasmid pBS3147 was constructed to generate the *InmO* gene replacement mutant in *S. atroolivaceus* S-140 via a double crossover homologous recombination. Briefly, a 1.1-kb DNA fragment was amplified from pBS3006 by PCR using primers 5'-TACAGATCTGGAAGCTCCTTCTTCCACCCGTG-3' (the *Bgl*II site underlined) and 5'-ATCTCTAGATGATCGAGGACAGGAAGTGCT-3' (the *Xba*I site underlined) and cloned into the same sites of pSET151 to yield pBS3144. A 2.2-kb *Kpn*I-*Bst*BI fragment, containing *InmO* from cosmid pBS3006, was cloned into the same sites of pUO9090 to afford pBS3145, from which a 1.8-kb *Bam*HI-*Sph*I fragment was excised and cloned into pBS3144 to afford pBS3146. Finally, a 1.5-kb *Bgl*II-*Bam*HI fragment containing the *acc(3)IV* gene was inserted into the *Bam*HI site of pBS3146 to afford pBS3147. Introduction of pBS3147 into the *S. atroolivaceus* S-140 wild-type by intergeneric conjugation and selection of the apramycin-resistant and thiostrepton-sensitive phenotype were carried out according to previously established procedures (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004) (Fig. 3a). This yielded the double-crossover *InmO* mutant strain *S. atroolivaceus* SB3048, whose genotype was confirmed by Southern analysis (Fig. 3b). A 0.5-kb DNA fragment, amplified with primers *InmO*-Sblot-F (5'-CCCTTCGTGCCGACCAC-3') and *InmO*-Sblot-R (5'-CATCCAGCAGGTTTCATCTGGC-3') and *S. atroolivaceus* S-140 wild-type genomic DNA as template, was used as the probe in Southern analysis (Fig. 3).

Generation of the *InmO* complementation strain *S. atroolivaceus* SB3049

A pBS8004 based plasmid pBS3150, in which the expression of *InmO* is under the control of the constitutive promoter *ErmE** (Kieser et al. 2000), was constructed to complement the *InmO* mutant strain SB3048. Thus, a 7.0-kb *Pst*I-*Kpn*I fragment from cosmid pBS3007, encoding the last module of LnmJ to LnmP (i.e., including LnmO), was first cloned into the same sites of Litmus 28 to afford pBS3148. A 3.5-kb *Aat*II fragment, including *InmO* and its flanking region from pBS3148, was then cloned into the same site of Litmus 28 and subsequently moved as a 3.5-kb *Eco*RI-*Eco*RV fragment into the same sites of pWHM79 to afford pBS3149, placing *InmO* under the control of *ErmE**. A 4.0-kb *Eco*RI-*Xba*I fragment from pBS3149 was finally cloned into the same sites of pBS8004, affording pBS3150. Introduction of pBS3150 into SB3048 was achieved by intergeneric conjugation, with

selection for the thiostrepton resistant phenotype, affording the *InmO* complementation strain *S. atroolivaceus* SB3049 (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004).

Generation of the *InmO*-overexpressing recombinant strains SB3050 and SB3051

A pSET152 based plasmid pBS3152, in which the expression of *InmO* is under the control of the constitutive promoter *ErmE*^{*} was constructed to generate the *InmO*-overexpressing recombinant strains SB3050 and SB3051. Briefly, a 2.2-kb *PstI*-*XhoI* fragment containing *InmO* from pBS3145 was first cloned into the same sites of pSP72 and then moved as a 2.2-kb *SphI*-*XbaI* fragment into the same sites of pWHM79 to afford pBS3151. A 2.7-kb *EcoRI*-*SphI* (blunt-ended) fragment from pBS3151 was cloned into the *EcoRI*-*EcoRV* sites of pSET152 to afford the *InmO* overexpression plasmid pBS3152. Introduction of pBS3152 to the *S. atroolivaceus* wild-type or the *InmE* mutant SB3033 was achieved by intergeneric conjugation, with selection for the apramycin resistant phenotype (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004) (Fig. 3a), affording the *InmO*-overexpressing recombinant strains SB3050 (i.e., wild-type with *InmO* overexpressing) and SB3051 (i.e., SB3033 with *InmO* overexpressing), respectively.

Production of LNM and LNM E1 and HPLC analysis

For LNM or LNM E1 production, the *S. atroolivaceus* wild-type and recombinant strains were cultured using a two-stage fermentation following previously published procedures (Cheng et al. 2002; Cheng et al. 2003; Huang et al. 2015; Tang et al. 2004). Briefly, 10 μ L of spore suspension was inoculated into a 250-mL baffled flask containing 50-mL seed medium (glucose 1%, soluble starch 1%, beef extract 0.3% yeast extract 0.5%, tryptone 0.5%, CaCO₃ 0.2%, pH 7.2), and fermentation was carried out on a shaking incubator at 28 °C with 250 rpm for 2 days to afford the seed culture. The seed culture (5 mL) was then transferred into 250-mL baffled flasks containing 50-mL production medium (soluble starch 3%, corn steep solids 1%, KH₂PO₄ 0.05%, MgSO₄ 0.025%, ZnSO₄·7H₂O 0.004 %, L-methionine 0.01%, vitamin B₁₂ 0.0001%, CaCO₃ 0.5%, pH 7.0), with 3% Diaion HP-20 resin added to the production medium 18 hrs after inoculation, and fermentation was carried out on a shaking incubator at 28 °C with 250 rpm for 5 days.

To analyze the production of LNM or LNM E1, the production culture was acidified to pH 2.0 with 5 N HCl. The Diaion HP-20 resins were recovered by filtration through two layers of cheese gauze and extracted twice with CH₃OH. The combined CH₃OH extracts were finally concentrated in vacuo and subjected to HPLC analysis. HPLC was carried out on a Varian HPLC system, consisting of Varian ProStar 210 pumps and a ProStar 330 photodiode array detector equipped with an Apollo C18 column (250 mm $\times$ 4.6 mm, 5 μ m), following previously published procedures (Huang et al. 2015). The peak areas with UV detection at 320 nm were used to quantify LNM or LNM E1 production on the basis of calibration curves with authentic LNM or LNM E1 standards. LNM or LNM E1 titers are the averages from at least three independent fermentations.

Results

Identification of LnmO as a new member of Crp/Fnr family transcriptional regulators by bioinformatics analysis

The *lnm* gene cluster consists of 27 genes including one putative regulatory gene *lnmO* (Fig. 1a). Blast analysis of LnmO (227 amino acids) revealed its sequence similarity to proteins annotated as the Crp/Fnr family transcriptional regulators. To probe the function of LnmO, we performed a phylogenetic analysis of LnmO against well-annotated Crp/Fnr regulators selected from Swiss-Prot database as well as three LnmO close homologues (sharing over 42% sequence identity with LnmO) from *Catenulispora acidiphila* DSM 44928, *Micromonospora aurantiaca* ATCC 27029 and *Saccharothrix espanaensis* DSM 44229, which we have suggested previously to contain gene clusters encoding the biosynthesis of LNM congeners (Ma et al. 2015). LnmO and the three homologues form a unique subgroup (LnmO-like proteins) phylogenetically, relatively close to the Crp-type regulators (Fig. 2a). LnmO shows slightly higher sequence similarity to Crp-type regulators, such as the well-studied *Mycobacterium tuberculosis* Crp (30% identity/47% similarity) (Rickman et al. 2005), *S. coelicolor* Crp SCO3571 (26% identity/46% similarity), and *E. coli* Crp (26% identity/45% similarity) than other types of Crp/Fnr regulators (Fig. 2a). LnmO-like proteins feature (i) an N-terminal domain (1–140 aa for LnmO), which is similar to those of Crp-type regulators, suggesting LnmO-like proteins might respond to cAMP-like signal molecules by direct binding, and (ii) a C-terminal DNA binding domain containing a HTH motif (147–221 aa for LnmO) (Fig. 2b). Taken together, the bioinformatics analysis results suggested that LnmO is a Crp/Fnr family regulator, most likely belonging to the Crp-type. To our knowledge, LnmO represents the first example of a Crp/Fnr regulator identified within a secondary metabolite biosynthetic gene cluster.

LnmO is indispensable and pathway-specific to LNM biosynthesis

To investigate the role of LnmO in LNM biosynthesis, the *lnmO* mutant *S. atroolivaceus* SB3048 was constructed by inserting the apramycin resistance cassette *aac(3)IV* into the *Bam*HI site of *lnmO* in *S. atroolivaceus* S-140 through double crossover homologous recombination (Fig. 3a). The *lnmO* genotype (i.e., *lnmO::aac(3)IV*) of SB3048 was confirmed by Southern analysis. Using a 0.5-kb DNA fragment as a probe, digestion of the genomic DNAs by *Xho*I resulted in detection of a 1.6-kb band in SB3048 and a 3.5-kb band in wild-type, respectively (Fig. 3b). SB3048 showed no apparent morphological difference in comparison with the *S. atroolivaceus* wild-type when cultivated on agar plates or liquid medium, suggesting that LnmO is not a regulator for morphological differentiation. When fermented under the standard conditions for LNM production (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004), with the wild-type strain as a control, HPLC analysis revealed that SB3048 completely lost its ability to produce LNM (Fig. 4a). The *lnmO* mutation in SB3048 was next complemented by introducing pBS3150, in which the expression of *lnmO* is under the control of *ErmE*^{*}, into SB3048 to generate SB3049. Upon fermenting under the standard conditions for LNM production (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004), HPLC analysis showed that LNM production in SB3049 was restored to the level comparable to that in the wild-type (Fig. 4a). Taken together, these results conclusively established the essential role of LnmO in LNM biosynthesis and supported the functional

assignment of LnmO as a Crp/Fnr family transcriptional regulator that positively regulates LNM biosynthesis. While all functionally characterized Crp/Fnr family regulators known to date are pleiotropic (Körner et al. 2003; Matsui et al. 2013), LnmO appears to be a pathway-specific for LNM biosynthesis.

Overexpression of *lnmO* in *S. atroolivaceus* wild-type and *lnmE* mutant SB3033 improving LNM and LNM E1 titers

Overexpression of pathway-specific positive regulators has been shown to be an effective way to improve secondary metabolite biosynthesis in *Streptomyces* (Chen et al. 2010). To explore the utility of LnmO in improving LNM and LNM E1 production, pBS3152, in which expression of *lnmO* is under the control of *ErmE**, was introduced into the *S. atroolivaceus* wild-type and *lnmE* mutant SB3033 to generate the *lnmO*-overexpressing recombination strains SB3050 and SB3051, respectively. Fermentations of SB3050, with wild-type as a control, and SB3051, with SB3033 as a control, under the standard conditions for LNM (Cheng et al. 2002; Cheng et al. 2003; Tang et al. 2004) and LNM E1 (Huang et al. 2015) production, followed by HPLC analysis revealed that LNM production in SB3050 (1.6 ± 0.3 mg/L) was increased by ~3-fold compared to that in wild-type (0.5 ± 0.2 mg/L) (Fig. 4a) and LNM E1 production in SB3051 (84 ± 9 mg/L) was increased by ~4-fold compared to that in SB3033 (21 ± 5 mg/L) (Fig. 4b), respectively.

Discussion

The Crp/Fnr family regulators function in a pleiotropic manner controlling a broad range of cellular functions, in response to internal or external environmental changes (Körner et al. 2003; Matsui et al. 2013). The *S. coelicolor* Crp, a pleiotropic regulator, plays a positive but not indispensable role in the biosynthesis of several antibiotics, in addition to regulate morphological development (Derouaux et al. 2004a; Gao et al. 2012; Piette et al. 2005). The *S. coelicolor* Crp is highly conserved in streptomycetes, sharing over 90% sequence identity with its homologues, and it therefore has been suggested that *S. coelicolor* Crp-like proteins are common pleiotropic regulators that regulate both morphological development and secondary metabolism in streptomycetes (Gao et al. 2012).

The *lnm* gene cluster, responsible for production of the antitumor drug lead LNM, contains one putative regulatory gene *lnmO* that encode a Crp/Fnr-type regulator (Tang et al. 2004). LnmO could now be assigned to the Crp/Fnr family positive transcriptional regulator based on the following experimental findings: (i) LnmO featured both the N-terminal cAMP/cGMP binding domain and a C-terminal HTH motif for DNA binding common to known members of the Crp/Fnr family regulators; (ii) gene inactivation and complementation results supported that LnmO is indispensable to LNM biosynthesis; and (iii) overexpression of *lnmO* led to significant titer improvement of LNM or LNM E1. While all Crp/Fnr family regulators characterized to date appeared to be pleiotropic, inactivation of *lnmO* only affected LNM production of LNM with no apparent morphological changes. Taken together, LnmO may represent the first Crp/Fnr family regulator that positively regulates secondary metabolite biosynthesis in a pathway-specific manner. LnmO shows >42% sequence identity to its homologues from *C. acidiphila* DSM 44928, *M. aurantiaca* ATCC 27029 and *S.*

espanaensis DSM 44229 (Fig. 2b), while it shows <30% sequence identity to all the characterized Crp/Fnr regulators. Further analysis revealed that the aforementioned three homologues of LnmO are all present within *Inm*-like secondary metabolite biosynthetic gene clusters (Ma et al. 2015). It is therefore tempting to propose that a small and unique group of Crp/Fnr regulators, as exemplified by LnmO and its homologues, may have evolved to be specific for regulating secondary metabolite biosynthesis in actinomycetes, although the exact mechanism of regulation requires future investigation. In addition, overexpression of *InmO* in *S. atroolivaceus* wild-type and *InmE* mutant allowed us to achieve approximately 3- and 4-fold increase in LNM and LNM E1 production, respectively. Engineered strains overproducing LNM and LNM E1 will facilitate further mechanistic studies and clinical evaluation of LNM and LNM E1 as novel anticancer drugs. LnmO joins the growing list of regulators that could be exploited to improve secondary metabolite production in *Streptomyces*.

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Fig. 1.

Biosynthesis of LNM in *S. atroolivaceus* S-140 with the *lnmE* mutant SB3033

accumulating LNM E1. **a** Genetic organization of the *lnm* biosynthetic gene cluster. **b** Proposed biosynthetic pathway for LNM featuring LNM E1 as a key intermediate. Color coding indicates the moieties installed by NRPS (blue), PKS (red), β -alkyl branch (green), and other tailoring enzymes (black). "SH", "SH" group proposed to be derived from L-Cys; SAM, *S*-adenosylmethionine; TE, thioesterase.

Fig. 2.

Bioinformatics analysis of LnmO suggesting it as a Crp-Fnr family regulator. **a** Phylogenetic analysis of LnmO and selected members of Crp/Fnr family regulators. LnmO is analyzed against 69 unique well-annotated Crp/Fnr family regulators selected from the Swiss-Prot database, the *S. coelicolor* Crp (SCO3571), and the three LnmO homologues from *C. acidiphila* DSM 44928, *M. aurantiaca* ATCC 27029 and *S. espanaensis* DSM 44229. Phylogenetic tree was generated by MEGA6 using the maximum likelihood method with a bootstrap test of 100 replicates (Tamura et al. 2013). **b** Sequence alignment of LnmO and

selected Crp-type regulators. WP_012786802_Caci, WP_013287048_Maau and WP_015102016_Saes correspond to proteins with accession number WP_012786802 (*C. acidiphila* DSM 44928), WP_013287048 (*M. aurantiaca* ATCC 27029), and WP_015102016 (*S. espanaensis* DSM 44229), respectively. Crp_Mytu, the Crp from *M. tuberculosis*; Crp_Sco, the Crp from *S. coelicolor*; Crp_Eco, the Crp from *E. coli*. Residues involved in cAMP binding in *E. coli* Crp were marked with asterisks. Sequences were aligned by ClustalW and then further analyzed on the ENDscript server (McWilliam et al. 2013; Robert and Gouet 2014).

Fig. 3.

Inactivation of *InmO* in *S. atroolivaceus* S-140 to generate the *InmO* mutant SB3048. **a** Inactivation of *InmO* by inserting the *aac(3)IV* cassette into the middle of *InmO* to afford the mutant plasmid pBS3147 and isolation of the *InmO* mutant SB3048 by double crossover homologous recombination between *S. atroolivaceus* S-140 and pBS3147. Apr^S , apramycin sensitive; Apr^R , apramycin resistance; Thio^S , thiostrepton sensitive; and Thio^R , thiostrepton resistance; X, *XhoI*. The predicted sizes of fragments from the wild-type and SB3048, upon *XhoI* digestion, hybridizing to the probe, are labeled. **b** Confirmation of the genotype of SB3048 by Southern analysis with the 0.5-kb probe. Lane 1, molecular weight marker; lane 2, wild-type (predicted size 3.5 kb); lane 3, SB3048 (predicted size 1.6 kb).

Fig. 4. Production of LNM or LNM E1 by *S. atroolivaceus* S-140 wild-type, SB3033, and recombinant strains derived thereof. **a** HPLC chromatograms of LNM production in SB3048, SB3049, and SB3050 with the wild-type strain as a control. **b** HPLC chromatograms of LNM E1 production in SB3051 with SB3033 as a control. (●), LNM; (▲), LNM E1.