Published in final edited form as:

Appl Microbiol Biotechnol. 2016 March ; 100(5): 2267–2277. doi:10.1007/s00253-015-7119-7.

Overproduction of lactimidomycin by cross-overexpression of genes encoding *Streptomyces* antibiotic regulatory proteins

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Abstract

The glutarimide-containing polyketides represent a fascinating class of natural products that exhibit a multitude of biological activities. We have recently cloned and sequenced the biosynthetic gene clusters for three members of the glutarimide-containing polyketides – isomigrastatin (iso-MGS) from *Streptomyces platensis* NRRL 18993, lactimidomycin (LTM) from *Streptomyces amphibiosporus* ATCC 53964, and cycloheximide (CHX) from *Streptomyces* sp. YIM56141. Comparative analysis of the three clusters identified *mgsA* and *chxA*, from the *mgs* and *chx* gene clusters, respectively, that were predicted to encode the PimR-like *Streptomyces* Antibiotic Regulatory Proteins (SARPs), but failed to reveal any regulatory gene from the *ltm* gene cluster. Overexpression of *mgsA* or *chxA* in *S. platensis* NRRL 18993, *S. sp.* YIM56141 or SB11024, a recombinant strain of *Streptomyces coelicolor* M145 carrying the intact *mgs* gene clusters, has no significant effect on iso-MGS or CHX production, suggesting that MgsA or ChxA regulation may not be rate limiting for iso-MGS and CHX production in these producers. In contrast, overexpression of *mgsA* or *chxA* in *S. amphibiosporus* ATCC 53964 resulted in significant increase in LTM production, with LTM titer reaching 106 mg/L, which is five-fold higher than that of the wild-type strain. These results support MgsA and ChxA as members of the SARP family of positive regulators for the iso-MGS and CHX biosynthetic machinery and demonstrate the feasibility to improve glutarimide-containing polyketide production in *Streptomyces* strains by exploiting common regulators.

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Conflict of Interests. The authors declare that they have no competing interests.

Animal and Human Subjects. This article does not contain any studies with human participants or animals performed by any of the authors.

Keywords

Biosynthesis; regulation; glutarimide-containing polyketides; *Streptomyces* Antibiotic Regulatory Proteins; antibiotic titer improvement

Introduction

Streptomycetes are Gram-positive filamentous soil bacteria, and are well known as prolific producers of a wide variety of natural products, including many important antimicrobial and anticancer drugs. The production of these molecules usually occurs in a growth phase or developmentally controlled manner, and is also influenced by various environmental and physiological signals (Hopwood et al. 1995; Bibb 2005). The expression of natural products biosynthetic gene clusters usually involves complex regulatory cascades (Bibb 2005; Liu et al. 2013). Pathway specific regulatory genes are prevalent in natural products biosynthetic gene clusters, and many of these regulators belong to the *Streptomyces* Antibiotic Regulatory Protein (SARP) family (Wietzorrek and Bibb 1997). Examples of well characterized SARP family regulators include RedD, which positively regulates the biosynthesis of undecylprodigiosin in *Streptomyces coelicolor* (Narva and Feitelson 1990), and DnrI, which is a positive regulator of daunorubicin biosynthesis in *Streptomyces peucetius* (Stutzman-Engwall et al. 1992). The manipulation of pathway specific regulatory elements has been widely exploited to improve the production of *Streptomyces*-derived natural products in both native producers and heterologous hosts (Chen et al. 2010).

The glutarimide-containing polyketides are a family of natural products including several members of important biological relevance (Ju et al. 2005a; Rajski and Shen 2010). The members of this family include cycloheximide (CHX), actiphenol (APN), iso-migrastatin (iso-MGS), migrastatin (MGS), the dorrigocins (DGNs), NK30424A and NK30424B, and lactimidomycin (LTM) (Fig. 1A). Best known as an inhibitor of eukaryotic protein translation, CHX was first isolated from *Streptomyces griseus* and then has been isolated from numerous *Streptomyces* species (Leach et al. 1947; Kornfeld et al. 1949; Huang et al. 2011). APN, sharing the same carbon skeleton as CHX but having a phenol in place of a cyclohexanone moiety, exhibits weak translation inhibition activity (Hight and Prelog 1959; Rao and Cullen 1960; Rao 1960). CHX, APN, and their congeners are often isolated together as their biosynthesis is governed by a single biosynthetic machinery (Yin et al. 2014). MGS was originally isolated from *Streptomyces* sp. MK929-43F1 as a new inhibitor of tumor cell migration (Nakae et al. 2000). DGNs were isolated from *Streptomyces platensis* NRRL 18993 as inhibitors of carboxyl methyltransferases involved in the processing of Ras-related proteins (Karwowski et al. 1994). Fermentation optimization subsequently resulted in the isolation of MGS and iso-MGS from *S. platensis* NRRL 18993 (Woo et al. 2002). Both MGS and DGNs have been shown to be degradation products of iso-MGS via H₂O-mediated rearrangements, whereas iso-MGS is the only bona fide natural product biosynthesized by the native producer *S. platensis* NRRL 18993 (Ju et al. 2005b). Identical to the cysteine adducts of iso-MGS, NK30424A and NK30424B were isolated from *Streptomyces* sp. NA30424 for their ability to inhibit PLS-induced TNF- α production by suppressing the NF- κ B signaling pathway (Takayasu et al. 2001). Finally, LTM was

originally isolated from *Streptomyces amphibiosporus* ATCC 53964 for its potent cytotoxicity activity against various tumor cells (Sugawara et al. 1992). We recently established the mode of action for LTM to block the translocation step in eukaryotic protein translation initiation (Schneider-Poetsch et al. 2010).

Among the multitude of activities exhibited by the glutarimide-containing polyketides, the inhibitory activities of MGS/iso-MGS against tumor cell migration and LTM against protein translation initiation, respectively, have captured the interests of biologists and chemists (Ju et al. 2005a; Rajski and Shen 2010). A library of glutarimide-containing polyketides was generated from optimized fermentations of *S. platensis* NRRL 18993 and *S. amphibiosporus* ATCC 53694, and preliminary evaluation revealed that 12-membered macrolides are extremely potent cell migration inhibitors with some members displaying activity on par or superior to that of MGS as exemplified by iso-MGS and LTM (Ju et al. 2005a; Ju et al. 2008; Ju et al. 2009).

The protein initiation inhibitory activity of LTM has been exploited in the development of the Global Translation Initiation Sequencing (GTI-seq) technology (Lee et al. 2012) and Quantitative Translation Initiation Sequencing (QTI-seq) technology (Gao et al. 2015). However, the low productivity of glutarimide-containing polyketides in their wild-type producing strains may impede their further mechanistic and clinical studies, thereby motivating the development of high-production strains.

We have recently cloned and sequenced the biosynthetic gene clusters of iso-MGS, CHX, and LTM from *S. platensis* NRRL 18993, *Streptomyces* sp. YIM56141, and *S. amphibiosporus* ATCC 53964, respectively (Lim et al. 2009; Yin et al. 2014; Seo et al. 2014). The biosynthetic machineries of iso-MGS, CHX, and LTM all feature an acyltransferase (AT)-less type I polyketide synthase (PKS). Comparative analysis of the iso-MGS, LTM, CHX AT-less type I PKSs established a unified paradigm for the biosynthesis of the glutarimide-containing polyketides (Lim et al. 2009; Yin et al. 2014; Seo et al. 2014). From sequence analysis of the three gene clusters, we identified *mgsA* from the *mgs* gene cluster and *chxA*, a homologue of *mgsA*, as well as *chxF*, from the *chx* gene cluster, that may encode regulatory proteins (Lim et al. 2009; Yin et al. 2014), but failed to identify any regulatory gene from the *ltm* gene cluster (Seo et al. 2014).

Here we report the overexpression of *mgsA* or *chxA* in *S. platensis* NRRL 18993, *S. sp* YIM56141, and SB11024, a recombinant strain of *Streptomyces coelicolor* M145 carrying the intact *mgs* gene cluster and their effect on iso-MGS, CHX, and LTM production. These results support MgsA and ChxA as members of the SARP family of positive regulators for the iso-MGS and CHX biosynthetic machinery. Most interestingly overexpression of *mgsA* or *chxA* in *S. amphibiosporus* ATCC 53964 resulted in a significant increase in LTM production, demonstrating the feasibility to improve glutarimide-containing polyketide production in *Streptomyces* strains by exploiting common regulators.

Materials and Methods

Bacterial strains and plasmids

All strains and plasmids used in this study are summarized in Table 1. All *Streptomyces* strains were grown on ISP4 agar plate at 28 °C. *Escherichia coli* strains were cultured in Luria-Bertani medium. *E. coli* DH5 α was used for the propagation of plasmids using standard protocols. Antibiotics were selectively used at the following concentrations: for *Streptomyces* strains, 25 $\mu\text{g mL}^{-1}$ apramycin or thiostrepton, and for *E. coli*, 25 $\mu\text{g mL}^{-1}$ apramycin, 50 $\mu\text{g mL}^{-1}$ chloramphenicol or kanamycin. To avoid the methyl-specific restriction, conjugation of plasmids into *Streptomyces* strains was performed using *E. coli* ET12567 carrying the conjugation-facilitating plasmid pUZ8002.

DNA manipulations

General DNA manipulations were carried out according to standard procedures. PCRs were performed using Q5 DNA polymerase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The resultant PCR products were treated with Taq DNA polymerase from Invitrogen for 10 min at 72°C to add the terminal 'A' overhangs. *Streptomyces* protoplasts preparation, transformation, and conjugation were performed as previously described (Kieser et al. 2000).

Overexpression of *mgsA* in *S. sp.* YIM56141, *S. platensis* NRRL 18993, and *S. amphibiosporus* ATCC 53964

Genomic DNA of *S. platensis* NRRL 18993 was used as a template to amplify *mgsA* by PCR, using primers 5'-GGAAATTCCATATGATGCCTTCATCCCTGACGCCGA-3' (*mgsA* upstream with the engineered *NdeI* site underlined) and 5'-CGGAATTCTCACCCGGCCGCCACCGCCT-3' (*mgsA* downstream with the engineered *EcoRI* site underlined), and was inserted into pGEM-T Easy after adding terminal 'A' overhangs by Taq DNA polymerase. After confirming its identity by DNA sequencing, the *mgsA* gene was excised as a 3654-bp *NdeI/EcoRI* fragment and inserted into the same sites of pUWL201PW to generate pBS11027, in which the expression of *mgsA* is under the control of the constitutive *ErmE** promoter. The *mgsA* fragment with the *ErmE** promoter was then excised and inserted into the *KpnI* and *EcoRI* sites of pUC19 to generate plasmids pBS11028. Finally, a 3666-bp fragment of *mgsA* with *ErmE** was excised and inserted into the *XbaI* and *EcoRI* sites of pKC1139 to generate plasmids pBS11029. pBS11029 was introduced into the *S. sp.* YIM56141, *S. platensis* NRRL 18993, and *S. amphibiosporus* ATCC 53964 by *Streptomyces* - *E. coli* conjugation to afford recombinant strains SB19013 (*mgsA* overexpressing in *S. sp.* YIM56141), SB11022 (*mgsA* overexpressing in *S. platensis* NRRL 18993), and SB15008 (*mgsA* overexpressing in *S. amphibiosporus* ATCC 53964), respectively.

Overexpression of *chxA* in *S. sp.* YIM56141, *S. platensis* NRRL 18993, and *S. amphibiosporus* ATCC 53964

Genomic DNA of *S. sp.* YIM56141 was used as a template to amplify *chxA* by PCR, using primers 5'-GGAAATTCCATATGATGGGGCAGGGGAATATGTTCGT-3' (*chxA*

upstream with the engineered *NdeI* site underlined) and 5'-CGGAATTCCTACTCGACCGCACCCACGGCT-3' (*chxA* downstream with the engineered *EcoRI* site underlined), and was inserted into pGEM-T Easy after adding terminal 'A' overhangs by Taq DNA polymerase. After confirming its identity by DNA sequencing, the *chxA* genes was excised as a 3372-bp *NdeI/EcoRI* fragment and inserted into the same sites of pUWL201PW to generate pBS19016, in which the expression of *chxA* is under the control of the constitutive *ErmE** promoter. The *chxA* fragment with the *ErmE** promoter was then excised and inserted into the *KpnI* and *EcoRI* sites of pUC19 to generate plasmids pBS19018. Finally, a fragment of *chxA* with *ErmE** was excised and inserted into the *XbaI* and *EcoRI* sites of pKC1139 to generate plasmids pBS19019. pBS19019 was introduced into the *S. sp.* YIM56141, *S. platensis* NRRL 18993, and *S. amphibiosporus* ATCC 53964 by *Streptomyces* - *E.coli* conjugation to afford recombinant strains SB19014 (*chxA* overexpressing in *S. sp.* YIM56141), SB11023 (*chxA* overexpressing in *S. platensis* NRRL 18993), and SB15009 (*chxA* overexpressing in *S. amphibiosporus* ATCC 53964), respectively.

Overexpression of *mgsA* or *chxA* in SB11024, a recombinant *S. coelicoloer* M145 strain carrying the intact *mgs* gene cluster

pBS11001, a pStreptoBAC-based expression construct that harbored the intact *mgs* gene cluster (Feng et al. 2009), was mobilized into *S. coelicoloer* M145 via conjugation to generate SB11024 for iso-MGS production in a heterologous host. pBS11029 or pBS19018 was subsequently introduced into SB11024 by protoplast transformation to afford SB11025 (*mgsA* overexpressing in SB11024) and SB11026 (*chxA* overexpressing in SB11024), respectively.

Production of iso-MGS, LTM, and CHX and HPLC Analysis

For CHX, iso-MGS, or LTM production, the wild-type and recombinant strains were cultured using a two-stage fermentation following previously published procedures (Lim et al. 2009; Feng et al. 2009; Yin et al. 2014; Seo et al. 2014). Briefly, for CHX production, fresh spores of *S. sp.* YIM56141 wild-type or recombinant strains were cultured in 250-mL baffled flasks containing 50 mL of TSB (Tryptic Soy Broth) medium at 28°C and 250 rpm for 40–48 hrs. The resultant seed cultures (0.5 mL) were then inoculated into 250-mL baffled flasks containing 50 mL of the production medium (0.5% malt extract, 0.2% dextrose, 0.2% yeast extract, pH 7.0), and fermentation continued at 28°C and 250 rpm for 5 days (Yin et al. 2014). For iso-MGS production, both stages utilized the same medium (2% glycerol, 2% dextrin, 1% soy peptone, 0.3% yeast extract, 0.2% (NH₄)₂SO₄, and 0.2% CaCO₃, pH 7.0). Thus, fresh spores of *S. platensis* NRRL 18993 wild-type and recombinant strains were cultured in 250-mL baffled flasks containing 50 mL of medium at 28°C and 250 rpm for 48 hrs. The resultant seed cultures (2.5 mL) were then inoculated into 250-mL baffled flasks containing 50 mL of the same medium with the addition of XAD-16 resin (4%), and fermentation continued at 28°C and 250 rpm for 5 days (Lim et al. 2009; Feng et al. 2009). For LTM production, both stages also utilized the same medium (2% dextrin, 0.5% pharmedia, 0.8% soy peptone, 0.2% yeast extract, 0.2% and CaCO₃, pH 7.0). Thus, fresh spores of *S. amphibiosporus* ATCC 53964 wild-type and recombinant strains were cultured in 250-mL baffled flasks containing 50 mL of medium at 28°C and 250 rpm for 48

hrs. The seed cultures (2.5 mL) were then inoculated into 250-mL baffled flasks containing 50 mL of the same medium with the addition of XAD-16 resin (4%), and fermentation continued at 28°C and 250 rpm for 7 days (Seo et al. 2014).

HPLC was carried out on a Varian high performance liquid chromatography system consisting of Varian ProStar 210 pumps and a ProStar 330 photodiode array detector equipped with an Apollo C18 column (250 mm × 4.6 mm, 5 µm, Grace Davison Discovery Sciences, Deerfield, IL, USA). HPLC analyses of CHX, iso-MGS, or LTM were performed following previously published procedures, respectively (Lim et al. 2009; Yin et al. 2014; Seo et al. 2014). The peak areas, with UV detection at 210 nm (CHX), 205 nm (iso-MGS), or 210 nm (LTM), were used to quantify production on the basis of calibration curves with authentic CHX, iso-MGS, or LTM standards. To measure cell growth, cultures were harvested by centrifugation, and mycelia were then collected, dried upon heating at 100°C to constant weight, and finally weighted to determine cell masses.

Results

Identification of MgsA and ChxA as members of the SARP family of regulators by bioinformatics analysis of the *mgs*, *chx*, and *ltm* clusters

Comparative analysis of the *mgs*, *chx*, and *ltm* gene clusters had previously revealed three putative regulatory genes (Fig. 1B). The *mgsA* gene, which encoded a protein of 1217 amino acids (aa), was the only regulatory gene within the *mgs* gene cluster. From the *chx* gene cluster, two regulatory genes, *chxA* and *chxF*, were identified – ChxA is a homologue of MgsA (46% identity/59% similarity) and ChxF belongs to the LysR-family of transcriptional regulators (Maddocks and Oyston 2008). Intriguingly, no regulatory genes were found within the *ltm* gene cluster. Bioinformatics analysis revealed that MgsA and ChxA belonged to a unique group of SARP family regulators, the “PimR-like” SARPs (Anton et al. 2004). Typical SARP family regulators contain a transcriptional regulatory protein (TRC) domain, which is also known as OmpR-like DNA-binding domain, and an adjacent bacterial transcriptional activator domain (BTAD) (Wietzorrek and Bibb 1997; Anton et al. 2004; Tanaka et al. 2007) (Fig. 2A). SARPs bind repeated motifs through their TRC domain which features a winged helix-turn-helix DNA-binding motif and the adjacent BTAD domain turns on the expression of target antibiotic biosynthetic genes (Wietzorrek and Bibb 1997; Liu et al. 2013). Compared with typical SARP family of regulators (consisting of 250–400 aa), PimR-like SARPs are larger in size (consisting of 1000–1300 aa). They share two similar domain architectures (Fig. 2A) – (i) the N-terminus containing a TRC-BTAD di-domain (SARP-like region) and (ii) the middle regions featuring an AAA-domain (domain characteristic of ATPases associated with diverse cellular activities), which contain conserved Walker A and B motifs (Patel and Latterich 1998; Leipe et al. 2003).

MgsA and ChxA show end to end sequence similarity to PimR-like SARPs. For example, MgsA show sequence homology to PnR2 (87% identity), PimR (67% identity), ScnRI (67% identity), SanG (37% identity) and PolR (37% identity), which are characterized as positive regulators from the phoslactomycin, pimaricin, natamycin, nikkomycin, and polyoxin biosynthetic pathways, respectively (Chen et al. 2012; Anton et al. 2004; Du et al. 2011; Liu et al. 2005; Li et al. 2009) (Fig. 2B and Fig. 2C). The SARP-like region of MgsA (63–270

aa) and ChxA (64–271 aa) also show high similarity to that of typical SARP family regulators, such as AurD, RedD, and CcaR, which are positive regulators from the aureothin, undecylprodigiosin, and clavulanic acid biosynthetic pathways, respectively (He and Hertweck 2003; Narva and Feitelson 1990; Perez-Llarena et al. 1997) (Fig. 2B). Like many other regulatory genes from natural product biosynthetic pathways, both *mgsA* and *chxA* feature two rare TTA codons (codon 448 and 1060 for *mgsA* and 326 and 347 for *chxA*) for leucine, indicating that expression of *mgsA* and *chxA* may depend on *bldA*, a tRNA encoding gene responsible for translating UUA into leucine (Leskiw et al., 1991).

Overexpression of *mgsA* and *chxA* gene in the CHX producer *S. sp.* YIM56141

Overexpression of *mgsA* and *chxA* does not significantly affect the CHX titer in *S. sp.* YIM56141. Two high-copy-number pKC1139-based vectors, pBS11029 and pBS19018, were constructed to overexpress *mgsA* or *chxA*, respectively, under the control of the constitutive *Erme** promoter. Conjugation of pBS11029 and pBS19018 into the CHX producer *S. sp.* YIM56141 afforded recombinant strains SB19013 (*mgsA* overexpressing) and SB19014 (*chxA* overexpressing), respectively. Fermentation of the recombinant strains and evaluation of the metabolic profiles of each of the recombinant strains by HPLC analysis revealed that CHX production in SB19013 and SB19014 was comparable to that of the wild-type strain (20 ± 5 mg/L) (Fig. 3A).

Overexpression of *mgsA* and *chxA* gene in the iso-MGS producers *S. platensis* NRRL 18993 and *S. coelicolor* SB11024

No significant improvement of iso-MGS titer was observed by overexpressing *mgsA* or *chxA* in either *S. platensis* NRRL 18993, a native producer of iso-MGS, or SB11024, an *S. coelicolor* heterologous host that carried the intact *mgs* gene cluster and produced iso-MGS. pBS11029 and pBS19028 were first introduced into *S. platensis* NRRL 18993 individually by conjugation to yield the recombinant strains *S. platensis* SB11022 and SB11023, respectively. Fermentation of the recombinant strains and HPLC analyses of the metabolite profiles revealed that iso-MGS titers in SB11022 (52 ± 9 mg/L) and SB11023 (54 ± 12 mg/L) were only slightly higher than that in the wild-type (48 ± 5 mg/L) (Fig. 3B).

We previously demonstrated the production of iso-MGS in heterologous hosts by expressing the intact *mgs* cluster in five *Streptomyces* strains (Feng et al. 2009). To investigate the effect of *mgsA* or *chxA* overexpression for iso-MGS production in heterologous host, pBS11011, which harbor the intact *mgs* cluster, was first introduced into *S. coelicolor* M145 by conjugation to afford SB11024. pBS11029 and pBS19018 were then introduced into SB11024 to yield SB11025 (*mgsA* overexpressing) and SB11026 (*chxA* overexpressing), respectively, each of which was then cultured under iso-MGS fermentation conditions with SB11024 as a control. Both SB11025 (20.5 ± 1.3 mg/L) and SB11026 (21.3 ± 0.9 mg/L) produced iso-MGS at titers that were slightly higher than that in SB11024 (19.6 ± 1.7 mg/L).

Overexpression of *mgsA* or *chxA* gene in LTM producer *S. amphibiosporus* ATCC 53964

Finally, pBS11029 and pBS19018 were individually introduced into the LTM producer *S. amphibiosporus* ATCC 53964, which lacks any apparent regulatory gene within the *ltm* gene

cluster, to generate recombinant strains SB15008 (*mgsA* overexpression) and SB15009 (*chxA* overexpression), respectively. The wild-type and recombinant strains were cultured using a two-stage fermentation following previously published procedures to evaluate the production of LTM. Both SB15008 (81 ± 3 mg/L) and SB15009 (85 ± 4 mg/L) produced significantly more LTM (approximately six fold higher) than the wild-type strain (14 ± 3 mg/L) (Fig. 3C).

To further investigate the effect of *mgsA* or *chxA* overexpression on LTM production, we examined the time course of LTM production in SB15008 and SB15009 in comparison with the wild-type strain. As showed in Fig. 4A, LTM production reached 25 mg/L on day 2, entered the steady phase until day 4, and started a slow decrease on day 5 by the wild-type strain. In contrast, SB15008 and SB15009 exhibited an increase of LTM production from day 2, reached the maximum at around 106 mg/L on day 5, and slowly decreased until day 7. Yet, comparison between the wild-type and recombinant strains confirmed that introduction of *chxA* or *mgsA* into the wild-type strain had little effect on cell growth (Fig. 4B). Taken together, the findings that overexpression of *mgsA* and *chxA* in *S. amphibiosporus* ATCC 53964 significantly improved the production of LTM support MgsA and ChxA as members of the SARP family of positive regulators for the iso-MGS and CHX biosynthetic machinery.

Discussion

Manipulation of pathway regulation has been demonstrated as an efficient strategy to increase specific secondary metabolite production (Chen et al. 2010). Overexpression of SARPs has been shown to successfully increase natural products production in many cases (Bruheim et al. 2002; Liu et al. 2005; Chen et al. 2008; Li et al. 2009; Yin et al. 2015). The glutarimide-containing polyketides represent a fascinating class of natural products that exhibit a multitude of biological activities. The low productivity of glutarimide-containing polyketides in the wild-type *Streptomyces* strains may impede their availability for mechanistic and clinical studies. Sequence analysis of the *mgs*, *chx*, and *ltm* gene clusters resulted in the identification of *mgsA* and *chxA* encoding members of the PimR-like SARPs.

All members of PimR-like SARPs characterized to date are positive regulators. We therefore predicted that MgsA and ChxA should positively regulate the iso-MGS and CHX biosynthetic machinery, respectively. This, in fact, is consistent with our early report that disruption of *mgsA* abolished the iso-MGS production in *S. platensis* NRRL 18993 (Lim et al. 2009). We have recently succeeded in producing iso-MGS by expressing the *mgs* cluster in five selected heterologous *Streptomyces* hosts (Feng et al. 2009). Intriguingly, while *mgsA* is essential for iso-MGS production in *S. platensis* NRRL 18993, the transcription of *mgsA* in heterologous hosts could not be detected by quantitative reverse-transcription PCR (qRT-PCR) analysis (Yang et al, 2011). While the genetic organization of the *chx*, *mgs*, and *ltm* biosynthetic gene clusters, as well as the deduced functions thereof, shows high similarity to each other (Fig. 1B), the PimR-like SARP regulators (MgsA and ChxA) are present in the *mgs* and *chx* clusters, respectively, but is absent in the *ltm* cluster. These enigmatic observations prompted us to test if overexpression of MgsA and ChxA in their native strains will improve the yield of iso-MGS and CHX, respectively, and if so, could MgsA and ChxA

be exploited to cross-regulate the production of other members of the glutarimide-containing polyketides.

The titers of iso-MGS and CHX were not significantly increased by overexpressing *mgsA* or *chxA* in their native producers or heterologous hosts (Fig. 3). We speculated that transcriptions of *mgsA* and *chxA* in the native producers or homologues in the heterologous hosts are sufficient to satisfy the requirement for the iso-MGS and CHX biosynthetic machinery, therefore MgsA and ChxA regulation are not rate limiting for iso-MGS and CHX production. In contrast, the LTM biosynthetic machinery *S. amphibiosporus* ATCC 53964 apparently is not under the control of any pathway specific regulator within the gene cluster, and overexpression of *mgsA* or *chxA* ensures efficient expression of the LTM biosynthetic machinery, thereby improving the production of LTM in *S. amphibiosporus* ATCC 53964. These results support MgsA and ChxA as members of the SARP family of positive regulators for the iso-MGS and CHX biosynthetic machinery and demonstrate the feasibility to improve glutarimide-containing polyketide production in *Streptomyces* strains by exploiting common regulators.

SARPs are known to bind repeated motifs through their TRC domain and activate the expression of target antibiotic biosynthetic genes (Wietzorrek and Bibb 1997; Liu et al. 2013). The fact that MgsA and ChxA can cross-regulate the production of LTM implies that certain regions within the *ltm* gene cluster are the DNA binding targets of MgsA and ChxA. And this is possible because the *mgs*, *chx*, and *ltm* gene clusters share similar genetic organization. It would be interesting to further investigate the target DNA sequences of MgsA and ChxA in these three gene clusters. The lack of an *mgsA* or *chxA* homologue within the *ltm* cluster in *S. amphibiosporus* ATCC 53964 also raises the question that if there are regulatory gene(s) outside of the *ltm* gene cluster responsible for activation of LTM biosynthesis.

In conclusion, we demonstrated that two homologous regulators, MgsA and ChxA, identified in the *mgs* and *chx* biosynthetic gene clusters, respectively, have positive regulatory roles in the production of glutarimide-containing polyketides. Although overexpression of *mgsA* or *chxA* did not significantly improve iso-MGS and CHX production, we succeeded in improving LTM production by overexpressing *mgsA* or *chxA* in *S. amphibiosporus* ATCC 53964, with a LTM titer that is five-fold higher than that of the wild-type strain. These results highlight that cross-regulation provides an opportunity to improve natural products production among close related pathways. Although these experiments fall short of addressing the direct mechanism by which MgsA or ChxA regulates antibiotic production, our data underscores the effectiveness of titer improvements by metabolic pathway engineering. The improved LTM titer should greatly facilitate its production for applications such as a protein translation initiation inhibitor.

Acknowledgments

This work is supported in part by National Institute of Health Grant CA106150. B.Z and Y.Y were supported in part by scholarships from the China Scholarship Council.

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Figure 1.

(A) Structures of selected members of the glutarimide-containing polyketide family of natural products and (B) genetic organization of the *chx*, *mgs*, and *ltm* biosynthetic gene clusters.

Figure 2.

Domain architecture of SARPs and amino acid alignment of MgsA and ChxA with other members of SARPs. (A) Domain architecture of typical SARP and 'PimR-like' SARP. TRC, transcriptional regulatory protein, C terminal; BTAD, bacterial transcriptional activator domain; AAA, ATPase domain, characteristic of ATPases associated with a variety of cellular activities. (B) Alignment of the SARP-like regions of MgsA and ChxA with those of other related SARP family regulators. Numbers indicate amino acid residues from the N-terminus of the protein. Conserved amino acid residues are highlighted. (C) Comparison of the AAA domains of MgsA and ChxA with those of other related proteins. The Walker A and B motifs are underlined. Numbers indicate amino acid residues from the N-terminus of the protein. Conserved amino acid residues are highlighted.

Figure 3. Production of glutarimide-containing polyketides by wild-type and recombinant strains. (A) HPLC traces of CHX (●) and APN (○) production in recombinant strains with the wild-type strain *S. sp.* YIM56141 as a control. (B) HPLC traces of iso-MGS (▼) production in recombinant strains with the wild-type strain *S. platensis* NRRL 18993 as a control. (C) HPLC traces of LTM (◆) production in recombinant strains with the wild-type strain *S. amphibiosporus* ATCC 53964 as a control.

Figure 4. Time course of (A) LTM production in and (B) cell mass of *S. amphibiosporus* ATCC 53964 wild-type (▲) and recombinant strains SB15008 (○) and SB15009 (□).

Table 1

Strains and plasmids used in this study

Plasmids/Strains	Relevant characteristics ^a	References
Plasmids		
pKC1139	<i>E. coli-Streptomyces</i> shuttle vector, pSG5 replicon (high copy number); Am ^r	Bierman et al. 1992
pUWL201PW	<i>E. coli-Streptomyces</i> shuttle vector, pIJ101 replicon (high copy number); <i>ErnE</i> [*] promoter; Thio ^r	Kieser et al. 1982
pGEM-T Easy	<i>E. coli</i> T-cloning vector for PCR products cloning; Amp ^r	Promega
pUC19	General cloning vector; Amp ^r	NEB
pBS11001	Derived from pStreptoBAC vector, containing the complete <i>mgs</i> gene cluster; Am ^r	Feng et al. 2009
pBS11027	pUWL201PW based plasmid containing <i>mgsA</i> with <i>ErnE</i> [*] promoter; Thio ^r	This study
pBS11028	pUC19 based plasmid containing <i>mgsA</i> with <i>ErnE</i> [*] promoter; Amp ^r	This study
pBS11029	pKC1139 based plasmid containing <i>mgsA</i> with <i>ErnE</i> [*] promoter; Am ^r	This study
pBS19016	pUWL201PW based plasmid containing <i>chxA</i> with <i>ErnE</i> [*] promoter; Thio ^r	This study
pBS19017	pUC19 based plasmid containing <i>chxA</i> with <i>ErnE</i> [*] promoter; Amp ^r	This study
pBS19018	pKC1139 based plasmid containing <i>chxA</i> with <i>ErnE</i> [*] promoter; Am ^r	This study
<i>E. coli</i> strains		
DH5α	General cloning host	Sambrook et al. 1989
ET12567/pUZ8002	Non-methylating ET12567 containing non-transmissible RP4 derivative plasmid pUZ8002; Cm ^r , Tet ^r , Kan ^r	Kieser et al. 2000
<i>Streptomyces</i> strains		
<i>S. sp.</i> YIM56141	CHX wild-type strain	Yin et al. 2014
SB19013	Derived from <i>S. Sp.</i> YIM 56141, containing pBS11029, for <i>mgsA</i> overexpression; Am ^r	This study
SB19014	Derived from <i>S. Sp.</i> YIM 56141, containing pBS19018, for <i>chxA</i> overexpression; Am ^r	This study
<i>S. platensis</i> NRRL18993	iso-MGS wild-type strain; Thio ^r	Lim et al. 2009
SB11022	Derived from <i>S. platensis</i> NRRL18993, containing pBS11029, for <i>mgsA</i> overexpression; Am ^r	This study
SB11023	Derived from <i>S. platensis</i> NRRL18993, containing pBS19018, for <i>chxA</i> overexpression; Am ^r	This study
<i>S. coelicolor</i> M145	Host for heterologous expression of iso-MGS gene cluster	Gomez-Escribano and Bibb 2010
SB11024	<i>S. coelicolor</i> M145 with pBS11001, iso-MGS heterologous producing; Am ^r	This study
SB11025	Derived from SB11024, containing pBS11029, for <i>mgsA</i> overexpression; Am ^r , Thio ^r	This study
SB11025	Derived from SB11024, containing pBS19018, for <i>chxA</i> overexpression; Am ^r , Thio ^r	This study
<i>S. amphibiosporus</i> ATCC 53964	LTM wild-type strain	Seo et al. 2014

Plasmids/Strains	Relevant characteristics ^a	References
SB15008	Derived from <i>S. amphibiosporus</i> ATCC 53964, containing pBS11029, for <i>mgxA</i> overexpression; Am ^r	This study
SB15009	Derived from <i>S. amphibiosporus</i> ATCC 53964, containing pBS19018, for <i>chxA</i> overexpression; Am ^r	This study

^a Cm^r, chloramphenicol resistance; Thio^r, thioestrepton resistance; Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; Am^r, apramycin resistance; Amp^r, ampicillin resistance