Asm8, a specific LAL-type activator of 3-amino-5-hydroxy-benzoate biosynthesis in ansamitocin production

PAN WenQin, KANG QianJin, WANG Lei, BAI LinQuan[*] & DENG ZiXin

State Key Laboratory of Microbial Metabolism and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

The highly potent antitumor agent ansamitocin P3 is a macrolactam isolated from *Actinosynnema pretiosum* ATCC 31565. A 120-kb DNA fragment was previously identified as the ansamitocin biosynthetic gene cluster, and contains genes for polyketide assembly, precursor synthesis, post-polyketide synthesis modification, and regulation. Within the biosynthetic gene cluster, *asm8* encodes an 1117-amino-acid protein with a high degree of similarity to the large ATP-binding LuxR family-type regulators. In the current study, we determined that inactivation of *asm8* by gene replacement in ATCC 31565 resulted in the complete loss of ansamitocin production, and that complementation with a cloned *asm8* gene restored ansamitocin biosynthesis. Interestingly, the disruption of *asm8* decreased the transcription of genes responsible for 3-amino-5-hydroxybenzoate (AHBA) formation, the starter unit required for ansamitocin biosynthesis. Subsequently, feeding of exogenous AHBA to the *asm8* mutant restored ansamitocin biosynthesis, which showed that Asm8 is a specific positive regulator in AHBA biosynthesis. In addition, investigation of *asm8* homologs identified two new ansamitocin producers, and inactivation of the *asm8* homolog in *A. pretiosum* ATCC 31280 abolished ansamitocin production in this strain. Characterization of the positive regulator Asm8 and discovery of the two new ansamitocin producers paves the way for further improving production of this important antitumor agent.

ansamitocins, regulation, AHBA, *Actinosynnema pretiosum*, LuxR family

Like their plant-derived maytansinoid counterparts [1], ansamitocins (e.g., ansamitocin P-3; AP-3) demonstrate potent antitumor activity by specifically inhibiting eukaryotic mitosis [2]. The antibody-conjugated drug Kadcyla uses maytansinoid as the active compound, and was approved in February 2013 by the United States Food and Drug Administration for treating breast cancer [3]. Produced by *Actinosynnema pretiosum* subsp. *auranticum* ATCC 31565 (hereafter referred to as ATCC 31565), these microbial metabolites belong to an important class of polyketide natural products called ansamycins [2]. All ansamycin antibiotics are macrolactams, and are characterized by an amide-linkage-terminated benzoic acid or a naphthalenic chromophore bridged by an aliphatic polyketide. Typical ansamycins include rifamycin, ansamitocin, and macbecins.

Biosynthesis of ansamycin antibiotics involves the formation of 3-amino-5-hydroxybenzoate (AHBA) as the starter unit for the assembly of the polyketide chain. The first three steps of the AHBA pathway are very similar to the shikimate pathway, but later steps are differentiated by the introduction of glutamine-derived nitrogen, to give 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP). Cyclization and dehydration of amino-DAHP generates 5-deoxy-5-amino-3-dehydroquinic acid

---

*Corresponding author (email: bailq@sjtu.edu.cn)

(aminoDHS), which is finally aromatized by the enzyme AHBA synthase to give AHBA [4,5]. Sequence analysis of the ansamitocin biosynthetic gene cluster identified 52 open reading frames in two separate regions of 83 and 12 kb [6]. Among these genes, *asm47*, *-23*, *-44*, *-45*, and *-22* are homologs of *rifG*, *-J*, *-L*, *-M*, and *-N*, respectively, from the rifamycin biosynthetic gene cluster [6]. Both *asm24* and *asm43* are homologs of *rifK* [5] (Figure 1A). Moreover, genes *asm43–47* form an operon, and *asm23* and *24* are transcribed from a divergent promoter region (Figure 1B).

The biosynthetic mechanism of ansamitocins has been studied extensively [6–9]. However, little is known about the regulation of ansamitocin biosynthesis. Previous sequencing of the ansamitocin biosynthetic gene cluster has revealed the presence of a set of regulatory genes. Three genes, *asm2*, *-29*, and *-34*, encode TetR family proteins, while *asm31*, *-39*, and *-40* encode a sigma factor, sigma factor antagonist, and anti-sigma antagonist, respectively. The protein encoded by *asm18* is homologous to a SARP (*Streptomyces* antibiotic regulatory protein) family protein. The remaining two proteins, encoded by *asm8* and *asm48*, are similar to LuxR or LAL family (large ATP-binding regulator of the LuxR family) regulatory proteins.

A previous study showed that in-frame deletion of *asm2* caused an over-production of ansamitocins [10]. More interestingly, AP-3 production was also increased by constitutive over-expression of *asm2* and *asm39* [11].

Here, the function of *asm8* was studied through gene inactivation, complementation, transcriptional analysis, and feeding experiments. Results showed that *asm8* is a positive regulator of ansamitocin production, specifically the formation of the AHBA starter unit, in ATCC 31565. Further investigation of *asm8* homologs through gene comparison and gene inactivation identified two additional novel ansamitocin producers.

1 Materials and methods

1.1 Bacterial strains, cloning vectors, and cultivation

Strains and plasmids used in this study are listed in Table S1. *Actinosynnema* strains were cultivated at 30°C and 220 r min^{-1} in liquid tryptic soy broth medium (supplemented with 1% yeast extract and 10.3% sucrose) for 2 d. Fermentation was carried out on yeast and malt extract with glucose (YMG) agar plates at 30°C for 7 d. The same medium was supplemented with apramycin when used for growth and/or metabolite production of the *asm8* mutant, PW201. *E. coli* strains DH10B (Invitrogen, New York) and ET12567 (pUZ8002) were used as hosts for plasmid construction and *E. coli-Streptomyces* conjugation [12], respectively. pJTU1278 was used for gene inactivation [13], and pBluescript KS(−) (Agilent Technologies Inc., Santa Clara, CA, USA) and pUC18 (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used for gene cloning and DNA sequencing. pRSET-B (Invitrogen, New York) was used for protein over-expression
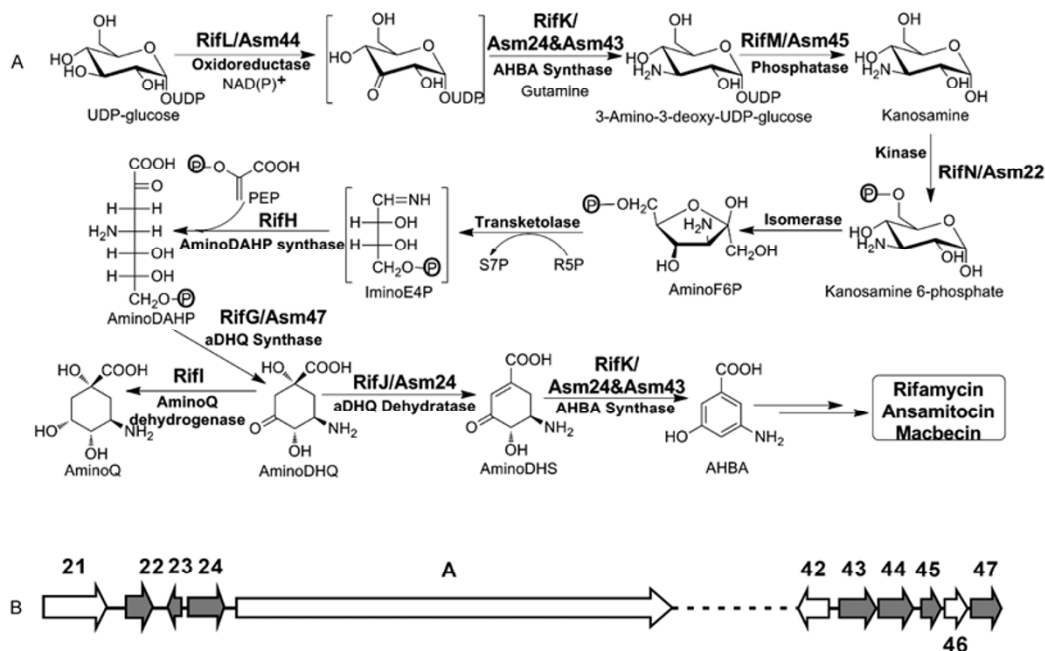

Figure 1 Proposed biosynthetic pathway of AHBA, and organization of the AHBA biosynthetic gene cluster. A, Proposed pathway for AHBA biosynthesis. Gln (glutamine), aminoF6P (3-amino-3-deoxy-D-fructose 6-phosphate), iminoE4P (1-deoxy-1-imino-D-erythrose 4-phosphate), PEP (phosphoenolpyruvate), aminoDAHP (3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate), aminoDHQ (5-deoxy-5-amino-3-dehydroquinic acid), aminoDHS (5-deoxy-5-amino-3-dehydroshikimic acid), AHBA (3-amino-5-hydroxybenzoate). B, Organization of AHBA biosynthetic gene cluster (*asm21–24* and *asm42–47*) in ATCC 31565.

in *E. coli*. Shuttle vectors pIB139 [14] and pSET152 [15] were used for gene complementation.

1.2   Genetic manipulation

Standard genetic techniques using *E. coli* and *in vitro* DNA manipulation were performed as described by Sambrook et al. [16]. Recombinant DNA techniques in *Actinosynnema* species and isolation of *Actinosynnema* DNA were performed as described previously [17].

1.3   Disruption of *asm8* using Redirect Technology

A 7.0-kb *Sac* I/*Bam*H I fragment from cosmid 3C11 [6] was cloned into pJTU1278, to generate pJTU851. The 3.40-kb *asm8* gene within pJTU851 was replaced with an *aac3(IV)-oriT* cassette, to generate pJTU852, using Redirect Technology according to a previously published method [18]. The *aac(3)IV-oriT* cassette was amplified from pIJ773 using primer pair asm8-tgt-F (5′-ATGCGGCTGGTGGAC-CGCGACGGGCAGTGCGAGGCGCTGattccggggatccgtc-gacc-3′) and asm8-tgt-R (5′-TCAGGCGGGGGTGGTCA-GGTCGGCGTGCAGGGTGGTGGGtgtaggctggagctgcttc-3′) (pIJ773 homologous sequences are in lowercase). For a marker-free gene replacement, pJTU852 was digested with *Xba* I and then re-ligated to generate pJTU886, in which the *aac(3)IV-oriT* cassette was replaced with an 81-bp "scar" sequence. pJTU886 was used in conjugation with an apra-mycin-resistant *asm8* mutant to generate the marker-free mutant PW1, which was verified by PCR amplification with the primers asm8-Det-F (5′-AGTGGGGGTGCGGTGGT-3′) and asm8-Det-R (5′-CTGGTCGGGTCGGGGTC-3′).

1.4   Trans-complementation of the mutant PW1 with cloned *asm8*

For the complementation of the PW1 Δ*asm8* mutant, a 3.80-kb *Nru* I fragment from pJTU851, containing *asm8* and its promoter region, was cloned into pSET152. The resulting plasmid, pJTU3101, was then transferred by conjugation from *E. coli* ET12567 (pUZ8002) to PW1, as described previously [19].

1.5   Fermentation, purification, and analysis of ansa-mitocins

Seven-day YMG agar plate cultures of the wild-type ATCC 31565 strain and the PW1 mutant were extracted with ethyl acetate. The extract was concentrated under reduced pressure, and was further extracted with 1 mL methanol. The methanol extract was analyzed by liquid chromatog-raphy-mass spectrometry (LC-MS) using an Agilent 1100 series LC/MSD Trap system [8]. High performance liquid chromatography (HPLC) was operated at a flow rate of 0.45 mL min^{-1} with methanol-water (70/30, v/v) on a Calesil

ODS-100 (4.6 mm×150 mm, 5 μm) column. An ion-trap mass spectrometer was operated with the electrospray ioni-zation source in positive ion mode. The drying gas flow was 6 L min^{-1}, and the nebulizer pressure was 30 psi. The drying gas temperature was 325°C.

1.6   RNA isolation and quantitative reverse transcrip-tion-polymerase chain reaction (qRT-PCR) analysis

A two-day culture of *A. pretiosum* mycelia was collected from YMG agar plates and disrupted using glass beads (150–212 μm, Sigma) in a Precellys homogenizer (6500 r min^{-1}, 2×20 s; Peqlab). RNA was isolated using a Total RNA Isolation Kit (SBS Genetech, Shanghai) and then treated with RNase-free DNase I (Thermo Fisher Scientific Inc.) to eliminate possible chromosomal DNA contamination.

Equal amounts of purified RNA (3 μg) were used to generate cDNA using RevertAid H Minus reverse tran-scriptase and random hexamer primers, as described by the manufacturer (Thermo Fisher Scientific Inc.). The condi-tions were as follows: first strand cDNA synthesis, 42°C for 1 h, followed by 70°C for 10 min. One microliter of synthe-sized cDNA was then used as template for each qRT-PCR, which was performed following the Maxima SYBR Green/ROX qPCR master mix protocol (Thermo Fisher Sci-entific Inc.). The conditions were as follows: 50°C for 20 s, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The DNA gyrase gene *gyrB* was used as an internal control. The transcription of 45 genes in the biosynthetic gene cluster was analyzed.

2   Results

2.1   Asm8 is positively involved in ansamitocin biosyn-thesis

To investigate the function of *asm8*, an in-frame deletion of *asm8* was performed in *E. coli* using Redirect Technology [18], followed by two rounds of conjugation with ATCC 31565 (Figure 2A). Three thiostrepton-sensitive apramycin-sensitive (Thio^{S}Apr^S) mutants (designated PW1-1–PW1-3) were obtained. Total genomic DNA was extracted from the mutants and the wild-type was compared by PCR. The wild-type gave a 3.80-kb amplified product, whereas am-plicons from the mutants were only 0.60 kb (Figure 2B), confirming that a 3354-bp internal DNA fragment, corre-sponding to *asm8*, had been replaced by an 81-bp scar fragment in the mutant genomes. ATCC 31565 and the PW1 mutants were incubated in fermentation medium for 7 d. The fermentation broths were then analyzed by LC-MS. AP-3, with a retention time of 19.7 min and an *m/z* of 635, was not detected in the fermentation broths of the PW1 mu-tants (Figure 2C).

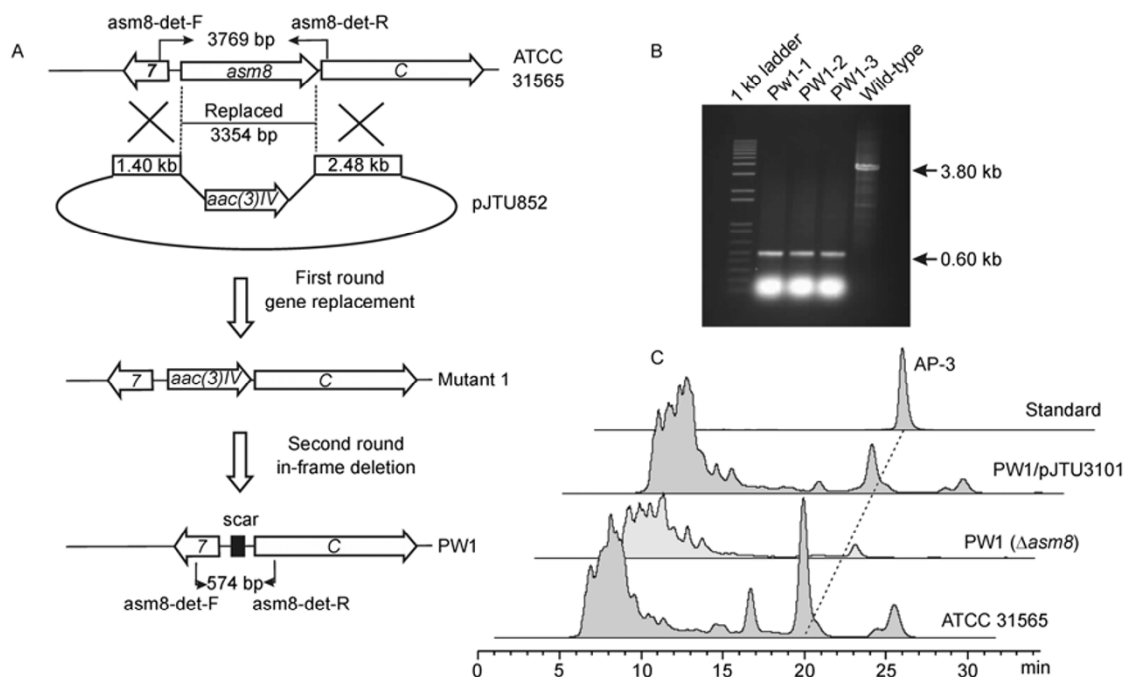To verify that the loss of AP-3 was caused only by the *asm8* inactivation, mutant PW1 was trans-complemented

Figure 2 Schematic representation of *asm8* inactivation and results of HPLC analysis of antibiotic production. A, Schematic representation of *asm8* disruption. B, PCR analysis of wild-type and the PW1 mutant using primers asm8-Det-F and asm8-Det-R. C, HPLC analysis of the fermentation broths of wild-type ATCC 31565, mutant PW1, and PW1 complemented with cloned *asm8* on pJTU3101. The peaks corresponding to AP-3 are marked by a dotted line.

with a cloned 3.80-kb DNA fragment containing *asm8* and its 280-bp upstream region. Plasmid pJTU3101, carrying the 3.80-kb fragment, was introduced into PW1 by conjugation. AP-3 productivity in the complemented mutant strain was restored to 30% of the wild-type (ATCC 31565) level (Figure 2C). The successful trans-complementation of PW1 verified that *asm8* is a pathway-specific activator of ansamitocin biosynthesis, and that the upstream 280-bp region of *asm8* contains the native promoter in ATCC 31565.

2.2 Severely reduced transcription of AHBA biosynthetic genes in PW1

The effect of *asm8* mutation on ansamitocin production was further analyzed at the transcription level by qRT-PCR. The 45 genes putatively related to ansamitocin biosynthesis were chosen for transcription analysis. Total RNA from two-day cultures of the wild-type and PW1 mutant were extracted and analyzed by qRT-PCR. Surprisingly, the transcription levels of *asm23*, *-24*, *-43*, *-44*, *-45*, *-46*, and *-47* were severely reduced in PW1, whereas transcription levels of the genes responsible for polyketide assembly, methoxymalonyl-ACP biosynthesis, post-PKS modification, and regulation were not affected. Other than *asm46*, the genes showing reduced expression in PW1 are all involved in AHBA biosynthesis [20] (Figure 3). In addition, disruption of *asm46* had no effect on AP-3 production (data not shown).

2.3 Feeding with AHBA restored ansamitocin biosynthesis

The qRT-PCR analysis suggested that Asm8 specifically regulates the expression of the AHBA biosynthetic genes, rather than acting on the transcription of other biosynthetic genes. More supporting evidence was obtained from feeding experiments, whereby exogenous AHBA was fed to cultures of the PW1 mutant strain. PW1 was cultivated in fermentation medium supplemented with 1, 10, or 20 mg L^{-1} AHBA. As shown in Figure 4, the production level of AP-3 in PW1 gradually increased in proportion to the amount of exogenous AHBA added to the fermentation broths.

2.4 Identification of *asm8* homologs and two new ansamitocin producers

BLAST analysis of Asm8 against the database identified homologous protein Amir_3178 in *Actinosynnema mirum* DSM 43827, which had an identity of 77%. As the genome sequence of DSM 43827 is available [21], the flanking sequences of *asm8* and *amir_3178* were compared. A strikingly high level of sequence similarity and organization, especially for the ansamitocin biosynthetic genes, was observed between ATCC 31565 and DSM 43827 (Figure S1 in Supporting Information). Moreover, genes homologous to the AHBA-specific genes *asm43–47* were also identified, not only in DSM 43827, but also in *A. pretiosum* ATCC 31280 [22], a strain known for ansamycin-type macbecin
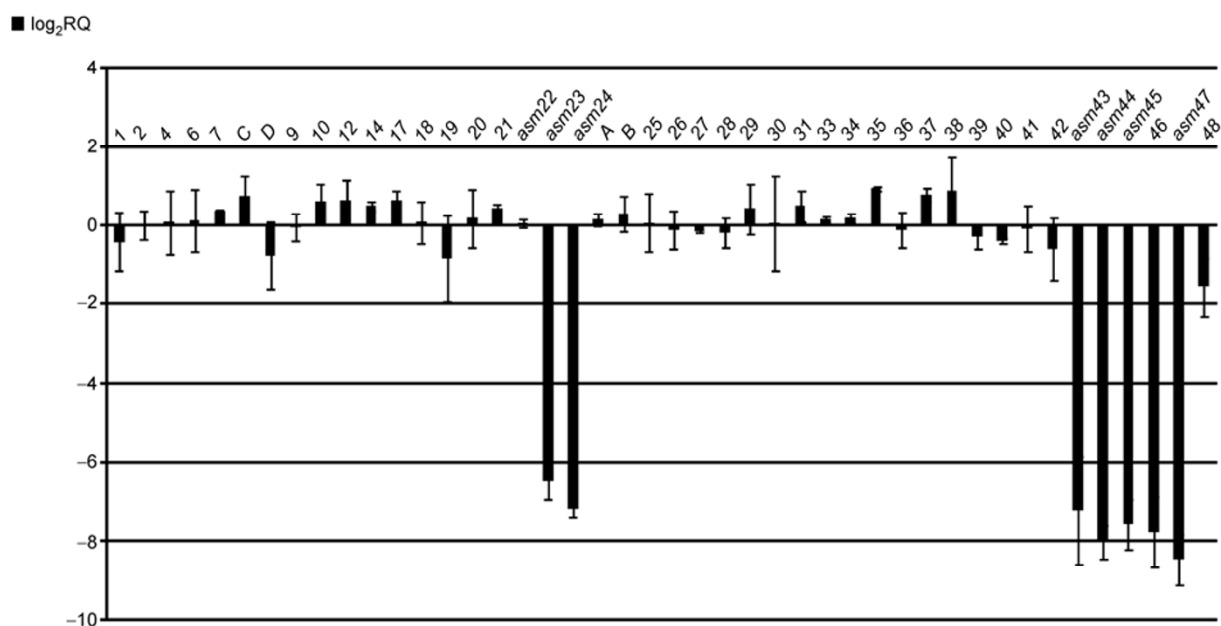
Figure 3 Transcriptional analysis of ansamitocin biosynthetic genes in PW1 compared with ATCC 31565. Transcription levels were determined by qRT-PCR, and putative AHBA biosynthesis genes are indicated in bold.
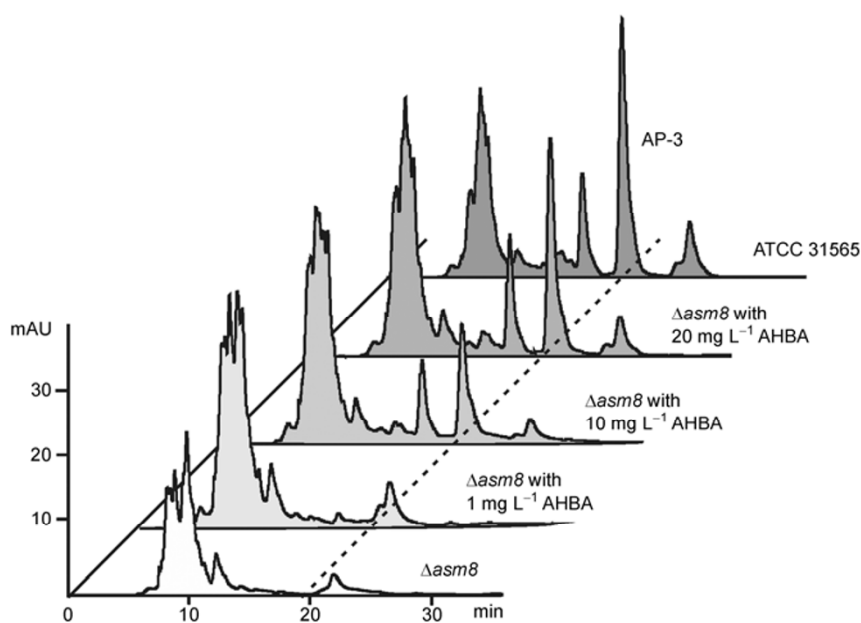

Figure 4 Feeding of exogenous AHBA to the PW1 mutant. The level of AP-3 production by the PW1 mutant was analyzed by HPLC when PW1 was cultivated in fermentation medium supplemented with 0, 1, 10, or 20 mg L^{-1} exogenous AHBA.

production (Figure S1). This gene organization suggested that the ansamitocin biosynthetic gene cluster may be present in the ATCC 31280 genome.

Several pairs of primers specific for the ansamitocin biosynthetic genes were used to try and PCR amplify the corresponding genes from ATCC 31280. The resultant products were sequenced and found to be identical to the homologous genes in ATCC 31565 (data not shown). In addition, all the three *Actinosynnema* strains were cultivated on YMG agar plates for 7 d, and the plates were then extracted with ethyl acetate for LC-MS analysis. Interestingly, peaks with the same retention time and mass as AP-3 were detected in both ATCC 31280 and DSM 43827 (Figure 5A), confirming the identification of two new ansamitocin-
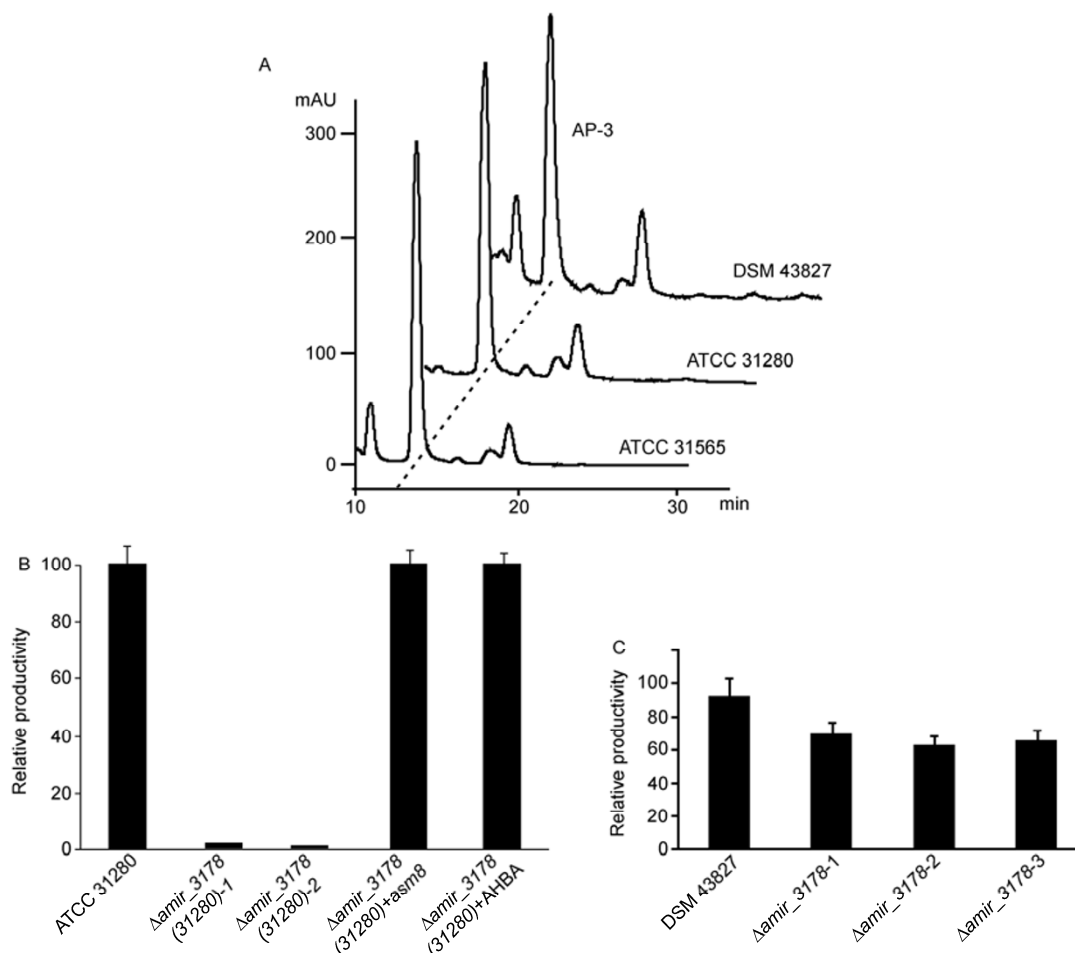
Figure 5 Detection of ansamitocins in the fermentation broths of ATCC 31280 and DSM 43827. A, LC-MS analysis of ansamitocins. The peaks corresponding to AP-3 are indicated by a dotted line. B, AP-3 production in ATCC 31280, an *amir_3178(31280)* mutant, an *amir_3178(31280)* mutant complemented with cloned *asm8*, and an *amir_3178(31280)* mutant supplemented with exogenous AHBA. Gene *amir_3178(31280)* was homologous to *asm8* from ATCC 31280. C, AP-3 production in DSM 43827 and the *amir_3178* mutants. Gene *amir_3178* is homologous to *asm8* from DSM 43827.

producing strains.

2.5   Inactivation of genes homologous to *asm8* in ATCC 31280 and DSM 43827

In strain ATCC 31280, a gene homologous to *asm8* was also identical to *amir_3178* from DSM 43827, and was subsequently named *amir_3178(31280)*. Gene *amir_3178 (31280)* was inactivated by gene replacement in ATCC31280 (Figure S2), and the production of AP-3 was completely abolished (Figure 5B). As expected, feeding with exogenous AHBA recovered the AP-3 productivity of the *amir_3178(31280)* mutant. A trans-complemented mutant with cloned *asm8* expressed from plasmid pJTU3101 had restored AP-3 productivity.

Similar gene inactivation of *asm8* homologous gene *amir_3178* was also performed in DSM 43827 (Figure S3). Even though *amir_3178* from DSM 43827 was identical to *amir_3178(31280)*, the *amir_3178* deletion mutant was

surprisingly found to be capable of producing AP-3, albeit with a reduced yield of 60%–70% of the wild-type level (Figure 5C).

3   Discussion

The LAL family regulatory proteins are characterized by a LuxR-type pattern, consisting of a helix-turn-helix DNA-binding motif (~65 amino acids) at the C terminus, and a distinctive P-loop at the N-terminus, which is specific for an ATP-binding site. With a size of up to 116 kD, Asm8 fits the profile of LAL family proteins, and has a high degree of sequence similarity to a number of *Streptomyces* regulators, such as GdmRII from *S. hygroscopicus* 17997 [23], and AveR from *S. avermitilis* K139 [24], which are pathway-specific transcriptional activators of geldanamycin biosynthesis and avermectin biosynthesis, respectively.

Our experiments demonstrated for the first time that the

transcriptional activator gene *asm8* is required for AP-3 production, and specifically controls the biosynthesis of the AHBA starter unit. Although most of the antibiotic biosynthetic gene clusters from actinomycetes have one or more regulatory genes [25], the presence of regulators controlling a complete set of genes with designated functions, such as precursor biosynthesis genes, is rare.

Lechner et al. [26] identified a LuxR regulatory gene, *salR2*, in the salinosporamide biosynthetic gene cluster from *Salinispora tropica*; this gene is specifically involved in the formation of the halogenated precursor chloroethylmalonyl-CoA. When the *salR2* mutant was supplemented with intermediate 4-chlorocrotonic acid, the productivity of salinosporamide was restored. In the clavulanic acid biosynthetic gene cluster, a LysR transcriptional regulator, ClaR, was specific for the expression of genes in the downstream steps of clavulanic acid production, i.e., in the conversion of clavaminic acid to clavulanic acid [27].

Transcriptional analysis using qRT-PCR determined that the transcription of seven genes was significantly reduced (Figure 3), six of which were implicated in AHBA biosynthesis (Figure 1A). *asm46* was not related to ansamitocin biosynthesis (data not shown). Moreover, the transcription of *asm22*, encoding a putative kinase and homologous to *rifN*, was not affected. Even though homologs of *asm22* are present in all identified AHBA biosynthetic gene sets [28], its involvement in AHBA formation in ATCC 31565 needs to be further confirmed, either through gene inactivation or by *in vitro* enzymatic catalysis with the substrate kanosamine.

To date, the negative regulatory gene *asm2* and the positive regulatory gene *asm8* are the only two genes characterized among the nine putative regulatory genes within the previously sequenced region [6]. Recently, eight more regulatory genes were identified by sequencing the 30-kb region between Cluster I and Cluster II (GenBank accession number of HQ441578) (Figure S1). The AP-3 yield was nearly doubled in a mutant with the 30-kb region deleted (data not shown). Because there are 18 regulatory genes within the 120-kb ansamitocin biosynthetic gene cluster, the biosynthesis of ansamitocin is assumed to be stringently regulated, which might cause difficulties for increasing the titer of ansamitocin.

Further investigation into the functions of other regulatory genes will assist with boosting the expression of ansamitocin biosynthetic genes, and ultimately increasing the titer. Already, introducing the cloned *asm8* plasmid construct into the wild-type ATCC 31565 strain has been shown to increase the AP-3 titer. Moreover, the identification of ATCC 31280 and DSM 43827 as new ansamitocin producers may provide better starting candidates for genetic manipulation and titer improvement.

1   Kupchan S M, Komoda Y, Court W A, et al. Maytansine, a novel antileukemic ansa macrolide from *Maytenus ovatus*. J Am Chem Soc, 1972, 94: 1354–1356

2   Higashide E, Asai M, Ootsu K, et al. Ansamitocin, a group of novel maytansinoid antibiotics with antitumour properties from *Nocardia*. Nature, 1977, 270: 721–722

3   Dhillon S. Everolimus in combination with exemestane: a review of its use in the treatment of patients with postmenopausal hormone receptor-positive, HER2-negative advanced breast cancer. Drugs, 2013, 73: 475–485

4   Kim C G, Yu T W, Fryhle C B, et al. 3-Amino-5-hydroxybenzoic acid synthase, the terminal enzyme in the formation of the precursor of mC$_7$N units in rifamycin and related antibiotics. J Biol Chem, 1998, 273: 6030–6040

5   Watanabe K, Rude M A, Walsh C T, et al. Engineered biosynthesis of an ansamycin polyketide precursor in *Escherichia coli*. Proc Natl Acad Sci USA, 2003, 100: 9774–9778

6   Yu T W, Bai L Q, Clade D, et al. The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. Proc Natl Acad Sci USA, 2002, 99: 7968–7973

7   Wu Y, Kang Q, Shang G, et al. *N*-methylation of the amide bond by methyltransferase *asm10* in ansamitocin biosynthesis. Chembiochem, 2011, 12: 1759–1766

8   Li Y, Zhao P, Kang Q, et al. Dual carbamoylations on the polyketide and glycosyl moiety by *asm21* result in extended ansamitocin biosynthesis. Chem Biol, 2011, 18: 1571–1580

9   Spiteller P, Bai L, Shang G, et al. The post-polyketide synthase modification steps in the biosynthesis of the antitumor agent ansamitocin by *Actinosynnema pretiosum*. J Am Chem Soc, 2003, 125: 14236–14237

10   Bandi S, Kim Y, Chang Y K, et al. Construction of *asm2* deletion mutant of *Actinosynnema pretiosum* and medium optimization for ansamitocin P-3 production using statistical approach. J Microbiol Biotechn, 2006, 16: 1338–1346

11   Ng D, Chin H K, Wong V V T. Constitutive overexpression of *asm2* and *asm39* increases AP-3 production in the actinomycete *Actinosynnema pretiosum*. J Ind Microbiol Biot, 2009, 36: 1345–1351

12   Paget M S, Chamberlin L, Atrih A, et al. Evidence that the extracytoplasmic function sigma factor sigmaE is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J Bacteriol, 1999, 181: 204–211

13   He Y, Wang Z, Bai L, et al. Two pHZ1358-derivative vectors for efficient gene knockout in streptomyces. J Microbiol Biotechnol, 2010, 20: 678–682

14   Wilkinson C J, Hughes-Thomas Z A, Martin C J, et al. Increasing the efficiency of heterologous promoters in actinomycetes. J Mol Microbiol Biotechnol, 2002, 4: 417–4126

15   Flett F, Mersinias V, Smith C P. High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol Lett, 1997, 155: 223–229

16   Sambrook J, Fritsch E F, Maniatis T. Molecular Cloning: a Laboratory Manual, 2nd ed. New York: Cold Spring Harbor, 1989

17   Kieser T, Bibb M J, Buttner M J, et al. Practical Streptomyces Genetics. Norwich: The John Innes Foundation, 2000

18   Gust B, Challis G L, Fowler K, et al. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA, 2003, 100: 1541–1546

19   Zhao P, Bai L, Ma J, et al. Amide *N*-glycosylation by Asm25, an *N*-glycosyltransferase of ansamitocins. Chem Biol, 2008, 15: 863–874

20   Cassady J M, Chan K K, Floss H G, et al. Recent developments in the maytansinoid antitumor agents. Chem Pharm Bull, 2004, 52: 1–26

21   Land M L A, Mayilraj S, Chen F, et al. Complete genome sequence

of *Actinosynnema mirum* type strain (101T). Stand Genom Sci, 2009, 1: 46–53

22 Ono Y, Kozai Y, Ootsu K. Antitumor and cytocidal activities of a newly isolated benzenoid ansamycin, macbecin I. Gann, 1982, 73: 938–944

23 He W Q, Lei J, Liu Y Y, et al. The LuxR family members GdmRI and GdmRII are positive regulators of geldanamycin biosynthesis in *Streptomyces hygroscopicus* 17997. Arch Microbiol, 2008, 189: 501–510

24 Kitani S, Ikeda H, Sakamoto T, et al. Characterization of a regulatory gene, *aveR*, for the biosynthesis of avermectin in *Streptomyces avermitilis*. Appl Microbiol Biot, 2009, 82: 1089–1096

25 Liu G, Chater K F, Chandra G, et al. Molecular regulation of antibiotic biosynthesis in streptomyces. Microbiol Mol Biol Rev, 2013, 77: 112–143

26 Lechner A, Eustaquio A S, Gulder T A, et al. Selective overproduction of the proteasome inhibitor salinosporamide A via precursor pathway regulation. Chem Biol, 2011, 18: 1527–1536

27 Paradkar A S, Aidoo K A, Jensen S E. A pathway-specific transcriptional activator regulates late steps of clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Mol Microbiol, 1998, 27: 831–843

28 Kang Q, Shen Y, Bai L. Biosynthesis of 3,5-AHBA-derived natural products. Nat Prod Rep, 2012, 29: 243–263

Supporting Information

Table S1   Strains and plasmids used in this study

Figure S1   Gene organization in the regions flanking *asm8* and its homologs in *A. pretiosum* ATCC 31565 and ATCC 31280, and in *A. mirum* DSM 43827.

Figure S2   Disruption of *amir_3178(31280)* in ATCC 31280. A, Schematic representation of the inactivation of *amir_3178(31280)* in ATCC 31280. *amir_3178(31280)* was first replaced by an apramycin resistance gene, *aac(3)IV*, through Redirect Technology, and then replaced by an 81-bp scar fragment. B, PCR amplification of the DNA templates from *amir_3178 (31280)* mutants, designated PW11.

Figure S3   Deletion of *amir_3178* in DSM 43827. A, Schematic representation of the inactivation of *amir_3178* in DSM 43827. Gene *amir_3178* was replaced by an apramycin resistance gene, *aac(3)IV*, using Redirect Technology. B, PCR amplification with two pairs of primers (3178-det-F1/R1 and 3178-det-F2/R2) using genomic DNA from the *amir_3178* mutant, PW21, as the template.

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.