J Ind Microbiol Biotechnol (2009) 36:1345–1351
DOI 10.1007/s10295-009-0619-7

ORIGINAL PAPER

Constitutive overexpression of *asm2* and *asm39* increases AP-3 production in the actinomycete *Actinosynnema pretiosum*

Daniel Ng · Hing Kah Chin · Victor Vai Tak Wong

Received: 2 February 2009 / Accepted: 30 June 2009 / Published online: 16 July 2009
© Society for Industrial Microbiology 2009

Abstract Constitutive overexpression of regulators in the ansamitocin biosynthetic cluster of *Actinosynnema pretiosum* was investigated as a strategy to increase the production of ansamitocin-P3 (AP-3), a clinically promising chemotherapeutic agent. Putative transcriptional regulators *asm2*, *asm29*, and *asm34* as well as the putative regulatory protein *asm39* were cloned into a single-site integrative vector and a multicopy replicative vector, pAP40 and pREP, respectively, and then transformed into *A. pretiosum*. Transformants overexpressing *asm2* and *asm39* in pREP showed an increase in ansamitocin production (1.3-fold over parental levels) in a bioassay screen. In shake-flask fermentations, the *asm2* and *asm39* overexpression transformants attained a maximum AP-3 titer of 33 and 52 mg/l, respectively, which were 1.6- and 2.5-fold higher than the blank vector control. The increase in AP-3 production for the *asm2* overexpression transformant was unexpected, since prior reports suggested that Asm2 was a transcriptional repressor. The increase in production appeared to be dependent on the high expression levels achieved with the replicative vector, which may have disrupted the normal function of Asm2. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) confirmed that *asm2* and *asm39* transcription levels were significantly higher in the transformants relative to the control, suggesting that the yield improvement was due to the transformed plasmids. This study demonstrates that deregulated overexpression of regulatory genes is a feasible strategy to increase AP-3 production in *A. pretiosum*.

Keywords Ansamitocin-P3 · Constitutive expression · Multicopy plasmid · Regulatory protein · Transcriptional regulator

D. Ng · H. K. Chin · V. V. T. Wong (✉)
Bioprocessing Technology Institute, Agency for Science, Technology and Research, A*STAR, 20 Biopolis Way, #06-01, Centros, Singapore 138668, Singapore
e-mail: victor_wong@bti.a-star.edu.sg

Introduction

The actinomycete *Actinosynnema pretiosum* is of industrial importance because of its production of ansamitocin P-3 (AP-3), a microtubule disruptor [8]. Immunoconjugates of AP-3 have shown much promise as target-specific cancer chemotherapeutic agents in clinical trials [2, 12, 13]. As the production of AP-3 in wild-type *A. pretiosum* is relatively low, there is interest in increasing the yield of AP-3.

Secondary metabolism in actinomycetes is regulated through a complex cascade of regulatory factors [1, 3]. In the model organism, *Streptomyces coelicolor*, several pleiotropic and pathway-specific transcriptional activators, such as *act*II-ORF4 and *redD*, have been identified and are relatively well studied [1]. Deregulated overexpression of *act*II-ORF4 and *redD* have been shown to significantly increase the expression of actinorhodin and undecylprodigiosin, respectively, in *S. coelicolor* [5, 19]. In contrast, transcriptional regulators in *A. pretiosum* have only been identified through sequence homology [21] and their functions are largely unknown.

Two lines of evidence suggest that regulators in the ansamitocin biosynthetic cluster (*asm*) may be promising targets for genetic manipulation to increase the yield of AP-3. Firstly, we had previously performed a microarray analysis on genes in the *asm* cluster of high AP-3 producers obtained through a rifampicin-resistance screen

(unpublished data). Interestingly, two of the genes that were significantly differentially expressed (>2-fold, P value < 0.05) in the high producers were the transcriptional regulators *asm2* and *asm29*. As a putative transcriptional repressor, *asm2* was downregulated, while *asm29* was upregulated. Secondly, deletion of *asm2* has been reported to increase AP-3 yield in *A. pretiosum* [18], but the effect of *asm2* overexpression is not known.

In this study, we investigated the effects of constitutive overexpression of transcriptional regulators in the *asm* cluster on AP-3 production. Although the original intent of overexpressing *asm2* was to confirm its role as a repressor, we show that overexpression of *asm2*, and the regulatory protein, *asm39*, on a replicative vector is a feasible strategy to increase AP-3 production in *A. pretiosum*.

Materials and methods

Bacterial strains and growth conditions

Media was prepared from DIFCO (BD Diagnostics) stocks and antibiotics were purchased from Sigma (Singapore). *A. pretiosum* subsp. *auranticum* 31565 and *E. coli* strains used in this study are listed in Table 1. *A. pretiosum* spore stocks were prepared as previously described [7]. *E. coli* XL10 Gold was used for general cloning; transformants were cultured in LB medium at 37°C supplemented with either apramycin (50 µg/ml) for pAP40 and pREP clones or ampicillin (100 µg/ml) for TOPO clones. *E. coli* ET12567/pUZ8002 transformants were maintained in chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), and apramycin (50 µg/ml).

Construction of overexpression mutants

The integrative expression vector for *A. pretiosum*, pAP40, had been described previously [7]. The plasmid contains the ermE* constitutive promoter and IS117 insertion sequence.

The new replicative expression vector for *A. pretiosum*, pREP, was constructed as follows: pAP40 was digested with *Nco*I and treated with Klenow fragment before digestion with *Nhe*I to excise the IS117 segment. Concurrently, the pIJ101 replicative segment was amplified from pIJ4090 [10] with primers pIJ101-NheI-F and pIJ101-EcoRV-R (Table 2) and digested with *Nhe*I and *Eco*RV. The pIJ101 PCR product was then ligated to the fragment from pAP40. The resulting vector contains the ermE* constitutive promoter as in pAP40, but with the IS117 insertion sequence replaced by the pIJ101 replicative segment.

Genes of interest (*asm2*, *asm29*, *asm34*, and *asm39*: accession number AF453501 and U33059) were amplified from *A. pretiosum* genomic DNA using respective primers listed in Table 2 and cloned into TOPO TA-vectors (Invitrogen, Singapore) for sequence verification. Each forward primer included the sequence of the *E. coli* consensus ribosome binding site (RBS) previously described [7] as well as the *Bam*HI restriction site. Each reverse primer included the *Eco*RI restriction site. For cloning into pAP40, each TOPO vector was digested with *Bam*HI and *Eco*RI and ligated to pAP40 that had been similarly digested. For cloning into pREP, each TOPO vector was digested with *Bam*HI and treated with Klenow fragment, before digestion with *Eco*RI. The fragment containing a gene of interest was then ligated with pREP that was digested with *Eco*RI and *Eco*RV.

Conjugation between *E. coli* ET12567/pUZ8002 transformants bearing the pAP40 or pREP plasmids and *A. pretiosum* was carried out as described previously [7].

Ansamitocin bioassay

The bioassay is based on the sensitivity of the fungus *Hamigera avellanea* (ATCC 10414) to microtubule

Table 1 Strains used in this study

Strain	Relevant characteristic	Source
Actinosynnema pretiosum subs. p*auranticum* 31565	Parental strain	ATCC
A. pretiosum pAP40-asm2, -asm29, -asm34, -asm39	Mutants overexpressing *asm2*, *asm29*, *asm34*, and *asm39* in the integrative vector pAP40	This study
A. pretiosum pREP-asm2, -asm29, -asm34, -asm39	Mutants overexpressing *asm2*, *asm29*, *asm34*, and *asm39* in the replicative vector pREP	This study
E. coli ET12567/pUZ8002	Methylation-deficient host with nontransmissible helper plasmid for conjugation	Westpheling lab
E. coli XL10 Gold	Tet^r *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB* $lacI^qZ\Delta M15$ *Tn10* (Tet^r) Amy Cam^r]	Stratagene
Hamigera avellanea 10414	Fungal strain for ansamitocin bioassay	ATCC

Table 2 Primers used in this study

Primer	Sequences (5′–3′)
pREP construction	
pIJ101-NheI-F	GCTAGCTTGCTTGATCTCCGAGCCGCA
pIJ101-EcoRV-R	GATATCCACGCCACCCGCTCCGCGAT
Amplification of asm genes	
asm2-BamRBS-F	GGATCCAAGCTAACGTAAGGAGGAAAAACACGTGACCGCGCCACCGGGCCGTCG
asm2-RI-R	GAATTCTCAGCGGTCCTGCGGCGCGC
asm29-BamRBS-F	GGATCCAAGCTAACGTAAGGAGGAAAAACACGTGAGCGCGGCCGTGCGGCG
asm29-RI-R	GAATTCTCAGCGCGTCAGGGCCGCGA
asm34-BamRBS-F	GGATCCAAGCTAACGTAAGGAGGAAAAACATATGGCGCGACGCAACCCGGA
asm34-RI-R	GAATTCTCATTTCCGCTCTCGTCGA
asm39-BamRBS-F	GGATCCAAGCTAACGTAAGGAGGAAAAACATATGGACGAGGTGGACGACCCGAC
asm39-RI-R	GAATTCTCAGGGCGCGGGGACGTTCG
qPCR	
asm2-qP-F	TGCGCCACGAGGTTCAC
asm2-qP-R	GTGGCGGGCGCACAT
asm39-qP-F	GACGCGGACGAGGAGTC
asm39-qP-R	GAGTGGGGAGGTGGTGTG
16S-F	CAGAAGAAGCACCGGCTAAC
16S-R	TTAAGCCCCAAGTTTTCACG

disruption by ansamitocin [20]. Spores from a single colony of either transformants or parental *A. pretiosum* 31565 were transferred onto YMG agar plates (4 g/l yeast extract, 10 g/l malt extract, 4 g/l glucose, 20 g/l agar, pH 7.2) with sterile toothpicks. The plates were incubated for 3 days at 26°C before $\sim 4 \times 10^5$ *H. avellanea* spores suspended in 5 ml molten potato-dextrose agar (BD) were overlaid on the *A. pretiosum* point colonies. After the overlay had solidified, the plate was further incubated for 3 days at 26°C before measurements were taken. The inhibition quotient was calculated by dividing the distance between the edges of the *A. pretiosum* colony and the growth-inhibition zone by the diameter of the *A. pretiosum* colony. At least 3–10 independent clones per transformant were screened using the bioassay and the mean inhibition quotient determined.

Shake-flask fermentation and quantification of AP-3

Preculture was initiated by inoculating single colonies of pREP-asm2, pREP-asm39, and pREP (*A. pretiosum* transformed with blank pREP vector) transformants in VM (5 g/l meat extract, 5 g/l peptone, 5 g/l yeast extract, 2.5 g/l enzyme hydrolysate of casein, 20 g/l glucose, 1.5 g/l NaCl) with 50 μg/ml apramycin and incubated at 26°C for 48 h at 180 rpm. The packed cell volume (PCV), representing cell growth, of the preculture was determined by centrifuging a 5 ml broth sample (3,273 g, 15 min) in a tapered graduated tube and measuring the volume of the cell pellet [17]. The inoculum volume was adjusted so that the same PCV was transferred into each flask containing 160 ml YMG with 50 μg/ml apramycin in 1-l flasks. The culture was incubated for 15 days at 26°C with shaking and aliquots were removed at regular intervals for PCV and AP-3 measurements. Extraction and quantification of AP-3 by high-performance liquid chromatography (HPLC) was described previously [7]. The results presented are the mean of two independent clones for each transformant, each fermented in duplicate flasks.

Quantitative RT-PCR

Total RNA was extracted using the RiboPure™-Bacteria Kit (Ambion, Foster City, CA). Cell pellets from day 7 cultures of pREP-asm2, pREP-asm39, and pREP were first resuspended in 0.5× PCV diethyl pyrocarbonate water (0.1% v/v), freeze-thawed five times in liquid nitrogen and a 55°C water bath, then treated with lysozyme (3.5 mg/ml) at 37°C for 20 min. RNA extraction using RNAwiz was then performed according to the manufacturer's protocol. The RNA obtained was treated with DNase I, electrophoresed in a 1% Tris-acetate-ethylenediamine tetraacetic acid (TAE) agarose gel to check for degradation, and quantified spectrophotometrically.

RNA (1 μg) was converted to complementary DNA (cDNA) using the iScript cDNA synthesis kit (Biorad, Hercules, CA) and random hexamers in a 20 μl reaction. Subsequently, 5 μl cDNA was used for *asm2* or *asm39*

amplification. For 16s rRNA (reference gene for normalization) amplification, cDNA was diluted 10^6 times and 5 μl was used for quantitative PCR (qPCR). Reactions were run on an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) with 12.5 μl iTaq SYBR Green Supermix with Rox (Bio-Rad) and 300 nM each of forward and reverse primers in a 25 μl reaction. The cycling conditions were 3 min at 95°C, followed by 45 cycles of 15 s at 95°C and 45 s at 58°C. qPCR primers (Table 2) were designed by Primer Express (Applied Biosystems). Fold change was analyzed by the Pfaffl method [15].

Results and discussion

Four putative regulators in the ansamitocin biosynthetic cluster were studied: *asm2*, *asm29*, and *asm34* show the signature consensus for the helix-turn-helix domain of the TetR-type transcriptional regulators at the N-terminus and *asm39* has similarity to regulatory proteins bearing the histidine kinase-like ATPase C-terminus (HATPase_c) domain.

The four regulators were cloned into pAP40, an integrative expression vector driven by the strong constitutive ermE* promoter [7]. This vector has been shown to integrate singly into specific sites in the *A. pretiosum* genome [7]. To further increase the expression of the transcriptional regulators, a new replicative vector, pREP, was constructed using the replicative segment from pIJ101, which occurs at around 300 copies per chromosome [10].

To assay the ansamitocin production of multiple clones from each transformant efficiently, a biological assay based on sensitivity of *H. avellanea* to ansamitocin was used. A quantitative score indicating the relative ansamitocin production normalized against colony size, the inhibition quotient, was determined for each clone. Figure 1 shows the average inhibition quotient of the transformants compared with parental *A. pretiosum* 31565 and blank vector controls. In general, clones of transformants with overexpression of *asm39* in the replicative vector (pREP-asm39) showed the largest increase (1.3 times) in inhibition quotient over parental levels. Transformants with overexpression of *asm29* in the replicative vector (pREP-asm29) and overexpression of *asm34* in the integrative vector (pAP40-asm34) showed modest increases of 1.1 times. Surprisingly, transformants with overexpression of *asm2* in the replicative vector (pREP-asm2) also showed an increase of about 1.3 times over parental levels. Analysis by two-sample *t* test showed that the differences were statistically significant ($\alpha = 0.05$). There was no significant difference between the parental and blank vector controls.

The two transformants showing the largest increase in ansamitocin production in the bioassay screen, pREP-asm2 and pREP-asm39, were selected for further study. AP-3 production in shake-flask fermentations of two clones for each transformant was compared with *A. pretiosum* transformed with the blank pREP vector. This was considered more appropriate than a wild-type strain for comparison, since it accounts for differences due to either the conjugation process or the presence of the replicative vector. After 15 days of fermentation, pREP-asm39 accumulated 52 mg/l AP-3, 2.5 times more than the pREP control (Fig. 2a). Similarly, pREP-asm2 transformants produced 1.6 times more AP-3 than the control. Analysis by two-sample *t* test showed that the difference in production between the overexpression mutants and the control was significant ($\alpha = 0.05$) from day 9 onwards. In contrast, there was no significant difference in PCV values between the mutants and control (Fig. 2b), indicating that the higher AP-3 production was not because of increased cell growth.

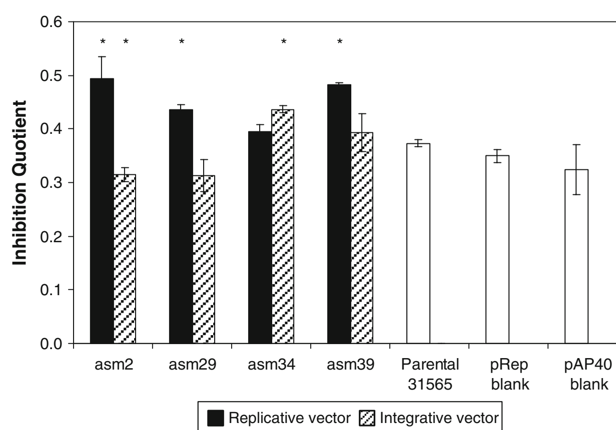

Fig. 1 Inhibition quotient from ansamitocin bioassay of *A. pretiosum* transformed with different transcriptional regulators cloned into either the replicative vector pREP (*black bars*) or the integrative vector pAP40 (*shaded bars*). *Unshaded bars* are for inhibition quotient of untransformed parental *A. pretiosum* 31565 and pREP or pAP40 blank vector controls. Colonies were grown on YMG agar for 3 days at 26°C before overlaying with *H. avellanea* spores suspended in molten potato-dextrose agar. *Asterisks* (*) indicate significant difference in inhibition quotient from parental based on two-sample *t* test, $\alpha = 0.05$

To investigate whether the yield improvement was due to the overexpression of the target genes, the transcription levels of *asm2* and *asm39* in pREP-asm2 and pREP-asm39, respectively, were determined by quantitative RT-PCR and compared with pREP. Although there was some variation in the transcription levels of each individual clone, the levels of *asm2* and *asm39* were significantly higher in the respective overexpression mutants compared with the blank vector control (Fig. 3). This suggests that the increased production was likely to be associated with the overexpression of the targeted regulator genes instead of a random mutation.

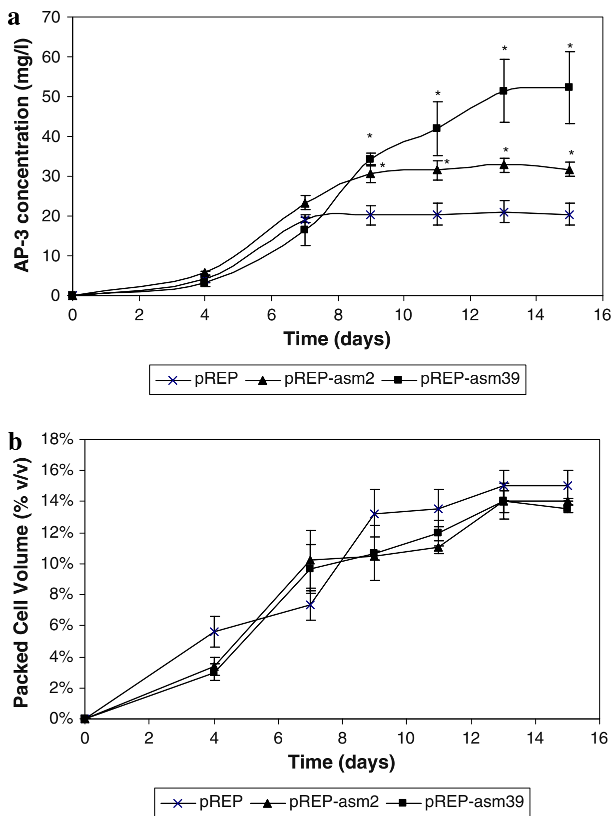

Fig. 2 AP-3 production (**a**) and growth (**b**) of *A. pretiosum* transformed with either *asm2* or *asm39* cloned in the replicative vector (pREP-asm2, pREP-asm39) or blank vector control (pREP). Samples were taken from shake-flask cultures fermented in YMG media with 50 µg/ml apramycin at 26°C. *Error bars* indicate the standard error of two independent clones for each transformant fermented in duplicate flasks each. *Asterisks* (*) indicate significant difference in AP-3 production compared to pREP based on two-sample t test, $\alpha = 0.05$

The increase in AP-3 production when *asm2* was overexpressed on the replicative vector was unexpected, as *asm2* is thought to be a transcriptional repressor [18, 21]. Previous microarray analysis of a spontaneous high-producer *A. pretiosum* mutant showed that *asm2* was downregulated in the mutant relative to the parental strain (unpublished data). It is worth noting that the increased AP-3 production was observed only when *asm2* was overexpressed from the replicative vector but not the integrative vector (Fig. 1). Overexpression of *asm2* from the integrative vector resulted in reduced ansamitocin production, consistent with its putative role as a transcriptional repressor. As pAP40 is known to integrate at a single site per transformant [7], the expression levels obtained with pAP40 is expected to be lower compared with the multicopy pREP vector, although this remains to be verified. Hence, the increase in AP-3 production associated with *asm2* overexpression appears to be dependent on the higher level of expression from the replicative vector.

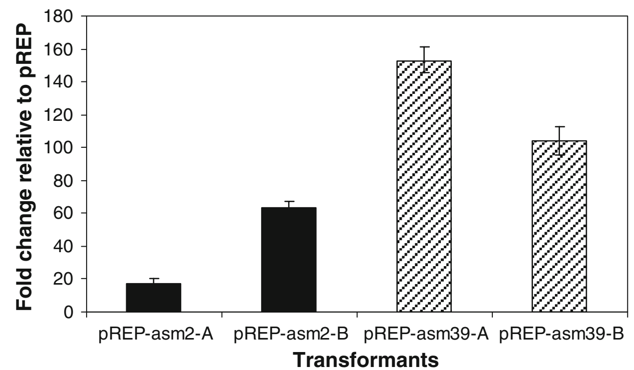

Fig. 3 Relative fold-change from quantitative RT-PCR of RNA extracted from *A. pretiosum* transformed with either pREP-asm2, pREP-asm39 or pREP. Cell pellets from day 7 samples of shake-flask cultures of two independent clones for each transformant (clone A and B) were used for RNA extraction. qRT-PCR was performed using primers specific for *asm2* and *asm39* on pREP-asm2 (*black bars*) and pREP-asm39 (*shaded bars*) samples, respectively, and compared with the corresponding samples from pREP. Ratios were normalized against the respective 16s ribosomal RNA. *Error bars* indicate the standard deviation of triplicate amplification reactions

The top four hits from a BLASTP of *A. pretiosum* Asm2 amino acid sequence identified TetR family transcriptional regulators in *S. ambofaciens*, *S. griseus*, *Streptomyces* sp. Mg1, and *S. coelicolor* (accession numbers: CAJ88084, YP001826556, ZP03170548, and NP628162, respectively). The identities were all around 70% over 187–194 aa overlap (E value $< 2e^{-56}$). However, very little is known about the function of these proteins. A number of TetR-type transcriptional regulators repress transcription by binding to operator sequences as dimers. This leads to the blocking of RNA polymerase binding (e.g., TetR), or hinders the transition of the RNA polymerase–promoter complex into a productively transcribing state (e.g., QacR) [16]. If the overabundance of Asm2 in the pREP-asm2 transformants somehow impeded its binding to the operator sequence, perhaps through the formation of nonfunctional multimers or allosteric hindrance at the binding site, then transcriptional repression at the operator sequence would be relieved, allowing increased AP-3 synthesis. At present, it is not known if Asm2 requires secondary modifications, such as phosphorylation, to act as a repressor. For instance, in the AbsA two-component signal transduction system in *S. coelicolor*, the response regulator AbsA2 acts as a repressor of antibiotic production upon phosphorylation by the adjacent sensor kinase, AbsA1 [14]. Phosphorylated AbsA2 represses antibiotic production by binding to the promoters of antibiotic activator genes, *act*II-ORF4, *redZ*, and *cdaR* [14]. A possible hypothesis is that constitutive overexpression of Asm2 may have disrupted its interaction with its upstream activator or its downstream targets. Further studies are required to understand the role of Asm2

and elucidate the underlying mechanism for the increase in AP-3 production.

The top four hits from a BLASTP of Asm39 identified regulatory proteins from several *Streptomyces* species, with identities ranging from 40% to 46%. All these hits have a characteristic C-terminal domain corresponding to the HATPase_c family, which includes the *S. coelicolor* antibiotic regulator AbaA. When Asm39 was aligned with AbaA-ORFA (SCO0702), there was 33% identity over 154 aa overlap (*E* value $9e^{-13}$). *abaA* is a pleiotropic regulatory locus for antibiotic production in *S. coelicolor* [6]. Both AbaA-ORFA and AbaA-ORFB have been implicated in antibiotic production in *S. coelicolor* and *S. lividans*, and overexpression of a fragment containing these regulators on a multicopy plasmid resulted in overproduction of actinorhodin [6]. However, the mechanism for these regulators is presently unknown. A recently reported regulator of sigma factor activity, RsfA, encoded by SCO4677, has some resemblance to AbaA-ORFA (36% identity over 118 aa, *E* value $6e^{-12}$), and contains the signature sequence for HATPase_c [11]. RsfA was shown to be an antisigma factor, interacting specifically with σ^F, and regulates antibiotic production and morphological differentiation in *S. coelicolor* [11]. When Asm39 was aligned with the RsfA sequence, there was 38% identity over 100 aa (*E* value $1e^{-9}$). Further work will be necessary to determine if Asm39 or AbaA also affects antibiotic production through sigma factors. The sequence similarity between Asm39 and AbaA-ORFA as well as the overproduction of antibiotics when these regulators are overexpressed in multicopy plasmids may indicate similar modes of action between these two regulators.

Alternatively, the effect of *asm2* or *asm39* overexpression on AP-3 production may be indirect. In *S. coelicolor*, cross-regulation between cluster-situated regulators (i.e., regulators located within biosynthetic gene clusters, such as *asm2* and *asm39*) and pleiotropic regulators, usually located outside of the biosynthetic gene clusters, have been reported [9]. Through microarray analysis, Huang et al. [9] showed that constitutive expression of the cluster-situated regulator, *redZ*, increased the expression of the pleiotropic regulator *afsR2*. Hence, it may be that constitutive overexpression of *asm2* and *asm39* increased AP-3 production indirectly via their effect on higher-level pleiotropic regulators.

Other putative transcriptional regulators in the *asm* cluster include *asm8* and *asm18*. However, these were not included in the present study as we were unable to amplify these genes from the *A. pretiosum* genomic DNA (gDNA) despite using a range of PCR conditions, possibly due to the presence of sequences in the *A. pretiosum* genome that are very similar to these genes. Nevertheless, the current results suggest that it is worthwhile to investigate the effects of overexpression of other regulators and sigma factors in the *asm* cluster. Given the current results, it would also be interesting to compare the AP-3 production of mutants with *asm2* and *asm39* deletions with the corresponding low-copy and multicopy overexpression mutants in the same genetic background.

The AP-3 production levels of the overexpression mutants were relatively modest using the current fermentation conditions. There is clearly room for further yield improvement through optimization of media or fermentation strategies. For example, using a statistical approach for media optimization, Srinivasulu et al. [18] reported an increase in AP-3 titer from 5 to 78.3 mg/l in their optimized media. In the patent literature, *A. pretiosum* mutants isolated from extensive rounds of random mutagenesis screens reportedly produce up to 401 mg/l of AP-3 [4]. It might be interesting to investigate whether constitutive overexpression of *asm2* and *asm39* may be applied to existing industrial strains to further improve yield.

In conclusion, we have shown that constitutive overexpression of the regulators *asm2* and *asm39* on the multicopy replicative vector, pREP, increased AP-3 production in *A. pretiosum*. In the case of *asm2*, the increase in production was unexpected, but appeared to be dependent on the high expression from the replicative vector. The vectors described in this study may be useful for the constitutive expression of other targets in *A. pretiosum*. This work demonstrates that overexpression of regulatory genes could be a useful strategy to increase AP-3 production in this industrially important organism.

Acknowledgments We thank Sabrina Yeo for help with vector construction; Corrine Wan, Ong Boon Tee, and Lee May May for help with AP-3 quantification; Janet Westpheling for the *E. coli* ET12567 strain and pKS1 vector; Andrea Camattari and Dave Ow for useful discussions throughout the course of the work; and Niki Wong for critical review of the manuscript. The work was supported by the Biomedical Research Council of A*STAR (Agency for Science, Technology and Research), Singapore.

References

1. Bibb MJ (2005) Regulation of secondary metabolism in streptomycetes. Curr Opin Microbiol 8:1–8
2. Cassady JM, Chan KK, Floss HG, Leistner E (2004) Recent development in the maytansinoid antitumor agents. Chem Pharm Bull 52:1–26
3. Chater KF, Bibb MJ (1996) Regulation of bacterial antibiotic production. In: Kleinkauf H, von Döhren H (eds) Products of secondary metabolism, biotechnology, 7. Weinheim-VCH, New York, pp 57–105
4. Chung J and Byng GS (2003) Mutant *Actinosynnema pretiosum* strain with increased maytansinoid production. US Patent 0157694A1
5. Fernández-Moreno MA, Caballero JL, Hopwood DA, Malpartida F (1991) The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell 66:769–780

6. Fernández-Moreno MA, Martín-Triana AJ, Martínez E, Niemi J, Kieser HM, Hopwood DA, Malpartida F (1992) *abaA*, a new pleiotropic regulatory locus for antibiotic production in *Streptomyces coelicolor*. J Bacteriol 174:2958–2967
7. Goh S, Camattari A, Ng D, Song R, Madden K, Westpheling J, Wong VVT (2007) An integrative expression vector for *Actinosynnema pretiosum*. BMC Biotechnol 7:72
8. Higashide E, Asai M, Ootsu K, Tanida S, Kozai Y, Hasegawa T, Kishi T, Sugino Y, Yoneda M (1977) Ansamitocin, a group of novel maytansinoid antibiotics with antitumor properties from *Nocardia*. Nature 270:721–722
9. Huang J, Shi J, Molle V, Sohlberg B, Weaver D, Bibb MJ, Karoonuthaisiri N, Lih CJ, Kao CM, Buttner MJ, Cohen SN (2005) Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. Mol Microbiol 58:1276–1287
10. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich
11. Kim ES, Song JY, Kim DW, Chater KF, Lee KJ (2008) A possible extended family of regulators of sigma factor activity in *Streptomyces coelicolor*. J Bacteriol 190:7559–7566
12. Kirschning A, Harmrolfs K, Knobloch T (2008) The chemistry and biology of the maytansinoid antitumor agents. C R Chimie 11:1523–1543
13. Liu C, Chari RV (1997) The development of antibody delivery systems to target cancer with highly potent maytansinoids. Expert Opin Investig Drugs 6:169–172
14. McKenzie NL, Nodwell JR (2007) Phosphorylated AbsA2 negatively regulates antibiotic production in *Streptomyces coelicolor* through interactions with pathway-specific regulatory gene promoters. J Bacteriol 189:5284–5292
15. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
16. Ramos JL, Martinez-Bueno M, Molina-Henares AJ, Teran W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R (2005) The TetR family of transcriptional repressors. Microbiol Mol Biol Rev 69:326–356
17. Shuler ML, Kargi F (1992) Bioprocess engineering—basic concepts. Prentice-Hall, New Jersey
18. Srinivasulu B, Kim YJ, Chang Y-K, Shang G, Yu T-W, Floss HG (2006) Construction of *asm2* deletion mutant of *Actinosynnema pretiosum* and medium optimization for ansamitocin P-3 production using statistical approach. J Microbiol Biotechnol 16:1338–1346
19. Takano E, Gramajo HC, Strauch E, Andres N, White J, Bibb MJ (1992) Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). Mol Microbiol 6:2797–2804
20. Tanida S, Hasegawa T, Hatano K, Higashide E, Yoneda M (1980) Ansamitocins, maytansinoid antitumor antibiotics producing organism, fermentation and antimicrobial activities. J Antibiot (Tokyo) 33:192–198
21. Yu T-W, Bai L, Clade D, Hoffmann D, Toelzer S, Trinh KQ, Xu J, Moss SJ, Leistenr E, Floss HG (2002) The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. Proc Natl Acad Sci USA 99:7968–7973