Transcriptional Regulation and Increased Production of Asukamycin in Engineered *Streptomyces nodosus* subsp. *asukaensis* Strains

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ABSTRACT

Asukamycin, a member of the manumycin family of antibiotics, exhibits strong anti-bacterial, antifungal, and anti-neoplastic activities. However, its production in the wild-type strain of *Streptomyces nodosus* subsp. *asukaensis* ATCC 29757 is relatively low. Recently, the biosynthetic gene cluster for asukamycin was identified in the producing organism and among the 36 genes reported in the cluster, six (*asuR1-asuR6*) were proposed to encode proteins that function as transcriptional regulators. To investigate their involvement in asukamycin biosynthesis and to engineer mutant strains of *S. nodosus* that are able to produce large amounts of asukamycin, we carried out *in vivo* gene inactivation, transcriptional analysis of the biosynthetic genes in the mutants, and gene duplication in the producing strain of *S. nodosus*. The results show that two of the putative regulatory genes (*asuR1* and *asuR5*) are critical for asukamycin biosynthesis, whereas others regulate the pathway at various levels. Overexpression of a gene cassette harboring *asuR1*, *asuR2*, *asuR3*, and *asuR4* in *S. nodosus* resulted in changes in morphology of the producing strain and an approximately 14-fold increase of asukamycin production. However, overexpression of the individual genes did not give a comparable cumulative level of asukamycin production, suggesting that some, if not all, of the gene products act synergistically to regulate the biosynthesis of this antibiotic.

Key words: asukamycin, transcriptional regulation, biosynthesis, genetic engineering

INTRODUCTION

The actinomycetes, especially those of the genus *Streptomyces,* have been known to be a prolific source of bioactive natural products. More than one-half of known antibiotics discovered during the past half century are of *Streptomyces* origin (Berdy 2005). However, very often efforts to develop these antibiotics for clinical uses are hampered by the low availability of the compounds, mainly due to their suboptimal production under laboratory conditions. Therefore, strain and culture improvements are often necessary to achieve production suitable for further studies and clinical uses.

Among potential antibiotics with interesting biological activities are the asukamycins, members of the manumycin family of antibiotics produced by *Streptomyces nodosus* subsp. *asukaensis* ATCC 29757 (Figure 1). They have pronounced antibacterial, antifungal, and antineoplastic activities (Omura et al. 1976, Hu and Floss 2004, Shipley et al. 2009), but their production by *S. nodosus* ATCC 29757 under known culture conditions is relatively low (Omura et al. 1976, Hu and Floss 2004). Our initial attempts to improve asukamycin production by ribosome engineering (Wang et al. 2008) and pH shock (Kim et al. 2007) methodologies gave little success (data not shown). More recently, Rui *et al.* reported the biosynthetic gene cluster of asukamycin from *S. nodosus* ATCC 29757 (Rui et al. 2010). Annotation of the cluster resulted in a total of 36 *orf*s (Figure 2a), of which six (*asuR1-asuR6*) were proposed to encode proteins that function as transcriptional regulators. The *asuR1-4* genes are clustered in an operon located upstream (on the left arm) of the cluster, whereas *asuR5 and asu6* are in separate operons located downstream of the cluster (on the right arm) (Figure 2a).

Some of these proteins may be critical for the regulation of asukamycin production or acting as response mechanisms to a variety of environmental signals or nutritional conditions. Proper manipulations of the expression of these proteins may enhance the production level of this important antibiotic.

The genes *asuR1* and *asuR6* encode LuxR family regulators with a conserved LuxR_C DNA binding domain in their C terminal region (Table 1). This domain contains a helix-turn-helix motif and is responsible for DNA binding. Proteins belonging to this group are mostly response regulators, some act as transcriptional activators, others as transcriptional repressors (Sitnikov et al. 1995, Baikalov et al. 1996). The gene *asuR2* encodes a protein similar to TetR family of transcriptional regulators, which are widely distributed among bacteria with an HTH DNA-binding motif. Members of this family commonly negatively regulate antibiotic biosynthesis, efflux pumps, osmotic stress, and others (Ramos et al. 2005). The gene *asuR3* encodes an integral membrane protein with no meaningful similarity to known regulators. On the other hand, *asuR4* and *asuR5* encode a cold-shock protein and a SARP family protein, respectively. Whereas cold shock proteins more likely function as response mechanisms to temperature-related environmental conditions (Horn et al. 2007), members of the SARP family are normally involved in the regulation of secondary metabolite biosynthesis (Wietzorrek and Bibb 1997, Sheldon et al. 2002). To investigate the putative involvement of these regulatory genes in asukamycin biosynthesis we carried out *in vivo* gene inactivation and determined by RT-PCR their implications on the transcription of a select set of structural genes and their effects on the production level of asukamycin in *S. nodosus*. We also

cloned and introduced the regulatory genes, either in combination or individually, into the asukamycin producer and analyzed their effects on asukamycin production. In this paper, we report our findings on the role of these putative regulatory genes and their implications in the phenotypic changes of the bacteria and the production of asukamycin.

MATERIALS AND METHODS

Strains, plasmids and general methods

The strains used in this study are listed in Table S1. The general techniques for PCR and cloning followed Sambrook *et al.* (Sambrook and Russell 2001). Genetic manipulations of *Streptomyces* followed Kieser *et al.* (Kieser et al. 2000). For spore production, *S. nodosus* strains were cultured on YM agar medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, dextrose 1%, agar 2%) for 4 days at 30 °C. Taq polymerase, high-fidelity Taq polymerase, T4 DNA ligase, restriction enzymes and other related enzymes were purchased from Invitrogen.

Construction of mutant strains of *S. nodosus*

For gene deletion of *asuR1*, *asuR2*, *asuR3*, *asuR4*, *asuR5* and *asuR6* in *S. nodosus*, plasmids pTMPX57, pTMPX67, pTMYS008, pTMPX68, pTMPX58 and pTMPX30 were constructed (Table S1). To construct Δ*asuR1*, Δ*asuR2*, Δ*asuR3*, Δ*asuR4* and Δ*asuR5* mutants, the in-frame deletion strategy was employed to decrease the possibility of polar effects. For Δ*asuR1*, 2-kb downstream and upstream DNA fragments of *asuR1* were amplified by PCR with the primer pairs asuR1_left and asuR1_right (Table S2),

which were cloned into the *Hin*dIII/*Kpn*I and *Xba*I/*Hin*dIII sites of the cloning vector pBluescript II SK (-), respectively. After confirmation by DNA sequencing, the upstream DNA fragment was inserted into the *Xba*I and *Hin*dIII sites of the plasmid vector pJTU1278 (He et al. 2010) followed by the second insertion of the downstream DNA fragment into the *Hin*dIII and *Kpn*I sites. The resulting plasmid pTMPX57 was introduced into *S. nodosus* by the standard *E. coli-Streptomyces* conjugation procedure (Kieser et al. 2000), and exconjugants were subsequently selected using 20 μg/mL thiostrepton. Six colonies resistant to thiostrepton were picked and re-streaked onto YM medium without antibiotics for a second round of recombination. Double crossover mutants were screened by replica from the colonies grown on the YM medium. Mutants that lost resistance to thiostrepton were selected for further screening and genotypic confirmation by PCR. The same procedure was applied to the construction of Δ*asuR2*, Δ*asuR4* and Δ*asuR5* mutants by their specific primer pairs for PCR amplification (Table S2).

For Δ*asuR3*, 2-kb of the downstream and the upstream DNA fragments of *asuR3* were amplified by PCR with the primer pairs asuR3_left and asuR3_right (Table S2). The downstream DNA fragment was cloned into the *Hin*dIII and *Xba*I sites of the cloning vector pIJ2925 (Janssen and Bibb 1993), followed by the second insertion of the upstream DNA fragment into the *Xba*I/*Nde*I sites. After confirmation by DNA sequencing, the plasmid was first digested with *Nde*I and the protruding ends were filled with Large (Klenow) Fragment (Promega). The Klenow-treated plasmid was then digested with *Hin*dIII and the downstream/upstream fragment was inserted into the *Xba*I (blunt-ended with Klenow fragment) and *Hin*dIII sites of the plasmid vector pTMN002

(Ito et al. 2009). The same transformation procedure was followed to introduce this plasmid into *S. nodosus*. Four colonies resistant to apramycin were picked and re-streaked onto YM medium without antibiotics for a second round of recombination. Double crossover mutants were screened by replica from the colonies grown on the YM medium. Mutants that lost resistance to apramycin were selected for further screening and genotypic confirmation by PCR.

The construction strategy for the Δ*asuR6* mutant was modified slightly due to the difficulties of getting mutants by in-frame deletion. After cloning of the 2-kb downstream and upstream DNA fragments into the *Eco*RI/*Hin*dIII and *Xba*I/*Eco*RI sites of pJTU1278, an additional ~1-kb gene cassette for apramycin resistance was further inserted into the *Eco*RI site of the vector to give pTMPX30. The same transformation procedure was followed to introduce this plasmid into *S. nodosus*. Around 50 colonies resistant to apramycin were inoculated onto two types of YM medium plates with only apramycin or both thiostrepton and apramycin for double crossover mutants screening. Colonies resistant to apramycin but sensitive to thiostrepton were picked and subjected to further PCR confirmation.

Fermentation, HPLC, and LC-MS analysis of the metabolites

S. nodosus wild-type and mutant strains were grown on solid YM medium (2% agar) for spore production, and harvested. For asukamycin production, strains were inoculated in 30-mL seed medium for 2 days followed by a 3-day cultivation in 50 mL production medium (Hu and Floss 2004). Each strain was cultured in triplicate. The fermentation broth was collected after 5000-rpm centrifugation for 15 min, and extracted twice with

equal volumes of EtOAc. After evaporation, the EtOAc extracts were dissolved in 10 mL MeOH and subjected to HPLC or LC-MS analysis. All fermentation experiments and analyses were performed twice to confirm consistency.

HPLC was performed as follows: A reversed-phase octadecyl silica column (YMC-pack ODS-A 250 x 4.6 mm, YMC) was equilibrated with 50% solvent B (acetonitrile, 0.1% formic acid) and 50% solvent A (water, 0.1% formic acid) for 15 min, then developed with a gradient of 50-100% solvent B in solvent A for 30 min, and washed with 100% solvent B for 10 min. The flow rate was 0.4 mL/min and 10 µL of crude extract was injected each time. The compounds were monitored at 315 nm. For LC-MS analysis, a negative ion mode electrospray mass spectrometry was used. For quantification of asukamycin in the wild-type and the mutant samples, areas under the curves of HPLC peaks were directly compared with those of a standard asukamycin sample (0.1 mg/mL), and the yields of asukamycin produced by the wild-type and the mutant strains were calculated accordingly.

RNA isolation and reversed-transcription PCR (RT-PCR)

Wild-type and mutant strains of *S. nodosus* were inoculated into production medium and cells were harvested for RNA extraction after 1-3 days incubation. RNAs were extracted from the cells using Trizol (Invitogen) after a 30 min treatment of the cells with lysozyme. RNAs were precipitated by an equal volume of isopropanol and washed with 70% ethanol. The RNA samples were then treated with DNase (RNase-free, Invitrogen) to remove residual genomic DNA and, after quantification, reverse-transcribed into

cDNA using “RevertAid™ First Strand cDNA Synthesis” kit (MBI Fermentas). The products (20 ng) were then subjected to PCR amplification (25 cycles). Primers P1 – P8 (Table S1) were used to determine the transcription levels of genes from the asukamycin pathway, and primers for *hrdB* gene were used as an internal control. PCR conditions were: template DNA denatured at 94 °C for 5 min, then 94 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec, for 25 cycles.

Overexpression of regulatory genes in *S. nodosus*

Plasmid pTMW50, an integrative vector that harbors the constitutive expression promoter *ermE* (Figure 5a), was used as the overexpression vector for the regulatory gene(s) in *S. nodosus*. The 3-kb PCR product of regulatory genes *asuR1-4* was amplified from genomic DNA by PCR with primers described in Table S2 and cloned into the *Xba*I and *Bam*HI sites of pBluescript II SK(-) for DNA sequencing. After confirmation, this fragment was inserted immediately downstream of the *ermE* promoter at the corresponding sites of pTMW50 and the resulting plasmid pTMPX75 was introduced into *S. nodosus* by conjugation. Colonies resistant to apramycin were selected and confirmed by PCR using primers specific to *acc(3)IV* gene. For the co-overexpression of *asuR5* and *asuM1*, a 2.8-kb fragment containing these two genes was amplified from genomic DNA and cloned into *Xba*I and *Bam*HI sites of pBluescript II SK(-). The fragment was subcloned into pTMW50 and the resulting plasmid pTMPX76 was introduced into *S. nodosus* by conjugation following the same procedure described above.

For the overexpression of a single regulatory gene in *S. nodosus*, *asuR1*, *asuR2*, *asuR3* and *asuR4* were cloned individually into the *Xba*I and *Bam*HI sites of pBluescript II SK(-), and after DNA sequencing confirmation the genes were subcloned into pTMW50 and overexpressed in *S. nodosus* following the same procedures described above. The yields of asukamycin production in the transformants were quantified by HPLC.

Bioactivity assay

Antibacterial activity of metabolites isolated from the mutants was determined by an agar diffusion assay. The test strain *Staphylococcus aureus* was streaked on N medium (0.5% peptone, 0.5% meat extract, 1.2% agar) and grown at 37 °C overnight. A single colony was inoculated into N liquid medium and incubated in a shaker (200 rpm) at 37°C overnight. The cell suspension (1 mL) was mixed thoroughly with warm N medium (100 mL) and poured onto Petri Dishes (100*15 mm, VWR). A methanolic solution of each sample (15 μL) was loaded onto a sterile blank paper disk (Becton-Dickinson) and allowed to dry in the sterile hood for 30 min. The sample-loaded paper disks were then placed onto the solidified agar plates and incubated at 37 °C for 12 h. When needed, 1% MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (1 mL) was added onto the plates to better visualize the inhibition zone.

RESULTS

Genetic Analysis of the Putative Regulatory Genes in the Asukamycin Biosynthetic Gene Cluster

Recent studies on the biosynthetic gene cluster of asukamycin and careful inspection of the bioinformatics data revealed that at least six putative regulatory genes are located within the cluster. To determine the involvement of these putative regulatory genes (*asuR1*, *asuR2*, *asuR3*, *asuR4*, *asuR5* and *asuR6*) in asukamycin biosynthesis, we inactivated the genes individually by in-frame gene deletion (for *asuR1, -R2, -R3, -R4, -R5*) or gene replacement with *aac(3)IV* (for *asuR6*). The mutants were confirmed by PCR (Figures 2b and 3a).

Cultivation of the six resulting mutant and the wild-type strains of *S. nodosus* was carried out in triplicate and the yields of asukamycin were quantified and compared by HPLC analysis. As shown in Figure 3b, mutant strains lacking *asuR1* or *asuR5* were no longer able to produce asukamycin, suggesting that AsuR1 and AsuR5 are essential regulators, most likely involved in the transcriptional initiation of structural genes, or act as switches specific for asukamycin production. On the other hand, Δ*asuR2*, Δ*asuR4* and Δ*asuR6* mutants still produced asukamycin, but at reduced levels (5%, 13% and 33% of that of the wild-type, respectively), suggesting that the corresponding three proteins also play a role in regulating asukamycin biosynthesis, albeit less significantly than AsuR1 and AsuR5. Finally, no significant differences in asukamycin production were observed in Δ*asuR3* mutant compared to the wild-type, suggesting that this unknown integral membrane protein is not directly involved in asukamycin biosynthesis.

Transcriptional Analysis of Asukamycin Biosynthetic Genes in the Mutants

The decreased production level of asukamycin in the mutants indicates possible positive regulatory roles of AsuR1, -R2, -R4, -R5, and -R6 in the pathway. To determine the range of the transcriptional regulation of these proteins RT-PCR was employed to establish the transcription levels of various *asu* structural genes. Eight pairs of primers (1-8) representing eight operons that cover the majority of the asukamycin biosynthetic cluster (Figure 4a), and one primer pair for the *hrdB* gene (the internal control, (Kieser et al. 2000)) were used to generate cDNAs. The selected genes have been proposed to be involved in the formation of the core unit 3-amino-4-hydroxybenzoic acid (*asuA2*), the cyclohexane ring (*asuB1*), the polyketide chains (*asuC3*, *asuC9*, *asuC12*), and the 2-amino-3-hydroxycyclopent-2-enone moiety (*asuD2*) (Rui et al. 2010). In addition, genes that encode a tailoring enzyme (*asuE1*) and a putative export protein (*asuM1*) were also examined. The RNA templates were isolated from the mutants and the wild-type cells from 1-day, 2-day, and 3-day cultivations in the production medium. The results showed that compared to the wild-type strain most of the structural genes in Δ*asuR1* and Δ*asuR5* were down-regulated during the 3-day period, suggesting wide-ranging regulatory spectra for AsuR1 and AsuR5. The results are consistent with those of the metabolic analyses, in which no asukamycin production was observed in the Δ*asuR1* and Δ*asuR5* mutants (Figure 3b). Decreased transcriptional levels of certain genes, e.g., *asuE1*, *asuC9*, *asuA2*, *and asuC12*, were also observed in the Δ*asuR6* mutant, particularly on Day 1 and Day 2, suggesting a narrower regulatory spectrum for AsuR6. However, the expression levels of the suppressed genes rebounded on Day 3, resulting in an overall 33% of asukamycin production in the mutant compared to the wild-type strain (Figure 3b).

Surprisingly, the transcription levels for all genes analyzed in Δ*asuR2* and Δ*asuR4* were comparable to or higher than those in the wild-type, but the overall production of asukamycin in those mutants was only 5% and 13% of the wild-type level, respectively. These results suggest that AsuR2 and AsuR4 do not directly regulate the transcription of the representative genes analyzed in this study, but may play a role in asukamycin biosynthesis by regulating other genes in the cluster or elsewhere in the genome. On the other hand, *asuC9* was not expressed in all the strains tested at different cultivation time. This may be due to a number of factors, e.g., the gene is silent or its expression level is too low to be detected by the assay. Further investigations are necessary to decipher this complex regulatory network.

Overexpression of *asuR1-R4* and *asuM1-R5* Cassettes in *S. nodosus*

The down-regulation of asukamycin biosynthesis in the Δ*asuR1-asuR6* mutants underscores the importance of these regulatory genes. Therefore, we carried out overexpression experiments using two sets of genes (excluding *asuR6,* as its deletion from the chromosome has the smallest effect on asukamycin production) in *S. nodosus*. The *asu1-asu4* gene locus, located upstream of the asukamycin biosynthetic gene cluster, was cloned into the integration vector pTMW50 (Figure 5a), a pSET152 derivative containing the *aac3(IV)* gene and an *ermE* promoter, to give pTMPX75 (Figure 5b). Similarly, *asuM1* and *asuR5*, located downstream of the cluster, were cloned into pTMW50 to give pTMPX76. The *asuM1* gene was included because it encodes an efflux protein similar to EmrB and is located within the same operon

adjacent to *asuR5*. The plasmids pTMPX75 and pTMPX76 were then introduced into *S. nodosus* and the morphological phenotypes of the transformants and the production level of asukamycin were determined. Distinct morphological phenotypes were observed in the pTMPX75 transformant after 3-day cultivation on the solid CM medium (Figures 6a and 6b). The strain displayed sparse aerial mycelia and reduced spore production. Interestingly, it also produced a large amount of visible yellowish substance(s); this was later identified as asukamycin, which had diffused into the agar medium. Cultivation of the transformant in the production medium followed by HPLC analysis of the culture broth revealed a significant increase of asukamycin production in this strain (264 $\pm$ *28* mg/L), approximately 14-fold higher than that of the wild-type (19.1 $\pm$ *3.3* mg/L) (Figure 6c). On the other hand, the pTMPX76 transformant did not show any recognizable morphological differences from the wild-type (Figures 6a and 6b) nor did it show significant increases in asukamycin production (22 $\pm$ *3.8* mg/L) (Figure 6c). Further confirmation of the relative production levels of asukamycin in these transformants was performed by comparing the antibacterial activity of the extracts using an agar diffusion assay with *Staphylococcus aureus* as the indicator. The results showed that samples from the pTMPX75 transformant had a much larger growth inhibition zone than that of the wild-type (2.84 cm^2 vs 0.78 cm^2, Figure 6d), consistent with the estimated production levels of asukamycin in those strains.

AsuR1, AsuR2, AsuR3, and AsuR4 Are Likely Pathway-Specific Regulators

As overexpression of pTMPX75 in *S. nodosus* significantly improved the production of asukamycin, we attempted to express the gene cassette in *Streptomyces pactum*,

another antibiotic-producing actinomycete, which produces the anticancer antibiotics pactamycin, the piericidins, and many others (Bhuyan 1962, Matsumoto et al. 1987). The *S. pactum* strain was transformed with pTMPX75 by conjugation, and the transformant was cultured and production of antibiotics analyzed. However, no significant increase of pactamycin and piericidin production was observed (data not shown), suggesting that these regulatory genes are likely pathway specific and do not function in *S. pactum*.

Dissecting the Role of Individual *asuR1, asuR2, asuR3,* and *asuR4* in *S. nodosus*

The significant effect of *asuR1-R4* cassette in asukamycin production prompted us to investigate the role of each of these genes in asukamycin production. The genes were individually cloned into the vector pTMW50 to generate plasmids pTMPX82, pTMPX83, pTMPX84, and pTMPX85 (Figure 5b). The plasmids were then introduced into *S. nodosus* and the resulting transformants were investigated for asukamycin production. The *asuR3* gene, which encodes a putative membrane protein, was included in the study to explore the possibility of its involvement in asukamycin biosynthesis. HPLC analysis and agar diffusion assay were employed to determine the production levels of asukamycin in those strains. Interestingly, the results showed that none of the transformants could produce asukamycin at the level of those harboring the *asuR1-R4* cassette (Figure 6c). Whereas the strain that contains pTMPX82 (*asuR1*) has a 3.5-fold increase in asukamycin production (70 ± 13 mg/L), other single gene transformants, including empty pTMW50, surprisingly showed reduced asukamycin production relative to the wild-type (Figure 6c). While the reason for the reduced production is unclear, the

result confirmed that AsuR1 is an important regulator in asukamycin biosynthesis, and may work synergistically with other proteins. Also, it is more likely that the slight reduction in asukamycin production of the other transformants (13%, 58%, and 54% of that of the wild-type for *asuR2*, *asuR3*, and *asuR4* overexpression, respectively) is due to some unknown factor related to the plasmid they carry, rather than the presence of the genes, as indicated by the similar reduction (11% of that of the wild-type) observed in the strain carrying only empty pTMW50 (Figure 6c).

DISCUSSION

In recent years, targeted manipulation of regulatory genes has become one of the major techniques to improve antibiotic production and has several advantages over laborious and time-consuming classical techniques. This molecular genetic approach becomes more applicable due to the improved knowledge of natural product biosynthesis and regulation, the more readily accessible genetic tools, and the relatively low costs of DNA sequencing (Chen et al. 2010). To date, numerous regulatory proteins involved in antibiotic production have been identified in *Streptomyces*. Pathway-specific regulators (*e.g*., ActII-ORF4, RedD, CdaR), many of which belong to the SARP-family proteins with a typical OmpR-like conserved domain in the N-terminus, specifically regulate the transcription of certain biosynthetic genes (Wietzorrek and Bibb 1997). On the other hand, pleiotropic regulators (*e.g*., two-component regulators, AfsR, AdpA) were found to have a much broader regulatory spectrum than pathway-specific regulators, as they usually control more than one biosynthetic pathway by activating or repressing pathway-specific regulators (Bibb 1996). In some cases, these regulators (for example, AdpA)

are also found to be associated with morphological differentiation or other cellular processes of *Streptomyces* (Lopez-Garcia et al. 2010).

In the present study, we have confirmed five positive regulators of asukamycin biosynthesis and discovered that each of them regulates the pathway by different degrees. Whereas inactivation experiments and transcriptional analysis by RT-PCR revealed that AsuR1 (a LuxR family regulator) and AsuR5 (a SARP family regulator) are critical for asukamycin biosynthesis, results from overexpression experiments suggest that, as in many transcriptional regulatory systems, these regulators operate with other proteins in a more complex system. This conclusion was based on the fact that over-expression of a gene cassette containing *asuR1, -R2, -R3, and -R4* in *S. nodosus* increased the production of asukamycin approximately 14-fold over that of the wild-type, whereas over-expression of the individual genes only gave a modest increase or no increase at all. Great enhancements of antibiotic production were also observed when pTMPX75, which contains the *asuR1-4* cassette, was incorporated into three other *S. nodosus* mutant strains that produce asukamycin congeners (data not shown). To our surprise, pTMPX76, which contains *asuR5* (a member of the SARP family), did not significantly increase the production of asukamycin.

In addition to the overproduction of asukamycin, abnormal growth with sparse aerial mycelia and loss of sporulation was also observed in the strain containing pTMPX75. This observation provided one more example of close relationships between the morphological differentiation and antibiotic production in *Streptomyces* (Xie et al. 2009,

Lu et al. 2011). It is believed that further elevation of antibiotic production can be achieved through proper coordination of morphological differentiation and secondary metabolism in *Streptomyces,* as well as the genetic manipulation of pathway specific regulatory genes. These results demonstrate the importance of targeted genetic manipulation as a powerful approach to improve antibiotic production in microorganisms.

Acknowledgements

The authors thank Linquan Bai for providing pJTU1278 and Yong Keun Chang for providing *sha4* and *sha8* genes. We also thank Kerry McPhail for her comments and assistance in the preparation of this manuscript. This work was supported by the Herman-Frasch Foundation and a General Research fund from College of Pharmacy, Oregon State University.

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FIGURE CAPTIONS

Figure 1. The chemical structure of asukamycin and manumycin. Asukamycin differs from manumycin in the upper hydrocarbon chains highlighted with bold lines.

Figure 2. Genetic disruption of the regulatory genes *asuR1*, *asuR2*, *asuR3*, *asuR4*, *asuR5* and *asuR6*. a Genetic organization of the asukamycin gene cluster. **b** Illustration of genetic disruption strategy and the sizes of PCR products before and after gene disruption.

Figure 3. Construction of Δ*asuR1*, Δ*asuR2*, Δ*asuR3*, Δ*asuR4*, Δ*asuR5*, Δ*asuR6* mutants and their asukamycin production. a PCR verification of the mutants. W, wild type; M, mutant. **b** Quantification of the asukamycin production in the mutant and wild type strains.

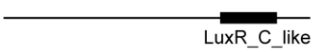
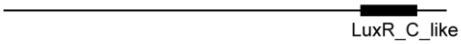
Figure 4. Transcriptional analysis of asukamycin structural genes. a The primers used in the RT-PCR experiments are indicated with short blue lines above the representative asukamycin biosynthetic genes, primers for *hrdB* gene used for the

internal control of RT-PCR. **b** RT-PCR analysis of the transcription of asukamycin biosynthetic genes from the wild type and the mutants. Three time points from day 1 to day 3 were used for RNA sample preparation.

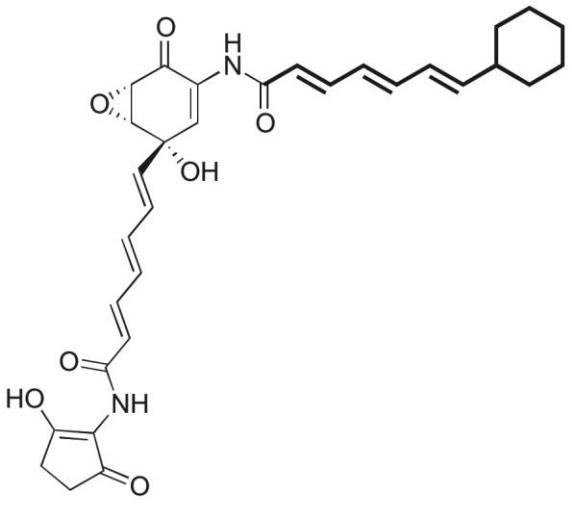
Figure 5. Overexpression of regulatory genes in *S. nodosus*. a The map of plasmid vector pTMW50 used for overexpression. This plasmid contains a constitutively expressed promoter *ermE*. The restriction sites *Bam*HI and *Xba*I were used for cloning. **b** Illustration of the plasmid construction strategy used in the overexpression of regulatory genes.

Figure 6. The phenotypes of wild-type *S. nodosus* and strains transformed with plasmid pTMW50, pTMPX75, pTMPX76, pTMPX82, pTMPX83, pTMPX84 and pTMPX85. **a** The front view of the plate: mutant and wild-type strains were cultivated on YM medium for 3 days; the pTMPX75 transformant displayed sparse aerial mycelia, reduced spore production, and increased asukamycin production. **b** The back view of the plate. **c** The quantification of asukamycin production from the above strains after fermentation. **d** Antibacterial assay of the metabolites isolated from the above strains. **1**, 50 μg/mL asukamycin standard; **2**, wild-type; **3**, pTMW50; **4**, pTMPX75; **5**, pTMPX76; **6**, pTMPX82; **7**, pTMPX83; **8**, pTMPX84; **9**, pTMPX85.

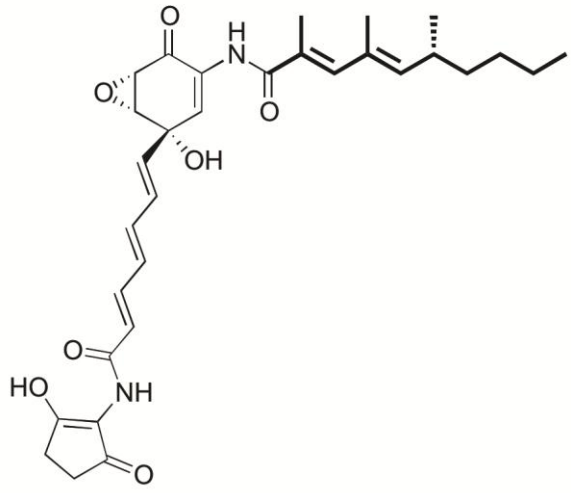
Table 1. The proposed biological functions and the conserved domains for the possible regulatory proteins encoded by asukamycin biosynthetic gene cluster

Protein name	Protein size (aa)	Proposed biological function	Conserved domain
AsuR1	216	Response regulator receiver protein, LuxR family	
AsuR2	191	Transcriptional regulator, TetR family	
AsuR3	301	Putative integral membrane protein	
AsuR4	141	Cold-shock DNA-binding domain protein	
AsuR5	254	Pathway specific regulator, SARP family	
AsuR6	280	LuxR family transcriptional regulator	

LuxR_C_like: C-terminal DNA-binding domain of LuxR-like proteins (Sitnikov et al. 1995, Baikalov et al. 1996).
CSP_CDS: Cold-Shock Protein (CSP) contains an S1-like cold-shock domain (CSD) (Horn et al. 2007).
Trans_reg_C: Transcriptional regulatory protein, C terminal (Wietzorrek and Bibb 1997, Sheldon et al. 2002).



Asukamycin



Manumycin

Figure 1

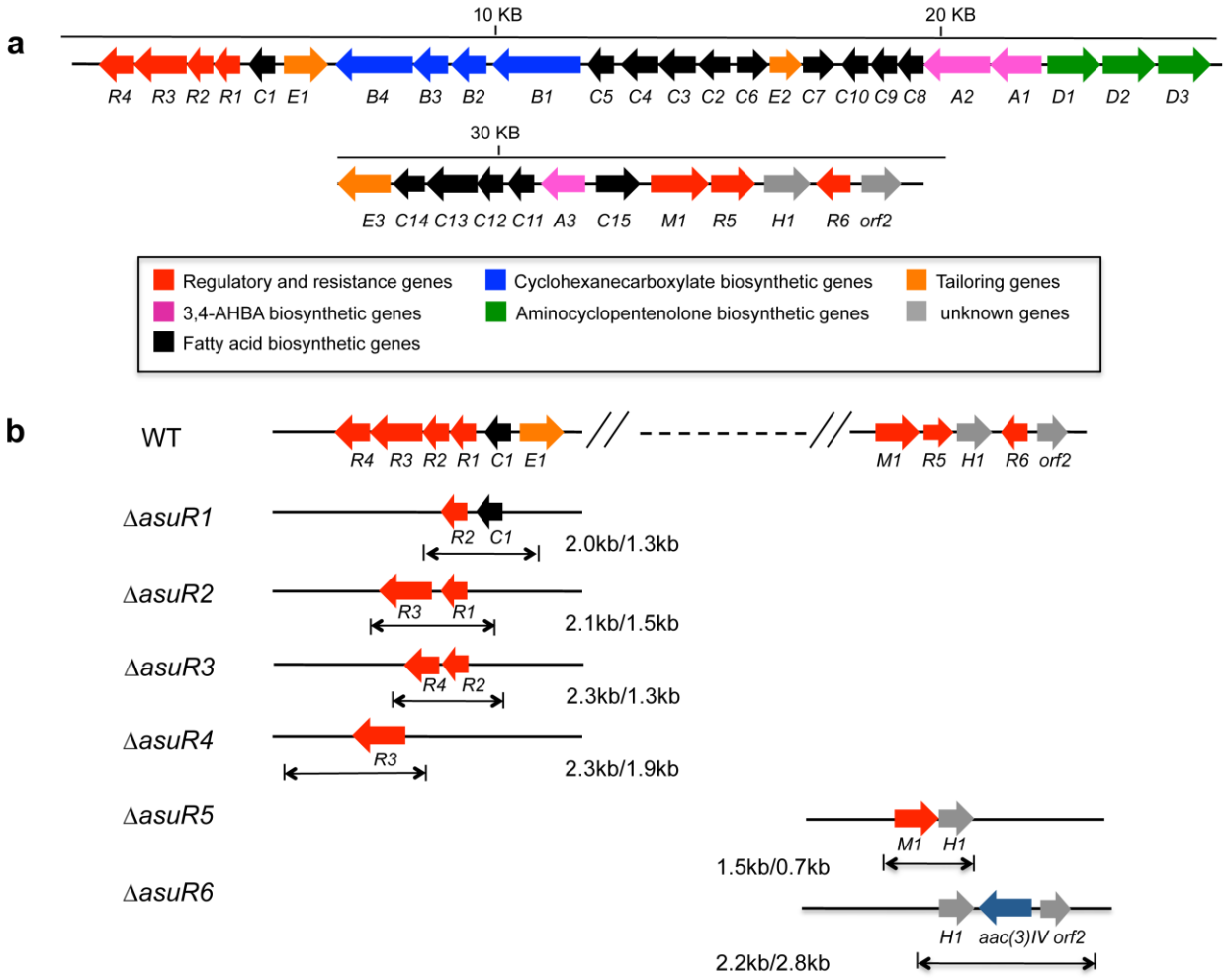

a
10 KB
20 KB
R4 R3 R2 R1 C1 E1 B4 B3 B2 B1 C5 C4 C3 C2 C6 E2 C7 C10 C9 C8 A2 A1 D1 D2 D3
30 KB
E3 C14 C13 C12 C11 A3 C15 M1 R5 H1 R6 orf2
Regulatory and resistance genes
Cyclohexanecarboxylate biosynthetic genes
Tailoring genes
3,4-AHBA biosynthetic genes
Aminocyclopentenolone biosynthetic genes
unknown genes
Fatty acid biosynthetic genes
b
WT
R4 R3 R2 R1 C1 E1
M1 R5 H1 R6 orf2
ΔasuR1
R2 C1
2.0kb/1.3kb
ΔasuR2
R3 R1
2.1kb/1.5kb
ΔasuR3
R4 R2
2.3kb/1.3kb
ΔasuR4
R3
2.3kb/1.9kb
ΔasuR5
M1 H1
1.5kb/0.7kb
ΔasuR6
H1 aac(3)IV orf2
2.2kb/2.8kb

Figure 2

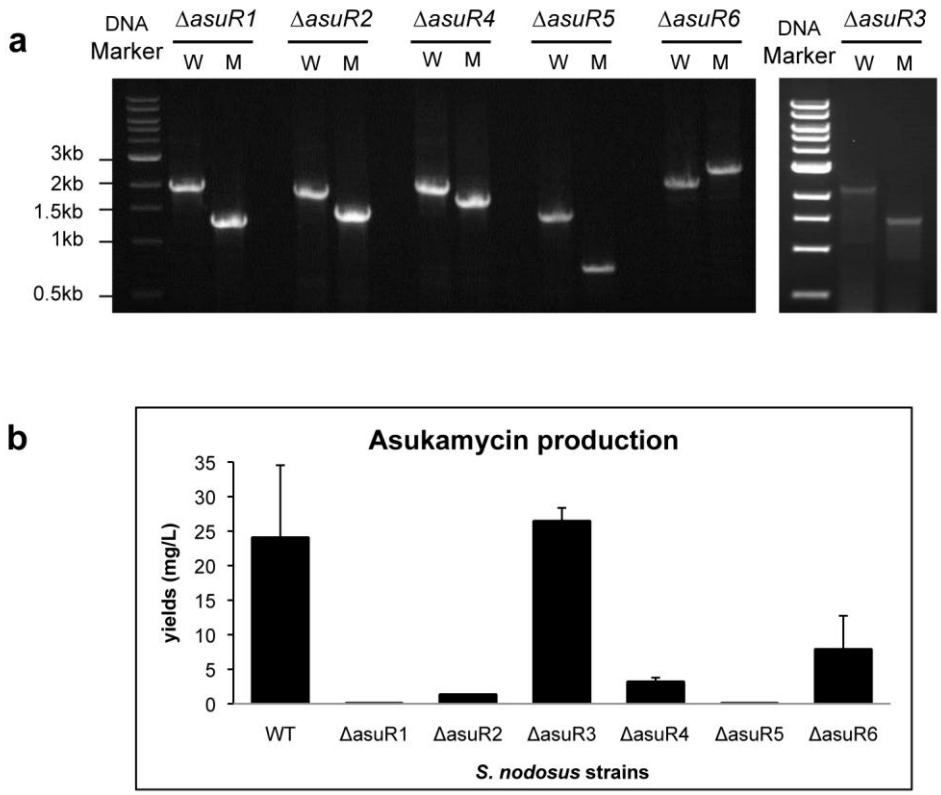

a
DNA Marker
ΔasuR1
ΔasuR2
ΔasuR4
ΔasuR5
ΔasuR6
DNA Marker
ΔasuR3
W M
3kb
2kb
1.5kb
1kb
0.5kb
b
Asukamycin production
yields (mg/L)
35
30
25
20
15
10
5
0
WT
ΔasuR1
ΔasuR2
ΔasuR3
ΔasuR4
ΔasuR5
ΔasuR6
S. nodosus strains

Figure 3

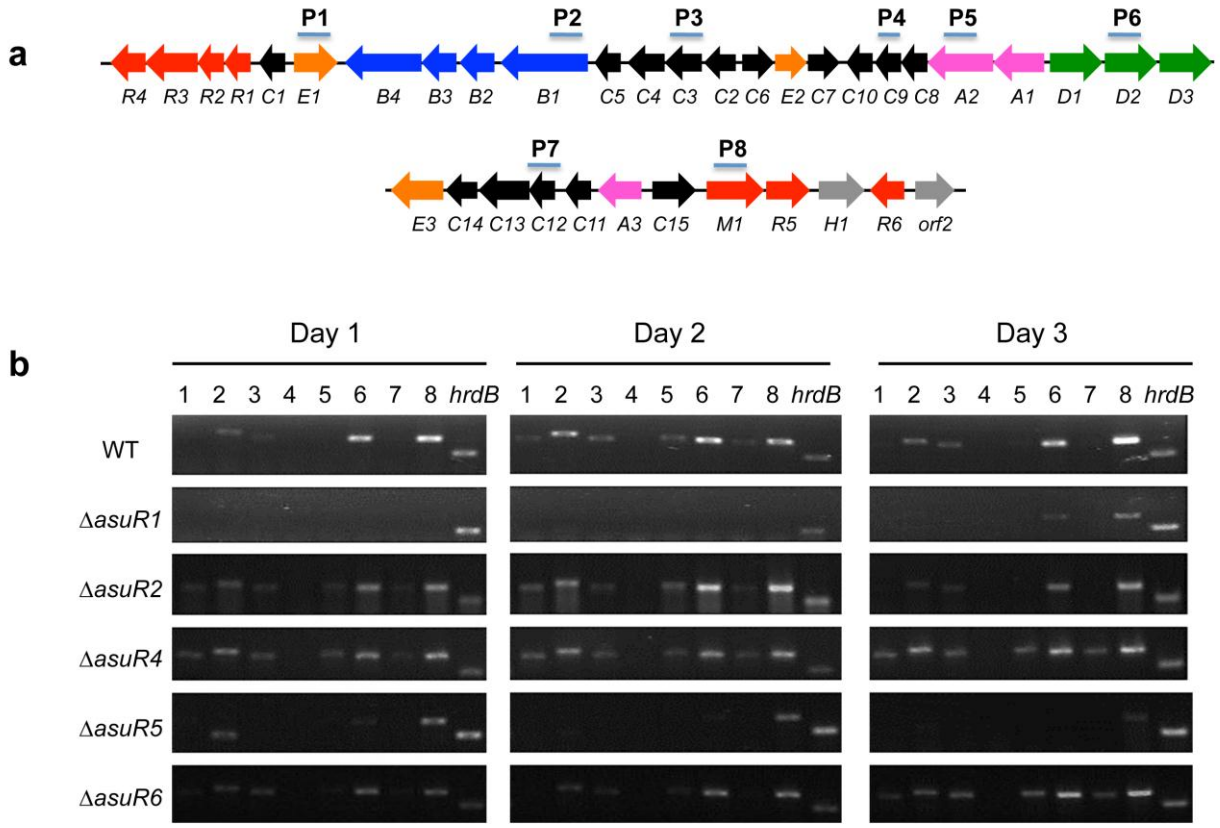

a
P1
P2
P3
P4
P5
P6
R4 R3 R2 R1 C1 E1 B4 B3 B2 B1 C5 C4 C3 C2 C6 E2 C7 C10 C9 C8 A2 A1 D1 D2 D3
P7
P8
E3 C14 C13 C12 C11 A3 C15 M1 R5 H1 R6 orf2
b
Day 1
Day 2
Day 3
1 2 3 4 5 6 7 8 hrdB
WT
ΔasuR1
ΔasuR2
ΔasuR4
ΔasuR5
ΔasuR6

Figure 4

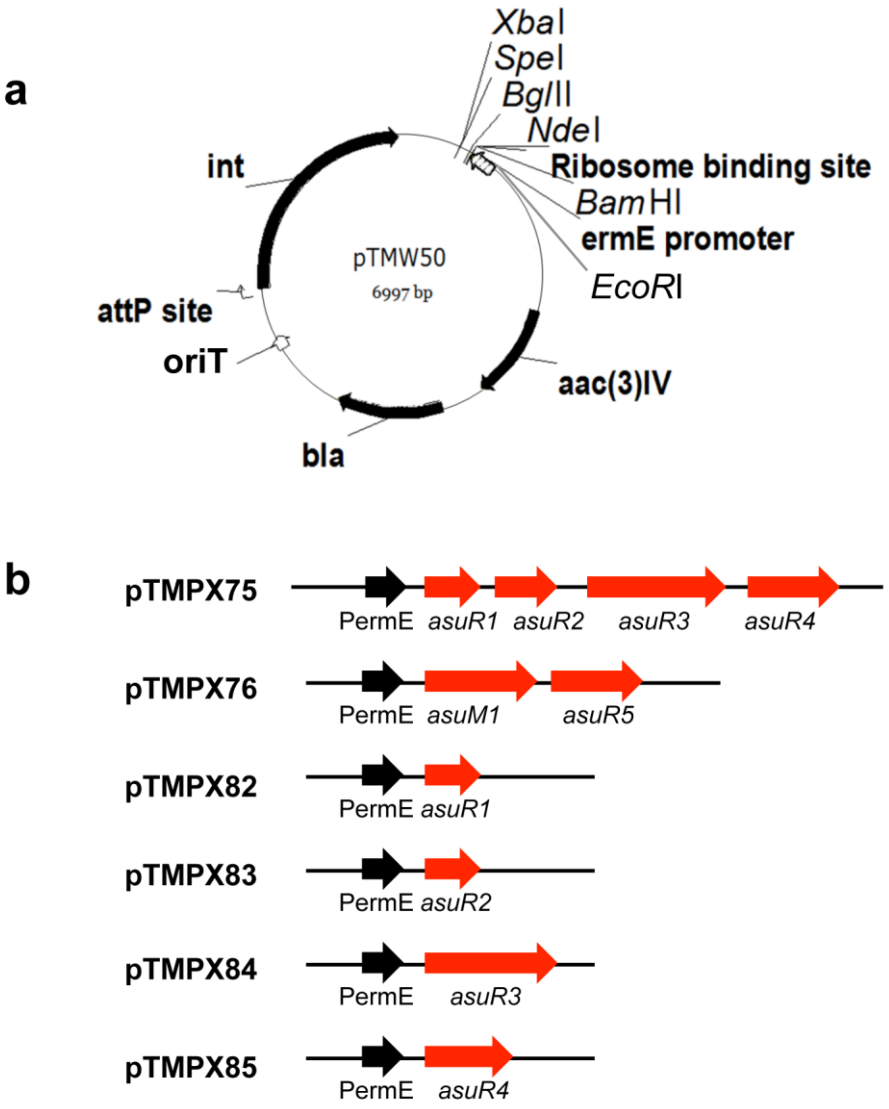

a
XbaI
SpeI
BglII
NdeI
Ribosome binding site
BamHI
ermE promoter
EcoRI
int
pTMW50
6997 bp
attP site
oriT
aac(3)IV
bla
b
pTMPX75
PermE asuR1 asuR2 asuR3 asuR4
pTMPX76
PermE asuM1 asuR5
pTMPX82
PermE asuR1
pTMPX83
PermE asuR2
pTMPX84
PermE asuR3
pTMPX85
PermE asuR4

Figure 5

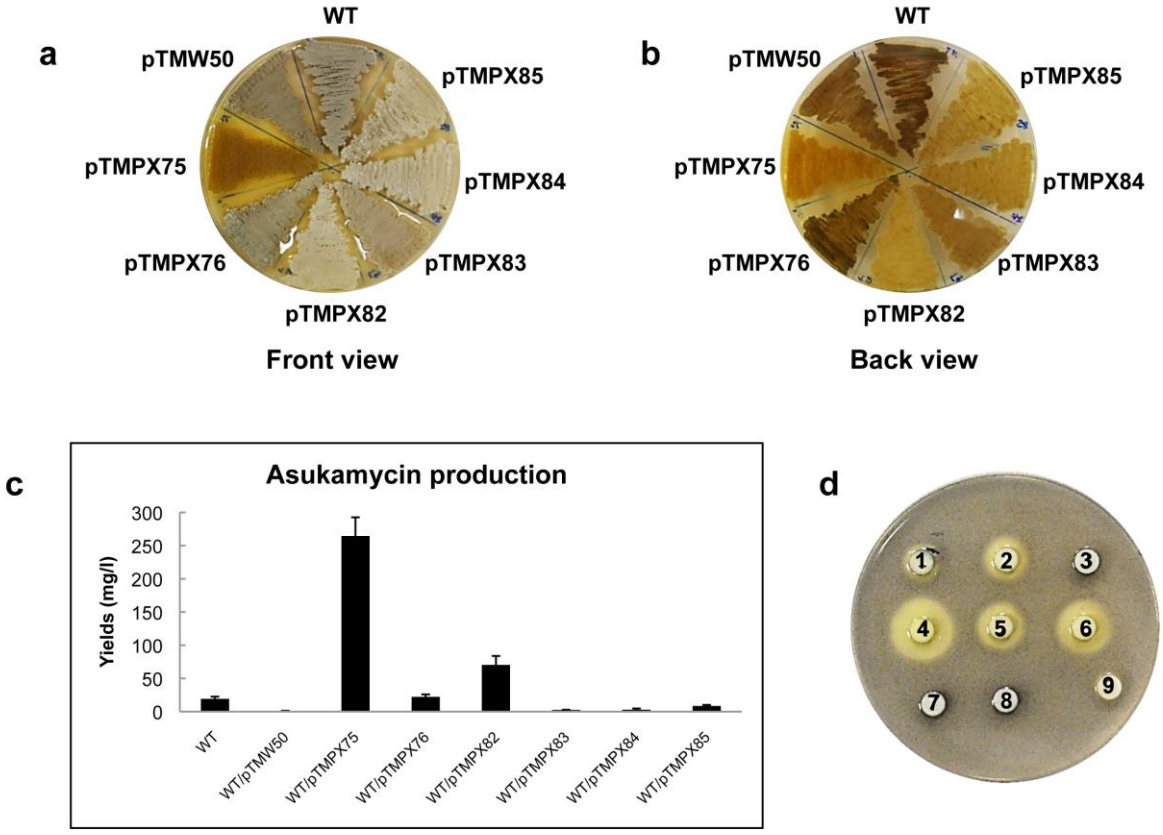

a
WT
pTMW50
pTMPX85
pTMPX75
pTMPX84
pTMPX76
pTMPX83
pTMPX82
Front view
b
WT
pTMW50
pTMPX85
pTMPX75
pTMPX84
pTMPX76
pTMPX83
pTMPX82
Back view
c
Asukamycin production
Yields (mg/l)
300
250
200
150
100
50
0
WT
WT/pTMW50
WT/pTMPX75
WT/pTMPX76
WT/pTMPX82
WT/pTMPX83
WT/pTMPX84
WT/pTMPX85
d
1
2
3
4
5
6
7
8
9

Figure 6