

A pathway-specific transcriptional regulatory gene for nikkomycin biosynthesis in *Streptomyces ansochromogenes* that also influences colony development

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Summary

DNA sequence analysis of a 7.5 kb *Xho*I DNA fragment from the region flanking the nikkomycin biosynthesis gene cluster in *Streptomyces ansochromogenes* revealed one 3.3 kb open reading frame (ORF), designated *sanG*. The deduced product of *sanG* (1061 amino acids), which is similar to PimR of *Streptomyces natalensis*, contains an OmpR-like DNA binding domain in its N-terminal portion and A- and B-type nucleotide binding motifs in the middle of the protein. Disruption of *sanG* abolished nikkomycin biosynthesis, reduced sporulation and led to brown pigment accumulation. All aspects of this complex phenotype were complemented by a single copy *sanG* which was integrated into the chromosome. The introduction of multiple copies of *sanG* resulted in increased nikkomycin production. S1 mapping results indicated that *sanG* is transcribed from at least three promoters (P1, P2 and P3), P1 being strongly upregulated when production of nikkomycins starts. Two putative transcription units for nikkomycin biosynthesis, starting from *sanN* and *sanO*, are dependent on the expression of *sanG*, whereas a putative transcription unit starting from *sanF* was not regulated by *sanG*. These results suggested that *sanG* encodes a transcriptional activator important for nikkomycin biosynthesis that, unusually, also has pleiotropic effects on secondary metabolism and development.

Introduction

Streptomycetes are filamentous soil bacteria with a complex life cycle. Spores germinate to form a substrate

mycelium (vegetative growth) that goes on to develop aerial hyphae, the tips of which form chains of spores. The production of a variety of secondary metabolites is considered to be closely coordinated with this morphological differentiation (Chater, 1993). In liquid medium, antibiotic production in *Streptomyces* is generally dependent on the growth phase, and involves the expression of clustered biosynthetic genes. Pathway-specific regulatory genes are typically located in these gene clusters (Martin and Liras, 1989). Expression of the pathway-specific regulatory genes is influenced by unlinked pleiotropic regulatory genes, some of which also control morphological differentiation (Ueda *et al.*, 2000).

Nikkomycins are a group of peptidyl nucleoside antibiotics that are structurally similar to the chitin synthase substrate UDP-*N*-acetylglucosamine (Fiedler *et al.*, 1982). These antibiotics show potent activity against phytopathogenic fungi and against human pathogens (Hector *et al.*, 1990). Nikkomycins are produced by both *Streptomyces ansochromogenes* (Chen *et al.*, 2000) and *Streptomyces tendae* (Brillinger, 1979). Their biosynthesis begins with the formation of the nucleoside moieties and the peptidyl moiety (aminohexuronic acid moiety) (Isono and Suzuki, 1979; Bormann *et al.*, 1989). The biosynthetic precursors of the nucleoside moieties of nikkomycin X and Z are ribose and histidine, or ribose and uracil with phosphoenolpyruvate (Isono *et al.*, 1978; Schuz *et al.*, 1992). The biosynthesis of the peptidyl moiety is thought to be similar to that of the related polyoxins, and begins with L-lysine (Bruntner and Bormann, 1998). Molecular analysis of the gene cluster revealed that more than 20 genes are involved in nikkomycin biosynthesis in *S. ansochromogenes* (Chen *et al.*, 2000; Li *et al.*, 2000; Zeng *et al.*, 2002). However, no report has been published so far on the regulation of nikkomycin biosynthesis. A better understanding of the molecular regulation mechanisms is of utmost interest in both academic research and industrial applications, as it will contribute insights into the fundamental issue of temporal regulation of differentiation and secondary metabolism in *Streptomyces* and the construction of overproducing strains.

In this study, physical and functional analysis of a 7.5 kb DNA fragment located downstream of *sanV* (Li and Tan,

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2003) revealed one antibiotic pathway-specific regulatory gene in *Streptomyces*.

Results

The sanG gene product resembles positive regulators involved in antibiotic biosynthesis

A 2.0 kb *XhoI*-*Bam*HI fragment containing *sanV* gene was used as a probe to identify a recombinant cosmid, COS19, which contains 18 kb of the nikkomycin biosynthesis gene cluster plus about 10 kb downstream of *sanV*. A 7.5 kb *XhoI* DNA fragment containing *sanV* and the downstream DNA was inserted into the *XhoI* site of pBlue-script KS⁺ to generate pGL101 (Fig. 1).

The cloned fragment was sequenced. It included one large open reading frame (ORF) designated *sanG* (Fig. 1). *sanG* contained 3186 nucleotides and started with an ATG codon at position 3171 of the sequenced fragment, preceded immediately upstream by a possible ribosome-binding site. *sanG* was separated from the near-

est upstream gene (a diverging gene, *orf3*) by about 2 kb of apparently non-coding DNA. The overall G + C content of the ORF was about 73.4%, which is typical in the genes of *Streptomyces* (Bibb *et al.*, 1984). The deduced *SanG* protein consists of 1061 amino acids and has a relative mass of 116 kDa.

In searches of databases, the deduced protein (*SanG*) of *sanG* showed end-to-end similarity to several proteins: 89% identity amino acids with the deduced product of *orfR* (nucleotide sequence accession number AJ250878), annotated as a putative nikkomycin regulatory gene from *S. tendae*; 32% identity with PimR of *Streptomyces natalensis* (Anton *et al.*, 2004) and 33% identity with PteR of *Streptomyces avermitilis* (Ikeda *et al.*, 2003). PimR is the positive regulator for pimarin biosynthesis and PteR is a putative regulatory protein involved in the pentane filipin biosynthesis. Like PimR, *SanG* is composed of two portions (Fig. 2A). Notably, the N-terminal portion of *SanG* showed significant sequence similarity to so-called *Streptomyces* antibiotic regulatory proteins (SARPs) (Wietzorrek and Bibb, 1997), containing one *trans*-Reg-C domain

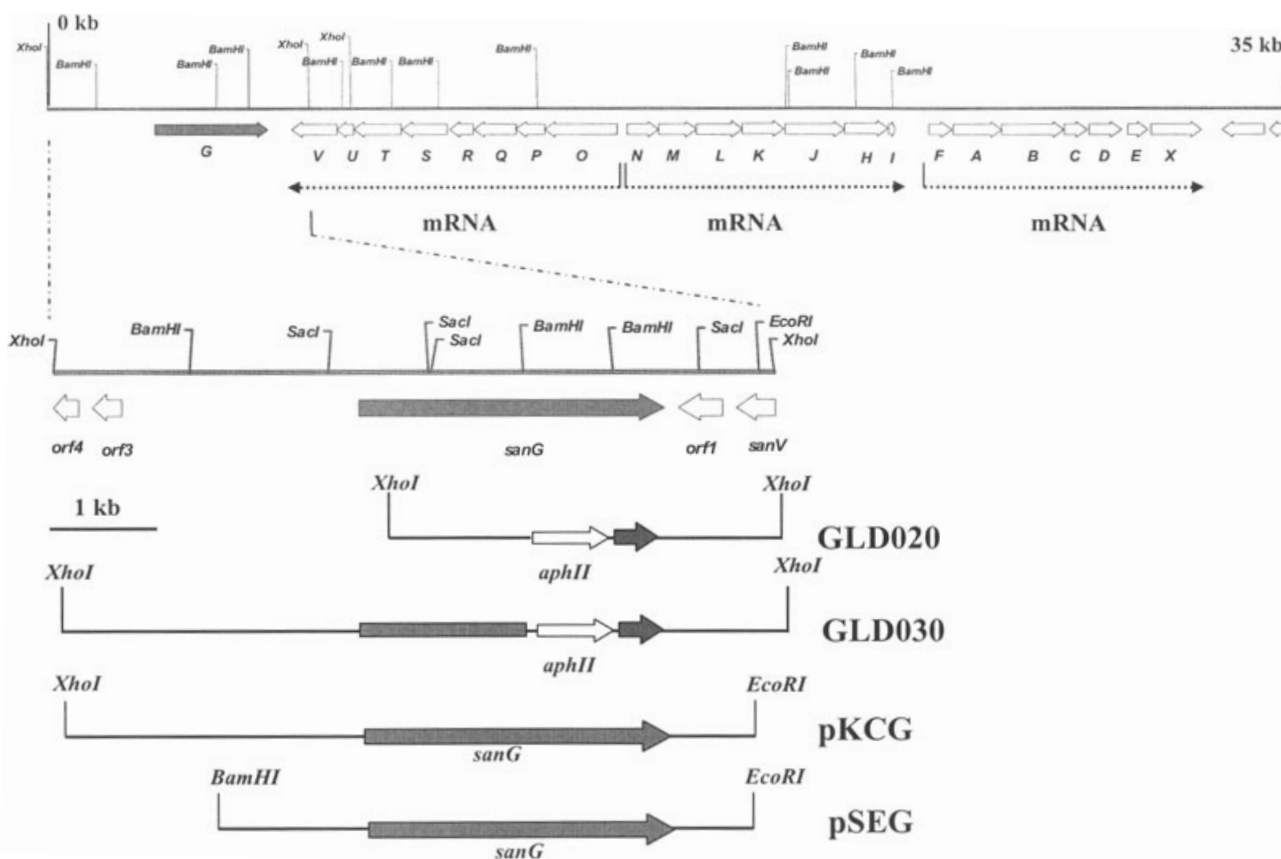


Fig. 1. Organization of the gene cluster for nikkomycin biosynthesis. The solid arrow shows *sanG* and its orientation. The dashed arrows indicate the different mRNAs transcribed by specific transcription units. The DNA fragment containing most of *sanG* and its promoter region was replaced by a kanamycin resistance gene (*aphII*) in GLD020. The 0.9 kb *Bam*HI fragment in *sanG* was replaced by *aphII* in GLD030. A 7.3 kb *XhoI*-*Eco*RI DNA fragment was inserted into the same sites of pK1139 to generate pKCG, and a 5.1 kb *Bam*HI-*Eco*RI DNA fragment was inserted into the same sites of pSET152 to generate pSEG.

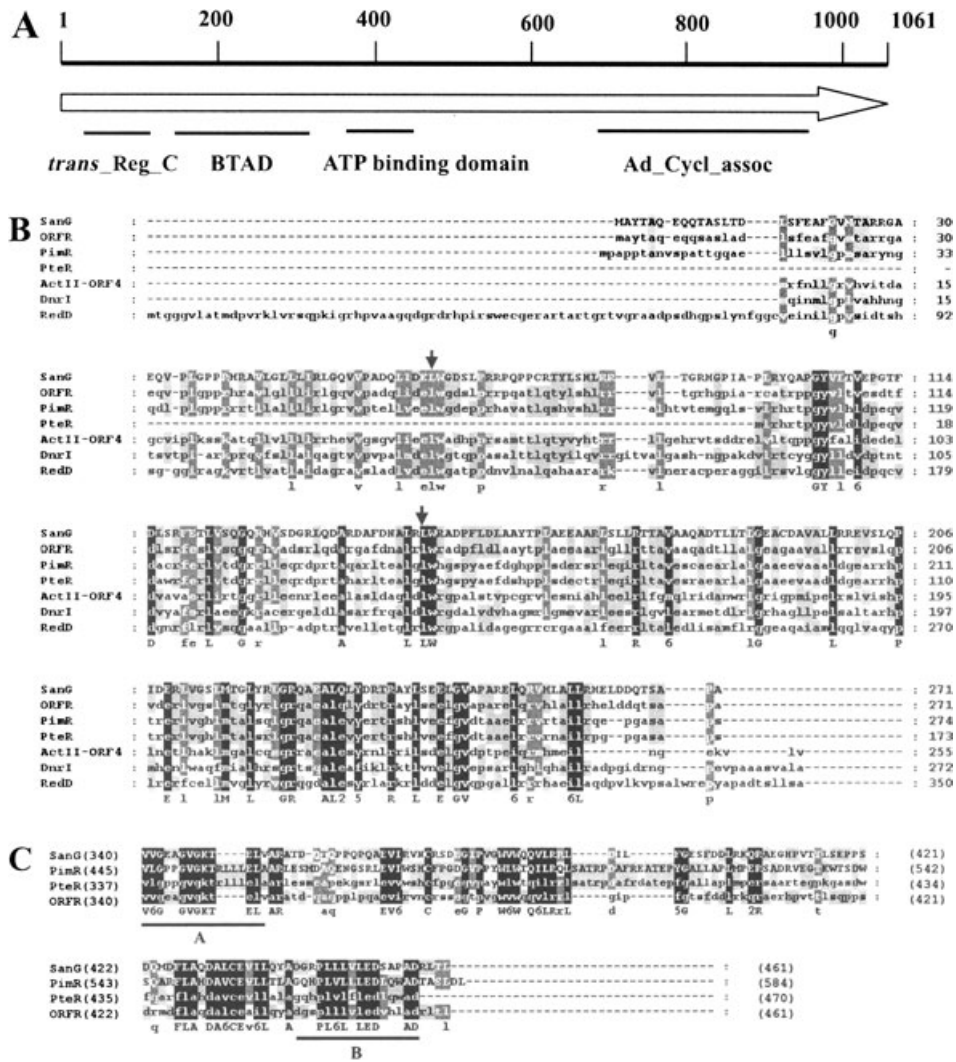


Fig. 2. Domain structure and amino acid alignment of parts of the SanG protein. A. The predicted domain structure of SanG. The N-terminal (*trans*-Reg-C) region resembles the DNA binding domain of OmpR; BATD, bacterial transcriptional activator domain; Ad_Cycl_assoc, Adenylate cyclase-associated domain. B. Alignment of the N-terminal portion of the SanG, ORFR, PimR, PteR, ActII-ORF4, DnrI and RedD proteins. ORFR, the deduced product of *orfR* (AJ250878) from *S. tendae*; PimR, Pimaricin biosynthesis regulator from *S. natalensis*; PteR, a putative regulatory protein from *S. avermitilis*; ActII-ORF4, a transcriptional activator for actinorhodin biosynthesis of *S. coelicolor*; DnrI, a transcriptional activator for daunorubicin biosynthesis of *S. peuceitius*; RedD, a transcriptional activator for undecylprodigiosin biosynthesis of *S. coelicolor*. The arrowheads over the alignment indicate the residues encoded by the rare TTA codon in *Streptomyces*. C. Comparison of the Walker A and B motifs of SanG with those of ORFR, PimR and PteR. Amino acid residues that are identical are shown in black, and similar residues are shaded.

(transcriptional regulatory protein, C terminal) and one BTAD domain (the bacterial transcriptional activator domain). The *trans*-Reg-C domain resembles the helix–turn–helix DNA binding domain at the C-terminus of the *Escherichia coli* activator OmpR (Martinez–Hackert and Stock, 1997). Thus, this region of SanG showed 32% identity to ActII-ORF4 of *Streptomyces coelicolor* (Fernandez-Moreno *et al.*, 1991), 31% identity to DnrI of *Streptomyces peuceitius* (Madduri and Hutchinson, 1995) and 31% identity to RedD of *S. coelicolor* (Narva and Feitelson, 1990) (Fig. 2B). The C-terminal portion of SanG

showed the characteristics of ATPases associated with diverse cellular activities, and contains an ATP/GTP-binding domain with Walker A and B motifs (Fig. 2C). It showed high similarity to several regulators of the LuxR-family, including a putative transcriptional regulator PteR (32% identity) in *S. avermitilis* (Ikeda *et al.*, 2003).

Interestingly, two leucines in the N-terminal portion of SanG were encoded by the rare TTA codon. TTA codons are also found in some other SARP genes including ActII-ORF4 (Fernandez-Moreno *et al.*, 1991). This suggests that the translation of *sanG* is controlled by *bldA* (Leskiw

et al., 1991; White and Bibb, 1997), which determines the tRNA for this codon.

Deletion mutants of sanG have lost the ability to produce nikkomycin

In order to identify the function of *sanG*, disruption mutants were constructed via homologous recombination. *sanG* was completely replaced in the *S. ansochromogenes* chromosome by a kanamycin resistance gene (*aph II*) as described in *Experimental procedures*. Seven individual *sanG* disruption mutants were selected randomly and confirmed by restriction digestion and Southern hybridization. To assess nikkomycin production, duplicate cultures from the same time-course experiments were subjected to bioassay against *Alternaria longipes*. Culture filtrates from the wild-type strain after 24 h incubation showed clear inhibition zones, whereas no inhibition was shown by culture filtrates of the *sanG* deletion mutant (Fig. 3). High-performance liquid chromatography (HPLC) analysis revealed no peaks of nikkomycin X and Z in culture filtrates of *sanG* deletion mutants (Fig. 4), in contrast to the culture filtrates of the wild-type strain, even though the biomasses were similar. These results suggested that *sanG* plays an important role in nikkomycin biosynthesis.

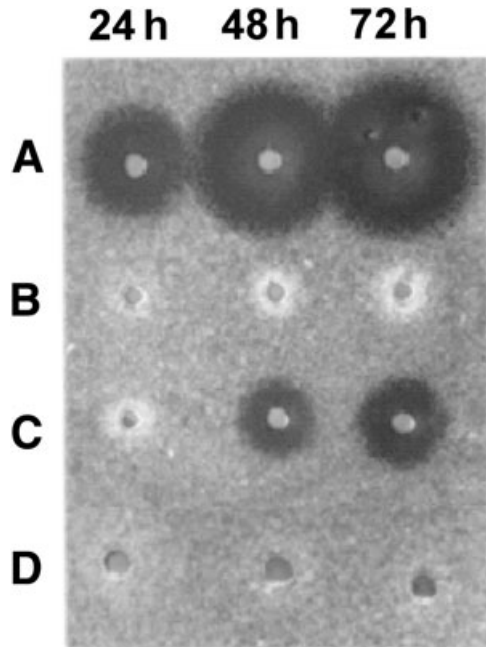


Fig. 3. Effect of *sanG* disruption on production of nikkomycin. Nikkomycin bioassay of fermentation filtrates with different incubation times and different strains. (A) *S. ansochromogenes* 7100; (B) *sanG* disruption mutant GLD020; (C) *sanG* complementation strain; (D) *sanG* truncation mutant GLD030. *Alternaria longipes* was used as the indicator strain.

Removal of the 3'-terminus of sanG abolishes nikkomycin production

Many SARPs lack extended C-terminal regions such as that found in SanG. To evaluate the importance of this region, a strain was constructed that contained a 3'-truncated copy of *sanG*. Thus, by homologous recombination, a 0.9 kb *Bam*HI internal fragment in the 3'-terminus of *sanG* gene was replaced by the *aph II* gene as described in *Experimental procedures*. Two individual *sanG* disruption mutants were selected randomly and confirmed by Southern blotting, and one (GLD030) was selected for further study. The growth of GLD030 was similar to that of the wild-type strain. When supernatants from SP medium cultures at different times during growth were analysed for nikkomycin production by bioassay (Fig. 3) or quantified by comparing peak areas obtained following HPLC analysis (Fig. 4), the wild-type strain showed production of nikkomycin X and Z, but the truncation mutant GLD030 did not even after 168 h (data not shown), indicating that the 3'-terminus is essential for the function of *sanG*.

sanG complementation restores nikkomycin production to a sanG disruption mutant

All seven *sanG* replacement mutants and both truncation mutants were nikkomycin-deficient, and there are no downstream genes potentially co-transcribed with *sanG* that could be subject to polar effects, making it highly probable that SanG deficiency was responsible for the mutant phenotypes. To confirm that the disruption of *sanG* was responsible for the abolition of nikkomycin production, a 5.1 kb DNA fragment containing *sanG* and its promoters was reintroduced into the *sanG* disruption mutant GLD020 on pSET152, a vector that integrates at the ϕ C31 prophage attachment site in the chromosome. The recombinant strain restored nikkomycin production in the SP medium, albeit at a very low level (Fig. 3). These results showed that *sanG* is essential for nikkomycin biosynthesis, and suggest that the location of *sanG* in the chromosome may affect the function of *sanG* significantly.

Pleiotropic effects of sanG on colonial morphology

Mutants of the GLD020 deleted for *sanG* formed colonies that grew at the same rate as the wild-type strain and formed aerial mycelium at the same time. However, the mutant colonies showed little of the grey pigmentation which was associated with sporulation in wild-type strain, even after prolonged incubation for 7–10 days on MM solid medium with either mannitol or glucose as the carbon source. The reduction of their spore numbers were observed by using phase-contrast microscopy. Meanwhile, they produced a large number of diffusible brown pigment in contrast to the wild-type strain, which produced

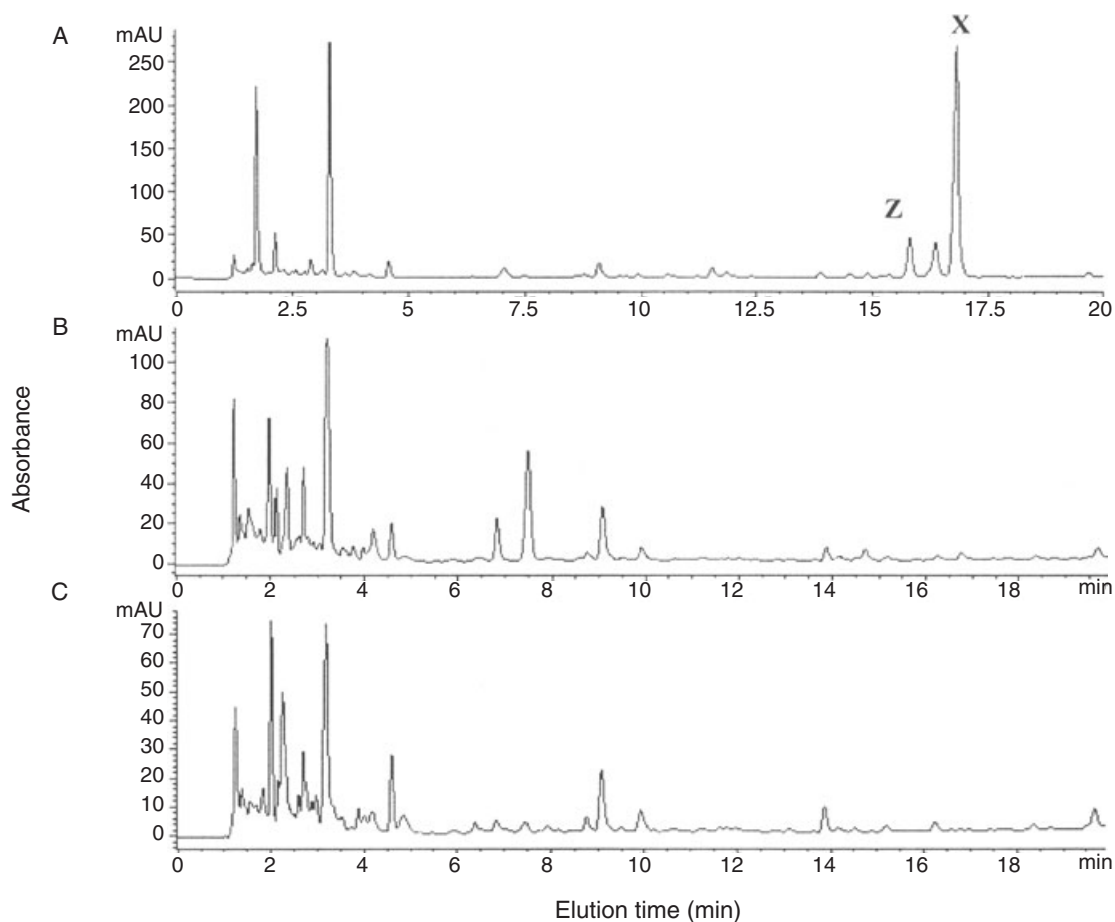


Fig. 4. Fermentation products from specifically disrupted mutants and the wild-type strain. HPLC analysis of culture filtrates from (A) wild-type strain, (B) *sanG* deletion mutant GLD020, and (C) *sanG* truncation mutant GLD030. X, nikkomycin X; Z, nikkomycin Z.

a small amount of brown pigment. As a control, a *sanN* disruption mutant constructed exactly in the same way displayed the phenotype of the wild-type strain (H. Tan, unpublished). In complementation experiments, the cloned fragment containing *sanG* and its promoter region rescued the morphological deficiency in these mutants and also resulted in loss of the brown pigment (Fig. 5). These results suggested that *sanG* has pleiotropic functions for both nikkomycin biosynthesis and morphological differentiation in *S. ansochromogenes*.

It is interesting that 3'-truncation of *sanG* did not change the morphology or pigmentation of *S. ansochromogenes* (data not shown), even though this mutation eliminated nikkomycin production. This indicated that sporulation and pigmentation were only affected by the N-terminal region of SanG.

Increasing the copy number of sanG results in increased nikkomycin production

In many species of *Streptomyces*, antibiotic biosynthesis is precisely controlled by regulatory proteins, especially

by transcriptional activators. Overexpression of these transcriptional activators is often associated with a concomitant increase in titres of the corresponding antibiotics. When pKCG (containing *sanG* on the multicopy vector pKC1139) was introduced into the wild-type strain, nikkomycin production was increased, even though the biomass of the transformants was similar to that of the wild-type strain (Fig. 6). This result reinforced the evidence that *sanG* is an important activator gene for nikkomycin production.

Three transcription start points are located unusually far upstream of sanG

To determine the transcription start point of *sanG*, S1 nuclease protection assay was performed as described in *Experimental procedures*. Initial attempts with probes covering the region close to the coding region revealed only full-length protection making it necessary to investigate regions further upstream. In the end, three signals were detected (Fig. 7A). The transcription start points of the corresponding promoters *sanG*-P1, *sanG*-P2 and *sanG*-P3, were localized at the nucleotides C (1016 base posi-

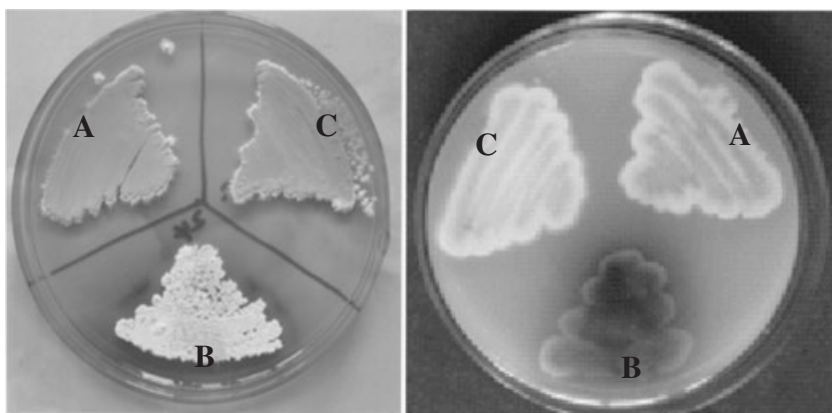


Fig. 5. Effect of *sanG* disruption on morphological differentiation. (A) *S. ansochromogenes* 7100; (B) *sanG* disruption mutant GLD020; (C) *sanG* complementation strain. Note that the phenotype of GLD020 mutant is much lighter than the wild-type and the complementation strain, and shows more diffusible brown pigment on the reverse side of the plate.

tion), A (1064 base position) and G (1182 base position) in the middle of the long non-coding region separating *sanG* from *orf3* and about 1 kb upstream of the *sanG* translation start codon (Fig. 7B).

The temporal regulation of these three signals was not the same, *sanG*-P3 transcript being rather constant throughout the time-course, while *sanG*-P1 was strongly induced when the strain started to produce nikkomycin. *sanG*-P2 had a similar profile to that of *sanG*-P1, but was much weaker (Fig. 7A). After the start of nikkomycin production, the transcripts of *sanG* from P1 and P2 declined significantly. As a control, the transcript of the *hrdB*-like gene (nucleotide sequence accession number AY628703) expected to encode the principal sigma factor of *S. ansochromogenes* was essentially constant during the time-course (Fig. 7A).

SanG as a transcriptional activator for nikkomycin biosynthetic genes

As the deduced product of *sanG* showed similarities to pathway-specific regulators of the SARP family, SanG was expected to act as a transcriptional activator of the nikkomycin biosynthetic genes. We therefore evaluated the effects of *sanG* deletion on promoters of the three putative transcription units encoding the enzymes of nikkomycin biosynthesis including the *sanO*, *sanN* (Wang *et al.*, 2003) and *sanF* promoters (G. Liu, unpublished). S1 mapping experiments were carried out according to the method of Kieser *et al.* (2000).

Transcripts from the divergently oriented promoters in the *sanN*–*sanO* intergenic region (Lauer *et al.*, 2001; Wang *et al.*, 2003) were abolished in the *sanG* mutant, but surprisingly, the *sanF* transcript was found in both the wild-type strain and *sanG* disruption mutants (Fig. 8). These results confirmed the regulatory importance of SanG, but also showed that there are differences in the transcriptional requirements of different transcription units in the *san* gene cluster.

Discussion

So far as we know, the nikkomycin biosynthesis genes are located in three putative transcription units in which *sanN*, *sanO* and *sanF* are the respective first genes. Pathway-specific regulatory genes are usually clustered with the antibiotic biosynthetic genes. In this study, a large pathway-specific regulatory gene, *sanG*, was found next to the nikkomycin biosynthetic genes. The deduced product of

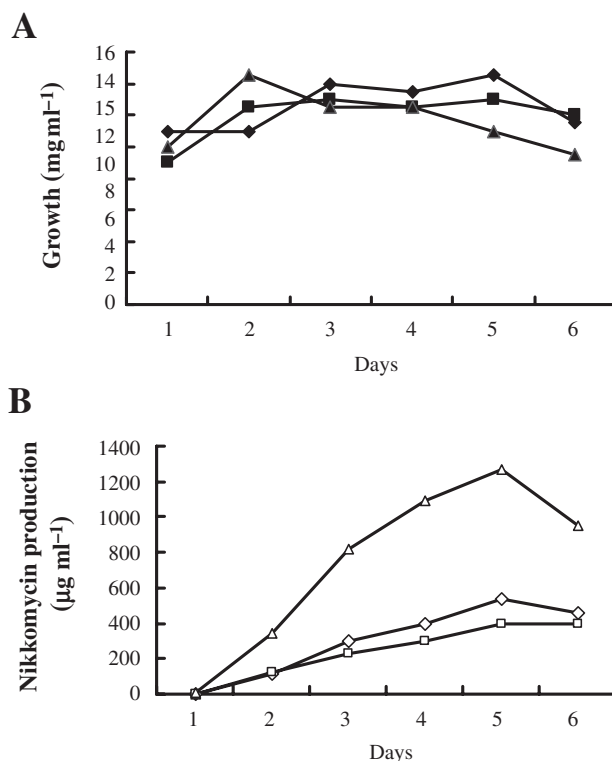


Fig. 6. Growth and nikkomycin production of *S. ansochromogenes* containing *sanG* on a multicopy plasmid. Growth curves: (◆) wild-type strain; (■) wild-type strain with pKC1139; (▲) transformants with pKCG. Nikkomycin production: (◇) wild-type strain; (□) wild-type strain with pKC1139; (△) transformants with pKCG.

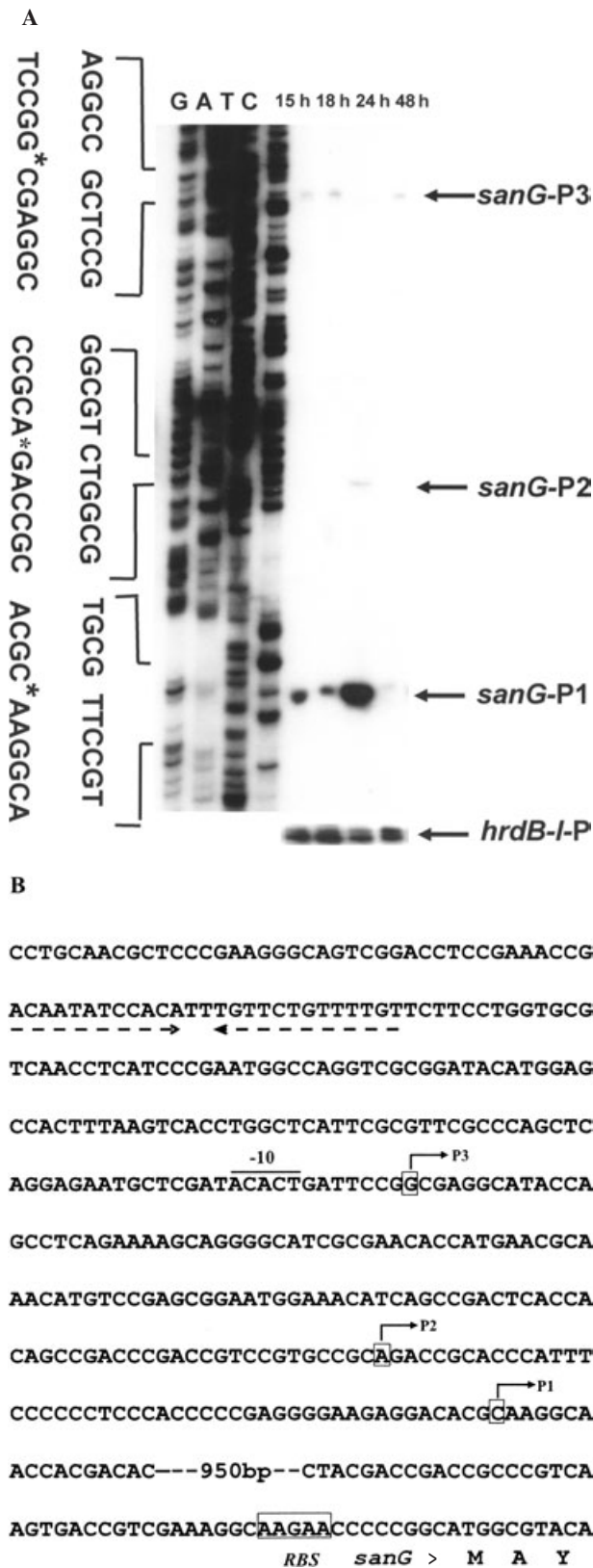


Fig. 7. Transcriptional analysis of *sanG*.

A. High resolution S1 nuclease mapping of *sanG*. Three *sanG* transcripts P1, P2 and P3 were detected at 15, 18, 24 and 48 h in the wild-type strain. The arrowheads indicate the transcription start points. The *S. ansochromogenes* principal sigma factor gene (*hrdB-I*) (AY628703) was used as an internal control.

B. The nucleotide sequence covering the promoter region of *sanG* is shown, together with its N-terminal amino acid sequence.

sanG showed end-to-end similarity to the pimaricin biosynthetic regulator PimR in *S. natalensis* and the putative biosynthetic regulator PteR involved in filipin biosynthesis in *S. avermitilis*. Pimaricin and filipin belong to the polyene antibiotics, but nikkomycins are peptidyl nucleoside antibiotics. Although their structures and biosynthesis pathways are different, all of them can be used against fungi. As the regulators showed high similarities, the regulation mechanisms may be similar. It seems plausible that these regulators might have been acquired in the process of evolution against fungi. Very few is known about the regulation of pimaricin and filipin biosynthesis. In this paper, we showed that SanG regulates nikkomycin production by controlling the transcription of the *sanO* and *sanN* operons, but interestingly, does not seem to be needed for expression of the *sanF* operon.

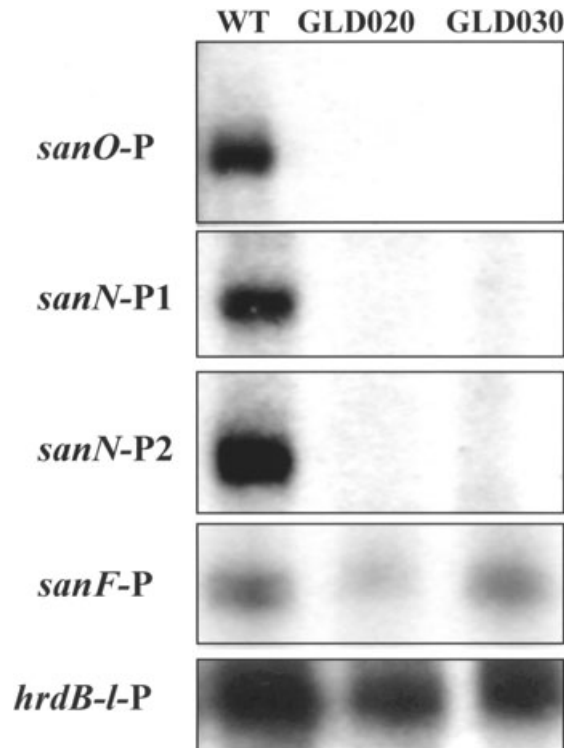


Fig. 8. Transcriptions of *sanN*, *sanO* and *sanF* genes in *S. ansochromogenes* wild-type strain (wt), *sanG* disruption mutants GLD020 and GLD030. *S. ansochromogenes hrdB-I* was used as an internal control. RNAs were extracted from strains for 48 h incubation in SP medium.

Translation of sanG may be controlled by the bldA gene

In *S. coelicolor*, mutation of *bldA*, which encodes the tRNA for the rare leucine codon UUA, causes pleiotropic deficiencies in both morphological differentiation and antibiotic production on some media. *bldA* mutants are generally unable to translate UUA codon-containing mRNA. A TTA codon of the *adpA* gene is the principal target through which *bldA* influences morphological differentiation of *S. coelicolor* (Takano *et al.*, 2003); and a TTA codon has been proved to be involved in translational control of *actII-ORF4*, the pathway-specific regulatory gene for actinorhodin biosynthesis in *S. coelicolor* (Fernandez-Moreno *et al.*, 1991). Genes controlling undecylprodigiosin biosynthesis and methylenomycin biosynthesis also contain TTA codons (White and Bibb, 1997; S. O'Rourke, unpublished). Phenotypically similar *bldA* mutants have also been found in *S. griseus* and may be widespread in actinomycetes (Leskiw *et al.*, 1991). We also found that some bald mutants of *S. ansochromogenes* have lost nikkomycin production (G. Liu, unpublished). The presence of two rare TTA codons in *sanG* suggested that they may be targets through which *bldA* influences nikkomycin biosynthesis. However, the TTA-containing *ccaR* gene for clavulanic acid and cephamycin C biosynthesis can be translated efficiently in a *bldA* mutant of *Streptomyces clavuligerus* (Trepanier *et al.*, 2002). Thus, the translational regulation by *bldA* is complex in *Streptomyces*.

Transcription of sanG is growth phase-regulated

Antibiotic production is generally dependent on growth phase. Nikkomycin was first detected after *S. ansochromogenes* was cultured in SP medium for 24 h. In *S. tendae*, transcriptions of *nikA-G* and *nikP1-V* genes reached the maximal level at stationary phase after 25 h incubation (Bruntner *et al.*, 1999; Lauer *et al.*, 2001). Our data showed that *sanG* was regulated at the level of transcription during *S. ansochromogenes* growth in SP medium and *sanG*-P1 exhibited the strongest signal for

24 h incubation and then reduced quickly with further incubation, indicating that transcription of *sanG* gene is growth phase-regulated, but what kinds of factors triggered metabolic changes are still unknown.

sanN and sanO genes are controlled by SanG

The N-terminal portion amino acid sequence of the SanG has a high similarity with proteins of SARP family, such as DnrI (Madduri and Hutchinson, 1995), ActII-ORF4 (Fernandez-Moreno *et al.*, 1991) and RedD (Narva and Feitelson, 1990). DnrI binds to promoters of the daunorubicin biosynthesis genes (Tang *et al.*, 1996) and ActII-ORF4 target sites were also found in the *act* cluster (Arias *et al.*, 1999). It is reasonable to predict that they may maintain the precise control of antibiotic biosynthesis by a similar mechanism of DNA binding and transcriptional activation. As transcriptions of *sanN* and *sanO* genes depending on the expression of *sanG*, their promoter regions may contain binding sites for SanG. It has been recognized that proteins of SARP family contain an OmpR-like DNA binding domain which binds to direct repeat sequences within the regulatory region of target genes (Sheldon *et al.*, 2002). As expected, an intergenic region between the *sanN* and the *sanO* genes carries the divergently oriented promoters and contains two intact sets of tandem five-base pair repeat sequences of the kind proposed specific region (Wang *et al.*, 2003) for DNA recognition and binding by SARPs (Fig. 9). Meanwhile, the promoter region of *sanF* gene which transcribes independently does not contain the similar tandem repeat sequence. So the tandem repeat sequences in *sanN*–*sanO* intergenic region are candidates for such binding sites by SanG.

How might SanG activity be regulated?

Analysis of the nucleotide sequence of *sanG* promoter region revealed an A-T rich region (5'-GACAATATCCAC ATTTGTTCTGTTTTGTT-3'), which are partially palindromic with A and T rows at ends and share several highly



Fig. 9. The intergenic region between the *sanN-I* and *sanO-V* operons within the nikkomycin biosynthesis gene cluster. The arrowheads indicate the transcription start points of *sanN* and *sanO*. The boxes showed tandem five-base pair repeat sequences preceding the promoters of *sanN* and *sanO*.

conserved residues of the experimentally verified autoregulatory elements (AREs), including *scbR* (Takano *et al.*, 2001), *ccaR* (Pérez-Llarena *et al.*, 1997), *spbR* (Folcher *et al.*, 2001) and *sabR* (Li *et al.*, 2003). In *Streptomyces*, γ -butyrolactone autoregulators and the corresponding receptors play a crucial role in controlling the production of antibiotics and the morphological differentiation (Chater and Horinouchi, 2003). In *Streptomyces pristinaespiralis*, the pristinamycin biosynthetic pathway-specific regulator *PapR1* was regulated by a butyrolactone receptor *SpbR*. In our previous studies, *SabR*, a γ -Butyrolactone receptor homologous gene, was identified in *S. ansochromogenes* (Li *et al.*, 2003), and nikkomycin biosynthesis was delayed conditionally in the *sabR* disruption mutant. These suggested that *sabR* positively controls nikkomycin production. Thus, *sanG* promoter region may be potentially the ultimate target for DNA binding by the *SabR* protein.

A developmental role for SanG

Remarkably, the complete elimination of SanG resulted in a markedly reduced efficiency of sporulation, and in the appearance of an uncharacterized brown pigment (Fig. 5). These aspects of the mutant phenotype were complemented by the introduction of a wild-type copy of *sanG*, and are therefore verified to be causally associated with the mutant locus. We are not aware of any other example in the literature of such a pleiotropic effect of a pathway-specific regulator of antibiotic biosynthesis, though there are numerous examples of mutations in genes unlinked to pathway gene sets that have effects on both secondary metabolism and development (notably, most of the so-called *bld* mutants: Chater, 2001). This effect is unlikely to be caused indirectly through the switching off of nikkomycin production, because the loss of nikkomycin production through a mutation in a biosynthetic gene *sanN* (which encodes acetyl dehydrogenases involved in nikkomycin biosynthesis) did not have the same pleiotropic effects. Moreover, the deletion of the C-terminal domain of SanG eliminated nikkomycin biosynthesis, but did not have any associated pigmentation or morphological phenotype. Thus, it seems that the region including the SARP domain influences multiple distinct characteristics, whereas the C-terminal domain, which contains a nucleotide binding motif, is needed only for the regulation of nikkomycin biosynthesis. The interesting fact that the pathway-specific regulators of the biosynthetic pathways of two other antifungal antibiotics (but in those cases, polyene compounds) have end-to-end similarity to SanG raises the question of whether all three producing organisms use such regulators to permit them to respond to particular environmental circumstances, associated with fungal competitors, by both producing the antifungal antibiotics and by sporulating efficiently.

Experimental procedures

Strains, plasmids and growth conditions

Streptomyces ansochromogenes 7100 (wild-type strain), a nikkomycin producer, was grown at 28°C and also as a host strain for gene propagation and gene disruption. Sporulation was achieved on minimal medium (MM) using mannitol as sole carbon source. For nikkomycin production, SP medium (3% mannitol, 1% soluble starch, 0.75% yeast extract, and 0.5% soy peptone, pH 6.0) was used as described previously (Zeng *et al.*, 2002). *Streptomyces* liquid medium YEME and solid medium R2YE were prepared as described (Hopwood *et al.*, 1985) and used for the regeneration of protoplasts and for the selection of the transformants. For routine subcloning, *E. coli* DH5 α and JM109 were grown at 37°C in Luria–Bertani (LB) medium containing ampicillin or apramycin when necessary for propagating plasmids. *E. coli* ET12567 (*dam dcm hsdS*) was used to propagate non-methylated DNA when it was to be introduced into *S. ansochromogenes* (MacNeil *et al.*, 1992). *E. coli* ET12567 (pUZ8002) was used for conjugal transfer of DNA from *E. coli* to *Streptomyces* (Paget *et al.*, 1999).

Plasmids pBluescript KS⁺ (Stratagene) and pUC19 were used for routine cloning and subcloning experiments. Cosmid, COS19 containing part of the nikkomycin biosynthetic gene cluster was used to isolate *sanG* (Li *et al.*, 2000). pSET152 (Bierman *et al.*, 1992), which can integrate into the *Streptomyces* chromosome by site-specific recombination at the bacteriophage ϕ C31 attachment site (*attB*) (Kuhstoss and Rao, 1991), was used to introduce a single copy of *sanG* into *S. ansochromogenes*. *E. coli*–*Streptomyces* shuttle vector pKC1139, which contains a *Streptomyces* temperature-sensitive origin of replication, was used for gene expression and gene disruption, as it can be used efficiently in gene replacement by homologous recombination at the non-permissive temperature (Kieser *et al.*, 2000).

DNA manipulation and sequencing

Plasmid and chromosomal DNA were isolated according to the standard techniques from *Streptomyces* (Kieser *et al.*, 2000) or *E. coli* (Sambrook *et al.*, 1989). Preparation of protoplasts and transformation of *S. ansochromogenes* were performed as described previously (Li *et al.*, 2003). Intergenic conjugation from *E. coli* ET12567 (pUZ8002) to *S. ansochromogenes* was carried out as described previously (Kieser *et al.*, 2000). Southern hybridization was taken place with probes labelled with a digoxigenin DNA labelling kit (Roche Biochemicals).

DNA sequencing was performed by Takara Biotechnology Cooperation (Dalian, China). Database searching and sequence analysis were made using Artemis program (Sanger, UK), FramePlot 2.3 (Ishikawa and Hotta, 1999) and the program PSI-BLAST (Altschul *et al.*, 1997).

Construction of *sanG* disruption mutants

To construct *sanG* disruption mutants, a 7.5 kb *XhoI* DNA fragment containing *sanG* was cloned into the *SalI* site of a

pUC18 derivative from which the *Bam*HI site had been deleted. The resulting plasmid was digested with *Bam*HI, and the largest fragment containing the vector and the flanking DNA fragment of *sanG* was recovered after agarose gel electrophoresis and self-ligated to yield pGLD018, it was subsequently digested with *Bam*HI and ligated with the *Bam*HI fragment containing the kanamycin resistance cassette (*aph II*) from pUC119::KanR to generate pGLD019, in which *sanG* was replaced by *aph II*. A 4.3 kb insert of pGLD019 was isolated after *Hind*III and *Eco*RI digestion, and ligated into the same sites of pKC1139 to generate pGLD020. The same strategy was used to construct pGLD030, in which an internal 0.9 kb *Bam*HI fragment of *sanG* was replaced by *aph II*. Subsequently, pGLD020 and pGLD030 were introduced into *S. ansochromogenes*, and the transformants were confirmed by plasmid isolation and restriction digestion. The spores of transformant containing pGLD020 or pGLD030 were harvested and spread on agar MM containing kanamycin (Kan). After growing for 4 days at 39°C, colonies were replicated on MM containing apramycin (Apr). The *sanG* disruption mutants were selected by both apramycin sensitivity (Apr^s) and kanamycin resistance (Kan^r). Their chromosomal DNAs and wild-type DNA were isolated and digested with *Xho*I. Southern blotting experiments were performed using a 7.5 kb *Xho*I fragment containing *sanG* as a probe. A 7.5 kb hybridizing band was found for the wild-type strain, whereas a 4.3 kb positive signal was found for *sanG* disruption mutant GLD020. GLD030 was confirmed by the same way except that the chromosomal DNA was digested with *Xho*I-*Bam*HI. The 3.5 kb, 1.7 kb, 1.4 kb and 0.9 kb hybridizing bands were found for the wild-type strain, whereas only 6.1 kb and 1.4 kb positive signals were found for *sanG* disruption mutant GLD030 (Fig. 1). This result indicated that a double cross-over event had occurred.

Complementation of specifically disrupted strains

For complementation analysis, the integrative vector pSET152 was used. A 3.3 kb *Bam*HI fragment containing a partial *sanG* gene and its flanking DNA was isolated from pGL101 and inserted into pSET152 to give pSET152::3.3 kb, which was then digested with *Bgl*II and *Eco*RI, and subsequently ligated with a 4.1 kb *Bgl*II-*Eco*RI fragment containing a partial *sanG* gene from pGL101. The resulting recombinant plasmid, pSEG, contained *sanG* and its flanking DNA sequence together with the apramycin resistance gene, *aac3* (IV). Then, pSEG was integrated into the chromosomal *attB* site of *S. ansochromogenes* after conjugal transfer from *E. coli*.

Overexpression of *sanG*

A 7.5 kb *Xho*I DNA fragment carrying *sanG* from pGL101 was subcloned into the *Sal*I site of pUC19, and the resulting plasmid was digested with *Hind*III and *Eco*RI to give a 7.3 kb *Hind*III-*Eco*RI DNA fragment containing *sanG*. This was ligated into the same sites of pKC1139 to give pKCG, which was then introduced into *S. ansochromogenes* 7100.

Transcriptional analysis

To investigate transcription during nikkomycin biosynthesis, total RNA was isolated from strains grown in SP medium for different times. Mycelium was collected, frozen quickly in liquid nitrogen and ground into a fine white powder. Ground mycelial samples were frozen at -20°C until the time-course was completed. RNA was then extracted using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. S1 protection assays were performed using the *hrdB*-like gene (*hrdB-I*) (AY628703) probe as a control. The *hrdB-I* probe was generated by polymerase chain reaction (PCR) using the unlabelled oligonucleotide 5'-GGGTACGCC CCGTCAGTG-3' and the radiolabelled oligonucleotide 5'-AGCCTTTCCCGCTCAAT-3', which was uniquely labelled at its 5' end with [³²P]-ATP using T4 polynucleotide kinase. The probe to detect *sanG* transcripts was generated by PCR using the radiolabelled oligonucleotide 5'-CTTTCGTCACGG TCTCGGA-3'. The sequence ladder was made using an *fmo*I DNA cycle sequencing kit (Promega, USA) with the same labelled primer. For *sanO*, the probe was generated by PCR using the radiolabelled oligonucleotide 5'-CGACCTGGGCG GCGAACA-3' and the unlabelled oligonucleotide 5'-CGACG AGGGACTGGATGC-3'. For *sanN*, the probe was generated by PCR using the radiolabelled oligonucleotide 5'-CGACG AGGGACTGGATGC-3' and the unlabelled oligonucleotide 5'-CGACCTGGGCGGCGAACA-3'. For *sanF*, the probe was amplified using the radiolabelled oligonucleotide 5'-TACTG CTTCTCGTGCTTCGGGT-3' and the unlabelled oligonucleotide 5'-CGCGCAGGTCGGCCAGGT-3'.

Nikkomycin bioassay and HPLC analysis

Nikkomycins produced by *S. ansochromogenes* 7100 were measured by a disk agar diffusion method using *Alternaria longipes* as indicator strain. Nikkomycins in culture filtrates were identified by HPLC analysis. For HPLC analysis, Agilent 1100 HPLC and RP C-18 were used. The detection wavelength was 290 nm, the reference wavelength was 350 nm. Chemical reagent, mobile phase and gradient elution process were as described by Fiedler (1984).

Nucleotide sequence accession number

The nucleotide sequence of *sanG* determined in this study has been submitted to the GenBank database under accession number AY631852.

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