SanG, a transcriptional activator, controls nikkomycin biosynthesis through binding to the *sanN*–*sanO* intergenic region in *Streptomyces ansochromogenes*

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Streptomyces ansochromogenes SanG is a pathway-specific regulator that mainly controls the transcription of two transcriptional units involved in nikkomycin biosynthesis. SanG consists of three major functional domains: an N-terminal *Streptomyces* antibiotic regulatory protein (SARP) domain, a central ATPase domain, and a C-terminal half homologous to guanylate cyclases belonging to the LuxR family. SanG was expressed in *Escherichia coli* as a C-terminally His₆-tagged protein. The purified SanG-His₆ was shown to be a dimer in solution by dynamic light scattering. An electrophoretic mobility-shift assay showed that the purified SanG protein could bind to the DNA fragment containing the bidirectional *sanN*–*sanO* promoter region. The SanG-binding sites within the bidirectional *sanN*–*sanO* promoter region were determined by footprinting analysis and identified a consensus-directed repeat sequence 5'-CGGCAAG-3'. SanG showed significant ATPase/GTPase activity *in vitro*, and addition of ATP/GTP enhanced the affinity of SanG for target DNA, but ATP/GTP hydrolysis was not essential for SanG binding to the target DNA. However, real-time reverse transcription PCR showed that mutation of the ATPase/GTPase domain of SanG significantly decreased the transcriptional level of *sanN*–*I* and *sanO*–*V*. These results indicated that the ATPase/GTPase activity of SanG modulated the transcriptional activation of SanG target genes during nikkomycin biosynthesis.

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INTRODUCTION

Streptomyces species produce a wide variety of secondary metabolites, which are strictly coordinated with their producer's growth and environmental conditions (Bibb, 2005). Complex regulatory cascades link environmental and physiological signals through pleiotropic and pathway-specific regulatory proteins that control the expression of individual antibiotic gene clusters (Takano, 2006). Intracellular secondary messenger levels also modulate their production (Chater & Chandra, 2008). Thus, the regulation of antibiotic biosynthesis is complex and finely balanced.

Many pathway-specific regulatory proteins belong to the *Streptomyces* antibiotic regulatory proteins (SARPs), which bind to a direct repeat sequence overlapping with the –35 region of the target promoter (Tanaka *et al.*, 2007).

Abbreviations: EMSA, electrophoretic mobility-shift assay; SARP, *Streptomyces* antibiotic regulatory protein.

Members of the SARP family include ActII-Orf4, which regulates transcription of the actinorhodin biosynthetic genes in *Streptomyces coelicolor* (Fernández-Moreno *et al.*, 1991; Arias *et al.*, 1999); DnrI, which controls the daunorubicin biosynthetic *dnr* cluster of *Streptomyces peucetius* (Madduri & Hutchinson, 1995; Tang *et al.*, 1996); TylS, which activates the positive regulatory gene *tylR* involved in tylosin biosynthesis of *Streptomyces fradiae* (Bate *et al.*, 2002); ThnU, the transcriptional activator of the cephamycin C genes of *Streptomyces cattleya* (Rodríguez *et al.*, 2008); and FdmR1, which positively controls the transcription of most genes in the fredericamycin A biosynthetic gene cluster of *Streptomyces griseus* (Chen *et al.*, 2008). Some pleiotropic regulatory proteins also belong to the SARP family, such as the pleiotropic global regulator AfsR (Lee *et al.*, 2002). External signals related to nutrient conditions or physiological conditions probably stimulate the production of secondary metabolites through these pathway-specific regulatory proteins.

Nikkomycins are peptidyl nucleoside antibiotics that are produced by *Streptomyces ansochromogenes* and *Streptomyces tendae* (Ling *et al.*, 2007; Lauer *et al.*, 2001). As for many antibiotics, nikkomycin biosynthesis is dependent on growth phase and it is produced at stationary phase in SP liquid medium. SanG, a multi-domain protein, is a pathway-specific transcriptional regulator of nikkomycin biosynthesis in *S. ansochromogenes*. Deletion of *sanG* abolished nikkomycin biosynthesis, reduced sporulation and led to brown pigment accumulation (Liu *et al.*, 2005). Two putative transcription units for nikkomycin biosynthesis, starting from *sanN* and *sanO*, are dependent on the expression of *sanG*, whereas another putative transcription unit starting from *sanF* was not regulated by *sanG*. Consistent with nikkomycin production, the transcription of the *sanG* gene is also growth-phase regulated (Liu *et al.*, 2005).

Sequencing analysis showed that SanG exhibits significant sequence similarity with PolR, PimR and PteR (Li *et al.*, 2009b; Antón *et al.*, 2004; Ikeda *et al.*, 2003). All these proteins contain three major functional domains: an OmpR-like DNA-binding domain, a central ATPase domain with a potential ATP-binding motif and a C-terminal half homologous to the guanylate cyclase domain of the LuxR family. The N-terminal 300 aa residues of SanG containing the OmpR-like DNA-binding domain showed significant sequence similarity with several pathway-specific regulators of the SARP family that appear to turn on the expression of at least some of the genes of their respective clusters, and in turn to control antibiotic production. The central region of SanG displays an ATP/GTP-binding AAA domain that has characteristics of a large family of ATPases associated with diverse cellular activities, including cell-cycle regulation, protein degradation and protein transport (Leipe *et al.*, 2004). Deletion of *sanG* had pleiotropic effects on secondary metabolism and development, but the C-terminal region was not essential for the development of *S. ansochromogenes* (Liu *et al.*, 2005).

In this study, the target gene of SanG was identified by electrophoretic mobility-shift assay (EMSA) and the SanG-binding sequence was determined by DNase I footprinting. Our results indicate that the ATPase/GTPase activity of SanG modulates the transcription level of *sanN-I* and *sanO-V* during nikkomycin biosynthesis.

METHODS

Bacterial strains, plasmids and culture conditions. *Streptomyces ansochromogenes* 7100 (wild-type strain), a nikkomycin producer, was grown at 28 °C and used 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). For routine subcloning, *Escherichia coli* DH5 α and JM109 were grown at 37 °C in Luria-Bertani (LB) medium containing ampicillin or apramycin for propagating

plasmids. *E. coli* BL21(DE3) was used for protein expression (Sambrook *et al.*, 1989). *E. coli* ET12567(pUZ8002) was used for conjugal transfer of DNA from *E. coli* to *Streptomyces* (Paget *et al.*, 1999). pET44b (Pharmacia Biotech) containing an ampicillin-resistance gene was used for gene overexpression in *E. coli*.

Primers and PCR. All primers used in this study are listed in Table 1. The PCRs were performed using Taq DNA polymerase (Sino-American Biotechnology Corporation) or KOD-plus (TOYOBO). An initial denaturation at 95 °C for 5 min was followed by 30 cycles of amplification (95 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min) and an additional 10 min at 72 °C (68 °C for KOD-plus). In some cases, the annealing temperature and time, as well as the elongation time, were changed to accommodate different DNA templates and primers.

Overexpression and purification of SanG-His₆. The entire *sanG* gene was amplified from the genomic DNA of *S. ansochromogenes* 7100 with primers P1 and P2. The amplified fragment was digested with *Nde*I and *Eco*RI, and was inserted into the corresponding sites of pET44b to generate a recombinant plasmid (pET44b::*sanG*), which was subsequently introduced into *E. coli* BL21(DE3) for overexpression of SanG-His₆ under the control of the T7 promoter. *E. coli* BL21(DE3) containing plasmid pET44b::*sanG* was grown at 37 °C overnight in LB medium supplemented with 100 μ g ampicillin ml⁻¹. One millilitre of the above overnight culture was inoculated into 200 ml LB medium and incubated at 37 °C on a rotary shaker to an OD₆₀₀ of 0.6. The culture was induced by 0.1 mM IPTG and incubated at 18 °C for an additional 20 h. Cells harvested by centrifugation were washed twice with binding buffer (20 mM Tris/HCl, 500 mM NaCl, 5 mM imidazole, 5 % glycerol, pH 7.9), then resuspended in 20 ml of the same buffer and treated by mild sonication. The lysate was centrifuged at 12 000 g for 20 min, and the soluble fraction was purified with an Ni-NTA spin column (Novagen) according to the manufacturer's manual. The concentration of the purified SanG-His₆ was determined by the method of Bradford using BSA as standard (Smith *et al.*, 1985).

EMSAs. The EMSAs were performed according to the method of Yamazaki *et al.* (2000) and Elliot *et al.* (2001) with some modifications. To prepare the 335 bp DNA fragment containing the *sanN-sanO* intergenic region, P5 and P6 were used. P7 and P8 were used to amplify the control probe. A probe ³²P-labelled at the 5' end was incubated with various amounts of SanG at 25 °C for 20 min in a total volume of 20 μ l reaction mixture containing 20 mM Tris/HCl (pH 7.9), 1 mM DTT, 5 % glycerol, 0.04 μ g BSA μ l⁻¹ and 10 mM MgCl₂. After incubation, complexes and free DNA were separated on 4 % non-denaturing polyacrylamide gels (mono/bis, 80:1) with a running buffer containing 22.25 mM Tris/H₃BO₃ (pH 7.9), 20 mM sodium acetate and 1 mM EDTA. Then the gel was dried and exposed to Biomax radiographic film (Kodak).

DNase I footprinting. In order to characterize the binding sites of SanG on both strands of the *sanN-sanO* intergenic region, two probes were prepared separately by labelling the 5'-ends of sense or antisense strands. For preparation of the labelled antisense strand, unlabelled P5 and 5'-end-labelled P6 were used in the PCR amplification. For the sense strand, 5'-end-labelled P5 and unlabelled P6 were used. The DNase I footprinting was performed according to the procedures of Elliot *et al.* (2001). The reaction mixture (50 μ l) contained 10 kc.p.m. ³²P-labelled DNA probe, 0.08–4.8 μ M SanG, 25 mM HEPES/KOH (pH 7.9), 0.5 mM EDTA/NaOH (pH 8.0), 50 mM KCl and 10 % glycerol. After incubation for 20 min at 25 °C, 0.5 U DNase I (Promega) was added to each reaction mixture, followed by further incubation at 37 °C for 70 s. The reaction was stopped by the addition of 50 μ l stop solution (3 M ammonium acetate, 0.25 M EDTA, 0.1 mg tRNA ml⁻¹) and 100 μ l phenol/chloroform. DNA

Table 1. Primers used in this study

Primer	Positions	Sequence (5'–3')
P1	Containing the translational start codon of <i>sanG</i>	GGGAATTC <u>CATATGG</u> CGTACACAGCTCAAG (<i>NdeI</i> site underlined)
P2	Containing the translational stop codon of <i>sanG</i>	CGGAATTC <u>GGG</u> CGAAGCGGACGTCAGGGGA (<i>EcoRI</i> site underlined)
P3	Nucleotide positions +1029 to +1058 from the translational start codon of <i>sanG</i>	CGGCGAGGCGGGCGTCTTCGAAACCGAACT (GGC→TTC; AAG→GAA)
P4	Nucleotide positions +1019 to +1044 from the translational start codon of <i>sanG</i>	GACGCCCCGCTCGCCGGACGACGGCGG
P5	Nucleotide positions –262 to –246 from the transcriptional start point of <i>sanO</i>	TCTTGGTCGCCAGGTCC
P6	Nucleotide positions +35 to +53 from the transcriptional start point of <i>sanO</i>	CTTCGGATGCTGAATGTGC
P7	Nucleotide positions 221 to 240 from the translational start codon of <i>sanG</i>	AGGCCACCCTGCAGACGTAC
P8	Nucleotide positions +541 to +558 from the translational start codon of <i>sanG</i>	GAGGAGCGTGTCGGCTTG
MaL	Nucleotide positions –44 to –56 from the transcriptional start point of <i>sanO</i>	TCGCGAATTC <u>CGCCCGCGGCGCC</u> (<i>EcoRI</i> site underlined)
MaR	Nucleotide positions –24 to –7 from the transcriptional start point of <i>sanO</i>	GGCGGAATTC <u>GCGAGGCTCTAGAGT</u> CCTTGCAGGGACGCGG (<i>EcoRI</i> and <i>XbaI</i> sites underlined)
MbL	Nucleotide positions –70 to –58 from the transcriptional start point of <i>sanO</i>	CGGCAAGCTTTGCACTGACCGGCCGGC (<i>HindIII</i> site underlined)
MbR	Nucleotide positions –106 to –88 from the transcriptional start point of <i>sanO</i>	TGCAAAGCTTGCCGTGGATCCGGGCCCCAGCTCCAGGCTG (<i>HindIII</i> and <i>BamHI</i> sites underlined)
PsanNL	Nucleotide positions +751 to +769 from the transcriptional start point of <i>sanN</i>	GAGAGCCGCTGACGAAATC
PsanNR	Nucleotide positions +812 to +831 from the transcriptional start point of <i>sanN</i>	CTAGAGGCCGTGGAGTCGTT
PsanOL	Nucleotide positions +744 to +762 from the transcriptional start point of <i>sanO</i>	GCCTACGTCATCCACACCT
PsanOR	Nucleotide positions +833 to +851 from the transcriptional start point of <i>sanO</i>	GAAGGCGAAGTAGCGGTTC
PhrdBL	Nucleotide positions +631 to +650 from the translational start codon of <i>hrdB</i>	AACCTCCGTCTGGTGGTCTC
PhrdBR	Nucleotide positions +746 to +767 from the translational start codon of <i>hrdB</i>	GAGAACTTGTAGCCCTTGGTGT

fragments in the aqueous phase were precipitated by adding 3 vols ethanol, and washed with 75 % ethanol. After the DNA fragment was dried, it was directly suspended in 10 µl of 90 % formamide-loading gel buffer (10 mM Tris/HCl, pH 8.0, 20 mM EDTA, pH 8.0, 0.05 % bromophenol blue, 0.05 % xylene cyanol). Samples were then denatured at 95 °C for 2 min and separated on a 6 % polyacrylamide/urea gel. The sequence ladder was made using an *fmol* DNA cycle sequencing kit (Promega) with the labelled primer P5 or P6 for the sense or antisense strand. After electrophoresis, the gels were dried and exposed to Kodak X-ray film. Experiments were repeated at least three times and the same results were obtained.

Alterations of the SanG-binding sequence. To assess the importance of the identified SanG-binding sequence, the two direct repeats (see Fig. 2a, b) in the SanG-binding region were replaced by restriction sites (*XbaI*, *EcoRI*, *HindIII* and *BamHI*, respectively) using overlapping primers MaL and MaR or MbL and MbR (Table 1). The binding activity of SanG to the mutated probes was measured by EMSA.

Assay of ATPase/GTPase activity. ATPase/GTPase activity was measured by the modified malachite green ATPase assay described by Lanzetta *et al.* (1979). The reaction mixture (450 µl) contained 1–

2 µg SanG or SanGM, 5 mM HEPES, 30 mM KCl, 30 mM NH₄Cl, 1 mM DTT, 5 mM magnesium acetate, 2 mM ATP or GTP and 5 µg BSA ml^{–1}. The components were mixed together on ice, and then incubated at 30 °C. Samples (50 µl each) were withdrawn at different time points (10, 20, 30, 40, 50 and 60 min), and the reaction was stopped by adding 800 µl malachite green/ammonium molybdate reagent. This reagent contained 3 vols 0.045 % malachite green hydrochloride (Sigma), 1 vol. of ammonium molybdate (4.2 % in 4 M HCl), and 1/50 vol. of 1 % Triton X-100. After 1 min at room temperature, 100 µl 34 % citric acid was added to stop the colour development. To quantify the amounts of enzymically released phosphate (P_i), the samples (in triplicate) were measured photometrically by referring to a standard curve, which was prepared with dilutions of a standard solution.

Site-directed mutagenesis. Primers P3 and P4 were used to construct the site-directed mutation of SanG according to the method of the QuikChange Site-Directed Mutagenesis kit (Stratagene). Plasmid pET44b::*sanG* was used as template for the mutagenesis. The mutation generated a diagnostic *BstBI* site that was designed to screen for the presence of the mutation. The expected mutations were confirmed by DNA sequencing. The constructed plasmid pET44b::*sanGM* was used to express the mutated protein SanGM

in which two amino acids (Gly-349 and Lys-350) were respectively replaced by Phe and Glu in the A-type ATP-binding motif. The entire *sanGM* gene with its promoter region was inserted into pSET152 and the resulting plasmid was used to complement the *sanG* disruption mutant GLD020 (Liu *et al.*, 2005).

Preparation of antibody and Western blotting analysis.

Approximately 2 mg purified SanG-His₆ mixed with Freund's complete adjuvant was injected three times (once per week) into one healthy rabbit. Antisera were collected from the rabbit after 1 month and used as polyclonal antibodies against SanG.

For Western blotting analysis, the supernatants of cell lysates were boiled for 5 min, subjected to 8% SDS-PAGE, and electro-blotted to a nitrocellulose membrane. Non-specific binding was blocked with 5% fat-free milk in PBS overnight at 4 °C, followed by rinsing twice with 0.1% Tween 20/PBS. Then the membrane was incubated with anti-SanG antibody at room temperature for 1 h, followed by AP-labelled anti-rabbit IgG antibody for 1 h. The reaction product was visualized with a solution containing BCIP (5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt)/NBT (nitro-blue tetrazolium chloride).

Real-time quantitative PCR analysis. Total RNAs were isolated from strains grown in SP medium as described previously (Liu *et al.*, 2005). Quality and quantity of RNAs were examined by UV spectroscopy and checked by agarose gel electrophoresis. To remove the chromosomal DNA contamination, each sample was treated with DNase and tested by PCR to ensure that there was no chromosomal DNA left. After DNase treatment, RNA samples (1 µg) were reverse transcribed using SuperScript III and random hexamers (N15) as described by the vendor of the enzyme (Invitrogen). Samples of each of the reversed-transcribed RNAs (and dilutions) were then amplified and detected with the ABI-PRISM 7000 Sequence Detection System (Applied Biosystems) using optical-grade 96-well plates. Each reaction (20 µl) contained 0.1–10 ng reverse-transcribed RNA depending on dilution, 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), and 0.6 µM of both forward and reverse primers for *sanN* (81 bp), *sanO* (107 bp) and *hrdB* (136 bp), respectively (the size of each amplicon is provided in parentheses). The PCR conditions were 50 °C for 2 min, 95 °C for 10 min,

followed by 40 cycles of 96 °C for 30 s, 60 °C for 1 min; fluorescence was measured at the end of each cycle. Data analysis was done with the Sequence Detection software supplied by Applied Biosystems.

RESULTS

Overexpression, purification and characterization of SanG

The full-length *sanG* gene from *S. ansochromogenes* was cloned into plasmid pET44b and then overexpressed in *E. coli* after induction with IPTG. The resulting protein (SanG-His₆) carrying a C-terminal His-tag with a predicted molecular mass of 121 kDa was purified to near-homogeneity by Ni²⁺-NTA affinity chromatography (data not shown). Dynamic light scattering was performed to determine the possible subunit structure of SanG-His₆ under non-denaturing conditions. SanG-His₆ was shown to have a hydrodynamic radius of 6.3 nm, corresponding to a molecular mass of 246 kDa. As the calculated molecular mass of SanG-His₆ is 121 kDa, this protein is unambiguously a dimer in solution under the conditions of this experiment. These measurements were made on a monodisperse solution of SanG-His₆ in which 99.6% of the mass was accounted for by the single peak at 6.3 nm.

Binding of SanG to the intergenic region between *sanO* and *sanN*

As SanG contains an OmpR-like DNA-binding domain and the transcription of *sanN-I* and *sanO-V* is dependent on SanG, the bidirectional *sanN-sanO* promoter region was chosen as a target for EMSA. As expected, the purified SanG bound to the 335 bp DNA fragment containing the bidirectional *sanN-sanO* promoter region. EMSAs with

Fig. 1. EMSAs for binding of SanG to the intergenic region between *sanO* and *sanN*. The 335 bp γ -³²P-labelled DNA fragment P_{O-N} (1 nM) was incubated with increasing concentrations of SanG protein (lanes 1–7; lanes contain 0, 5, 15, 30, 50, 100 and 150 nM, respectively). Lane 8, 30 nM SanG with 200-fold molar excess of unlabelled P_{O-N}; lane 9, 30 nM SanG with 400-fold molar excess of unlabelled P_{O-N}; lane 10, free non-specific competitor probe (Pck); lane 11, 30 nM SanG with Pck (1 nM). The unbound free probes are indicated by the solid arrow and SanG–P_{O-N} complexes are indicated by the brace. The position of the well of the gel is shown by the open arrowhead.

Fig. 2. Determination of the SanG-binding sites in the intergenic region between *sanO* and *sanN*. (a) DNase I footprinting experiments. A P_{O-N} fragment was labelled in the sense and antisense strands respectively, and about 30 nM DNA probe was used in each reaction. The amounts of SanG-His₆ used in lanes 1–6 were 0, 0.08, 0.72, 2.4, 4.8 and 0 μ M, respectively. Reaction mixtures were migrated alongside sequencing reactions of the labelled fragments (lanes G, A, T and C). Regions protected against DNase I digestion by SanG are indicated by brackets. The positions of the protected regions are given relative to the transcriptional start point of *sanO*. (b) Nucleotide sequence of *sanO*–*sanN* promoter region and SanG-binding sites. The SanG-binding site is underlined and the direct repeats are indicated by arrows. The bent arrows indicate the transcription start points and transcription orientation of *sanO* and *sanN*, and the –10 and –35 regions are boxed. (c) EMSAs with a probe containing either the intact SanG-binding sites (wt) or the mutated sites, lacking both of the two divergent SanG-binding direct repeats (*m_{ab}*). Lanes 1–4 contain 0, 10, 15, 30 nM SanG, respectively.

Fig. 3. Assay of ATPase (a) and GTPase (b) activity of SanG, assayed with 1 µg (●) and 2 µg (■) SanG. The release of phosphate (P_i) was measured by the malachite green method every 10 min for 60 min.

unlabelled specific and non-specific competitor DNA were used as controls (Fig. 1). This binding was enhanced by increasing the amount of SanG (up to 50 nM). These results suggest that SanG controlled nikkomycin biosynthesis through direct interaction with the *sanN*–*sanO* promoter region.

Identification of SanG-binding sites

To identify the specific binding sites of SanG, a DNase I footprinting assay was used to analyse the interaction of SanG with the bidirectional *sanN*–*sanO* promoter region. On the sense strand, SanG protected two regions stretching from positions –9 to –46 and from –63 to –93 bases relative to the transcription start point of *sanO*. On the antisense strand, two regions stretching from positions –7 to –47 and from –58 to –89 bases relative to the transcription start point of *sanO* were protected by SanG (Fig. 2a).

Analysis of the SanG-binding region revealed two direct repeats 5'-CGGCAAG-3' that overlap with the –35 regions of the *sanN* promoter P1 and *sanO* promoter, respectively (Fig. 2b). This is a typical feature of SARP-binding targets.

To measure the relative contribution of each site to SanG binding, EMSAs were performed using a probe containing either the intact binding sites or the mutated sites (Fig. 2c). The affinity of SanG for the mutated DNA fragment lacking both SanG-binding direct repeats was abolished completely in comparison with the corresponding wild-type target. The affinity of SanG for a mutated DNA fragment lacking either of the two divergent SanG-binding direct repeats still remained (data not shown). These results imply that two divergent SanG-binding direct repeats are important for SanG-binding activity.

ATPase and GTPase activity of SanG

Analysis of the SanG sequence has revealed the existence of a central ATPase domain that is important for the function of many activators, so the possible ATPase/GTPase activities of SanG were determined by assaying ATP/GTP

hydrolysis and measuring the amount of phosphate (P_i) released at different time intervals (Fig. 3a). SanG showed an ATPase activity of $9.26 \text{ pmol phosphate min}^{-1} (\mu\text{g SanG protein})^{-1}$. Phosphate was released in the reaction mixture with the addition of SanG, whereas no phosphate was released in the reaction mixture without SanG or with the heat-inactivated SanG (data not shown). GTP was

Fig. 4. EMSAs for binding of SanG to the target DNA. The labelled DNA fragment P_{O-N} and 25 nM SanG were incubated with increasing concentrations of ATP (a) or GTP (b). The unbound free probe is indicated by the open arrowheads. The positions of SanG– P_{O-N} complexes are indicated by braces.

Fig. 5. Effects of mutation in the ATP/GTP-binding motif on the ATPase/GTPase activities and binding of SanG to the target. (a) ATPase activities of SanG (■) and SanGM (●) (1 μg of each protein) were measured by release of phosphate (P_i). (b) GTPase activities of SanG (■) and SanGM (●). (c) EMSAs, carried out using 1 nM labelled P_{O-N} and increasing concentrations of SanG or SanGM. Lanes: 1, 26 nM SanG with probe Pck as control; 2 and 18, free Pck; 3, 26 nM SanG with $\times 200$ unlabelled P_{O-N} ; 4–9, SanG at 150, 50, 35, 15, 10 and 5 nM, respectively; 10, free probe P_{O-N} ; 11–16, SanGM at 5, 10, 15, 30, 50 and 150 nM; 17, 26 nM SanGM with 200-fold molar excess of unlabelled P_{O-N} ; 19, 26 nM SanGM with Pck.

hydrolysed at almost the same rate as ATP in the presence of SanG (Fig. 3b). These results indicated that SanG possesses an intrinsic ATP/GTP cleavage activity.

Affinity of SanG for target DNA is increased by the addition of ATP or GTP

EMSAs were performed with SanG and the bidirectional *sanN*–*sanO* promoter region, and results showed that affinity of SanG for its targets was increased with the addition of ATP or GTP at different concentrations of 0.1–10 mM in the reaction mixture (Fig. 4), suggesting that ATP/GTP could modulate the affinity of SanG for its targets.

ATP hydrolysis is dispensable for SanG binding to the target DNA

To determine whether ATP/GTP hydrolysis is required for DNA binding by SanG, the conserved amino residues of the ATP/GTP-binding motif of SanG were replaced by site-directed mutagenesis. When G349K350 was changed to F349E350, the ATPase/GTPase activity of SanGM(G349F, K350E) was about one-third that of the wild-type SanG (Fig. 5). However, EMSAs showed that the mutated

SanGM still retained DNA-binding ability and its affinity was stronger than that of the wild-type SanG (Fig. 5). Addition of different concentrations of ATP/GTP in the reaction mixture did not enhance the affinity of SanGM for its targets, while the affinity was decreased by increasing the nucleotide concentration (Fig. 6). These results indicated that ATP/GTP hydrolysis was dispensable for SanG binding to its targets, but it could enhance the affinity of SanG. It is possible that ATP/GTP modulates nikkomycin biosynthesis through controlling SanG-binding ability.

ATP hydrolysis by SanG modulates the transcription of the target genes

To study the function of ATP/GTP hydrolysis in nikkomycin biosynthesis, the gene (*sanGM*) encoding SanGM was introduced into the *sanG* deletion mutant GLD020; GLD020 could be complemented by *sanGM*, but its nikkomycin production was only 15 % of that produced by the strain complemented with the wild-type *sanG* (Fig. 7b). Sporulation was fully restored and brown pigment accumulation disappeared in the strain complemented with *sanGM* (data not shown). Western blotting was performed using cell extracts from GLD020/*sanGM*, GLD020/*sanG*, GLD020 and the wild-type strain. As a

Fig. 6. EMSAs for binding of SanGM to the target DNA with ATP (a) or GTP (b). The labelled DNA fragment P_{O-N} and 10 nM SanGM were incubated with increasing concentrations of ATP or GTP. The unbound free probe is indicated by the open arrowheads. The positions of SanG-P_{O-N} complexes are indicated by braces.

control, GLD020 did not give an immunoblotting signal. GLD020/*sanGM*, GLD020/*sanG* and the wild-type gave rise to a 116 kDa immunoblotting signal (Fig. 7c). Thus, both the mutated SanGM (G349F, K350E) and the wild-type SanG were normally expressed in GLD020. Subsequently, the transcription of *sanN* and *sanO* was determined by real-time RT-PCR (Fig. 7d). The transcriptional level of *sanN* or *sanO* in GLD020/*sanGM* was much lower than that in the GLD020/*sanG*, suggesting that ATP/GTP hydrolysis did modulate the transcriptional level of *sanN-I* and *sanO-V* during nikkomycin biosynthesis.

DISCUSSION

ATP is the universal source of chemical energy used to catalyse molecular transactions of many proteins (Thomsen & Berger, 2008). In this study, we have shown that ATP/GTP hydrolysis affected transcription of *sanN-I* and *sanO-V* through modulating the activation of SanG during nikkomycin biosynthesis.

Fig. 7. Effects of mutation in the ATP/GTP-binding motif of SanG on the nikkomycin production and transcription of *sanN* and *sanO*. (a) Growth of GLD020/pSEG (◆) and GLD020/pSEGM (●) strains during fermentation. (b) Nikkomycin production by GLD020/pSEG (◇) and GLD020/pSEGM (□) strains during fermentation. (c) Western blotting analysis of SanG or SanGM in GLD020, wild-type strain, GLD020/pSEG and GLD020/pSEGM. Signals corresponding to SanG are arrowed. (d) Transcriptional analysis of *sanO* and *sanN* by real-time RT-PCR. WT, *S. ansochromogenes* wild-type strain; GLD020, *sanG* disruption mutant; GLD020/pSEG, *sanG* complementation strain; GLD020/pSEGM, *sanGM* complementation strain.

Some proteins of the SARP family contain a motif similar to those that characterize Walker-box ATPases, such as SanG, PimR, PolR, PolY and AfsR (Antón *et al.*, 2004; Li *et al.*, 2009a, b; Tanaka *et al.*, 2007). Although they all

belong to the SARP family, two groups are found among them based on structural analysis. SanG, PolR and PimR belong to one group, which contain a guanylate cyclase domain of the LuxR family in their C-terminal region. PolY and AfsR belong to another group, which contain a tetratricopeptide repeat (TPR) domain in their C-terminal region. SanG and PolR directly bind to the promoter region of structural genes during the biosynthesis of secondary metabolites, but PolY and AfsR bind to the promoter region of regulatory genes. Unlike AfsR, which is a monomer in solution, SanG was shown to be a dimer. The dimer may bind to the direct repeats and recruit RNA polymerase to their target promoters.

Adenine nucleotides can act as cofactors to regulate the activity of SanG protein, and the binding and hydrolysis of ATP are functionally separable. Some transcriptional activators with the ATPase domain oligomerize into ATPase-active rings that use the energy from ATP hydrolysis to physically remodel transcriptionally closed complexes in bacteria (Madiraju *et al.*, 2006). As SanG without ATP/GTP is easy to aggregate and ATP/GTP did enhance the affinity of SanG for the target DNA, it is possible that ATP/GTP could stabilize the conformation of SanG. When the A-type ATP-binding motif of the ATPase domain was mutated, the conformation of the resulting SanGM protein may be stabilized either with or without ATP/GTP. In our study, the affinity of SanGM was even stronger than that of SanG. It is possible that SanGM still could hydrolyse ATP/GTP and this could have a different effect on the DNA-binding activity of SanG. The dual actions of ATP binding and ATP hydrolysis can be considered as the means by which a polymerization/depolymerization cycle of SanG is set up to bring about transcriptional activation or inactivation of the target genes.

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