Conditionally positive effect of the TetR-family transcriptional regulator AtrA on streptomycin production by *Streptomyces griseus*

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AtrA, a transcriptional activator for *actII-ORF4*, encoding the pathway-specific transcriptional activator of the actinorhodin biosynthetic gene cluster in *Streptomyces coelicolor* A3(2), has been shown to bind the region upstream from the promoter of *strR*, encoding the pathway-specific transcriptional activator of the streptomycin biosynthetic gene cluster in *Streptomyces griseus* [Uguru *et al.* (2005) *Mol Microbiol* **58**, 131–150]. The *atrA* orthologue (*atrA-g*) in *S. griseus* was constitutively transcribed throughout growth from a promoter located about 250 nt upstream of the translational start codon, as determined by S1 nuclease mapping. DNase I footprinting showed that histidine-tagged AtrA-g bound an inverted repeat located upstream of *strR* at positions –117 to –142 relative to the transcriptional start point of *strR* as +1. This AtrA-g-binding site was between two AdpA-binding sites at approximately nucleotide positions –270 and –50. AdpA is a central transcriptional activator in the A-factor regulatory cascade and essential for the transcription of *strR*. AtrA-g and AdpA simultaneously bound the respective binding sites. In contrast to AdpA, AtrA-g was non-essential for *strR* transcription; an *atrA-g*-disrupted strain produced streptomycin on routine agar media to the same extent as the wild-type strain. However, the *atrA-g*-disrupted strain tended to produce a smaller amount of streptomycin than the wild-type strain under some conditions, for example, on Bennett agar containing 1 % maltose and on a minimal medium. Therefore, AtrA-g had a conditionally positive effect on streptomycin production, as a tuner, probably by enhancing the AdpA-dependent transcriptional activation of *strR* in a still unknown manner.

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INTRODUCTION

The genus *Streptomyces* consists of Gram-positive, filamentous, soil-dwelling bacteria that are characterized by complex morphological differentiation resembling that of fungi and by the ability to produce a wide variety of secondary metabolites. About two-thirds of all known biologically active substances, including antibiotics, are produced by streptomycetes. The biosynthetic and regulatory genes for a certain secondary metabolite are generally found in a gene cluster that includes a regulatory gene encoding a pathway-specific transcriptional activator. The pathway-specific transcriptional activator acts as a master switch for biosynthesis of the respective secondary metabolite. Therefore, a variety of external signals, such as nutrient conditions and physiological conditions, are probably gathered to the promoter of a pathway-specific transcriptional activator gene, for example, *actII-ORF4* for the actinorhodin biosynthesis gene cluster in *Streptomyces coelicolor* A3(2) (Fernández-Moreno *et al.*, 1991) and *strR*

for the streptomycin biosynthesis gene cluster in *Streptomyces griseus* (Retzlaff & Distler, 1995; Tomono *et al.*, 2005).

We have long studied the A-factor (2-isocapryloyl-3R-hydroxymethyl- γ -butyrolactone) regulatory cascade that leads to secondary metabolism and morphological differentiation in *S. griseus* (Horinouchi, 2007; Horinouchi & Beppu, 2007). As one of the target phenotypes controlled by A-factor, the regulation of streptomycin biosynthesis has been studied with a focus on the pathway-specific activator gene *strR*. The signal relay from A-factor to *strR* is as follows. A-factor is gradually accumulated in a growth-dependent manner by the activity of AfsA, which is the key enzyme for A-factor biosynthesis (Kato *et al.*, 2007). When the concentration of A-factor reaches a critical level at or near the middle of exponential growth (Ando *et al.*, 1997), it binds the A-factor receptor protein (ArpA), which has bound and repressed the promoter of *adpA*, and dissociates ArpA from the promoter, thus inducing transcription of *adpA* (Ohnishi *et al.*, 1999). AdpA then activates a number of genes required for morphological differentiation and secondary metabolism, forming an AdpA regulon (Horinouchi, 2002; Ohnishi *et al.*, 2005). *strR* was the first member of the AdpA regulon to be identified, as described below.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of *atrA-g* is AB365454.

We previously identified the *strR* promoter as an A-factor-dependent promoter (Vujaklija *et al.*, 1991) and therefore conducted experiments to detect a predicted activator protein(s) that might bind the region upstream from the *strR* promoter. In this experiment, we detected four proteins that did bind the region *in vitro* (Vujaklija *et al.*, 1993). One of them, which bound the region at approximately nucleotide position –270 relative to the transcriptional start point of *strR* as +1, was produced in an A-factor-dependent manner and named AdpA (A-factor-dependent protein). We purified AdpA, cloned the gene, and found that *adpA* was a target gene of ArpA (Ohnishi *et al.*, 1999). Because disruption of *adpA* resulted in no transcriptional activation of *strR* and no production of streptomycin, AdpA was proved to be essential for *strR* transcription (Ohnishi *et al.*, 1999). Furthermore, we identified an additional AdpA-binding site at nucleotide position –50 in front of the *strR* promoter and found that both AdpA-binding sites (positions –270 and –50) are necessary for full transcriptional activation of *strR* by AdpA (Tomono *et al.*, 2005). However, the three proteins other than AdpA that bound the region upstream from *strR*, and were apparently produced independently of A-factor, have not been studied any further. Therefore, whether or not these three proteins are involved in the regulation of the transcription of *strR* has not been elucidated.

Recently, McDowall and his colleagues identified a transcriptional activator for *actII-ORF4* encoding the pathway-specific transcriptional activator for the actinorhodin biosynthesis gene cluster in *S. coelicolor* A3(2) (Uguru *et al.*, 2005). They named this TetR-family regulator AtrA (actinorhodin-associated transcriptional regulator). Here we call it AtrA-c to discriminate it from its homologue (AtrA-g) in *S. griseus*. AtrA-c binds two sites flanking the promoter of *actII-ORF4*. Disruption of *atrA-c*, which is not associated with any secondary metabolite gene cluster, reduced production of actinorhodin but showed no detectable effects on production of other secondary metabolites, such as undecylprodigiosin and a calcium-dependent antibiotic (CDA). Furthermore, they investigated whether AtrA-c orthologues have a role in regulating transcription of some pathway-specific transcriptional activators for secondary metabolites in disparate *Streptomyces* strains and showed that AtrA-c bound *in vitro* the region upstream from the *strR* promoter of *S. griseus*. In their following paper (Hong *et al.*, 2007), they reported that expression of *atrA-c* in *S. griseus* caused DNA-binding-dependent reduction in streptomycin production and in the *strR* mRNA level. Because expression of *atrA-c* in *S. griseus* did not affect the *adpA* mRNA level, they proposed that the A-factor–ArpA–AdpA–StrR regulatory cascade represents only part of the full complexity of regulation of streptomycin biosynthesis in *S. griseus*. However, they did not characterize the function of the endogenous *atrA-g* gene and therefore it was unclear whether AtrA-g actually contributes to streptomycin biosynthesis in *S. griseus*.

The work by Uguru *et al.* (2005) and Hong *et al.* (2007) reminded us of the four proteins, including AdpA, that bound the region upstream from the *strR* promoter. On the assumption that AtrA-g could be one of the three proteins other than AdpA, we started characterizing *atrA-g* to determine whether it plays a role in streptomycin production in *S. griseus*. Here we report that AtrA-g is non-essential for streptomycin production but has a conditionally positive effect.

METHODS

General recombinant DNA studies. All the bacterial strains, plasmids and media used were described previously (Kato *et al.*, 2005; Hirano *et al.*, 2006). Bennett agar without glucose [yeast extract, 0.1%; meat extract (Kyokuto), 0.1%; NZ amine, 0.2%; agar, 2%; pH 7.2] and SMM agar [glucose, 0.9%; L-asparagine, 0.9%; (NH₄)₂SO₄, 0.2%; Trizma base, 0.24%; NaCl, 0.1%; K₂SO₄, 0.05%; MgSO₄·7H₂O, 0.02%; CaCl₂, 0.01%; trace element solution (Hopwood *et al.*, 1985), 1%; agar, 2%; pH 7.2] containing 2.5 mM KH₂PO₄ were used for streptomycin assay. The strategies used for gene disruption, alteration of the AtrA-g-binding site by PCR, gel mobility shift assay, DNase I footprinting and S1 nuclease mapping were also described previously (Yamazaki *et al.*, 2000; Hirano *et al.*, 2006).

S1 nuclease mapping. Total RNA was isolated with an RNAqueous kit (Ambion) from cells grown on cellophane on the surface of Bennett agar containing 1% maltose. Hybridization probes were prepared by PCR with a pair of ³²P-labelled and non-labelled primers. The PCR primers used for low-resolution S1 nuclease mapping were *atrA-g*-SLF and *atrA-g*-SLR* for *atrA-g* and *strR*-S1F and *strR*-S1R* for *strR* (Table 1). Primers indicated with an asterisk were labelled at the 5' end with [γ -³²P]ATP by using T4 polynucleotide kinase before PCR. Primers *atrA-g*-SHF and *atrA-g*-SHR* were used for high-resolution S1 nuclease mapping of *atrA-g*.

Production and purification of AtrA-g. The sequence (Met-1 to Ala-267) of AtrA-g was expressed as a fusion to a His-tag at the N terminus, as follows. The *atrA-g* sequence encoding Met-1 to Ala-267 was amplified by PCR with primers *atrA-g*-pF (containing *SphI* and *NdeI* sites) and *atrA-g*-pR (containing a *BamHI* site). The *atrA-g*-coding sequence was excised as an *SphI*–*BamHI* fragment and placed between the *SphI* and *BamHI* sites of pUC19. The absence of PCR errors was checked by nucleotide sequencing. The *NdeI*–*BamHI* fragment was excised from this plasmid and placed between the *NdeI* and *BamHI* sites of pET16b, resulting in pET16b-*atrA-g*. *Escherichia coli* BL21(DE3) containing pLysS harbouring pET16b-*atrA-g* was cultured at 26.5 °C for 3 h in the presence of 1 mM IPTG. AtrA-g with a His-tag at its N terminus was purified from the soluble fraction with a nickel-nitrilotriacetic acid spin column (Qiagen) according to the manual of the manufacturer. The AtrA-g protein was eluted by sodium phosphate buffer [50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole and 10% glycerol (pH 8.0)].

Gel mobility shift assay. The DNA fragments used for ³²P-labelled probes were amplified by PCR and ³²P-labelled with T4 polynucleotide kinase. Various regions upstream and in the coding sequence of *atrA-g* were used as ³²P-labelled probes. Table 1 lists the primer sequences used for preparing these probes. Four probes, S1 to S4, were prepared as follows: *strR*-F1 and *strR*-R1 were used for probe S1; *strR*-F2 and *strR*-R2 for probe S2; *strR*-F3 and *strR*-R3 for probe S3; and *strR*-F4 and *strR*-R4 for probe S4.

Table 1. Primers used in this study

Gene and primer	Position	Sequence (5'–3')
<i>atrA-g</i>		
atrA-g-SLF	–99 to –80	ACGATGTCCCGGTGCGGATC
atrA-g-SLR	+302 to +279	TCCTGTCGTCGCCGTTCCCGACGT
atrA-g-SHF	–204 to –185	CGGCAAATTGTGTGACCGGA
atrA-g-SHR	+106 to +87	TGAATATGCATAACTTCCCC
datrA-g-LF	+996 to +1026	CCCAAGCTTCACGTGTTGCTGGACATCCTGCTGGAGGGGCTGCGGT
datrA-g-LR	+3166 to +3137	GGAATTCGATGGACACTTGGGAACGCTGCCAGATCCA
datrA-g-RF	–1944 to –1915	GCTCTAGAGACCGACCGTACTGACAGTCGCCGCCCGGT
datrA-g-RR	+302 to +273	CCCAAGCTTTCCTGTCGTCGCCGTTCCCGACGTCTGTCGC
atrA-g-pF	+237 to +266	ACATGCATGCCATATGGGTTCGGCCGTTGCCGGACACGCCCCG
atrA-g-pR	+1037 to +1009	CGGGATCCGCCGGGCGCAGCCGACGCCCTCCAGCA
catrA-g-F	–374 to –345	CGGAATTCAGTCTCTCCTTCAACTTCCGTTGGAGACG
catrA-g-R	+1476 to +1447	CGGGATCCTCTTCCTGCTGCTGCTCGTTACCGCTCATC
<i>strR</i>		
strR-F1	–482 to –463	GCGGCACGTATGGCCTCCAG
strR-R1	–160 to –180	CGCACCGCTGTCAGCGATT
strR-F2	–344 to –324	TCCTCGCGTGGTCTTGGGCCG
strR-R2	+19 to –5	AGAGCAATGCTTTCGCACTTCGCG
strR-F3	–225 to –202	ATGTATTCGGCAATCAAACCTGCGG
strR-R3	–50 to –71	CCGCATGACCGAAAAGCGACCT
strR-F4	–68 to –46	TCGCTTTTCGGTCATGCGGACAG
strR-R4	+136 to +117	TTCACTTTCCTCGACCGCAC
strR-S1F	–184 to –162	AATGAAATCGCTGACAGGCGGTG
strR-S1R	+218 to +199	CGACATCCTCGCCGGCACTG
strR-LmtF	–488 to –468	CGGGATCCATCCCGGCGGCACGTATGGCC
strR-LmtR	–138 to –159	CCCAAGCTTAGCCAGCGCCTGCGCCCGATCA
strR-RmtF	–131 to –101	CCCAAGCTTGGCAGCGCTGGTCTCTCCGCCCTGGAGGAT
strR-RmtR	+334 to +314	TCCGCGCATTTTGGTGGCGCG

DNase I footprinting. The method of DNase I footprinting was described previously (Yamazaki *et al.*, 2000). For analysis of AtrA-g-binding sites, a 32 P-labelled fragment was prepared by PCR with primers strR-F3 and strR-R3.

Alteration of the AtrA-g-binding sequence by PCR. Two mutations were introduced by PCR into the AtrA-g-binding site upstream of *strR*. The region upstream from the AtrA-g-binding site was amplified with primers strR-LmtF (containing a *Bam*HI sequence at the 5' end) and strR-LmtR (containing a *Hind*III sequence at the 5' end), and digested with *Bam*HI plus *Hind*III. The region downstream from the AtrA-g-binding site was amplified with primers strR-RmtF (containing *Hind*III and *Aor*51HI sequences at the 5' end) and strR-RmtR, and digested with *Hind*III plus *Sph*I (position +305). The *Bam*HI–*Hind*III fragment and the *Hind*III–*Sph*I fragment were inserted between the *Bam*HI and *Sph*I sites of pUC19 by three-fragment-ligation, generating pUC-mt-*strR*. The absence of PCR errors was checked by nucleotide sequencing. The GGAGGG and CGTTCC sequences in the AtrA-g-binding site were changed to *Hind*III (AAGCTT) and *Aor*51HI (AGCGCT) cleavage sequences, respectively. Mutated probe mS2 used for gel mobility shift assay was amplified by PCR using pUC-mt-*strR* as the template.

Gene disruption. For disruption of *atrA-g* on the chromosome, the *atrA-g* sequence was replaced by the neomycin resistance gene *aphII*. Briefly, a 2.2 kb sequence upstream of the *atrA-g* coding sequence and a 2.2 kb sequence downstream of the *atrA-g* coding sequence, together with *aphII*, were assembled in pUC19. This mutagenic plasmid was linearized by digestion with *Dra*I (Oh & Chater, 1997) and introduced by transformation into *S. griseus* IFO13350, and neomycin (10 µg

ml^{–1})-resistant colonies were isolated. Correct disruption was checked by Southern hybridization with the *atrA-g* sequence (positions –1944 to +302, prepared with primers datrA-g-RF and datrA-g-RR) and the *aphII* sequence as 32 P-labelled probes against the chromosomal DNA digested with *Hind*III plus *Pvu*II.

Construction of a complementation plasmid. For construction of pKU209-*atrA-g*, the *atrA-g* sequence with its promoter region was amplified by PCR with primers catrA-g-F (containing an *Eco*RI sequence at the 5' end) and catrA-g-R (containing a *Bam*HI sequence at the 5' end), and digested with *Eco*RI plus *Bam*HI. The *Eco*RI–*Bam*HI fragment was inserted between the *Eco*RI and *Bam*HI sites of pUC19, generating pUC-*atrA-g*. The absence of PCR errors was checked by nucleotide sequencing. The *Pvu*II fragment was excised from pUC-*atrA-g* and inserted in the *Sma*I site of pUC19. The *Pst*I fragment including the *atrA-g* sequences was excised from this plasmid and inserted in the *Pst*I site of pKU209, generating pKU209-*atrA-g*.

Streptomycin assay. The amount of streptomycin produced was measured by a bioassay using *Bacillus subtilis* ATCC 6633 as an indicator (Horinouchi *et al.*, 1984).

RESULTS

atrA orthologue (*atrA-g*) in *S. griseus*

We searched the genome sequence of *S. griseus* IFO13350 (our unpublished data) for ORFs encoding an AtrA-c-like

Fig. 1. Amino acid sequence of AtrA (a), gene organization in the neighbourhood of *atrA-g* (b), and transcriptional analysis of *atrA-g* (c, d). (a) Alignment of amino acid sequences of AtrA-c from *S. coelicolor* A3(2) and AtrA-g from *S. griseus*. The TetR-type DNA-binding domain is indicated with a dotted line. (b) Positions and directions of ORFs predicted by the *S. griseus* genome sequence are indicated by arrows. The predicted ORFs are: SGR3903, hypothetical protein; SGR3904, hypothetical protein; SGR3906, putative NADH dehydrogenase; and SGR3907, hypothetical protein. (c) Time-course of *atrA-g* transcription, as determined by low-resolution S1 mapping with RNA prepared from cells grown at 28 °C on cellophane on the surface of Bennett agar containing 1% maltose for the times indicated. As an internal control, transcription of *hrdB* (constitutive) was also determined. SM, substrate mycelium; AM, aerial mycelium; wt, wild-type. Mutant $\Delta adpA$ grew as substrate mycelium throughout growth. (d) High-resolution S1 mapping to determine the transcriptional start point of *atrA-g*. RNA prepared from mutant $\Delta adpA$ grown at 28 °C for 12 h on Bennett agar was used. The arrowhead indicates the position of the S1-protected fragment. The 5' terminus of the mRNA was assigned to position +1, because the fragments generated by the chemical sequencing reactions migrated 1.5 nt further than did the corresponding fragments generated by S1 digestion of the DNA–RNA hybrids (0.5 residue from the presence of the 3'-terminal phosphate group and 1 residue from elimination of the 3'-terminal nucleotide). The nucleotide sequence, including the –35 and –10 elements of the *atrA-g* promoter, is also shown. The transcriptional start point is indicated as +1.

sequences of AtrA-c and AtrA-g. In these two proteins, the sequences of about 110 aa in the N-terminal portion are highly homologous to each other. This region contains a TetR-type DNA-binding domain (46 aa), shown by a dotted line in Fig. 1(a). The amino acid sequences of the DNA-binding domains are identical between AtrA-g and AtrA-c, suggesting that both proteins would recognize and bind the same DNA sequence. The C-terminal regions of about 70 aa are also highly homologous to each other, although the function of this domain is unknown. The gene organization in the neighbourhood of *atrA-g* is shown in Fig. 1(b). *atrA-g* is located between the ORFs encoding a putative NADH dehydrogenase (SGR3906) and a hypothetical protein (SGR3904). This gene organization is the same in the region around *atrA-c* in *S. coelicolor* A3(2) (Bentley *et al.*, 2002), indicating that *atrA-g* is the *S. griseus* orthologue of *atrA-c* in *S. coelicolor* A3(2).

We analysed the transcription of *atrA-g* by S1 nuclease mapping by using RNA prepared from cells grown on cellophane on the surface of Bennett agar containing 1% maltose (Fig. 1c). Under these conditions, *S. griseus* grew as substrate mycelium at 12 and 18 h, and as a mixture of substrate mycelium and aerial mycelium at 24 and 36 h. The *hrdB* gene, which is transcribed throughout growth, was used as an internal control to check the integrity and amount of mRNA used. We found that *atrA-g* was constitutively transcribed throughout growth not only in the wild-type strain but also in an *adpA*-disrupted strain (mutant $\Delta adpA$). The transcriptional start point was

protein. A single ORF, designated *atrA-g*, which encoded a protein exhibiting end-to-end similarity to AtrA-c, was found. Fig. 1(a) shows the alignment of the amino acid

determined to be 236 nt upstream of the translational start codon, as determined by high-resolution S1 nuclease mapping (Fig. 1d). We propose that the –35 and –10 sequences are 5'-TTCTGA-3' and 5'-TATGTT-3', respectively, with a space of 17 nt between them (Fig. 1d). The –35 and –10 sequences are similar to those (5'-TTGACR-3' and 5'-TAGRRT-3', respectively; R=A or G) recognized probably by the house-keeping sigma factor HrdB (Strohl, 1992). This fact is consistent with the constitutive transcription of *atrA-g*.

Binding of AtrA-g to the region upstream from the *strR* promoter

For purification of AtrA-g as a His-tagged protein from *E. coli*, we constructed pET16b-*atrA-g* carrying the *atrA-g* sequence under the control of the T7 promoter. The recombinant AtrA-g protein (His-AtrA-g), having the structure Met-Gly-His₁₀-Ser₂-Gly-His-Ile-Glu-Gly-Arg-His-AtrA-g, was produced in the soluble fraction of *E. coli* harbouring pET16b-*atrA-g* and purified with a nickel-nitrilotriacetic acid spin column (Fig. 2a). Using this protein, we examined whether AtrA-g bound the region upstream from the *strR* promoter by this assay. We designed four ³²P-labelled probes (S1 to S4) for various regions and used them for gel mobility shift assay (Fig. 2b). Two of the four probes tested, S2 (nucleotide positions –344 to +19, relative to the transcriptional start point of *strR* as +1) and S3 (positions –225 to –50), gave a single retarded signal (Fig. 2b). A very faint signal observed when a large amount of His-AtrA-g was used for probe S1 presumably resulted from non-specific binding. Because only one retarded signal was detected with the two probes, a single AtrA-g-binding site in the overlapping region was expected.

To determine the exact location of the AtrA-g-binding site, we performed DNase I footprinting with a ³²P-labelled probe covering the region from –225 to –50. AtrA-g protected from DNase I digestion a sequence from positions –117 to –142 on the sense and antisense strands (Fig. 2d). In this region, there was an imperfect inverted repeat sequence, as shown in Fig. 2(d). This AtrA-g-binding site was the same as the AtrA-c-binding site determined by Uguru *et al.* (2005), consistent with the fact that the TetR-type DNA-binding domains of AtrA-g and AtrA-c are completely conserved. It should be noted that the binding site of AtrA-g overlaps with that (positions –188 to –74) of one of the three proteins which we previously detected by gel mobility shift assay as being able to bind the region upstream of *strR* (Vujaklija *et al.*, 1993). AtrA-g presumably corresponds to this protein.

An inverted repeat sequence as an AtrA-g-binding site

To confirm the importance of the imperfect inverted repeat as the AtrA-g-binding site, we introduced mutations into

the sequence by site-directed mutagenesis, as shown in Fig. 2(c). One half of the palindrome (positions –137 to –132) was changed to a *HindIII* recognition sequence and the other half of the palindrome was changed to an *Aor51HI* recognition sequence. The effect of the mutation on AtrA-g binding was examined by gel mobility shift assay (Fig. 2c). The ³²P-labelled probe (mS2; positions –344 to +19) containing the mutation gave no retarded signal, whereas a similar probe (S2) containing the intact AtrA-g-binding sequence gave a distinct signal (Fig. 2b). These data showed that the imperfect inverted repeat sequence from positions –123 to –137 was the AtrA-g binding site.

Simultaneous binding of AdpA and AtrA-g to the region upstream from the *strR* promoter

AdpA binds two sites upstream from the *strR* promoter, at approximately positions –270 and –50, and activates the transcription of *strR* (Tomono *et al.*, 2005). The AtrA-g-binding site was found between the two AdpA-binding sites (Fig. 3a). To determine whether AdpA and AtrA-g can simultaneously bind the DNA fragment containing the respective binding sites, we performed a gel mobility shift assay using the ³²P-labelled probe covering the region from –344 to +19 (probe S2). The binding of two molecules of AdpA dimer to this fragment was confirmed (Fig. 3b, lanes 1–5). Although the AdpA–DNA complex seemed to be readily aggregated and stacked in the gel well, two retarded signals were detected, as indicated by arrows. We next examined the binding of AdpA to this probe S2 in the presence of an excess of AtrA-g (Fig. 3b, lanes 6–10). In the absence of AdpA, almost all the probe DNA fragments were retarded by AtrA-g to give a single distinct signal (Fig. 3b, lane 6). When AdpA at different concentrations was added to the reaction mixture (Fig. 3b, lanes 7–10), two additional retarded signals were detected. Because an excess of AtrA-g sufficient to bind all the probe DNA fragments was present in these reaction mixtures and because the AtrA-g-binding site was far enough apart from the two AdpA-binding sites to avoid competitive binding, almost all the probes were assumed to be bound by at least AtrA-g under the experimental conditions. We therefore assumed that the fast-moving one of the two additional retarded signals represented a ternary complex consisting of DNA, AtrA-g and one molecule of AdpA dimer. The upper one presumably represented a ternary complex consisting of DNA, AtrA-g and two molecules of AdpA dimer. This result showed that two molecules of AdpA dimer and one molecule of AtrA-g were capable of simultaneously binding the DNA fragment.

In this experiment, we noticed that the presence of AtrA-g in the reaction mixture lowered the minimum concentration of AdpA to bind the DNA fragment. For example, when 0.1 µg AdpA was used, no retarded signal was detected in the absence of AtrA-g (Fig. 3b, lane 2), whereas half of the DNA fragment bound by AtrA-g was further retarded by the binding of AdpA (Fig. 3b, lane 7). These

Fig. 3. Binding of AtrA-g and AdpA to the region upstream from the *strR* promoter. (a) Schematic diagrams of the *strR* promoter. The binding sites of AtrA-g and AdpA are shown. (b) Gel mobility shift assays with AtrA-g and AdpA. The ^{32}P -labelled probe S2 (positions -344 to $+19$) containing the intact AtrA-g-binding sequence was used. The amounts of AdpA used in lanes 1, 2, 3, 4 and 5 were 0, 0.1, 0.2, 0.4 and 0.6 μg , respectively. In lanes 6, 7, 8, 9 and 10, 0.2 μg AtrA-g was used, in addition to the same amounts of AdpA as in lanes 1, 2, 3, 4 and 5, respectively. (c) Similar gel mobility shift assays using the mutated probe mS2 containing the mutations in the AtrA-g-binding sequence. The amounts of proteins were the same as those in (b).

(Fig. 3c, lane 2), whereas a distinct retarded signal by binding of AdpA was detected in the presence of AtrA-g (Fig. 3c, lane 7). Similar enhancement of AdpA binding was also observed with GriR, the pathway-specific transcriptional activator for the grixazone biosynthesis gene cluster (Higashi *et al.*, 2007), indicating that the enhancement of AdpA binding was not specific to AtrA-g (data not shown). These observations made unlikely the possibility that binding of AtrA-g to probe S2 facilitates the *in vitro* binding of AdpA or recruits AdpA to its binding sites, but showed that the presence of even AtrA-g unbound to its binding site facilitated the AdpA binding in an unknown manner. One possible explanation is that

AtrA-g, probably together with BSA in the reaction buffer, increased the stability of AdpA *in vitro*.

Streptomycin production by an *atrA-g*-disrupted strain

To determine the possible role of *atrA-g* in streptomycin biosynthesis *in vivo*, *atrA-g* was inactivated in such a way that most of *atrA-g* was replaced with *aphII*. The correct disruption of *atrA-g* was checked by Southern hybridization with appropriate probes (data not shown). The *atrA-g*-disrupted strain (mutant $\Delta\text{atrA-g}$) grew normally and formed aerial mycelium and spores with the same time-course as the wild-type strain on YMPD agar (data not shown). Mutant $\Delta\text{atrA-g}$ produced a yellow pigment, grixazone, which is one of the secondary metabolites under the control of A-factor (Ohnishi *et al.*, 2004), to the same extent as the wild-type strain (data not shown). We examined the ability to produce streptomycin of mutant $\Delta\text{atrA-g}$ on Bennett agar without glucose. This medium has been routinely used for streptomycin production assay in our laboratory because, due to glucose catabolite repression, *S. griseus* produces streptomycin in a very small amount on Bennett agar containing 1% glucose. Mutant $\Delta\text{atrA-g}$ produced streptomycin to the same extent as the wild-type strain (data not shown), indicating that AtrA-g was non-essential for streptomycin production and exerted no detectable effect on streptomycin production when examined with the strain grown on the routine agar medium. This is a vivid contrast to the fact that mutant ΔadpA never produces streptomycin on any media.

We next examined streptomycin production by mutant $\Delta\text{atrA-g}$ under some different culture conditions, because there was the possibility that AtrA-g might contribute to the transcription of *strR*, to some extent, under some specific culture conditions. First, we lowered the culture temperature. The wild-type strain and mutant $\Delta\text{atrA-g}$ were cultivated for 1–7 days at 18 $^{\circ}\text{C}$ on Bennett agar and streptomycin production was examined by a bioassay using *B. subtilis* as an indicator. No difference was observed between the amounts of streptomycin produced by the two strains (data not shown). Second, the osmotic pressure of the medium was increased by addition of 0.03–0.5 M sucrose. As the sucrose concentration of the medium increased, the yields of streptomycin production reduced. However, there was no difference between the amounts of streptomycin produced by the two strains in any cases (data not shown). Finally, some different carbon sources (glucose, maltose and glycerol) were added to Bennett agar. On Bennett agar containing 1% glucose, no difference in streptomycin production was detected, although the yields were greatly reduced due to glucose catabolite repression. However, on Bennett agar containing either 1% maltose or glycerol, mutant $\Delta\text{atrA-g}$ produced a smaller amount of streptomycin than the wild-type strain. Fig. 4 shows a typical result of the bioassay for streptomycin production of the two strains on Bennett agar containing 1% maltose.

Fig. 4. Streptomycin production by the *atrA-g*-disrupted strain. The wild-type (wt) strain and mutant $\Delta atrA-g$ were grown at 28 °C on Bennett agar containing 1 % maltose for the number of days indicated, and soft agar containing spores of *B. subtilis* was overlaid. The plates were incubated at 28 °C overnight, and streptomycin production was detected as a growth inhibition zone of the indicator around the colony.

The growth inhibition zones around the 4 and 5 day colonies of mutant $\Delta atrA-g$ were somewhat smaller than those of the wild-type strain. We isolated four $\Delta atrA-g$ strains independently and all of these strains showed a similar phenotype. We repeated this assay four times and calculated the amounts of streptomycin produced. The wild-type strain produced 3.7 ± 1 and 110 ± 60 μg streptomycin per colony on days 4 and 5, respectively, whereas mutant $\Delta atrA-g$ produced 0.89 ± 0 and 39 ± 10 μg streptomycin per colony on days 4 and 5, respectively. The reduced streptomycin production by mutant $\Delta atrA-g$ was partially complemented by pKU209-*atrA-g* carrying *atrA-g* with its own promoter on low-copy-number plasmid pKU209. Mutant $\Delta atrA-g$ harbouring the empty vector pKU209 produced 0.54 ± 1 and 26 ± 20 μg streptomycin per colony on days 4 and 5, respectively (data not shown). On the other hand, mutant $\Delta atrA-g$ harbouring pKU209-*atrA-g* produced 1.7 ± 1 and 52 ± 30 μg streptomycin per colony on days 4 and 5, respectively (data not shown). The reduced streptomycin production of mutant $\Delta atrA-g$ in comparison with the wild-type strain was also observed clearly when the strains were cultured on SMM minimum medium (data not shown). These data showed that AtrA-g exerted a positive effect on streptomycin production at least under some culture conditions.

Because the yield of streptomycin significantly reflects the *strR* transcriptional level (Tomono *et al.*, 2005) and because AtrA-g binds within the upstream regulatory region of the *strR* promoter, AtrA-g affects the transcriptional level of *strR*, but not the diverging *strD*. On the assumption that *strR* transcription in mutant $\Delta atrA-g$ might be reduced when grown on Bennett agar containing 1 % maltose or glycerol, we determined transcription of *strR* in mutant $\Delta atrA-g$ by S1 mapping. S1 mapping with RNA prepared from mutant $\Delta atrA-g$ cells grown on Bennett agar without glucose showed that the extent of the *strR* transcription was the same as in the wild-type strain (data not shown). This was in agreement with the finding that the yields of streptomycin produced by mutant $\Delta atrA-g$ and the wild-type strain were the same on the routine agar medium. We then prepared RNA from mutant $\Delta atrA-g$ grown on Bennett agar with 1 % maltose

and performed similar S1 mapping. Contrary to our expectation, however, no detectable difference in the transcription of *strR* was observed between mutant $\Delta atrA-g$ and the wild-type strain (data not shown). We assume that the difference in *strR* transcription between mutant $\Delta atrA-g$ and the wild-type strain was too small to be detected by the S1 mapping method employed.

DISCUSSION

We thought that the four proteins which we previously found to bind the region upstream of the promoter of *strR*, the pathway-specific activator gene for the streptomycin biosynthesis gene cluster in *S. griseus*, would provide useful information on the regulation of streptomycin biosynthesis by A-factor. Because *strR* is the pathway-specific activator, a variety of external signals, such as nutrient conditions and physiological conditions, are assumed to be gathered to the *strR* promoter and to affect its transcription. One of the four proteins was AdpA, a key transcriptional activator in the A-factor regulatory cascade, which we have extensively and intensively studied. The recent discovery by Uguru *et al.* (2005) that AtrA-c of *S. coelicolor* A3(2) binds not only the flanking regions of the *actII-ORF4* promoter but also the region upstream from the *strR* promoter in a heterologous host reminded us of the proteins and led us to search for a homologue of AtrA-c in *S. griseus* and to study its possible role in the regulation of streptomycin biosynthesis. As expected, AtrA-g, which turns out to be the orthologue of AtrA-c in *S. griseus*, was identified and shown to bind the inverted repeat (positions –123 to –137 with respect to the transcriptional start point of *strR*) in front of the *strR* promoter. We assume that AtrA-g corresponds to one of the four proteins we previously detected (Vujaklija *et al.*, 1993), on the basis of the location of its binding site and its constitutive production independently of A-factor. Our careful experiments for determination of the role of AtrA-g showed that it affected streptomycin biosynthesis in a positive way under some specific conditions just as a tuner. We observed the positive effect of AtrA-g on streptomycin production only when the strains were grown on certain media, such as Bennett agar containing either 1 % maltose or glycerol and SMM agar. The reason why the positive effect of AtrA-g on streptomycin production was observed only on some media is not clear. However, maltose or glycerol seem to have no direct relation with the function of AtrA-g, because mutant $\Delta atrA-g$ produced a smaller amount of streptomycin than the wild-type strain even on SMM.

Hong *et al.* (2007) observed a negative effect of the *S. coelicolor* A3(2) *atrA-c* on streptomycin production by a heterologous host, *S. griseus*, and discussed the possibility that expression of *atrA-c* in *S. griseus* may cause DNA-binding-dependent reduction in streptomycin production and in the *strR* mRNA level. Our results obtained with *atrA-g* in the original host are apparently in contradiction

to their observations: AtrA-g has a positive effect on streptomycin production, although the effect is only conditional and rather small. The reason why *atrA-c* exerts the negative effect on streptomycin production in the heterologous host *S. griseus* is unclear, but it may fail to exert the positive function that is observed for actinorhodin production in the original *S. coelicolor* A3(2) host. The negative effect of *atrA-c* on streptomycin production in *S. griseus* that Hong *et al.* (2007) observed may be due to interference of *strR* transcription resulting from competitive binding to the inverted repeat in front of the *strR* promoter between the cognate AtrA-g and the heterologous, overproduced AtrA-c. Alternatively, different culture conditions or different *S. griseus* strains used by the two groups may explain this apparent contradiction. In any case, it is important and to be emphasized that the effect of AtrA-g on streptomycin production that we observed is much smaller than that discussed by Hong *et al.* (2007); the A-factor–ArpA–AdpA–StrR regulatory cascade is the major and decisive regulation for streptomycin production in *S. griseus* and *atrA-g* is a tuner under some specific conditions.

How does AtrA-g contribute to streptomycin production under these specific conditions? It is probable that co-existence of AdpA and AtrA-g enhances the transcriptional activation of *strR* by an as yet unclear mechanism. However, the level of the transcriptional enhancement by AtrA-g seems to be too low to be detected by S1 nuclease mapping, even with RNAs prepared from cells grown under the specific conditions. For full activation of the *strR* transcription, AdpA is required to occupy the two sites upstream from the *strR* promoter, at approximately nucleotide positions –270 and –50 (Tomono *et al.*, 2005). AdpA recruits RNA polymerase to the promoter region of *strR*, and perhaps facilitates the isomerization of the RNA polymerase–DNA complex into an open complex competent for transcriptional initiation. In this process, we speculate that the DNA sequence upstream from the *strR* promoter bends so that the two molecules of AdpA dimer bound at positions –270 and –50 become accessible to the promoter region to recruit RNA polymerase. In fact, we showed that the DNA fragment upstream from the *strR* promoter contains multiple bent sites even in the absence of any DNA-binding proteins (Vujaklija *et al.*, 1993). Because an AdpA dimer does bind the two AdpA-binding sites simultaneously with AtrA-g in such a way that AtrA-g sits between the two AdpA dimers, we speculate that AtrA-g causes further DNA bending so that the AdpA dimer bound to the site at position –270 can interact with the AdpA dimer bound to the site at position –50. For full transcriptional activation of *strR*, the two AdpA-binding sites should be occupied by AdpA (Tomono *et al.*, 2005), which suggests that the two AdpA dimers should interact with each other to assist RNA polymerase in initiating the transcription of *strR*. Uguru *et al.* (2005) also speculated a DNA bending caused by AtrA-c for transcriptional activation of *actII-ORF4* (see below).

AtrA-c binds two sites in the flanking region of the *actII-ORF4* promoter at nucleotide positions –162 and +86, both of which are not usual as the binding sites of transcriptional activators (Uguru *et al.*, 2005). Concerning the transcriptional activation of *actII-ORF4* by AtrA-c, Uguru *et al.* (2005) speculated that the DNA-bound AtrA-c might induce DNA bending in such a way that another transcriptional factor bound in the vicinity of the AtrA-c-binding site can readily make an interaction with RNA polymerase. AtrA-g bound the region upstream from the *strR* promoter at approximately position –130, which is also unusual for a transcriptional activator. Considering (i) the AtrA-g-binding site relative to the two AdpA-binding sites, (ii) simultaneous binding of AdpA and AtrA-g to the respective binding sites, and (iii) a conditionally positive effect of AtrA-g on streptomycin production, we assume that AtrA-g helps AdpA to initiate transcription of *strR* in an unknown way. A speculative mechanism by which AtrA-g helps AdpA in transcriptional activation of *strR* is DNA bending, as described above. Further analyses of AtrA-c in activation of the *actII-ORF4* transcription and of AtrA-g in activation of the *strR* transcription will give us new insight into the function of the AtrA regulators for secondary metabolism.

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