Conditional Effect of the Deletion of *eshA* on Streptomycin Production in *Streptomyces griseus* IFO13350

Takeaki Tezuka, Yasuo Ohnishi* and Sueharu Horinouchi†

Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo,
Bunkyo-ku, Tokyo 113-8567, Japan

(Received Sep. 29, 2010 / Accepted Oct. 22, 2010 / Published Dec. 25, 2010)

The *eshA* gene was originally found to encode a protein required for the extension of sporogenic hyphae during submerged spore formation in *Streptomyces griseus* NRRL B-2682. An *eshA*-disrupted strain of *S. griseus* IFO13189 was reported to be conditionally deficient in streptomycin production and aerial mycelium formation. Our previous transcriptomic analyses indicated that AdpA, a global transcriptional regulator of morphological and physiological differentiation, induced *eshA* (*SGR1270*) transcription in *S. griseus* IFO13350. Here, we examined the transcriptional regulation of *eshA* by AdpA and the involvement of *eshA* in the morphological and physiological differentiation of *S. griseus* IFO13350. Transcriptional analysis by S1 nuclease mapping showed that *eshA* was transcribed throughout growth on solid medium. In contrast, no *eshA* transcription was detected in an *adpA* deletion mutant. Recombinant His-tagged AdpA bound to a region upstream from the *eshA* promoter *in vitro*. However, mutation of the AdpA-binding sequence did not affect the transcription of *eshA* *in vivo*, indicating that AdpA indirectly activates *eshA* transcription. Streptomycin production by an *eshA* deletion mutant grown on TSB plates was lower than that of the wild-type strain. However, the *eshA* deletion mutant grew and formed aerial mycelia and spores following the same time course as the wild-type strain on various media.

INTRODUCTION

The morphological development and secondary metabolism of *Streptomyces griseus* are globally controlled by the chemical signaling molecule A-factor (2-isocaprolyl-3R-hydroxymethyl- γ -butyrolactone) (Horinouchi, 2007; Horinouchi & Beppu, 2007). AdpA, the central transcriptional regulator in the A-factor regulatory cascade, activates a number of genes required for morphological development and secondary metabolite formation, which together form an AdpA regulon (Ohnishi *et al.*, 2005). We previously determined the complete genomic sequence of *S. griseus* IFO13350, and, by DNA microarray analysis, the effects of AdpA on global gene expression (Ohnishi *et al.*, 2008). A comparison of the transcriptomes of the wild-type strain and an *adpA* deletion ($\Delta adpA$) mutant predicted *eshA* (extension of sporogenic hyphae; *SGR1270*) to be a possible AdpA-inducible gene (fold-change, 2.3; $p = 0.025$), which prompted us to examine the transcriptional regulation of *eshA* by AdpA in *S. griseus* IFO13350.

The *eshA* gene was originally found to encode a protein required for the extension of sporogenic hyphae during submerged spore formation in liquid cultures of *S. griseus* NRRL B-2682 (Kwak *et al.*, 2001). Subsequent analyses revealed that EshA had positive effects on actinorhodin

production and aerial mycelium formation in *S. coelicolor* A3(2) 1147 (Kawamoto *et al.*, 2001; Saito *et al.*, 2006). An *eshA* disrupted strain accumulated lower levels of ppGpp than the corresponding wild-type strain (Saito *et al.*, 2006). EshA contains a cyclic nucleotide-binding domain that is indispensable for its activation of actinorhodin production (Saito *et al.*, 2006). An *eshA* mutant strain of *S. griseus* IFO13189 was found to be conditionally deficient in streptomycin production and aerial mycelium formation, and displayed a lower rate of DNA synthesis than the wild-type strain (Saito *et al.*, 2003). EshA localizes to the cytoplasm, where it forms large multimeric cube-like structures (Saito *et al.*, 2003). Though many studies have sought to characterize EshA genetically and biochemically, its functional roles across *Streptomyces* species have not yet been fully elucidated. We therefore examined the effect of deletion of *eshA* on morphogenesis and secondary metabolism in *S. griseus* IFO13350.

MATERIALS AND METHODS

General recombinant DNA studies

The restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes used in this study, were purchased from Takara Biochemicals. [α - 32 P]dCTP (110

*Corresponding author. Mailing address: Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan. Tel: 81 3 5841-5123; Fax: 81 3 5841-8021; E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp

†Deceased on 12th July 2009

TBq/mmol) (for DNA labeling with the *Bca*Best DNA labeling system [Takara Biochemicals]) and [γ - 32 P]ATP (220 TBq/mmol) (for 5'-end labeling with T4 polynucleotide kinase) were purchased from PerkinElmer. DNA was manipulated in *Streptomyces* (Kieser *et al.*, 2000) and *Escherichia coli* (Ausubel *et al.*, 1987; Maniatis *et al.*, 2001) using previously described protocols. PCR products were confirmed to be error-free by DNA sequencing following cloning into pUC19 vector. Nucleotide sequences were determined by the dideoxy chain-termination method using a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (GE Healthcare).

Bacterial strains, media and plasmids

S. griseus IFO13350 was obtained from the Institute of Fermentation (Osaka, Japan). The *S. griseus* mutant Δ *adpA* has been described previously (Ohnishi *et al.*, 1999). The *Streptomyces* strains were grown in YMPD medium. YMPD agar contained 2.2% agar (Tezuka *et al.*, 2009). R2YE medium (Kieser *et al.*, 2000) was used for protoplast regeneration. Bennett, SMM (Hirano *et al.*, 2008) and TSB (Saito *et al.*, 2003) media were also used in this study. Neomycin (20 μ g/ml) and thiostrepton (20 μ g/ml) were added when necessary. *E. coli* JM109 and the vector pUC19 (for use in DNA manipulation) were purchased from Takara Biochemicals. *E. coli* JM110 cells carrying *dam* and *dcm* mutations were used to prepare non-methylated *Streptomyces* DNA for gene disruption. His-tagged AdpA was purified from *E. coli* BL21(DE3) harboring pET-*adpA* as described previously (Yamazaki *et al.*, 2000). The media and growth conditions for *E. coli* culture were as described by Maniatis *et al.* (2001). Ampicillin (50 μ g/ml) and kanamycin (50 μ g/ml) were used when necessary.

Plasmid construction

For cloning of the DNA fragment containing the *eshA* coding sequence and its upstream region, we first amplified this region by PCR using the *S. griseus* chromosome as template and the primers GF (5'-CCGGAATTCTCTACCTGTTCCGCGCGTTCGAC-3' [*Eco*RI site underlined]) and ACR (5'-GCCAAGCTTTGGACTCCTTGATGAGGTGTG-3' [*Hind*III site underlined]). The amplified fragment was digested with *Eco*RI and *Hind*III and cloned between the *Eco*RI and *Hind*III sites in pUC19, generating pUC-*eshAwt*. The *Eco*RI-*Hind*III fragment was then excised from pUC-*eshAwt* and cloned into the same sites in the integration vector pTYM19 (Onaka *et al.*, 2003), yielding pTYM-*eshAwt*.

A mutation was introduced into AdpA-binding site 2, located upstream from the *eshA* promoter. The sequence CTGAAA in site 2 was replaced with the *Bam*HI cleavage sequence GGATCC by PCR using pUC-*eshAwt* as the template (see Fig. 2A). A 140-bp DNA fragment containing the region upstream of the AdpA-binding site 2 was amplified with the primers GF and M2-R (5'-CGGGATCC-

CCGTGATCGAGGAGGGCTTT-3' [*Bam*HI site underlined]), and then digested with *Eco*RI and *Bam*HI. Separately, a 1,733-bp DNA fragment containing the region downstream of the AdpA-binding site 2 was amplified with the primers M2-F (5'-CGGGATCCGCGTTCGGCCCGGGGGCAAA-3' [*Bam*HI site underlined]) and ACR and then digested with *Bam*HI and *Hind*III. The 133-bp *Eco*RI-*Bam*HI fragment and the 1,726-bp *Bam*HI-*Hind*III fragment were inserted between the *Eco*RI and *Hind*III sites in pUC19 by three-fragment ligation, generating pUC-*eshAm2*. Finally, the 1,855-bp *Eco*RI-*Hind*III fragment was excised from pUC-*eshAm2* and cloned into the same sites in pTYM19, generating pTYM-*eshAm2*.

RNA isolation

Total RNA was isolated from cells grown at 28°C on cellophane on the surface of YMPD agar medium using ISOGEN (Nippon Gene) as described previously (Tezuka *et al.*, 2009).

S1 nuclease mapping

S1 nuclease mapping was performed as described by Bibb *et al.* (1986) and Kelemen *et al.* (1998). Hybridization probes were prepared by PCR with 32 P-labeled/unlabeled primer pairs. *hrdB*, which encodes the principal sigma factor of RNA polymerase, was used to determine the amount and purity of RNA used, as described previously (Ohnishi *et al.*, 1999). The PCR primers used for the *eshA* probe were AF (5'-TACGCGGGTGCTCTACCCTGTTC-3') and AR (5'-GAGGCATGCATAGCGGACATGAC-3'). The PCR primers used for the *eshB* probe were BF (5'-CACGTCGGTGAATCTCGTCAGCC-3') and BR (5'-TCACCAACGGACATCTGACGTCC-3'). Primers AR and BR were 5'-labeled with [γ - 32 P]ATP using T4 polynucleotide kinase prior to their use in PCR. Marker 10 (pBR322/*Msp*I digest, Nippon Gene) was 5'-labeled with [γ - 32 P]ATP and used as a DNA standard marker.

Gel mobility shift assay

The purification of His-tagged AdpA from *E. coli* BL21(DE3) cells and gel mobility shift assays were performed as described previously (Yamazaki *et al.*, 2000). The DNA fragments used as probes were amplified by PCR and 32 P-labeled with T4 polynucleotide kinase. All probes were designed to the region upstream of the *eshA* coding sequence.

Mutation of the AdpA-binding sequences by PCR

Mutations were introduced into the AdpA-binding sites by PCR. A 0.2-kb fragment (positions -232 to +10 relative to the transcription start site in *eshA*) was amplified by PCR using the primers GF and GR (5'-GCCAAGCTTCCAGATCCAGGGGAACACTAAGG-3' [*Hind*III site underlined]) using the *S. griseus* chromosome as template. The amplified fragment was digested with *Eco*RI and *Hind*III and cloned between the *Eco*RI and *Hind*III sites in pUC19, generating pUC-*eshAp*. The 252-bp *Eco*RI-*Hind*III fragment was

excised from pUC-*eshA* and used in gel mobility shift assays as an intact probe (probe W). The sequence CCGGAT in AdpA-binding site 1 was replaced with the *KpnI* cleavage sequence GGTACC by PCR using pUC-*eshA* as the template (see Fig. 2A). A 65-bp DNA fragment containing the region upstream of the AdpA-binding site 1 was amplified with primers GF and M1-R (5'-GGGGTACC-CCGGTCGGAGCGGGTGGGCG-3' [*KpnI* site underlined]) and digested with *EcoRI* and *KpnI*. A 205-bp DNA fragment containing the region downstream of the AdpA-binding site 1 was amplified with primers M1-F (5'-GGGGTACCTTCGCCGGGCATTCGCCGCT-3' [*KpnI* site underlined]) and GR and then digested with *KpnI* and *HindIII*. The 58-bp *EcoRI*-*KpnI* fragment and 198-bp *KpnI*-*HindIII* fragment were inserted between the *EcoRI* and *HindIII* sites in pUC19 by three-fragment ligation, generating pUC-*eshAm1*. The 252-bp *EcoRI*-*HindIII* fragment was then excised from pUC-*eshAm1* and used in gel mobility shift assays as the mutated probe M1. The six nucleotides in AdpA-binding sites 2 and 3 were similarly replaced with *BamHI* and *PstI* recognition sequences by PCR and used as the mutated probes M2 and M3, respectively (see Fig. 2A).

Gene disruption

To generate disruptants, most of the *eshA* or *eshB* coding sequence was deleted from the chromosome of *S. griseus*. Sequences from regions upstream and downstream of the coding region (each approximately 2 kb in length) were assembled in pUC19, together with the neomycin resistance gene *aphII*. The primers used for the *eshA* upstream region were AUF (5'-GCCAAGCTTCCCCGTCATGGTCGA-CAACGAC-3' [*HindIII* site underlined]) and AUR (5'-CGCGGATCCAACAGTCATCGGGCAGGCTCTCC-3' [*BamHI* site underlined]), while those for the *eshA* downstream region were ADF (5'-CGCGGATCCCAGATCGC-CAACTGGCCCAGGTAG-3' [*BamHI* site underlined]) and ADR (5'-CCGGAATTCGGTCCACGTACATGCTC-GACTCG-3' [*EcoRI* site underlined]). The primers used for the *eshB* upstream region were BUF (5'-TTTCCTGCAGGCCGAACACCTCGTAGTGGACGTC-3' [*Sse8387I* site underlined]) and BUR (5'-GCTCTAGAGCGAACCTCTT-CACCAACGGA-3' [*XbaI* site underlined]), while those for the *eshB* downstream region were BDF (5'-CGTCTAGAGTGCTGGAGAACGTCGAGGTCGG-3' [*XbaI* site underlined]) and BDR (5'-CCCTTGTTGTCCCGGTACCA-GCC-3'). The *aphII* gene cassette was inserted into the *HindIII* and *Sse8387I* sites on the *eshA* and *eshB* disruption plasmids, respectively. The plasmids were introduced by protoplast transformation into *S. griseus*, and neomycin-resistant transformants resulting from a single crossover of the plasmid into the chromosome were isolated. Neomycin-sensitive colonies, derived from a second crossover in one of the transformants, were identified as candidate *eshA*/*eshB* disruption strains. The correct replacements were confirmed by Southern hybridization using each gene and *aphII* as ³²P-labeled probes.

Streptomycin assay

The amount of streptomycin produced was measured in a bioassay in which *Bacillus subtilis* was used as the indicator (Horinouchi *et al.*, 1984).

RESULTS

Expression profiles of *eshA* and *eshB* in the wild-type and *adpA*-disrupted strains

A comparison of the transcriptomes in the wild-type and Δ *adpA* strains grown in YMPD liquid medium identified *eshA* (SGR1270) as a putative AdpA-inducible gene (fold-change, 2.3; $p = 0.025$). *S. griseus* IFO13350 has an *eshA* homolog, *eshB* (SGR2264). The amino acid sequence of EshB shows 64% identity with that of EshA. Because the transcription of *eshB* was suggested in the same analysis to be induced by AdpA (fold-change, 2.0; $p = 0.026$), we examined the transcription of *eshA* and *eshB* in the wild-type and Δ *adpA* strains by low-resolution S1 nuclease mapping. For this analysis, total RNA was isolated from *S. griseus* cells grown on YMPD agar at 28°C for 24, 48 and 72 h. On YMPD solid medium, the wild-type strain grew as substrate mycelia at 24 h, as a mixture of substrate and aerial mycelia at 48 h, and as a mixture of substrate mycelia and aerial hyphae with spores at 72 h. The Δ *adpA* mutant grew as substrate mycelia throughout the time course. In the wild-type strain, *eshA* transcription was detected throughout the time course; its expression appeared to decrease at 48 h, before increasing again at 72 h (Fig. 1). An additional transcript with a different 5'-end was also detected at 48 and 72 h. The approximate transcription start site in *eshA* (predicted from the larger band) agreed with the precise start point reported previously (Kwak *et al.*, 2001). The transcription of *eshB* was detected at 48 and 72 h, but not at 24 h. Importantly, in the Δ *adpA* mutant, the transcription of *eshA* and *eshB* was completely lost, suggesting the possible involvement of AdpA in the transcriptional activation of these two genes.

Effect of AdpA on *eshA* expression

We examined AdpA binding to the upstream region of *eshA* in a gel mobility shift assay using recombinant His-tagged AdpA purified from *E. coli*. The ³²P-labeled probe tested in this assay (positions -232 to +10 relative to the transcription start site in *eshA*), designated W, yielded a shifted band (Fig. 2A and B). When we searched region upstream from the *eshA* promoter for the AdpA-binding consensus sequence (5'-TGGCSNGWWY-3', where S = G or C; W = A or G; Y = T or C; N = any nucleotide) (Yamazaki *et al.*, 2004), allowing for up to three mismatches, we identified three possible sequences, which were denoted sites 1-3 (Fig. 2A). We introduced mutations into these three possible AdpA-binding sequences to replace the original six nucleotides with *KpnI*, *BamHI* and *PstI* recognition sequences, yielding the mutated probes M1, M2 and M3, respectively (Fig. 2A). The effect of these

Fig. 1. Time course of *eshA* and *eshB* transcription as determined by low-resolution S1 nuclease mapping. RNA was prepared from wild-type and $\Delta adpA$ cells grown at 28°C on cellophane on the surface of YMPD agar for the indicated time periods. *hrdB* was used as an internal control. SM, substrate mycelium; AM, aerial mycelium; SP, spore; wt, wild-type. The $\Delta adpA$ mutant grew only as substrate mycelia throughout the growth period.

mutations on AdpA binding was examined by a gel mobility shift assay. The M2 probe containing the mutation in site 2 produced no signal retardation, whereas the other two mutated probes yielded shifted bands that were similar to those produced by the intact probe (probe W). This result indicated that site 2 (positions –103 to –112 relative to the transcription start site in *eshA*) is important for the binding of AdpA to this region.

To evaluate the importance of the binding of AdpA to the upstream region of *eshA* for the transcription of *eshA* *in vivo*, *eshA* with a mutation in site 2 was integrated into the chromosome of an *eshA* deletion mutant using the integration vector pTYM19 (the *eshA* deletion mutant is described below). The *eshA* gene with an intact site 2 was also integrated into the chromosome as a control. RNA was extracted from $\Delta eshA$ mutants carrying the wild-type and site 2-mutated forms of *eshA*, which were grown for 24, 48 and 72 h on solid YMPD medium, and were then analyzed for *eshA* expression by S1 nuclease mapping. Contrary to our expectation, the levels of *eshA* transcription were similar in the two strains (Fig. 2C). This result indicated that the binding of AdpA to the upstream region of *eshA* did not have a significant effect on *eshA* transcription. There-

Fig. 2. AdpA binding to the region upstream from the *eshA* promoter. A) Schematic representation of the possible AdpA-binding sites in the region upstream of *eshA*, the probes used in B), and the mutations introduced into each site. Mutations were introduced into the putative AdpA-binding sequences (sites 1–3) by PCR to replace the original six nucleotides with *Kpn*I, *Bam*HI and *Pst*I recognition sites, respectively. The putative AdpA-binding sequence in each site is underlined. B) A gel mobility shift assay was performed using purified AdpA and 32 P-labeled DNA probes. Probe W, containing the intact sequence of the upstream region of the *eshA* gene (from –232 to +10), was used. The following amounts of AdpA were used: 0.2 μ g (lane 2) and 0.4 μ g (lane 3). Lane 1: negative control (no AdpA). Three mutated probes (M1, M2 and M3) were also used. C) Time course of *eshA* transcription as determined by low-resolution S1 nuclease mapping with RNA prepared from $\Delta eshA$ mutant cells harboring pTYM-*eshAw1* or pTYM-*eshAm2* and grown at 28°C on cellophane on the surface of YMPD agar for the indicated time periods. *hrdB* transcription was assessed as an internal control.

fore, we conclude that AdpA does not directly activate *eshA*. The molecular mechanism underlying the absence of *eshA* transcription in the $\Delta adpA$ mutant remains to be elucidated.

Functional analysis of *eshA* and *eshB*

To investigate the *in vivo* function of *eshA*, most of the *eshA* coding sequence was deleted without inserting a marker gene to avoid possible polar effects on the expression of the flanking open reading frames. Correct disruption of the gene was confirmed by Southern hybridization using appropriate probes (data not shown). First, the *eshA* deletion mutant ($\Delta eshA$) was incubated at 28°C on various media (YMPD, R2YE, SMM, Bennett and TSB agar). No difference in morphological differentiation was observed between the wild-type and $\Delta eshA$ strains. Next, we examined the production of streptomycin, a representative secondary metabolite produced by *S. griseus*, on glucose-depleted Bennett agar and TSB agar, using *B. subtilis* as an indicator. The growth-inhibition zones surrounding the five- and six-day-old $\Delta eshA$ colonies on TSB plates were smaller than those of the wild-type strain (Fig. 3). We repeated this assay three times and calculated the amount of streptomycin produced. The wild-type strain produced 3.0 ± 0 and $8.7 \pm 0.1 \mu\text{g}$ of streptomycin per colony on days 5 and 6, respectively, whereas the $\Delta eshA$ mutant produced only 1.1 ± 0 and $1.7 \pm 0.1 \mu\text{g}$ of streptomycin per colony on days 5 and 6, respectively. The reduced streptomycin production by the $\Delta eshA$ mutant was almost completely compensated by the integration of *eshA* with its own promoter into the *S. griseus* $\Delta eshA$ chromosome. Mutant $\Delta eshA$ harboring pTYM19-*eshA*_{wt} produced 2.4 ± 0 and $6.2 \pm 0.1 \mu\text{g}$ of streptomycin per colony on days 5 and 6, respectively ($n = 3$). In contrast, no differences in streptomycin production were observed between the wild-type and $\Delta eshA$ strains on glucose-depleted Bennett agar. These data showed that EshA exerts a positive, but conditional, effect on streptomycin production.

In addition to the $\Delta eshA$ mutant, we generated an *eshB* deletion ($\Delta eshB$) mutant and a double ($\Delta eshA\Delta eshB$) mutant. However, no phenotypic differences were observed

between the wild-type and $\Delta eshB$ strains, or between the $\Delta eshA$ and $\Delta eshA\Delta eshB$ mutants. These results indicated that EshB exerts no apparent effect on morphological differentiation or secondary metabolite formation, at least under the culture conditions used in this study.

DISCUSSION

In this study, we examined the transcriptional regulation of *eshA* by AdpA and the involvement of *eshA* in the morphological and physiological development of *S. griseus* IFO13350. Contrary to our expectation, the binding of AdpA to the upstream region of *eshA* was not responsible for the transcriptional activation of *eshA*. It is noteworthy that the affinity of AdpA for the upstream region of *eshA* was low compared with the affinities of AdpA to other functional AdpA-binding sites. However, the affinity of AdpA to an AdpA-binding site does not necessarily represent the importance of the AdpA-binding site in transcriptional regulation of its neighboring gene. For example, although the affinity of AdpA to the upstream region of *adsA* is low, AdpA-binding to this site is essential for the transcriptional activation of *adsA* (Yamazaki *et al.*, 2000). Therefore, it was necessary to examine the importance of the binding of AdpA to the upstream region of *eshA* by mutational analysis. Recently, we also found that the binding of AdpA to a region upstream from the *SGR2418* (*bldK*) promoter was not responsible for the activation of that promoter (Akanuma *et al.*, manuscript submitted). These results suggested that the *S. griseus* chromosome contains many AdpA-binding sites with little or no role in the transcriptional regulation of neighboring genes. The suspected involvement of AdpA binding in the activation of a putative target promoter should therefore be confirmed by mutational analyses.

Saito *et al.* (2003) found that the disruption of *eshA* abolished aerial mycelium formation in *S. griseus* IFO13189 grown on TSB agar. However, this phenotype was not observed in *S. griseus* IFO13350. Although it has been reported that EshA is only produced during submerged spore formation in *S. griseus* NRRL B-2682 (Kwak *et al.*, 2001) and during late exponential growth and stationary phase in *S. griseus* IFO13189 (Kawamoto *et al.*, 2001), we detected *eshA* transcription during vegetative growth in *S. griseus* IFO13350 on YMPD agar (Fig. 1). In *S. griseus* IFO13350 grown in liquid YMPD medium, *eshA* transcription was also detected at 24 (mid-exponential growth phase) and 48 h (transition phase) (data not shown). These conflicting findings may be ascribed to inter-strain differences. The fact that *S. griseus* IFO13189 has a greater ability to form aerial mycelium and spore than *S. griseus* IFO13350 may be the cause of the phenotypic differences observed between these two strains.

Streptomycin production was markedly reduced in the $\Delta eshA$ mutant grown on TSB agar. We assume that this is an indirect effect of the *eshA* deletion, because the difference in

Fig. 3. Streptomycin production by the $\Delta eshA$ mutant. Wild-type and $\Delta eshA$ cells were grown at 28°C on TSB agar for the indicated number of days, and were then overlaid with soft agar containing *B. subtilis* spores. Following incubation of the plates overnight at 28°C, streptomycin production was detected as the zone of growth inhibition of the indicator surrounding each colony. wt, wild-type.

streptomycin production between the wild-type and Δ eshA strains was observed only on TSB agar and not replicated when the two strains were grown on glucose-depleted Bennett agar. Although the TSB agar used in this study contained 10.3% sucrose and the glucose-depleted Bennett agar contained no sucrose, high osmotic pressure seemed not to be a critical factor in the phenotypic difference between the wild-type and Δ eshA strains on TSB agar; both strains produced almost the same amount of streptomycin on glucose-depleted Bennett agar containing 10.3% sucrose (data not shown). Glucose-depleted Bennett agar is used routinely in streptomycin production assays in our laboratory. Saito *et al.* reported that in *S. coelicolor* A3(2), an *eshA* disruptant accumulated lower levels of ppGpp than the parent strain (Saito *et al.*, 2006). The reduced streptomycin production by the Δ eshA strain on TSB agar may also be ascribed to lower levels of ppGpp in *S. griseus*.

Close homologs of *eshA* are widely distributed among *Streptomyces* species. A BLAST search using the *S. griseus* EshA primary sequence to query 24 *Streptomyces* genomic sequences registered in the NCBI genome database revealed that all of them contained at least one close homolog of *eshA*. The broad distribution of the *eshA* gene among *Streptomyces* species suggests that *eshA* has an important function, probably in the morphological and physiological differentiation of members of this genus. However, the loss of *eshA* function appears not to cause any apparent phenotypic changes under normal growth conditions. Further biochemical characterization of EshA may provide clues as to the culture conditions under which the effect of the deletion of *eshA* results in dramatic phenotypic changes.

ACKNOWLEDGMENTS

T. Tezuka was supported by the Japan Society for the Promotion of Science.

REFERENCES

- Ausubel, F.M., Brent, R., Kingstone, R.E., Moore, D.O., Seidman, J.S., Smith, J.A. and Struhl, K. (1987). Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
- Bibb, M.J., Janssen, G.R. and Ward, J.M. (1986). Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. *Gene* **41**, 357–368.
- Hirano, S., Tanaka, K., Ohnishi, Y. and Horinouchi, S. (2008). Conditionally positive effect of the TetR-family transcriptional regulator AtrA on streptomycin production by *Streptomyces griseus*. *Microbiology* **154**, 905–914.
- Horinouchi, S. (2007). Mining and polishing of the treasure trove in the bacterial genus *Streptomyces*. *Biosci. Biotechnol. Biochem.* **71**, 283–299.
- Horinouchi, S. and Beppu, T. (2007). Hormonal control by A-factor of morphological development and secondary metabolism in *Streptomyces*. *Proc. Jpn. Acad. Ser. B.* **83**, 277–295.
- Horinouchi, S., Kumada, Y. and Beppu, T. (1984). Unstable genetic determinant of A-factor biosynthesis in streptomycin-producing organisms: cloning and characterization. *J. Bacteriol.* **158**, 481–487.
- Kawamoto, S., Watanabe, M., Saito, N., Hesketh, A., Vachalova, K., Matsubara, K. and Ochi, K. (2001). Molecular and functional analyses of the gene (*eshA*) encoding the 52-kilodalton protein of *Streptomyces coelicolor* A3(2) required for antibiotic production. *J. Bacteriol.* **183**, 6009–6016.
- Kelemen, G.H., Brian, P., Flärdh, K., Chamberlin, L., Chater, K.F. and Buttner, M.J. (1998). Developmental regulation of transcription of *whiE*, a locus specifying the polyketide spore pigment in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **180**, 2515–2521.
- Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics. Norwich, UK: The John Innes Foundation.
- Kwak, J., McCue, L.A., Trczianka, K. and Kendrick, K.E. (2001). Identification and characterization of a developmentally regulated protein, EshA, required for sporogenic hyphal branches in *Streptomyces griseus*. *J. Bacteriol.* **183**, 3004–3015.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Ohnishi, Y., Ishikawa, J., Hara, H., Suzuki, H., Ikenoya, M., Ikeda, H., Yamashita, A., Hattori, M. and Horinouchi, S. (2008). Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J. Bacteriol.* **190**, 4050–4060.
- Ohnishi, Y., Kameyama, S., Onaka, H. and Horinouchi, S. (1999). The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* **34**, 102–111.
- Ohnishi, Y., Yamazaki, H., Kato, J.Y., Tomono, A. and Horinouchi, S. (2005). AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* **69**, 431–439.
- Onaka, H., Taniguchi, S., Ikeda, H., Igarashi, Y. and Furumai, T. (2003). pTOYAMAcos, pTYM18, and pTYM19, actinomycete-*Escherichia coli* integration vectors for heterologous gene expression. *J. Antibiot.* **56**, 950–956.
- Saito, N., Matsubara, K., Watanabe, M., Kato, F. and Ochi, K. (2003). Genetic and biochemical characterization of EshA, a protein that forms large multimers and affects developmental processes in *Streptomyces griseus*. *J. Biol. Chem.* **278**, 5902–5911.
- Saito, N., Xu, J., Hosaka, T., Okamoto, S., Aoki, H., Bibb, M.J. and Ochi, K. (2006). EshA accentuates ppGpp accumulation and is conditionally required for antibiotic production in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **188**, 4952–4961.
- Tezuka, T., Hara, H., Ohnishi, Y. and Horinouchi, S. (2009). Identification and gene disruption of small noncoding RNAs in *Streptomyces griseus*. *J. Bacteriol.* **191**, 4896–4904.
- Yamazaki, H., Ohnishi, Y. and Horinouchi, S. (2000). An A-factor-dependent extracytoplasmic function sigma factor (σ^{AdfA}) that is essential for morphological development in *Streptomyces griseus*. *J. Bacteriol.* **182**, 4596–4605.
- Yamazaki, H., Tomono, A., Ohnishi, Y. and Horinouchi, S. (2004). DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* **53**, 555–572.