Bidirectional Regulation of AdpA_{Ch} in Controlling the Expression of *scnRI* and *scnRII* in the Natamycin Biosynthesis of *Streptomyces chattanoogensis* L10

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AdpA, an AraC/XylS family protein, had been proved as a key regulator for secondary metabolism and morphological differentiation in *Streptomyces griseus*. Here, we identify AdpA_{Ch}, an ortholog of AdpA, as a "higher level" pleiotropic regulator of natamycin biosynthesis with bidirectional regulatory ability in *Streptomyces chattanoogensis* L10. DNase I footprinting revealed six AdpA_{Ch}-binding sites in the *scnRI–scnRII* intergenic region. Further analysis using the *xylE* reporter gene fused to the *scnRI–scnRII* intergenic region of mutated binding sites demonstrated that the expression of *scnRI* and *scnRII* was under the control of AdpA_{Ch}. AdpA_{Ch} showed a bi-stable regulatory ability where it firstly binds to the Site C and Site D to activate the transcription of the two pathway-specific genes, *scnRI* and *scnRII*, and then binds to other sites where it acts as an inhibitor. When Site A and Site F were mutated *in vivo*, the production of natamycin was increased by 21% and 25%, respectively. These findings indicated an autoregulatory mechanism where AdpA_{Ch} serves as a master switch with bidirectional regulation for natamycin biosynthesis.

Keywords: bidirectional regulation, AdpA, natamycin biosynthesis, *Streptomyces chattanoogensis* L10, pathway-specific gene

INTRODUCTION

The secondary metabolic process in *Streptomyces* is regulated by a complex regulatory network involving pathway-specific, pleiotropic, and global regulators which respond to a variety of physiological and environmental condition alterations (van Wezel and McDowall, 2011; Liu et al., 2013). The best characterized is the A-factor regulatory cascade in which AdpA is the most important transcriptional factor for the secondary metabolism (Horinouchi, 2002; Ohnishi et al., 2005). In early culture stages, the transcription of *adpA* in *Streptomyces griseus* is repressed by ArpA, the receptor protein for A-factor (Onaka and Horinouchi, 1997). When A-factor reaches a critical concentration, it binds to ArpA and confers the conformational change of ArpA (Ohnishi et al., 1999). This results in dissociation of ArpA from the *adpA* promoter, in turn switching on the expression of *adpA* (Ohnishi et al., 1999). The induced AdpA then activates the transcription of

various genes related to secondary metabolism such as *strR*, the pathway-specific regulatory genes for streptomycin in *S. griseus* (Retzlaff and Distler, 1995; Tomono et al., 2005).

AdpA is a member of the AraC/XylS family proteins (Gallegos et al., 1997). It has been suggested to form a dimer through the N-terminal portion which belong to the ThiJ/PfpI/DJ-1 family (Yamazaki et al., 2004; Ohnishi et al., 2005). To date, a number of AdpA orthologs have been described as having essential roles in the secondary metabolism in many *Streptomyces* species, such as *Streptomyces lividans* (Guyet et al., 2013), *Streptomyces coelicolor* A3(2) (Takano et al., 2001; Nguyen et al., 2003), *Streptomyces ansochromogenes* (Pan et al., 2009), *Streptomyces avermitilis* (Komatsu et al., 2010), *Streptomyces hygroscopicus* 5008 (Tan et al., 2015), and *Streptomyces clavuligerus* (López-García et al., 2010).

Typically, AdpA is regarded as an activator for downstream regulated genes, except itself which is proved to be negatively auto-regulated by binding to its own promoter region (Kato et al., 2005b; Hara et al., 2009). The molecular mechanism of transcriptional activation begins as a dimer of AdpA binds to the target sites with consensus sequences which then recruit RNA polymerase to the promoter for transcriptional initiation (Yamazaki et al., 2004; Kato et al., 2005a). For different target genes, AdpA showed a different number of binding sites in the promoter regions. For example, there are two AdpA-binding sites in the promoter of *strR* (Tomono et al., 2005), whereas there are three AdpA-binding sites for regulation of *ssgA* (Yamazaki et al., 2003a). However, the precise regulation mechanism how the AdpA binds to multiple sites to activate transcription has not been experimentally determined. Based on the importance of AdpA in the biosynthesis of the secondary metabolism, it is necessary to elucidate details of its regulatory mechanisms.

Natamycin, an antifungal polyene macrolide antibiotic, is synthesized by a type I polyketide synthase gene cluster. Previous analysis of the gene cluster of natamycin in *Streptomyces chattanoogensis* L10 revealed the existence of 17 open-reading frames, including two pathway-specific genes, *scnRI* and *scnRII* (Du et al., 2011a). These two genes showed high sequence identity to *pimR* and *pimM* of *Streptomyces natalensis*, respectively (Antón et al., 2007; Santos-Aberturas et al., 2012). Gene disruption of *scnRI* resulted in a large decrease in the expression of biosynthetic genes, indicating its role as a pivotal activator for the biosynthesis of natamycin (Du et al., 2011a). *scnRII*, adjacent but divergently transcribed transcriptional regulatory genes, was shown to act as a second positive regulator for natamycin production (Du et al., 2009). We also had proved that AdpAch controls the production of natamycin, but the detailed relationship among AdpAch, ScnRI, and ScnRII had not been well characterized (Du et al., 2011a).

Here, we reveal the sophisticated regulatory characteristics of AdpAch in the natamycin biosynthesis of *S. chattanoogensis* L10. AdpAch acts as a "higher level" pleiotropic regulator for transcription of the two divergently transcribed pathway-specific genes, *scnRI* and *scnRII*. In this regulatory process, AdpAch shows a bi-stable regulatory ability, where it firstly acts as an activator,

then a repressor. Moreover, natamycin production was enhanced by mutating the AdpAch-binding sites which had an inhibitory effect. This work not only advances the understanding of detailed regulatory mechanism of AdpA, but also provides a potential target for the enhancement of other antibiotic production levels by manipulating the regulatory network.

RESULTS

AdpAch Identified as a "Higher Level" Pleiotropic Regulator for Natamycin Biosynthesis

In our previous study, the biosynthetic gene cluster of natamycin has been cloned and characterized in *S. chattanoogensis* L10. Within this there are two divergently transcribed genes, *scnRI* and *scnRII*, encoding proteins that resemble pathway-specific regulators (Du et al., 2009, 2011a). Although the functions of these two regulators have been well characterized, an important question remains as to whether there are multiple levels of control in the biosynthesis of natamycin. Based on our previous study that AdpAch affected the transcription of these two pathway-specific genes (Du et al., 2011a), we speculated that AdpAch may act as a "higher level" pleiotropic regulator for regulating the natamycin biosynthesis.

To test this hypothesis, electrophoretic mobility shift assays (EMSAs) were applied. As shown in **Figure 1**, retardation was readily detected upon the addition of 50 pM AdpAch with the probe RI–RII, while the addition of 50- to 100-fold excess of unlabeled specific PCR product reduced the proportion of the labeled promoter-containing fragment (**Figure 1**). These data clearly demonstrate that AdpAch could specifically bind to the *scnRI–scnRII* intergenic region and could control the expression of these two pathway-specific genes.
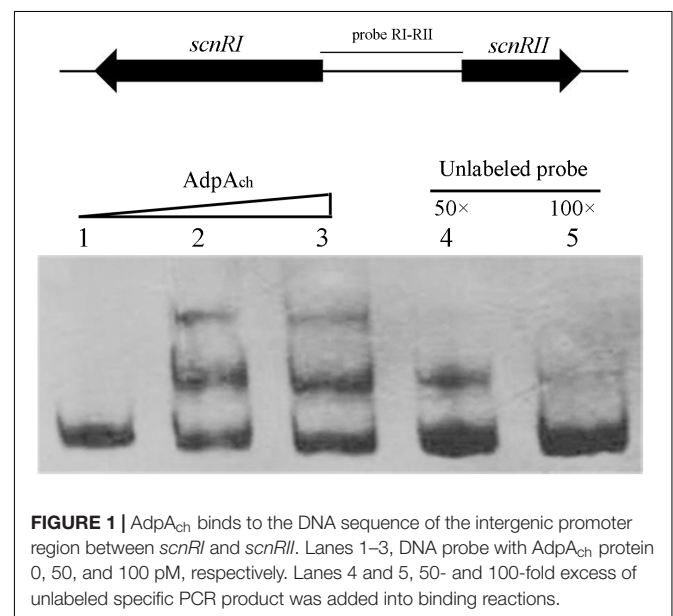

FIGURE 1 | AdpAch binds to the DNA sequence of the intergenic promoter region between *scnRI* and *scnRII*. Lanes 1–3, DNA probe with AdpAch protein 0, 50, and 100 pM, respectively. Lanes 4 and 5, 50- and 100-fold excess of unlabeled specific PCR product was added into binding reactions.
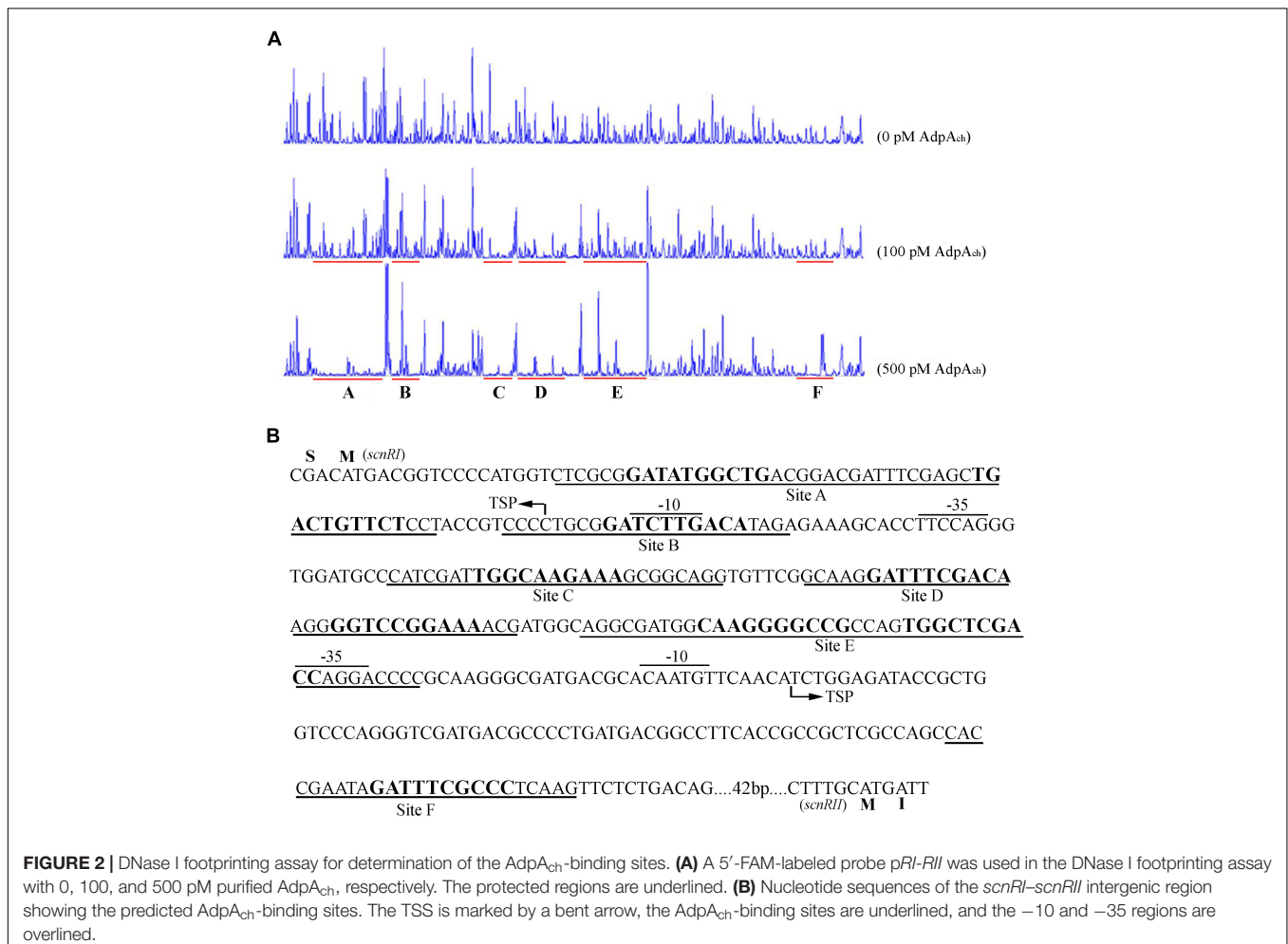
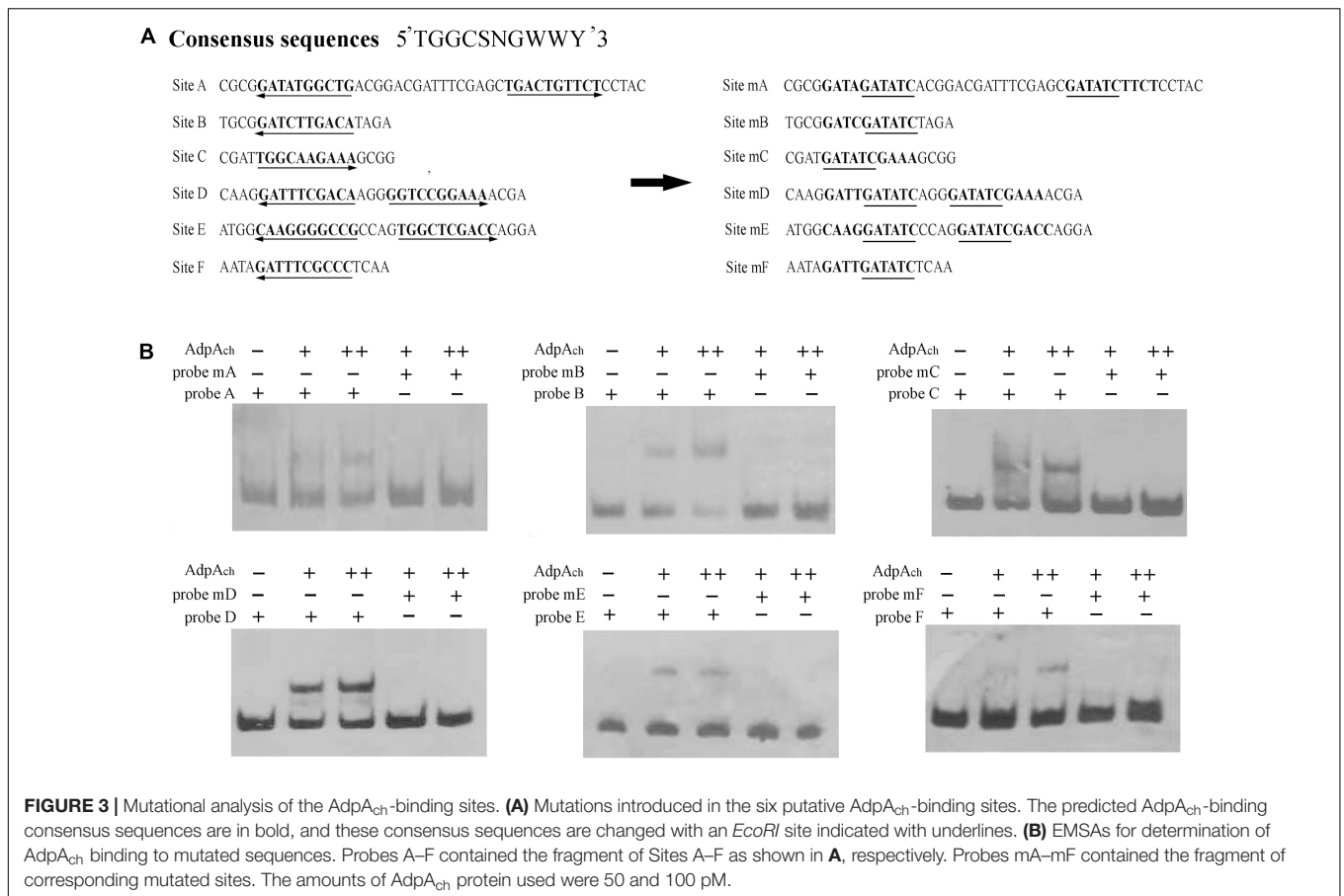## DNase I Footprinting Assay Reveals Six AdpA$_{ch}$-Binding Sites in the *scnRI–scnRII* Intergenic Region

To identify the exact DNA sequences that AdpA$_{ch}$ protected in the *scnRI–scnRII* intergenic region, DNase I footprinting assays, in absence or presence of purified recombinant AdpA$_{ch}$, were performed. In our previous studies, we had determined the transcription start site (TSS) of the two pathway-specific genes, *scnRI* and *scnRII* (Du et al., 2011a). As seen in **Figure 2A**, at a lower AdpA$_{ch}$ protein concentration of 100 pM, the DNA strands of the *scnRI–scnRII* intergenic region showed two protected regions, Site C and Site D, extending from positions −69 to −44 and −106 to −74 relative to the TSS of *scnRI*. When increasing the protein concentration to 500 pM, another four protected regions (Sites A, B, E, and F) were observed. With respect to the *scnRI* TSS, the AdpA$_{ch}$-binding Site A locates at positions +8 to +54, Site B at positions −20 to +2, Site E at positions −161 to −114, and Site F at positions −283 to −259 (**Figure 2B**). The six AdpA$_{ch}$-binding sites were spread over the *scnRI–scnRII* intergenic region. Notably, Site A was located downstream of the *scnRI* TSS, while Site B overlapped the −10 region of the *scnRI* promoter. Site F was located downstream of the *scnRII* TSS,

and Site E overlapped the −35 region of the *scnRII* promoter. This data suggest that AdpA$_{ch}$ might have a negative regulatory ability for the expression of these two pathway-specific genes. Additionally, the results from the DNase I footprinting assay also reveal that AdpA$_{ch}$ may have higher affinity to Site C and Site D than to the others.

The Consensus AdpA$_{ch}$-Binding Sequence in the AdpA$_{ch}$-Binding Sites

The orthologs of AdpA$_{ch}$ identified in *S. griseus* and *S. coelicolor* have been reported to have the consensus binding sequence, 5-TGGCSNGWWY-3 (S: G or C; W: A or T; Y: T or C; N: any nucleotide) (Yamazaki et al., 2004). After alignment of these six protected regions, we also found that there were highly conserved AdpA$_{ch}$-binding sequences in each binding site (**Figure 3A**). To further study the roles of these consensus sequences in the AdpA$_{ch}$-binding ability, EMSAs were carried out using the probes containing either the sequences of wild-type (wt) binding sites or the mutated sites (**Figure 3A**). As shown in **Figure 3B**, no binding shift was detected for the mutated sites A–F when compared with their corresponding wt targets. Taken together, these data demonstrated that AdpA$_{ch}$ indeed

FIGURE 2 | DNase I footprinting assay for determination of the AdpA$_{ch}$-binding sites. **(A)** A 5′-FAM-labeled probe p*RI-RII* was used in the DNase I footprinting assay with 0, 100, and 500 pM purified AdpA$_{ch}$, respectively. The protected regions are underlined. **(B)** Nucleotide sequences of the *scnRI–scnRII* intergenic region showing the predicted AdpA$_{ch}$-binding sites. The TSS is marked by a bent arrow, the AdpA$_{ch}$-binding sites are underlined, and the −10 and −35 regions are overlined.

FIGURE 3 | Mutational analysis of the $AdpA_{ch}$-binding sites. **(A)** Mutations introduced in the six putative $AdpA_{ch}$-binding sites. The predicted $AdpA_{ch}$-binding consensus sequences are in bold, and these consensus sequences are changed with an *EcoRI* site indicated with underlines. **(B)** EMSAs for determination of $AdpA_{ch}$ binding to mutated sequences. Probes A–F contained the fragment of Sites A–F as shown in **A**, respectively. Probes mA–mF contained the fragment of corresponding mutated sites. The amounts of $AdpA_{ch}$ protein used were 50 and 100 pM.

has six binding sites in the *scnRI–scnRII* intergenic region and the consensus sequence is essential for the binding activity of $AdpA_{ch}$.
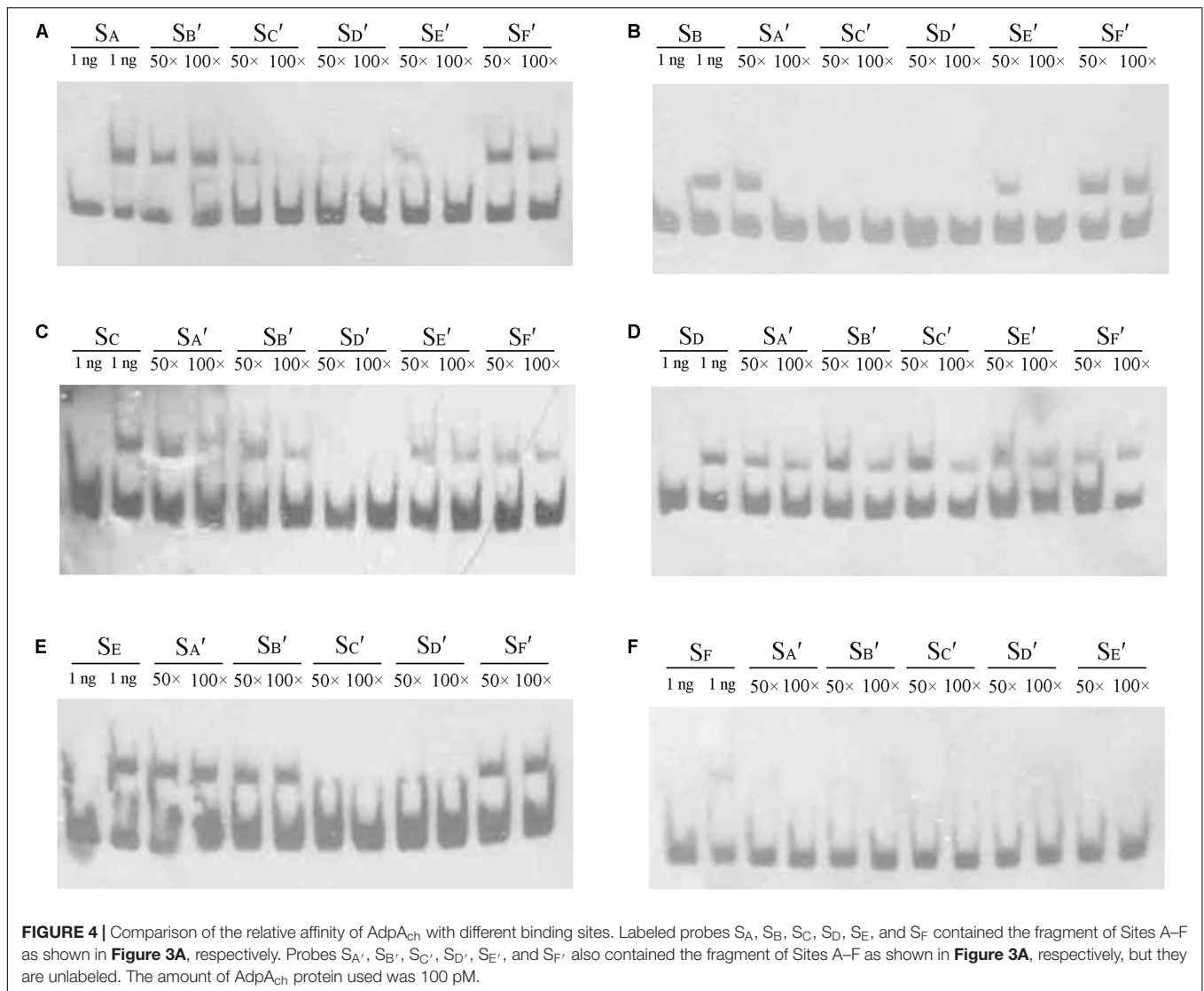
$AdpA_{ch}$ Has Differing Affinities for Different Binding Sites

In the DNase I footprinting analysis, Site C and Site D were occupied with a lower concentration of $AdpA_{ch}$ than the other sites. This suggests that there may be affinity differences for $AdpA_{ch}$ between the six binding sites. To test this possibility, competitive EMSAs with 50- to 100-fold excess of unlabeled fragments of six $AdpA_{ch}$-binding sites were used to compete with each labeled fragment. As shown in **Figure 4A**, 100-fold excess of unlabeled S_B' (Site B) and S_F' (Site F) could not completely abolish $AdpA_{ch}$ complex formation with the labeled probe S_A (Site A). However, the same amount of unlabeled S_C' (Site C), S_D' (Site D), and S_E' (Site E) outcompeted the labeled probe S_A. This result indicated that $AdpA_{ch}$ binds to Site A more tightly than Site B and Site F, but less tightly than Site C, Site D, and Site E. Following this way, we could conclude that Site B has less affinity for $AdpA_{ch}$ than others, except for Site F (**Figure 4B**), which was the weakest affinity among the six binding sites (**Figure 4F**), and Site D was the strongest affinity of these six sites (**Figure 4D**). The affinity of Site E for $AdpA_{ch}$ was between that of Site C and Site A (**Figures 4A,C,E**). Therefore, we determined the affinity

of $AdpA_{ch}$ to different binding sites in the following order: Site D > Site C > Site E > Site A > Site B > Site F.

Promoter-Probe Assays of the $AdpA_{ch}$-Binding Sites in the *scnRI–scnRII* Intergenic Region

The binding sites of $AdpA_{ch}$ in the *scnRI–scnRII* intergenic region were adjacent to either the *scnRI* or the *scnRII* start codon. This raised the possibility that this intergenic region might harbor a bidirectional promoter allowing $AdpA_{ch}$ to regulate transcriptions of the divergently transcribed flanking genes, *scnRI* and *scnRII* (**Figure 2B**). To investigate the promoter activities of the two pathway-specific genes with each of the $AdpA_{ch}$-binding sites, we used the promoter-probe plasmid pIJ8601 carrying the *xylE* gene, encoding catechol 2,3-dioxygenase, as the reporter. As shown in **Figure 5A**, the transcriptional profiles of *scnRI* were severely decreased when the $AdpA_{ch}$-binding Site C and Site D were mutated. Conversely, its transcriptional activity was increased when Site A and Site B were mutated and remained almost unchanged when Site E and Site F were mutated. For the promoter activity of *scnRII*, we did not detect any consistent differences when Sites A, B, and C were mutated, but mutation in the Sites D and E resulted in a large decreases of up to 70 and 40%, respectively, compared to those of the wt. The mutation in Site F resulted in a statistically significant increase

FIGURE 4 | Comparison of the relative affinity of AdpA$_{ch}$ with different binding sites. Labeled probes S$_A$, S$_B$, S$_C$, S$_D$, S$_E$, and S$_F$ contained the fragment of Sites A–F as shown in **Figure 3A**, respectively. Probes S$_{A'}$, S$_{B'}$, S$_{C'}$, S$_{D'}$, S$_{E'}$, and S$_{F'}$ also contained the fragment of Sites A–F as shown in **Figure 3A**, respectively, but they are unlabeled. The amount of AdpA$_{ch}$ protein used was 100 pM.

(**Figure 5B**). These findings indicated that expressions of *scnRI* and *scnRII* are both under the control of AdpA$_{ch}$, which has a completely different regulatory ability (activation or inhibition) when binding to different binding sites.
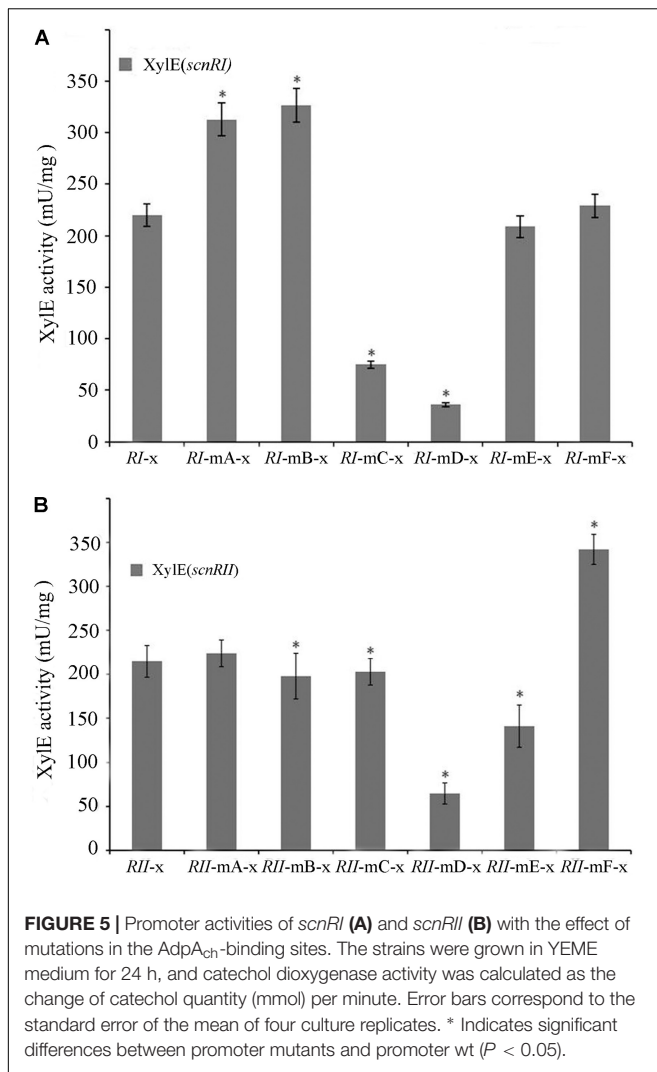
Effect of Mutated AdpA$_{ch}$-Binding Sites on Natamycin Production *in Vivo*

There have been some reports where effects upon DNA-binding sites were found *in vitro* that failed to be exhibited *in vivo*. In order to test this possibility and reveal the function of the six AdpA$_{ch}$-binding sites in natamycin biosynthesis *in vivo*, a series of mutants were constructed as described in Experimental procedures. As shown in **Figure 6A**, compared to the WT strain, the level of natamycin production in the R-mA (mutation in Site A) and R-mF (mutation in Site F) had increased by 21 and 25%, respectively. However, the constructed strains of R-mC (mutation in Site C), R-mD (mutation in Site D), and R-mE (mutation in Site E) showed up to 31, 42 and 15% reductions,

respectively. The natamycin production of R-mB (mutation in Site B) mutant exhibited almost no change. This finding indicated that the AdpA$_{ch}$-binding Sites A and F play negative roles for natamycin biosynthesis, while the functions of the Sites C, D, and E were positive. Quantitative real-time PCR (qRT-PCR) analysis showed that the promoting effect of site mutation on natamycin production was due to alteration of the pathway-specific genes at the transcriptional level (**Figure 6B**).

DISCUSSION

Streptomyces spp. have developed complicated mechanisms to adapt to altered circumstances (Santos-Beneit et al., 2009; Yu et al., 2012). Among these mechanisms, the multiple levels of regulation in controlling the expression of the genes responsible for the formation of the secondary metabolism are drawing increased attention. In this study, we focused on the regulatory network of natamycin biosynthesis in *S. chattanoogensis* L10,

FIGURE 5 | Promoter activities of *scnRI* **(A)** and *scnRII* **(B)** with the effect of mutations in the AdpA$_{ch}$-binding sites. The strains were grown in YEME medium for 24 h, and catechol dioxygenase activity was calculated as the change of catechol quantity (mmol) per minute. Error bars correspond to the standard error of the mean of four culture replicates. * Indicates significant differences between promoter mutants and promoter wt ($P < 0.05$).

FIGURE 6 | (A) The effect of mutated AdpA$_{ch}$-binding sites on the natamycin production *in vivo*. The strains were grown in YEME medium for 96 h. Vertical error bars correspond to the standard error of the mean of four replicated cultures. **(B)** Real-time RT-PCR analysis of the *scnRI* and *scnRII* transcript in the wt strain and mutated AdpA$_{ch}$-binding sites strain. The expression level of *scnRI and scnRII* is presented relative to the wt sample from 24 h, which was arbitrarily assigned a value of 1. The transcription of *hrdB* was assayed as an internal control. Error bars were calculated by measuring the standard deviation among three replicates of each sample. * Indicates significant differences between AdpA$_{ch}$-binding site mutants and wt ($P < 0.05$).

an industrial strain for natamycin production. In our previous study, we determined that gamma-butyrolactones (GBLs) serve as quorum-sensing signaling molecules for activating natamycin production in *S. chattanoogensis* L10 (Du et al., 2011b), and ScnRII acts as a positive regulator by directly binding to the promoters of natamycin biosynthetic genes (Du et al., 2009) where ScnRI acts as a positive regulator for the transcription of *scnRII* (Du et al., 2011a). However, the deletion of *scnRI* did not result in a complete halt of the transcription of *scnRII* (our unpublished data). This is quite different from the function of PimR in *S. natalensis* where the deletion of *pimR* almost completely destroys the transcription of *pimM* (Antón et al., 2004; Santos-Aberturas et al., 2012). As the regulation of antibiotic biosynthesis involves numerous transcription factors (McKenzie and Nodwell, 2007; van Wezel and McDowall, 2011), participation of other regulator(s) is possible, in the regulation of *scnRII*.

With AdpA$_{ch}$ being able to regulate the expression of both of the pathway-specific genes, *scnRI* and *scnRII*, it provides a possible explanation that there is a coordinate regulation in
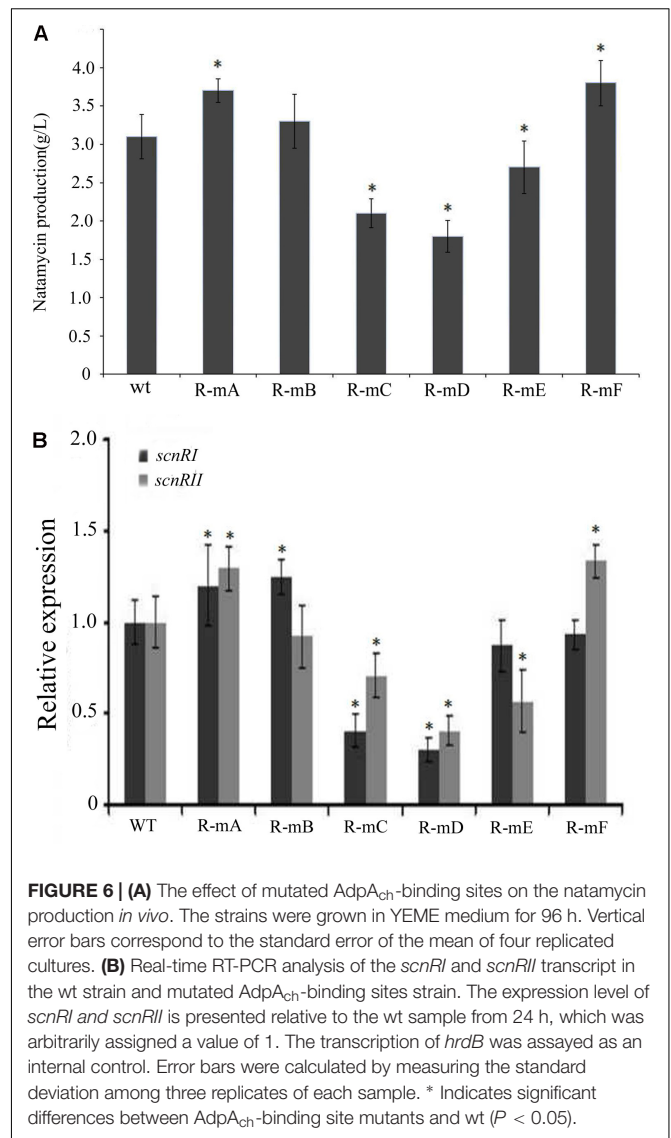
controlling expression of *scnRII* by AdpA$_{ch}$ and ScnRI. This regulatory pattern may occur in following steps. Firstly, AdpA$_{ch}$ binds to the *scnRI–scnRII* intergenic region and activates both transcription of *scnRI* and *scnRII*. Then ScnRI also binds to the *scnRI–scnRII* intergenic region which, in turn, promotes the transcriptional level of *scnRII*. However, these two genes were not completely controlled by AdpA$_{ch}$. Trace expression of *scnRI* was observed in the *adpA$_{ch}$* mutant, and then ScnRI would promote the transcription of *scnRII* (Du et al., 2011a). Notably, a certain amount of AdpA$_{ch}$ is required for binding to the *scnRI–scnRII* intergenic region (~50 pM). This is why we did not detect the shifted band with low concentration AdpA$_{ch}$ (~1 pM) in the binding reaction of our previous study (Du et al., 2011a).

In most cases, AdpA acts as an activator for the target genes, except for itself where it exhibits an autorepression (Kato et al., 2005b). In this study, we concluded from promoter-probe assays *in vivo* that AdpA$_{ch}$ could not only regulate both

pathway-specific genes, but also displayed completely opposite regulatory abilities in control of them. The AdpA_{ch}-binding Site C and Site D were involved in activating the transcription of *scnRI*, while AdpA_{ch} binding to Sites A and B resulted in repression. For the promoter activity of *scnRII*, mutation in the Site C and Site D resulted in a decrease of transcriptional profiles, while a mutation in the Site F led to a statistically significant increase. A similar phenotype was observed in *S. ansochromogenes* where transcription of *sanG* decreased when Site I and Site V were mutated but increased when other three AdpA-L-binding sites were mutated (Pan et al., 2009). However, when combinations of binding site mutations were carried out, the promoter activities were not in accordance with our predictions. For example, mutations in both Sites E and F reduced the transcriptional level of *scnRII* (data not shown). Based on the short distances between the AdpA_{ch}-binding sites which are spread over the *scnRI–scnRII* intergenic region, there may be complicated interactions between different AdpA_{ch} dimmers to explain this.

With further analysis using competitive gel shift assays, we could conclude that AdpA_{ch} binds to Sites A–F with the following affinities: Site D > Site C > Site E > Site A > Site B > Site F (**Figure 4**). These data are consistent with the footprinting assay where the regions of Site C and Site D were previously protected at a lower AdpA_{ch} protein concentration (**Figure 2A**). This gives a hint that the regulatory ability of AdpA_{ch} may occur in a growth phase-dependent manner. In the early stage, AdpA_{ch} firstly binds to the Site C and D to recruit RNA polymerase to the promoter and initiates the transcription of *scnRI* and *scnRII*. This in turn triggers natamycin production (**Figure 7**). When AdpA_{ch} is accumulated to a certain critical level, it will bind to other binding sites located near the TSS. A DNA loop may be formed via the interaction between different AdpA_{ch} dimmers, thus preventing RNA polymerase from access to the promoter of

the pathway-specific genes (**Figure 7**). Reduced transcription of the pathway-specific genes will result in a low rate of natamycin production.

The discovery of this bidirectional regulation of AdpA_{ch} in the control of natamycin biosynthesis reveals an artful adaptive mechanism in microbial cells. Microorganisms produce molecules with antibiotic activity and expel them into the environment, presumably enhancing their ability to compete with their neighbors (Berdy, 2005; Hopwood, 2007). However, most of these molecules are toxic to the producer (Mak et al., 2014; Moody, 2014). Mechanisms must exist to ensure that antibiotic production reaches a reasonable level. The proposed model of AdpA_{ch} in **Figure 7** may provide a fresh mechanistic insight into how *S. chattanoogensis* controls the production level of natamycin via AdpA_{ch}. However, further work will be needed to prove the proposed model and the detailed mechanism of how AdpA_{ch} responds to the signal of natamycin. In all, the complicated regulatory network involving AdpA_{ch}, ScnRI, and ScnRII helps advance our understanding of the molecular regulation mechanisms of antibiotic biosynthesis and provides an effective strategy to help improve yields in industrial strains.

MATERIALS AND METHODS

Media, Plasmids, Strains, and Growth Conditions

All plasmids and bacterial strains used in this study are listed in **Table 1**. General techniques for the manipulation of nucleic acids and bacterial growth were carried out according to the standard protocols as previously described (Kieser et al., 2000). *Escherichia coli* DH5α was the general cloning host. Vectors used were pSET152, pIJ8660, pTA2. *S. chattanoogensis* L10 strains were grown at 28°C on YMG agar for sporulation and at 30°C
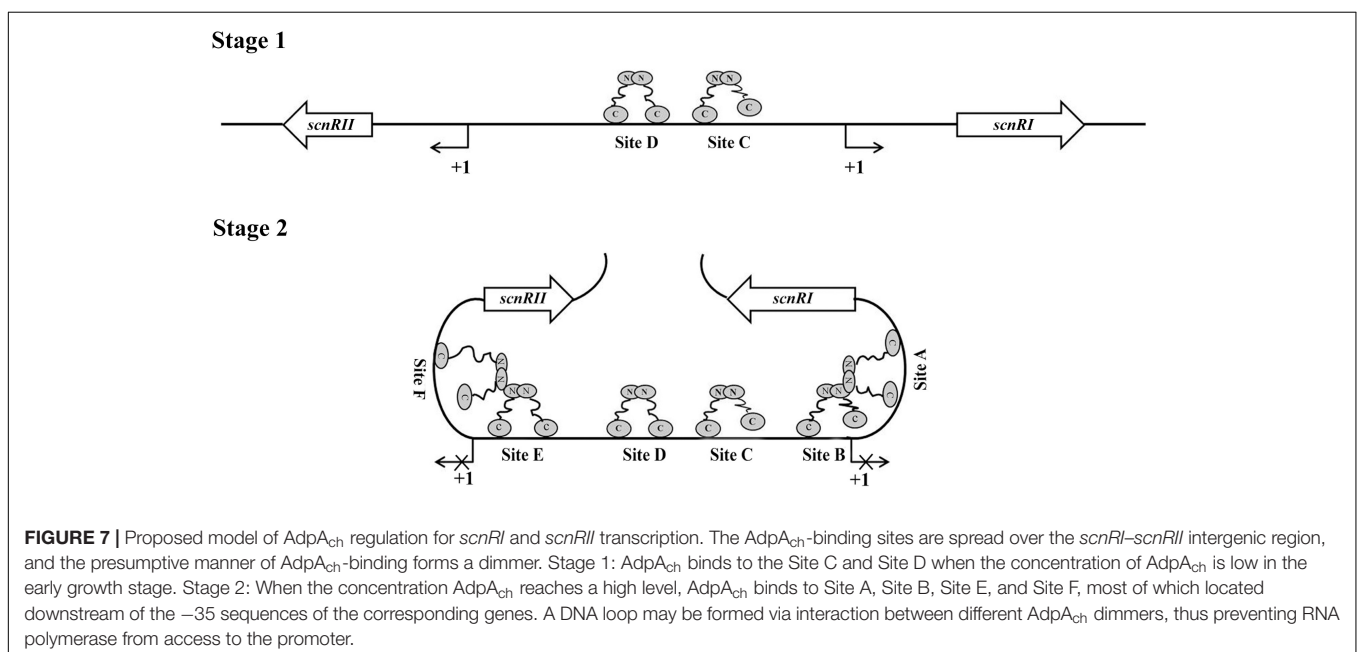

FIGURE 7 | Proposed model of AdpA_{ch} regulation for *scnRI* and *scnRII* transcription. The AdpA_{ch}-binding sites are spread over the *scnRI–scnRII* intergenic region, and the presumptive manner of AdpA_{ch}-binding forms a dimmer. Stage 1: AdpA_{ch} binds to the Site C and Site D when the concentration of AdpA_{ch} is low in the early growth stage. Stage 2: When the concentration AdpA_{ch} reaches a high level, AdpA_{ch} binds to Site A, Site B, Site E, and Site F, most of which located downstream of the −35 sequences of the corresponding genes. A DNA loop may be formed via interaction between different AdpA_{ch} dimmers, thus preventing RNA polymerase from access to the promoter.

TABLE 1 | Bacterial strains and plasmids used in this work.

Strains/plasmids	Characteristics	Reference
Strains		
E. coli TG1	General cloning host	Novagen
E. coli ET12567/pUZ8002	Methylation-deficient *E. coli* for conjugation with the helper plasmid	Macneil and Klapko, 1987
E. coli BL21 (DE3)	A host for protein expression	Novagen
E. coli BW25113/pIJ790	Strain used for PCR-targeted mutagenesis	Gust et al., 2003
Wt	*S. chattanoogensis* L10 wt; natamycin producer	Du et al., 2009
RI-x	wt with pIJ8601-*pRI*	This study
RI-mA-x	wt with pIJ8601-*pRI-mA*	This study
RI-mB-x	wt with pIJ8601-*pRI-mB*	This study
RI-mC-x	wt with pIJ8601-*pRI-mC*	This study
RI-mD-x	wt with pIJ8601-*pRI-mD*	This study
RI-mE-x	wt with pIJ8601-*pRI-mE*	This study
RI-mF-x	wt with pIJ8601-*pRI-mF*	This study
RII-x	wt with pIJ8601-*pRII*	This study
RII-mA-x	wt with pIJ8601-*pRII-mA*	This study
RII-mB-x	wt with pIJ8601-*pRII-mB*	This study
RII-mC-x	wt with pIJ8601-*pRII-mC*	This study
RII-mD-x	wt with pIJ8601-*pRII-mD*	This study
RII-mE-x	wt with pIJ8601-*pRII-mE*	This study
RII-mF-x	wt with pIJ8601-*pRII-mF*	This study
R-mA	wt with mutation in Site A	This study
R-mB	wt with mutation in Site B	This study
R-mC	wt with mutation in Site C	This study
R-mD	wt with mutation in Site D	This study
R-mE	wt with mutation in Site E	This study
R-mF	wt with mutation in Site F	This study
Plasmids		
pTA2 vector	General cloning vector	TOYOBO
p-*RI-RII*	pTA2 containing the fragment of the *scnRI–scnRII* intergenic region	This study
pIJ8601	Streptomyces integrative shuttle vector with *xylE* reporter gene	This study
pIJ8601-*pRI*	pIJ8601 with the promoter of *scnRI*	This study
pIJ8601-*pRI-mA*	pIJ8601-*pRI* with mutation in Site A	This study
pIJ8601-*pRI-mB*	pIJ8601-*pRI* with mutation in Site B	This study
pIJ8601-*pRI-mC*	pIJ8601-*pRI* with mutation in Site C	This study
pIJ8601-*pRI-mD*	pIJ8601-*pRI* with mutation in Site D	This study
pIJ8601-*pRI-mE*	pIJ8601-*pRI* with mutation in Site E	This study
pIJ8601-*pRI-mF*	pIJ8601-*pRI* with mutation in Site F	This study
pIJ8601-*pRII*	pIJ8601 with the promoter of *scnRII*	This study
pIJ8601-*pRII-mA*	pIJ8601-*pRII* with mutation in Site A	This study
pIJ8601-*pRII-mB*	pIJ8601-*pRII* with mutation in Site B	This study
pIJ8601-*pRII-mC*	pIJ8601-*pRII* with mutation in Site C	This study
pIJ8601-*pRII-mD*	pIJ8601-*pRII* with mutation in Site D	This study
pIJ8601-*pRII-mE*	pIJ8601-*pRII* with mutation in Site E	This study
pIJ8601-*pRII-mF*	pIJ8601-*pRII* with mutation in Site F	This study

in YEME medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l tryptone, 10 g/l glucose) for natamycin production.

Electrophoretic Mobility-Shift Assays (EMSAs)

His-AdpA$_{ch}$, histidine-tagged protein was purified from the soluble fractions of *E. coli* BL21 (DE3) harboring the plasmids pET32a-*adpA*$_{ch}$, as previously described (Du et al., 2011a).

The Bradford reagent (Bio-Rad) was used to determine the protein concentration. For probe preparation, all primers used in this study are listed in Supplementary Table S1. The EMSA DNA probe RI–RII (517 bp) spanning the entire *scnRI–scnRII* intergenic region was amplified by PCR using primer pair RI–RII-F and RI–RII-R. The PCR product was then cloned into a pTA2-vector (TOYOBO) to generate the plasmid pT-*RI–RII*. The biotin-labeled probe RI–RII was made with 5′-biotin-labeled M13 universal primer pair using pT-*RI–RII* as a template by PCR

amplification. The probes A (295 bp), B (281 bp), C (294 bp), D (282 bp), E (288 bp), F (284 bp), mA (295 bp), mB (281 bp), mC (294 bp), mD (282 bp), mE (288 bp), and mF (284 bp) were prepared following the above-mentioned method. In the EMSAs assay, 1 ng of the probe was incubated with varying quantities of $AdpA_{ch}$, at 25°C for 30 min in the buffer (20 mM Tris, pH 7.5, 5% glycerol, 0.01% BSA, 50 μg ml^{-1} sheared sperm DNA). For the competition assay, 100 times of excessive un-labeled probes and non-specific DNA were added to the reaction buffer, respectively. Reactions were displayed on 5% acrylamide gels for separation in 0.5× TBE buffer. EMSA gels were then electro-blotted onto the nylon membrane and UV-fixed by UV crosslinker. Labeled DNA was detected with streptavidin-HRP and BeyoECL plus (Beyotime, China) as described by the manufacturer.

DNase I Footprinting Assay

DNase I footprinting assay was performed as previously described (Mao et al., 2009). Firstly, $AdpA_{ch}$ protein was ultra-filtered with YM-10 (Millipore) for 10 kD cut-off and eluted in 20 mM Tris buffer, pH 7.5. Then, FAM-labeled probe was amplified using 5′-(6-FAM)-labeled M13 universal primers from plasmid pT-*RI-RII*, followed by gel recovery. About 50 ng of fluorescently labeled probe was added to the reaction mixture to a final volume of 50 μl. After binding of the $AdpA_{ch}$ protein to 5′-(6-FAM)-labeled probe (30°c, 30 min), 0.01 U of DNase I (Promega) was added for 1 min at 30°C, followed with equal volume of 100 mM EDTA to stop the reactions and extracted by phenol/chloroform. After precipitation with 40 μg of glycogen, 0.75 M ammonium acetate (NH_4Ac), and ethanol, the digested DNA mixture was loaded into ABI 3130 DNA sequencer with Liz-500 DNA marker (MCLAB). DNA sequencing ladder was prepared according to Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (USB).

Alterations of the Consensus Sequence for $AdpA_{ch}$-Binding Sites

The consensus sequence of $AdpA_{ch}$-binding sites A–F was replaced by the sequence of EcoRV restriction sequence sites using overlapping primers (Supplementary Table S1). The PCR product was then cloned into a pTA2-vector (TOYOBO). The resulted plasmids were used as template for PCR to amplify mutated probes using 5′-biotin-labeled M13 universal primers, and the binding ability was measured by EMSAs.

Construction and Analysis of Transcriptional Fusions to the *xylE* Reporter Gene

For *xylE* fusions, the *xylE* gene was PCR amplified with the primers *xylE*-F and *xylE*-R. This fragment was digested with *Nde*I and *Not*I, and introduced into the likewise-digested pIJ8660 (Sun et al., 1999) to construct pIJ8601. To probe *scnRIp* and *scnRIIp* activities with the mutation of $AdpA_{ch}$-binding sites, the wt and mutated promoter regions were amplified by PCR using upstream primers carrying a BamHI site listed in Supplementary Table S1. These promoter fragments were cloned into BamHI-cut pIJ8601 and transferred by conjugation into *S. chattanoogensis*

L10. Plasmid-containing strains were grown on YEME medium for 24 h. Cell pellets from 1 ml culture samples were kept on ice and measured immediately. Assays of catechol 2,3-dioxygenase were performed as previously described (Kieser et al., 2000).

Mutational Analysis of the $AdpA_{ch}$-Binding Sites on Natamycin Biosynthesis

The 1.8 kb DNA fragment containing the sequence of *scnRI–scnRII* intergenic region was amplified by PCR using primers scnRI-F and scnRII-R. The resulted 1.8 kb sequence was used as template to amplify the DNA fragment for construction of mutated $AdpA_{ch}$-binding sites *in vivo* using overlapping primers (Supplementary Table S1), then PCR product was purified and ligated into pKC1139. The resulting plasmids containing DNA fragment of mutated sites was conjugated by *E. coli* ET12567/pUZ8002 into *S. chattanoogensis* L10. The mutants were selected by replica plating for apramycin-sensitive colonies and they were used as template for PCR with primer pairs RI-RII-F and RI-RII-R. The amplified sequences were digested with EcoRV to confirm the mutants.

Determination of Natamycin Production by HPLC Analysis

Natamycin production was confirmed by HPLC analysis with the Agilent 1100 HPLC system. HC-C_{18} column (5 μm, 4.6 by 250 mm) was used with UV detector set at 303 nm. Mobile phase and gradient elution process were as described previously (Du et al., 2009).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00316/full#supplementary-material

REFERENCES

Antón, N., Santos-Aberturas, J., Mendes, M. V., Guerra, S. M., Martín, J. F., and Aparicio, J. F. (2007). PimM, a PAS domain positive regulator of pimaricin biosynthesis in *Streptomyces natalensis*. *Microbiology* 153, 3174–3183. doi: 10.1099/mic.0.2007/009126-0

Antón, N., Vendes, M. V., Martin, J. F., and Aparicio, J. F. (2004). Identification of PimR as a positive regulator of pimaricin biosynthesis in *Streptomyces natalensis*. *J. Bacteriol.* 186, 2567–2575. doi: 10.1128/JB.186.9.2567-2575. 2004

Berdy, J. (2005). Bioactive microbial metabolites : a personal view. *J. Antibiot.* 58, 1–26. doi: 10.1038/ja.2005.1

Du, Y. L., Chen, S. F., Cheng, L. Y., Shen, X. L., Tian, Y., and Li, Y. Q. (2009). Identification of a novel *Streptomyces chattanoogensis* L10 and enhancing its natamycin production by overexpressing positive regulator ScnRII. *J. Microbiol.* 47, 506–513. doi: 10.1007/s12275-009-0014-0

Du, Y. L., Li, S. Z., Zhou, Z., Chen, S. F., Fan, W. M., and Li, Y. Q. (2011a). The pleiotropic regulator AdpAch is required for natamycin biosynthesis and morphological differentiation in *Streptomyces chattanoogensis*. *Microbiology* 157, 1300–1311. doi: 10.1099/mic.0.046607-0

Du, Y. L., Shen, X. L., Yu, P., Bai, L. Q., and Li, Y. Q. (2011b). Gamma-butyrolactone regulatory system of *Streptomyces chattanoogensis* links nutrient utilization, metabolism and developmental programme. *Appl. Environ. Microbiol.* 77, 8415–8426. doi: 10.1128/AEM.058 98-11

Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K., and Ramos, J. L. (1997). Arac/XylS family of transcriptional regulators. *Microbiol. Mol. Biol. Rev.* 61, 393–410.

Gust, B., Challis, G. L., Fowler, K., Kieser, T., and Chater, K. F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1541–1546. doi: 10.1073/pnas.033754 2100

Guyet, A., Gominet, M., Benaroudj, N., and Mazodier, P. (2013). Regulation of the clpP1clpP2 operon by the pleiotropic regulator AdpA in *Streptomyces lividans*. *Arch. Microbiol.* 195, 831–841. doi: 10.1007/s00203-013-0918-2

Hara, H., Ohnishi, Y., and Horinouchi, S. (2009). DNA microarray analysis of global gene regulation by A-factor in *Streptomyces griseus*. *Microbiology* 155, 2197–2210. doi: 10.1099/mic.0.027862-0

Hopwood, D. A. (2007). *Streptomyces in Nature and Medicine. The Antibiotic Makers*. New York, NY: Oxford University Press Inc.

Horinouchi, S. (2002). A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. *Front. Biosci.* 7, d2045–d2057.

Kato, J. Y., Chi, W. J., Ohnishi, Y., Hong, S. K., and Horinouchi, S. (2005a). Transcriptional control by A-factor of two trypsin genes in *streptomyces griseus*. *J. Bacteriol.* 187, 286–295.

Kato, J. Y., Ohnishi, Y., and Horinouchi, S. (2005b). Autorepression of AdpA of the AraC/XylS family, a key transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *J. Mol. Biol.* 350, 12–26.

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000). *Practical Streptomyces Genetics*. Norwich: John Innes Foundation.

Komatsu, M., Uchiyama, T., Omura, S., Cane, D. E., and Ikeda, H. (2010). Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2646–2651. doi: 10.1073/pnas. 0914833107

Liu, G., Chater, K. F., Chandra, G., Niu, G., and Tan, H. (2013). Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiol. Mol. Biol. Rev.* 77, 112–143. doi: 10.1128/MMBR.00054-12

López-García, M. T., Santamarta, I., and Liras, P. (2010). Morphological differentiation and clavulanic acid formation are affected in a *Streptomyces clavuligerus adpA*-deleted mutant. *Microbiology* 156, 2354–2365. doi: 10.1099/ mic.0.035956-0

Macneil, D. J., and Klapko, L. M. (1987). Transformation of *Streptomyces avermitilis* by plasmid DNA. *J. Ind. Microbiol.* 2, 209–218. doi: 10.1007/ BF01569542

Mak, S., Xu, Y., and Nodwell, J. R. (2014). The expression of antibiotic resistance genes in antibiotic-producing bacteria. *Mol. Microbiol.* 93, 391–402. doi: 10.1111/mmi.12689

Mao, X. M., Zhou, Z., Cheng, L. Y., Hou, X. P., Guan, W. J., and Li, Y. Q. (2009). Involvement of SigT and RstA in the differentiation of *Streptomyces coelicolor*. *FEBS. Lett.* 583, 3145–3150. doi: 10.1016/j.febslet.2009. 09.025

McKenzie, N. L., and Nodwell, J. R. (2007). Phosphorylated AbsA2 negatively regulates antibiotic production in *Streptomyces coelicolor* through interactions with pathway-specific regulatory gene promoters. *J. Bacteriol.* 189, 5284–5292. doi: 10.1128/JB.00305-07

Moody, S. C. (2014). Microbial co-culture: harnessing intermicrobial signaling for the production of novel antimicrobials. *Future Microbiol.* 9, 575–578. doi: 10.2217/fmb.14.25

Nguyen, K. T., Tenor, J., Stettler, H., Nguyen, L. T., Nguyen, L. D., and Thompson, C. J. (2003). Colonial differentiation in *Streptomyces coelicolor* depends on translation of a specific codon within the *adpA* gene. *J. Bacteriol.* 185, 7291–7296. doi: 10.1128/JB.185.24.7291-7296.2003

Ohnishi, Y., Kameyama, S., Onaka, H., and Horinouchi, S. (1999). The A-factor regulatory cascade leading to streptomycin production in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* 34, 102–111. doi: 10.1046/j.1365-2958.1999. 01579.x

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. doi: 10.1271/bbb. 69.431

Onaka, H., and Horinouchi, S. (1997). DNA-binding activity of the A-factor receptor protein and its recognition DNA sequences. *Mol. Microbiol.* 24, 991–1000. doi: 10.1046/j.1365-2958.1997.4081772.x

Pan, Y., Liu, G., Yang, H., Tian, Y., and Tan, H. (2009). The pleiotropic regulator AdpA-L directly controls the pathway-specific activator of nikkomycin biosynthesis in *Streptomyces ansochromogenes*. *Mol. Microbiol.* 72, 710–723. doi: 10.1111/j.1365-2958.2009.06681.x

Retzlaff, L., and Distler, J. (1995). The regulator of streptomycin gene expression, StrR, of *Streptomyces griseus* is a DNA binding activator protein with multiple recognition sites. *Mol. Microbiol.* 18, 151–162. doi: 10.1111/j.1365-2958.1995. mmi_18010151.x

Santos-Aberturas, J., Vicente, C. M., Payero, T. D., Martín-Sánchez, L., Cañibano, C., Martín, J. F., et al. (2012). Hierarchical control on polyene macrolide biosynthesis: PimR modulates pimaricin production via the PAS-LuxR transcriptional activator PimM. *PLoS One* 7:e38536. doi: 10.1371/ journal.pone.0038536

Santos-Beneit, F., Rodriguez-Garcia, A., Sola-Landa, A., and Martin, J. F. (2009). Cross-talk between two global regulators in Streptomyces: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription. *Mol. Microbiol.* 72, 53–68. doi: 10.1111/j.1365-2958.2009. 06624.x

Sun, J., Kelemen, G. H., Fernandez-Abalos, J. M., and Bibb, M. J. (1999). Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* 145, 2221–2227. doi: 10.1099/ 00221287-145-9-2221

Takano, E., Chakraburtty, R., Nihira, T., Yamada, Y., and Bibb, M. J. (2001). A complex role for the gamma-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 41, 1015–1028. doi: 10.1046/j.1365-2958.2001.02562.x

Tan, G. Y., Peng, Y., Lu, C., Bai, L., and Zhong, J. J. (2015). Engineering validamycin production by tandem deletion of γ-butyrolactone receptor genes in *Streptomyces hygroscopicus* 5008. *Metab. Eng.* 28, 74–81. doi: 10.1016/j. ymben.2014.12.003

Tomono, A., Tsai, Y., Yamazaki, H., Ohnishi, Y., and Horinouchi, S. (2005). Transcriptional control by A-factor of strR, the pathway-specific transcriptional activator for streptomycin biosynthesis in *Streptomyces griseus*. *J. Bacteriol.* 187, 5595–5604. doi: 10.1128/JB.187.16.5595-5604.2005

van Wezel, G. P., and McDowall, K. J. (2011). The regulation of the secondary metabolism of Streptomyces: new links and experimental advances. *Nat. Prod. Rep.* 28, 1311–1333. doi: 10.1039/c1np00003a

Yamazaki, H., Ohnishi, Y., and Horinouchi, S. (2003a). Transcriptional switch on of ssgA by A-factor, which is essential for spore septum formation in *Streptomyces griseus*. *J. Bacteriol.* 185, 1273–1283.

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. doi: 10.1111/j.1365-2958.2004.04153.x

Yu, Z., Zhu, H., Dang, F., Zhang, W., Qin, Z., Yang, S., et al. (2012). Differential regulation of antibiotic biosynthesis by DraR-K, a novel two-component system in *Streptomyces coelicolor*. *Mol. Microbiol.* 85, 535–556. doi: 10.1111/j.1365-2958.2012.08126.x