Activation and mechanism of a cryptic oviedomycin gene cluster via the disruption of a global regulatory gene, *adpA*, in *Streptomyces ansochromogenes*

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Genome sequencing analysis has revealed at least 35 clusters of likely biosynthetic genes for secondary metabolites in *Streptomyces ansochromogenes*. Disruption of *adpA* encoding a global regulator (AdpA) resulted in the failure of nikkomycin production, whereas other antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus subtilis* were observed with the fermentation broth of ΔadpA but not with that of the wild-type strain. Transcriptional analysis showed that a cryptic gene cluster (*pks7*), which shows high identity with an oviedomycin biosynthetic gene cluster (*ovm*), was activated in ΔadpA. The corresponding product of *pks7* was characterized as oviedomycin by MS and NMR spectroscopy. To understand the molecular mechanism of *ovm* activation, the roles of six regulatory genes situated in the *ovm* cluster were investigated. Among them, proteins encoded by co-transcribed genes *ovmZ* and *ovmW* are positive regulators of *ovm*. AdpA directly represses the transcription of *ovmZ* and *ovmW*. Co-overexpression of *ovmZ* and *ovmW* can relieve the repression of AdpA on *ovm* transcription and effectively activate oviedomycin biosynthesis. The promoter of *ovmOI–ovmH* is identified as the direct target of OvmZ and OvmW. This is the first report that AdpA can simultaneously activate nikkomycin biosynthesis but repress oviedomycin biosynthesis in one strain. Our findings provide an effective strategy that is able to activate cryptic secondary metabolite gene clusters by genetic manipulation of global regulatory genes.

The crisis of antibiotic resistance has become an impending global problem, and it brings a significant threat to human health, so novel antibiotics are required to combat the evolving pathogens and new emerging diseases. Traditional large-scale screening of environmental microorganisms for bioactive molecules has been unable to meet the need (1). Therefore, it has become necessary to develop new technologies to discover novel bioactive compounds from diverse natural resources (2). Genome sequencing and *in silico* methods based on biological databases can rapidly pinpoint potential gene clusters for secondary metabolites, such as clustscan (3), CLUSEAN (4), SBSPKS toolbox (5), antiSMASH (6), etc. Thus, the productive capability of secondary metabolites in *Streptomyces* has been greatly underestimated. Cryptic secondary metabolite gene clusters have become a significant natural product reservoir, from which many more novel antibiotics could be discovered.

The expression of cryptic gene clusters is conditioned and can be activated by genetic manipulations of essential genes (7, 8). One of the approaches is to replace the native promoters of biosynthetic structural genes with constitutive promoters. For example, productions of the blue pigment indigoidine and two novel members of the polycyclic tetramate macrolactam family were activated by insertion of a strong and constitutive promoter in front of the transcriptional unit of structural genes of a small NRPS cluster and a hybrid type I PKS-NRPS cluster in *Streptomyces albus* J1074 (9). Another activation approach is to overexpress positive regulatory genes or to disrupt the negative regulatory genes situated in the cluster. For example, a giant type I modular PKS gene cluster of *Streptomyces ambofaciens* ATCC23877 spanning almost 150 kb was activated by overexpression of a LAL family regulatory gene, leading to the discovery of stambomycins, unusual glycosylated macrolides with unique chemical structures and promising antiproliferative activity against human cancer cell lines (10). Similarly, the production of chattamycins A and B in *Streptomyces chattanoogensis* was activated by overexpression of a pathway-specific activator gene *chaI* (11). Disruption of a putative repressor gene, *pgaY*, activated the production of UWM6 and rabelomycin in *Streptomyces* sp. PGA64 (12), and disruption of *jadR2* activated jadomycin B production in *Streptomyces venezuelae* (13). Activation of cryptic gene clusters can also be achieved by genetic manipulation of global regulatory genes because they usually play a key role in controlling the check point for the onset of secondary metabolite biosynthesis. For example, disruption of *dasR* caused a significant increase in transcription of *cpk* genes in *Streptomyces coelicolor* (14). Two novel tylosin analogues were generated by disruption of *wblA* in *Streptomyces ansochromogenes* (15). Integration of a functional *bldA* gene activated the production of a polyneoic acid amide in *Streptomyces calvus* (16). As one of the conserved global regulators in

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most of *Streptomyces*, AdpA can function as activator or repressor in terms of different target genes (17–19). In *Streptomyces griseus*, AdpA regulates more than 1000 genes, including those related to morphological development (20–22) and secondary metabolism, such as activator StrR for streptomycin (23) and GriR for grixazone biosynthesis (24). In *Streptomyces lividans*, the mutation of *adpA* affected the expression of more than 300 genes as revealed by transcriptomic analyses (25). Thus, the genetic manipulation of *adpA* could be an effective approach for the activation of cryptic gene clusters to gain novel antibiotics in *Streptomyces*. *S. ansochromogenes* is a natural producer of antifungal nikkomycin. Our previous results indicated that AdpA not only influenced morphological differentiation but also significantly affected nikkomycin production by directly controlling *sanG*, which encodes a pathway-specific transcriptional activator (26). antiSMASH analysis of *S. ansochromogenes* draft genome sequence indicated that 35 secondary metabolite gene clusters exist in the genome (27), of which only two corresponding secondary metabolites (nikkomycin and tylosin analogues) were identified. Therefore, it is expected to obtain more bioactive metabolites from the cryptic gene clusters by *adpA* manipulation.

In this work, we demonstrated that the cryptic oviedomycin biosynthetic gene cluster could be activated by disruption of *adpA* in *S. ansochromogenes*, and the activation mechanism was investigated. It is intriguing that the cluster-situated regulators (OvmZ and OvmW) are the targets of AdpA, and they collaboratively control oviedomycin biosynthesis.

Results

Activation of a cryptic oviedomycin biosynthetic gene cluster by disruption of adpA in S. ansochromogenes

A previous study (26) indicated that disruption of *adpA* in *S. ansochromogenes* (the wild-type strain) resulted in the failure of nikkomycin production. Due to the pleiotropic role of AdpA on target genes, it is interesting to know whether new bioactive compounds could be generated by *adpA* disruption. The fermentation broth of ΔadpA and the WT strain was subjected to bioassays against pathogenic bacteria. As a result, clear inhibition zones against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus subtilis*) were observed in the fermentation broth of ΔadpA (Fig. 1), which do not occur in the broth of WT strain. The results indicated that disruption of *adpA* led to the biosynthesis of other bioactive compound, whereas nikkomycin production was completely abolished. The bioactivity profiles of complemented strain (ΔadpAc) were restored to the same level as WT strain (Fig. 1), indicating that disruption of *adpA* is essential for both the production of new compound and the abolishment of nikkomycin biosynthesis.

To verify the corresponding gene cluster(s) responsible for the newly emerged bioactive compound(s) in ΔadpA, we isolated the total RNA from both WT strain and ΔadpA grown in MS, SP, and GP media at different time courses, respectively. Transcription of the core genes (27) in each PKS and NRPS secondary metabolic gene cluster in *S. ansochromogenes* genome was analyzed by reverse transcription-PCR (RT-PCR). The gene clusters silent in WT strain but activated in ΔadpA
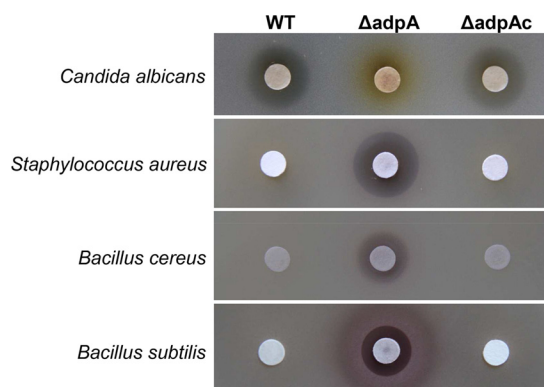

Figure 1. Bioassays of fermentation broth of wild-type strain and its derivatives. *WT*, *S. ansochromogenes*; ΔadpA, *adpA* disruption mutant; ΔadpAc, strain complemented by integrating a copy of *adpA* into the chromosome of ΔadpA. The strains were cultured in liquid MS for 4 days, and 200-μl supernatants from culture broths were assayed for bioactivity against *C. albicans*, *S. aureus*, *B. cereus*, and *B. subtilis*.

are potentially responsible for the biosynthesis of the new bioactive compound. A core gene from *pks7* in ΔadpA strain was transcribed in all detected media at each time course but not in WT strain (Fig. 2). Meanwhile, as we anticipated, the expression of nikkomycin biosynthetic gene cluster was impeded in ΔadpA (Fig. 2). These results indicated that *pks7* was activated and may be responsible for the production of the bioactive metabolite in ΔadpA.

Isolation, identification, and characterization of oviedomycin

BLAST analysis revealed that *pks7* contains 26 complete ORFs spanning 27 kb and shares more than 95% DNA sequence identities with the oviedomycin biosynthetic gene cluster in *Streptomyces antibioticus* ATCC 11891 (28). To verify if *pks7* can also synthesize similar product in ΔadpA, the fermentation broth was analyzed by HPLC, and the active metabolite was isolated and characterized. On HPLC chromatograms, there was a distinct peak at a retention time of 28 min in the extract of ΔadpA, compared with that of WT strain (Fig. 3*A*). It exhibited inhibitory activity against *S. aureus*, whereas the HPLC solute methanol did not show inhibition zone (Fig. 3*B*). To determine the chemical structure of this compound, 3 liters of fermentation broth of ΔadpA in MS medium was harvested and extracted with chloroform. The organic phase was applied onto a Sephadex LH-20 column to remove most impurities, and finally the compound was separated and collected by semi-preparative HPLC. The chemical structure of purified compound was determined by MS and NMR spectroscopy. A molecular ion peak at m/z^- 349.0 was shown on the electrospray ionization-MS spectrum. MS/MS analysis revealed that most fragments were identical with those of the known angucyclinone oviedomycin (Fig. 3*C*) (29). Furthermore, ^{1}H NMR and ^{13}C NMR data were also consistent with those of oviedomycin (supplemental Fig. 1). Although the sugar side chain is not present in the structure as in other augucyclinone antibiotics, the purified oviedomycin showed good inhibitory activity against A549 (non-small-cell lung cancer), HepG2 (liver cancer) and MCF-7 (breast cancer) with $IC_{50} < 20$ μM (Fig. 3*E*). Thus, the disruption of *adpA* elicited the production of a compound potentially applicable as drug.
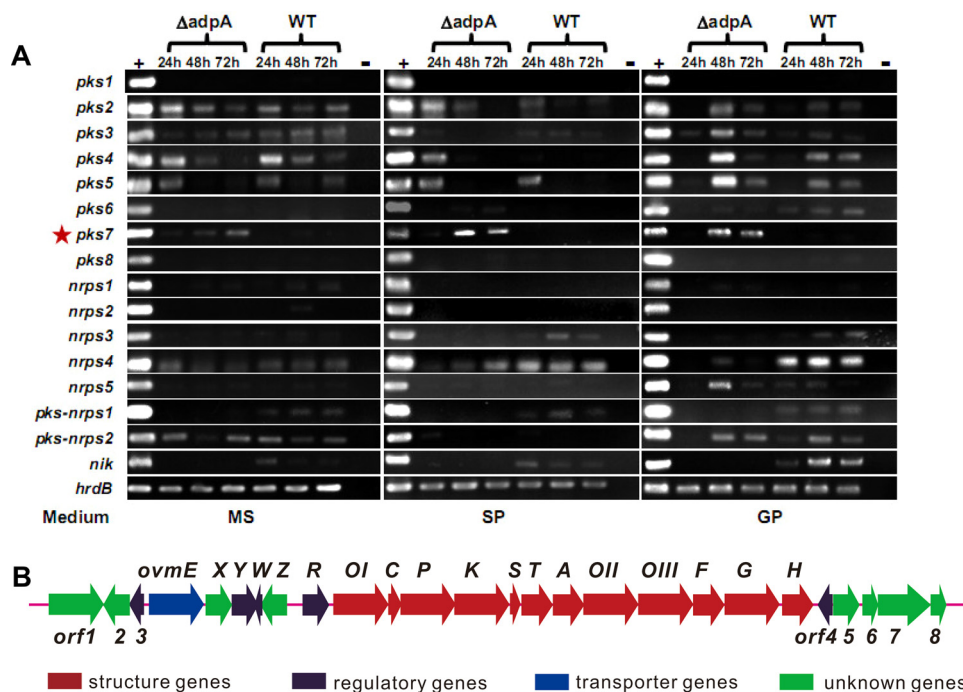
Figure 2. Transcriptional analysis of PKS and NRPS biosynthetic gene clusters in *S. ansochromogenes* and ΔadpA by RT-PCR. *A*, cDNA templates were synthesized from RNA of *S. ansochromogenes* cultivated in three different liquid media (*MS*, *SP*, and *GP*). The strain name is indicated at the *top* of the gel, and growth time is *below* the strain name. The media used are indicated at the *bottom* of the gels. *S. ansochromogenes* genomic DNA (+) and double-distilled H₂O (−) were used as positive and negative controls for each primer pair. Each gene cluster is given at the *left* of the gels. *B*, genetic organization of *pks7* cluster in *S. ansochromogenes*. The gene cluster contains 26 ORFs, and the gene *ovmW* was marked in this study.

There are 12 genes (*ovmOI–ovmH*) assembling as one transcriptional unit responsible for oviedomycin biosynthesis in *S. antibioticus* ATCC 11891 (28). To verify whether *pks7* is directly responsible for oviedomycin biosynthesis, the structural genes (*ovmOI– ovmH*) spanning >14-kb DNA fragments in this cluster were cloned into a *Streptomyces* integration vector pSET152 through Gibson assembly under the control of promoter P_{hrdB} (Fig. 4*A*). The resulting construct was then introduced into *S. coelicolor* M1146 by conjugation for heterologous expression. As a result, oviedomycin was successfully produced in M1146 (Fig. 4*B* and supplemental Fig. 2), demonstrating that *pks7* is undoubtedly responsible for oviedomycin biosynthesis in *S. ansochromogenes*, and then *pks7* was designated as *ovm*.

AdpA represses the expression of oviedomycin biosynthetic genes in *S. ansochromogenes*

To reveal the activation mechanism of *ovm* by disruption of *adpA*, the transcription of structural and regulatory genes in *ovm* were analyzed by quantitative real-time PCR (qRT-PCR)[3] with the total RNA extracted from mycelia of WT strain and ΔadpA grown for 24 h.

In *ovm*, structural genes, *ovmOI– ovmH*, are in the same transcriptional direction and probably constitute one operon. Among the structural genes, *ovmC*, *ovmP*, *ovmK*, *ovmS*, *ovmT*, and *ovmA* are responsible for the ring skeleton formation; *ovmOI*, *ovmOII*, and *ovmOIII* are oxygenases responsible for

the introduction of those oxygens; and *ovmF*, *ovmG*, and *ovmH* are involved in polyketide metabolism (30). qRT-PCR showed that the transcription of four representative structural genes (*ovmOI*, *ovmP*, *ovmS*, and *ovmT*) from the operon (*ovmOI-ovmH*) in charge of oviedomycin biosynthesis increased 1000– 4500-fold in ΔadpA compared with that in WT strain (Fig. 5*A*), indicating that AdpA plays a negative role in these genes' expression.

In addition, there are six putative regulatory genes (*orf3*, *ovmY*, *ovmW*, *ovmR*, *orf4*, and *ovmZ*) in *ovm*. Orf3 shares 49% amino acid sequence identity with the ArsR family regulator Rv2642 from *Mycobacterium tuberculosis* H37Rv. OvmY shows 39% amino acid sequence identity with the TetR family regulator CprB from *S. coelicolor*. OvmR shares 61% amino acid sequence identity with the IclR family regulator WP_ 044364315 from *Streptomyces natalensis*. Orf3, OvmY, and OvmR contain a helix-turn-helix DNA-binding domain at the N terminus. Orf4 shares 62% amino acid sequence identity with the MarR family regulator SFJ27529 from *Amycolatopsis sacchari* and contains a helix-turn-helix domain at its interior. *ovmW* encoding a small protein with 63 amino acids was not annotated in *S. antibioticus* ATCC 11891, and OvmW shares 65% identity with MerR family regulator EpaH from *Kitasatospora* sp. HKI 714 (31). However, both OvmW and EpaH have no identity with MerR family proteins in the sequence of amino acids by alignment (see "Discussion"). There is another putative regulatory gene *ovmZ* adjacent to *ovmW*. Its encoding product shares 59% identity with ChaP from chattamycin biosynthetic gene cluster in *S. chattanoogensis* L10 (11), 43% identity with Aur1O from the auricin biosynthetic gene cluster in *Streptomyces aureofaciens* CCM 3239 (32), 40% identity with TylU from

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[3] The abbreviations used are: qRT-PCR, quantitative real-time PCR; CSR, cluster-situated regulator; GUS, β-glucuronidase enzyme; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucoronide; YEME, yeast extract-malt extract; nt, nucleotide.
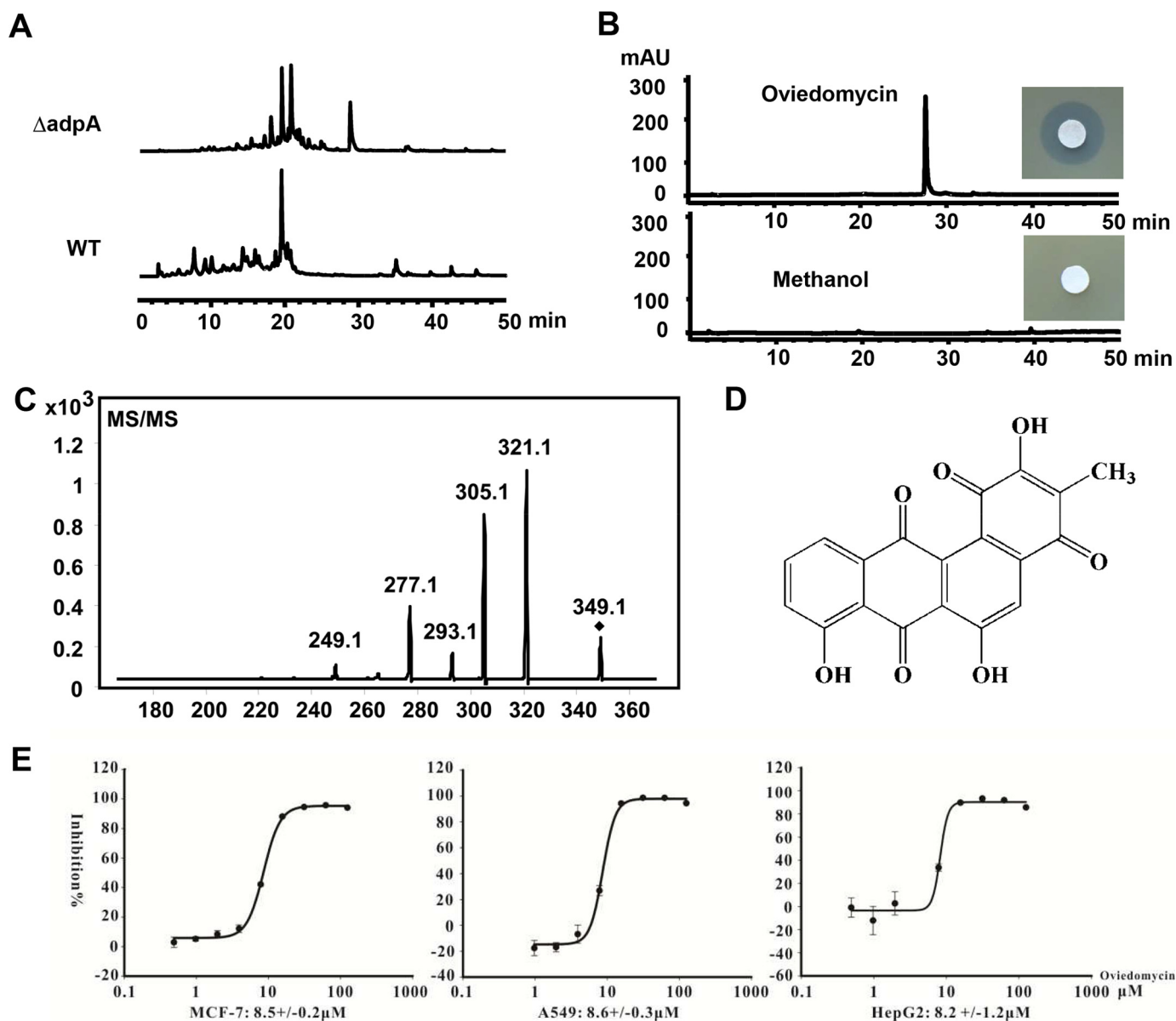
A

B

C

D

E

Figure 3. Identification and bioassay of oviedomycin. *A*, HPLC analysis of oviedomycin production in WT strain and ΔadpA. *B*, HPLC analysis of purified oviedomycin and the bioassay against *S. aureus*. The solute methanol was used as the negative control. *mAU*, milli-absorbance unit. *C*, MS/MS analysis of oviedomycin. *D*, structure of oviedomycin. *E*, cytotoxic assay of oviedomycin. The cell lines used in the cytotoxin assay were MCF-7, A549, and HepG2. *Error bars*, S.D.

the tylosin biosynthetic gene cluster in *Streptomyces fradiae* T59235 (33), 38% identity with Med-ORF27 from the medermycin biosynthetic gene cluster in *Streptomyces* sp. AM-7161 (34), 37% identity with PgaK from the *pga* gene cluster in *Streptomyces* sp. PGA64 (35), and 34% identity with EpaI from the endophenazines biosynthesis gene cluster in *Kitasatospora* sp. HKI 714 (31). Despite the fact that OvmZ homologous proteins are widespread in *Streptomyces*, their functions have not been elucidated except for TylU, a regulator relating to tylosin production (33). It is noteworthy that most regulatory genes of this type situate in PKS biosynthetic gene clusters. Both *ovmW* and *ovmZ* were preliminarily predicted as regulatory genes. Their role in oviedomycin biosynthesis was assessed by qRT-PCR. The transcription of *ovmY*, *ovmW*, *ovmR*, *orf4*, and *ovmZ* was increased in ΔadpA compared with that in WT strain, and particularly the transcription of *ovmZ*, *ovmW*, and *ovmY* was increased >100-fold

(Fig. 5*B*), whereas the transcription of *orf3* was not affected. It was concluded that both structural and regulatory genes in *ovm* were up-regulated in ΔadpA, suggesting that AdpA plays a negative regulatory role in the transcription of cluster-situated regulator (CSR) genes except *orf3*. AdpA might regulate oviedomycin biosynthesis by controlling these CSR genes.

Coordinative regulation of OvmZ and OvmW on the oviedomycin biosynthetic gene cluster

To identify the roles of putative CSR genes on oviedomycin biosynthesis, disruptions of these genes were carried out via homologous recombination in both WT strain and ΔadpA. HPLC analysis of fermentation broth showed that oviedomycin production was not detected in ΔovmZ, ΔovmW, ΔovmR, ΔovmY, Δorf3, and Δorf4 strains as well as in WT strain (Fig. 6*A*), suggesting that none of the six regulatory genes plays the
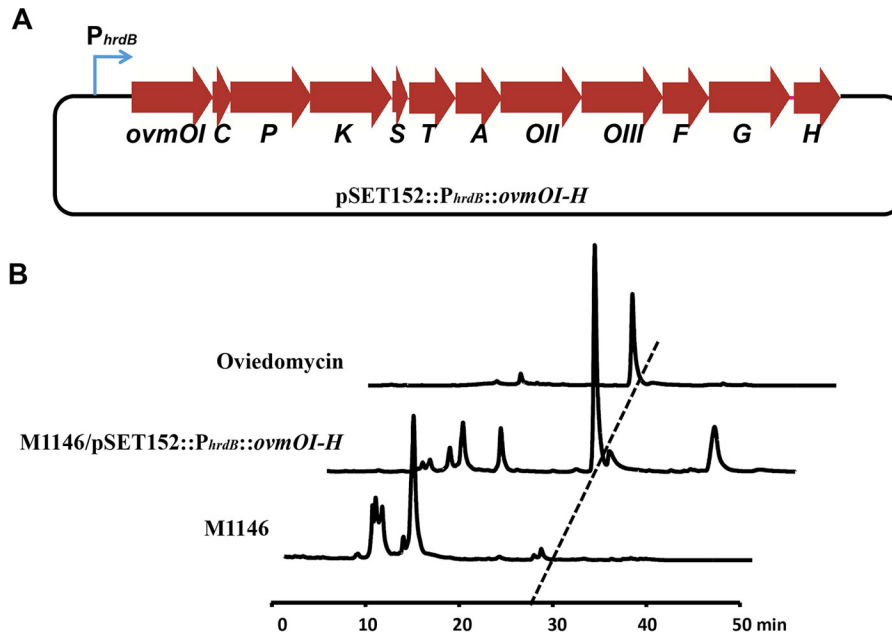
Figure 4. Heterologous expression of oviedomycin biosynthesis gene cluster. *A*, schematic representation of the plasmid used for heterologous expression of oviedomycin. *B*, heterologous expression of oviedomycin in M1146.
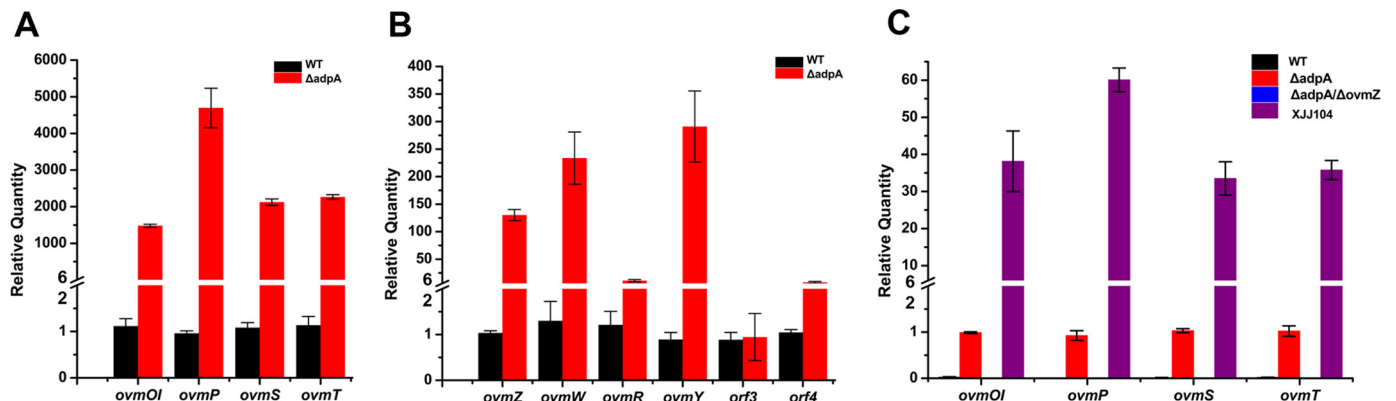

Figure 5. Transcriptional analysis of oviedomycin biosynthetic genes by qRT-PCR. Total RNAs were isolated from mycelia of *S. ansochromogenes* and its derivatives after fermentation for 24 h. The constitutive *hrdB* transcript was used as an internal control. *Error bars*, S.D. *A*, transcriptional analysis of structural genes in WT strain and ΔadpA. *B*, transcriptional analysis of regulatory genes in WT strain and ΔadpA. *C*, transcriptional analysis of structural genes in WT strain, ΔadpA, XJJ104, and ΔadpA/ΔovmZ.

repressor role in oviedomycin biosynthesis. Moreover, oviedomycin produced in ΔadpA was completely abolished in the double mutants ΔadpA/ΔovmZ and ΔadpA/ΔovmW (Fig. 6*B*). A moderate decrease of oviedomycin production was detected in the double mutant ΔadpA/ΔovmR, whereas oviedomycin production in ΔadpA/ΔovmY, ΔadpA/Δorf3, and ΔadpA/Δorf4 strains was almost the same as in the ΔadpA strain (Fig. 6*B*). These results demonstrated that *ovmZ* and *ovmW* are essential for activation of oviedomycin biosynthetic gene cluster.

To further clarify whether OvmZ, OvmW, and OvmR are positive regulators in oviedomycin biosynthesis, *ovmZ*, *ovmW*, or *ovmR*, respectively, was inserted into pKC1139 under the control of a strong constitutive expressed *hrdB* promoter (P*hrdB*) (36). The resulting recombinant plasmids were introduced into WT strain to generate XJJ101, XJJ102, and XJJ103, respectively. However, no oviedomycin was detected in these constructed strains, indicating that the overexpression of *ovmZ*, *ovmW*, or *ovmR* individually was not sufficient for acti-

vation of oviedomycin biosynthesis in WT strain. Because *ovmZ* and *ovmW* are adjacent and situated in the same operon in the cluster, we attempted to make co-overexpression of the two genes synchronously in WT strain by inserting them into pKC1139 as mentioned above. As anticipated, the resulting strain XJJ104 gave rise to much higher production of oviedomycin than ΔadpA (Fig. 6*C*).

Subsequently, the transcriptional analyses of structural genes (*ovmOI*, *ovmP*, *ovmS*, and *ovmT*) in WT, ΔadpA, ΔadpA/ΔovmZ, and XJJ104 strains were performed by qRT-PCR. The biosynthetic genes were not transcribed in ΔadpA/ΔovmZ as in WT strain, whereas the transcriptional levels were 30–60 times higher in XJJ104 than those in ΔadpA (Fig. 5*C*). Therefore, co-overexpression of *ovmZ* and *ovmW* can derepress oviedomycin biosynthesis in WT strain. OvmZ and OvmW proved to be positive regulators for oviedomycin biosynthesis, and the expression of structural genes is dependent on the co-expression of OvmZ and OvmW.
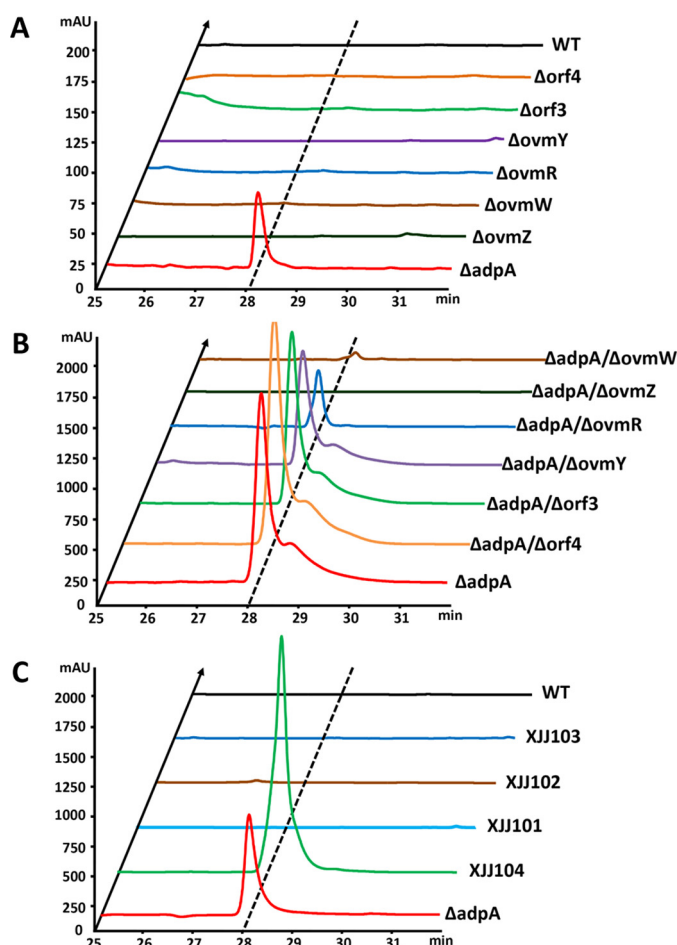
Figure 6. HPLC analysis of oviedomycin produced by *S. ansochromogenes* and its derivatives. *A*, the injection volume for ΔadpA is 10 *μ*l, and the others are 100 *μ*l. *B*, injection volume is 100 *μ*l. *C*, injection volume is 50 *μ*l.

Cascade regulation of oviedomycin biosynthesis

The above results showed that *ovmZ* and *ovmW* are required for oviedomycin biosynthesis, and global regulator AdpA exerts negative regulatory effect on oviedomycin biosynthesis by repressing the expression of *ovmZW*. To determine whether AdpA could directly regulate its target gene, AdpA-His$_6$ was expressed in *Escherichia coli* BL21 (DE3) and purified through chromatography on nickel-nitrilotriacetic acid resin. EMSAs were performed by using AdpA-His$_6$ with the promoter P$_{ovmZ}$ as the probe. As anticipated, AdpA could form a complex with P$_{ovmZ}$ in a concentration-dependent manner (Fig. 7*A*). EMSA suggested that AdpA inhibits oviedomycin biosynthesis in WT strain via directly repressing *ovmZW* transcription.

To determine the specific binding sites of AdpA on the upstream region of *ovmZ*, DNase I footprinting was performed to analyze the protected regions of FAM/HEX-labeled probe in the presence or absence of AdpA. Three AdpA protected binding sites (sites I, II, and III) spanning the translation start codons of *ovmZ* were clearly observed (Fig. 7, *B* and *C*). Site I is consistent with the consensus AdpA-binding sequence in *S. griseus* (37), and the other two sites showed some similarities to the consensus sequence in *S. griseus* and to that of the *sanG* promoter in *S. ansochromogenes* (26).

To confirm whether OvmZ and OvmW directly regulate the transcription of oviedomycin biosynthetic genes, the promoter region (P$_{ovmOI}$) of the first gene (*ovmOI*) of the co-transcribed structural gene operon in *ovm* was used as a probe. OvmZ and OvmW were expressed and purified from *E. coli* as described above. EMSAs were performed to show the binding activity of OvmZ and OvmW to the probe. However, no binding shift pattern was observed by using OvmZ-His$_6$ or OvmW-His$_6$ individually or both proteins together (supplemental Fig. 3). We suspected that OvmZ and OvmW might have no function when expressed in *E. coli*, or the binding conditions *in vitro* might be inappropriate so the *gusA* guided *in vivo* reporting system was taken into account. *gusA* encodes β-glucuronidase enzyme (GUS), which can hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-Gluc) to form a blue precipitate, 5,5′-dibromo-4,4′-dichloro-indigo (38). An integrating *gusA* reporter plasmid, pIJ10500::P$_{ovmOI}$::*gusA*, was constructed and then introduced into *S. coelicolor* M1146 via conjugation to generate the XJJ105 strain. Subsequently, plasmids pKC1139::P$_{hrdB}$ZW, pKC1139::P$_{hrdB}$Z, and pKC1139::P$_{hrdB}$W were respectively introduced into M1146 to give XJJ106, XJJ107, and XJJ108 and also introduced into XJJ105 to give XJJ109, XJJ110, and XJJ111. After culture on an AS-1 plate containing X-Gluc for 3 days, the blue pigment only appeared in XJJ109, which carries both pKC1139::P$_{hrdB}$ZW and pIJ10500::P$_{ovmOI}$::*gusA*, whereas the other constructs had no obvious GUS activity (Fig. 8). This result confirmed that collaborated OvmZ-OvmW could directly activate P$_{ovmOI}$ and in turn positively regulate the transcription of oviedomycin biosynthetic genes.

Taken together, the molecular mechanism activating a cryptic oviedomycin biosynthetic gene cluster in *S. ansochromogenes* was proposed (Fig. 9). In the wild-type strain, AdpA positively regulates nikkomycin production by activating the expression of the pathway-specific regulatory gene *sanG*. Meanwhile, it represses oviedomycin biosynthesis by repressing the positive regulatory genes *ovmZ* and *ovmW*. OvmZ and OvmW, as partner, coordinately regulate the transcription of structural genes in *ovm*. Co-overexpression of *ovmZ* and *ovmW* or disruption of *adpA* leads to the activation of *ovm* and, in turn, oviedomycin production.

Discussion

Sequencing of *Streptomyces* genomes revealed the presence of a large number of secondary metabolic gene clusters for previously unsuspected products (39) and thus the potential to produce many more natural products than had previously been recognized. It has therefore become necessary to establish new methods to activate these "cryptic" pathways (7, 40, 41). Mining of genome sequences has revealed 35 gene clusters for secondary metabolite biosynthesis in *S. ansochromogenes*, a nikkomycin producer. Global regulatory genes, such as *adpA*, are also present in this strain. Although AdpA was proved to be an activator for nikkomycin production in our previous work (26), presumably it may play a repressor role for some other secondary metabolic gene clusters based on its pleiotropic roles on gene regulations and the vast target. Thus, disruption of global regulatory genes, including *adpA*, may be an effective strategy to activate cryptic gene clusters. In this work, disruption of

A

B

GCCTGAATTTACACACTGATAAACCGCGATT
TCACCGCACGTATCCGGCACATTCGAAACCA
TCATTCAGCGCG*CGTCGCGCCA*TTCCGCCGC
               III
GCGGCGCCGTCCTGCGCCGCAGCAGGCATCC
*GCGCCCGGCCA*GGACCA*TGGCCGGCCA*GCCA
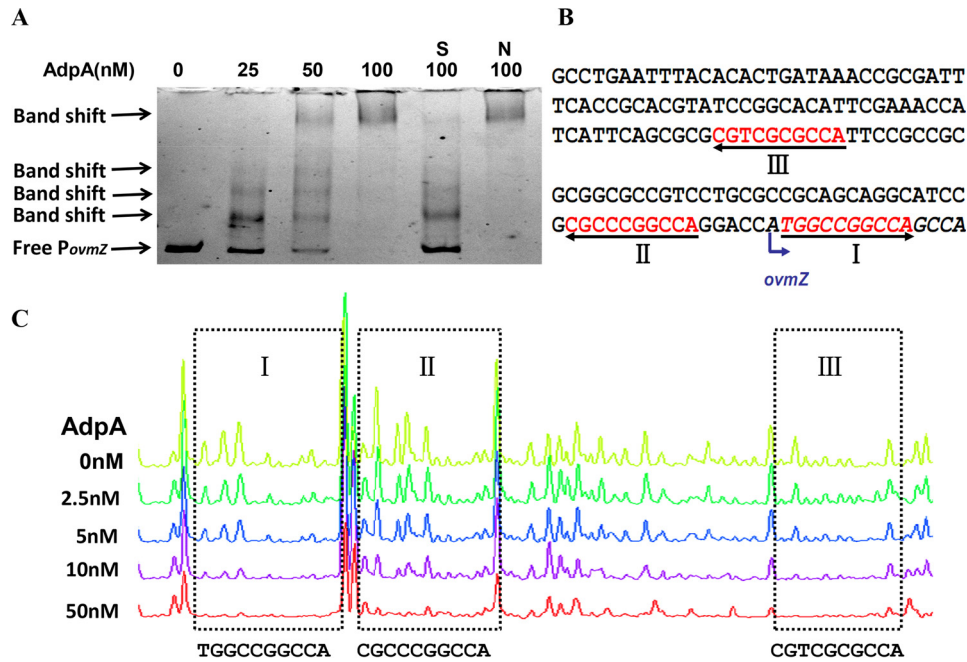   **II**   **I**

ovmZ

C

Figure 7. EMSA and DNase I footprinting assays. *A*, EMSA of His$_6$-tagged AdpA protein with the probe P$_{ovmZ}$ containing the promoter of *ovmZ*. Each lane contains 50 ng of DNA probes and 2 μg of poly(dI-dC). *S*, unlabeled specific probe (10-fold) was added; *N*, nonspecific probe (10-fold) was added. *B*, binding sequence (sites I, II, and III) of AdpA on the upstream region of *ovmZ*. The binding sites are *shaded* in *red*, and the translation start codons are indicated in *italic type*. *C*, DNase I footprinting assays of the His$_6$-tagged AdpA protein binding to the probe P$_{ovmZ}$. Each *line* contains 200 ng of DNA probe incubated with increasing concentrations of AdpA protein (0, 2.5, 5, 10, and 50 nM). The binding sites are marked in the *dashed rectangle*, and the sequences are given *below* the *dashed rectangle*.
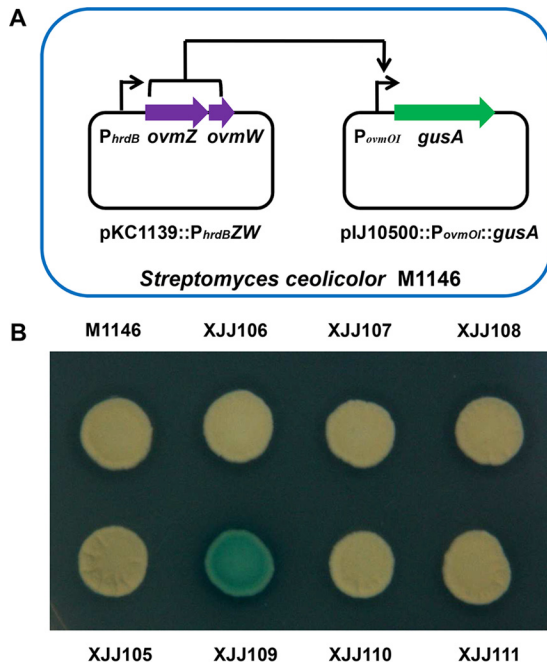
A

B

Figure 8. *In vivo* determination of the interaction between OvmZW and P$_{ovmOI}$. *A*, the plasmid pKC1139::P$_{hrdB}$ZW was used to express OvmZ and OvmW. The plasmid pIJ10500::P$_{ovmOI}$::*gusA* contains the promoter of *ovmOI* and the reporter gene *gusA*. They were introduced into M1146 to detect the activity of the *ovmOI* promoter. *B*, chromogenic assay on AS-1 plate containing the substrate X-Gluc. XJJ105 contains the plasmid pIJ10500::P$_{ovmOI}$::*gusA*; XJJ106 contains the plasmid pKC1139::P$_{hrdB}$ZW; XJJ107 contains the plasmid pKC1139::P$_{hrdB}$Z; XJJ108 contains the plasmid pKC1139::P$_{hrdB}$W; XJJ109 contains the plasmids pKC1139::P$_{hrdB}$ZW and pIJ10500::P$_{ovmOI}$::*gusA*; XJJ110 contains the plasmids pKC1139::P$_{hrdB}$Z and pIJ10500::P$_{ovmOI}$::*gusA*; and XJJ111 contains the plasmids pKC1139::P$_{hrdB}$W and pIJ10500::P$_{ovmOI}$::*gusA*.

adpA combined with transcription screening, bioassay, and compound characterization facilitated rapid localization of relevant gene clusters. Also, this is the first experimental evidence that AdpA positively and negatively regulates the expression of two antibiotic biosynthetic gene clusters (activating nikkomycin but repressing oviedomycin production) in the same strain. Manipulation of global regulatory genes can promote the discovery of novel bioactive compounds.

In elucidation of the regulatory mechanism of cryptic *ovm* gene cluster, two novel CSR genes encoding positive regulators OvmW and OvmZ were identified, which are essential for the activation of oviedomycin production. Interestingly, these two activators are dependent on each other and cooperatively function as partners. Constitutive expression of *ovmZ* and *ovmW* individually in WT strain could not activate oviedomycin biosynthesis, whereas co-overexpression of OvmZ and OvmW could relieve the repression of AdpA. OvmZ, consisting of 248 amino acids, shows no conserved domains in the database, and all of the orthologues are hypothetical proteins. OvmW comprises 63 amino acids, and its homologous gene is present but not annotated in the *ovm* gene cluster in *S. antibioticus* ATCC11891 (28). Although some of its orthologues are annotated as MerR family transcriptional regulators, alignment analysis of the OvmW amino acid sequence showed no homology to those of typical MerRs. Thus, this kind of small protein is also hypothetical. To date, six candidate orthologues of OvmZ (PgaK, Med-ORF27, ChaP, Aur1O, TylU, and EpaI) have been reported; however, their functions remain unclear. Most of the OvmZ orthologues are present in the type II PKS biosynthetic gene cluster, such as PgaK from the *pga* gene cluster (35), Med-ORF27 from the medermycin biosynthetic gene cluster
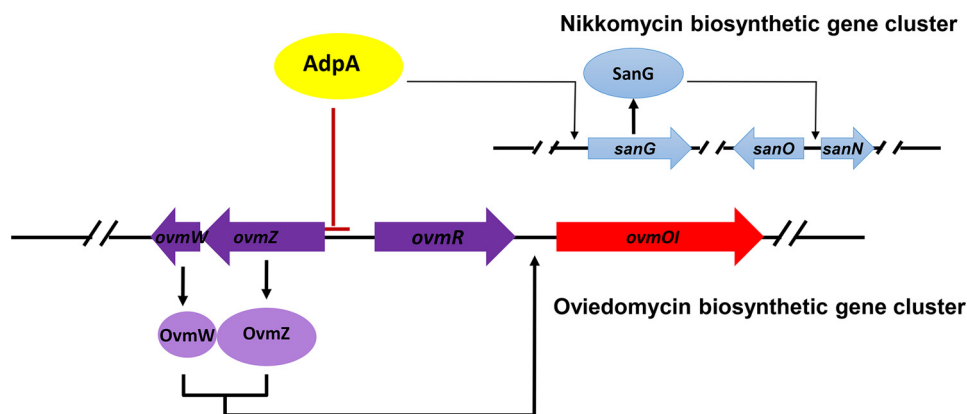
Figure 9. Model for the regulation of nikkomycin and oviedomycin biosynthesis by AdpA in *S. ansochromogenes*.

(34), ChaP from the chattamycin biosynthetic gene cluster (11), Aur1O from the auricin biosynthetic gene cluster (32), and EpaI from the endophenazines biosynthesis gene cluster (31). Unlike OvmZ, there is no *ovmW* orthologue adjacent to them in the gene clusters except for *epaI* and *tylU*. *epaH*, an *ovmW* ortho logue, is located downstream of *epaI*. In the type I PKS tylosin biosynthetic cluster, *tylU*, the so-called SARP "helper" gene, has homology with *ovmZ*, and there is another unannotated gene adjacent to *tylU* that is homologous to *ovmW*. In a previous report (33), *tylU* disruption caused a decrease of tylosin pro duction, but the overexpression of *tylU* did not improve tylosin production in *S. fradiae*. Probably, the function of TylU was not elucidated clearly because the equivalent homologous gene of *ovmW* in tylosin gene cluster was not taken into consideration. Interestingly, a BLAST search against a database revealed that this kind of coupled regulatory gene is widespread in actinomy cetes. Moreover, most of them are present in secondary metabo lite biosynthetic gene clusters, as revealed by analyzing the flanking sequence of these coupled genes with antiSMASH (supplemental Table 1 and Fig. 4). Although the definite func tion and regulation mechanism remains a mystery, there is no doubt that they may play essential roles in different types of secondary metabolisms with an uncharacterized mechanism. It would be helpful to elucidate the mechanism of close cooperation of OvmZ and OvmW in the future.

Because genetic manipulations of other regulatory genes in the cluster have no effect on oviedomycin biosynthesis, it seems that OvmZ and OvmW are the direct effectors and dominate the transcription of the structural genes. Gene disruption of *ovmZ* and *ovmW* in ΔadpA and their overexpression in WT strain revealed that they are positive regulatory genes. How ever, EMSAs showed that the OvmZ and OvmW expressed in *E. coli* failed to bind to their target P$_{ovmOI}$, suggesting that the additional factors or ligands may be required for their activity or for proper protein folding. To verify this assumption, *in vivo* genetic construction with *gusA* reporter gene was performed, indicating that the *ovmOI* promoter is the direct target of co expressed OvmZ and OvmW. Unfortunately, we failed to detect OvmZ and OvmW proteins by using Western blot hybridization and electrospray ionization-MS detection of the whole proteins of cell lysates in ΔadpA or even in the wild-type strain containing the plasmid for overexpression of OvmZ and OvmW, suggesting that the fast degradation of expressed pro

tein or posttranslational modification might take place in *Strep tomyces*. This could be a serious obstacle in resolving the regu lation mechanisms for this kind of regulator. Development of adequate techniques to overcome this issue will be helpful.

Angucycline antibiotics have become the largest group of type II PKS-engineered products because they possess diverse chemical structures and potent biological activities against a number of pathogenic bacteria as well as cancer cell lines (42). Intriguingly, most angucycline antibiotic gene clusters are usu ally cryptic or silent, and their activation mechanisms still remain unknown (13, 43, 44). For example, jadomycin, as one of the intensively studied angucycline members, is produced under stress conditions, such as heat shock, phage infection, and ethanol treatment. But there is no *ovmZ* orthologous within the gene cluster. *pga* cluster is also a cryptic angucycline cluster, and genetic manipulations of all CSR genes could not activate its expression (35). A functionally unknown *ovmZ* ho mologous gene, *pgaK*, has not been genetically manipulated. It would not be surprising that PgaK could not work alone because a partner protein gene is absent in the cluster. Another cryptic angucycline cluster is the chattamycin biosynthetic gene cluster. Although there is *ovmZ* orthologous in this clus ter, the direct effector of regulation seems to be another positive regulator, ChaI, because the transcription of the cluster could be activated by constitutive expression of ChaI (11). Thus, it would be more complicated to activate cryptic gene clusters for angucycline antibiotics by manipulating the CSR genes. In our study, the biosynthesis of an angucycline antibiotic, oviedomy cin, was activated by disruption of *adpA*. This study indicated that genetic manipulation of the global regulatory genes for activating cryptic gene clusters could be an effective approach.

Experimental procedures

Strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1, and primers are listed in supplemental Table 2. *S. anso chromogenes* (the wild-type strain), *S. coelicolor* M1146, and their derivative strains were grown on mannitol soya flour medium agar or in yeast extract-malt extract (YEME) liquid medium at 28 °C (45). Mannitol soya flour liquid medium was used as the fermentation medium for oviedomycin production. *E. coli* JM109, C41 (DE3), ET12567/pUZ8002, and their deriv

Table 1
Strains and plasmids used in this study

Name	Description	Sources
Strains		
S. ansochromogenes	Wild-type strain	Ref. 49
ΔadpA	*adpA* disruption mutant	This study
ΔovmZ	*ovmZ* disruption mutant	This study
ΔovmW	*ovmW* disruption mutant	This study
ΔovmR	*ovmR* disruption mutant	This study
Δorf3	*orf3* disruption mutant	This study
Δorf4	*orf4* disruption mutant	This study
ΔadpA/ΔovmZ	*adpA* and *ovmZ* disruption mutant	This study
ΔadpA/ΔovmW	*adpA* and *ovmW* disruption mutant	This study
ΔadpA/ΔovmR	*adpA* and *ovmR* disruption mutant	This study
ΔadpA/ Δorf3	*adpA* and *orf3* disruption mutant	This study
ΔadpA/Δorf4	*adpA* and *orf4* disruption mutant	This study
XJJ101	*ovmZ* constitutive expression strain	This study
XJJ102	*ovmW* constitutive expression strain	This study
XJJ103	*ovmR* constitutive expression strain	This study
XJJ104	*ovmZ* and *ovmW* constitutive expression strain	This study
S. coelicolor		
M1146	Δ*act* Δ*red* Δ*cpk* Δ*cda*	Ref. 50
XJJ105	M1146 derivative containing *ovmOI* promoter-*gusA* fusion plasmid	This study
XJJ106	M1146 derivative containing *ovmZW* overexpression plasmid	This study
XJJ107	M1146 derivative containing *ovmZ* overexpression plasmid	This study
XJJ108	M1146 derivative containing *ovmW* overexpression plasmid	This study
XJJ109	M1146 derivative containing *ovmOI* promoter-*gusA* fusion plasmid and *ovmZW* overexpression plasmid	This study
XJJ110	M1146 derivative containing *ovmOI* promoter-*gusA* fusion plasmid and *ovmZ* overexpression plasmid	This study
XJJ111	M1146 derivative containing *ovmOI* promoter-*gusA* fusion plasmid and *ovmW* overexpression plasmid	This study
E. coli		
JM109	F,' *proA*$^+$B$^+$, *lacI^q*, Δ(*lacZ*)M15/Δ(*lac-proAB*), *gyrA96*, *recA1*, *relA1*, *endA1*, *hsdR17*	Ref. 51
ET12567/pUZ8002	*recE*, *dam*, *dcm*, *hsdS*, Cm^R, Str^R, Tet^R, Km^R	Ref. 52
C41(DE3)	F$^-$, *ompT*, *gal dcm hsd S$_B$* (r$_B^-$, m$_B^-$) (DE3)	Lucigen
S. aureus CGMCC1.89	Indicator strain for bioassays	CGMCC^a
B. cereus CGMCC1.1626	Indicator strain for bioassays	CGMCC
B. subtilis CGMCC1.1630	Indicator strain for bioassays	CGMCC
C. albicans CGMCC2.4159	Indicator strain for bioassays	CGMCC
Plasmids		
pKC1139	*E. coli-Streptomyces* shuttle plasmid contains a *Streptomyces* temperature-sensitive origin of replication	Ref. 53
pIJ10500	Hyg^R, a derivative of pMS82 containing ΦBT1 integrase gene	Ref. 54
pET23b::*adpA*	pET23b derivative with *adpA* coding region	Ref. 26
pAD4	Plasmid used for disruption of *adpA*	Ref. 26
pAD5	pSET152 derivative with intact adpA and its native promoter	Ref. 26
pUZ101	Plasmid used for construction of pKC1139::ZUD	This study
pZP101	Plasmid used as an amplification template for probe P$_{ovmZ}$	This study
pKC1139::ovmZUD	Plasmid used for disruption of *ovmZ*	This study
pKC1139::ovmWUD	Plasmid used for disruption of *ovmW*	This study
pKC1139::ovmRUD	Plasmid used for disruption of *ovmR*	This study
pKC1139::orf3UD	Plasmid used for disruption of *orf3*	This study
pKC1139::orf4UD	Plasmid used for disruption of *orf4*	This study
pKC1139:: P$_{hrdB}$Z	pKC1139 derivative with intact *ovmZ* and *hrdB* promoter	This study
pKC1139:: P$_{hrdB}$W	pKC1139 derivative with intact *ovmW* and *hrdB* promoter	This study
pKC1139::P$_{hrdB}$ZW	pKC1139 derivative with intact *ovmZW* and *hrdB* promoter	This study
pKC1139::P$_{hrdB}$R	pKC1139 derivative with intact *ovmR* and *hrdB* promoter	This study
pIJ10500::P$_{ovmOI}$::*gusA*	pIJ10500 derivative with intact *gusA* and *ovmOI* promoter	This study

^{a} CGMCC, China General Microbiological Culture Collection.

ative strains were grown at 37 °C in LB medium containing necessary antibiotics for propagating plasmids. The final concentrations of antibiotics used were as follows: 100 µg/ml ampicillin, 100 µg/ml apramycin, 100 µg/ml kanamycin, and 12.5 µg/ml chloramphenicol in LB for *E. coli*; 25 µg/ml nalidixic acid in mannitol soya flour medium for selection of *Streptomyces* exconjugants; 10 µg/ml apramycin and 10 µg/ml kanamycin in YEME and mannitol soya flour medium for *S. ansochromogenes*; and 50 µg/ml apramycin in YEME and mannitol soya flour medium for *S. coelicolor* M1146.

Gene disruptions

To construct an *adpA* disruption mutant (ΔadpA) in *S. ansochromogenes* and its derivatives, the plasmid pAD4 (26) was first introduced into *E. coli* ET12567/pUZ8002 by transformation and then introduced into *S. ansochromogenes* by conjugal transfer. The transformants resistant to kanamycin (Kan^r) but

sensitive to apramycin (Apr^s) were selected as the double crossover disrupted strains and confirmed by PCR with primer pair ad1F/ad2R and using their genomic DNA as the template. For complementation analysis, the plasmid pAD5 (26) was introduced into ΔadpA by conjugal transfer from ET12567/pUZ8002, and the complemented strain was confirmed by PCR with primer pair ad1F/ad2R. To construct recombinant plasmid for *ovmZ* disruption, the 1468-bp DNA fragment corresponding to the upstream region of *ovmZ* was amplified by PCR with primer pair UZF/UZR. The PCR product was purified by gel purification (Qiagen) and digested with HindIII and XbaI and then inserted into the HindIII/XbaI site of pKC1139 to generate pUZ101. The 1441-bp DNA fragment corresponding to the downstream region of *ovmZ* was amplified by PCR with primer pair DZF/DZR, followed by digestion with HindIII and EcoRI after purification, and then inserted into the HindIII/

EcoRI site of pUZ101 to generate pKC1139::ovmZUD. The resulting plasmid was introduced into *S. ansochromogenes* by conjugal transfer from ET12567/pUZ8002. The transformants conferring apramycin resistance were obtained, and then the colonies, which had lost the apramycin resistance after growing for 4 days at 42 °C, were selected as the double crossover disrupted mutant of *ovmZ*. This mutant strain (ΔovmZ) was confirmed by PCR using primer pair UZF/DZR and its chromosome DNA as the template to generate a 2909-bp fragment. For the double disruptions of *adpA* and *ovmZ*, the plasmid pAD4 was introduced into ΔovmZ by conjugal transfer from ET12567/pUZ8002. The double mutant strain (ΔadpA/ΔovmZ) conferring kanamycin resistance (Kan^r) but sensitive to apramycin (Apr^s) was selected and further confirmed by PCR as described above. The construction of single disruption strains of *ovmW*, *ovmR*, *ovmY*, *orf3*, and *orf4* and the double disruption strains of these genes together with *adpA* were performed as mentioned above.

Construction for constitutive overexpression of regulatory genes

To construct constitutive overexpression plasmids for regulatory genes, *Streptomyces* multicopy number plasmid pKC1139 was used to clone the regulatory genes under the control of the *hrdB* promoter (P$_{hrdB}$). The *ovmZ* coding region was amplified from the genomic DNA of *S. ansochromogenes* with primer pair ovmZF/ovmZR, and the *hrdB* promoter was amplified from the genomic DNA of *S. coelicolor* M145 using primer pair hrdBpF/hrdBpR. The primer hrdBpR was phosphorylated with T4 polynucleotide kinase (New England Biolabs) before PCR amplification to facilitate subsequent ligation reactions. The PCR product of the *ovmZ* coding region was digested with EcoRI, and the PCR product of the *hrdB* promoter was digested with XbaI. After purification from gel, two fragments were ligated together with XbaI/EcoRI-digested plasmid pKC1139 to generate pKC1139::P$_{hrdB}$Z. The plasmids pKC1139::P$_{hrdB}$W, pKC1139::P$_{hrdB}$ZW, and pKC1139::P$_{hrdB}$R were constructed in a similar way. The primer pairs used in the constructions are listed in supplemental Table 1. The resulting plasmids pKC1139::P$_{hrdB}$Z, pKC1139::P$_{hrdB}$W, pKC1139::P$_{hrdB}$ZW, and pKC1139::P$_{hrdB}$R, respectively, were introduced into *S. ansochromogenes* to obtain XJJ101, XJJ102, XJJ103, and XJJ104.

Heterologous expression of ovm in S. coelicolor M1146

The plasmid for heterologous expression of *ovm* was generated by Gibson assembly. The *ovm* cluster containing 12 structural genes (*ovmOI–ovmH*) was separately amplified by PCR to split into three DNA fragments (F1 with 5005 bp, F2 with 5000 bp, and F3 with 4418 bp) using three sets of primer pairs (F1F/F2R, F2F/F2R, and F2F/F2R) and *S. ansochromogenes* genomic DNA as the template. The *hrdB* promoter was amplified with the primer pair hrdBpF′/hrdBpR′ from genomic DNA of *S. coelicolor* M145. It contains a 30-nt sequence at the 5′-end of P$_{hrdB}$ overlapped with the one end of pSET152 digested with EcoRV and a 36-nt sequence at the 3′-end of P$_{hrdB}$ overlapped with the 5′-end of F1 fragment. F1, F2, and F3 fragments contain a 30-nt sequence overlapped with each other as the genetic organization order of the *ovm* cluster, and the F3 fragment has a 30-nt

sequence at its 3′-end overlapped with the other end of pSET152 as mentioned above. 15 μl of Gibson assembly mix, ~180 ng of purified *ovm* fragments, and ~20 ng of P$_{hrdB}$ fragment were added in a final volume of 20 μl of reaction mixture. The reactions were performed at 50 °C for 1 h (46). Then the assembled DNA was transformed into the *E. coli* JM109 competent cells. The resulting plasmid, pSET152::P$_{hrdB}$::*ovmOI-H*, was further transferred into *S. coelicolor* M1146 by conjugation from ET12567/pUZ8002. The regenerated recombinant strain was used for heterologous expression of oviedomycin.

Isolation, purification, and characterization of oviedomycin

ΔadpA was cultivated in a 5-liter fermentor containing 3 liters of mannitol soya flour liquid medium for 3 days at 28 °C and 200 rpm. The cultures were filtered through gauze, extracted with chloroform, dried *in vacuo*, and then redissolved in a small amount of methanol. Sephadex LH-20 column was applied for further purification. Active fractions were collected by semipreparative HPLC equipped with ZORBAX SB-C18 reverse-phase column (9.4 × 250 mm, 5 μm; Agilent) at 3 ml/min using a linear gradient elution. The elution solution was acetonitrile from 5 to 100% in water with 0.1% trifluoroacetic for 40 min followed by 100% acetonitrile with 0.1% trifluoroacetic for 10 min. MS analysis was performed on an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a Dionex Ultimate 3000 nano-low system and a nano-electrospray ion source. NMR spectra were recorded on a 500-MHZ Bruker spectrometer using DMSO-d_6 as the solvent. Normal detection of oviedomycin was performed by HPLC with a ZORBAX SB-C18 reverse phase column (4.6 × 250 mm, 5 μm; Agilent). Samples were eluted at 1 ml/min with the same linear gradient as mentioned above.

Expression and purification of AdpA, OvmZ, and OvmW

The plasmid pET23b::*adpA* used for AdpA expression was constructed previously by Pan *et al.* (26). To construct recombinant plasmids for OvmZ and OvmW expression, the coding regions of *ovmZ* and *ovmW* were amplified from genomic DNA of *S. ansochromogenes* by PCR using primer pairs ovmZ1F/ovmZ1R and ovmW1F/ovmW1R. The PCR products were digested with NdeI and XhoI and then inserted into the NdeI/XhoI site of pET23b to generate plasmids pET23b::*ovmZ* and pET23b::*ovmW*. To construct the plasmid for the co-overexpression of OvmZ-OvmW, the *ovmZ* and *ovmW* coding regions with His$_6$ label at the 3′-end were amplified from genomic DNA of *S. ansochromogenes* by PCR using primer pairs ovmZ1F/ovmZ2R and ovmW2F/ovmW1R. The PCR product of *ovmZ* was digested with NdeI/BbsI, and the PCR product of *ovmW* was digested with BbsI/XhoI. The purified fragments were ligated together with NdeI/XhoI linear plasmid pET23b to generate pET23b::*ovmZW*. The resulting plasmids were introduced into *E. coli* C41 (DE3) for the expression of OvmZ and OvmW.

Electrophoretic mobility shift assays and DNase I footprinting

The EMSAs were performed as described previously (47). The upstream regions (probes P$_{ovmZ}$ and P$_{ovmOI}$) of *ovmZ* and *ovmOI* were amplified from genomic DNA of *S. ansochromogenes*

Activation of cryptic oviedomycin production

by PCR using primer pairs ovmZpF/ovmZpR and ovmOIpF/ovmOIpR, respectively. The probe P_{ovmZ} was inserted into the EcoRV site of pBluescript KS+ to generate the plasmid pZP101, and then it was used as the template for PCR amplification using primers FAM-M13F/HEX-M13R to prepare fluorescently labeled probe P_{ovmZ}. The probes P_{ovmZ} and P_{ovmOI} were subsequently incubated with various concentrations of the protein individually at 25 °C for 30 min in 20 μl of reaction mixture. For competition assays, the unlabeled probe (specific competitor) or the nonspecific probe (a DNA fragment of the internal sequence of *ovmOI* amplified by PCR with primer pair OIF/OIR), respectively, was added to the binding reaction mixtures. After incubation, the samples were loaded on 4% (w/v) native polyacrylamide gels for electrophoresis in 4 °C. The fluorescently labeled DNA was detected by Tanon-5200 Multi. The unlabeled probe DNA in the gel was stained with SYBR Gold nucleic acid gel stain for 30 min and photographed under UV transillumination using Quantity One.

DNase I footprinting assays were performed as described previously (47). The probes (200 ng) and different amounts of AdpA were added to a final reaction volume of 50 μl and incubated at 25 °C for 30 min. DNase I (Promega) digestions were carried out for 50 s at 25 °C and stopped by adding 10 μl of EGTA. The purified samples were added to 9.5 μl of HiDi formamide and 0.5 μl of GeneScan-LIZ500 size standard, and the mixture was then analyzed with a 3730XL DNA analyzer. Subsequently, the results were analyzed with the software GeneMarker version 2.2.

Construction of plasmids and generation of strains for gusA reporter system

The promoter of *ovmOI* was amplified from genomic DNA of *S. ansochromogenes* by PCR using primer pair P_{ovmOI}F/P_{ovmOI}R. The *gusA* coding region was amplified from pGUS (48) with primers gusAF/R. The primer gusAF was phosphorylated with T4 polynucleotide kinase before PCR amplification to facilitate subsequent ligation reactions. The PCR products of *gusA* coding region and the *ovmOI* promoter were digested with NotI and XbaI, respectively, and were subsequently ligated together with XbaI/NotI-digested pIJ8660 plasmid to generate pIJ8660::P_{ovmOI}::*gusA*. The fragment tfd-P_{ovmOI}-*gusA* was then amplified from the plasmid pIJ8660::P_{ovmOI}::*gusA* by PCR with primer pair tfdF/gusA2R. The PCR product was digested with XhoI/NotI and then inserted into the XhoI/NotI site of pIJ10500 to generate the plasmid pIJ10500::P_{ovmOI}::*gusA*. The resulting plasmid was transformed into *S. coelicolor* M1146 to obtain XJJ105. Three plasmids (pKC1139::P_{hrdB}ZW, pKC1139::P_{hrdB}Z, and pKC1139::P_{hrdB}W) were introduced into M1146, respectively, to obtain XJJ106, XJJ107, and XJJ108, and also the three plasmids were introduced into XJJ105 to obtain XJJ109, XJJ110, and XJJ111, respectively. The strains were cultured on AS-1 plates with X-Gluc at a final concentration of 40 μM at 30 °C for 3 days to allow the blue color formation.

RNA isolation, RT-PCR, and qRT-PCR

Total RNA was isolated from *S. ansochromogenes* and its derivatives, and RT-PCR and qRT-PCR were performed as described previously (27, 44).

Bioassays of oviedomycin

To detect the biological activity against *S. aureus*, *B. cereus*, and *B. subtilis*, 1-ml overnight cultures of indicator strains were well dispersed in 100 ml of predissolved LB agar and poured into 15-cm plates, respectively. Oxford cups were placed on the plates, and fermentation extracts were then added into the Oxford cups. After incubation for 12 h at 37 °C, the inhibition zones were observed and assessed. Bioassays against *Candida albicans* were carried out by a disk diffusion method as described previously (15). Bioassays for anticancer activity were performed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (see supplemental material).

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