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 Bacillus cereus, and 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 ovmO1–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 fight against these pathogenic 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 significant natural product reservoirs, 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 albicus J1074 (9). Another activation approach is to co-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 chattamyccins A and B in Streptomyces chattanoogenesis 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 polyeneic acid amide in Streptomyces calvus (16). As one of the conserved global regulators in
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 grixazoine 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 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. 3A). It exhibited inhibitory activity against S. aureus, whereas the HPLC solute methanol did not show inhibition zone (Fig. 3B). 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. 3C) (29). Furthermore, 1H NMR and 13C 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 angucyclinone antibiotics, the purified oviedomycin showed good inhibitory activity against A549 (non-small-cell lung cancer), HepG2 (liver cancer) and MCF-7 (breast cancer) with IC50 < 20 µM (Fig. 3E). Thus, the disruption of adpA elicited the production of a compound potentially applicable as drug.
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_{uraB} (Fig. 4A). 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. 4B 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) with the total RNA extracted from mycelia of WT strain and AdpA 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. 5A), 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 TyI from...
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 Epal 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, orf3, 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. 5B), 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. 6A), suggesting that none of the six regulatory genes plays the
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**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. anschromogenes* 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 ∆adpA. B, transcriptional analysis of regulatory genes in WT strain and ∆adpA. C, transcriptional analysis of structural genes in WT strain, ∆adpA, XJJ104, and ∆adpA/∆ovmZ.

Repressor role in oviedomycin biosynthesis. Moreover, oviedomycin produced in ∆adpA was completely abolished in the double mutants ∆adpA/∆ovmZ and ∆adpA/∆ovmW (Fig. 6B). A moderate decrease of oviedomycin production was detected in the double mutant ∆adpA/∆ovmR, whereas oviedomycin production in ∆adpA/∆ovmY, ∆adpA/∆orf3, and ∆adpA/∆orf4 strains was almost the same as in the ∆adpA strain (Fig. 6B). 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 activation 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 ∆adpA (Fig. 6C).

Subsequently, the transcriptional analyses of structural genes (*ovmOI, ovmP, ovmS, and ovmT*) in WT, ∆adpA, ∆adpA/∆ovmZ, and XJJ104 strains were performed by qRT-PCR. The biosynthetic genes were not transcribed in ∆adpA/∆ovmZ as in WT strain, whereas the transcriptional levels were 30–60 times higher in XJJ104 than those in ∆adpA (Fig. 5C). 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.
To confirm whether OvmZ and OvmW directly regulate the transcription of oviedomycin biosynthetic genes, the promoter region (P\text{ovmO1}) of the first gene (ovmO1) 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\_\text{S} or OvmW-His\_\text{S} 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, pJJ10500::P\text{ovmO1}::gusA, was constructed and then introduced into *S. coelicolor* M1146 via conjugation to generate the XJJ105 strain. Subsequently, plasmids pKC1139::P\text{hrdB}::P\text{ovmZ}, pKC1139::P\text{hrdB}::P\text{ovmW}, and pKC1139::P\text{hrdB} 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, whereas the other constructs had no obvious GUS activity (Fig. 8). This result confirmed that collaborated OvmZ-OvmW could directly activate P\text{ovmO1} 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 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.
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 nikkomyacin 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 ovn 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 ovn 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.
(34), ChaP from the chattamycin biosynthetic gene cluster (11), Aur1O from the auricin biosynthetic gene cluster (32), and Epal from the endophenazines biosynthesis gene cluster (31). Unlike OvmZ, there is no ovmW orthologue adjacent to them in the gene clusters except for epal and tyllU, epaH, an ovmW orthologue, is located downstream of epal. In the type I PKS tylosin biosynthetic cluster, tyllU, the so-called SARP “helper” gene, has homology with ovmZ, and there is another unannotated gene adjacent to tyllU that is homologous to ovmW. In a previous report (33), tyllU disruption caused a decrease of tylosin production, but the overexpression of tyllU did not improve tylosin production in S. fradiae. Probably, the function of TyllU 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 actinomycetes. Moreover, most of them are present in secondary metabolite 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 function 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. However, EMSAs showed that the OvmZ and OvmW expressed in E. coli failed to bind to their target PovmO1 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 ovmO1 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 protein or posttranslational modification might take place in Streptomyces. This could be a serious obstacle in resolving the regulation 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 usually 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 orthologue 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 homologous 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 cluster, 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, oviedomycin, 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. ansochromogenes (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-
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### Table 1

**Strains and plasmids used in this study**

| Name | Description | Sources |
|------|-------------|---------|
| **Strains** | | |
| *S. ansochromogenes* | Wild-type strain | Ref. 49 |
| ∆adpA | adpA disruption mutant | This study |
| ∆ovmZ | ovmZ disruption mutant | This study |
| ∆ovmW | ovmW disruption mutant | This study |
| ∆ovmR | ovmR disruption mutant | This study |
| ∆orf3 | orf3 disruption mutant | This study |
| ∆orf4 | orf4 disruption mutant | This study |
| ∆adpA/∆ovmZ | adpA and ovmZ disruption mutant | This study |
| ∆adpA/∆ovmW | adpA and ovmW disruption mutant | This study |
| ∆adpA/∆ovmR | adpA and ovmR disruption mutant | This study |
| ∆adpA/∆orf3 | adpA and orf3 disruption mutant | This study |
| ∆adpA/∆orf4 | adpA and orf4 disruption mutant | This study |
| XJ1101 | ovmZ constitutive expression strain | This study |
| XJ1102 | ovmW constitutive expression strain | This study |
| XJ1103 | ovmR constitutive expression strain | This study |
| XJ1104 | ovmZ and ovmW constitutive expression strain | This study |
| **S. coelicolor** | | |
| M1146 | ∆act DEL del ∆cpk ∆ada | Ref. 50 |
| XJ1105 | M1146 derivative containing ovmOI promoter-gusA fusion plasmid | This study |
| XJ1106 | M1146 derivative containing ovmZW overexpression plasmid | This study |
| XJ1107 | M1146 derivative containing ovmZ overexpression plasmid | This study |
| XJ1108 | M1146 derivative containing ovmW overexpression plasmid | This study |
| XJ1109 | M1146 derivative containing ovmOI promoter-gusa fusion plasmid and ovmZW overexpression plasmid | This study |
| XJ1110 | M1146 derivative containing ovmOI promoter-gusa fusion plasmid and ovmZW overexpression plasmid | This study |
| **E. coli** | | |
| JM109 | F' proA B lacI, lacIΔZJM15, Δ(lac-proAB), gyrA96, recA1, relA1, endA1, hsdR17 | Ref. 51 |
| ET12567/pUZ8002 | recE, dam, dcm, hsdS, CmR, StrR, TetR, KmR | Ref. 52 |
| C41(DE3) | F', ompT, gal dcm hsd S, orf3, Tet R, Km R | Lucigen |
| **S. aureus CGMCC1.89** | Indicator strain for bioassays | CGMCC |
| **B. cereus** | | |
| **C. albidus** | | |
| **C. albidus** | | |
| **Plasmids** | | |
| pKC1139 | E. coli-Streptomyces shuttle plasmid contains a Streptomyces temperature-sensitive origin of replication | Ref. 53 |
| pl110500 | HygR, a derivative of pMS82 containing PhT1 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 |
| pZIP101 | Plasmid used as an amplification template for probe P<sub>ovmZ</sub> | 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<sub>ovmZ</sub> | pKC1139 derivative with intact ovmZ and hrdB promoter | This study |
| pKC1139-P<sub>ovmW</sub> | pKC1139 derivative with intact ovmW and hrdB promoter | This study |
| pKC1139-P<sub>ovmZ</sub> | pKC1139 derivative with intact ovmZW and hrdB promoter | This study |
| pKC1139-P<sub>ovmR</sub> | pKC1139 derivative with intact ovmR and hrdB promoter | This study |
| pIJ10500-P<sub>ovmZ</sub> | pIJ10500 derivative with intact gusA and ovmZ promoter | This study |

*CGMCC, China General Microbiological Culture Collection.*

**Gene disruptions**

To construct an adpA disruption mutant (∆adpA) 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 (Km<sup>+</sup>) but sensitive to apramycin (Apr<sup>+</sup>) were selected as the double cross-over 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 ∆adpA 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 chromosomal 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 (KanR) but sensitive to apramycin (AprS) 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 multiplicity 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_{hrdBZ}, pKC1139::P_{hrdBZ}W, and pKC1139::P_{hrdBZ}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_{hrdBZ}W, pKC1139::P_{hrdBZ}W, and pKC1139::P_{hrdBZ}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 (ovmOl–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::ovmOl-H}, was further transferred into S. coelicolor M1146 by conjugation from ET12567/pUZ8002. The regenerated recombinant strain was used for heterologous expression of ovmoycin.

**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 Xhol and then inserted into the NdeI/Xhol 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/ovmZ1R and ovmW2F/ovmW1R. The PCR product of ovmZ was digested with Ndel/BbsI, and the PCR product of ovmW was digested with BbsI/Xhol. The purified fragments were ligated together with Ndel/Xhol 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_{ovmO}l) of ovmZ and ovmOl were amplified from genomic DNA of S. ansochromogenes...
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by PCR using primer pairs ovmZpF/ovmZpR and ovmOlpF/ovmOlpR, 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/HYX-M13R to prepare fluorescently labeled probe P_{ovmZ}. The probes P_{ovmZ} and P_{ovmO} 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 ovmO) 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 ovmO was amplified from genomic DNA of S. ansochromogenes by PCR using primer pair P_{ovmO}F/P_{ovmO}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 ovmO promoter were digested with NotI and XbaI, respectively, and were subsequently ligated together with XbaI/NotI-digested pIJ8660 plasmid to generate pIJ8660::P_{ovmO}::gusA. The fragment tdf-P_{ovmO}::gusA was then amplified from the plasmid pIJ8660::P_{ovmO}::gusA by PCR with primer pair tdfF/gusAR. The PCR product was digested with XhoI/NotI and then inserted into the XhoI/NotI site of pIJ10500 to generate the plasmid pIJ10500::P_{ovmO}::gusA. The resulting plasmid was transformed into S. coelicolor M1146 to obtain XJ1105. Three plasmids (pKC1139::P_{hrdB}ZW, pKC1139::P_{hrdB}Z, and pKC1139::P_{hrdB}W) were introduced into M1146, respectively, to obtain XJ1106, XJ1107, and XJ1108, and also the three plasmids were introduced into XJ1105 to obtain XJ1109, XJ1110, and XJ1111, 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).

Author contributions—J. X., Y. T., and H. T. designed the study. J. X. performed most of the experiments, analyzed the primary data, and wrote the draft of the manuscript. J. Zhang participated in the bioassay and structure analysis of oviedomycin. J. Zhuo assisted with strain fermentation. Y. L. participated in the protein purification and analysis. Y. T. revised the manuscript and wrote partial contents. H. T. supervised the whole research work and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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