

Inactivation of the positive LuxR-type oligomycin biosynthesis regulators *OlmRI* and *OlmRII* increases avermectin production in *Streptomyces avermitilis*

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Oligomycins are a group of 26 macrocyclic lactones that exhibit broad biological activities, including antifungal, anticancer and nematocidal activities. Analysis of the oligomycin biosynthetic gene cluster (*olm*) in *S. avermitilis* revealed 2 tandem LuxR-type regulators, *OlmRI* (931 aa) and *OlmRII* (941 aa), with shared identity of 38%. Gene replacement of *olmRI* or *olmRII* abolished oligomycin production, and this production could be partially restored in the disruptants by introducing cloned *olmRI* and *olmRII* with their native promoters, demonstrating the essential role of *OlmRI* and *OlmRII* for oligomycin biosynthesis. Quantitative real-time RT-PCR analysis revealed that transcription of 14 *olm* genes was differentially affected by the deletion of *olmRI* and *olmRII*. Unexpectedly, avermectin production in both mutants was enhanced at least 4-fold. The identification of the positive cluster-situated regulators, *OlmRI* and *OlmRII*, paves the way for the transcriptional analysis of oligomycin biosynthesis and for the enhancement of oligomycin and avermectin production through regulator engineering.

Streptomyces, oligomycin, regulation, avermectin, enhanced production

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Streptomyces are Gram-positive soil bacteria that undergo morphological differentiations from substrate mycelia to aerial hyphae and chains of spores, and have an amazing ability to produce a great variety of valuable biologically active secondary metabolites, including hundreds of antibiotics [1]. Antibiotics are usually produced during the late growth phase, and the coordinated expression of all the genes in an antibiotic biosynthetic gene cluster is tightly regulated in response to environmental conditions through a complex regulatory network [2].

The regulatory hierarchies employed by some representative biosynthetic pathways have been elucidated, and a great number of regulatory genes involved in antibiotic production and morphological differentiation have been identified [3]. These genes take part in the regulation at dif-

ferent levels to respond to various external environmental changes and to the internal growth-phase transition. Some pleiotropic regulators execute global functions not only in secondary metabolism, but also in morphological differentiation [4]. *bldA*, encoding a tRNA recognizing the rare leucine codon UUA, is such a regulator. Mutation of *bldA* causes loss of aerial hyphae and of actinorhodin and undecylprodigiosin production [5–7]. Other important regulatory cascades are switched on by hormone-like molecules, such as the A-factor of *S. griseus* [8] or γ -butyrolactone in *S. virginiae* [9,10]. In *S. griseus*, A-factor binds to the A-factor receptor protein, ArpA, and initiates the transcription of *adpA* by dissociating the DNA-bound ArpA from the *adpA* promoter. AdpA then plays a central regulatory role in morphological development and secondary metabolism pathways by binding the transcriptional start sites of target genes and recruiting RNA polymerase to the promoter re-

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gions. In contrast, pathway-specific regulators only affect the transcription of a single antibiotic biosynthetic pathway, within which the genes are clustered. However, cluster-situated regulators, which are considered to be pathway-specific, have also been shown to influence colony development [11].

Some well-known pathway-specific regulators situated in Type I polyketide synthase gene clusters belong to a novel LAL family (large ATP-binding regulators of the LuxR family) of activators [12]. They are large proteins that feature an N-terminal ATP/GTP-binding domain containing the Walker A and B motifs [13], and a C-terminal LuxR family helix-turn-helix (HTH) DNA-binding domain [14]. Examples of LAL transcriptional activators include PikD, required for pikromycin production by *S. venezuelae* [15], NysRI and NysRIII for nystatin biosynthesis in *S. noursei* [16], GdmRI and GdmRII for geldanamycin biosynthesis in *S. hygroscopicus* 17997 [17], and PimR for pimarinin biosynthesis in *S. natalensis* [18]. In particular, the LAL family regulator, AveR, is not only the key pathway-specific activator for avermectin biosynthesis but also negatively affects oligomycin production by down-regulating *olmRI* and *olmRII* transcription [19].

Oligomycins are a group of 26 macrolides that exhibit antifungal, antitumor and nematocidal activities [20–22]. They act as inhibitors of mitochondrial F1F0-ATPase leading to ATP depletion [23]. They can induce apoptosis in a variety of cell types, make cells more susceptible to cell death and can also cause a switch in the death mode from apoptosis to necrosis [24,25]. Recently, much research has focused on the potent anti-cancer activity of oligomycin [22,26,27]. The genome of *S. avermitilis* ATCC 31267, has been sequenced and contains more than 20 secondary metabolite biosynthetic clusters [28]. Oligomycin A and avermectin are the two main antibiotics synthesized by *S. avermitilis* ATCC 31267 [29]. The oligomycin biosynthetic gene cluster extends to about 90 kb, including seven long open reading frames (ORFs) encoding multifunctional Type I polyketide synthases (PKSs) (Figure 1 from [30]).

Genes *olmRI* (GI15823972) and *olmRII* (GI15823973) of *S. avermitilis* ATCC 31267, located upstream of the first PKS gene *olmA1*, were predicted to be LuxR-family regulators. Through gene inactivation and complementation followed by transcriptional analysis, we describe here the in-

volvement of *olmRI* and *olmRII* in oligomycin biosynthesis and their influence on avermectin production.

1 Materials and methods

1.1 Bacterial strains, media, growth conditions, DNA manipulation and sequence analysis

All strains and plasmids used in this study are listed in Table S1. Culture and standard bacteriological methods were performed as described by Sambrook et al. [31] and Kieser et al. [32]. According to refs. [33,34], the wild-type strain, *S. avermitilis* ATCC 31272, represents a lyophilized culture obtained by ultraviolet irradiation of *S. avermitilis* ATCC 31267. The avermectin and oligomycin production, and the DNA sequence in the oligomycin cluster, were confirmed in this study to be consistent between the two strains. *E. coli* strains were grown at 37°C in Luria-Bertani medium. *S. avermitilis* ATCC 31272 and its mutants were grown at 30°C on SFM [32] agar for oligomycin fermentation and for conjugation with *E. coli* ET12567/pUZ8002. YD agar [35] was used for sporulation, and TSBY liquid medium [32] supplemented with 10.3% (w/v) sucrose was used for mycelial growth. For *Streptomyces*, apramycin and thiostrepton were respectively used at 30 and 20 µg/mL in agar plates and at 15 and 10 µg/mL in liquid medium.

General techniques for DNA manipulation were used as described in refs. [31,32]. Synthesis of oligonucleotide primers and DNA sequencing of PCR products were performed by Invitrogen Biotechnology (China). Restriction digestions were carried out according to the protocols for the enzymes (Fermentas Inc., Canada). Analyses of amino acid sequences were performed using Blastp and ClustalW, and Pfam at <http://pfam.janelia.org/> [36].

1.2 Inactivation of *olmRI* and *olmRII* and complementation of the mutants

Fragments (7.1–7.3 kb) were recovered from *Bam*H I-digested total genomic DNA separated by agarose gel electrophoresis, and cloned into pBluescript SK(+). After screening clones by *Pvu* II and *Xho* I digestion, a plasmid containing the 7266-bp *olmRI-olmRII* fragment was confirmed and named pJTU1714. The fragment was then

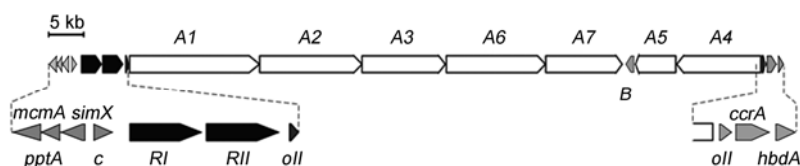


Figure 1 Oligomycin biosynthetic gene cluster of *S. avermitilis*. Arrows indicate ORFs and the orientation of transcription. *RI* and *RII* (black arrows, *olmRI* and *olmRII*), LAL-family regulators; *A1*–*A7* (blank arrow boxes, *olmA1*–*olmA7*), type I polyketide synthases. The grey arrows indicate the post-modification genes: *mcmA*, methylmalonyl-CoA mutase; *pptA*, phosphopantetheinyl transferase; *simX*, *simX4* homolog; *C* (*olmC*), thioesterase; *B* (*olmB*), cytochrome P450; *OII* (*olmOII*), hypothetical protein; *ccrA*, crotonyl-CoA reductase; *hbdA*, 3-hydroxyacyl-CoA dehydrogenase.

*Bam*H I-digested and transferred to pJTU412 to generate plasmid pJTU1715. To inactivate *olmRI* and *olmRII*, two *aac(3)IV-oriT* cassettes were amplified with the template pIJ773, using the primers *olmRI*-tgtP1 & P2, and *olmRII*-tgtP3 & P4 respectively (Table S2). They were then separately introduced into *E. coli* BW25113/pIJ790/ pJTU1715 by electroporation and recombined with pJTU1715 under temperature pressure [37] to generate the apramycin-resistant disruptants, pJTU1710 for Δ *olmRI* and pJTU1727 for Δ *olmRII*. pJTU1710 and pJTU1727 were individually conjugated into *S. avermitilis* ATCC 31272 as described [32]. Apramycin-resistant thiostrepton-sensitive ($\text{Am}^R \text{Tsr}^S$) double-crossover exconjugants YQ6 (Δ *olmRI*) and YQ18 (Δ *olmRII*) were selected and confirmed by PCR with primers *olmRI*-test-P1 & P2 and *olmRII*-test-P3 & P4, respectively (Table S2).

For complementation, the plasmid pJTU1739 was constructed by inserting the 7266-bp fragment from pJTU1714 into the *Bam*H I-digested pIB139-derived vector, pJTU824 (Table S1). pJTU1739 was introduced through conjugation into both of the mutants. YQ6: pJTU1739 and YQ18: pJTU1739 were selected by an $\text{Am}^R \text{Tsr}^R$ phenotype.

1.3 Oligomycin and avermectin production assay

Thawed suspensions of *S. avermitilis* ATCC 31272 and its derivatives were inoculated into 10 mL TSBY liquid medium and grown at 30°C for 18 h to produce seed cultures. After detecting the wet weight of mycelia, the same amounts of seed cultures were grown on two SFM agar plates (40 mL melted medium spread to 56.5 cm²) at 30°C for 72 h for antibiotic production. The solid fermentation cultures were extracted twice for 8 h with two volumes of methanol. The methanol was evaporated and crude extracts were re-dissolved in 1 mL of methanol, passed through 0.22 μm nylon filters, and analyzed using an Agilent 1100 series LC/MSD Trap system using a ZORBAX Extend-C18 column (5 μm , 2.1 mm \times 150 mm, Agilent, USA). Eluent A was Milli-Q (Millipore, USA) deionized water with 0.2% formic acid, and eluent B was acetonitrile. The LC was monitored at 230 nm and the flow rate was at 0.27 mL min⁻¹ using a gradient mobile phase starting with 60% B (v/v) and ending with 80% B for 15 min, followed by the isocratic mobile phase containing 80% B for 2 min, and finally by an increasing gradient phase ending with 90% B for 10 min. Oligomycin A from *S. diastatochromogenes* (Sigma-Aldrich Corp., Saint Louis, MO, USA) was used as the standard with a retention time of 15.3 min.

1.4 Isolation of total RNA and gene expression analysis by quantitative real-time reverse transcriptase PCR (qPCR)

For RNA isolation, *S. avermitilis* was grown for 3–4 d at 30°C on SFM agar plates covered with cellophane sheets.

Mycelia were scraped from the cellophane and disrupted in a Precellys Homogenizer (5500 \times g, 1 \times 30 s; Peqlab, Germany) with glass beads (150–212 μm , Sigma). RNA was isolated with the total RNA Isolation Kit from SBS Genetech and treated with DNase I (RNAase-free, Fermentas) to eliminate genomic DNA.

RNA (3 μg) was used to generate cDNA using RevertAidTM H Minus Reverse Transcriptase and Random Hexamer Primer (Fermentas) in 20- μL reactions, as described in the manufacturer's protocol. The synthesized cDNA (0.6 μL) was used as template for each reaction using the primers listed in Table S3. The sigma factor gene *hrdB* and the 16S rRNA gene were used as references and the primers were designed according to the 16S rRNA gene of *S. lividans* (X86354.1) and the *hrdB* gene of *S. coelicolor* (X52983.1), respectively. The primers for the target genes within the oligomycin cluster were designed to match the sequence of the *S. avermitilis* oligomycin biosynthetic gene cluster (AB070940.1, GI15823967). The qPCR reaction mixtures were prepared according to the protocol of Maxima[®] SYBR Green/ROX qPCR Master Mix (Fermentas). The qPCR assays were carried out in triplicate using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems Inc., USA) and then repeated 3 times with RNA isolated from independent batches. For a given target gene, the relative transcript copy number was the ratio of its transcribed copies in mutant samples to its copies in the wild-type control. The ratio was calculated using the comparative C_t method with the formula $2^{-\Delta\Delta C_t}$ ($\Delta\Delta C_t = \Delta C_t \text{ sample (target-reference)} - \Delta C_t \text{ control (target-reference)}$) [38]. All the key genes involved in oligomycin biosynthesis were analyzed. For each gene, the transcript copy number in the control wild-type strain was assigned a value of 1.0. A value higher than 2, suggested that the transcription of a target gene was distinctly activated or increased in the mutant. A value lower than 0.5 showed decreased or depressed transcription.

2 Results and discussion

2.1 *In silico* analysis of regulatory genes *olmRI* and *olmRII*

Two putative regulatory genes, *olmRI* and *olmRII*, are located upstream of *olmA1*, the first Type I PKS gene of the oligomycin biosynthesis pathway in *S. avermitilis* ATCC 31272 [30]. Blastp analysis revealed that *OlmRI* (931 amino acids) and *OlmRII* (943 amino acids) share 38% identity, and are putative members of the LAL family (large ATP-binding regulators of the LuxR family) of transcriptional activators [12]. Multiple alignments of LAL regulators in the polyketide biosynthesis pathway revealed conserved motifs that are also contained in *OlmRI* and *OlmRII* (Figure 2(a)). Walker A and B motifs are present at the N termini (Figure 2(b)) and LuxR-type HTH DNA binding motifs [14] are located at the C termini (Figure 2(c)). Close

to the HTH motifs are tetratricopeptide repeats (TPR) [39] at amino acid residues 615–834 in *OlmRI* and at 687–821 in *OlmRII* (Figure 2(a)).

The Walker A and B motifs of *OlmRI* and *OlmRII* are characteristic of the triphosphate-binding domain of a large family of ATPases associated with diverse cellular activities. The two regulators were therefore proposed to hydrolyze NTP to activate oligomycin biosynthesis. Interestingly, Walker A motifs in *OlmRI* (aa 32–39) and *OlmRII* (aa 17–24) have a truncated core motif GXGK(R)T(S), compared to the consensus ATP-binding sequence, GXXG-XGKT [13]. The native S32_{*OlmRI*} and A17_{*OlmRII*} are different from the highly conserved glycine residue of other well-known Walker A motifs (indicated by a grey circle in Figure 2(b)). This reveals the diversity of the conserved sequences of the functional Walker A core motifs. The two regulators also have TPR domains implicating protein-protein interactions and the C-terminal LuxR-family DNA-binding helix-turn-helix domains. The known LuxR-family proteins, such as NysRI, NysRIII and PimR, can activate the biosynthesis of macrocyclic lactones. It was thus expected that inactivation of *olmRI* or *olmRII* would reduce or abolish oligomycin production by reducing the transcription of the cognate genes, which specify the enzymes involved in oligomycin biosynthesis.

2.2 *OlmRI* is essential for oligomycin production

To ascertain the involvement of *olmRI* in oligomycin biosynthesis, 300 bp of the coding region of *olmRI* was replaced by an *aac(3)IV-oriT* cassette using Redirect Technology [37] (Figure 3(a)). Six apramycin-resistant and thiostrepton-sensitive (Am^R Tsr^S) mutants were confirmed by PCR amplification using primers *olmRI*-test-P1 and P2 and named as YQ6. The wild type gave a 1.16-kb amplified fragment, whereas YQ6 produced a 2.29-kb amplicon (Figure 3(b)).

By comparing the size and morphological features of the single colonies on MM and SFM agar, the growth rate and morphology of wild type and YQ6 were exactly alike. We determined oligomycin yields of the wild type and mutant after 120 h of growth on SFM plates at 30°C when oligomycin production was maximal. In two independent mutants, HPLC profiles did not show any detectable peak corresponding to oligomycin (Figure 3(c)). Complemented with the plasmid pJTU1739, the oligomycin productivity of YQ6 was restored to about 10% of the wild-type level. Therefore, *olmRI* was found to execute positive control on oligomycin biosynthesis.

2.3 Inactivation of *olmRII* abolished oligomycin production

Using a similar strategy as above, a 2040-bp internal region of *olmRII* was replaced by the *aac(3)IV-oriT* cassette (Figure 4(a)). Nine Am^R Tsr^S mutants were verified by PCR

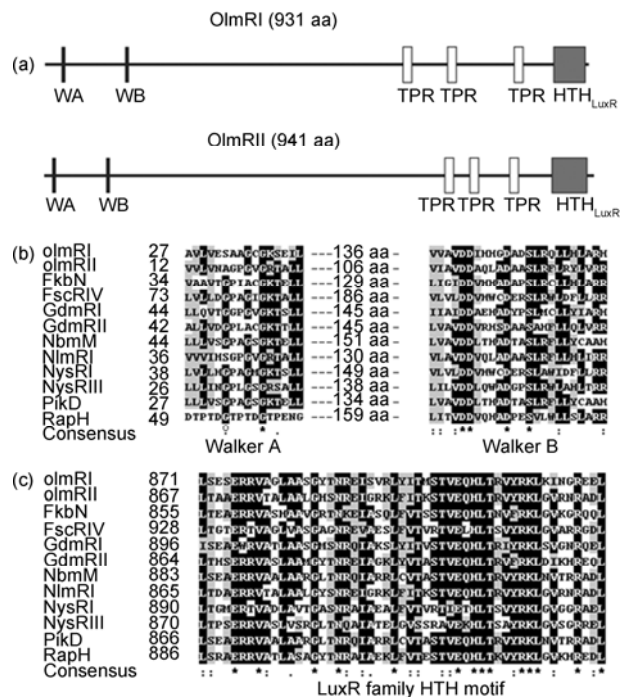


Figure 2 Structures and alignment of the *OlmRI* and *OlmRII* regulators. (a) Conserved motifs in *OlmRI* and *OlmRII*. WA and WB, Walker A and B NTP binding motifs; TPR, tetratricopeptide repeats; HTH_{LuxR}, LuxR-family HTH DNA-binding domains. (b) Alignment of the Walker A and B motifs from proteins resembling *OlmRI* and *OlmRII*. The circle indicates the conserved glycine residue that is divergent in *OlmRI* and *OlmRII*. The stars and spots indicate highly conserved amino acid residues. The aligned proteins include the known and putative LuxR-type regulators from various streptomyces sources: FkbN from *S. hygrosopicus* subsp. *ascomyceticus*, FscRIV from *Streptomyces* sp. FR-008, GdmRI and GdmRII from *S. hygrosopicus* 17997, NbmM from *S. narbonensis*, NlmRI from *S. nanchangensis*, NysRI and NysRIII from *S. noursei*, PikD from *S. venezuelae* and RapH from *S. cinnamonensis*. (c) Alignment of LuxR-type HTH DNA-binding motifs.

amplification with the primers *olmRII*-test-P3 & P4 and named YQ18 (Figure 4(b)).

On MM and SFM agar plates, YQ18 showed a similar growth rate and morphology to the wild type. Similar to the Δ *olmRI* mutant YQ6, HPLC assays showed no detectable peak corresponding to oligomycin in YQ18 (Figure 4(c)). pJTU1739 complementation of YQ18 also restored oligomycin biosynthesis, but only up to 10% of the wild-type level. Therefore, *OlmRII* was also identified as an activator of the LAL regulator family for oligomycin biosynthesis. Moreover, the lack of oligomycin productivity in both *olmRI* and *olmRII* mutants of *S. avermitilis* ATCC 31267 was also mentioned as unpublished data, without any experimental details, by Guo et al. [19].

2.4 Transcriptional analysis of oligomycin biosynthetic genes in *olmRI* and *olmRII* mutants

The transcription of all the key biosynthetic genes in the oligomycin cluster was analyzed by quantitative real-time

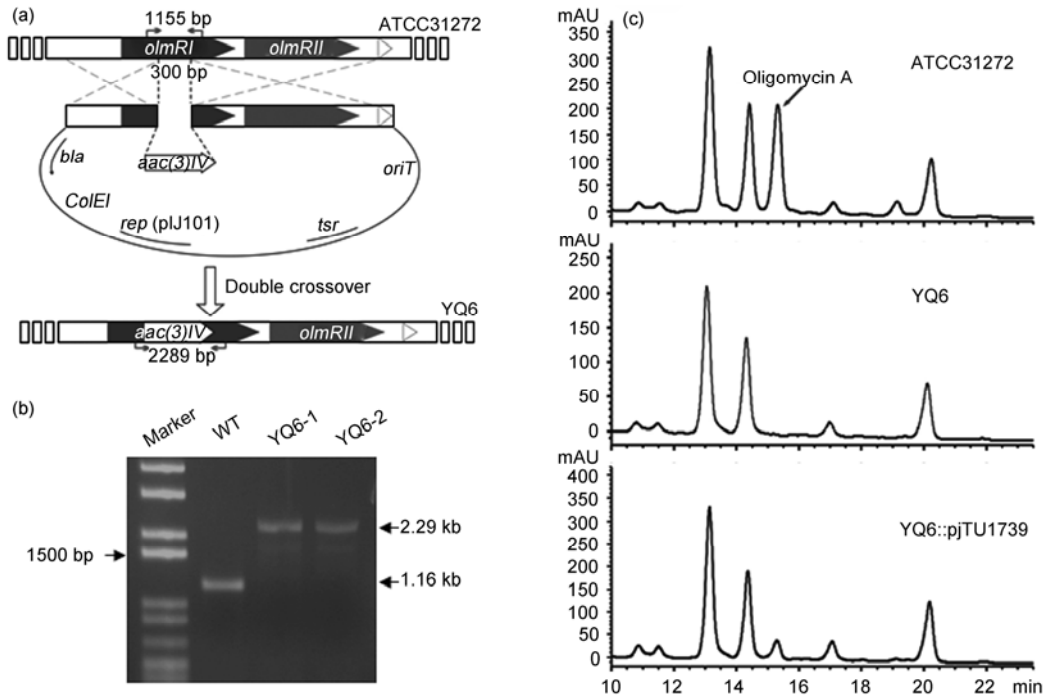


Figure 3 Inactivation of *olmRI* by gene replacement. (a) The strategy of generating the *olmRI* mutant YQ6 in *S. avermitilis* by replacing the 300-bp internal part of *olmRI* with the apramycin resistance gene *aac(3)IV*. (b) The desired mutants were confirmed by PCR analysis using primers *olmRI*-test P1 & P2 (angled arrows in (a)). (c) HPLC assay of oligomycin production. Top, extract of the wild-type ATCC31272; middle, extract from YQ6; bottom, the extract of YQ6: pJTU1739. All the other peaks represent avermectins produced by *S. avermitilis*.

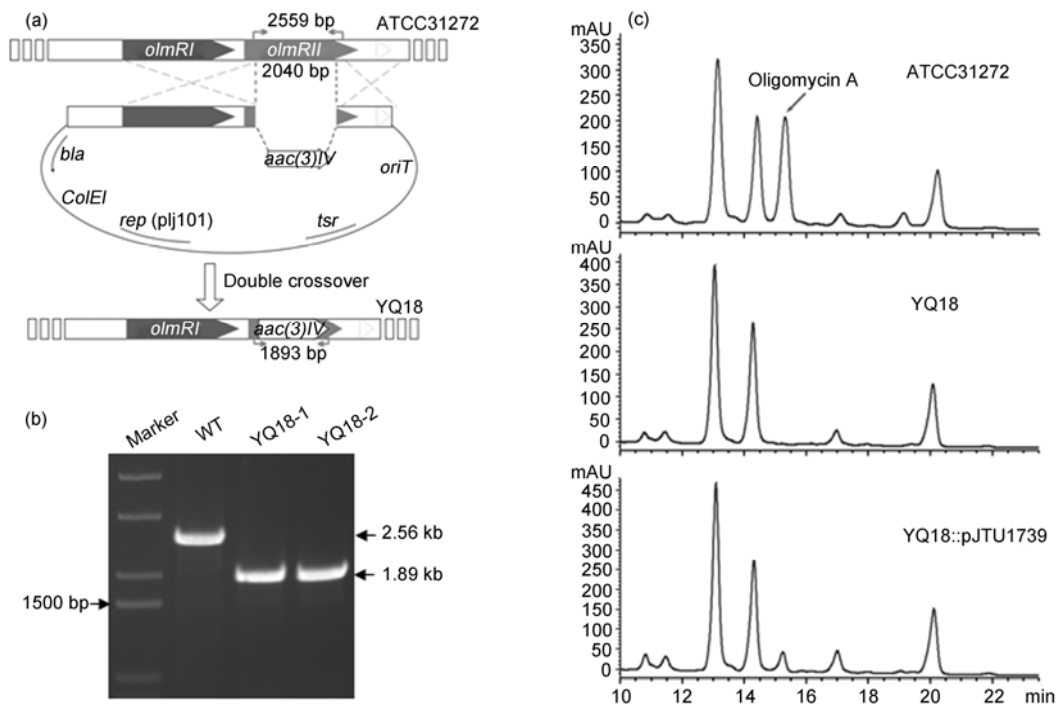


Figure 4 Inactivation of *olmRII*. (a) The strategy of generating *olmRII* mutant YQ18 in *S. avermitilis*. (b) The desired mutants were confirmed by PCR analysis using primers *olmRI*-test P3 & P4 (angled arrows in (a)). (c) HPLC assay of oligomycin production. Top, extract of the wild-type ATCC31272; middle, extract from YQ18; bottom, the extract of YQ18: pJTU1739. All the other peaks represent avermectins.

RT-PCR. Total RNA was isolated from the wild type, *olmRI* mutant YQ6 strains and *olmRII* mutant YQ18 strains grown for 120 h on SFM agar covered with cellophane. The selected key genes included PKS genes, *olmA1-olmA7*, cytochrome P450 gene, *olmB*, the post-modification genes, *olmOI*, *ccrA* and *hbdA*, and the thioesterase gene, *olmC*. Moreover, *hrdB*, encoding the constitutive vegetative sigma factor and 16S rRNA gene were used as internal references. The quantitative PCR primers were specifically designed within the first 1 kb of each gene. The primers, *qolmRI* fw-rv and *qolmRII* fw-rv (inset in Figure 5), were designed in the regions upstream of their corresponding inserted cassettes to assess the possibility of interplay between the two regulators.

Figure 5 illustrates the general influence on the oligomycin cluster by the disruption of *olmRI* and *olmRII*. The *olmRI* remnant in YQ6 was transcribed to a similar extent as in the wild type. Similarly, the remnant of *olmRII* was transcribed at a slightly higher level than in the wild type (value 1.5–1.8). These results indicate that these two regulators did not execute auto-regulation on themselves; compared with the wild type, the transcription ratios of *olmRI* in YQ18 and of *olmRII* in YQ6 were approximately 1.2 and 1.1, respectively. This revealed no interplay between the two tandem LAL regulators.

Obviously, in YQ6, inactivation of *olmRI* caused a sharp reduction in the transcription of all the PKS and post-modification genes. The expression of the PKS genes, *olmC* and *olmA1-A7*, located downstream and in the same orientation as the regulatory genes, was reduced to 20%–30% of the wild type. Expression of the genes located in the reverse orientation, *olmA4-olmB*, *olmOI-ccrA*, but not *hbdA*, was severely declined to 2% of wild type levels. In short, *OlmRI* is a crucial positive transcriptional regulator controlling almost the whole oligomycin biosynthetic gene cluster.

In contrast, compared to the wild type, inactivation of *olmRII* in YQ18 exerted different effects on *olmC* and *olmA1-A7* transcription: *olmC* was expressed at almost wild type levels; *olmA1*, the first ORF encoding the polyketide skeleton, had increased its transcription more than 2-fold; the transcription of *olmA2-A7* was reduced to approximately 50%. These observations indicate that when *OlmRII* is disrupted, *OlmRI* exerts very weak activation on the first four genes and strong positive control on the initial PKS genes. Meanwhile, the transcription of the reverse-direction PKS genes was nearly absent in YQ18, as in YQ6. This indicated that *OlmRII* was also a key positive transcriptional regulator of the reverse-direction genes in the oligomycin cluster.

Different from other homologues, structurally similar *OlmRI* and *OlmRII* are both indispensable for oligomycin biosynthesis. Bioinformatic prediction reveals a putative protein-interacting TPR motif in *OlmRI* and *OlmRII*. This motif is capable of forming homodimers or heterodimers to activate the transcription of genes essential for oligomycin biosynthesis. Because the transcription of *A1* increased and decreased with the respective disruption of *RII* and *RI*, *RI* is demonstrated to form a homodimer to activate the first PKS gene, *A1*. For the rest of the genes whose transcription levels were severely impaired in both YQ6 and YQ18, a heterodimer of *OlmRI* and *OlmRII* is required to function in the activation of gene transcription. Oligomycin and its derivatives are potent candidates for cancer therapy. Research on the positive regulators *OlmRI* and *OlmRII* may shed light on the over-production of this candidate compound.

2.5 Enhanced avermectin production in both mutants

Streptomyces avermitilis produces a series of avermectin analogues, including A2a, B1a, A1b and A1a (Figure 6(a)).

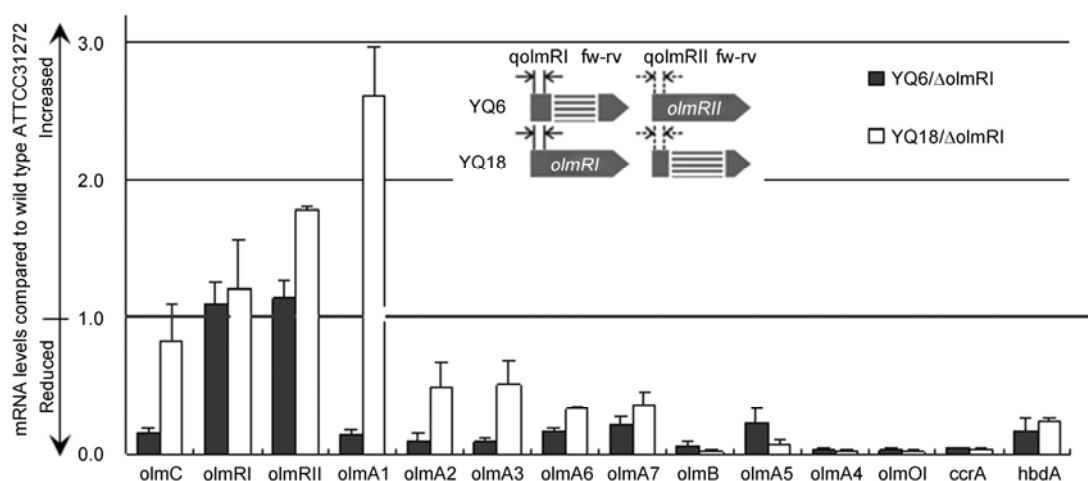


Figure 5 Transcriptional analysis of the oligomycin biosynthetic cluster using real-time RT-PCR. The histograms represent the individual target gene's transcriptional change as the ratio of the expression level in the mutants to that in the wild-type strain. Value of 1.0 (the bold line) indicates, for a certain target gene, that the mutant transcribes the same amount of mRNA as the wild type. Error bars were calculated from three independent experiments each with duplicates to validate the reproducibility. Solid bars, YQ6; white bars, YQ18. The inset shows the binding sites of the primers in *olmRI* and *olmRII* used for the quantitative real-time PCR, the solid arrow lines indicate primers *qolmRI* fw-rv and the dotted arrow lines indicate primers *qolmRII* fw-rv. The striped boxes marked the position of the replaced region in the ORFs.

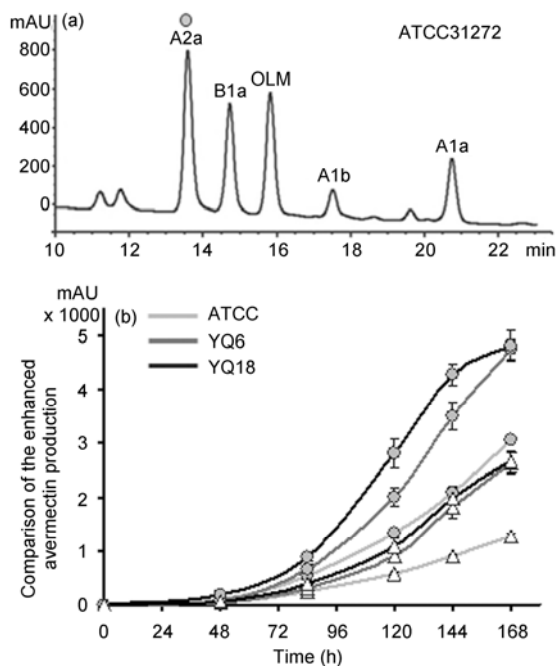


Figure 6 The avermectin production in *S. avermitilis* and its mutants. (a) HPLC analysis of oligomycin and the series of avermectins in wild-type *S. avermitilis* ATCC31272. A2a, B1a, A1b and A1a are the main components of the avermectins. OLM refers to the peak representing oligomycin. Corresponding to (b), A2a is marked with grey filled circles and A1b with hollow triangles. (b) The variation in antibiotic production in wild type and mutants.

In both of the LuxR-like regulator mutants, the production of avermectin was obviously enhanced. Component A2a was increased 1.5-fold in the mutants and component A1a was increased 2-fold (Figure 6(b)). However, further quantitative assays confirmed that *aveR* and *aveA3*, the known crucial regulatory and structural genes of avermectin biosynthesis, were transcribed at wild type levels (data not shown). Thus, *OlmRI* and *OlmRII* are cluster-situated specific activators of oligomycin biosynthesis. The overproduction of avermectins in the mutants may be the result of increased metabolic flux towards avermectins while the oligomycin production was switched off.

3 Conclusion

Using gene inactivation and complementation in *S. avermitilis* we have identified two positive cluster-situated regulators for oligomycin biosynthesis that show typical structural features of LuxR-type regulators, including an N-terminal ATP/GTP-binding domain and a C-terminal HTH DNA-binding domain. As detected by quantitative real-time RT-PCR, the transcription of genes in the oligomycin biosynthetic cluster was altered to different extents in the *olmRI* or *olmRII* mutants, suggesting similar but different regulatory mechanisms for *OlmRI* and *OlmRII*. Moreover, even though *olmRI* and *olmRII* share high similarity and are lo-

cated close to one another, the effect of one mutant on the transcription of a particular gene was not observed in the other mutant. Meanwhile, disruption of the activator genes *olmRI* and/or *olmRII* unexpectedly enhanced the production of avermectin A1a and A2a components. This not only provides understanding of the competitive utilization of substrates in *Streptomyces avermitilis*, but also provides a new strategy to enhance the productivity of antibiotics of interest.

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Supporting Information

Table S1 Bacterial strains and plasmids used in this study

Table S2 Primers used for construction and confirmation of disrupted mutants

Table S3 Primers used for quantitative real-time PCR

The supporting information is available online at csb.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.