




FadR1, a pathway-specific activator of fidaxomicin biosynthesis in *Actinoplanes deccanensis* Yp-1

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Abstract

Fidaxomicin, an 18-membered macrolide antibiotic, is highly active against *Clostridium difficile*, the most common cause of diarrhea in hospitalized patients. Though the biosynthetic mechanism of fidaxomicin has been well studied, little is known about its regulatory mechanism. Here, we reported that FadR1, a LAL family transcriptional regulator in the fidaxomicin cluster of *Actinoplanes deccanensis* Yp-1, acts as an activator for fidaxomicin biosynthesis. The disruption of *fadR1* abolished the ability to synthesize fidaxomicin, and production could be restored by reintegrating a single copy of *fadR1*. Overexpression of *fadR1* resulted in an approximately 400 % improvement in fidaxomicin production. Electrophoretic mobility shift assays indicated that fidaxomicin biosynthesis is under the control of FadR1 through its binding to the promoter regions of *fadM*, *fadA1-fadP2*, *fadS2-fadC*, and *fadE-fadF*, respectively. And the conserved binding sites of FadR1 within the four promoter regions were determined by footprinting experiment. All results indicated that *fadR1* encodes a pathway-specific positive regulator of fidaxomicin biosynthesis and upregulates the transcription levels of most of genes by binding to the four above intergenic regions. In summary, we not only clearly elucidate the regulatory mechanism of FadR1 but also provide strategies for the construction of industrial high-yield strain of fidaxomicin.

Keywords *Clostridium difficile* infection · LAL family · Pathway-specific transcriptional regulator · Regulatory mechanism · Industrial high-yield strain

Introduction

Actinomycetes are of particular interest as producers of a variety of antibiotics and other valuable secondary metabolites, such as antibiotics, anti-tumor agents, immunosuppressors,

and enzyme inhibitors (Newman and Cragg 2012; Harvey 2008; Hopwood 2007). The regulatory network of these secondary metabolites is highly complex and is pivotal for responding to the changes in physiological and environmental conditions (Martin and Liras 2010; Liu et al. 2013; van Wezel and McDowall 2011). Typically, the biosynthesis of natural product is determined by a large gene cluster, within which there is always one or more pathway-specific regulatory genes (Malpartida and Hopwood 1986; Nett et al. 2009). Analysis of these regulatory genes is important to understand the molecular mechanisms of regulation, and this would be of great value in the pharmaceutical industry.

A number of regulators responsible for initiating secondary metabolite production have already been identified. For instance, the *Streptomyces* antibiotic regulatory proteins (SARPs) containing a DNA-binding domain at the N-terminus are well-characterized pathway-specific regulators and usually act as transcriptional activators (Wietzorrek and Bibb 1997; Arias et al. 1999; Narva and Feitelson 1990; Yang et al. 2015). The LAL regulators (large ATP-binding regulators of the LuxR family) are another important pathway-

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specific regulatory family, most of which have been reported to play positive roles in antibiotic production (Martín and Liras 2012). The LAL family contains two functional domains, an N-terminal ATP/GTP-binding domain with conserved Walker A motif and a C-terminal DNA-binding domain (DBD) with a conserved helix-turn-helix (HTH) motif (Walker et al. 1982; Chen and Xie 2011). So far, several LAL family regulators have been identified, including FkbN of tetracycline gene cluster in *Streptomyces tsukubaensis* L19 (Zhang et al. 2016a, b), AveR of avermectin pathway in *Streptomyces avermitilis* (Guo et al. 2010), and AstG1 of ansatrienins gene cluster in *Streptomyces* sp. XZQH13 (Xie et al. 2015). In most cases, overexpression of these pathway-specific regulators resulted in an increased production of corresponding antibiotics (Zhang et al. 2016a, b; Qu et al. 2015), so it is very necessary to investigate the regulatory mechanisms of these regulators for accelerating the process of industrialization of value-added drugs.

Fidaxomicin, also known as tiacumicin B, lipiarmycin A3, PAR-101, OPT-80, and difimicin, is an 18-membered macrolide glycosidic polyketide with a halogenated moiety. It can act as an RNA polymerase inhibitor and is highly active against various gram-positive pathogenic bacteria (Gualtieri et al. 2006). It was approved by the FDA for the treatment of *Clostridium difficile* infection (CDI) which has become a significant problem in health care (Sullivan and Spooner 2010). Due to its importance and expensiveness in clinical medicine, it is necessary to focus more research on the biosynthesis of fidaxomicin to enhance the production titers and decrease the cost of fidaxomicin production. To date, several strains have been reported as fidaxomicin producers like *Dactylosporangium aurantiacum* NRRL 18085 (Hochlowski et al. 1987), *Micromonospora echinospora* subsp. *Armeniaca* (Omura et al. 1986), *Actinoplanes deccanensis* ATCC 21983 (Arnone et al. 1987), and *Catellatospora* sp. Bp3323-81 (Kurabachew et al. 2008). The biosynthetic gene cluster of fidaxomicin has been described in *D. aurantiacum* NRRL 18085 and there are two regulatory genes, *tiaR1* and *tiaR2*, which have been deduced to encode putative regulators homologous to LuxR-type transcriptional activator and ArsR-type repressor, respectively. However, no biological experiments have yet been performed to verify their functions in the production of fidaxomicin (Xiao et al. 2011; Niu et al. 2011).

In this work, we sequenced and annotated the whole genome of the *A. deccanensis* Yp-1 from China General Microbiological Culture Collection Center (CGMCC 4.2098). One gene cluster responsible for fidaxomicin biosynthesis was located in the genome and the coding gene for the putative regulator involved in fidaxomicin biosynthesis was annotated as *fadR1* which was highly homologous to *tiaR1* gene; however, no homolog of *tiaR2* was found in the chromosome. Here, we provided genetic and biochemical

evidences which suggested that FadR1 is a pathway-specific transcriptional activator for fidaxomicin biosynthesis. We also investigated the regulatory mechanism in which FadR1 upregulates the transcription levels of most of key genes by binding to four intergenic regions. Furthermore, we increased the yield of fidaxomicin by 400% through genetically engineering the *fadR1* gene which will lay a good foundation for industrialization of fidaxomicin.

Materials and methods

Media, plasmids, strains, and growth conditions

All bacterial strains and plasmids used in this study were listed in Table 1. The manipulation of nucleic acids and bacterial growth was carried out according to the standard protocols as previously described (Bierman et al. 1992). *Escherichia coli* TG1 (Novagen, Madison, WI, USA) was the general cloning host. *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) was used for protein expression. Vectors used were pSET152, pKC1139, pIJ8630, and pTA2. *A. deccanensis* Yp-1 strain (CGMCC 4.2098) was cultured at 30 °C on ISP4 agar for sporulation and at 30 °C in YEME medium [3 g/L yeast extract, 3 g/L malt extract, 5 g/L tryptone, 10 g/L glucose] for fidaxomicin production.

In-frame deletion and complementation of *fadR1*

Disruption of *fadR1* was performed by gene replacement according to the previously described method (Yuan et al. 2016). The upstream and downstream regions of *fadR1* were amplified with the primer pairs *fadR1*-A-F and *fadR1*-A-R as well as *fadR1*-B-F and *fadR1*-B-R (Table S1). The PCR products were purified and ligated into pKC1139 using a ClonExpress II One Step Cloning Kit (Vazyme Biotech Co, Nanjing, Jiangsu, China) to generate the disruption plasmid *pdel-fadR1*. The resulting plasmid *pdel-fadR1* was then conjugated by *E. coli* ET12567/*pUZ8002* into *A. deccanensis* Yp-1. The mutants were selected by replica plating for apramycin-sensitive colonies and confirmed by PCR amplification using primer pair P1 and P2 (Table S1).

fadR1 with the *fadR1* promoter fragment amplified using primers *fadR1*-HB-F and *fadR1*-HB-R (Table S1) was cloned into pSET152 to generate the complementation plasmid *comR1*, which was integrated into the Δ *fadR1* mutant to obtain the complementation strain.

Overexpression of *fadR1*

The *fadR1* ORF was amplified using primers *fadR1*-GP-F and *fadR1*-GP-R (Table S1). This PCR product was then ligated into the pIJ8630 containing the *ermE** promoter by the

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>A. deccanensis</i> Yp-1		
WT	<i>A. deccanensis</i> Yp-1 wild type	CGMCC 4.2098
$\Delta fadR1$	<i>fadR1</i> -disrupted mutant	This study
Com- <i>R1</i> / $\Delta fadR1$	$\Delta fadR1$ complemented with <i>fadR1</i> and its own promoter	This study
OE- <i>R1</i> /WT	Wild-type strain carrying <i>fadR1</i> overexpression	This study
<i>E. coli</i>		
TG1	General cloning host	Novagen
ET12567/pUZ8002	Methylation-deficient <i>E. coli</i> strain for conjugation	MacNeil and Klapko 1987
BL21(DE3)	Host for protein expression	Novagen
Plasmids		
pTA2	General cloning vector	TOYOBO
pKC1139	Temperature-sensitive shuttle vector	Bierman et al. 1992
pSET152	Integrative shuttle vector	Bierman et al. 1992
pIJ8630	Integrative shuttle vector	Sun et al. 1999
com- <i>R1</i>	pSET152 carrying <i>fadR1</i> and its promoter	This study
OE- <i>R1</i>	pIJ8630 carrying <i>fadR1</i> and <i>ermE</i> * promoter	This study
pex- <i>fadR1</i>	For expression of FadR1	This study
pdel- <i>fadR1</i>	For deletion of <i>fadR1</i>	This study

ClonExpress II One Step Cloning Kit (Vazyme Biotech Co, Nanjing, Jiangsu, China). The resulting plasmid OE-*R1* was then introduced into *A. deccanensis* Yp-1.

Electrophoretic mobility shift assays

The primer pair pex-FadR1-F and pex-FadR1-R (Table S1) was used to amplify *fadR1*. The *fadR1* fragment was cloned into pET28a to get the expression plasmid pex-*fadR1*, which was then introduced into *E. coli* BL21 (DE3). The resulting strain was grown at 37 °C to an optical density (OD₆₀₀) of 0.6 and then induced with 0.1 mM isopropyl-thiogalactopyranoside (IPTG) at 16 °C for 8 h. The soluble histidine-tagged FadR1 was purified with Ni²⁺-nitrilotriacetic acid (NTA) resin (Qiagen) according to the manufacturer's instructions.

The electrophoretic mobility shift assay (EMSA) DNA probes P1 (236bp), P2 (277bp), P3 (432bp), P4 (278bp), P5 (191bp), P6 (570bp), P7 (304bp), and P8 (336bp) were amplified by PCR using the primer pairs as listed in Table S1. The PCR products were firstly cloned into a pTA2-vector (TOYOBO, Kita-ku, Osaka, Japan). Then, the 5'-FAM-labeled probes were made using 5'-FAM-labeled M13 universal primers by PCR amplification. The probes PA (64 bp), PAmI (64 bp), PAmII (64 bp), PAmI-II (64 bp), PB (60 bp), PBmI (60 bp), PBmII (60 bp), PBmI-II (60 bp), PC (50 bp), PCmI (50 bp), PCmII (50 bp), PCmI-II (50 bp), PD (60 bp), PD(1)mII (60 bp), PD(2)mII (60 bp), PDmII (60 bp), PE (44 bp), and PEmII (44 bp) were prepared following the above

method. The EMSAs assay were carried out as previously described with a little modification (Yu et al. 2014). In the EMSAs assay, 80 ng of the probes was incubated with varying quantities of FadR1, at 30 °C for 30 min in the buffer (20 mM Tris, pH 7.5, 5% glycerol, 0.01% BSA, 50 $\mu\text{g mL}^{-1}$ sheared sperm DNA). The reactions were displayed on 5% acrylamide gels for separation in a 0.5 \times TBE buffer. The shift bands were then detected using the ImageQuant LAS 4000 (GE Healthcare Life Sciences, Piscataway, NJ, USA).

DNase I footprinting assay

The DNase I footprinting assays were carried out as previously described (Yu et al. 2014). In brief, 5'-FAM-labeled M13 universal primers were used to amplify FAM-labeled probes. Next, the probes were purified by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and were incubated with different quantities of FadR1 protein at 30 °C for 30 min. Then, 0.01 U of DNase I (Promega, Madison WI, USA) with 10 mM MgCl₂ and 1 mM CaCl₂ was added for digesting exactly 1 min at 30 °C. The reaction mixtures were stopped by adding 50 μL of 0.1 M EDTA (pH 8.0). After extraction with phenol/chloroform and precipitation with 0.75 M NH₄Ac, 40 μg of glycogen, and 70% ethanol, DNAs mixed with Liz-500 DNA marker (MCLAB, South San Francisco, CA, USA) were loaded into an ABI 3130 sequencer and electropherograms were analyzed by Genemapper v4.0 software (Applied Biosystems, Foster City, CA, USA) to align and determine the protected

region. The DNA sequencing ladder was prepared according to Thermo Sequence Dye Primer Manual Cycle Sequencing Kit (Affmetrix, Santa Clara, CA, USA). The alignment and consensus of the binding sequences was analyzed by DNAMAN software (Lynnon, San Ramon, CA, USA).

RNA isolation, reverse transcriptase PCR and quantitative real-time PCR

In order to prepare genomic DNA-free RNA, RNA isolation was performed as follows. In brief, *A. deccanensis* Yp-1 incubated in YEME medium at 30 °C, 220 rpm. Mycelia were collected and washed twice with RNase-free TE buffer. Total RNAs were prepared from mycelia using EASYspin Plus bacteria RNA extract kit (Aidlab Biotech, Beijing, China) according to the manufacturer's instructions. In order to confirm the absence of genomic DNA contamination, the RNase-free DNase I (Takara, Shiga, Japan)-treated RNA samples were amplified by PCR with three different primer pairs. Then, the concentration of genomic DNA-free RNA was measured by Thermo Scientific™ NanoDrop™. The cDNA was prepared using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan) following the manufacturer's instructions.

To analyze transcription units in fidaxomicin biosynthesis gene cluster, the cDNA of *A. deccanensis* Yp-1 was used as templates for reverse transcriptase PCR (RT-PCR) by rTaq polymerase with primer pairs (Table S1). The co-transcription assay primers were designed based on the following principle: the upstream primers were selected from the end of genes and the downstream primers were designed at the middle of the adjacent genes. And the genomic DNA was also performed PCR amplification as a positive control. Subsequently, the PCR products were analyzed by 1% agarose gel electrophoresis after 31 cycles of amplification. These analyses were carried out at least three times for each primer pair.

To analyze the transcription level of the fidaxomicin gene cluster in *A. deccanensis* Yp-1, $\Delta fadR1$, and OE-*R1*/WT, quantitative real-time PCR (RT-qPCR) was carried out on Roche LightCycler 480 (Roche, Mannheim, Germany) with the SYBR Premix Ex Taq (Takara, Shiga, Japan) in a 20- μ L volume following the manufacturer's instructions. The sequence-specific primers used for each gene are listed in Table S1. And the expression level of *hrdB* was used as the internal reference. The relative expression level was calculated by normalizing to the expression level of *hrdB*. Each experiment was performed in triplicate.

Determination of fidaxomicin production by HPLC analysis

Fidaxomicin production was confirmed by HPLC analysis using the Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). A HC-C₁₈ column (5 μ m, 4.6 by 250

mm) was used with UV detector set at 254 nm. The mobile phase and gradient elution process were as previously described (Xiao et al. 2011). All experiments were performed in quadruplicate.

Accession number of nucleic acid sequence

The sequence of the fidaxomicin cluster in the *A. deccanensis* Yp-1 has been deposited in Genbank (accession number MG972807).

Results

Identification and analysis of the fidaxomicin gene cluster

A fidaxomicin-producing strain was purchased from China General Microbiological Culture Collection Center (CGMCC). 16S rRNA sequence analysis showed that the sequence shared 99%, 98%, and 97% nucleotide identity with the strain *A. deccanensis* IFO 13994, *A. deccanensis* JCM9916, and *A. deccanensis* Y16, respectively. We therefore designated this strain as *A. deccanensis* Yp-1 (CGMCC 4.2098). Illumina & PacBio sequencing techniques were used to determine the complete nucleotide sequence of the *A. deccanensis* Yp-1 genome at Beijing Genomics Institute (BGI) in Shenzhen, China. The size of the *A. deccanensis* Yp-1 draft genome was determined to be approximately 10.5 Mb. The genome was submitted to RAST Annotation Server (Aziz et al. 2008) to analyze putative protein-coding sequences (CDSs) and their functions which revealed about 9730 CDSs in the genome. After carrying out antiSMASH analysis (Blin et al. 2017), the fidaxomicin gene cluster was revealed, showing a 90% DNA sequence identity to that of *D. aurantiacum* NRRL 18085 (GenBank accession number HQ011923). And the sequence of the fidaxomicin cluster in the *A. deccanensis* Yp-1 has been deposited in GenBank (accession number MG972807). The cluster contained 33 ORFs, spanning 84,962 bp of DNA sequence, was shown in Fig. 1. The deduced functions of the ORFs in the fidaxomicin cluster from *A. deccanensis* Yp-1 and *D. aurantiacum* NRRL 18085 are presented in Table S2.

As compared with *D. aurantiacum* NRRL 18085, the fidaxomicin cluster in *A. deccanensis* Yp-1 has five more genes, including *fadW1*, *fadW2*, *fadW3*, *fadY1*, and *fadY2*. The deduced products of *fadW1*, *fadW2*, and *fadW3* are methylmalonyl-CoA mutase-related enzymes, which catalyze the reversible isomerization of methylmalonyl-CoA to succinyl-CoA. The macrocyclic aglycone of fidaxomicin was reported to be biosynthesized from one propionyl-CoA (starter unit), 4 malonyl-CoAs, 4 methylmalonyl-CoAs, and one ethylmalonyl-CoA by TiaA1-TiaA4 (Xiao et al. 2011). So, it

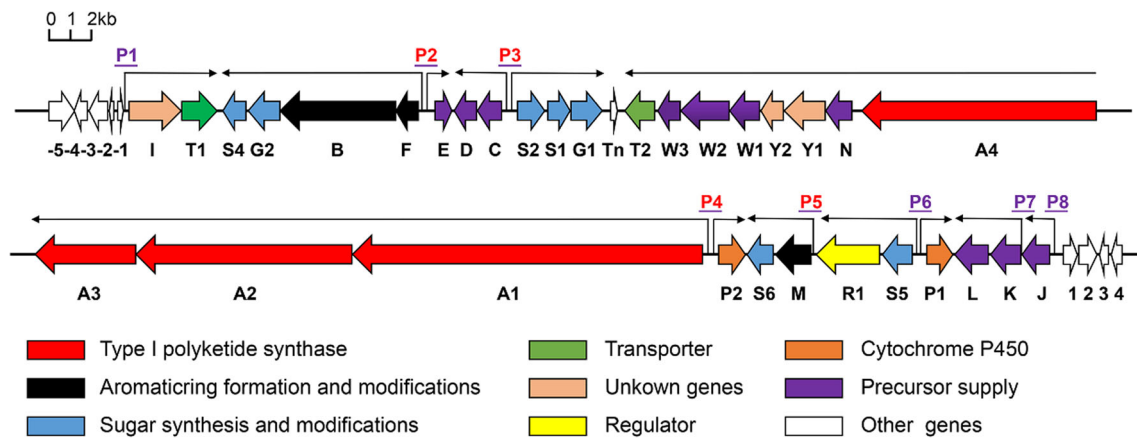


Fig. 1 Genetic organization of the fidaxomicin cluster. The genetic organization of the 33 ORFs plus 5 upstream ORFs and 4 downstream ORFs of the fidaxomicin cluster. Arrows indicate deduced transcriptional units. Purple lines above the ORFs indicate DNA fragments for probes

(P1–P8) in the EMSA experiments. Red probes (P2, P3, P4, and P5) showed retarded bands, and purple probes (P1, P6, P7, and P8) indicate that probes did not show retarded bands in the EMSA experiments

seems that succinyl-CoA does not participate in the process of fidaxomicin biosynthesis. This raises the question about the function of methylmalonyl-CoA mutase in the fidaxomicin cluster. *fadY1* and *fadY2* have been shown to encode 2-oxoacid oxidoreductase-related enzymes. The functions of these enzymes were suggested to use ferredoxin as an electron acceptor in the formation of a free radical intermediate (Gibson et al. 2016; Pierce et al. 2010). However, the fidaxomicin cluster of *A. deccanensis* Yp-1 did not contain homologous genes corresponding to *tiaS3*, *tiaT3*, and *tiaT4* in *D. aurantiacum* NRRL 18085 (Table S2). *tiaS3* was reported to encode a GDP-D-mannose 4,6-dehydratase, which converts GDP-D-mannose to D-rhamnose. *TiaT3* and *TiaT4* are ABC transporter-related proteins and may be involved in the transport of fidaxomicin and its analogues. It has been suggested that the functions of these enzymes may be replaced by other proteins or that they are just not involved in the fidaxomicin biosynthesis. It is also worth noting that the ArsR-type repressor, *TiaR2*, is not present in the fidaxomicin cluster or anywhere else in the chromosome of *A. deccanensis* Yp-1, indicating a different regulatory mechanism of fidaxomicin between *A. deccanensis* Yp-1 and *D. aurantiacum* NRRL 18085.

FadR1 is a putative pathway-specific transcriptional regulator

In the fidaxomicin gene cluster of *D. aurantiacum* NRRL 18085, there are two pathway-specific regulators, *TiaR1* and *TiaR2*. In contrast, only one pathway-specific regulatory gene, *fadR1*, was found in the corresponding gene cluster of *A. deccanensis* Yp-1. Sequence analysis of the *fadR1* gene product (873aa) showed a strikingly high sequence identity (88%) to *TiaR1*. FadR1 contained C-terminal DNA-binding domain of LuxR-like proteins with a helix-turn-helix motif

and an N-terminal AAA ATPase domain with a P-loop motif involved in binding ATP, which suggested that FadR1 belongs to LAL family of transcription factors. To investigate the contribution of *fadR1* to fidaxomicin biosynthesis in *A. deccanensis* Yp-1, partial coding region of *fadR1* was deleted via homologous recombination as described in the “Materials and methods” section. The disruption mutant Δ *fadR1* was confirmed by PCR analysis (see Fig. S1) and further verified by DNA sequencing (data not shown). Meanwhile, the complementary strain was constructed by introducing Com-R1, containing *fadR1* and its own promoter into the deleted mutant Δ *fadR1*. High-performance liquid chromatography (HPLC) analysis (Fig. 2) showed that deletion of *fadR1* totally blocked fidaxomicin production, while the complementary strain Com-R1/ Δ *fadR1* was found to restore the production of fidaxomicin to a similar level compared with that of the WT strain (Fig. 2). Nevertheless, there were no obvious differences in the growth rates and morphological differentiation among these three strains (Fig. S1). These results indicated that FadR1 is a pivotal positive regulator in fidaxomicin biosynthesis.

Overexpression of *fadR1* results in an increased fidaxomicin production

In order to further evaluate the effect of increased expression of *fadR1* to the yield of fidaxomicin, a *fadR1* overexpression plasmid OE-R1 was constructed, in which *fadR1* was cloned into pIJ8630 under the control of the strong constitutive *ermE** promoter. This plasmid was then introduced into the wild-type strain by conjugation, resulting in *fadR1* overexpression strain OE-R1/WT. The overexpression of *fadR1* was confirmed by RT-qPCR analysis, and its effects on cell growth, aerial mycelium formation, and fidaxomicin productivity in the fermentation medium were also determined. As

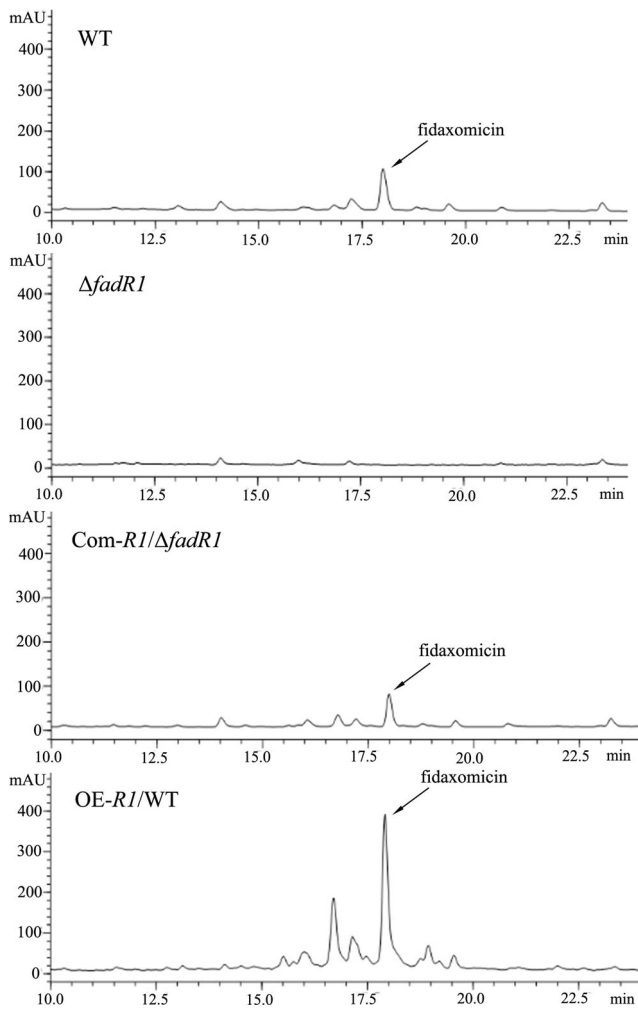


Fig. 2 The effect of *fadR1* disruption and overexpression on production of fidaxomicin. The strains were grown in YEME medium for 120 h. HPLC analysis of fermentation filtrates from the WT strain, the *fadR1* deletion mutant, the *fadR1*-complementary mutant, and the *fadR1* overexpression mutant. The peak of fidaxomicin is marked by an arrow. mAU milli-absorbance units

seen in Fig. 3, the presence of an extra copy of *fadR1* under *ermE**p (OE-*R1*/WT) led to the increase of fidaxomicin production by approximately 400 % compared with that of the wild-type strain. And no apparent difference in the cell growth and morphology between the overexpression strain and wild type was observed (Fig. S1). These results further demonstrated the positive regulatory roles of FadR1 in the pathway.

Effects of FadR1 on the transcription levels of fidaxomicin biosynthetic genes

To test whether FadR1 positively regulates fidaxomicin production by tuning the transcription levels of associated biosynthetic genes, we performed RT-qPCR analysis. The results suggested that the transcription levels of most fidaxomicin-associated biosynthetic genes exhibited an obvious increase in the *fadR1* overexpression strain. Firstly, the expressions of

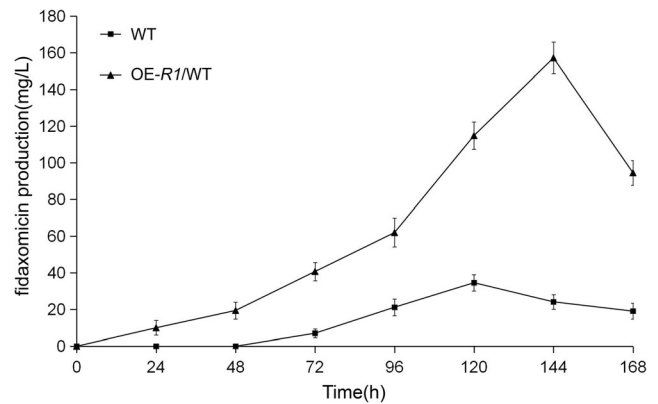


Fig. 3 The effect of *fadR1* overexpression on production of fidaxomicin. The strains were grown in YEME medium for 7 days and fermentation broth was sampled with a 24-h interval. Vertical error bars correspond to the standard error of the mean of four replicated cultures

precursor-associated genes like *fadJ*, *fadN*, and *fadK* involved in synthesis of EM-CoA extender units and *fadE* encoding thioesterase responsible for eliminating abnormal precursors were upregulated by 5–20-fold. The transcription levels of *fadB*, *fadF*, and *fadM* involved in the biosynthesis and modification of the aromatic moiety were increased by 20–30-fold. Secondly, we can see that the transcription levels of four PKS genes *fadA1*–*fadA4* that encode modular polyketide synthase responsible to assemble precursor units like propionyl-CoA to form fidaxomicin aglycone were increased by 10–40-fold. Thirdly, the transcription levels of *fadS1*–*S6* encoding a series of modifying enzymes that were involved in the biosynthesis of deoxygenated sugars and *fadG1* and *fadG2* that modified these deoxygenated sugars at C-11 and C-20 were increased by 8–30-fold. Finally, FadR1 also significantly improved the expression of other genes such as *fadW1*, *fadW2*, and *fadW3* (methylmalonyl-CoA mutase-related enzymes), *fadP2* (cytochrome P450), *fadT1* (transport protein), *fadC* (E1 alpha subunit), and *fadD* (E1 beta subunit). The increase of expression levels of these precursors or skeleton modification steps often helps to improve the yield of antibiotics (Matthews and Wurtzel 2000; Hemmerlin 2013). However, the transcription levels of all fidaxomicin-associated biosynthetic genes were decreased by at least 50% and some genes like *fadS4*, *fadE*, *fadS2*, *fadS1*, and *fadJ* were even barely expressed in the *fadR1* deletion mutant (Δ *fadR1*) (Fig. 4). Accordingly, we can conclude that FadR1 positively regulates the transcription levels of most of genes in the fidaxomicin gene cluster and its overexpression resulted in the increase of fidaxomicin production. These results further confirmed that FadR1 is a pathway-specific positive transcriptional regulator of fidaxomicin biosynthesis. And in view of the regulatory mechanisms of most of pathway-specific regulators (Bibb 2005; Chen et al. 2010), we proposed that the binding sites of FadR1 were distributed in the promoter regions of fidaxomicin-associated biosynthetic genes.

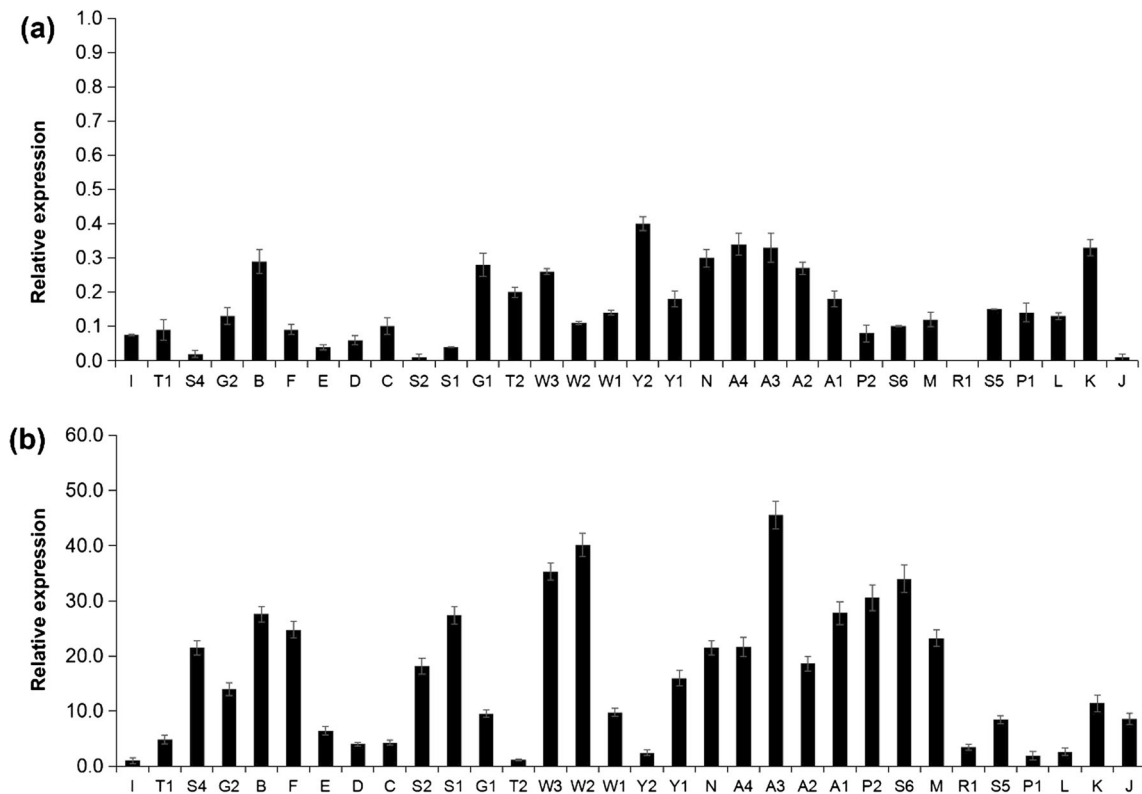


Fig. 4 Effect of *fadR1* deletion and overexpression on the transcription level of fidaxomicin cluster genes. Compared with wild-type strain, the relative expression of fidaxomicin biosynthetic genes in *fadR1* deletion mutant was shown in **a** and in *fadR1* overexpression strain was shown in **b**. The RNA samples were obtained from cultures grown in YEME medium. The expression levels of fidaxomicin cluster genes are presented

relative to the levels of corresponding genes in the wild-type sample, which were arbitrarily assigned a value of 1. The transcription level of *hrdB* was assayed as an internal control, and error bars were calculated by measuring the standard deviations among data from three replicates of each sample

Identification of FadR1 binding sites in the fidaxomicin gene cluster

In order to investigate the detailed regulatory mechanism of FadR1, we firstly characterized the organization of transcription units in the fidaxomicin cluster by RT-PCR (Materials and methods). cDNA samples were prepared from mycelia of *A. deccanensis* Yp-1 after being incubated in YEME medium at 30 °C, and primers covering the intergenic regions between two genes were designed (Table S1). As shown in Fig. 5, an amplified cDNA band was observed using the primers covering the intergenic region between *fadK* and *fadL*. However, no transcript was detected between *fadJ* and *fadK*. The results indicated that *fadJ* and *fadK* genes had their own promoters while *fadK* and *fadL* were co-transcribed as an operon. However, an overlapping sequence was observed between *fadK* and *fadJ* according to the nucleotide sequence of fidaxomicin gene cluster. So, in order to confirm that *fadJ* and *fadK* genes were transcribed separately, another two primer pairs were used to amplify the cDNA of *A. deccanensis* Yp-1 and still no transcripts were observed (data not shown). Finally, we concluded that *fadK* had its own promoter, and the promoter may be located at the inner of *fadJ*, and *fadK* and

fadL were co-transcribed as an operon. Besides, based on the RT-PCR results, *fadA1*, *fadA2*, *fadA3*, *fadA4*, *fadN*, *fadY1*, *fadY2*, *fadW1*, *fadW2*, *fadW3*, and *fadT2* were deduced to encode a polycistronic transcript, while *fadE*, *fadP1*, *fadP2*, and *fadJ* were transcribed separately. And the transcriptional features of other genes in the fidaxomicin cluster were also characterized. In view of the chromosomal arrangement, the fidaxomicin gene cluster was proposed to have twelve transcription units (Fig. 1).

To identify the specific binding sites of FadR1 in the fidaxomicin gene cluster, we carried out EMSAs with eight 5'-FAM-labeled probes (P1-P8), which covered all possible promoter regions according to the transcriptional organization of fidaxomicin gene cluster. As shown in Fig. 6, when FadR1 was incubated with probes P2 (intergenic region of *fadE-fadF*), P3 (intergenic region of *fadS2-fadC*), P4 (intergenic region of *fadA1-fadP2*), and P5 (promoter region of *fadM*), retarded bands were observed which suggested that FadR1 could directly bind to these promoter regions. However, no retarded bands appeared (Fig. S2) when FadR1 was incubated with other probes, indicating that FadR1 could not directly interact with these promoter regions. Besides, the pTA2 vector probe was used as a negative control which can confirm the

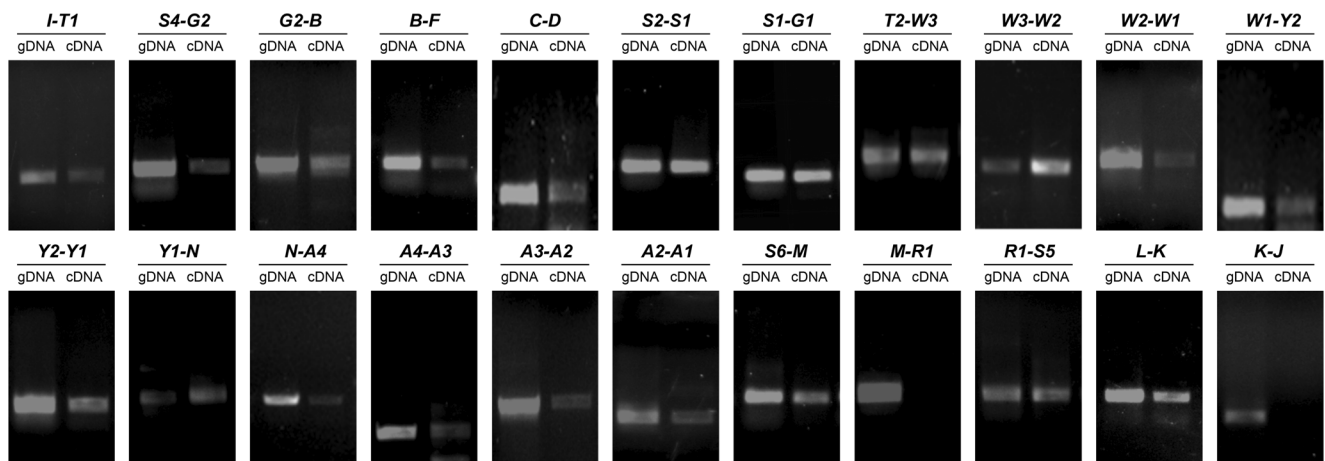


Fig. 5 Transcriptional organization analysis of the fidaxomicin cluster by RT-PCR. cDNA for RT-PCR was prepared from *A. deccanensis* Yp-1

after incubated in YEME medium at 30 °C. genomic DNA (gDNA) was used as a template for controls

binding specificity of FadR1 to the above four promoter regions.

To further define the specific binding sites of FadR1 in each promoter region, DNase I footprinting assays were carried out. The experiments revealed that there are two binding sites in the intergenic region of *fadS2-fadC*, while there is one binding site in the intergenic regions of *fadE-fadF* and *fadA1-fadP2* and the promoter of *fadM* (Fig. 7a, c, e, g). The nucleotide sequences of the promoter regions of *fadS2-fadC*, *fadE-fadF*, *fadA1-fadP2*, and *fadM*, and the FadR1 binding sites were showed in Fig. 7b, d, f, and h. These results further confirmed the direct binding of FadR1 to the multiple binding sites of the fidaxomicin cluster.

Based on the results from EMSA and footprinting, we can see that the binding sites of FadR1 were located at the intergenic regions of *fadE-fadF*, *fadS2-fadC*, and *fadA1-fadP2* and the promoter of *fadM*. Combined with the analysis of transcription units, we can know that the promoters located at the above four intergenic regions were important for controlling the most of key genes of fidaxomicin biosynthesis like PKS skeleton-associated genes, genes for precursor supply, and genes for glycosyl synthesis and modification. And this result is consistent with the changes of transcription level

learned from RT-qPCR. Finally, we concluded that *fadR1* positively regulates the transcription of most of genes in the fidaxomicin gene cluster directly or indirectly by binding to the four intergenic regions, which resulted in the improvement of fidaxomicin production. According to the above results, we proposed the regulatory mode of FadR1 controlling the biosynthesis of fidaxomicin (Fig. 8).

Characterization of the consensus FadR1-binding sequence

By aligning the sequences within the protected regions of the five FadR1-binding regions, a consensus inverted repeat (IR) 5'-CCCTN_xAGGG-3' was identified in the intergenic regions of *fadE-fadF* and *fadS2-fadC*, and a consensus direct repeat (DR) 5'-AGGGN_xAGGG-3' was observed in the intergenic region of *fadA1-fadP2*, while only a consensus sequence 5'-AGGG-3' was revealed in the promoter of *fadM* (Fig. 7i). To further study the roles of these consensus sequences for their FadR1-binding ability, EMSAs were carried out using the probes containing either the sequences of wild-type binding sites or the mutated sites (Fig. S3). When one of the consensus sequences in site A, site B, site C, or site D were mutated,

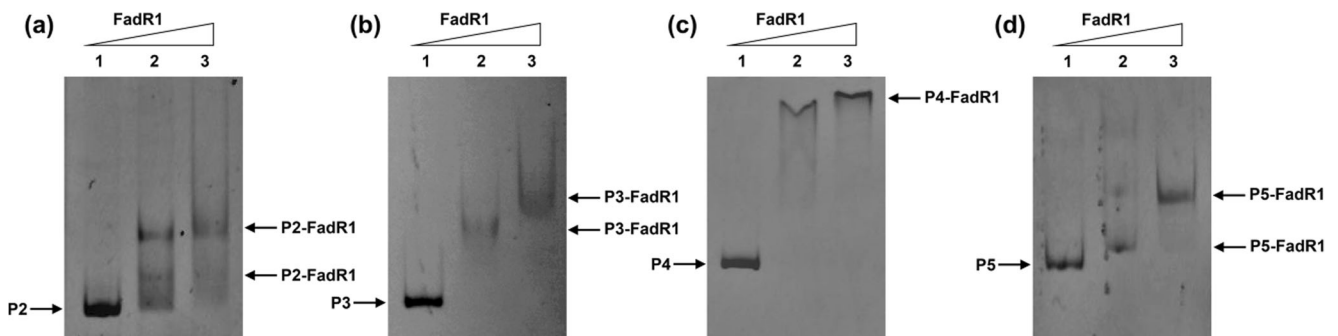


Fig. 6 EMSAs using labeled DNA fragments (P2, P3, P4, and P5) and the FadR1 protein. P2: Probe 2, P3: Probe 3, P4: Probe 4, P5: Probe 5.

Lanes 1–3 are labeled fragment with 0 μg, 0.05 μg, and 0.2 μg of purified FadR1, respectively. The probe-FadR1 complex is marked by an arrow

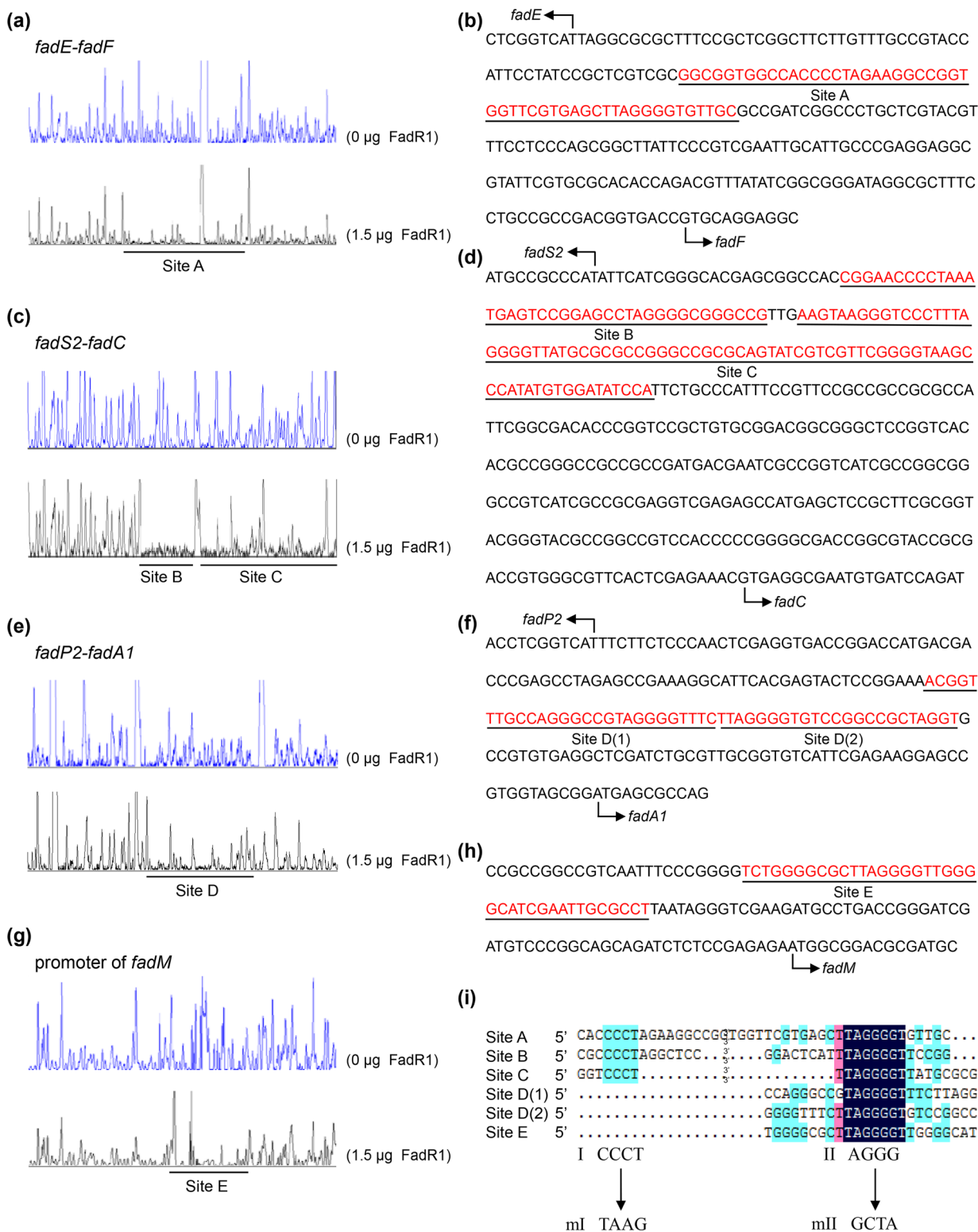


Fig. 7 DNase I footprinting assay for determination of the FadR1-binding sites. 5'-FAM-labeled P2 covering the promoter region of *fadE-fadF* (a), P3 covering the promoter region of *fadS2-fadC* (c), P4 covering the promoter region of *fadA1-fadP2* (e), and P5 covering the promoter region of *fadM* (g) were used in the DNase I footprinting assays, with or without purified FadR1, respectively. The protected regions are underlined. Nucleotide sequences of the promoter regions of *fadE-fadF*,

fadS2-fadC, *fadA1-fadP2*, and *fadM* were shown in b, d, f, and h, respectively. In site D, two consensus AGGG motifs were found, named site D(1) and site D(2). The FadR1-binding sites are overlined and the initiation codons are marked with arrows. Sequences of the determined FadR1-binding sites and the consensus FadR1-binding sequence (in bold-face type) were shown in g

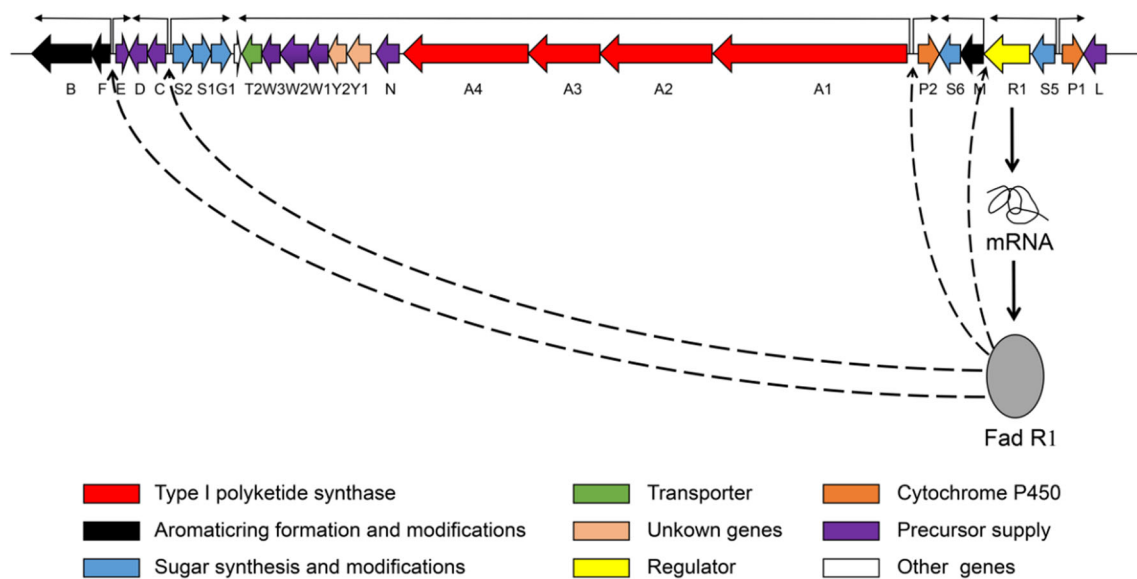


Fig. 8 Hypothetical regulatory model of FadR1 to fidaxomicin biosynthetic gene cluster in *A. deccanensis* Yp-1. The transcription units and their orientations were indicated with solid and black arrows. The binding sites of FadR1 were shown with dotted arrows

binding shifts were still detected (Fig. 9b, c, f, g, j, k, n, o). However, when we carried out mutation in both consensus sequences, there were no evident banding shifts (Fig. 9d, h, l, p). On the other hand, there was only one consensus sequence in the site E, and the mutation of this sequence could completely abolish FadR1 complex formation with the labeled probe (Fig. 9r). The above data validated the FadR1 consensus binding sequences and confirmed that these consensus sequences are essential for the binding activity of FadR1. Based on these results, we proposed that FadR1 probably recognized the structures derived from the consensus sequence and then recruited RNA polymerase by binding to the target promoter regions to enhance the expression of fidaxomicin biosynthetic genes.

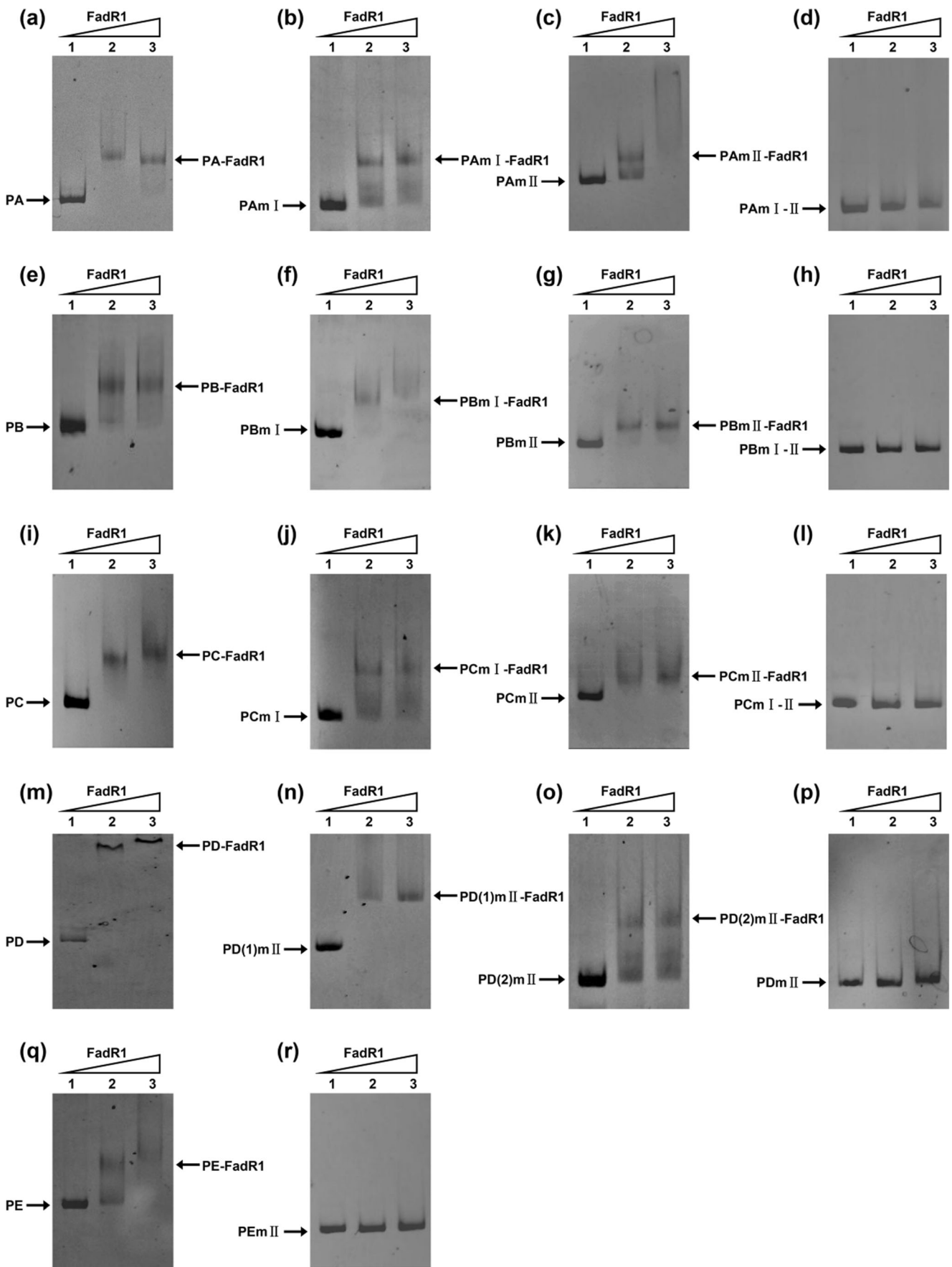
Discussion

It has been reported that many pathway-specific positive regulators from polyene macrolide biosynthesis gene clusters belong to the LAL (large ATP-binding regulators of the LuxR) family, such as PimM, NysR-RIII, AmphRI-RIII, FscRI, PikD, and AveR. And they always share similar binding sites and functional conservation (Santos-Aberturas et al. 2011). However, only several LAL family regulators have been well-characterized, which is not enough for us to fully comprehend the significant roles of this protein family (Chen and Xie 2011).

In our study, complete genome sequencing and bioinformatic analysis reveal a typical LAL family regulator named FadR1 in fidaxomicin gene cluster from *A. deccanensis* Yp-1. And we have proven that FadR1 can bind to four intergenic regions of several key genes, thereby activating the

transcription of most of genes in the fidaxomicin gene cluster directly or indirectly and finally leading to an increase in the production of fidaxomicin. In addition, the analysis of binding sites reveals a conserved sequence 5'-AGGG-3' which is common and important for lots of LuxR regulators (Santos-Aberturas et al. 2011). Overall, the elucidation of the regulatory role of FadR1 contributes to the understanding of the entire fidaxomicin biosynthetic process and the investigation of important function of analogical LAL family regulators in the gene clusters of other macrolide antibiotics. However, according to our results, there are still some protein-probe complexes displaying a relatively smeared pattern although we have optimized several conditions for EMSA. One of the main possible reasons is that the binding of FadR1 to conserved sites occurred but the protein-probe complexes may be unstable in vitro, resulting in dissociation during electrophoresis. Similar phenomena are also observed for other various regulators like the LuxR transcriptional regulator PimM from *Streptomyces natalensis* (Santos-Aberturas et al. 2011), the SARP family regulator AfsR from *S. coelicolor* A3(2) (Tanaka et al. 2007), the osmotic regulator OmpR from *E. coli* (Harlocker et al. 1995), the global response regulator CtrA from *Caulobacter crescentus* (Spencer et al. 2009), the quorum-sensing regulator RsaL from *Pseudomonas aeruginosa* (Rampioni et al. 2007), and the developmental

Fig. 9 Mutational analysis of consensus sequences in the FadR1-binding sites. EMSAs for the determination of FadR1 binding to mutated sequences. Probes containing the FadR1-binding sequences of wild-type and mutants are as shown in Fig. S3. PA: Probe A, PAm: Probe A mutant, PB: Probe B, PBm: Probe B mutant, PC: Probe C, PCm: Probe C mutant, PD: Probe D, PDm: Probe D mutant, PE: Probe E, PEm: Probe E mutant. Lanes 1–3 are labeled fragment with 0 μ g, 0.05 μ g, and 0.2 μ g of purified FadR1, respectively. The probe-FadR1 complex is marked by an arrow



regulator BldD from *Saccharopolyspora erythraea* (Chng et al. 2008). Besides, as shown in Fig. 9, we also notice that when the conserved sites are mutated, the FadR1-probe complexes become more smeared, such as Fig. 9 c-3 or f-3. Similar phenomenon is also observed in the *Pseudomonas* quorum-sensing regulator RsaL (Rampioni et al. 2007). So, we suppose that the secondary structure of FadR1 binding sites formed by inverted repeat (IR) or direct repeat (DR) sites may also be required for the formation of stable complexes between FadR1 and its target DNAs. Certainly, further studies are needed to investigate the underlying causes.

Furthermore, it is particularly interesting to note that the regulatory mechanism of FadR1 is very similar to other LuxR regulators like PimM, AmphRIV, NysRIV, and PteF from polyene macrolide gene clusters. A previous study by Santos-Aberturas et al. (2011) proves that heterologous LuxR regulators like AmphRIV, NysRIV, and PteF can complement the function of PimM in *S. natalensis* Δ pimM, and the yield of pimaricin displays a marked difference, which suggests that these functionally conserved regulators are fully exchangeable and may display different regulatory intensities in different hosts. Therefore, we suppose that replacing FadR1 with other LuxR regulators may further improve the production of fidaxomicin and we will consider performing more relevant experiments in the future. Besides, Martínez-Burgo et al. (2019)'s research proves that heterologous expression of pimM from *S. natalensis* in *S. clavuligerus* can activate cryptic gene clusters. Similarly, Yushchuk's (Yushchuk et al. 2018) study suggests that heterologous AdpA transcription factors of different origins can activate landomycin biosynthesis in *S. cyanogenus* S136 harboring a nonfunctional AdpA. Thus, we suppose that some gene clusters are silent in native strains but are active in heterologous hosts which may be due to the influence of regulatory networks. Since FadR1 as a pathway-specific positive regulator displays functional conservation with other LuxR transcriptional activators, it also has great potential to activate cryptic gene clusters in heterologous strains for drug discovery. Also, further researches are needed to prove this hypothesis. For instance, according to the conserved binding sites CTVGGGAWWTCCCBAG of some LuxR regulators, we can perform whole genome scanning to locate the conserved sites by the MEME suite (Santos-Aberturas et al. 2011; Bailey et al. 2015) and predict the target cryptic gene clusters. Then, FadR1 regulator can be expressed in the corresponding heterologous host to activate silent gene clusters, which may accelerate the excavation of novel natural products.

In summary, we have characterized FadR1 as a LuxR-type pathway-specific activator in controlling fidaxomicin biosynthesis, analyzed its regulatory mechanism, and proposed the action mode of FadR1 regulator in fidaxomicin biosynthesis. Our future research goals are to determine how FadR1 responds to environmental signals to initiate fidaxomicin

biosynthesis and to look into whether there is a higher level regulatory network (Liu et al. 2013) directly involved in the regulation of FadR1. Also, we will try to discover more efficient LuxR regulators to replace FadR1 for further enhancing the production of fidaxomicin as well as to activate cryptic gene clusters by heterologous expression of FadR1. This study not only sets the stage for an increased understanding of the genetic control of fidaxomicin biosynthesis which would have general implications about the regulatory mechanism of other macrolide antibiotics harboring similar LAL family regulator in the gene clusters but also will provide an effective strategy to improve the yield of fidaxomicin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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