Redox-sensing regulator Rex regulates aerobic metabolism, morphological differentiation, and avermectin production in *Streptomyces avermitilis*

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The regulatory role of redox-sensing regulator Rex was investigated in *Streptomyces avermitilis*. Eleven genes/operons were demonstrated to be directly regulated by Rex; these genes/operons are involved in aerobic metabolism, morphological differentiation, and secondary metabolism. Rex represses transcription of target genes/operons by binding to Rex operator (ROP) sequences in the promoter regions. NADH reduces DNA-binding activity of Rex to target promoters, while NAD$^+$ competitively binds to Rex and modulates its DNA-binding activity. Rex plays an essential regulatory role in aerobic metabolism by controlling expression of the respiratory genes *atpIBEFHAGDC*, *cydA1B1CD*, *nuoA1-N1*, *rex-hemAC1DB*, *hppA*, and *ndh2*. Rex also regulates morphological differentiation by repressing expression of *wblE*, which encodes a putative WhiB-family transcriptional regulator. A rex-deletion mutant (Drex) showed higher avermectin production than the wild-type strain ATCC31267, and was more tolerant of oxygen limitation conditions in regard to avermectin production.
Even though Rex was first characterized in *S. coelicolor* and its regulatory mechanism has been extensively studied, few target operons/genes of Rex in *Streptomyces* have been confirmed4, and the overall regulatory function of Rex in this genus remains to be elucidated.

*S. avermitilis* is an important species used for industrial production of avermectins, a group of anthelmintic antibiotics widely used in the medical, veterinary, and agricultural fields11. We investigated the regulatory role of Rex in the expression of operons/genes involved in aerobic metabolism, morphology, and secondary metabolism of *S. avermitilis*. Our findings have potential application to novel genetic engineering strategies for high antibiotic-producing strains and hypoxia-tolerating strains of this genus.

**Results**

**Expression of atpBEFAGDC, cydA1B1CD, nuoA1-N1, and rex-hemAC1DB is negatively regulated by Rex.** The rex gene is conserved within the genus *Streptomyces* and is cotranscribed with the heme synthesis genes hemACD4. To evaluate the regulatory role of Rex in *S. avermitilis*, we constructed a rex-deletion mutant (termed Drex) by homologous recombination in wild-type strain ATCC31267. rex deletion had no effect on growth in liquid fermentation medium (Fig. 1).

The promoter regions of the operons atpBEFAGDC, cydA1B1CD, nuoA1-N1, and rex-hemAC1DB in *S. avermitilis* all contain a putative Rex-binding motif, 5′-TGTTGANNTTTCACAA-3′ (Table 1). *nuoA1-N1* (SAV4837-4850) encodes putative NADH dehydrogenase I (complex I), *cydA1B1CD* (SAV4260-4258) encodes putative cytochrome bd-1 oxidase (cytochrome bd complex), *atpBEFAGDC* (SAV2888-2880) encodes putative F-type proton-transporting ATPase, and *hemAC1DB* (SAV4739-4742) is cotranscribed with rex and encodes heme synthesis enzymes (Fig. S1). NADH dehydrogenase I, cytochrome bd-1 oxidase, and F-type proton-transporting ATPase are essential components of the respiratory chain. Heme is most abundant in cytochromes, which are electron transfer proteins involved in the final reduction of oxygen during aerobic respiration. We performed qRT-PCR to determine whether expression of these genes involved in aerobic respiration is regulated by Rex.

Expression of these genes differed greatly when cells were static-cultured following 3 days' culture on a rotary shaker (250 rpm). Transcription level of *cydA1* under oxygen limitation condition in ATCC31267 increased steadily during 60 min, whereas the level in Drex increased to a maximal value during the first 30 min, then gradually declined during the subsequent 30 min (Fig. 2). These findings suggest that induction of *cydA1* under
Table 1. Putative Rex target genes. aGenomic position. bNumber of mismatches with respect to the consensus. PWM, positive weight matrix. cValues are distances (in nucleotides) to the predicted start codon of the downstream gene.

| Gene                        | Function                                          | Nucleotide position | PMW Score | Sequence | ATG Distance |
|-----------------------------|---------------------------------------------------|---------------------|-----------|----------|--------------|
| cydA1B1CD (SAV4260-4258)    | putative cytochrome bd-I oxidase (cytochrome bd complex) | 5226395  5226412  | 1.00      | ATGGAAACGGTTCCAA  | 81           |
| rex-hemAC1DB (SAV4738-4742) | redox-sensing transcriptional repressor; heme biosynthetic enzymes | 5776959  5776976  | 1.00      | TTGGTCAACGGTTCCAA  | 77           |
| atpBEFHAGDC (SAV2888-2880) | putative F-type proton-transporting ATPase | 3533322  3533349  | 2.00      | TTGGTATACGGTTCCAGA  | 137          |
| whbE (SAV3016)             | putative WhiB-family transcriptional regulator | 3771378  3771395  | 2.00      | ATGGAAACGGTTCCAA  | 43           |
| fppA (SAV4616)             | putative inorganic H+ pyrophosphatase | 5632612  5632629  | 2.00      | TCGTGAATCTTCCAA  | 195          |
| muoA1-N1 (SAV4837-4850)    | putative NADH dehydrogenase I (complex I) | 5880239  5880256  | 2.00      | ATGGAAACGGTTCCAA  | 147          |
| pbp3-f (SAV3603-SAV3604)   | putative peptidil-binding protein | 4400250  4400267  | 2.00      | TTGGTACGGTTCAGA  | 37           |
| SAV288                    | putative rhinomannosidase | 982811  982828  | 3.00      | CTTGGAAATGTCACCT  | 137          |
| echA7 (SAV2316)            | putative enoyl-CoA hydratase | 2820643  2820660  | 3.00      | TCGTGAACGACGAACT  | 66           |
| SAV2652                   | putative regulatory protein | 3252402  3252419  | 3.00      | TCGTGAACGCTCCACCA  | 288          |
| ndh2 (SAV3529)            | putative NADH dehydrogenase (complex III) | 4369478  4369495  | 3.00      | TCGTGAACGACGAACT  | 119          |
| ectABCD (SAV6398-6395)     | putative L-2,4-diaminobutyrate acetyltransferase, ectoine biosynthesis | 7673586  7673603  | 3.00      | TCGTGAACGAACTCCACAT  | 155          |
| SAV6368                   | putative multiple sugar ABC transporter permease protein | 7638483  7638500  | 3.00      | TCGTGAACGACGAACT  | 81           |
| avaB-avaL2 (SAV2267-2268) | putative gamma-butyrolactone- dependent transcriptional regulator | 2766273  2766290  | 3.00      | TCGTGAACGACGAACT  | 29           |
| SAV3213                   | putative nitroreductase family protein, NADH dehydrogenase/NADPH nitroreductase | 4004553  4004570  | 3.00      | TCGTGAACGACGAACT  | 96           |
| SAV1351                   | putative fatty acid CoA racemase | 2820643  2820660  | 3.00      | TCGTGAACGACGAACT  | 66           |
| tmk (SAV4622)             | putative thymidylate kinase | 5643741  5643761  | 3.00      | TCGTGAACGACGAACT  | 73           |
| folD2 (SAV543)            | putative methylenetetrahydrofolate | 688370  688387  | 3.00      | TCGTGAACGACGAACT  | 231          |
| parA (SAV6508)            | putative partitioning or sporulation protein | 7797460  7797477  | 3.00      | TCGTGAACGACGAACT  | 114          |
| SAV7415-7416              | putative sugar isomerase putative simple sugar ABC transporter | 8846427  8846444  | 3.00      | TCGTGAACGACGAACT  | 203          |
| SAV897                    | putative alpha-amylase inhibitor | 1075479  1075496  | 3.00      | TCGTGAACGACGAACT  | 73           |

Determination of Rex operator (ROP) sequences on promoter regions of atpBEFHAGDC, cydA1B1CD, nuaA1-N1, and rex-hemAC1DB. Rex binding sequences in 5′-end fluorescein-labeled promoter regions of the above operons were determined by DNase I footprinting analysis. One protected region was detected in the above promoter region in the presence of 1.2 or 2.4 μM Rex-His, which contains a ROP site. In contrast, expression of atpI under oxygen limitation declined steadily during 60 min in both ATCC31267 and Drex (Fig. 2). Transcription levels of cydA1, nuaA1, hemA, and atpI were consistently higher for Drex than for ATCC31267 under equivalent treatments, confirming that these genes are negatively regulated by Rex.
(5′-TTGTGACCTGCTTCACAT-3′) (Fig. S2D). ROP in the *nuoA1* and *atpI* promoter regions is located between −35 region and −10 region, and encompasses −35 region. These findings suggest that Rex blocks attachment of RNA polymerase to the promoters or inhibits the progress of RNA polymerase by binding to ROP in or
downstream of the promoters of atpBEFHAGDC, cydA1B1CD, nuoA1-N1, and rex-hemAC1DB, and blocks transcription of these operons.

**DNA-binding activity of Rex is modulated by NADH/NAD⁺ ratio.** In S. coelicolor, NADH at concentrations <5 μM inhibits DNA-binding activity of Rex, whereas 1 mM NAD⁺ has no inhibitory effect. NAD⁺ competes with NADH for Rex binding⁴. In B. subtilis and S. aureus, NAD⁺ enhances binding of Rex to putative Rex-binding sites, while NADH competes with NAD⁺ for Rex binding and reduces Rex activity⁵. We examined the effects of NAD⁺ and NADH on DNA-binding activity of Rex to upstream regions of cydA1 in S. avermitilis. DNA-binding activity of Rex was reduced by addition of NADH, but not by NAD⁺ concentrations up to 1 mM (Fig. 4A,B; Fig. S3). NADH and NAD⁺ were added to EMSA binding buffer to assess the effect of NAD⁺/NADH ratio on DNA-binding activity of Rex in vitro. At NAD⁺ concentration 0.2 mM, 5 μM NADH was sufficient to dissociate the Rex-DNA complex (Fig. 4C). At NAD⁺ concentration 1 mM, dissociation of DNA-Rex complex required 25 μM NADH, suggesting that Rex-binding activity was recovered by addition of increasing amounts of NAD⁺ (Fig. 4D). These findings indicate that NAD⁺ and NADH bind competitively to Rex and modulate its DNA-binding activity. These findings also imply that Rex exploits the similar regulatory mechanism in Streptomyces.

**Rex regulates morphological differentiation.** In comparison to ATCC31267, Drex showed delayed morphogenesis on SFM agar at day 2, when aerial mycelium was initiated. Spore formation at day 6 did not differ notably between the two strains. Morphogenesis of the Drex complementation strain was similar to that of ATCC31267 (Fig. 5), indicating that the delayed morphogenesis was due solely to rex deletion.

The promoter region of wblE in S. avermitilis contains a putative Rex-binding motif (Table 1). wblE encodes a putative WhiB-family transcriptional regulator, which may be involved in morphological differentiation²,¹³. qRT-PCR analysis revealed notable increases of wblE transcription level in Drex. Levels under oxygen limitation condition declined gradually during 60 min for both ATCC31267 and Drex, and were consistently higher for Drex than for ATCC31267 (Fig. 2). EMSAs showed that Rex-His, bound to the wblE promoter region in vitro (Fig. 3A). In in vivo ChIP assays, PCR product of the wblE promoter region was selectively enriched from immunoprecipitated DNA of ATCC31267, whereas no such PCR band was amplified from immunoprecipitated DNA of Drex (Fig. 3B). These findings indicate that wblE is negatively regulated by Rex. Binding sequence in the wblE promoter region was determined by DNase I footprinting analysis. A 28-nt region protected by Rex-His⁶ was detected, extending from positions +108 to +135 relative to TSS of wblE (Fig. S4). The protected region contains a consecutive ROP site (5'-TTGTGGAAGCCGTTACAT-3') and a half-site ROP (5'-TTTCAACAC-3') located downstream of TSS. Rex may inhibit the progress of RNA polymerase by binding to ROP downstream of the wblE promoter, and thereby repress transcription.

To test the possibility that overexpression of wblE in Drex results in delayed morphogenesis, we attempted to delete wblE in S. avermitilis. However, this attempt was unsuccessful. wblE is evidently an essential gene in Streptomyces; an attempt to delete it in S. coelicolor was also unsuccessful¹⁵. When wblE was overexpressed in ATCC31267, the resulting strain had a phenotype similar to that of Drex (Fig. 5), suggesting that Rex regulates morphological differentiation through its effect on wblE expression.

**Rex negatively regulates avermectin production.** The overexpression of rex caused a decrease in avermectin production to 33% of ATCC31267 level. Drex had avermectin production ~3-fold higher than that of ATCC31267. The mycelial dry weight of Drex was similar to that of ATCC31267, indicating that the improved avermectin yield was not achieved by improved growth. In the Drex complementation strain, avermectin production was similar to that of ATCC31267 (Fig. 1; Fig. 6A). Oxygen limitation for 60 min reduced expression of the biosynthetic genes aveD, relative to ATCC31267. Oxygen limitation for 60 min reduced expression of the biosynthetic genes aveD and aveA1 and aveD, relative to ATCC31267. Oxygen limitation for 60 min reduced expression of these genes in ATCC31267; however, Drex showed lower fold repression, and a slight induction of aveA1 and aveD (Fig. 6C). EMSAs revealed that Rex-His₉ did not bind to the aveR promoter region or the aveD-A1 intergenic region (Fig. S5). Although no retarded band was observed when aveR promoter region was probed with Rex-His₉ protein, DNase I footprinting analysis showed one protected region extending for 15 nucleotides on the aveR coding strand in the presence of 10 or 15 μM Rex-His₉ (Fig. 7). No consecutive ROP site was observed in the protected region; however, two adjacent half-site ROP (5'-TTGTGGA-3' and 5'-TTTCAACAC-3') were found in the protected region and downstream region. Rex can evidently interact with the half-site ROP; however, because the interaction is weak and easily dissociated in vitro, EMSA did not reveal a clear shifted band.

**Confirmation of putative Rex target genes.** A genome-wide search of consensus motif 5'-TTTGGAANNNTTCACAA-3' using the genome sequence of ATCC31267 revealed the presence of 36 motifs up to 350 bp upstream of predicted genes: 2 motifs with one mismatch, 10 motifs with two mismatches, and 24 motifs with three mismatches. Our previous experiments showed that wblE, cydA1B1CD, rex-hemAC1DB, atpBEFHAGDC, and nuoA1-N1 are directly controlled by Rex. To investigate whether Rex binds to promoter regions of other putative target genes, we selected 16 genes with predicted gene function for EMSAs (Table 1). Of these, Rex bound to the probes of hppA (encodes an inorganic H⁺ pyrophosphatase), ndh2 (encodes a NADH
Figure 4. DNA-binding activity of Rex is modulated by NADH/NAD^+ ratio. (A) EMSAs of *cydA1* promoter region using Rex-His<sub>6</sub> and 0.1 or 1 mM pyridine nucleotides. (B) EMSAs of *cydA1* promoter region using Rex-His<sub>6</sub> with various NADH concentrations. (C,D) Assay mixtures contained NADH at indicated concentration and 0.2 mM (C) or 1 mM (D) NAD^+. Arrow: free probe.
dehydrogenase (complex I)), echA7 (encodes an enoyl-CoA hydratase), ectABC (encodes ectoine biosynthesis enzymes), SAV828 (encodes a rhamnosidase), and SAV2652 (encodes a regulatory protein). Probes whose binding motif had one or two mismatches showed higher affinity than probes whose binding motif had three mismatches (Fig. 3A, Fig. 8). These findings demonstrated that 5′-TTGTGAANNNNTTCACAA-3′ is the consensus motif of Rex in *S. avermitilis*.

**Discussion**

Results of this study show that Rex in *S. avermitilis* acts as a repressor of aerobic metabolism, morphological differentiation, and secondary metabolism (summarized schematically in Fig. 9). Results of EMSAs demonstrated that at least 11 genes/operons are directly regulated by Rex. Among these, atpIBEFHAGDC, cydA1B1CD, nuoA1-N1, and rex-hemAC1DB operons encode key components of the electron transfer chain and play crucial roles in aerobic metabolism14–17. hppA encodes a putative pyrophosphate-energized proton pump that converts energy from pyrophosphate hydrolysis into active H+ transport across the plasma membrane18. ndh2 encodes a NADH dehydrogenase involved in NAD+ regeneration19,20. echA7 encodes an enoyl-CoA hydratase that catalyzes the second step of the β-oxidation pathway of fatty acid metabolism21. SAV828 encodes a rhamnosidase that hydrolyzes L-rhamnose from L-rhamnoside22. Under oxygen limitation condition, the increase of intracellular NADH/NAD+ ratio in *S. avermitilis* dissociates binding of Rex from its target binding sites and derepresses its target genes/operons, and upregulation of cydA1B1CD, nuoA1-N1, rex-hemAC1DB, ndh2, and hppA increases oxygen utilization, NAD+ regeneration, and ATP synthesis (Fig. 9). On the other hand, expression of atpIBEFHAGDC in *Corynebacterium glutamicum* is regulated by ECF σ23. A sigH homolog is present in *Streptomyces*; whether it regulates atpIBEFHAGDC expression remains to be tested.

WhiB-like family transcription factors are widely present in actinomycetes, but not found in other bacterial orders. WhiB was first identified as a small transcription factor-like protein essential for sporulation in *S. coelicolor*24. Genome sequencing revealed that *Streptomyces* species have multiple whiB-like genes (designated “wbl”). Eleven wbl genes (including whiB and whiD) have been identified in *S. coelicolor*13. Among these, wblA, whiB, and whiD are essential for sporulation, and WblA also negatively regulates antibiotic biosynthesis in *Streptomyces*13,25–27. Other wbl genes are not involved in morphological development, with the exception of wblE.
Fowler-Goldsworthy et al.\textsuperscript{13} reported that \textit{wblE} could not be deleted in various strains of \textit{S. coelicolor}, and we made a similar observation in \textit{S. avermitilis}. Thus, \textit{wblE} appears to be essential in this genus. The homolog of \textit{wblE} in \textit{Mycobacterium tuberculosis} is \textit{whiB1}, which encodes an essential transcription factor in response to nitric oxide exposure\textsuperscript{28}. We demonstrated that \textit{wblE} is directly negatively regulated by Rex, and that \textit{wblE} overexpression results in delayed morphogenesis similar to that of Drex. Expression of \textit{wblE}, like that of \textit{atpIBEFHAGDC}, is downregulated by oxygen limitation in both \textit{ATCC31267} and Drex, suggesting that (i) \textit{wblE} is jointly regulated by Rex and some other regulator, or (ii) \textit{wblE} itself responds to low oxygen concentration via its own redox-sensitive [4Fe-4S] cluster. Under oxygen limitation condition, \textit{wblE} expression in \textit{Streptomyces} is downregulated, with consequent stimulation of sporulation and production of a large number of spores to maintain viability under conditions of little or no oxygen. Another Rex target, \textit{ectABC}, encodes enzymes for biosynthesis of ectoine (a compatible solute) that serves as an osmolyte and promotes survival under osmotic or temperature stress\textsuperscript{29}. By regulating \textit{ectABC} transcription, Rex facilitates ectoine biosynthesis to enhance viability under these types of stress.

In Drex, expression of regulatory gene \textit{aveR} and biosynthetic genes, and avermectin production, were notably increased. Although EMSA showed no clearly retarded band between \textit{aveR} promoter region probe and Rex-His\textsubscript{6}, DNase I footprinting analysis revealed one 15-nt protected region consisting of two adjacent half-site ROP on the coding strand of \textit{aveR} by Rex-His\textsubscript{6}. Thus, Rex may directly regulate \textit{aveR} expression by interacting with the half-site ROP in the \textit{aveR} promoter region. Expression of electron transfer chain components was enhanced in Drex, thus promoting aerobic respiration rate, ATP production, and secondary metabolism. The notable increase of \textit{atpIBEFHAGDC}, \textit{cydA1B1CD}, \textit{muoA1-N1}, and \textit{rex-hemAC1DB} expression in Drex enhanced the tolerance of cells to oxygen limitation. The findings described here provide a basis for construction of new \textit{Streptomyces} strains with high antibiotic production and hypoxia tolerance.

\begin{figure}
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\includegraphics[width=\textwidth]{fig6.png}
\caption{Effect of \textit{rex} deletion on avermectin production in \textit{S. avermitilis}. (A) Avermectin production in \textit{rex}-related mutant strains. \textit{ATCC31267}, wild-type; Drex, \textit{rex}-deletion mutant; 31267 (pSET152) and 31267 (pKC1139), empty plasmid-containing controls; Drex-C, \textit{rex}-deletion complementation strain 31267 (pSET-rex); Orex, \textit{rex} overexpressing strain 31267 (pKC-rex). (B) Avermectin production in \textit{ATCC31267} and Drex with various oxygen limitation conditions. *Agitation speed 230 rpm (control, 250 rpm); **Static culture for 2 h at day 5 during fermentation (250 rpm). (C) RT-qPCR analysis of \textit{aveR}, \textit{aveA1}, and \textit{aveD} transcription levels in \textit{ATCC31267} and Drex. RNA samples were the same ones used for experiments shown in Fig. 1. Quantitative data were normalized to \textit{hrdB} expression value. Values shown are mean ± SD from three replicates. Statistical significance of differences was determined using Student’s \textit{t}-test. ***\(P < 0.001\); NS, not significant.}
\end{figure}
Materials and Methods

Bacterial strains and growth conditions. The *S. avermitilis* strains used were ATCC31267 (wild-type), Drex (*rex*-deletion strain), Drex-C (*rex*-deletion complementary strain harboring plasmid pSET-rex), and Orex (ATCC31267 harboring *rex* overexpressing plasmid pKC-rex). *E. coli* strains JM109 and BL21 (DE3) were used for routine cloning and protein expression, respectively. YMS medium and SFM medium were used for sporulation and phenotype studies. Culture conditions for mycelial growth, protoplast preparation, and regeneration of *S. avermitilis* were as described previously. Seed medium and fermentation medium FM-I were used for avermectin production and for RNA isolation, and soluble fermentation medium FM-II was used for ChIP analysis.

Gene deletion, complementation, and overexpression. A *rex* (SAV4738) gene deletion mutant was generated through targeted gene deletion mediated by homologous recombination. A 566-bp fragment upstream of *rex* (position −460 to +87 from start codon) was amplified by primers rex-up-Fw and rex-up-Rev, and a 579-bp fragment downstream of *rex* (position +539 to +1098) was amplified by primers rex-dw-Fw and rex-dw-Rev, using ATCC31267 genomic DNA as template (Table S1; Fig. 1). The two fragments, after recovery, were digested respectively by *Bam*HI/*Hin*II and *Bam*HI/*Eco*RI, and ligated together into *Eco*RI/*Hin*II-digested pKC1139 to produce *rex*-deletion vector pKCD-rex. pKCD-rex was introduced into ATCC31267 protoplasts. Double-crossover recombinant strains were selected as described previously. Culture conditions for mycelial growth, protoplast preparation, and regeneration of *S. avermitilis* were as described previously. Seed medium and fermentation medium FM-I were used for avermectin production and for RNA isolation, and soluble fermentation medium FM-II was used for ChIP analysis.

RNA extraction and qRT-PCR analysis. RNA was isolated using Trizol reagent (Tiangen; China) from *S. avermitilis* mycelia grown in FM-I as described previously. Transcription levels of various genes were determined by qRT-PCR using the primer pairs listed in Table S1. An RNA sample without prior reverse transcription was used as negative control to rule out chromosomal DNA contamination. *hrdB* gene (SAV4738) was used as internal control.

Chromatin Immunoprecipitation (ChIP) assay. ChIP assay was performed as described previously. In brief, *S. avermitilis* cultures grown in FM-II for 2 or 6 days were fixed in cross-linking buffer (0.4 M sucrose, 10 mM Tris-Cl [pH 8.0], 1 mM EDTA) containing 1% formaldehyde for 20 min at 28 °C. ChIP was performed using anti-Rex antibody. After DNA extraction, pellets were washed with 70% ethanol and resuspended in 50 μl Tris-EDTA buffer. 1 μl DNA solution was subjected to PCR using the primer sets listed in Table S1.
Overexpression and purification of Rex-His$_6$. The rex coding region was amplified by PCR using primers His-rex-Fw and His-rex-Rev. The purified fragment was cut with NcoI/HindIII and cloned into NcoI/HindIII-digested pET28a (+) to generate expression plasmid pET-rex. pET-rex was introduced into E. coli BL21 (DE3) for overexpression of C-terminal His$_6$-tagged Rex. Rex-His$_6$ was induced by 0.2 mM IPTG at 37 °C and purified from whole-cell lysate by Ni-NTA agarose chromatography (Bio-works; Sweden) according to the manufacturer’s instructions.

Electrophoretic mobility gel shift assays (EMSAs). EMSAs were performed according to the manufacturer’s instructions (DIG Gel Shift Kit, 2nd Generation, Roche) as described previously$^{35}$. DNA probes were obtained by PCR using the primers listed in Table S1, and labeled with Digoxigenin-11-ddUTP at the 3′ end using recombinant terminal transferase. DIG-labeled DNA probe was incubated with various quantities of Rex-His$_6$ for 30 min at 25 °C in a total volume of 20 μl containing 1 μg poly[d(I-C)]. Electrophoresis (5.0% native polyacrylamide gel; 0.5 × TBE as running buffer) was performed to separate protein-bound probes from free probes. DNA was electroblotted onto a positively charged nylon membrane, and retarded and unbound bands were detected by chemiluminescence and recorded on X-ray film.

DNase I footprinting assays. A fluorescent labeling procedure was used for DNase I footprinting assays$^{37}$. DNA fragments were obtained by PCR using FAM-labeled primers (Table S1), and purified from agarose gel. Labeled DNA fragments (400 ng) and various quantities of Rex-His$_6$ were incubated in a 25-μl volume for 30 min at 25 °C. DNase I digestion was performed for 40 sec at 37 °C, and terminated by addition of 10 μl 0.2 M EDTA.
(pH 8.0). Samples were subjected to phenol/chloroform extraction, ethanol precipitation, and capillary electrophoresis. Electrophoreograms were analyzed using GeneMarker software v2.2.0.

**Fermentation and HPLC analysis of avermectin production.** Fermentation of *S. avermitilis* strains and estimation of avermectins yields by HPLC analysis were performed as described previously.\(^{32}\)

**Determination of transcriptional start sites.** Transcriptional start sites (TSS) of *rex* and *wlbE* were mapped by 5′-RACE using a 5′/3′ RACE Kit (2nd Generation, Roche). Total RNA was extracted from ATCC31267 grown in FM-I for 2 days. A gene-specific primer (sp1) was used to synthesize CDNA, and template RNA was degraded with RNase H. A homopolymeric A-tail was purified and added to the 3′-end of cDNA using terminal transferase. Tailed cDNA was PCR amplified through 35 cycles with a specific nested primer (sp2) and an oligo (dT)-anchor primer (Table S1). PCR products were electrophoresed, purified using a DNA agarose gel recovery kit (BioTek; China), and sequenced.

**Prediction of Rexputative targets.** To search for putative Rex target genes, Rex consensus motif 5′-TTGTAANNNNTCAAA-3′ was used to scan the intergenic regions of the *S. avermitilis* genome using Virtual Footprint software.\(^{39}\)

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Designed the experiments: Z.C. Performed the experiments: X.L., Y.C., and M.L. Analyzed the data: X.L., Y.C., and M.L. Contributed reagents/materials/analysis tools: Y.W., Y.S., and J.L. Wrote the paper: Z.C., X.L., and Y.C.

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