



# AccR, a TetR Family Transcriptional Repressor, Coordinates Short-Chain Acyl Coenzyme A Homeostasis in *Streptomyces avermitilis*

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**ABSTRACT** Malonyl coenzyme A (malonyl-CoA) and methylmalonyl-CoA are the most common extender units for the biosynthesis of fatty acids and polyketides in *Streptomyces*, an industrially important producer of polyketides. Carboxylation of acetyl- and propionyl-CoAs is an essential source of malonyl- and methylmalonyl-CoAs; therefore, acyl-CoA carboxylases (ACCases) play key roles in primary and secondary metabolism. The regulation of the expression of ACCases in *Streptomyces* spp. has not been investigated previously. We characterized a TetR family transcriptional repressor, AccR, that mediates intracellular acetyl-, propionyl-, methylcrotonyl-, malonyl-, and methylmalonyl-CoA levels by controlling the transcription of genes that encode the main ACCase and enzymes associated with branched-chain amino acid metabolism in *S. avermitilis*. AccR bound to a 16-nucleotide palindromic binding motif (GTAA-N6-TTAA) in promoter regions and repressed the transcription of the *accD1A1-hmgL-fadE4* operon, *echA8*, *echA9*, and *fadE2*, which are involved in the production and assimilation of acetyl- and propionyl-CoAs. Methylcrotonyl-, propionyl-, and acetyl-CoAs acted as effectors to release AccR from its target DNA, resulting in enhanced transcription of target genes by derepression. The affinity of methylcrotonyl- and propionyl-CoAs to AccR was stronger than that of acetyl-CoA. Deletion of *accR* resulted in increased concentrations of short-chain acyl-CoAs (acetyl-, propionyl-, malonyl-, and methylmalonyl-CoAs), leading to enhanced avermectin production. Avermectin production was increased by 14.5% in an *accR* deletion mutant of the industrial high-yield strain *S. avermitilis* A8. Our findings clarify the regulatory mechanisms that maintain the homeostasis of short-chain acyl-CoAs in *Streptomyces*.

**IMPORTANCE** Acyl-CoA carboxylases play key roles in primary and secondary metabolism. However, the regulation of ACCase genes transcription in *Streptomyces* spp. remains unclear. Here, we demonstrated that AccR responded to intracellular acetyl-, propionyl-, and methylcrotonyl-CoA availability and mediated transcription of the genes related to production and assimilation of these compounds in *S. avermitilis*. When intracellular concentrations of these compounds are low, AccR binds to target genes and represses their transcription, resulting in low production of malonyl- and methylmalonyl-CoAs. When intracellular acetyl-, propionyl-, and methylcrotonyl-CoA concentrations are high, these compounds bind to AccR to dissociate AccR from target DNA, promoting the conversion of these compounds to malonyl- and methylmalonyl-CoAs. This investigation revealed how AccR coordinates short-chain acyl-CoA homeostasis in *Streptomyces*.

**KEYWORDS** *Streptomyces*, TetR family transcriptional regulator, acyl-CoA carboxylase, polyketides, short-chain acyl-CoAs

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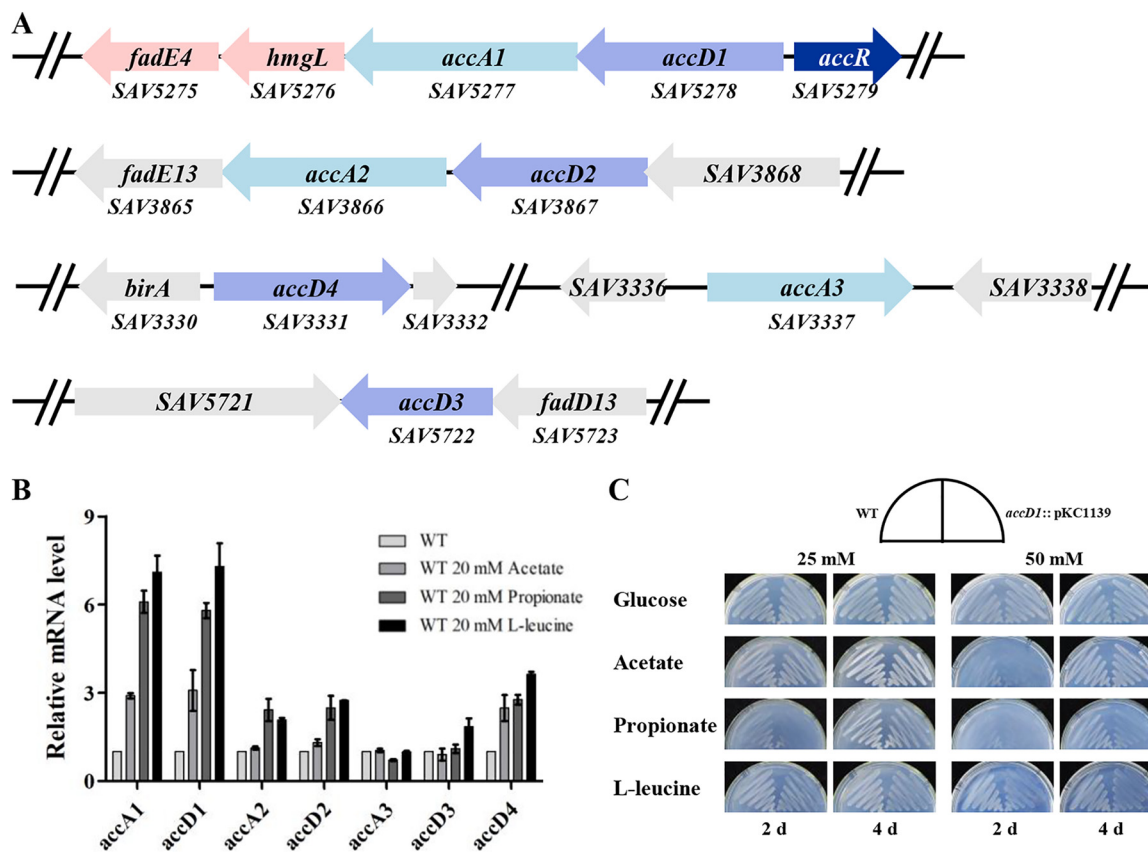
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Polyketides are a large class of natural products with diverse structures and biological activities. Many of them (e.g., avermectin, erythromycin, lovastatin, rifamycin, and tetracycline) are widely used in medicine, agriculture, and animal husbandry (1). The formation of polyketides is initiated by polyketide synthases, which condense acyl coenzyme A (acyl-CoA) extender units onto the starter unit through repeated decarboxylative Claisen condensation reactions (2, 3). Malonyl- and methylmalonyl-CoAs are the most common extender units for polyketide biosynthesis (4). Malonyl-CoA is also a building block for fatty acid biosynthesis. Production of malonyl- and methylmalonyl-CoAs usually involves the carboxylation of acetyl- and propionyl-CoAs (5).

In actinobacteria, including *Streptomyces*, *Mycobacterium*, and *Corynebacterium*, the enzymes that carboxylate acetyl-CoA and propionyl-CoA are termed acyl-CoA carboxylases (ACCases) (6). Actinobacterial ACCases have broad substrate specificity and are able to simultaneously catalyze acetyl-CoA, propionyl-CoA, and butyryl-CoA to generate malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA, respectively (7–9). The two major subunits of ACCase complex are a larger  $\alpha$  subunit that contains biotin carboxylase (BC) and biotin carboxyl carrier protein (BCCP) domains and a smaller  $\beta$  subunit that contains the carboxyltransferase (CT) domain (10). In some cases, a noncatalytic  $\varepsilon$  subunit is required for holo-carboxylase activity (11). ACCases are categorized as acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), or methylcrotonyl-CoA carboxylase (MCC) depending on substrate preference. Two ACCases have been characterized in *Streptomyces coelicolor* A3(2) (12–14). They have the same  $\alpha$  subunit, AccA1 or AccA2 (encoded by identical genes *accA1* or *accA2*). The  $\beta$  and  $\varepsilon$  components are, respectively, AccB and AccE for ACC and PccB and PccE for PCC (12–14). ACC is able to carboxylate acetyl-, propionyl-, and butyryl-CoAs with similar specificity, whereas PCC carboxylates only propionyl- and butyryl-CoAs. The major determinant of substrate specificity for ACCases is the  $\beta$  subunit (9). ACCases provide the building blocks for polyketide biosynthesis. Overexpression of ACC enhances intracellular malonyl-CoA levels, leading to the overproduction of actinorhodin in *S. coelicolor* A3(2) and of antitumor mithramycin in *S. argillaceus* (15, 16). PCC overexpression in *S. hygroscopicus* increased the rapamycin yield (17).

Despite the essential roles of ACCases in primary and secondary metabolism, the mechanisms that regulate their expression in actinobacteria are poorly understood. Several TetR family regulators (TFRs) have been identified to regulate the transcription of ACCase-encoding genes. TFRs are a ubiquitous group of transcriptional regulators, consisting of a highly conserved helix-turn-helix DNA binding motif at the N terminus and a diverse ligand binding domain at the C terminus (18). In *Corynebacterium glutamicum*, a highly conserved TFR FasR acts as a repressor for the gene *accD1*, which encodes the  $\beta$  subunit of the essential ACC. FasR also plays an important role in regulation of fatty acid synthesis by repressing the transcription of two fatty acid synthase encoding genes, *fasA* and *fasB* (19). In *Saccharopolyspora erythraea*, another TFR, PccD, represses PCC expression by binding to the upstream regions of the *pccBC* operon and *pccA* which encode the  $\beta$ ,  $\varepsilon$ , and  $\alpha$  subunits of PCC separately (20). Methylmalonic acid acts as a ligand of PccD, inhibiting the binding of PccD to its target DNA (20). In mycobacteria, the TetR family repressor BkaR (also known as Fad35R) binds to 16-nucleotide (nt) palindromic motifs (GTTANT-N4-ANTAAC) in upstream regions of target genes and represses the transcription of itself and of a divergently oriented operon, *accD1A1-fadE19-Rv2499c-citE-bkdABC*, involved in branched-chain keto-acid metabolism (21, 22). Regulation of ACCase expression in *Streptomyces* species has not been studied previously.

Avermectins, a series of 16-membered macrocyclic polyketides produced by *S. avermitilis*, are widely used in agricultural, veterinary, and medical fields because they display low host toxicity and broad-spectrum nematicidal, acaricidal, and insecticidal activities (23). Synthesis of avermectin aglycone is initiated by starter unit 2-methylbutyryl-CoA or isobutyryl-CoA and proceeds by the addition to the starter unit of seven acetate extender units from malonyl-CoA and five propionate extender units from methylmalonyl-CoA (23, 24). Here, we describe the identification of a TFR, AccR (SAV5279), that functions as a



**FIG 1** Analysis of acyl-CoA carboxylase (ACCase)-encoding genes in *S. avermitilis*. (A) Organization of ACCase genes in *S. avermitilis*. *accA* and *accD* encode the  $\alpha$  and  $\beta$  subunits of ACCase, respectively. *accR* encodes a TetR family regulator. *hmgL* and *fadE4* were cotranscribed with *accD1A1*. Gray, adjacent genes of *acc* genes. (B) Induction of *acc* genes by acetate, propionate, or L-leucine in *S. avermitilis*. The transcription levels of *acc* genes were determined by RT-qPCR. RNAs were extracted from cells cultured in FM-I with supplemental acetate, propionate, or L-leucine for 24 h. (C) Phenotypic analyses of WT and *accD1::pKC1139*. The strains were cultured on MM supplemented with glucose, acetate, propionate, or L-leucine as the sole carbon source.

repressor for the control of intracellular short-chain acyl-CoA levels in *S. avermitilis* by inhibiting the transcription of ACCase coding genes and genes related to branched-chain amino acid metabolism. Methylcrotonyl-, propionyl-, and acetyl-CoAs act as ligands and interact with AccR to dissociate it from its target DNA. Deletion of *accR* resulted in an increase of short-chain acyl-CoA pools and consequent enhancement of avermectin production in *S. avermitilis* wild-type (WT) and industrial strains.

## RESULTS

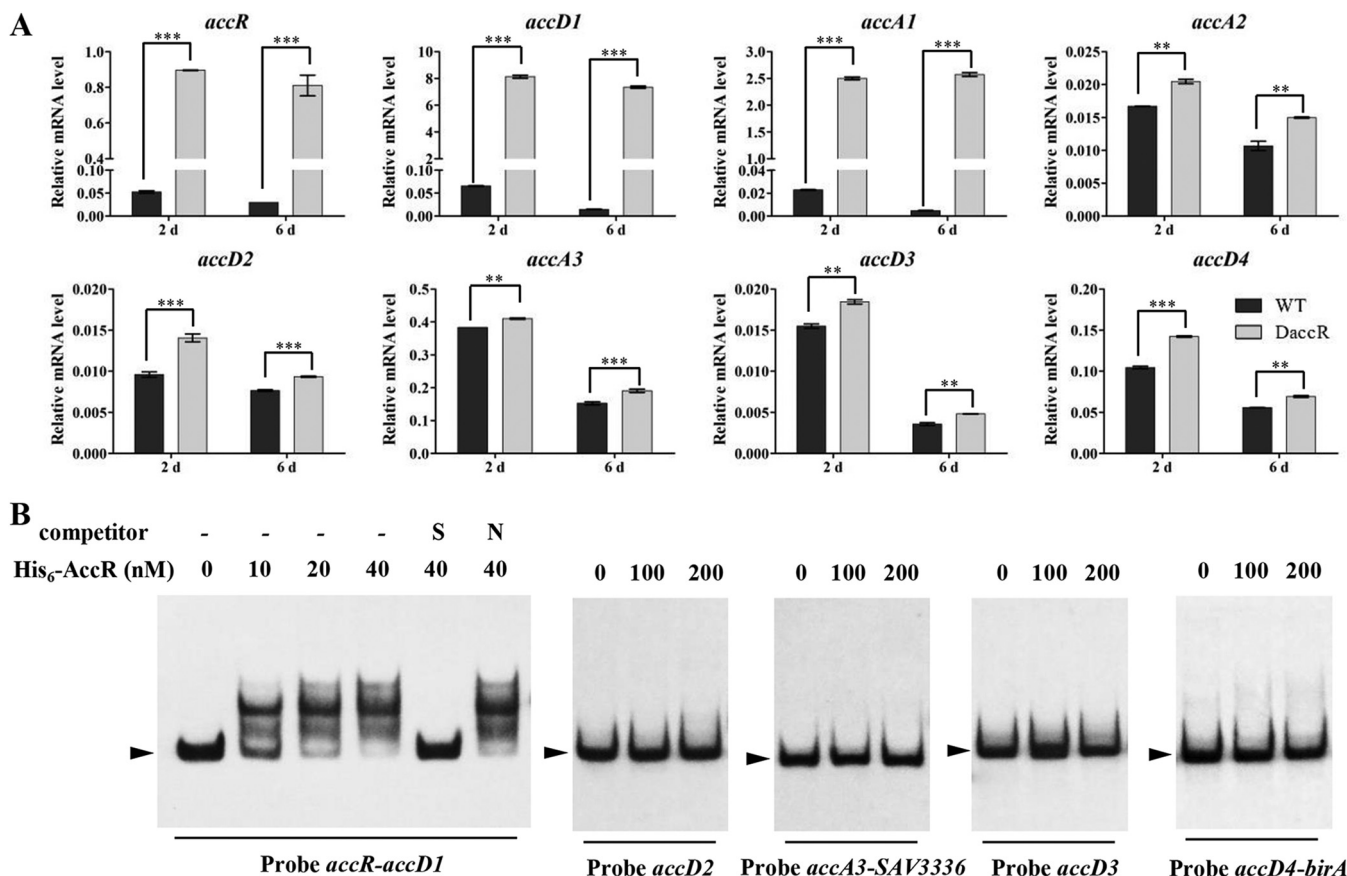
**The expression of *accD1* and *accA1* was strongly induced by acetate, propionate, and L-leucine.** Seven ACCase coding genes were found in the *S. avermitilis* genome. Among these, it is predicted that *accA1* (SAV5277), *accA2* (SAV3866), and *accA3* (SAV3337) encode  $\alpha$  subunits of ACCases and that *accD1* (SAV5278), *accD2* (SAV3867), *accD3* (SAV5722), and *accD4* (SAV3331) encode  $\beta$  subunits of ACCases (Fig. 1A). *S. avermitilis* AccA1, AccA2, AccA3, AccD1, AccD2, and AccD3 share 78, 82, 93, 90, 92, and 78% sequence identities to SCO2777, SCO4381, AccA1/AccA2 (SCO6271/SCO4921, identical proteins), AccD1 (SCO2776), SCO4380, and SCO2445 in *S. coelicolor* A3(2), respectively. AccD4 has 91% identity with *S. coelicolor* A3(2) PCC  $\beta$  subunit (PccB, SCO4926) and presumably functions as a PCC in *S. avermitilis*. *accD1*, *accA1*, and their downstream genes (*hmgL* and *fadE4*) are oriented in the same direction, with very short intergenic regions, and were shown by reverse transcription-PCR analysis to be organized in the same operon (see Fig. S1 in the supplemental material). *accD2* and adjacent

*accA2* are also oriented in the same direction. It is reasonable to presume that AccD1 and AccA1 form a heteromeric multisubunit ACCase, whereas AccD2 and AccA2 form another. The functions of these ACCases in *S. avermitilis* remain to be elucidated.

In bacteria, acetate and propionate are converted to acetyl-CoA and propionyl-CoA by acetyl/propionyl-CoA synthetase and then utilized by ACCases (25). To determine which *acc* gene encodes the main ACCase that carboxylates acetyl- and propionyl-CoAs in *S. avermitilis*, we analyzed the transcription levels of all *acc* genes in the WT strain ATCC 31267 after the addition of 20 mM acetate or propionate. In acetate-induced cells, the transcription levels of *accA1* and *accD1* were ~3-fold higher than in cells without acetate induction, which was the highest fold increase of all *acc* genes under the same condition (Fig. 1B). The transcription levels of *accA1* and *accD1* were increased ~6-fold under treatment with propionate. *accD4* expression was enhanced (~2.5-fold) by acetate and (~2.8-fold) by propionate, indicating that the ACCase consisting of AccD4 functions mainly as a PCC. Other *acc* genes shown no notable induction by acetate, and only *accA2* and *accD2* were slightly induced by propionate. In *Mycobacterium tuberculosis*, the homolog of AccD1A1 was identified as an MCC involved in leucine metabolism, which catalyzes carboxylation of methylcrotonyl-CoA during leucine degradation (26). Therefore, we also detected the leucine induction of the *acc* genes. *accD1A1* was significantly induced (~7-fold) by L-leucine, which is much higher than the fold increases for other *acc* genes (Fig. 1B). These findings collectively indicate that acetate, propionate, and L-leucine induced transcription of *accD1A1*, and the induction of propionate and L-leucine was stronger than that of acetate. The fold increases of *accA1* and *accD1* were more pronounced than the others under all treatments. Thus, ACCase consisting of AccD1 and AccA1 appears to play a crucial role in the assimilation of acetyl-CoA, propionyl-CoA, and methylcrotonyl-CoA in *S. avermitilis*.

**Disruption of *accD1* affected growth on media with acetate, propionate, or L-leucine as the sole carbon source.** The  $\beta$  subunit of the ACCase determines substrate specificity. To identify the substrate specificity of AccD1A1, we disrupted the *accD1* gene in the *S. avermitilis* genome by inserting plasmid pKC1139 into the *accD1* open reading frame (ORF). The growth of WT and *accD1::pKC1139* was investigated on minimal medium (MM) with glucose, acetate, propionate, or L-leucine as the sole carbon source. Both *accD1::pKC1139* and WT grew well on MM supplemented with glucose. The growth of the WT was slightly inhibited by acetate and propionate but not by L-leucine. Compared to the WT, the growth of *accD1::pKC1139* on MM with acetate, propionate, or L-leucine was significantly inhibited, and inhibition was more pronounced on the media with higher concentrations (50 mM) of these compounds (Fig. 1C). L-Leucine and propionate had more obvious inhibitory effects on *accD1::pKC1139* than acetate, which was consistent with the higher fold inductions of *accD1* and *accA1* by L-leucine and propionate (Fig. 1B and C). These findings indicated that AccD1A1 functions as the main ACCase assimilating acetyl-CoA, propionyl-CoA, and methylcrotonyl-CoA in *S. avermitilis*.

**AccR directly represses the expression of *accD1A1* and its own gene.** *SAV5279* (designated *accR*) shares a bidirectional promoter region with the *accD1A1-hmgL-fadE4* operon and was predicted to encode a TetR family regulator. AccR is highly conserved in actinobacteria, with 76 to 91% sequence identities to its homologs in *Streptomyces*, 53% identity to *Nocardia farcinica* Nfa50360, and 51% identity to *M. tuberculosis* BkaR (Fig. S2A). Its adjacent *acc* operon is also conserved in actinobacteria, except for the absence of the *hmgL* gene in *N. farcinica* and *M. tuberculosis*. To investigate possible regulation of adjacent *acc* operon by AccR, we constructed an *accR* deletion strain (termed DaccR) with an internal 421-bp deletion in the *accR* ORF by homologous recombination in the WT. The expression of *accR* and *acc* genes in WT and DaccR was analyzed by reverse transcription-quantitative PCR (RT-qPCR) during the exponential (day 2) and stationary (day 6) phases (Fig. 2A). *accR* expression in DaccR was, respectively, ~17- and ~27-fold higher than in the WT on days 2 and 6. The transcription levels of *accA1* and *accD1* in DaccR, relative to WT, were ~120-fold higher on day 2 and



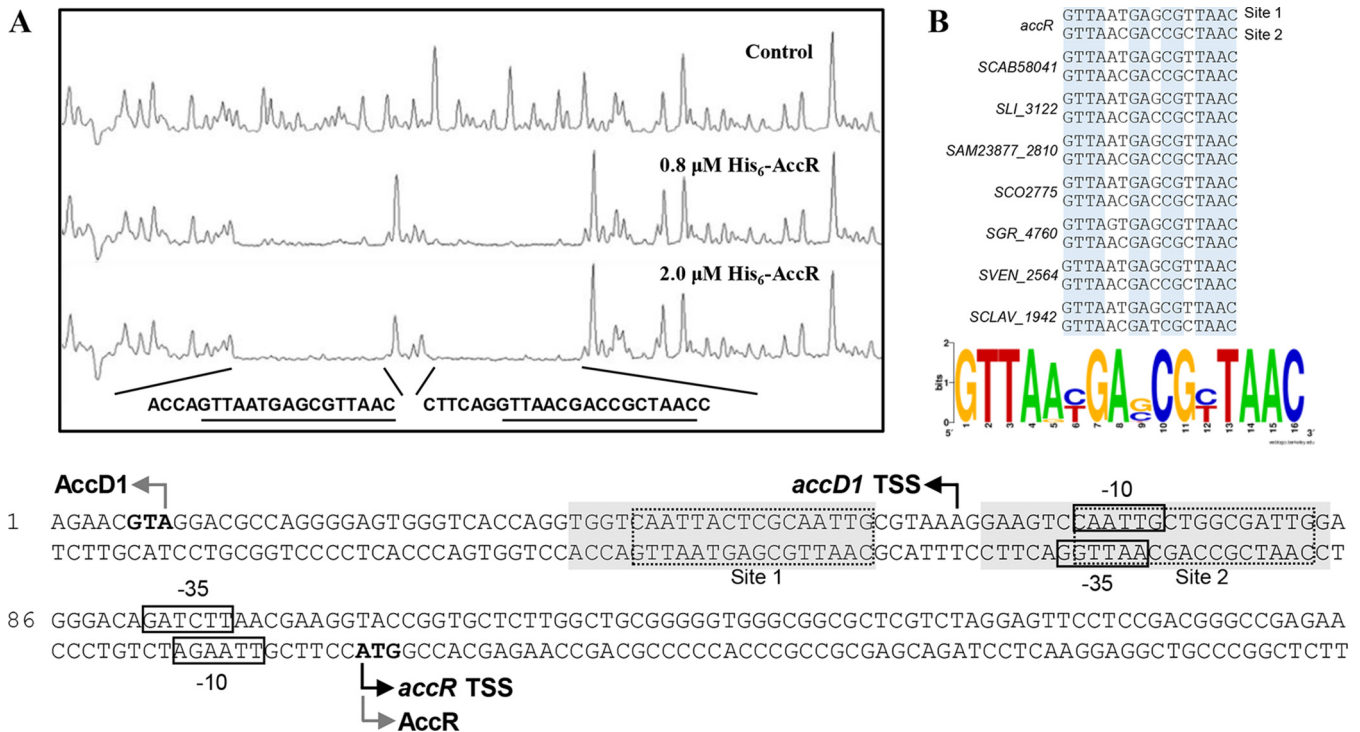
**FIG 2** Interaction of AccR with *acc* genes in *S. avermitilis*. (A) Transcriptional levels of *acc* genes in WT and DaccR were determined by RT-qPCR. Values are means ± the standard deviations from three replicate experiments. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (Student *t* test). (B) EMSAs of AccR with promoter regions of *acc* genes. A 200-fold excess of specific (S) or nonspecific (N) unlabeled probes was used as a competitor of the labeled probe. Arrowheads indicate free DNA probes.

>500-fold higher on day 6. The expression of other *acc* genes in DaccR increased to much smaller degrees. These findings indicated that AccR represses the expression of its own gene and of *acc* genes, particularly *accD1* and *accA1*.

To determine whether AccR directly regulates *acc* genes, we performed electrophoretic mobility shift assays (EMSAs) using upstream regions of *acc* genes and His<sub>6</sub>-AccR purified from *Escherichia coli* (Fig. S3). His<sub>6</sub>-AccR bound to the intergenic region of *accR-accD1*, leading to clear shifting of the bands (Fig. 2B). Shifting of the bands was abolished by an unlabeled specific probe but not by a nonspecific probe (Fig. 2B), indicating that interaction of AccR with the *accR-accD1* intergenic region is specific. No shifted bands were observed in upstream regions of *accD2*, *accD3*, or the intergenic regions of *accA3-SAV3336* and *accD4-birA* with His<sub>6</sub>-AccR. These findings, taken together, indicate that AccR directly represses the expression of *accR*, *accD1*, and *accA1* and indirectly inhibits the expression of other *acc* genes.

**Determination of the AccR binding site in the *accR-accD1* intergenic region.** To clarify the regulatory mechanism of AccR on *accD1A1* expression, we determined the binding sites of AccR by DNase I footprinting assays, using His<sub>6</sub>-AccR and a FAM-labeled DNA probe of the *accR-accD1* intergenic region. Two DNA regions in the *accR-accD1* intergenic region were clearly protected by increased concentrations of AccR (0.8 and 2.0 μM) (Fig. 3A). Analysis of the protected regions revealed two similar 16-nt palindromic sequences (GTAA-N6-TTAA), consistent with previous findings that TetR family regulators usually form homodimers and bind to palindromic sequences (27). Similar palindromic sequences were found in upstream regions of its homologous genes in other *Streptomyces* species (Fig. 3B; see also Fig. S2B in the supplemental material), indicating that the 16-nt GTAA-N6-TTAA palindromic sequence is the

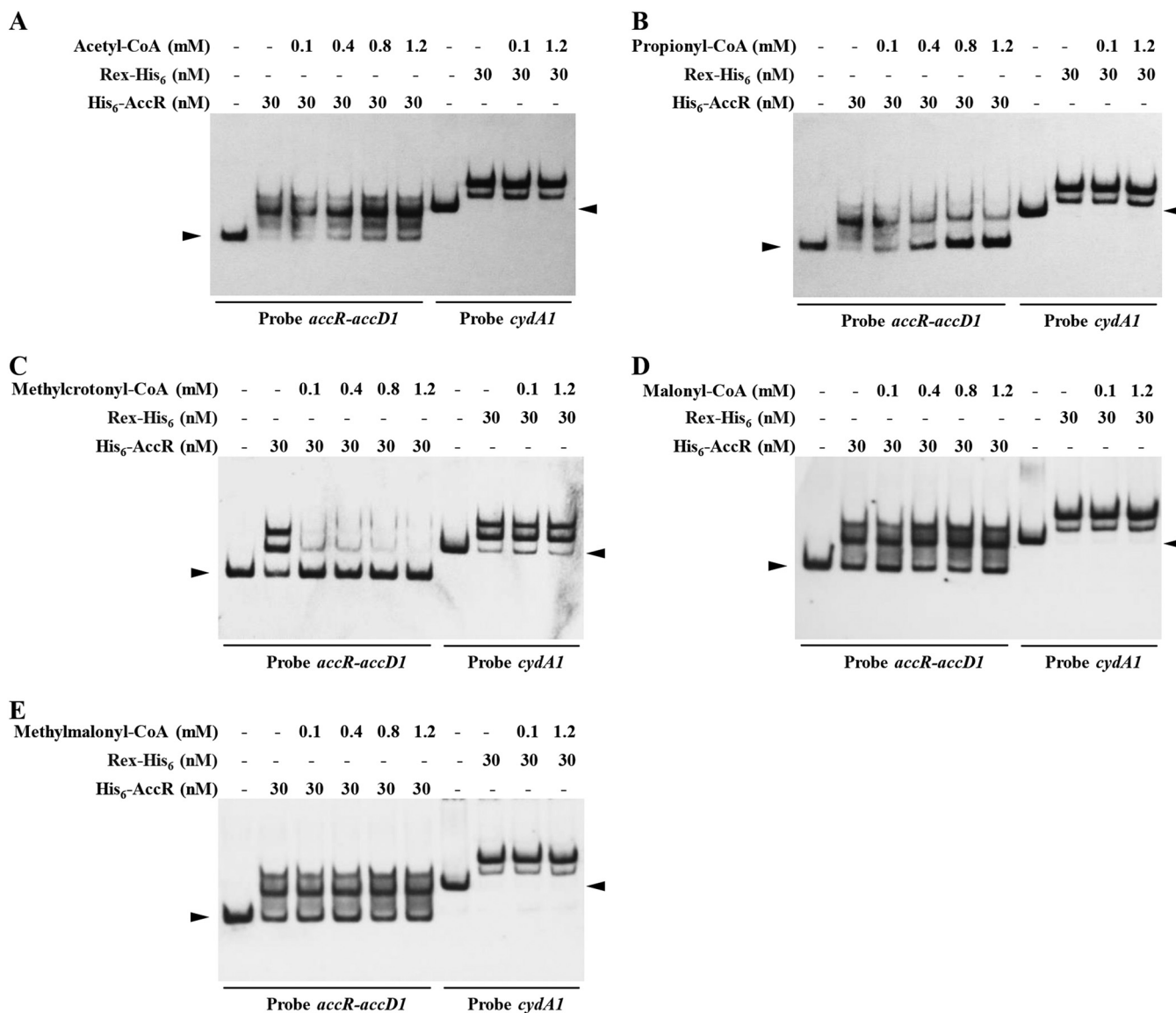




**FIG 3** Identification of AccR binding sites in the intergenic region of *accR-accD1*. (A) DNase I footprinting assay of AccR with the *accR-accD1* intergenic region. Gray-shaded boxes indicate DNA regions protected by AccR; dotted frames indicate palindromic binding sites. Dark and gray curved arrows indicate transcriptional start sites and translational initiation codons, respectively. Boxes indicate putative -10 and -35 regions. (B) Alignment of AccR binding sites of *accR-accD1* intergenic regions in *Streptomyces* species. The consensus sequence logo was created using WebLogo.

consensus sequence of AccR binding sites. Transcriptional start sites of *accR* and *accD1* were determined by 5' RACE and found to be located at nt -52 (A) for *accD1* and nt +1 (A) for *accR* relative to their translation initiation codons. The AccR binding sites are located at the -35 region and upstream of the -35 region for *accR* and in the -10 region and downstream of the -10 region for *accD1*. Thus, AccR represses transcription of the *accD1A1* operon and *accR* by blocking attachment of the RNA polymerase to their promoters, thereby preventing transcription initiation and extension.

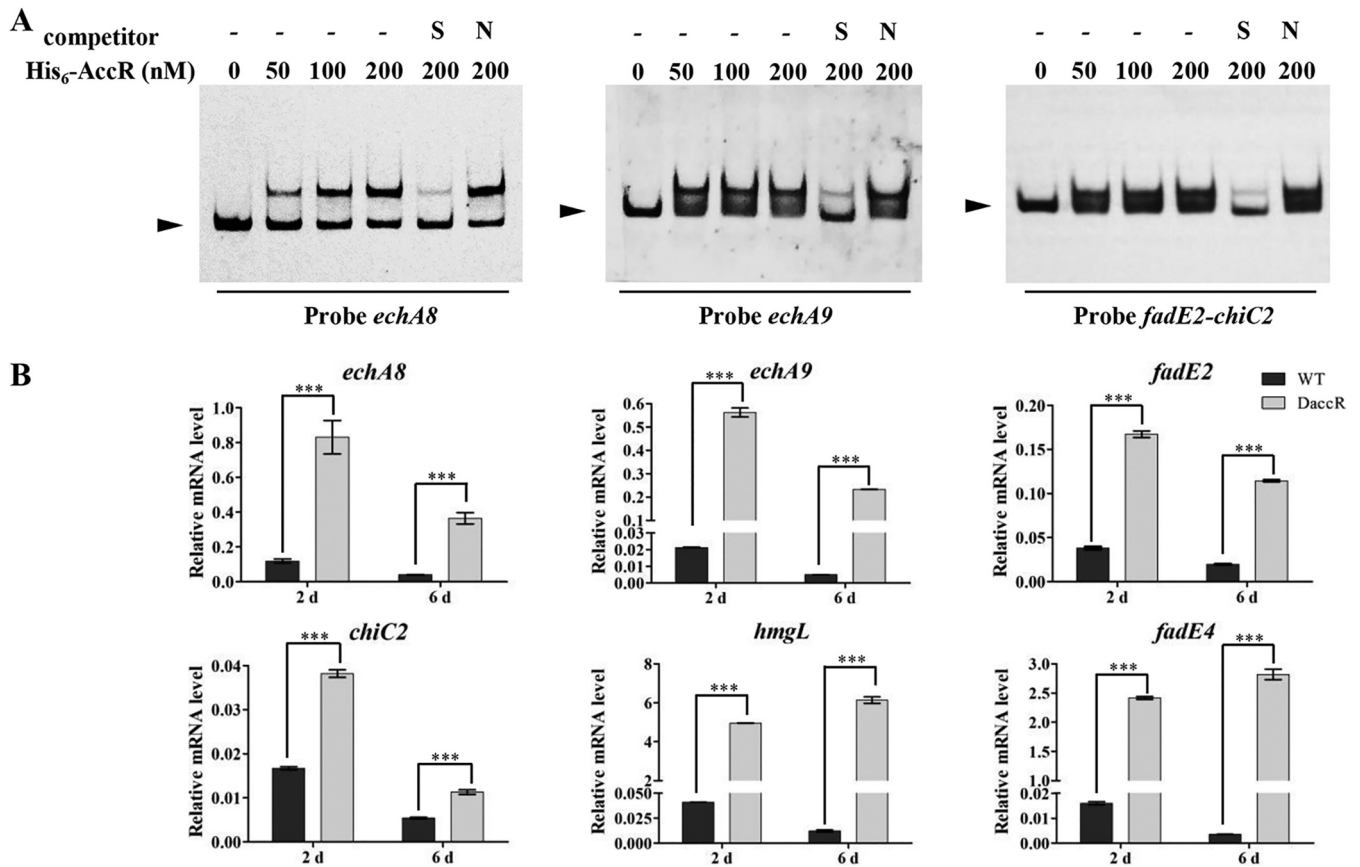
**Methylcrotonyl-CoA, propionyl-CoA, and acetyl-CoA are ligands of AccR.** TetR family regulators bind and respond to one or more ligands, which change their DNA binding ability for DNA targeting, thus coordinating expression of target genes. AccR represses the transcription of *accD1A1*, which encodes the ACCase catalyzing assimilation of acetyl-, propionyl-, and methylcrotonyl-CoAs. Therefore, we performed EMSAs to determine which ACCase substrate (acetyl-, propionyl-, and methylcrotonyl-CoAs) or product (malonyl- and methylmalonyl-CoAs) affects the binding ability of AccR to the *accR-accD1* intergenic region. Methylcrotonyl-, propionyl-, and acetyl-CoAs inhibited interaction of AccR with the *accR-accD1* intergenic region (Fig. 4A to C), whereas malonyl- and methylmalonyl-CoAs had no effect on the DNA-binding activity of AccR (Fig. 4D and E). The effect of methylcrotonyl- and propionyl-CoAs on AccR activity was much more pronounced than that of acetyl-CoA. Methylcrotonyl-CoA at 5 μM or propionyl-CoA at 50 μM was sufficient to attenuate the interaction of AccR with *accR-accD1* intergenic region, whereas a much higher concentration of acetyl-CoA was required to achieve a similar effect (Fig. 4A; see also Fig. S4 in the supplemental material). The redox-sensing regulator Rex of *S. avermitilis*, which recognizes NADH as a ligand (28), was used as a negative control. Taken together, these findings indicate that the ACCase substrates methylcrotonyl-CoA, propionyl-CoA, and acetyl-CoA are the true effectors of AccR to release AccR from its target genes. When intracellular methylcrotonyl-, propionyl-, and acetyl-CoA pools are abundant, these compounds



**FIG 4** Effects of acetyl-CoA (A), propionyl-CoA (B), methylcrotonyl-CoA (C), malonyl-CoA (D), and methylmalonyl-CoA (E) on *in vitro* binding of AccR to the *accR-accD1* intergenic region. The concentrations of the five acyl-CoAs are shown. Binding of Rex-His<sub>6</sub> to *cydA1* promoter region was used as a negative control. Arrowheads indicate free probes.

bind to AccR to dissociate AccR from DNA, resulting in the enhanced expression of *accD1A1* by derepression, which then promotes the utilization of these compounds.

**Identification of other target genes regulated by AccR.** To further clarify the mechanisms for regulation of AccR in *S. avermitilis*, a consensus sequence was created (29) by aligning AccR binding sites of *accR-accD1* intergenic regions from eight *Streptomyces* species (including *S. avermitilis*) (Fig. 3B) and used to predict AccR target genes in the *S. avermitilis* genome by PREDetector (30). With a score set to 10, a total of 13 genes were revealed (see Table S1 in the supplemental material), including *accR* and *accD1*. Interestingly, 8 of these 13 genes are involved in lipid and carbohydrate metabolism (*chiC2* and *udgA* in carbohydrate metabolism; *echA8*, *echA9*, *fadE1*, *fadE2*, and *icmB* in lipid metabolism; and *SAV1133* in lipid transport). The upstream regions of these 8 genes were used for EMSAs. AccR bound to promoter regions of *echA8* and *echA9* and the *fadE2-chiC2* intergenic region but not to *SAV1133* promoter region or intergenic regions of *fadE1-udgA* and *icmB-SAV3461* (Fig. 5A; see also Fig. S5 in the supplemental material).

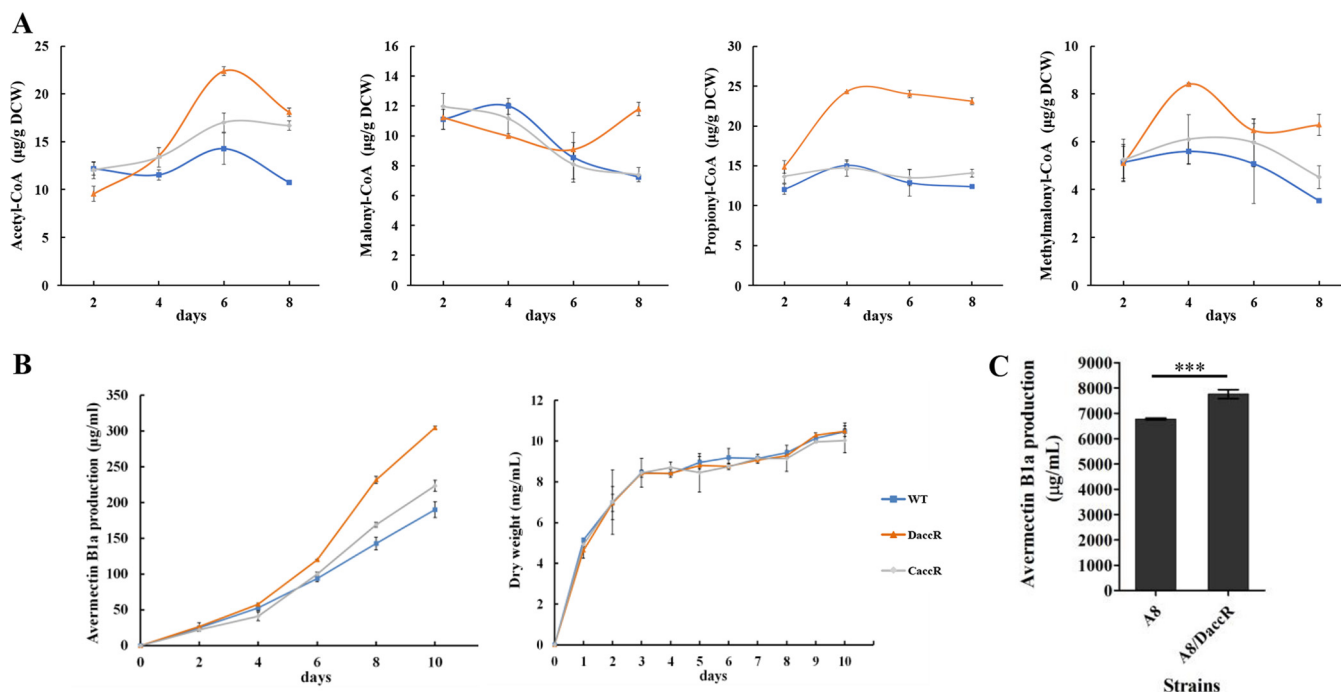


**FIG 5** Verification of putative AccR target genes. (A) EMSAs of AccR with promoter regions of putative target genes. Arrowheads indicate free DNA probes. (B) RT-qPCR analysis of the expression of putative target genes in WT and DaccR strains. \*\*\*,  $P < 0.001$  (Student *t* test).

The transcription levels of *echA8*, *echA9*, *fadE2*, and *chiC2* in DaccR were much higher than in the WT (Fig. 5B). Because *hmgL* and *fadE4* are in the same operon as *accD1A1*, we analyzed the transcriptional levels of *hmgL* and *fadE4*. *hmgL* and *fadE4* levels in DaccR were more than 100-fold higher than in the WT strain, similarly to *accD1A1* (Fig. 2A and 5B). Thus, AccR also represses the expression of *chiC2*, *echA8*, *echA9*, *fadE2*, *fadE4*, and *hmgL*. *chiC2* encodes a chitinase that degrades chitin. *echA8* and *echA9* encode enoyl-CoA hydratase, and *fadE2* and *fadE4* encode acyl-CoA dehydrogenase. These enzymes are involved in the  $\beta$ -oxidation process and branched-chain amino acid metabolism, an important source of acetyl- and propionyl-CoAs. *hmgL* encodes a hydroxymethylglutaryl-CoA cleavage enzyme that cleaves HMG-CoA (downstream product of carboxylation of methylcrotonyl-CoA in leucine degradation) to produce acetyl-CoA and acetoacetate. Thus, in addition to the repression of ACCase genes, AccR also represses the expression of genes related to acetyl- and propionyl-CoA production.

**AccR mediates homeostasis of intracellular short-chain acyl-CoAs.** As described above, AccR represses the transcription of acyl-CoA metabolism genes involved in the production and assimilation of acetyl- and propionyl-CoAs. To elucidate the regulatory role of AccR in the metabolism of short-chain acyl-CoAs related to avermectin biosynthesis, we assessed the intracellular availability of malonyl- and methylmalonyl-CoAs and of their substrates, acetyl- and propionyl-CoAs, in WT, DaccR, and complemented CaccR strains. Titrers of acetyl- and propionyl-CoAs were maintained at relatively constant levels in the WT but increased in DaccR in the early and middle stages of fermentation and then were maintained at much higher levels than in the WT strain (Fig. 6A). Malonyl- and methylmalonyl-CoA titers in WT increased in the early stage and reached maximal values on day 4 but, in DaccR, declined in the middle stage of





**FIG 6** Determination of intracellular short-chain acyl-CoA contents in WT, DaccR, and CaccR strains. (A) Intracellular contents of acetyl-, malonyl-, propionyl-, and methylmalonyl-CoAs at the indicated times. (B) Avermectin production by WT, DaccR, and CaccR strains in FM-I and growth curves in FM-II. Error bars represent the standard deviations of three replicates. (C) Effect on avermectin production of *accR* deletion in the industrial high-yield strain *S. avermitilis* A8. \*\*\*,  $P < 0.001$  (Student *t* test).

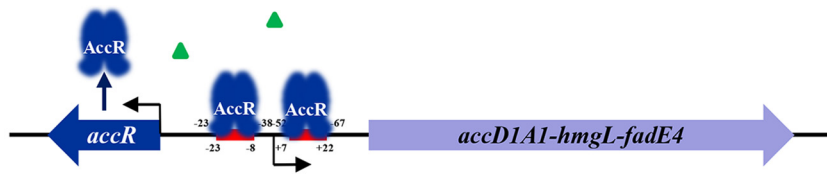
fermentation, reached minimal values on day 6, and increased thereafter. In particular, malonyl- and methylmalonyl-CoA levels in DaccR were much higher than those in the WT in late-stage fermentation when secondary metabolites were rapidly biosynthesized (Fig. 6A). The titer curves of CaccR were similar to those of WT, indicating that altered levels of short-chain acyl-CoAs in DaccR were due to the loss of *accR*. Overall, the levels of short-chain acyl-CoAs in DaccR were strongly increased, an observation consistent with the notably increased expression of *accD1A1*, *echA8*, *echA9*, *fadE2*, *fadE4*, and *hmgL* in DaccR, which promoted the accumulation and conversion of acetyl- and propionyl-CoAs to malonyl- and methylmalonyl-CoAs (Fig. 2A and 5B), suggesting that AccR plays an important role in maintaining the homeostasis of intracellular short-chain acyl-CoAs.

**Deletion of *accR* increased avermectin production.** DaccR showed increased pools of intracellular malonyl- and methylmalonyl-CoAs, the precursors of avermectin biosynthesis. We evaluated avermectin production in related mutants to determine whether acyl-CoA metabolism affects secondary metabolism. Avermectin B1a production in DaccR ( $304 \mu\text{g ml}^{-1}$ ) was higher than in WT ( $190 \mu\text{g ml}^{-1}$ ) and in CaccR it was close to the WT level. *accR* deletion had no effect on growth rate and biomass (Fig. 6B). The findings indicated that the enhanced avermectin yield was attributed to the deletion of *accR*. Overexpression of *accD1A1* led to enhanced avermectin production in the WT strain but not in DaccR, and production in the WT/*accD1A1*-overexpressing strain was less than in DaccR (Fig. S6), indicating that enhanced expression of other target genes besides *accD1A1* contributed to the increase of avermectin production in DaccR. Although the transcription levels of the avermectin pathway-specific regulatory gene *aveR* and the biosynthetic genes *aveA1*, *aveA4*, and *aveD* were increased in DaccR, AccR did not bind to the upstream regions of these genes. Taken together, these findings indicate that AccR indirectly regulates avermectin production (see Fig. S7A and B in the supplemental material), mainly by affecting intracellular metabolic flux.

To experimentally increase avermectin production in an industrial context, we deleted *accR* in the industrial high-yield strain *S. avermitilis* A8. The avermectin B1a yield of the A8/DaccR strain ( $7,757.23 \mu\text{g ml}^{-1}$ ) was 14.5% higher than that of the parental

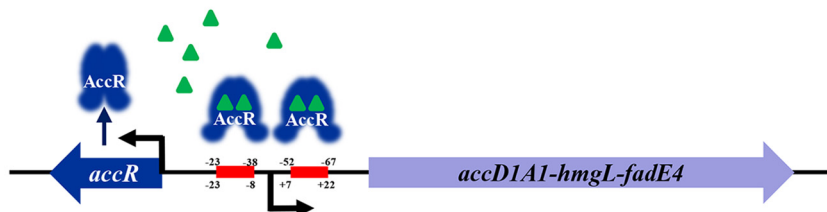
## A Repression

Low pools of M-CoA, MM-CoA



## B Derepression

High pools of M-CoA, MM-CoA



**FIG 7** Proposed model of AccR-mediated regulation of ACCase (AccD1A1) in *S. avermitilis*. (A) When the intracellular concentrations of methylcrotonyl-, propionyl-, or acetyl-CoA are low, AccR binds to the intergenic region of *accR-accD1* and represses the expression of the *accD1A1-hmgL-fadE4* operon and of its own gene, resulting in low production of malonyl- and methylmalonyl-CoAs. (B) When the methylcrotonyl-, propionyl-, or acetyl-CoA levels are sufficient, these compounds form complexes with AccR, leading to dissociation of AccR from DNA, and the expression of target genes is enhanced by derepression. Red bars indicate AccR binding sites. Numbers indicate the position relative to the transcriptional start site. The thickness of the arrows indicates the transcriptional levels of *accR* and *accD1A1-hmgL-fadE4*. Green triangles indicate methylcrotonyl-, propionyl- or acetyl-CoA.

strain ( $6,770.46 \mu\text{g ml}^{-1}$ ) (Fig. 6C), indicating that industrial avermectin production can be effectively enhanced by genetic manipulation of *accR*.

## DISCUSSION

Malonyl- and methylmalonyl-CoAs are the building blocks for biosynthesis of polyketides and fatty acids. We demonstrated in this study that intracellular pools of malonyl- and methylmalonyl-CoAs are regulated by a TetR family regulator, AccR. Through binding to a 16-nt palindromic sequence (GTAA-N<sub>6</sub>-TTAAC) in upstream regions of target genes, AccR represses the expression of branched-chain amino acid metabolism genes and the ACCase coding gene *accD1A1*, which is involved in the production and assimilation of acetyl- and propionyl-CoAs (see Fig. S8 in the supplemental material). AccR represses *accD1A1* expression but does not directly control the expression of other *acc* genes. Methylcrotonyl-CoA, propionyl-CoA, and acetyl-CoA act as the ligands of AccR, inhibiting the interaction of AccR with its target DNA. When the intracellular concentrations of methylcrotonyl-, propionyl-, and acetyl-CoAs are low, AccR binds to target genes and represses the expression of these genes and also its own gene, resulting in low production of malonyl- and methylmalonyl-CoAs. Conversely, when intracellular methylcrotonyl-, propionyl-, and acetyl-CoA concentrations are high, these compounds form complexes with AccR, resulting in the dissociation of AccR from target DNA. The expression of target genes is thereby enhanced through derepression, promoting the conversion of methylcrotonyl-, propionyl-, and acetyl-CoAs to malonyl- and methylmalonyl-CoAs (Fig. 7; see also Fig. S8 in the supplemental material). In summary, AccR responds to intracellular methylcrotonyl-, propionyl-, and acetyl-CoA availability and plays a key role in regulating short-chain acyl-CoA pools in *S. avermitilis*.

Several TetR family repressors have been shown to play essential roles in controlling expression of ACCase in bacteria; examples include CcrR in *Methylobacterium extorquens*, FasR in *Corynebacterium glutamicum*, PccD in *S. erythraea*, and BkaR (Fad35R)

in mycobacteria (19–21, 31). In this study, we demonstrated that the TetR family regulator AccR in *S. avermitilis* represses the expression of *echA8*, *echA9*, *fadE2*, and the *accD1A1-hmgL-fadE4* operon, which are involved in the generation and assimilation of acetyl- and propionyl-CoAs. The diversity of C-terminal structures found in TetR family regulators allows them to interact with a wide variety of small molecules, including signaling molecules, metabolites, and antibiotics. Such interaction will cause a structural change in the C-terminal helices and release the regulator from the operator, allowing the transcription of target genes (27, 32). Fatty acids and activated fatty acids (acyl-CoAs) have been identified as effector molecules for several TetR family regulators involved in regulation of acyl-CoA metabolism. Long-chain acyl-CoA ( $C_{14-20}$ ) acts as a ligand of YsiA (a functional homolog of *E. coli* FadR) and inhibits the binding of YsiA to promoter regions of genes involved in fatty acid degradation in *Bacillus subtilis* (33). Methylmalonic acid inhibits the interaction of the propionyl-CoA assimilation repressor PccD with the upstream regions of *pccBC* operon and *pccA* in *S. erythraea* (20). The present study shows that AccR in *Streptomyces* utilizes methylcrotonyl-, propionyl-, and acetyl-CoAs, the substrates of target genes, as ligands. Our findings expand the known ligand classes of TetR family regulators. AccR acts as a repressor in response to intracellular methylcrotonyl-, propionyl-, and acetyl-CoA levels and coordinates the gene expression of ACCase, which is responsible for methylcrotonyl-, propionyl-, and acetyl-CoA assimilation, in a substrate-dependent manner.

AccR is highly conserved in *Streptomyces* species, sharing 75 to 91% amino acid sequence identities and exhibiting high identities for the DNA binding domain (>97%) and the ligand binding domain (>78%) (Fig. S2A). AccR is an ortholog of BkaR from *Mycobacterium* (50% identity) that also plays a vital role in regulating catabolism of branched-chain amino acids (21, 22), although the genomic context of *accR* in *Streptomyces* is quite different from that of *bkaR* in *Mycobacterium*. In *Mycobacterium* spp., the neighboring genes *fadD35*, *scoAB*, *accD1A1*, *fadE19*, *Rv2499c*, *citE*, and *bkdABC* (*fadD35* and *scoAB* are absent from some species), divergently oriented from *bkaR*, are also under the negative control of BkaR (21, 22). *accD1A1* encodes an MCC which catalyzes the carboxylation of methylcrotonyl-CoA (26). In *S. avermitilis*, transcription of *accD1A1* was strongly induced by acetate, propionate, or L-leucine (Fig. 1B), indicating that the ACCase encoded by *accD1A1* has MCC, PCC, and ACC activities, which is consistent with the broad substrate specificity of actinobacterial ACCases. The 16-nt palindromic binding motif of AccR (GTAA-N6-TTAA) is similar to the motif (GTTANT-N4-ANTAAC) recognized by BkaR, consistent with the high N-terminal sequence identity of the two regulators (Fig. S2A and B). Two conserved motifs are also present in the intergenic regions of *accR* and *bkaR*, suggesting a similar regulatory mechanism of AccR (BkaR) in actinobacteria. *S. avermitilis* harbors the homologous genes *fadD35*, *scoAB*, *Rv2499c*, *citE*, and *bkdABC*, which are apparently not under the direct control of AccR, because neither conserved AccR motif was found in their promoter regions, and AccR did not bind to them (see Fig. S9 in the supplemental material). *bkdABC* expression is under the negative control of the AsnC family regulator BkdR in *Streptomyces* (34, 35). Thus, it appears that only *accD1A1* and *fadE19* (the homolog of *fadE4* in *S. avermitilis*) are conserved genes of the AccR/BkaR operon in actinobacteria.

Malonyl- and methylmalonyl-CoAs are extender units for avermectin production. AccR modulates avermectin production by controlling intracellular pools of malonyl- and methylmalonyl-CoAs. The intracellular pools of acetyl-, propionyl-, malonyl-, and methylmalonyl-CoAs were increased in DaccR because of the derepression of target genes, and the enhanced supply of precursors led to avermectin overproduction. *accR* and target genes (*accD1A1*, *echA*, *fadE*, and *hmgL*) are highly conserved in *Streptomyces* species; we therefore expect that future studies will provide improved, effective strategies for increasing the yields of polyketides for which malonyl- and methylmalonyl-CoAs are precursors, through the genetic manipulation of *accR* in natural or recombinant *Streptomyces* strains.

**TABLE 1** Plasmids and strains used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>S. avermitilis</i>		
ATCC 31267	Wild-type strain (WT)	Laboratory stock
<i>accD1::pKC1139</i>	<i>accD1</i> disruption strain by insertion of pKC1139	This study
DaccR	<i>accR</i> deletion mutant of strain ATCC 31267	This study
CaccR	DaccR with insertion of plasmid pSET-CaccR	This study
A8	Industrial high-yield avermectin-producing strain	Qilu Pharmaceutical
A8/DaccR	<i>accR</i> deletion mutant of strain A8	This study
WT(pKC-ermE- <i>accD1A1</i> )	<i>accD1A1</i> overexpression strain based on WT	This study
DaccR(pKC-ermE- <i>accD1A1</i> )	<i>accD1A1</i> overexpression strain based on DaccR	This study
<i>E. coli</i>		
JM109	Host for DNA cloning	Laboratory stock
BL21(DE3)	Host for protein overexpression	Laboratory stock
Plasmids		
pKC1139	Temperature-sensitive shuttle vector for <i>E. coli-Streptomyces</i>	41
pSET152	Integrated shuttle vector in <i>Streptomyces</i>	41
pET-28a(+)	Expression vector for His <sub>6</sub> -tagged protein purification in <i>E. coli</i>	Novagen
pKC-DaccD1	<i>accD1</i> disruption plasmid based on pKC1139	This study
pKC-DaccR	<i>accR</i> deletion plasmid based on pKC1139	This study
pSET-CaccR	<i>accR</i> complementation vector based on pSET152	This study
pET-AccR	<i>accR</i> overexpression vector based on pET-28a(+)	This study
pKC-ermE- <i>accD1A1</i>	<i>accD1A1</i> overexpression vector based on pKC1139 carrying promoter <i>ermE</i> <sup>*</sup> <i>p</i>	This study

## MATERIALS AND METHODS

**Strains and culture conditions.** Plasmids and strains of *E. coli* and *S. avermitilis* used in this study are listed in Table 1. *E. coli* was cultured in Luria-Bertani (LB) medium at 37°C. *S. avermitilis* was grown on solid YMS (yeast extract-malt extract-soluble starch) medium at 28°C for sporulation. Minimal medium (MM) supplemented with different carbon sources was used for phenotype observation of *S. avermitilis*. Modified YEME liquid medium and RM14 solid medium were used to culture mycelia for protoplast preparation and regeneration, respectively (36). Insoluble fermentation medium FM-I and soluble fermentation medium FM-II were used for avermectin production and biomass measurement, respectively (37). Apramycin at 100 μg ml<sup>-1</sup> or kanamycin at 50 μg ml<sup>-1</sup> was used to culture *E. coli*, and apramycin at 10 μg ml<sup>-1</sup> was used to culture *S. avermitilis*.

**Construction of *S. avermitilis* derived strains.** For the disruption of *accD1*, a 594-bp internal fragment of *accD1* was amplified with the primer pair *accD1*-Fw/*accD1*-Rev (Table 2). The fragment was cloned into pKC1139 to produce disruption plasmid pKC-DaccD1. Disruption plasmid was transformed into protoplasts of WT strain, and the disruption strain (*accD1::pKC1139*) was generated by single crossover. For the deletion of *accR*, a 534-bp upstream fragment (from positions -479 to +55 relative to the *accR* translation start codon) and a 541-bp downstream fragment (from positions +477 to +1017) were amplified by PCR using WT genomic DNA as the template and the primer pairs DaccR-up-Fw/DaccR-up-Rev and DaccR-down-Fw/DaccR-down-Rev, respectively. Fragments were digested with EcoRI/KpnI and KpnI/BamHI, respectively, and then ligated into EcoRI/BamHI-cleaved pKC1139 to construct the *accR* deletion vector (termed pKC-DaccR). The deletion vector was transformed into protoplasts of the WT strain and industrial strain A8, and the deletion strain (DaccR) was screened as described previously (38). Putative *accR* deletion strains were verified using the primer pairs DaccR-ex-Fw/DaccR-ex-Rev and DaccR-int-Fw/DaccR-int-Rev, which were flanking and located within the exchange region. For complementation, an 839-bp fragment, including the *accR* coding region and its promoter, was amplified from the WT genomic DNA with the primer pair DaccR-c-Fw/DaccR-c-Rev. The fragment was digested with EcoRI/BamHI and cloned into pSET152 to generate the complement vector pSET-CaccR, which was then transferred into protoplast of DaccR to obtain a complemented strain (termed CaccR). For the overexpression of *accD1A1* in *S. avermitilis*, a 3,746-bp DNA fragment containing the full ORF of *accD1A1* was amplified from WT genomic DNA with the primer pair *EaccD1A1*-Fw/*EaccD1A1*-Rev. The fragment was cloned into pKC1139 under the strong constitutive promoter *ermE*<sup>\*</sup>*p* to generate the *accD1A1* overexpression plasmid pKC-ermE-*accD1A1*. The resulting plasmid was transformed into WT and DaccR to obtain the WT(pKC-ermE-*accD1A1*) and DaccR(pKC-ermE-*accD1A1*) overexpression strains.

**Overexpression and purification of His<sub>6</sub>-AccR.** The *accR* coding region was amplified from WT genomic DNA using the primer pair His<sub>6</sub>-AccR-Fw/His<sub>6</sub>-AccR-Rev (Table 2) and inserted into pET-28a(+) to construct pET-AccR. pET-AccR was transformed into *E. coli* BL21(DE3) for overexpression of His<sub>6</sub>-AccR. The cells were cultured in LB medium with kanamycin at 50 μg ml<sup>-1</sup> at 37°C until the optical density at 600 nm reached 0.6. His<sub>6</sub>-AccR was then induced by 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. The cells were collected, resuspended in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole [pH 8.0]), sonicated on ice, and centrifuged. His<sub>6</sub>-AccR in the supernatant was purified by Ni<sup>2+</sup>-NTA agarose chromatography (Novagen) according to the manufacturer's protocol.

**Electrophoretic mobility shift assays.** EMSAs were performed using a DIG gel shift kit (2nd Generation; Roche) as described previously (39). DNA probes were amplified by PCR with primers

**TABLE 2** Primers used in this study

Purpose	Primer	Sequence (5'–3') <sup>a</sup>
Construction of <i>accD1::pKC1139</i>	accD1-Fw	CCGGAATTCCTCTACAACCAGGCACGGATGTC (EcoRI)
	accD1-Rev	CCCAGCTTGGTGCACAGCTCGATGAAGT (HindIII)
Construction and verification of DaccR	DaccR-up-Fw	CGGAATTCGCTTCTTACCGGTCATCGGGTA (EcoRI)
	DaccR-up-Rev	GGGGTACCCAGCCTCCTTGAGGATCTGCTC (KpnI)
	DaccR-down-Fw	GGGGTACCCCTGTGAACTCCACGCCACAC (KpnI)
	DaccR-down-Rev	CGGGATCCCGCTTCTTCTGCTCGTCGGT (BamHI)
	DaccR-ex-Fw	CGACATCCGTGCCTGGTTGTAG
	DaccR-ex-Rev	GCCACCCGTGATGAAGGCTTTG
	DaccR-Int-Fw	GGTCTGTACCCGGCACTTC
	DaccR-Int-Rev	CACCACCTCCACCCACAG
Complementation of DaccR mutant	DaccR-c-Fw	CGGAATTCCTCCGGTGCCTCTTGTCATCCT (EcoRI)
	DaccR-c-Rev	CGGGATCCCGCCTCGTGATCCTCGTTGAAC (BamHI)
Construction of pKC-ermE- <i>accD1A1</i>	EaccD1A1-Fw	GTGCCGGTTGGTAGGATCCAGCGGTGAGCATGCAAGAGGCACCGGAG
	EaccD1A1-Rev	GACGTTGTAACGACGGCCAGTGCCAAGCTTGCTCATGCCGCTCCTCCT
Construction of His <sub>6</sub> -AccR	His6-AccR-Fw	CGGGATCCATGGCCACGAGAACCGAC (BamHI)
	His6-AccR-Rev	CGGAATTCACAGTCGTCCAGGGCAACC (EcoRI)
DNase I footprinting	accR-FAM-Fw	GTCAGCTCCGGTGCCTCTT
	accR-Rev	TTGAGGATCTGCTCGCGG
EMSA	accR- <i>accD1</i> -Fw	GTCAGCTCCGGTGCCTCTT
	accR- <i>accD1</i> -Rev	TTGAGGATCTGCTCGCGG
	accD2-Fw	CCTGGCGGTGGCTCGTCC
	accD2-Rev	TGCTCGGCGGTTCAGTTGG
	accA3-SAV3336-Fw	AGACCTTGACCACATCGG
	accA3-SAV3336-Rev	ATGACTCCTCTCCTTGAAC
	accD3-Fw	CCGAACCTCTTACCAGTACC
	accD3-Rev	GTCAGCCATGCGTCAGTG
	accD4-birA-Fw	GCACCTCGGACGGCTTCAG
	accD4-birA-Rev	CGGCTCGGACATCGGGATG
	echA8-Fw	CGGCTACGGCGGCGAGTA
	echA8-Rev	GACGGTCGAGGCGGATGGT
	echA9-Fw	ATCCCGCCCGCCGTCAG
	echA9-Rev	GTCCGCTCGCTCCGCCATG
	fadE2- <i>chiC2</i> -Fw	GCGGACTGAGCATCGTGTCTC
	fadE2- <i>chiC2</i> -Rev	GGCATGGCGACGCTCTCCT

<sup>a</sup>Underlining is used to indicate restriction enzyme sites.

(Table 2) and then labeled with digoxigenin (DIG) using terminal transferase. Reaction mixtures contained 0.15 nM DIG-labeled probe, 0.5  $\mu$ l of poly(dI-dC) (2  $\mu$ g  $\mu$ l<sup>-1</sup>), and various amounts of His<sub>6</sub>-AccR. For the ligand binding assays, His<sub>6</sub>-AccR and various amounts of acyl-CoAs were preincubated for 15 min and then added to the reaction mixtures.

**RNA extraction and RT-qPCR analysis.** Total RNAs were extracted from mycelia grown in FM-I for various durations using TRIzol reagent (Tiangen; China) as described previously (39). To examine the induction of *acc* genes by acetate, propionate, or L-leucine, *S. avermitilis* cells were cultured in FM-I for 36 h. Then, 20 mM acetate, propionate, or L-leucine was added, and the sample was cultured for 24 h. The transcription levels of the tested genes were detected by RT-qPCR, using the *hrdB* transcription level as an internal control.

**DNase I footprinting assays.** These assays were performed using a fluorescence labeling procedure. A 167-bp fragment containing the *accR-accD1* intergenic region was amplified using the primers accR-FAM-Fw and accR-Rev (Table 2). A FAM-labeled DNA fragment and various amounts of His<sub>6</sub>-AccR were mixed, incubated at 25°C for 30 min, and digested by DNase I (0.016 U) at 37°C for 50 s. The reaction was terminated by the addition of EDTA (final concentration, 50 mM), and the reaction mixture was incubated for 10 min at 80°C. DNA samples were purified by chloroform extraction and ethanol precipitation and then sequenced by using a 3730XL DNA analyzer (Applied Biosystems).

**HPLC analysis of avermectin.** Fermentation of *S. avermitilis* strains and high-pressure liquid chromatography (HPLC) analysis of avermectin were performed as described previously (37).

**Detection of intracellular acyl-CoAs.** Extraction and HPLC analysis of acyl-CoAs were performed as described by Dayem et al. (40). In brief, *S. avermitilis* mycelia cultured in FM-I were collected at various times, frozen rapidly in liquid nitrogen, and ground to fine powder. Then, 0.1 g of the powder was extracted with 135  $\mu$ l of 10% trichloroacetic acid at 4°C, followed by centrifugation. Supernatants were analyzed by HPLC using a C<sub>18</sub> reverse-phase column (5  $\mu$ m; 4.6  $\times$  250 mm) and a multiple-gradient



elution procedure. HPLC buffer A contained 75 mM sodium acetate and 100 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 4.6), and buffer B contained 70% buffer A and 30% methanol. The column was equilibrated with 90% buffer A and 10% buffer B at a flow rate of 1 ml min<sup>-1</sup>. Samples (20 μl) were injected, and the percentage of buffer B was increased linearly to 40% over 35 min, increased linearly to 90% over 20 min, and then reduced linearly to 10% over 10 min. Acyl-CoAs were detected at a wavelength of 260 nm. Malonyl-, methylmalonyl-, acetyl-, and propionyl-CoAs (Sigma-Aldrich) were used as standards.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.6 MB.

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