Acyltransferase AniI, a Tailoring Enzyme with Broad Substrate Tolerance for High-Level Production of Anisomycin

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AB AC Anisomycin (compound 1), a pyrrolidine antibiotic, exhibits diverse biological and pharmacologic activities. The biosynthetic gene cluster of compound 1 has been identified previously, and the multistep assembly of the core benzylpyrrolidine scaffold was characterized. However, enzymatic modifications, such as acylation, involved in compound 1 biosynthesis are unknown. In this study, the genetic manipulation of *anil* proved that it encoded an indispensable acetyltransferase for compound 1 biosynthesis. Bioinformatics analysis suggested AniI as a member of maltose (MAT) and galactoside O-acetyltransferases (GAT) with C-terminal left-handed parallel beta-helix (LbH) subdomain, which were referred to as LbH-MAT-GAT sugar O-acetyltransferases. However, the biochemical assay identified that its target site was the hydroxyl group of the pyrrolidine ring. AniI was found to be tolerant of acyl donors with different chain lengths for the biosynthesis of compound 1 and derivatives 12 and 13 with butyryl and isovaleryl groups, respectively. Meanwhile, it showed comparable activity toward biosynthetic intermediates and synthesized analogues, suggesting promiscuity to the pyrrolidine ring structure of compound 1. These data may inspire new viable synthetic routes for the construction of more complex pyrrolidine ring scaffolds in compound 1. Finally, the overexpression of *anil* under the control of strong promoters contributed to the higher productivities of compound 1 and its analogues. These findings reported here not only improve the understanding of anisomycin biosynthesis but also expand the substrate scope of O-acetyltransferase working on the pyrrolidine ring and pave the way for future metabolic engineering construction of high-yield strains.

I , A CE Acylation is an important tailoring reaction during natural product biosynthesis. Acylation could increase the structural diversity and affect the chemical stability, volatility, biological activity, and even the cellular localization of specialized compounds. Many acetyltransferases have been reported in natural product biosynthesis. The typical example of the LbH-MAT-GAT sugar O-acetyltransferase subfamily was reported to catalyze the coenzyme A (CoA)-dependent acetylation of the 6-hydroxyl group of sugars.

atural products (NPs) are a powerful source of chemical defenders to survive in the ever-changing environments and ecosystems (1). The whole NP pool is comprised of countless natural compounds falling into different classes including polyketides (PKs) (2), nonribosomal peptides (NRPs) (3), ribosomally synthesized and posttranslationally modified peptides (RiPPs) (4), saccharides, terpenoids, isoprenoids, alkaloids, aminoglycosides, nucleosides, etc. Structural diversity and complexity of NPs not only contribute to the various biological or ecological activities but also benefit the motivations to develop medicinally important new drugs (5). The immense structural characteristics are introduced during the biosynthetic assembly (6). Typically, for most of the NPs, the biosynthetic assembly line mainly involves the construction of the basic scaffold and various postassembly modifications on the nascent scaffold. The modifications play important roles in rigidifying the molecule and fixing the three-dimensional conformation, introducing polar groups to increase the water solubility, revealing structural motifs and reactive sites indispensable for target inhibition, and appending oligosaccharides that bind to specific DNA sequences (6). Based on the tailoring enzyme chemistries, the modification reactions can be grouped into two broad categories: oxidative transformations and group transfer reactions (7). The group transfer reactions, like methylation, acylation, halogenation, and glycosylation, play important roles in diversifying the molecu(4(ac)22..o)22.D5li(l)16u.3(e.3(.4(an)22.8(d)]TJf.511-1.3.68.05517TD()77

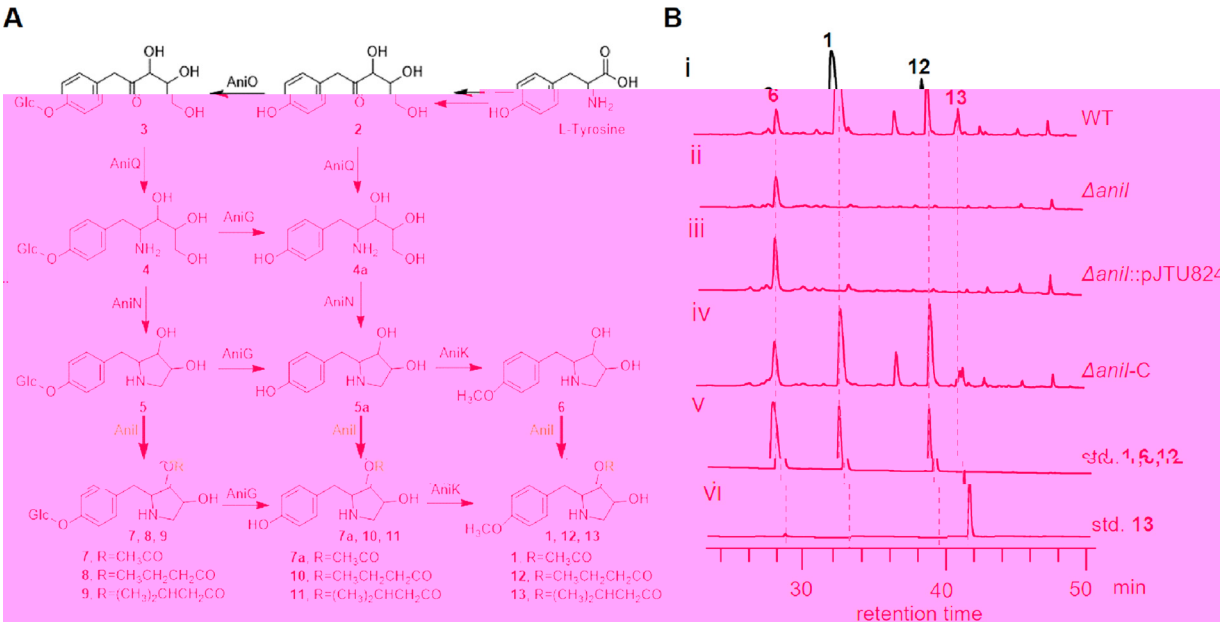

FIG 1 The biosynthetic pathway of compound 1 and complementation of *anil*. (A) The biosynthetic pathway of compound 1. The reactions catalyzed by Anil are highlighted in red. (B) The HPLC analysis of the fermentation products in genetic-interruption Δ*anil* strain (ii), complementary strain Δ*anil*-C (iv), the wild-type (WT) strain (i), and the control Δ*anil*-pJTU824 strain (iii). Standards 1, 6, and 12 (v) and standard 13 (vi) were used as controls.

deglycosylation must precede O-methylation on the phenol moiety. This likely accounts for the observation that the compound 1 producer strain is very "clean" (as no intermediates were accumulated in the wild-type [WT] strain), as the flexible enzymes can redirect shunt intermediates back into the pathway to compound 1. Actually, the tolerance of AniK and AniG has been demonstrated before (15). But, in the case of Anil, the possible flexibility and tolerance of different substrates still await discovery. Meanwhile, whether Anil could catalyze the transformation to compounds 12 and 13 is unknown (Fig. 1A).

The function of *anil* was characterized in this study. Anil exhibited tolerance of acyl group donors and acceptors, which might set the stage for efficient production of compounds 1, 12, and 13. Meanwhile, the activities of Anil toward synthesized compound 1 analogues provide a potential approach to construction of diverse pyrrolidine ring scaffolds in compound 1. Actually, the overexpression of *anil* contributed to the productivity improvement of compounds 1, 12, and 13. These findings reported here might supply good material for metabolic engineering of high-yield strains.

E L

A l d d 1, 12, d 13 . The previous genetic deletion of *anil* has completely abolished the production of compound 1 and resulted in the accumulation of compound 6 (Fig. 1B). Meanwhile, no accumulation of compounds 12 and 13 was detected (Fig. 1B). To exclude other possible explanations of the phenotype of the Δ*anil* mutant, a single copy of *anil* on the integrative plasmid pJTU824 was transferred into the Δ*anil* strain to construct the complementary strain Δ*anil*-C (see Fig. S1 in the supplemental material). The fermentation products of Δ*anil*-C were analyzed by liquid chromatography-mass spectrometry (LC-MS), and the production of compounds 1, 12, and 13 was restored (Fig. 1B), while the introduction of pJTU824 exhibited no effect on the biosynthesis of compound 6 (Fig. 1B). These data confirmed the indispensability of *anil* for biosynthesis of compound 1 and its derivatives (compounds 12 and 13) (Fig. 1B).

The *ani* gene cluster encoded an acetyltransferase with 222 amino acids (Anil, GenBank accession no. ARE72418.1), which belongs to the LbH-MAT-GAT sugar O-acetyltransferase

(thiogalactoside transacetylase) subfamily (based on the NCBI BLASTP analysis). Maltose O-acetyltransferase (MAT) (like LacA [22] in *Escherichia coli*) acetylates maltose and glucose exclusively at the C-6 position of the nonreducing end glucosyl moiety. Galactoside O-acetyltransferase (GAT) specifically acetylates galactopyranosides. Active MAT and GAT are homotrimers, with each subunit consisting of an N-terminal alpha-helical region and a C-terminal left-handed parallel alpha-helix (LbH) subdomain with six turns, each containing three imperfect tandem repeats of a hexapeptide repeat motif ([LIV]-[GAED]-X-X-[STAV]-X) (23, 24). These repeating sequence motifs were termed "isoleucine patch" or "hexapeptide repeat" and have since been found as an easily identifiable and characteristic feature of this expanding family of acyltransferases (24). AniI showed 40% sequence identity with *E. coli* maltose acetyltransferase (PDB: 1OCX) (23) and 36% with maltose O-acetyltransferase (PDB: 3HJJ) from *Bacillus anthracis* and 37% with galactoside O-acetyltransferase (PDB: 3FTT) from *Staphylococcus aureus* (25). Moreover, a similar "hexapeptide repeat" can be found in AniI (Fig. 2). However, AniI exhibited no sequence identity with acetyltransferases reported in natural product biosynthesis like LovD (26), BmmI (27), 1,13-dihydroxy-*N*-methylcandine 13-O-acetyltransferase AT1 (28), and chloramphenicol acetyltransferase (CAT) (29). No member of the LbH-MAT-GAT sugar O-acetyltransferases has been found involved in natural product biosynthesis, and the lack of sequence identity with those acetyltransferases responsible for natural product biosynthesis suggested the different reaction site of AniI.

A I d d . To verify that AniI was indeed responsible for the acylation modification reaction, we first cloned the intact *anil* gene into the pET28a plasmid for the construction of overexpressing plasmid pET28a-*anil* (Table 1) by one-step cloning using primers listed in Table 2. Then, the recombinant plasmid was transferred into *E. coli* BL21(DE3) for overexpression. The recombinant AniI was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography to near-homogeneity, and the purity and size (27.4 kDa) of resultant protein were determined by SDS-PAGE (Fig. S2).

For the biochemical demonstration on the acylation activity of AniI, we first conducted the *in vitro*

ABLE 2 Primers used in this study

Primer	Sequence (5′–3′)	Use(s)
824-I-fw	GTTGGTAGGATCCACATATGATGAAACCGACGCCCCCCGA (NdeI site)	Complementation of Δ*anil*
824-I-rv	TATGACATGATTACGAATTCTCACAGCTCCCGGACGATGC (EcoRI site)	
thioF	ATGCGGGGATCGACCGCGCG	Validation of Δ*anil*-C
thioR	TCATCAGCTGCATACCGCTG	
I-A	AGCAAATGGGTCGCGGATCCATGAAACCGACGCCCCCCGA (BamHI site)	Overexpression of His-tagged AniI protein in *E. coli*
I-S	TGTCGACGGAGCTCGAATTCTCACAGCTCCCGGACGATGC (EcoRI site)	
pSET152-pkasOp-F	GGGCTGCAGGTCGACTCTAGATGTTCACATTCGAACGGTCTCTG (XbaI site)	Overexpression of *anil* in WT strain
pSET152-pkasOp-R	CGCGGCCGCGGATCCTCTAGACTCCCCCAGTCCTGCACG (XbaI site)	
pSET152-AniI-F	TGGGGGAGTCTAGAGGATCCATGAAACCGACGCCCCCCGA (BamHI site)	Overexpression of *anil* in WT strain
pSET152-AniI-R	TCGATATCGCGCGCGGCCGCCATATGTCACAGCTCCCGGACGATGC (NotI site)	
pIB139-AniI-F	GTTGGTAGGATCCACATATGATGAAACCGACGCCCCCCGA (NdeI site)	Overexpression of *anil* in WT strain
pIB139-AniI-R	GCGGCCGCGGATCCTCTAGATCACAGCTCCCGGACGATGC (XbaI site)	
16S-RT-F	CCGCAAGGCTAAAACTCAAA	Detection of the transcriptional level of 16S rRNA
16S-RT-R	AACCCAACATCTCACGACAC	
AniI-RT-F	CCCCGCTTCCTGTGCGAGTT	Detection of the transcriptional level of *anil*
AniI-RT-R	GGTGGTTGGCGGTGAAGAGC	

that of compound 6, the value of which was triple that for compound 5a (Table 3 and Fig. S5). Based on the comparison, compound 5 seemed to be the preferred substrate of AniI when the acetyl group was used as acyl donor.

A I d d d 12 d 13 d . From the results mentioned above, AniI could catalyze the transfer of the acetyl group to the hydroxyl group in the pyrrolidine ring of compound 1. Considering that the presence of the other two derivatives (compounds 12 and 13) accumulated in the WT strain, the butyryl group and isovaleryl group were proposed to be transferred onto the same hydroxyl group of compound 6 for compound 12 and 13 production, respectively. To verify the proposal, butyryl-CoA and isovaleryl-CoA were used as acyl group donors, respectively, in the *in vitro* reaction system of compound 6. As can be seen from Fig. 4A, the butyryl group and isovaleryl group could actually be recognized by AniI, and compounds 12 and 13 were indeed produced based on the MS/MS analysis (Fig. S3).

The broad substrate specificity toward the acyl donors and the acyl receptors was common for acyltransferase (26, 27). To further characterize the substrate tolerance of AniI, compounds 5 and 5a were used as the acceptors again for the butyryl group and isovaleryl group donated by butyryl-CoA and isovaleryl-CoA, respectively. The results depicted in Fig. 4B showed that AniI could catalyze the total transformation of compound 5 into the butyrylated and isovalerylated products 8 and 9, respectively. But only partial compound 5a was transformed into butyrylated and isovalerylated products 10 and 11, respectively. The MS and MS/MS value analysis of compounds 8, 9, 10, and 11 confirmed the successful loading of the butyryl and isovaleryl groups (Fig. S3 and S4). The results mentioned above showed that compound 5 was the preferred acyl acceptor. To compare the preferences on the acyl donors, compound 5 was selected and incubated with three kinds of acyl donors (acetyl-CoA, isovaleryl-CoA, and butyryl-CoA), respectively. The reaction was quenched at 10 min with the addition of an equal volume of chloroform. As shown in Table 3, the K_m values of AniI for three kinds of acyl group donors were similar, and the k_{cat}/K_m values for acetyl-CoA (0.24 μM^{-1} min^{-1}) and butyryl-CoA (0.28 μM^{-1} min^{-1}) were similar, too. However, the k_{cat}/K_m for isovaleryl-CoA (μM^{-1} min^{-1}) was half of them (Table 3 and Fig. S5). Taken together, all these results demonstrated the promiscuity of AniI for different donors and acceptors of different acyl groups.

. It has been reported that the isotopically labeled compound 1 binds yeast and human tonsil ribosomes (30). Especially, the acetyl group at the C-3 position of the pyrrolidine ring in compound 1 was important for the ribosome interaction, since compound 6 showed 350-times-lower affin-

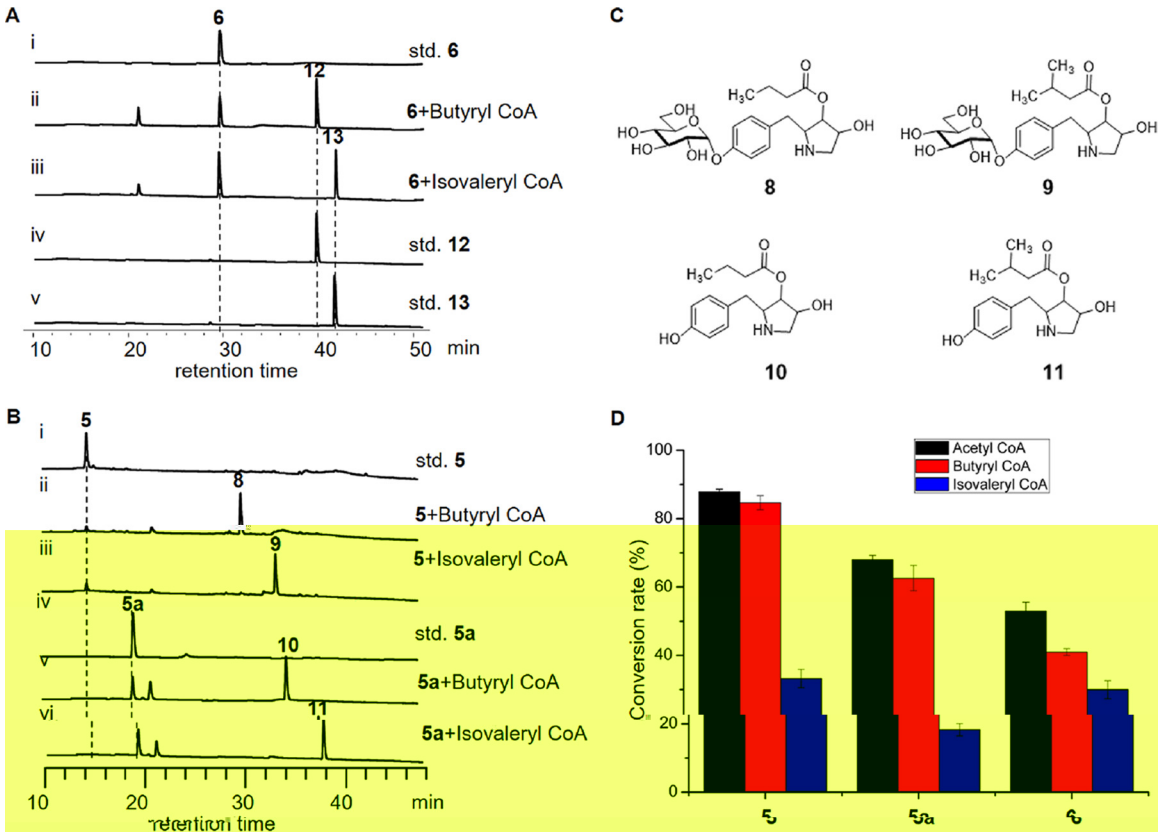

FIG 4 The characterization of substrate tolerance of AniI on acyl donors and acceptors. (A) HPLC profile of reaction products catalyzed by AniI with compound 6 with butyryl-CoA (ii) and isovaleryl-CoA (iii) group. Standards 12 and 13 were used as control. (B) Reactions of compounds 5 and 5a with butyryl-CoA (ii and v) and isovaleryl-CoA (iii and vi). Standards 5 (i) and 5a (iv) were used as control. (C) Molecular structures of compounds 8, 9, 10, and 11. (D) Comparative acylation activities analysis of compound 5, 5a, and 6 reaction with acetyl-CoA, butyryl-CoA, and isovaleryl-CoA. Typical 100-μl reaction systems of 20 μM AniI, 500 μM compounds (5, 5a, and 6, respectively), and 1 mM acyl donors (acetyl-CoA, butyryl-CoA, and isovaleryl-CoA, respectively) in PBS buffer were incubated at 30°C for 30 min.

role in the maturation of desired compounds. Engineering of the modification bottlenecks would lead to an efficient process to the desired metabolites, and the overexpression of tailoring genes (such as genes for hydroxylase [11], O-methyltransferase [31], and halogenase [13]) has been reported to produce remarkable improvement in purity and productivity of desired products. According to the metabolic profile of the WT strain, compound 6 could not be transformed into the active acylated products *in vivo* completely (Fig. 1B). To promote the active acylated product transformation, the gene *anil* was cloned into the integrative vector pIB139 under the control of *p*ermE* (Fig. 6A) and introduced into the WT strain through conjugation. Quantitative real-time reverse transcription-PCR (RT-qPCR) confirmed that *anil* was overexpressed in the recombinant strains (WT::pIB139-*anil*), and its transcription reached the highest level of 14.5-fold over that of the WT strain at 48 h (Fig. 6B). During the monitoring process, compound 1 was found to undergo deacetylation spontaneously in water (Fig. S6). That means that during the fermentation and detection process, the spontaneous inevitably occurs. So, the total production levels of compound 1 and its analogues (compounds 6, 12, and 13) were compared by high-pressure liquid chromatography (HPLC) analysis (Fig. 6C). As can be seen from Fig. 6D, the production of those four compounds was decreased slightly in the WT::pIB139 strain, suggesting a negative effect of the pIB139 integration. Fortunately, deducting the negative effect of empty plasmid, the productivity increased obviously and was 2.69 times that in the WT::pIB139-*anil* strain (Fig. 6D). Considering that the efficiency of promoter might be a limiting factor, we also tried another potent

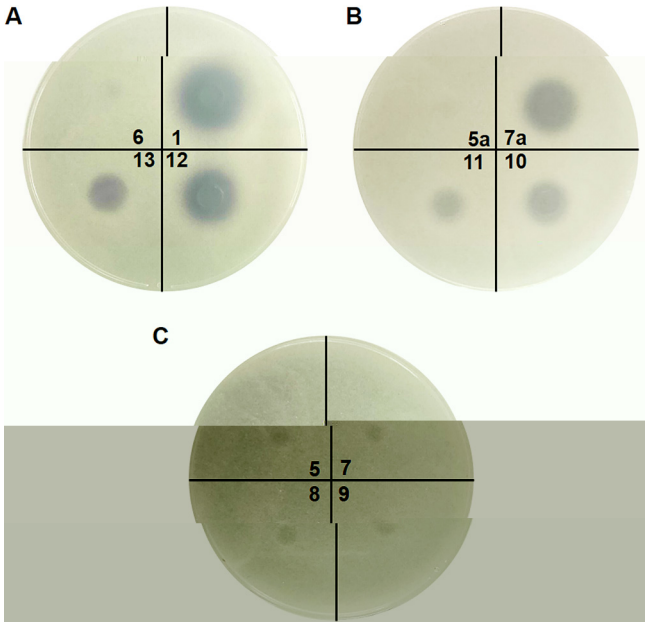

FIG 5 Bioactivity assay of compounds 6, 1, 12, and 13 (A); 5a, 7a, 10, and 11 (B); and 5, 7, 8, and 9 (C) used in this study. All the tested compounds were dissolved in water with the final concentration of 1 mM, 30 μl of which was used for assay.

promoter, *p*kasO*, and constructed the plasmid pOST, by integrating *p*kasO* into pSET152 (Fig. 6A). Then, a copy of intact *anil* was introduced into this plasmid, and the resultant pOST-*anil* was then transferred into the WT strain. Similarly, the transcription of *anil* was also increased in the resultant overexpressing strain, about 11-fold that observed in the WT strain (Fig. 6B). Meanwhile, plasmid pOST exerted a negative effect on compound accumulation and the production was increased in the WT::pOST-*anil* strain (about 2.03-fold that in the WT::pOST strain) (Fig. 6D). These data showed that the overexpression of *anil* was an efficient way to improve the fermentation titers of anisomycin and its analogues. So, more efficient promoters should be tried for further productivity improvement in future.

DI C I

The acylation of oxygen-containing substrates is one of the most common modification types of secondary metabolites. Acyl-sugars, acylated acyl carrier proteins, or acyl-activated coenzyme A thioesters can serve as activated acyl donors. Acetylation could increase the structural diversity and affect the chemical stability, volatility, biological activity, and even the cellular localization of specialized compounds (32). During the biosynthesis of NPs, the loaded acetyl group could serve as a stabilizing group that prevents ring opening and facilitates subsequent reactions (33), as the protective group makes the biosynthesis a unique and highly ordered process (28) and mediates the transport of biosynthetic intermediates (34). Recently, acetylation has also been reported for the condensation of the polyketide and ribosomally synthesized and posttranslationally modified peptides RiPP moieties (PK/RiPPs) involved in goadvionin biosynthesis (35).

Little is known about the acylation of anisomycin. In this study, the complementation of an intact *anil* gene into the Δ*anil* mutant successfully restored the production of compounds 1, 12, and 13, suggesting the indispensability of this gene (Fig. 1B). To verify the acyl group transferring activity, compound 6 and acetyl-CoA were first used as the substrates for AniI, and compound 6 could be transformed to compound 1 (Fig. 3). These data confirmed that AniI acted on the pyrrolidine ring, which was different from the typical reported sites on sugar targets of the LbH-MAT-GAT sugar O-acetyltransferase

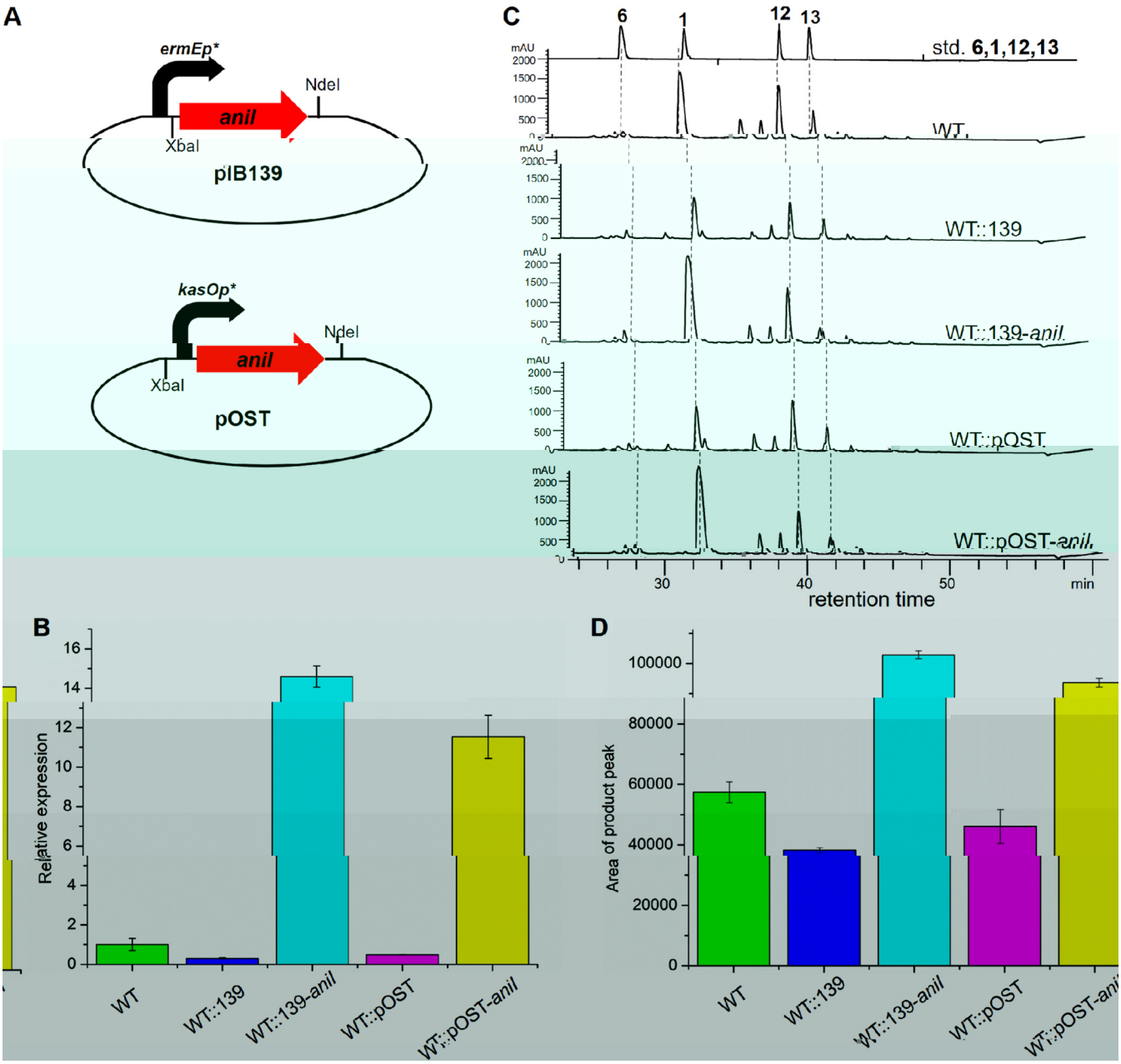

FIG 6 The construction and characterization of *anil*-overexpressing strains. (A) The depiction of construction of *anil* overexpression plasmids using pIB139 and pOST. (B) The RT-qPCR analysis of the transcription of *anil* in the WT::pIB139-*anil* and WT::pOST-*anil* strains. (C) The HPLC analysis of the products accumulated in different strains. The standard compounds 1, 6, 12, and 13 were used as controls. (D) The productivity analysis of compound 1 and its analogues in the overexpressing strains.

subfamily (Fig. 2). Considering the complex biosynthetic network of compound 1 (Fig. 1A), the biosynthetic intermediates 2/3, 4/4a, and 5/5a were also tested as the substrates for Anil. Only compounds 5 and 5a could be acetylated (Fig. 3), suggesting the necessity of the pyrrolidine ring. The catalytic efficiency (k_{cat}/K_m) of compound 5 was about 4.5-fold that of compound 5a (Table 3). To test the promiscuity of Anil on the structure of the pyrrolidine ring, analogues with different modifications of the pyrrolidine ring were prepared. The -OH group at C-4 of the pyrrolidine ring was modified with a methyl and benzyl group in analogues 15 and 17, respectively (see Fig. S7 and S8 in the supplemental material). Then, compounds 15 and 17 were separately used as the substrate for Anil, and the acetyl group was successfully loaded onto the C-3-OH (Fig. S9), which confirmed the promiscuity of Anil on the structure of the pyrrolidine ring. These data suggested that

AniI might be developed by protein engineering for biocatalytic synthesis of pyrrolidine rings with diverse modifications.

Moreover, AniI showed promiscuity of other acyl donors, such as the butyryl-CoA group and isovaleryl-CoA, and even the acetylated biosynthetic intermediates like compound 7 (Fig. S10). When the butyryl-CoA group and isovaleryl-CoA were used as acyl donor for compound 6, AniI could catalyze the transformation of compounds 6 to 12 and 13, respectively (Fig. 4A). The conversion rate suggested the preference of AniI on acyl donors, and the preference could be ordered as acetyl-CoA $>$ butyryl-CoA $>$ isovaleryl-CoA. When biosynthetic intermediates 5 and 5a were used as acceptors for butyryl and isovaleryl groups, respectively, the conversion rates of compound 5 to acylated products 8 and 9 were obviously higher than that of compounds 5a to 10 and 11 (Fig. 4D). Consistently, using compound 5 as the acceptor, AniI exhibited similar reaction efficiencies toward acetyl-CoA (k_{cat}/K_m of 0.24 $\mu M^{-1} min^{-1}$) and butyryl-CoA (k_{cat}/K_m of 0.28 $\mu M^{-1} min^{-1}$) and showed relatively low activity on isovaleryl-CoA (k_{cat}/K_m of 0.1 $\mu M^{-1} min^{-1}$). Moreover, acetylated compound 7 was found to act as acetyl donor and was transformed into deacetylated compound 5, resulting in the formation of acetyl-CoA (Fig. S10A). So, AniI catalyzed the reversible interconversion of acetylated compound 7 and acetyl-CoA (Fig. S10B). Taken together, AniI exhibited promiscuity of acyl donors and acceptors but showed preference simultaneously. However, more acyl donors should be tested in future for biocatalyst development.

Additionally, the acylation of compounds 5a and 6 turned out to be essential for the antifungal activity. Interestingly, the loading of the acetyl group gave the corre-

plasmid pSET152 for the construction of pOST, using the primers pSET152-pkasOp-F/R (Table 2). Subsequently, fragments of *anil* with flanking restriction sites of BamHI/NotI were amplified using corresponding primers (Table 2) and successively cloned into pOST-*kasOp** to generate pOST-*anil*. Similarly, fragments of *anil* with restriction sites of NdeI/NotI were amplified using corresponding primers (Table 2) and then inserted into pIB139 to generate pIB139-*anil*. The recombinant plasmids were introduced into the Δ*anil* strain or *S. hygrospinosus* subsp. *beijingensis* via intergeneric conjugation. The double-crossover strains were obtained through antibiotic selection and confirmed by PCR verification.

B **.** The bioinformatics analysis of AniI was conducted with the online software NCBI BLASTP (https://blast.ncbi.nlm.nih.gov/). Multiple sequences were aligned using ClustalW (41). Proteins used for sequence alignment are as follows: maltose O-acetyltransferase (PDB: 1OCX) from *E. coli*, maltose O-acetyltransferase (PDB: 3HJJ) from *Bacillus anthracis*, galactoside O-acetyltransferase (PDB: 3FTT) from *Staphylococcus aureus*, hexapeptide-repeat-containing acetyltransferase VCA0836 (PDB: 3NZ2) from *Vibrio cholerae* O1 biovar eltor, and hexapeptide-repeat-containing acetyltransferase (PDB: 3ECT) from *Vibrio cholerae*.

pretreatment at 50°C (2 min) and initial denaturation at 95°C (30 s) followed by 40 cycles of 95°C (30 s) and 60°C (30 s). The primers AniI-RT-F/R (listed in Table 2) were used for *aniI* analysis. The *hrdB* gene encoding the major sigma factor in *Streptomyces* was used as the internal control.

d **15** **d 17.** For the preparation of compound 15, a 500-μl reaction system composed of 5 mg compound 1, 2.11 mg *t*-BuOK, and 2.68 mg MeI was incubated at room temperature overnight, following the reported procedure (42). The reaction mixture was concentrated to dryness and then dissolved in methanol. The purification of compound 14 (the C-4-OH was methylated) was conducted with a semipreparative Agilent Zorbax SB-C_{18} column (5 μm, 9.4 by 250 mm). The column was equilibrated with 80% (vol/vol) solvent A (H_2O with 0.1% [vol/vol] trifluoroacetic acid) and 20% (vol/vol) solvent B (acetonitrile), developed with the isocratic elution for 25 min, and then kept at 100% B for 10 min at a flow rate of 1.5 ml/min and UV detection at 223 nm. The fraction with molecular weight 280.2 (*m/z*, $[M+H]^+$) was determined by LC-MS analysis with an Agilent 1100 series LC/MSD trap system (drying gas flow, 10 ml/min; nebulizer, 30 lb/in^2

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