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High-Yield Natural Vanillin Production by *Amycolatopsis* sp. after CRISPR-Cas12a-Mediated Gene Deletion

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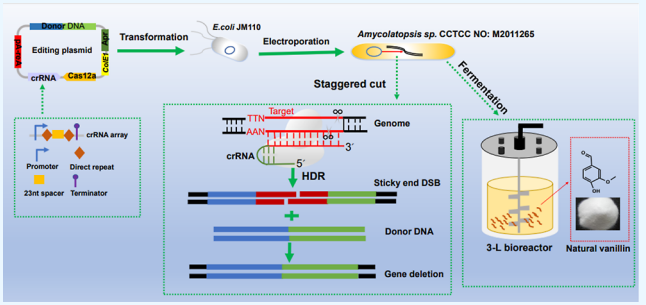
ABSTRACT: Vanillin is an aromatic compound, which is widely used in food flavoring, beverages, perfumes, and pharmaceuticals. *Amycolatopsis* sp. is considered a good strain for the production of vanillin from ferulic acid by fermentation; however, its high genomic guanine−cytosine (GC) content (>70%) and low transformation and recombination efficiency limit its genetic modification potential to improve vanillin production. Efficient genome editing of *Amycolatopsis* sp. has been challenging, but this study developed a CRISPR-Cas12a system for efficient, markerless, and scarless genome editing of *Amycolatopsis* sp. CCTCC NO: M2011265. A mutant, Δ*vdh*Δ*phdB*, was obtained by the deletion of two genes coding byproduct enzymes from the vanillin biosynthetic pathway. The gene deletion increased vanillin production from 10.60 g/L (wild-type) to 20.44 g/L and reduced byproduct vanillic acid from 2.45 to 0.15 g/L in a 3 L fed-batch fermentation, markedly enhancing vanillin production and reducing byproduct formation; the mutant has great potential for industrial application.



1. INTRODUCTION

Vanillin is an aromatic aldehyde and flavor compound, which is widely used in food, beverages, perfumes, and pharmaceuticals. The extraction of natural vanillin from cured seed pods of the orchid *Vanilla planifolia* is costly and labor-intensive and cannot meet the market demand for natural vanillin. Microbially produced vanillin is regarded as "nature-identical", is well-accepted by consumers, and is relatively inexpensive.[1] Microorganisms such as *Pseudomonas putida*, *Pseudomonas fluorescens*, *Escherichia coli*, *Pycnoporus cinnabarinus*, *Amycolatopsis* sp., *Streptomyces* sp., *Pediococcus acidilactici*, and *Bacillus subtilis* have been metabolically engineered to biotransform ferulic acid to produce vanillin.[2] Of these, *Amycolatopsis* sp. ATCC 39116 and *Streptomyces* sp. strain V-1 are used in the fermentative production of natural vanillin from ferulic acid, at yields above 10 g/L, and are possible for industrial-scale production.[3,4] Chemically synthesized vanillin is much cheaper (less than US $15 per kg) than natural or microbial vanillin, but its use for food purposes is not permitted by UK and EU legislation. The current vanillin yields from microbial fermentation are insufficient for economically viable industrial production, so increasing fermentation yields remains a challenge.

Amycolatopsis spp. are Gram-positive, non-sporulating, and aerobic actinomycetes that generate a variety of secondary metabolites.[5] Some members of this genus are also well known for the production of the medically important antibiotics, rifamycin[6] and vancomycin.[7] Although genetic manipulation tools have been developed for *Amycolatopsis* spp., including a site-specific recombination system, shuttle vector, suicide vectors, and reporter genes,[8−10] compared with that of other actinobacterial genera such as *Streptomyces*,[11,12] efficient genome editing of *Amycolatopsis* spp. remains challenging. The high guanine−cytosine (GC) content of the DNA (>70%), the relatively low efficiency of both DNA transformation and homologous recombination, and the limited range of antibiotics effective against *Amycolatopsis* spp. make it difficult to perform genetic/metabolic engineering. The conventional non-CRISPR gene-editing method for *Amycolatopsis* spp. is through a two-step homologous recombination procedure involving a suicide plasmid.[3,11] There is a low probability of double cross-over when performing the second recombination step, resulting in a requirement for extensive and time-consuming screening of transformants. The *rpsL* gene has been used as a counter-selectable marker to select for double-cross-over recombinants and increase the probability of the second recombination stage,[11] greatly improving the efficiency of the second homologous replacement in the presence of streptomycin. Nevertheless, the entire process of two-step homologous recombination, including gene editing and plasmid curing, is still very time-consuming. Use of the pSAM2 site-specific recombination system to perform anti-

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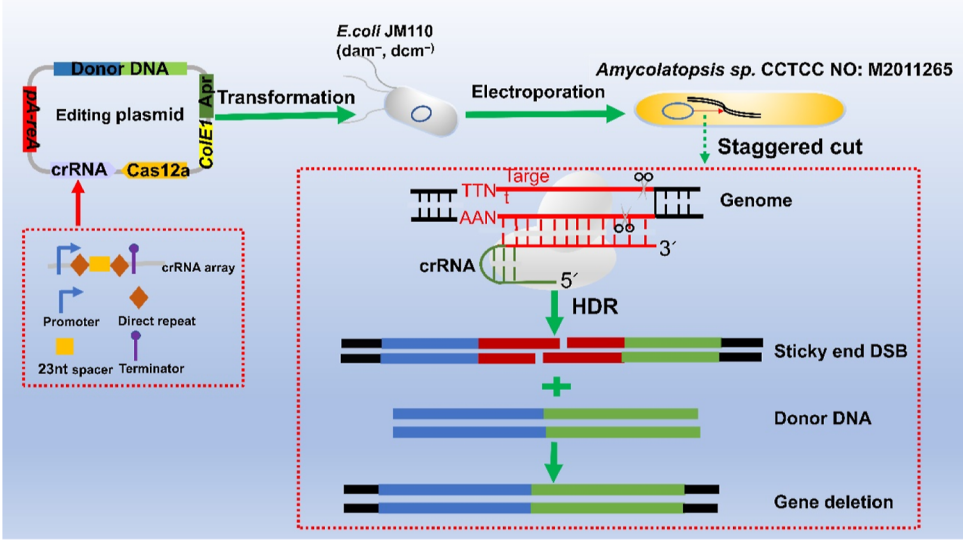

Figure 1. Schematic of CRISPR-Cas12a-assisted genome editing in *Amycolatopsis* sp. CCTCC NO: M2011265.

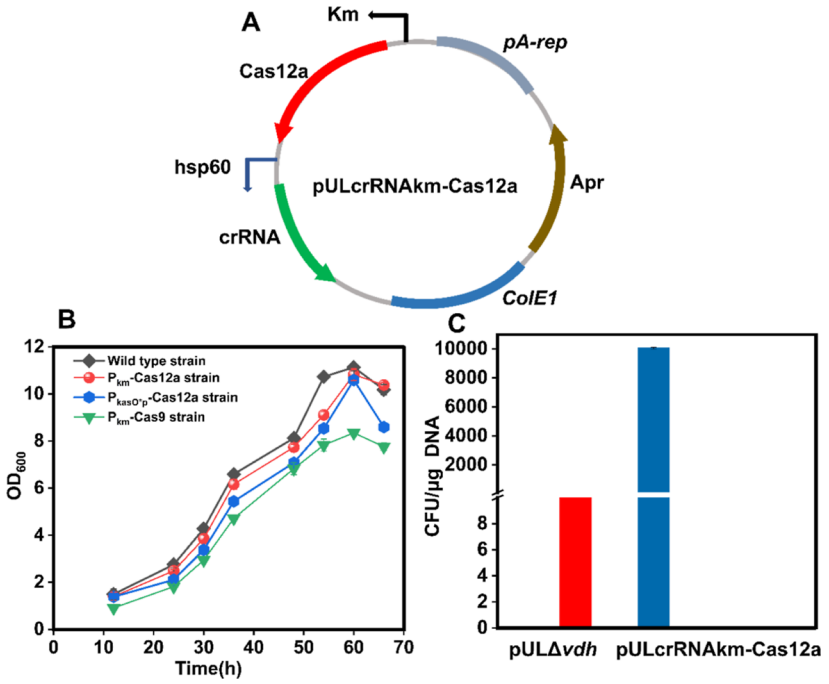

Figure 2. The CRISPR-Cas12a system is functional in *Amycolatopsis sp*. CCTCC NO: M2011265. (A) Structure of the pULcrRNAkm-Cas12a plasmid. Cas12a: Cas12a from *Francisella novicida*, driven by the constitutive promotor P_{km}; crRNA: the crRNA expression cassette driven by the constitutive promotor P_{hsp60}; *pA-rep*: the replicon used in CCTCC NO: M2011265; *ColE*1: the replicon used in *E. coli*; and Apr: apramycin resistance gene. (B) Effects of Cas gene expression on the growth of CCTCC NO: M2011265 determined by OD_{600} measurement. Error bars (mean ± SD) were derived from triplicate samples. (C) Number of transformant colonies obtained with plasmid pULΔ*vdh* (*vdh* guide sequence inserted in pULcrRNAkm-Cas12a) and control plasmid pULcrRNA-km-Cas12a (no *vdh* guide sequence). The data were obtained from three independent transformation experiments.

biotic resistance markerless gene deletion and large-fragment DNA knockout in *Amycolatopsis* spp.[12] was successful, but a "scar", a short (33 bp) section of residual DNA, remained on the genome, and this would impede the introduction of new genes into other targeted sites.

The recent development of the CRISPR genome editing system was a major advance in genetic manipulation.[13,14] The CRISPR system is more efficient and faster than conventional homologous recombination editing because it can disrupt multiple genes simultaneously, greatly reducing the workload of gene editing.[15,16] The CRISPR/Cas9 system has been developed as a powerful genomic editing tool that has been used successfully in various species;[17−20] however, attempts to use it for genomic editing of *Amycolatopsis* spp. and some *Streptomyces* strains failed because of the high cytotoxicity of Cas9.[21,22] CRISPR-Cas12a is another CRISPR-Cas system with great potential for genome editing, which, unlike Cas9, can achieve pre-crRNA maturation[23] and then target T-rich protospacer adjacent motifs (PAMs) under the guidance of single-stranded mature crRNAs to complete the cleavage of the

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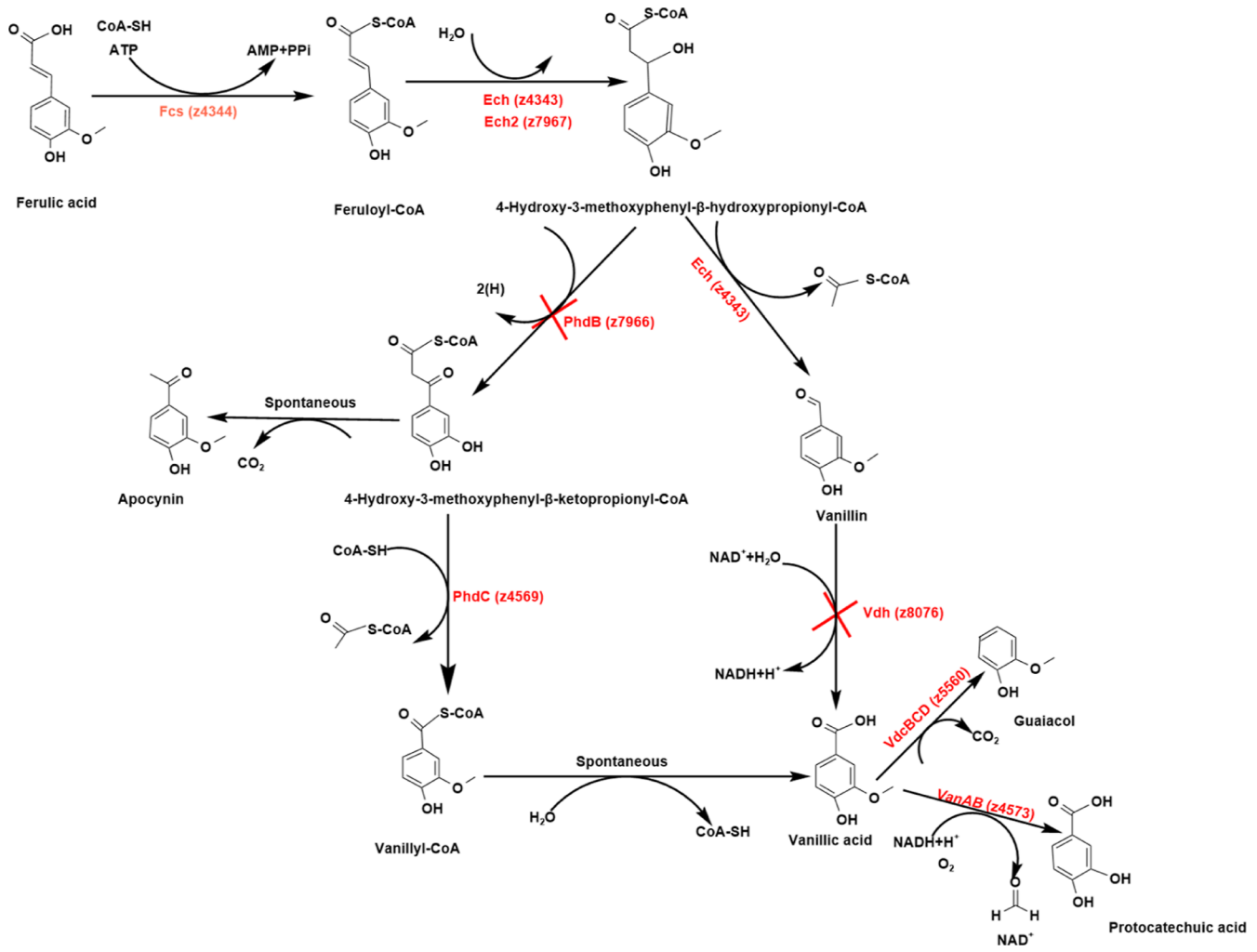

Figure 3. Schematic overview of ferulic acid metabolism in *Amycolatopsis* sp. CCTCC NO: M2011265. Fcs = feruloyl-CoA synthetase (z4344), Ech = enoyl-CoA hydratase/aldolase (z4343), Ech2 = putative enoyl-CoA hydratase (z7967), PhdB = HMPHP-CoA dehydrogenase (z7966), PhdC = HMPKP-CoA dehydratase (z4569), Vdh = vanillin dehydrogenase (z8076), VdcBCD = vanillate decarboxylase (z5560), and VanAB = vanillate demethylase (z4573).

target DNA.[24] Recently, Cas12a has been shown to be useful for efficient genome editing in *Amycolatopsis* spp.[22,25]

In this study, we successfully developed an all-in-one CRISPR-Cas12a system for efficient and scarless genome editing in *Amycolatopsis* sp. CCTCC NO: M2011265 (Figure 1). This system was used to produce a robust and efficient mutant *Amycolatopsis* sp. Δ*vdh*Δ*phdB*; production of natural vanillin and the fermentation characteristics of the mutant were compared with those of the wild-type strain. This study provides an essential reference for the development of CRISPR-Cas12a genome engineering tools for other industrial microorganisms.

2. RESULTS AND DISCUSSION

2.1. Establishment of a CRISPR-Cas12a-Mediated Genome Editing System for *Amycolatopsis* sp. *Amycolatopsis* sp. CCTCC NO: M2011265 can biotransform ferulic acid to vanillin.[26] Gene knockout and genomic integration have only been applied to one other vanillin-producing strain *Amycolatopsis* sp. ATCC 39116 because of the limited tools available for genetic manipulation.[3,11,27] This study focused on developing a CRISPR-Cas12a-mediated genome editing tool for *Amycolatopsis* sp. CCTCC NO: M2011265. An all-in-one CRISPR-Cas12a editing plasmid, pULcrRNAkm-Cas12a, was constructed, in which the expression of the Cas12a gene and target-specific crRNA was regulated by both constitutive promoters km and hsp60, respectively (Figure 2A). In this plasmid, replicon *pA-rep* was used for plasmid replication in CCTCC NO: M2011265 because it is the only one available for use in *Amycolatopsis* spp., being the endogenous plasmid pA387 in source *Amycolatopsis* sp. DSM 43387.[28] The apramycin resistance gene allows the selection of the plasmid in both species, and the *ColE1* replicon is used for replication in *E. coli*. The spacer sequences were integrated into the plasmid by reverse polymerase chain reaction (PCR) with designed primer pairs, and the *EcoRI* site was used for one-step cloning of repair arms via Gibson Assembly. Considering that a unique restriction modification system exists in *Actinomycetes*, transformation rates were reduced by 1000-fold when plasmid DNA was modified by *dam* or *TaqI* methylase.[29] This restriction modification system is also present in CCTCC NO: M2011265; electroporation of plasmids extracted from *E. coli* JM109 and *E. coli* JM110 (dam^-, dcm^-) into its cells resulted in no transformants from *E. coli* JM109 plasmids but many transformants from *E. coli* JM110 plasmids (dam^-, dcm^-) (Figure S1).

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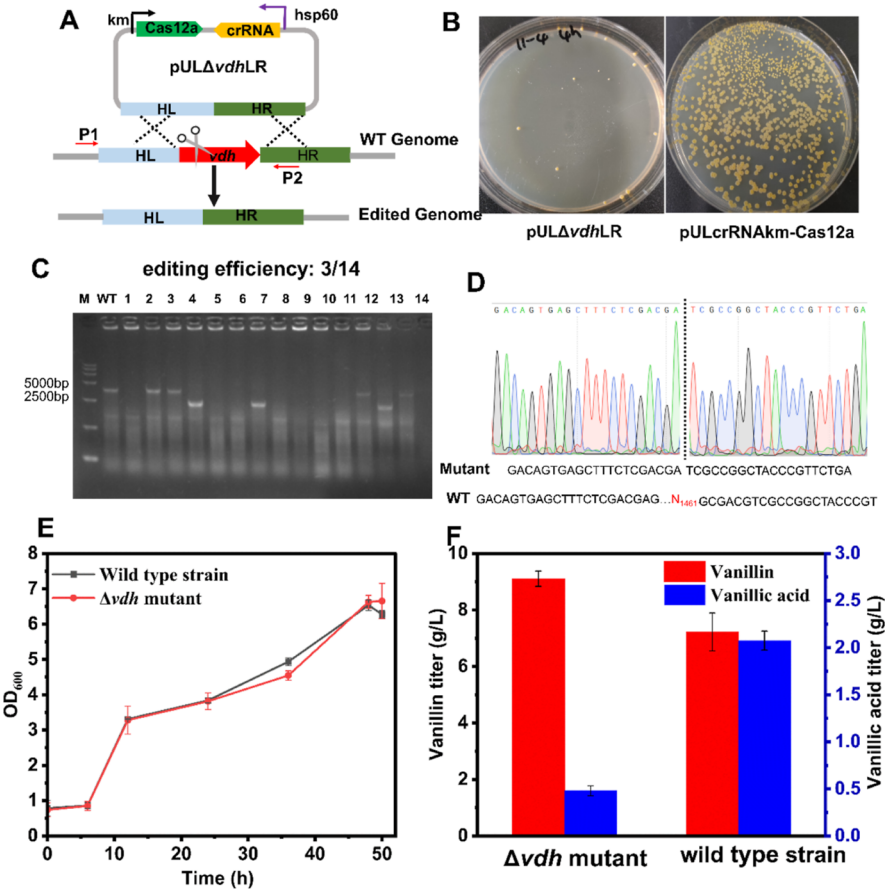

Figure 4. Markerless deletion of the *vdh* gene with CRISPR-Cas12a-assisted DNA cleavage and HDR-mediated recombination in *Amycolatopsis* sp. CCTCC NO: M2011265. (A) Schematic diagram illustrating the workflow of *vdh* deletion using the CRISPR-Cas12a system in CCTCC NO: M2011265. The editing template repaired the DSBs cut by Cas12a via HDR. P_{km} and P_{hsp60}: the promoters for expression of crRNA and Cas12a, respectively. (B) The transformants with pULΔ*vdh*LR are on the left and those with the pULcrRNAkm-Cas12a control plasmid (no *vdh* guide sequence) are on the right. The data are obtained from three independent transformation experiments, and only one replicate is shown. (C) Verification of *vdh* deletion mutants by colony PCR with paired primers P1 and P2. The PCR products for the wild type were 3925 bp and those for the mutants were 2464 bp. M represents the nucleotide size marker; WT represents the wild type; and 1−14 represent 14 colonies from the screening of the Bennet plate. (D) Confirmation of mutants by Sanger sequencing. All three amplicons are sequenced and found to be correct; only the results from one colony are shown. (E) Growth curves of the wild-type strain and Δ*vdh* mutant in shake flask fermentation. Error bars (mean ± SD) were derived from triplicate samples. (F) Vanillin and vanillic acid titers from shake flask fermentation of the wild-type strain and Δ*vdh* mutant.

The impact of Cas gene expression on the growth of CCTCC NO: M2011265 was assessed. pDZLkm-Cas12a, pDZLkasO*p-Cas12a, and pDZLkm-Cas9 were transferred into CCTCC NO: M2011265; both the wild type and recombinants that harbored the different promoter control Cas12a or Cas9 genes were cultured, and their growth curves were determined (Figure 2B). The P_{km}-Cas9 strain grew more slowly than the wild-type strain, whereas P_{km}-Cas12a and P_{kasO*p}-Cas12a (expressing Cas12a with the strong promoter kasO*p) grew similarly and slightly slower than the wild-type strain. This indicates that Cas12a expression had no detectable impact, whereas Cas9 expression had a harmful effect on CCTCC NO: M2011265 physiology.

To assess whether Cas12a can function as an effective RNA-guided nuclease in CCTCC NO: M2011265, pULcrRNAkm-Cas12a (no crRNA expression cassette) and pULΔ*vdh* were transformed into CCTCC NO: M2011265. A dramatic reduction (1000-fold) in colony forming units (CFUs) was observed when the pULΔ*vdh* plasmid was used for transformation compared with the absence of the crRNA expression cassette (Figure 2C), indicating the highly efficient programmable endonuclease activity of Cas12a in CCTCC NO: M2011265.

2.2. Vanillin Biosynthesis from Ferulic Acid in *Amycolatopsis* sp. When ferulic acid is used as a precursor for vanillin biosynthesis, there are four main pathways for ferulic acid consumption, namely, non-oxidative decarboxylation, side chain reduction, coenzyme-A-independent deacetylation, and coenzyme-A-dependent deacetylation; a CoA-dependent, non-β oxidative pathway was also identified in *Amycolatopsis* sp. strain HR167 (Figure 3).[30] Ferulic acid metabolism in *Amycolatopsis* sp. ATCC 39116 proceeds via the ferulic acid β-oxidation pathway, 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA and vanillyl-CoA, whereas the non-β-oxidative pathway proceeds via vanillin.[31] CCTCC NO: M2011265 can produce vanillin from ferulic acid as the precursor,[26] so the key enzyme genes involved in ferulic acid metabolism were annotated by genome sequencing and the nucleotide basic local alignment search tool (Tables S3 and S4), indicating the presence of the same metabolic pathways as those in ATCC 39116 (Figure 3). Therefore, the metabolic engineering strategy to increase production of vanillin by

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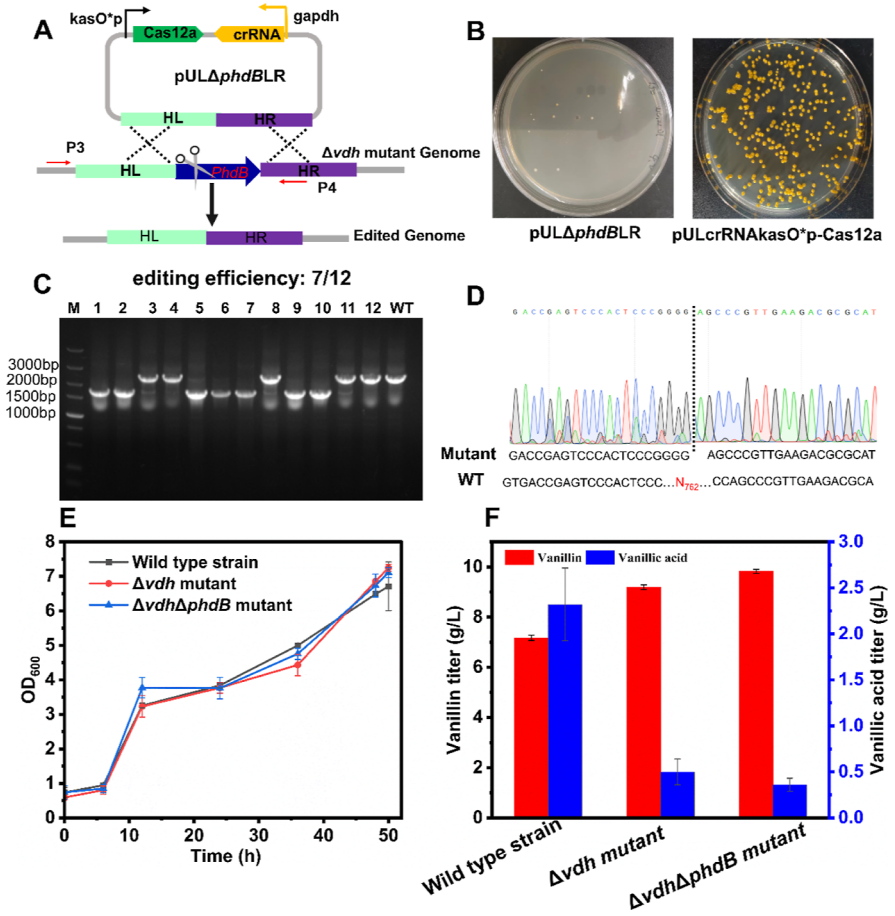

Figure 5. Markerless deletion of the *phdB* gene with CRISPR-Cas12a-assisted DNA cleavage and HDR-mediated recombination in the Δ*vdh* mutant. (A) Schematic for pULΔ*phdB*LR-mediated gene knockout from the Δ*vdh* mutant. The donor DNA is located upstream and downstream of the target region; $P_{kasO*p}$and P_{gapdh} are the promoters for expression of crRNA and Cas12a, respectively. (B) The transformants with pULΔ*phdB*LR are on the left and those with the pULcrRNAkasO*p-Cas12a plasmid (no *phdB* guide sequence) are on the right. The data are obtained from three independent transformation experiments, and only one replicate is shown. (C) Verification of *phdB* deletion mutants by colony PCR with paired primers P3 and P4. A fragment of 1674 bp was amplified from the edited colonies, and a fragment of 2436 bp was amplified from the wild type. M represents the nucleotide size marker, WT represents the wild type, and 1−12 represent randomly selected clone colonies from the Bennet plate. (D) Sanger sequencing results of the amplicon from (C). All seven amplicons are sequenced and found to be correct; only the results from one colony are shown. (E) Growth curves of the wild type strain, Δ*vdh* mutant, and Δ*vdh*Δ*phdB* mutant in shake flask fermentation. The error bars (mean ± SD) were derived from triplicate samples. (F) Vanillin and vanillic acid titers from shake flask fermentation of the wild type strain, Δ*vdh* mutant, and Δ*vdh*Δ*phdB* mutant. The error bars (mean ± SD) were derived from triplicate samples.

Amycolatopsis sp. was to knock out the *vdh* gene that converts vanillin into vanillic acid and the *phdB* gene that diverts the immediate precursor to vanillin into 4-hydroxy-3-methoxyphenyl-*β*-ketopropionyl-CoA, so the *vdh* and *phdB* genes were selected as the target genes for CRISPR editing.

2.3. Construction of the Δ*vdh* Mutant and Its Fermentation Performance. Vanillin dehydrogenase oxidizes vanillin to vanillic acid, which limits the accumulation of vanillin, so marker-free deletion of the *vdh* gene using the CRISPR-Cas12a system was performed. Careful spacer sequence design is essential for gene editing with the CRISPR-Cas12a system; the crRNA expression cassette is typically required to consist of a 19-nt direct repeat sequence and a 23-nt guide sequence for efficient genome editing,[24] so all crRNAs were designed according to this criterion. The 23-nt sequence immediately downstream of the TTN-PAM was selected as the target sequence in the coding region of *vdh*. The upstream and downstream homologous arms of the *vdh* gene were assembled by fusion, and the resulting donor fragment was introduced into the plasmids expressing *vdh*-targeted crRNA, generating plasmids pULΔ*vdh*LR and the control, pULcrRNAkm-Cas12a (no *vdh* guide sequence; Figure 4A), which were electroporated into CCTCC NO: M2011265 competent cells. The conjugation efficiency of pULΔ*vdh*LR was >3 orders of magnitude lower than that of the control, pULcrRNAkm-Cas12a (Figure 4B). The CRISPR-Cas12a system generates double-strand breaks in target genes, typically resulting in reduced survival rates.[22] Subsequently, 14 clones were randomly selected from the transformation with pULΔ*vdh*LR and verified by both PCR amplification and Sanger sequencing; the *vdh* gene was precisely and markerlessly deleted; however, the editing efficiency was only 3/14 (21.4%) (Figure 4C,D). To demonstrate clearance of the pULΔ*vdh*LR plasmid, one of the *vdh* deletion strains was cultured without antibiotic supplementation for two generations (48 h/generation), and then, diluted culture was spread on Bennet plates, with or without apramycin (Figure S2). Their failure to grow in the presence of apramycin indicated successful clearance of the editing plasmid, with its apramycin resistance marker gene, the removal of which allows the

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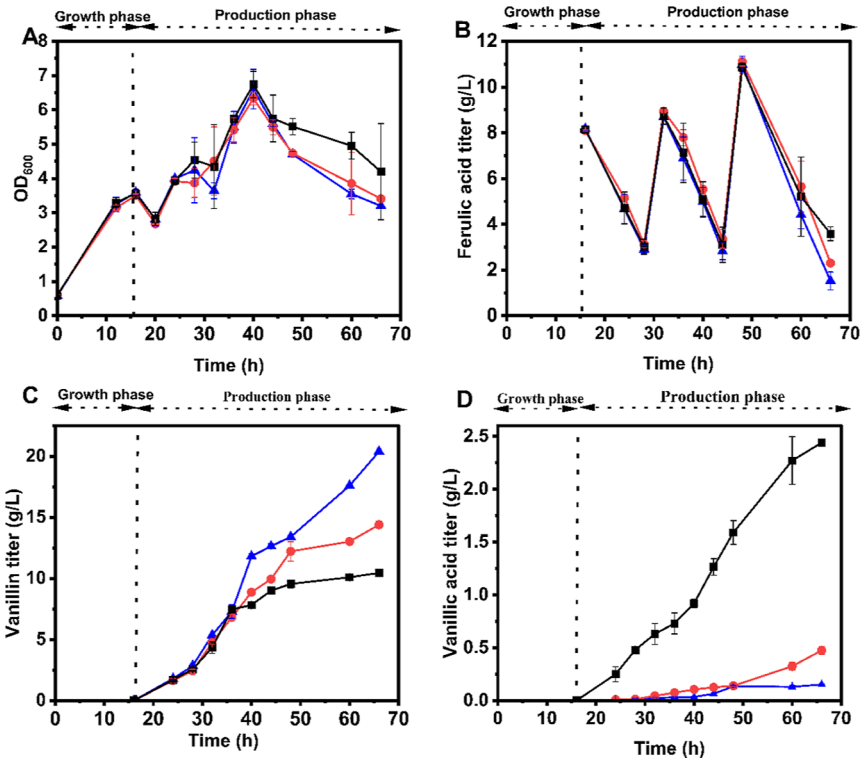

Figure 6. Fed-batch fermentation of CCTCC NO: M2011265 and the related mutants Δ*vdh* and Δ*vdh*Δ*phdB* in a 3 L bioreactor. (A) Cell growth curves. The error bars (mean ± SD) were derived from triplicate samples. (B) Ferulic acid titer; (C) vanillin titer; and (D) vanillic acid titer. (■) Wild-type strain; (red ●) Δ*vdh* mutant; and (blue ▲) Δ*vdh*Δ*phdB* mutant.

apramycin selection marker to be reused in this strain for subsequent pathway engineering, such as further genome editing, for example, deletion of the *phdB* gene (see below) or the introduction of heterologous genes. The clean mutant *Amycolatopsis* sp. CCTCC NO: M2011265 strain, with the whole *vdh* open reading frame deleted, was designated *Amycolatopsis* sp. Δ*vdh*.

The *vdh* deletion mutant was fermented on the shake flask scale to evaluate the effect of deleting vanillin dehydrogenase on the accumulation of vanillin. The *vdh* gene deletion had a negligible effect on cell growth, compared with the wild type (Figure 4E), but significantly increased vanillin accumulation from 7.22 to 9.11 g/L (26.2% increase) and markedly decreased vanillic acid accumulation, from 2.07 to 0.48 g/L, compared with the wild type (Figure 4F).

2.4. Construction of the Δ*vdh*Δ*phdB* Mutant and Its Fermentation Performance. Based on the ferulic acid metabolic pathway (Figure 3), deletion of the *phdB* gene should divert ferulic acid metabolic flux away from 4-hydroxy-3-methoxyphenyl-*β*-ketopropionyl-CoA and toward vanillin, so the *phdB* gene was deleted from the Δ*vdh* mutant. Efficient expression of Cas12a and crRNA is vital to achieve efficient CRISPR-Cas12a-mediated genome editing,[32] so the strong constitutive promoters, gapdh and kasO*p, are chosen to control the expression of the crRNA and Cas12a, respectively.[25] The Cas12a-crRNA complex that targets the *phdB* gene and the donor fragment (upstream and downstream homologous arms of the *phdB* gene) were first cloned into an expression plasmid, yielding the plasmid pULΔ*phdB*LR (Figure 5A) and the control plasmid pULcrRNAkasO*p-Cas12a (no *phdB* guide sequence), which were both transformed into the Δ*vdh* mutant competent cells. As expected, transformation with the pULΔ*phdB*LR plasmid resulted in markedly reduced CFU abundance than that with pULcrRNAkasO*p-Cas12a (Figure 5B). Subsequently, 12 clones from transformation with pULΔ*phdB*LR were randomly selected and verified by PCR amplification and Sanger sequencing, indicating that the *phdB* gene was precisely and markerlessly deleted, and the editing efficiency was 7/12 (Figure 5C,D). The increased efficiency of *phdB* gene knockout (58%) indicates that strong promoters improved the editing efficiency of the CRISPR-Cas12a system. The *phdB* gene-deletion mutant was designated *Amycolatopsis* sp. Δ*vdh*Δ*phdB*.

The *vdh* and *phdB* deletion mutants were fermented on the shake flask scale to evaluate the effect of the absence of the two dehydrogenase enzymes on the accumulation of vanillin (Figure 5E). The mutant had a very similar growth rate to the wild-type strain, indicating that deletion of the *vdh* and *phdB* genes had a negligible effect on the physiology of the mutant strain. The Δ*vdh*Δ*phdB*, Δ*vdh*, and wild-type strains had a vanillin production of 9.88, 9.13, and 7.25 g/L and a vanillic acid production of 2.04, 0.40, and 0.31 g/L, respectively, i.e. (Figure 5F), the Δ*vdh*Δ*phdB* mutant further increased vanillin and decreased vanillic acid production, compared with the Δ*vdh* single-deletion mutant.

2.5. Enhanced Vanillin Production Using Fed-Batch Fermentation in a 3 L Bioreactor. To optimize the fermentation performance of the above-mentioned strains, fed-batch fermentation was compared with that of the wild type in 3 L bioreactors (Figure 6). The fermentation proceeded in two phases, a growth phase in which cell biomass accumulated and a production phase, started by addition of ferulic acid, at the beginning of the stationary growth phase. The growth of the two mutants was essentially the same as that of the wild type. All three strains declined rapidly after 40 h, probably because

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of growth inhibition at higher vanillin concentrations, but the wild type declined less rapidly than the mutants (Figure 6A). Microbial cells can tolerate accumulated vanillin up to a threshold level, beyond which its accumulation decreases, resulting from its inhibition of cell growth and metabolic activity.[3] The ferulic acid concentration was maintained <8 g/L, to minimize its cytotoxicity, but >1 g/L, to ensure an adequate supply to the cells, by adding 60 g of ferulic acid, in three portions, during the fermentation (Figure 6B); the conversion of ferulic acid and subsequent accumulation of vanillin started after precursor addition. After fermentation for 50 h, the third portion of ferulic acid was added, and its concentration decreased to 1.5−3.5 g/L at 68 h. As expected, the ferulic acid metabolic flux was diverted predominantly to vanillin synthesis, and vanillin oxidation was inhibited in the Δ*vdh*Δ*phdB* mutant. The vanillin titer reached 20.4 g/L after 68 h, markedly higher than that of the Δ*vdh* mutant at 14.6 g/L and that of the wild type at 10.6 g/L (Figure 6C), whereas vanillic acid accumulation was 0.15 g/L (Δ*vdh*Δ*phdB*), 0.45 g/L (Δ*vdh*), and 2.45 g/L (wild type) (Figure 6D). Under fed-batch fermentation, vanillin production by the double deletion strain increased by 92.4% and the single deletion strain by 37.7%, compared with that by the wild type. As reported previously, optimizing the medium composition and other fermentation conditions, reducing the toxicity of vanillin to the producing microorganism using resins to adsorb vanillin, and using genetically engineered strains to avoid vanillin conversion to vanillic acid have been used to improve the vanillin yield.[3,4,27,33] Here, a vanillin high-producing mutant (Δ*vdh*Δ*phdB*) was engineered by genome editing and produced vanillin with a molar yield of 90.5% and also reached a vanillin titer high enough for viable industrial production.[3]

3. CONCLUSIONS

In summary, an all-in-one CRISPR-Cas12a system was developed in *Amycolatopsis* sp. CCTCC NO: M2011265 for efficient, markerless, and scarless genome editing. The genome editing tool was successfully utilized to engineer the high-yielding vanillin mutant strain *Amycolatopsis* sp. Δ*vdh*Δ*phdB*, which is robust and efficient, producing double the vanillin titer and 16-fold less vanillic acid in fed-batch fermentation than the wild type. With some modification, the CRISPR system should be applicable to other *Amycolatopsis* spp., and the resulting mutants may be even more efficient for production of natural vanillin.

4. EXPERIMENTAL SECTION

4.1. Strains, Plasmids, and Culture Conditions. Strains and plasmids used in this study are listed in Table S1. *Amycolatopsis* sp. CCTCC NO: M2011265 (China Center for Type Culture Collection no. M2011265) was used for vanillin production and genome editing. Its cells were grown at 30 °C in fresh Bennet medium (yeast extract, 1 g/L; glycerol, 10 g/L; glucose, 10 g/L; beef powder, 1 g/L; tryptone, 2 g/L; and agar, 20 g/L; pH 7.2) or yeast extract malt extract (YEME) medium (yeast extract, 3 g/L; malt extract, 3 g/L; peptone 5 g/L; glucose, 10 g/L; and sucrose, 220 g/L; pH 7.2.). *E. coli* JM110 (dam^-, dcm^-) and *E. coli* JM109, cultured at 37 °C in lysogeny broth medium, were used for amplification and extraction of plasmids. The plasmids pDZLCas12a and pULcrRNA were kindly gifted by Prof. Wang Jin, College of Life Sciences, Shanghai Normal University, Shanghai, China.

4.2. Construction of Editing Plasmids. Primers for plasmid construction, mutant verification, and Sanger sequencing are listed in Table S2. The Cas12a gene of *Francisella tularensis* subsp. *novicida* (FnCas12a, previously known as FnCpf1) was PCR-amplified from the plasmid, pDZLCas12a, using primers Cas12a-L and Cas12a-R. The pyruvate kinase promoter P_{km} was PCR-amplified from plasmid pLYZYP01 with primers km-L and km-R. The Cas12a gene P_{km}, and the linearized pULcrRNA vector were assembled using a ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech, Nanjing, China), to generate the recombinant plasmid pULcrRNAkm-Cas12a.

Generation of the knockout plasmid for pULΔ*vdh*LR was carried out as follows. The 23-nt crRNA guide sequences for coding regions in *vdh* (encoding vanillin dehydrogenase in *Amycolatopsis* sp.) immediately downstream of the TTN coding sequence were selected, pULcrRNAkm-Cas12a was used as an amplification template using the primer pair *vdh*-T1/*vdh*-T2 to introduce the target sequence, and then, the PCR products were purified and transformed into *E. coli* JM110, obtaining pULΔ*vdh*. Upstream and downstream homologous arms (1.5 kb) of the target genes *vdh* were PCR-amplified from the CCTCC NO: M2011265 genome with primer pairs *vdh*L-F/*vdh*L-R and *vdh*R-F/*vdh*R-R, the amplicons of which were used as homologous arms for homology-directed repair (HDR). The upstream and downstream homologous arms were linked by Gibson Assembly (ClonExpress MultiS One Step Cloning Kit) and introduced into the *NheI*-treated plasmids of pULΔ*vdh*, obtaining the knockout plasmid, pULΔ*vdh*LR.

Generation of the knockout plasmid pULΔ*phdB*LR was carried out as follows. The 23-nt crRNA guide sequences for coding regions in *phdB* (encoding HMPHP-CoA dehydrogenase in *Amycolatopsis* sp.) immediately downstream of the TTN were selected, pULcrRNAkasO*p-Cas12a was used as an amplification template using the primer pair *phdB*-T1/*phdB*-T2 to introduce the target sequence, and then, the PCR products were purified and transformed into *E. coli* JM110, obtaining pULΔ*phdB*. Subsequently, the plasmid was checked via sequencing to confirm that it contained the target sequence. The upstream and downstream homologous arms (1.5 kb) of the target gene *phdB* were PCR-amplified from the CCTCC NO: M2011265 genome with primer pairs *phdB*L-F/*phdB*L-R and *phdB*R-F/*phdB*R-R, the amplicons of which were used as homologous arms for HDR. In addition, the promoter hsp60, controlling the crRNA expression cassette on plasmid pULcrRNAkm-Cas12a, was replaced by promoter gapdh, and the promoter km was replaced by promoter kasO*p, which controls Cas12a expression by *HindIII* and *BamHI* digestion, and then ligated into pULΔ*phdB* to generate the plasmid pULΔ*phdB*LR.

The pDZLkm-Cas12a, pDZLkasO*p-Cas12a, and pDZLkm-Cas9 plasmids were constructed by linking the km and kasO*p promoters to the Cas12a and Cas9 genes, respectively, by a one-step cloning method.

4.3. Construction of *Amycolatopsis* sp. Mutants. *Amycolatopsis* sp. CCTCC NO: M2011265 was cultured at 30 °C in YEME liquid medium for 36−48 h until the stationary growth phase. The cell suspension (~5% v/v) was inoculated into YEME liquid medium (30 mL) with shaking at 30 °C and 220 rpm to an OD_{600} of 0.7−0.8, and then, the cells

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were collected by centrifugation at 5000*g* at 4 °C and washed three times with pre-cooled aqueous 15% glycerol/0.5 M sucrose. The resulting competent cells of CCTCC NO: M2011265 were resuspended in pre-cooled 15% glycerol/0.5 M sucrose (1.5 mL). The plasmid (0.5 μg) was mixed with a suspension of competent cells (50 μL) and loaded into an electroporation cuvette with a 1 mm gap. Electroporation was performed with a single pulse of 2500 V for 5 ms. The cells were immediately mixed with Bennet liquid medium (1 mL), left to recover at 30 °C for 4 h, and then cultured on Bennet agar plates supplemented with 50 μg/mL apramycin, at 30 °C for 7 days; then, the colonies were confirmed by colony PCR and Sanger sequencing.

4.4. Shake Flask and Fed-Batch Fermentation. The primary seed culture was obtained by transferring a single colony from Bennet agar plates to a 50 mL flask containing seed medium (30 mL; yeast extract, 10 g/L; glucose, 5 g/L; Na_2HPO_4, 4 g/L; KH_2PO_4,1 g/L; NaCl, 0.2 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; and $CaCl_2$, 0.05 g/L; pH 7.2) and culturing for 30 h at 30 °C with 220 rpm shaking. The seed culture medium was added to fermentation medium (300 mL; Na_2HPO_4, 4 g/L; KH_2PO_4, 1 g/L; NaCl, 0.2 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2$, 0.05 g/L; sucrose, 103 g/L; and yeast extract, 5 g/L; pH 7.2) to determine the vanillin production of the engineered strains. The cells were cultured until they reached the stationary growth phase, and then, the biotransformation was initiated by direct addition of ferulic acid to a final concentration of 12 g/L.

Fed-batch fermentation experiments were carried out in a 3 L bioreactor (Bailun Biochemical Technology, Shanghai, China) with automatic control of temperature, pH, dissolved oxygen, and mechanical stirring during the fermentation. Each inoculum was prepared by transferring a colony from agar to seed medium (150 mL) and culturing for 36 h at 30 °C and 220 rpm shaking. The bioreactor experiments consisted of two phases: the growth phase for biomass accumulation, controlling the pH at 7.2, followed by a fed-batch phase, controlling the pH at 8.0 for vanillin production. In the growth phase, the bioreactors contained 1.5 L of fermentation medium, maintained at 30 °C. The cells were grown until they reached the stationary growth phase, and then, the production phase was started by the addition of 150−200 mL of a ferulic acid solution (100 g/L ferulic acid in 0.5 M NaOH), resulting in a concentration of 8.0 g/L of ferulic acid in the culture medium, and the temperature was increased to 35 °C. When the ferulic acid in the culture medium was exhausted, a feeding solution (100 g/L ferulic acid) was added. The substrate, product, and metabolic intermediate concentrations were determined by high-performance liquid chromatography (HPLC) at the given time points. The fermentation was stopped when ferulic acid consumption ceased. The aeration rate was 2.0 volumes per volume per minute (vvm), and the stirrer speed was 500 rpm.

4.5. High-Performance Liquid Chromatography Method. Culture supernatants were obtained after centrifugation (5 min, 12,000*g*), dissolved in HPLC grade water, and filtered through a 0.22 μ syringe filter, before injection into an HPLC system (Waters, Milford, MA), fitted with an Amethyst RP-C18-H column (4.6 mm × 250 mm, 5 μM, Sepax Technologies, Newark, DE). The detection wavelength and column temperature were 280 nm and 30 °C, respectively. The isocratic mobile phase was methanol/0.1% aqueous acetic acid = 7:3; flow rate, 1.0 mL/min; and injection volume, 10 μL.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00790.

Additional experimental details, materials, and results; plasmids and strain used in this study; oligonucleotides used in this study; statistics of the sequencing, assembly, and annotation of *Amycolatopsis* sp. CCTCC NO: M2011265.with Illumina MiSeq paired-end whole-genome sequencing; sequence alignment results of key genes for ferulic acid metabolism and DNA sequences of key genes for ferulic acid metabolism; enhancing transformation efficiency by bypassing the RM system; and elimination of the self-replicable plasmid in *Amycolatopsis* sp. Δ*vdh* (PDF)

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Notes

The authors declare no competing financial interest.

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