Metabolic Engineering of *Escherichia coli* for High-Level Production of Salicin

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ABSTRACT: Salicin is a notable phenolic glycoside derived from plants including *Salix* and *Populus* genus and has multiple biological activities such as anti-inflammatory and antiarthritic, anticancer, and antiaging effects. In this work, we engineered production of salicin from cheap renewable carbon resources in *Escherichia coli* (*E. coli*) by extending the shikimate pathway. We first investigated enzymes synthesizing salicylate from chorismate. Subsequently, carboxylic acid reductases (CARs) from different resources were screened to achieve efficient reduction of salicylate. Third, glucosyltransferases from different sources were selected for constructing cell factories of salicin. The enzymes including salicylate synthase AmS from *Amycolatopsis methanolica*, carboxylic acid reductase CARse from *Segniliparus rotundus,* and glucosyltransferase UGT71L1 from *Populous trichocarpa* were overexpressed in a modified *E. coli* strain MG1655-U7. The engineered strain produced 912.3 ± 12.7 mg/L salicin in 72 h of fermentation. These results demonstrated the production of salicin in a microorganism and laid significant foundation for its commercialization for pharmaceutical and nutraceutical applications.
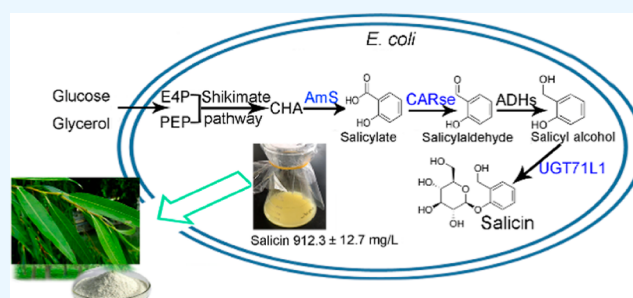
INTRODUCTION

Bioactive natural products are frequently glycosylated with monosaccharide or saccharide chains, which usually influence chemical properties and biological activities.[1,2] Phenolic glycosides are widely distributed in the plant kingdom, which demonstrate multiple biological and pharmacological activities.[3,4] Salicin is the glucoside of salicyl alcohol and was first discovered in willow bark in 1828.[5] Salicin is now mainly extracted from barks of various species of *Salix* (willow) and *Populus* genus.[3] Salicin has been used to treat pain and inflammation throughout the last centuries,[6−8] and salicin is also considered as a historic precursor to the medicine known today as aspirin.[5] Salicin gained renewed attention due to its potential bioactivities such as anticancer, antiaging, and neuroprotective effects in the past decades.[9−12]

The main source of phytochemicals is direct extraction from plants, which involves occupation of farming land and is time-consuming. Purification of salicin is a complicated and laborious process.[13] Furthermore, extracting from plants brings about concerns for environmental and ecological problems. In recent years, microbial synthesis has emerged as an alternative means for efficient, flexible production of phytochemicals.[14,15] A few important phenolic glycosides have been successfully synthesized in microorganisms by designing artificial pathways.[16,17] In the case of salicin production, microbial synthesis would offer an alternative route in addition to direct extraction from plants.

The biosynthetic pathway of salicin in plants is still poorly understood, even though the compound has been discovered and used to benefit human health for centuries. Earlier studies by isotope-labeled precursor feeding experiments with *Populus nigra* (*P. nigra*) leaf tissue shed light on the biosynthesis of salicin and complicated phenolic glycosides in *Populus*.[18] Salicylic alcohol is derived from phenylalanine and cinnamic acid via a *β*-oxidation-like pathway, and the exact pathway need to be further investigated. The feeding experiments with salicylic alcohol demonstrated that salicylic alcohol was turned into salicin by *P. nigra* leaf tissue.[18] Two UDP-glucose glycosyltransferases (UGT71L1 and UGT78M1) were discovered and characterized from *Populus tremuloides*, which accepted salicyl benzoate, salicylaldehyde, and 2-hydroxycinnamic acid as acceptor substrates.[19]

The microbial synthesis of the aglycon salicylic alcohol has been achieved in *Escherichia coli* (*E. coli*) with an artificial pathway via salicylic acid as the intermediate.[20] Salicylic acid is produced by plants as an important phytohormone[21] and some bacteria as an essential building block in siderophore

Table 1. Strains and Plasmids

	description	reference
	Plasmids	
pET-28a	pBR322ori with PT7KanR	Novagen
pETDuet-1	pBR322ori with PT7, AmpR	Novagen
pRed_Cas9_recA_T	For the T7 RNA polymerase gene (lacUV5 promoter) integrated into *nupG* gene site	this study
pET28a-svo189	pET28a carrying PT7-svo189	this study
pET28a-ugt71L1	pET28a carrying PT7-ugt71L1	this study
pET28a-ugt78M1	pET28a carrying PT7-ugt78M1	this study
pET28a-ugt73B6[FS]	pET28a carrying PT7-ugt73B6[FS]	this study
pETDuet-amS	pETDuet-1 carrying PT7-*amS*	this study
pETDuet-mbtI	pETDuet-1 carrying PT7-*mbtI*	this study
pETDuet-entC-pchB	pETDuet-1 carrying PT7-*entC- pchB*	this study
pETDuet-irp9	pETDuet-1 carrying P_{T7}-*irp9*	this study
pETDuet-amS-CARni-*sfp*	pETDuet-1 carrying P_{T7}-*amS*, P_{T7}-*CARni-sfp*	this study
pETDuet-amS-CARmm-*sfp*	pETDuet-1 carrying P_{T7}-*amS*, P_{T7}-*CARmm-sfp*	this study
pETDuet-amS-CARse-*sfp*	pETDuet-1 carrying P_{T7}-*amS*, P_{T7}-*CARse-sfp*	this study
pETDuet-amS- CARms-*sfp*	pETDuet-1 carrying P_{T7}-*amS*, P_{T7}-*CARms-sfp*	this study
pETDuet-amS-CARmav-*sfp*	pETDuet-1 carrying P_{T7}-*amS*, P_{T7}-*CARmav-sfp*	this study
pETDuet-amS-CARse-*sfp*-ugt71L1	pETDuet-1 carrying P_{T7}-*amS*, P_{T7}-*CARse-sfp*, PT7-*ugt71L1*	this study
	Strains	
BMGU	*E. coli* K-12 MG1655 with gene *tyrR, pykA, pykF, pheA, feaB, galE, galT* and *ugd* disrupted	in our lab (patent ZL201610361309.2)
U7	BMGU with T7 RNA polymerase gene (lacUV5 promoter) integrated into *nupG* gene site	this study
U7-01	U7 harboring pETDuet-amS	this study
U7-02	U7 harboring pETDuet-mbtI	this study
U7-03	U7 harboring pETDuet-entC-pchB	this study
U7-04	U7 harboring pETDuet-amS-CARni-*sfp*	this study
U7-05	U7 harboring pETDuet-amS-CARmm-*sfp*	this study
U7-06	U7 harboring pETDuet-amS-CARse-*sfp*	this study
U7-07	U7 harboring pETDuet-amS-CARms-*sfp*	this study
U7-08	U7 harboring pETDuet-amS-CARmav-*sfp*	this study
U7-09	U7 harboring pETDuet-amS-CARse-*sfp*-ugt71L1	this study
U7-10	U7 harboring pETDuet-irp9	this study
B-01	*E. coli* BL21 (DE3) harboring pET28a- ugt71L1	this study
B-02	*E. coli* BL21 (DE3) harboring pET28a-ugt73B6[FS]	this study
B-03	*E. coli* BL21 (DE3) harboring pET28a-ugt78M1	this study
B-04	*E. coli* BL21 (DE3) harboring pET28a-svo189	this study

biosynthesis.[22] In plants, the biosynthetic pathways of salicylic acid are complicated, which include two metabolic routes derived from chorismate and phenylalanine.[21,23] In *Pseudomonas aeruginosa*, salicylic acid is derived from chorismate catalyzed by an isochorismate synthase (IS) and an isochorismate pyruvate lyase (IPL) in the biosynthesis of pyochelin.[24,25] An IS EntC from *E. coli* and an IPL PchB from *Pseudomonas fluorescens* (*P. fluorescens*) were used for synthesis of salicylic acid as the precursor for 4-hydroxy-coumarin.[26] In recent years, carboxylic acid reductases have demonstrated significant potential to reduce aromatic acids to produce aromatic aldehydes and aromatic alcohols in microorganisms.[17,20,27,28] The synthesis of salicylic alcohol in *E. coli* was further achieved by introducing a carboxylic acid reductase [carboxylate reductase (CAR) from *Mycobacterium marinum* (*M. marinum*)].[20] The biosynthetic pathway of salicylic acid from chorismate catalyzed by a salicylate synthase was also known in the biosynthesis of yersiniabactin oramychelin.[22] The salicylate synthase Irp9 from *Yersinia enterocolitica* (*Y. enterocolitica*) has been used for the synthesis of yersiniabactin and glucoside of salicylate in *E. coli*.[29]

In the present study, we described the construction of a recombinant *E. coli* strain for production of salicin by using an artificial pathway. Specifically, the efficiency of IS/IPL was compared with that of salicylate synthases for synthesizing salicylate in an *E. coli* chassis cell MG1655-U7. Subsequently, CARs from different resources were screened for reducing salicylic acid to the aglycon salicyl alcohol. UDP-glycosyltransferases from bacteria or plants were tested using salicyl alcohol as the substrate. Finally, the salicin production was optimized with various concentrations of glycerol and glucose as the carbon resources for the fermentation. As a result, we achieved high-level production of salicin in the *E. coli* strain, and the work paves the way for large-scale production of salicin by fermentation.

■ MATERIALS AND METHODS

Plasmids, Strains, and Media. Cloning of genes and propagation of plasmids were performed in *E. coli* DH5α. *E. coli* BL21 (DE3) was utilized for glycosyltransferase expression to examine enzyme activity. *E. coli* K-12 MG1655 derivative strain U7 served as the starting strain, and its derivatives and plasmids used in this study are listed in Table 1. During plasmid construction and strain development, *E. coli* cells were routinely grown in Luria—Bertani (LB) medium or on LB agar plates. The M9Y medium containing 20 g/L glucose, 0.25 g/L
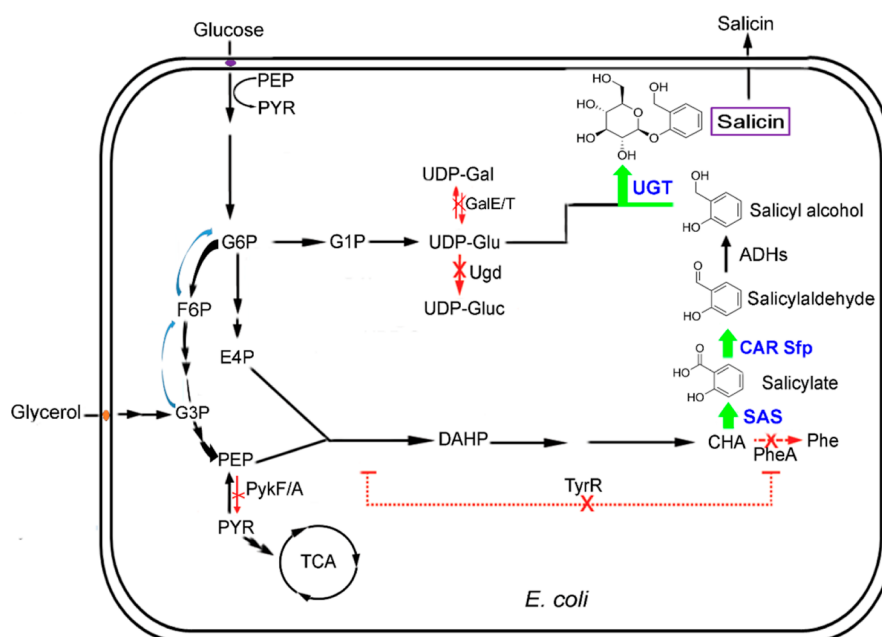
Figure 1. *de no* biosynthetic pathway of salicin in *E. coli*. Single arrows represent one-step conversion, double arrows represent multi-step conversion, red lines or X indicates gene deletion or elimination of feedback inhibition in *E. coli*, and enzymes overexpressed in the pathway are shown in blue text. Abbreviations: G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; G3P, glyceraldehyde 3-phosphate; F6P, fructose 6-phosphate; PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; PYR, pyruvate; DAHP, 3-deoxy-arabino-heptulonate-7-phosphate; CHA, chorismic acid; Phe: phenylalanine; Ugd, UDP-glucose 6-dehydrogenase; GalE, UDP-glucose 4-epimerase; GalT, galactose-1-phosphate uridylyltransferase; PykA, pyruvate kinase II; PykF, pyruvate kinase I; PpsA, phosphoenolpyruvate synthase; TktA, *E. coli* endogenous transketolase 1; AroG DAHP synthase; SAS, salicylate synthase; CAR, carboxylic acid reductase; *Sfp*, phosphopantetheinyl transferase; and UGT, UDP-glycosyltransferases.

yeast extract, 3 g/L KH$_2$PO$_4$, 1 g/L NH$_4$Cl, 17.1 g/L Na$_2$HPO$_4$·12H$_2$O, 0.5 g/L NaCl, 14.7 mg/L CaCl$_2$·H$_2$O, and 246.5 mg/L MgSO$_4$·7H$_2$O was used for feeding experiments and total biosynthesis of salicylate, salicyl alcohol, and salicin. Modified M9Y medium containing 5 g/L yeast extract, 3 g/L KH$_2$PO$_4$, 1 g/L NH$_4$Cl, 17.1 g/L Na$_2$HPO$_4$· 12H$_2$O, 0.5 g/L NaCl, 14.7 mg/L CaCl$_2$·H$_2$O, 246.5 mg/L MgSO$_4$·7H$_2$O, and different concentrations of glucose and glycerol supplemented was used for medium optimization. Standards containing salicylate, salicyl alcohol, and salicin were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

Plasmid Construction. All the primers used in this study were synthesized from GENEWIZ Bio, Inc. Heterologous genes of; *amS* (GenBank: WP_026153750.1) from *Amycolatopsis methanolica* (*A. methanolica*) 239T, *mbtI* (GenBank: YP_177877.1) from *Mycobacterium tuberculosis* (*M. tuberculosis*), *entC* (GenBank: NP_415125.1), and *pchB* (GenBank: WP_003106950.1) from *P. fluorescens*, were cloned into plasmid pETDuet-1 via *Nde*I and *Bgl*II sites to create plasmids pETDuet-ams, pETDuet-mbtI, and pETDuet-entC-pchB, respectively. Then, carboxylate reductases, CARni (GenBank: AAR91681.1) from *Nocardia iowensis* (*N. iowensis*), CARmm (GenBank: WP_012393886.1) from *M. marinum*, CARse (GenBank: WP_013138593) from *Segniliparus rotundus* (*S. rotundus*), CARms (GenBank: WP_011728718) from *Mycobacteriums megmatis*, or CARmav (GenBank: WP_011725490.1) from *Mycobacterium avium* with *sfp* (GenBank: WP_015715234.1) from *Bacillus subtilis*, were cloned into plasmid pETDuet-amS between *Eco*RI and *Hind*III to create pETDuet-amS-CARni-*sfp*, pETDuet-amS-CARmm-*sfp*, pETDuet-amS-CARse-*sfp*, pETDuet-amS-CARms-*sfp*, and

pETDuet-amS-CARmav-*sfp*, respectively. Glycosyltransferases, Sv0189 (GenBank: CCA53477.1) from *Streptomyces venezuelae* (*S. venezuelae*), UGT71L1 (GenBank: Potri.016G014500) and UGT78M1 (GenBank: Potri.006G171100) from *Populus trichocarpa* (*P. trichocarpa*), UGT73B6^{FS} (F389S)[28] which was a mutant of UGT73B6 (GenBank: AY547304.1) from *Rhodiola sachalinensis* (*R. sachalinensis*), were cloned into pET28a to create pET28a-sv0189, pET28a-ugt71L1, pET28a-ugt78M1, and pET28a-ugt73B6^{FS}, respectively. Eventually, *amS*, *CARse*, and *ugt71L1* were inserted into plasmid pETDuet-*sfp* to gain pETDuet-amS-CARse-*sfp*-ugt71L1 using one-step PCR cloning. All the genes were synthesized with *E. coli* codon optimization by GENEWIZ Bio, Inc. The sequences of *amS*, *CARse*, *sfp*, and *ugt71L1* with *E. coli* codon optimization are listed in Supporting Table S1.

U7 Strain Construction. The T7 RNA polymerase gene driven by the lacUV5 promoter was amplified from *E. coli* BL21 (DE3) and integrated into the *nupG* gene site of BMGU using the CRISPR/Cas9 systems. The plasmid of pRed_Cas9_recA was constructed, as shown in Supporting Figure S1, and the primers and N20 sequence used are shown in Supporting Table S2. The BMGU was an engineered strain with disruption of genes *tyrR*, *pykA/F*, *pheA*, *galE/T*, and *ugd* in *E. coli* K-12 MG1655 (constructed in our laboratory, patent ZL201610361309.2). The involved pathways of *tyrR*, *pykA/F*, *pheA*, *galE/T*, and *ugd*[30] are shown in Figure 1.

Feeding Experiments. The plasmids of pET28a-sv0189, pET28a-ugt71L1, pET28a-ugt78M1, and pET28a-ugt73B6^{FS} were individually transferred into *E. coli* BL21 (DE3). Single colonies of the transformed cells were inoculated into a 250 mL shake flask containing 5 mL of LB medium with kanamycin and cultivated at 37 °C overnight with shaking at
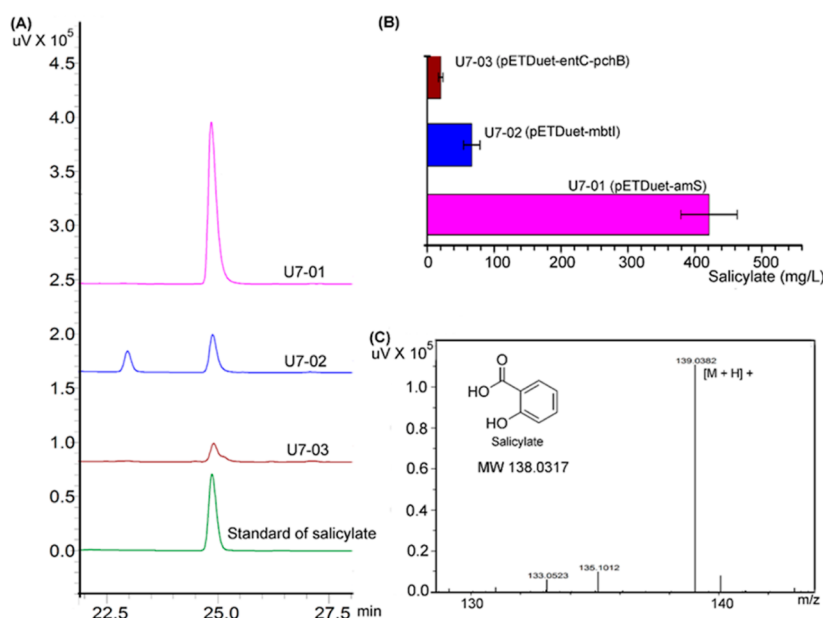
Figure 2. Identification of optimal salicylate synthase for salicylate biosynthesis. (A) HPLC analysis of recombinant strain fermentation broth and standard salicylate. (B) Production of salicylate in recombinant strains with different salicylate synthases expressed. (C) Mass analysis of salicylate produced by recombinant strains. Three replicates were performed, and the error bars represented standard deviation.

200 rpm. Then, 1 mL of seed culture was transferred into 50 mL of fresh LB medium with kanamycin and cultivated at 37 °C. Until the cell density reached 0.6–0.8 (OD_{600}), a final concentration of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) was added to the medium to induce gene expression at 16 °C for 12–16 h. The cells were harvested by centrifugation at 4000 rpm and resuspended in 50 mL of M9Y medium. Then, the substrate salicyl alcohol was added into the cultures at a final concentration of 2 mM. After 48 h cultivation at 30 °C at 220 rpm, the supernatant was sampled and analyzed by HPLC, LC-HRMS, and NMR. The data generated in this study were collected from three independent experiments.

Culture Conditions for Heterologous Production. M9Y medium was used for *de novo* production of salicylate, salicyl alcohol, and salicin. Modified M9Y medium was used for medium optimization to further improve salicin production. Then, 0.5 mL overnight seed cultures of recombinant strains in LB were respectively transferred into 50 mL of fresh M9Y or modified M9Y medium and cultivated at 30 °C with shaking at 270 rpm. After 4 h of cultivation, 0.1 mM IPTG was added to the medium. The cultivation was continued for 72 h at 30 °C with shaking at 270 rpm. Samples were taken at regular time intervals for HPLC or LC-HRMS analysis. The data generated in this study were collected from three independent experiments.

Chemical Analysis and Quantification. The target metabolites of the recombinant strains were analyzed by HPLC, LC-HRMS, and NMR. A SilGreen C18 column (4.6 × 250 mm, 5 μm) (Greenherbs, Beijing, China) was utilized for the HPLC analysis on a Shimadzu LC-20AD HPLC system, which was equipped with an SPD-M40 PDA detector. Solvent A was water with 0.1% formic and solvent B was methanol. The flow rate was 1 mL/min, and the gradient program was set as follows: 0–25 min, 10–50% B; 25–26 min, 50–100% B; 26–36 min, 100% B; 36–37 min, 100–10% B; and 37–47 min, 10% B. The standard calibration curves were generated with a series of known concentrations of standards. All

experiments were carried out in triplicate. The titers are presented as means ± SD. LC-HRMS analysis was carried out on an Agilent 1260 HPLC system with a Bruker micro-Q-TOF II mass spectrometer equipped with an electron spray ionization probe. The analysis programs were identical to HPLC analysis.

Fermentation broth (1 L) was centrifuged for isolation of salicin. The supernatant was incubated with resin (SP-825) for 2 h, and the resin was collected and washed with distilled water (two column volumes). Then, the resin was washed with ethanol. The 30% ethanol eluate was collected and concentrated under reduced pressure. The residue was dissolved in 2 mL of methanol and further purified by semi-preparative HPLC on a Shimadzu LC-6 AD with SPD-20A detector (solvent A = 0.1% methanoic acid in H_2O, solvent B = methanol, the gradient program was the same as mentioned above, and flow rate = 4 mL/min). The ^{1}H NMR spectrum for salicin was collected on a Quantum-I 400 MHz spectrometer (Q.One Instruments Ltd., Wuhan, China). D_2O was used as the solvent.

■ RESULTS AND DISCUSSION

Construction of the Salicylate Biosynthesis Pathway. In this study, construction of recombinant strains was performed using U7 as the starting strain. In some bacteria, isochorismate synthase (ICS) and IPL convert chorismate to salicylate. EntC (ICS) from *E. coli* and PchB (IPL) from *P. fluorescens* have better activities than their homologues MenF (ICS), PchA (ICS), or PfPchB (IPL).[31] The co-expression of EntC and PchB was used to establish the biosynthetic pathway of salicylate in *E. coli*.[20,32,33] Alternatively, single enzymes including MbtI from *M. tuberculosis* and AmS from *A. methanolica* convert chorismate into salicylate in one step. To select suitable enzymes for salicylate biosynthesis, a single gene of *amS* or *mbtI* under T7 promoter control was introduced into recombinant *E. coli* strain U7, generating U7-01 and U7-02. The *entC-pchB* were also expressed in *E. coli*
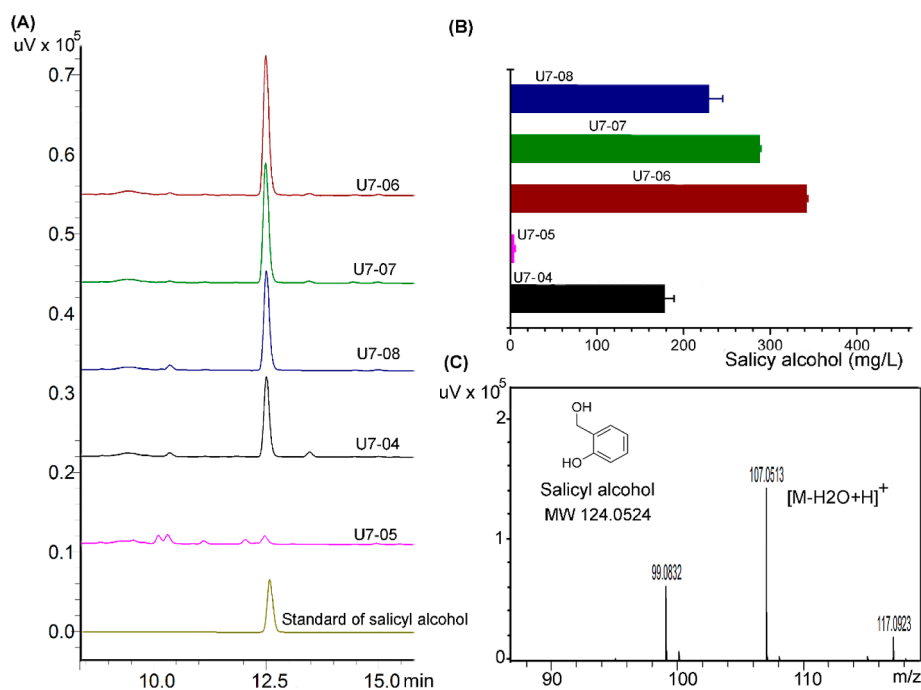
Figure 3. Selection of optimal CARs for salicyl alcohol biosynthesis. (A) HPLC analysis of recombinant strain fermentation broth and standard salicyl alcohol. (B) Production of salicyl alcohol in recombinant strains with CARs expressed. (C) Mass analysis of salicyl alcohol produced by recombinant strains. Three replicates were performed, and the error bars represented standard deviation.

strain U7, resulting in a strain U7-03. After cultivation in M9Y medium for 72 h, all the strains showed similar growth cell density at the end of 72 h. HPLC and LC-HRMS analysis of metabolites in fermentation broth showed that there was a salicylate peak (t_R = 25 min) with the molecular ion at m/z 139.0382 $\{[M + H]^+\}$ produced by these three strains, as shown in Figure 2A,C. The quantity of salicylate produced by these recombinant strains is shown in Figure 2B. The recombinant strain U7-01 carrying *amS* produced 421.2 ± 42.1 mg/L salicylate. The salicylate production by U7-02 with MbtI expression was 66.4 ± 12.3 mg/L, which was much lower than that of U7-01. The co-expression of EntC and PchB in *E. coli* showed low catalytic efficiency to synthesize salicylate, and the U7-03 strain produced only 20.1 ± 3.4 mg/L salicylate. The catalytic efficiency of AmS was much higher than that of MbtI or EntC/PchB and was the optimal enzyme for converting chorismate to salicylate in chassis strain U7.

The k_{cat}/K_m value of Irp9[34] from *Y. enterocolitica* was higher than that of AmS.[22] In our study, Irp9 under T7 promoter control was also introduced into recombinant *E. coli* strain U7, generating strain U7-10. Qi et al. (2018) reported that the strain containing Irp9 expression in the higher-copy number T7 system showed relatively lower salicylate glucoside production and higher plasmid loss.[35] In our work, the cell death appeared in some flasks with white precipitate during fermentation of the strain U7-10 containing plasmid pETDuet-T7-irp9 in M9Y medium. Occasionally, the U7-10 strain could grow normally and produce 500.8 ± 13.7 mg/L salicylate, as shown in Supporting Figure S5. In view of cell growth stability, we selected AmS as the optimal salicylate synthase for pathway construction.

CAR Selection for Salicyl Alcohol Biosynthesis. CARs catalyze the reduction of a carboxylic acid to an aldehyde, together with a phosphopantetheine transferase (producing an active CAR). CARs were found in many different organisms, including bacteria, fungi, and plants and display a broad substrate scope, varying from aromatic/hetero-aromatic acids to aliphatic acids. We sourced five CARs, including CARni from *N. iowensis*, CARmm from *M. marinum*, CARse from *S. rotundus,* and CARms from *Mycobacterium smegmatis*, which were reported to be capable of catalyzing different aromatic substrates to their corresponding aldehydes. In this work, these CARs were selected as candidates to synthesize salicylaldehyde. The CARmm has been used to construct the salicyl alcohol biosynthetic pathway in *E. coli* by Shen et al.[20] Plasmids containing *amS*, CAR genes from different sources, and *sfp* from *Bacillus* under the control of the T7 promoter were individually transferred into U7, yielding a series of U7 strains. After these strains were cultured in M9Y for 72 h, fermentation broths were sampled for HPLC and LC-HRMS analysis. In *E. coli*, salicylaldehyde can be readily reduced to salicyl alcohol by endogenous alcohol dehydrogenases. As the results shown in Figure 3A,C, salicyl alcohol was detected in fermentation broth of these recombinant strains, with t_R = 12.5 min and the molecular ion peak at m/z 107.0513 $[M-H_2O + H]^+$, which indicated that all the five CARs could convert salicylate into salicylaldehyde. Salicyl alcohol production by shake flask cultivation is shown in Figure 3B. The recombinant strain U7-06 with CARse produced 342.0 ± 1.6 mg/L salicyl alcohol (Figure 3B), which was 87 times higher than that produced by U7-05 with CARmm. Titers of salicyl alcohol produced by other three recombinant strains were also lower than those of U7-06 (Figure 3B). Thus, the CARse from *S. rotundus* was the optimal enzyme for producing salicyl alcohol in this work and was selected for subsequently constructing the salicin biosynthetic pathway.

UGT Selection for Salicin Biosynthesis. Uridine sugar glycosyltransferases (UGTs) catalyze glucosylation of salicyl alcohol to form salicin. Sv0189 from *S. venezuelae* is a promiscuous glycosyltransferase, and planar polyaromatic
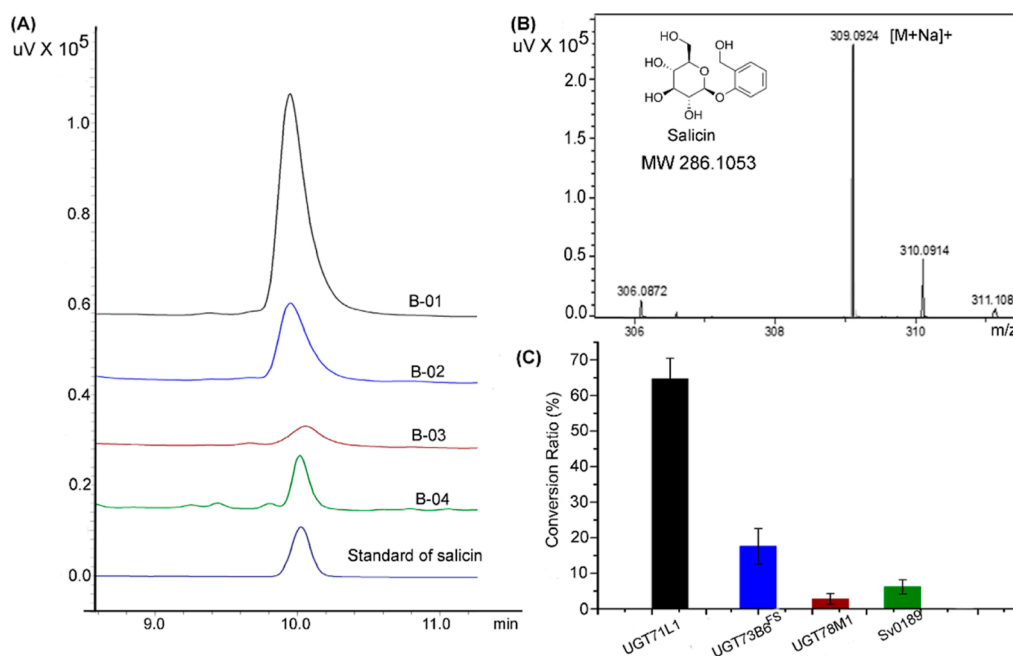
Figure 4. Comparison of biotransformation of salicyl alcohol with formation of salicin employing different UGTs with 2 mM substrate feeding. (A) HPLC analysis of recombinant strain fermentation broth and standard salicin. (B) Mass analysis of the salicin produced. (C) Bio-conversion rates of different UGTs to salicyl alcohol. Three replicates were performed, and the error bars represented standard deviation.

phenols were identified as its best substrates.[36] UGT73B6 from *R. sachalinensis* could glucosylate both aromatic and aliphatic alcohols of tyrosol, and its mutant UGT73B6[FS] selectively glucosylated aromatic alcohol of 4-hydroxybenzyl alcohol with high efficiency. Given the similarity in structures of these substrates to salicyl alcohol, we hypothesized that Sv0189 and UGT73B6[FS] might be able to catalyze the glucosylation of salicyl alcohol. In 2020, Fellenberg et al.[19] reported that UGT71L1 and UGT78M1 from poplar accept salicyl alcohol as glucose acceptors.[37] In this work, these four UGTs were tested by feeding salicyl alcohol as the aglycon to screen a more efficient UGT for salicin biosynthesis in *E. coli* BL21 (DE3). Genes encoding for the above-selected UGTs were cloned into pET28a. The transformants were cultured and fed with 2 mM salicyl alcohol. After 48 h of culturing, fermentation broths were sampled. HPLC analysis of metabolites showed that there was a new product with t_R = 10.0 min produced by all the recombinant strains, as shown in Figure 4A. LC-HRMS analysis showed that the new product had a molecular ion at m/z 309.0924 [M + Na]⁺, which was identical to that of the salicin standard (Figure 4B). Subsequently, the structure of this compound was further confirmed as salicin by ¹H NMR spectroscopy analysis (Supporting Figure S2) and comparison with the salicin standard.[38] The titers of salicin were 370.4 ± 33.2, 100.8 ± 20.3, 16.0 ± 3.2, and 35.5 ± 5.3 mg/L produced by strains B-01, B-02, B-03, or B-04, respectively. The conversion ratios of salicyl alcohol into salicin by UGTs are shown in Figure 4C. UGT78M1 and Sv0189 had conversion ratios under 10%. UGT73B6[FS] showed a conversion rate at about 20%. The substrate conversion rate of UGT71L1 was 64.7 ± 5.8%, which was 23 times higher than that of UGT71M1. The results demonstrated that UGT71L1 exhibited much higher enzymatic activity than the other three UGTs and was chosen for salicin biosynthesis.

De Novo Biosynthesis of Salicin in E. coli and Production Improvement. Salicin biosynthesis by the cell

factory approach has not been reported yet. In this work, the selected enzymes in the salicin biosynthesis including AmS, CARse-*Sfp*, and UGT71L1 were assembled into the U7 chassis strain. The recombinant strain U7-09 harboring pETDuet-amS-CARse-*sfp*-71L1 was cultured in M9Y medium. As shown in Figure 5, U7-09 carries the entire pathway-synthesized 368.7
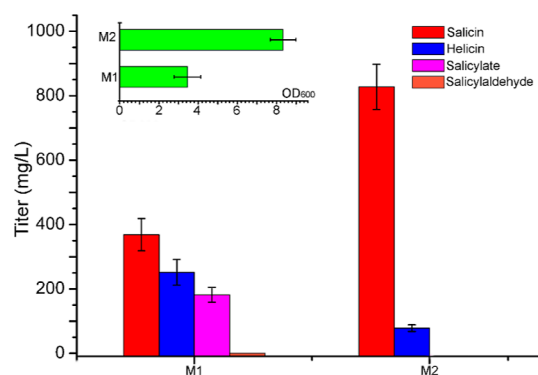

Figure 5. Titers of main metabolites with strain U7-09 in M9Y medium (M1) and modified M9Y medium with 20 g/L glycerol (M2). Three replicates were performed, and the error bars represented standard deviation.

± 50 mg/L salicin after 72 h of fermentation. Herein, we achieved the *de novo* production of salicin in *E. coli*. HPLC analysis showed that there was a peak with t_R = 12.0 min, as shown in Supporting Figure S3. LC-HRMS analysis showed that the new metabolite had a molecular ion peak at m/z 307.0805 [M + Na]⁺, which was identical to that of the helicin standard. The titer of the byproduct helicin reached 251.8 ± 40.0 mg/L in broth. About 181.5 ± 23.0 mg/L salicylate and 28.8 ± 4.0 mg/L salicylaldehyde were accumulated in broth. The final OD₆₀₀ of U7-09 reached 3.5 ± 0.6 in M9Y medium. When we enhanced yeast extract concentration to 5 g/L in
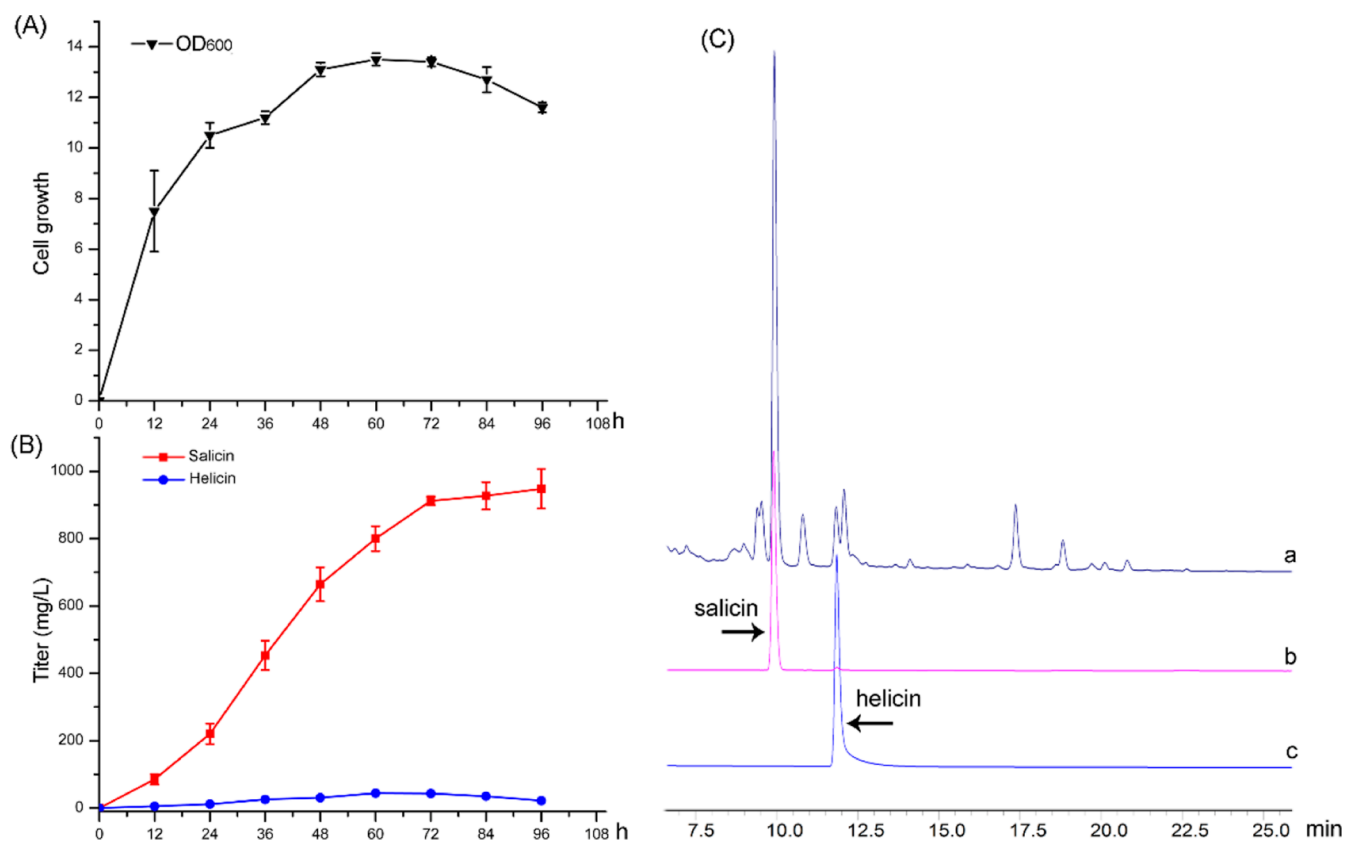
Figure 6. Strain U7-09 was fermented in modified M9Y medium with 20 g/L glycerol and 5 g/L glucose. (A) Time profiles of cell density. (B) Time profiles of salicin and helicin titers. (C) HPLC analysis (detected at 269 nm) of metabolites in broth (a), the standard of salicin (b), and the standard of helicin (c). Three replicates were performed, and the error bars represented standard deviation.

modified M9Y medium, the OD_{600} of cell density increased to 5.6 ± 0.2, while the production of salicin decreased to 203.0 ± 17.8 mg/L from 368.7 ± 50.0 mg/L, shown in Supporting Figure S4.

E. coli can utilize glucose and glycerol as carbon sources to produce various chemicals. Significant production enhancement could be realized through synergetic utilization of glucose and glycerol, which has been successfully applied to many valuable chemical biosynthesis processes such as myo-inositol,[38] lycopene,[39] N-acetylglucosamine,[40] and so forth. To achieve higher salicin production, the optimization of medium was conducted by varying the carbon sources in modified M9Y medium. First, we cultured strain U7-09 in modified M9Y media with 20 g/L glycerol as the sole carbon resource. After 72 h of fermentation, cell growth and the production of main metabolites were detected, and results are shown in Figure 5. The final OD_{600} of U7-09 improved to 8.3, and the titer of salicin reached up to 827.7 ± 65.0 mg/L. There was no accumulation of salicyl alcohol, salicylaldehyde, and salicylate in broth. The byproduct helicin production decreased to 78.3 ± 10.3 mg/L. Glycerol might be an optimal carbon source to produce salicin during U7-09 fermentation in modified M9Y media. Meanwhile, we speculated that glucose would be beneficial to enhance UDP-glucose biosynthesis in cell. Subsequently, 5 or 10 g/L glucose was respectively supplemented in modified M9Y media with 20 g/L glycerol. The results are shown in Supporting Figure S4. Also, there was no accumulation of salicylate and salicylaldehyde in broth. The time profiles of cell density and titers of main metabolites were made, as shown in Figure 6. After cultivation for 48 h, cell

growth reached the stationary phase with OD_{600} = 13.1 ± 0.2. The production of salicin continued to improve until 72 h. After that, salicin production remained stable. The titer of salicin reached up to 912.3 ± 12.7 mg/L in modified M9Y media with 20 g/L glycerol and 5 g/L glucose. The accumulation of byproduct helicin was low with the highest titer of 43.3 ± 5.1 mg/L. The production of helicin remained low during cultivation. Additionally, the engineered strain U7-09 was also cultured in modified M9Y with 15 g/L glycerol and 5 g/L glucose. The titers of main metabolites are shown as Supporting Figure S4; the production of salicin sharply decreased to 520.6 ± 32.2 mg/L after cultivation for 72 h, although the cell growth had no apparent decline (OD_{600} = 11.5 ± 0.2). Thus, the combination of 20 g/L glycerol and 5 g/L glucose as carbon sources supplemented in modified M9Y was optimal for salicin production.

■ CONCLUSIONS

There is great interest in the development of microbial cell factories for the sustainable production of phytochemicals using renewable carbon resources. In the study, we engineered a recombinant *E. coli* strain for high-level production of salicin. We utilized genetic components from both microbes and plants to construct an artificial pathway for salicin biosynthesis. Specifically, the enzymes including salicylate synthase AmS from *A. methanolica*, carboxylic acid reductase CARse from *S. rotundus,* and the glucosyltransferase UGT71L1 from *P. trichocarpa* were selected for overexpression in a modified *E. coli* strain MG1655-U7. The engineered strain produced 912.3 ± 12.7 mg/L salicin with optimized carbon resources in shake

flask fermentation. The work provides a green and sustainable way for large-scale production of salicin.

■ AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this article and its additional file.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03347.

> Map of pRed_Cas9_recA_T plasmid, ^{1}H NMR spectrum of isolated salicin in D_2O, HPLC analysis of U-09 fermentation broth in M9Y medium and standard, titers of salicin and helicin produced by strains U7-09 in modified M9Y medium with glucose and glycerol, production of salicylate in U-10 and other recombinant strains with different salicylate synthases being expressed, sequences of genes with *E. coli* codon optimization in this study, and primers and N20 sequence used in construction of plasmid pRed_Cas9_-recA_T (PDF)

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Author Contributions

T.L. and H.Y. conceived and supervised the project. H.Y., T.L., and M.Q.Z. wrote the manuscript. M.Q.Z., C.L., and D.Y.X. performed the experiments. Y.B.Z. performed compound analysis. All authors read and approved the final manuscript. All authors commented on the manuscript.

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Notes

The authors declare no competing financial interest.

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