RESEARCH ARTICLE

Heterologous expression of *abx* gene cluster in *E. coli* gives production of 2-heptyl-4(1H)-quinolone

Miao Wang[1, †], Xueli Li[2, 4, †], Xuemei Wang[2, 3], Ruirui Guo[2, 3], Xinyue Sun[2, 3], Rui Sun[2, 3], Zexin Li[1, 2], Lanlin Wang[2, 3], Changwu Yue[1, 2, 3, *], Yuhong Lü[2, 3, *]

[1]Guizhou Key Laboratory of Microbial Resources and Drug Development, Zunyi Medical University, Zunyi 563003, Guizhou, China. [2]Yan'an Key Laboratory of Microbial Drug Innovation and Transformation, School of Basic Medicine, Yan'an University, Yan'an 716000, Shaanxi, China. [3]Shaanxi Engineering & Technological Research Center for Conversation & Utilization of Regional Biological Resources, Yan'an University, Yan'an 716000, Shaanxi, China. [4]Shaanxi Key Laboratory of Chemical Reaction Engineering, College of Chemistry and Chemical Engineering, Yan'an University, Yan'an 716000, Shaanxi, China.

Received: June 30, 2021; **accepted:** August 27, 2021.

Anthrabenzoxocinones (ABX) are a class of type II aromatic polyketides, which were derived from actinomycetes, with unique chemical structure and important biological activities. Several ABX biosynthesis gene clusters (*abx*) from *Streptomyces* sp. FJS31-2, *Streptomyces* sp. FXJ1.264, *Streptomyces* sp. MA66575, and *Actinomycete* MA7150 have been cloned. Ten (10) genes from *abx* gene clusters including the structural skeleton synthesis genes of *abx*P, *abx*K, and *abx*S and the modification genes of *abx*A, *abx*O, *abx*R, *abx*P, *abx*K, *abx*S, *abx*C, *abx*D, *abx*H, and *abx*M have been identified to be related to the biosynthesis of ABXs and some of them may also involve in the biosynthesis of the 2-heptyl-4(1H)-quinolone (HHQ), a precursor and a signal molecule of quinolone. In order to further clarify the biological functions of these genes and provide theoretical and experimental basis for the biosynthesis of active natural products, those 10 *abx* genes were cloned into pET32a vector and transformed into *E. coli* Rosetta (DE3), respectively. Among the six genetic engineering bacteria strains with tandem combinations of different genes, the engineered strain pabxAORPKSCDHMRo resulted in obvious biosynthesis of HHQ while no such chemical was detected in the other five engineering strains. Since there is no report that the HHQ compounds were biosynthesized from genetic engineered *E. coli* bacteria, we report, for the first time, the production of HHQ by heterologous tandem expression of the full length *abx* gene cluster from a *Streptomycete* in *E. coli* bacteria, which may pave a way for biosynthesis of quinoline compounds in future.

Keywords: *abx* gene cluster; heterologous expression; 2-n-heptane-4-hydroxyquinoline; biosynthesis.

[*]**Corresponding author:** Changwu Yue, Guizhou Key Laboratory of Microbial Resources and Drug Development, Zunyi Medical University, Zunyi 563003, Guizhou, China. Phone: +86 911 233 2067. Email: changwuyue@126.com. Yuhong Lü, Yan'an Key Laboratory of Microbial Drug Innovation and Transformation, School of Basic Medicine, Yan'an University, Yan'an 716000, Shaanxi, China. Email: yuhonglyu@126.com.

[†]These authors contributed equally.

Introduction

As the important class of N-based heterocyclic compounds, quinoline moiety offers an easily access, well-understood scaffold, and the potency for structure optimization synthetic pathway for new drugs designing and pharmaceutical synthesis. Since the identification and purification of quinine from Cinchona bark in 1820, quinolines and their

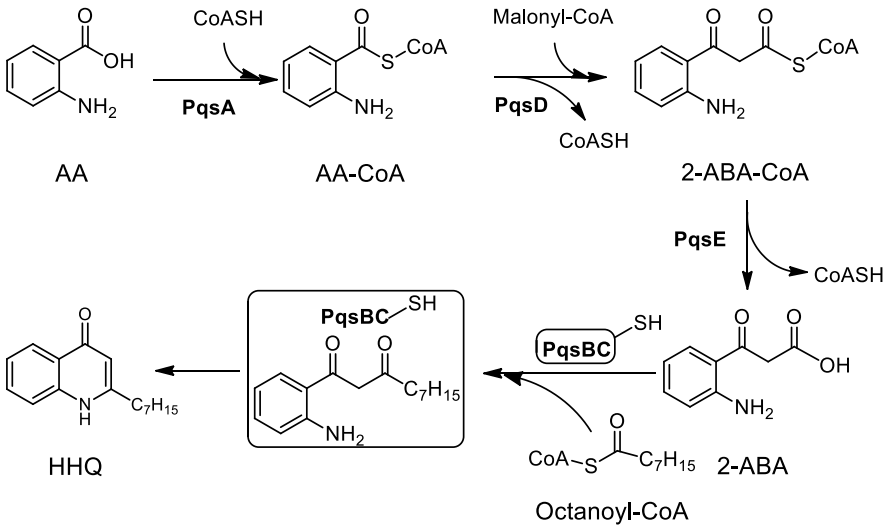

Figure 1. Proposed pathway for HHQ biosynthesis in *Pseudomonas aeruginosa*.

derivatives have attracted tremendous attention of researchers worldwide [1, 2]. Over the past 200 years, many quinoline derivatives with a broad range of bioactivities including antitumor, antimalarial, antibacterial, antifungal, antiparasitic, insecticidal, antiviral, antiplatelet, anti-inflammatory, herbicidal, antioxidant, and other activities have been isolated from natural sources [3-7]. Several different 4-hydroxyquinoline derivatives that predominantly exist as 4(1H)-quinolone and form the core structure of several alkaloids with medicinal properties were isolated from plant, animal, and bacterial species. Among them, 2-alkyl-4(1H)-quinolone and 2-heptyl-3-hydroxy-4(1H)-quinolone were isolated from *Pseudomonas aeruginosa* and related bacteria [8, 9]. Recently, the pqsABCDE operon that encodes 2-heptyl-4(1H)-quinolone (HHQ) biosynthetic proteins responded to the biosynthesis of HHQ and its derivatives was characterized, and the molecular biosynthesis mechanisms were discovered from the opportunistic pathogen *Pseudomonas aeruginosa* [10-14]. It has been known that anthranilic acid (AA) is a precursor of HHQ and is activated into anthraniloyl-coenzyme A (AA-CoA) by anthranilate-coenzyme A ligase (pqsA), and then, is condensed with malonyl-CoA to form 2-aminobenzoylacetyl-CoA (2-ABA-CoA) by pqsD [15]. The thioesterase (pqsE) and other thioesterases of *Pseudomonas aeruginosa* catalyze the hydrolysis of 2-ABA-CoA to 2-aminobenzoylacetate (2-ABA), which is the branch point metabolite in HHQ biosynthesis as it is shuttled into different reaction pathways. However, in the main biosynthetic route of HHQ, the heterodimeric pqsBC complex transfers 2-ABA onto octanoate to synthesize HHQ in a decarboxylative Claisen condensation after an acyl-enzyme intermediates pqsC and octanoyl CoA. In a final step, HHQ is hydroxylated at carbon position C3 by the monooxygenase pqsH [16-17] (Figure 1). As a precursor and a signal molecule of the *Pseudomonas aeruginosa* quinolone, HHQ has been considered to be the important target of anti-*Pseudomonas aeruginosa* infection drugs and important pharmaceutical and chemical raw materials [18]. In addition, HHQ is mainly synthesized by the opportunistic pathogen *Pseudomonas aeruginosa*, which is considered necessary for engineered biosynthesis of HHQ.

Anthrabenzoxocinones (ABX) are a class of type II aromatic polyketides, which were derived from actinomycetes, with unique chemical structure and important biological activities. In our previous studies, biosynthesized *abx* gene cluster

Table 1. Primers for colony PCR reactions.

Primer	Sequence 5'-3'	DNA fragments name	Products length (nt)
AORR	ATCAGCAGCACCATCTTCG	*abx*AOR	214
AORF	GCCGGGCCCAGGCGCCCGC		
HMR	AAGGAGTTCTTCGCCTACC	*abx*HM	245
HMF	CAGCAGGTTCTTCAGGATG		
PKSCDR	ACCAAGGGCTTCTGGGAG	*abx*PKSCD	268
PKSCDF	GAGGTGATGGGCGAGATG		

(accession no. KU243130) which encodes anthrabenzoxocinone biosynthetic proteins respond to the biosynthesis of Zunyimycin A, B, C, and their derivatives from *Streptomyces* sp. FJS31-2 were characterized [19, 20]. In this study, prokaryotic expression plasmids containing different genes from *abx* gene cluster were constructed and transformed into *E. coli* bacterial strains. Tandem expression of the full length of *abx* gene cluster in *E. coli* was examined to clarify the biological functions of these genes and provide theoretical and experimental basis for the biosynthesis of active natural products.

Material and methods

Construction of expression plasmids

In our former studies, *Streptomyces* sp. FJS31-2 containing *abx* gene cluster was isolated from a soil sample collected from the Fanjing Mountain of the Guizhou Province, China and deposited into China General Microbiological Culture Collection Center (accession no.: CGMCC 4.7321). Biosynthesis of *abx* gene cluster (accession no.: KU243130) which encodes anthrabenzoxocinone biosynthetic proteins for the biosynthesis of Zunyimycin A, B, C, and their derivatives has been characterized [19-20]. The genomic DNA of *Streptomyces* sp. FJS31-2 used for gene cloning was extracted directly from the liquid culture of International *Streptomyces* Project-2 medium (ISP-2) (4 g of Yeast extract, 10 g of Malt extract, 4 g of Dextrose, 20 g of Agar, in 1 L dH_2O) [21]. Molecular cloning manipulation was carried out according to standard protocols described by Sambrook and Russell [22]. For construction of recombinant expression plasmids, primers were designed based on the draft genome sequence of *Streptomyces* sp. FJS31-2. The predictive analysis of secondary metabolite gene clusters was carried out by using antiSMASH database (https://antismash.secondarymetabolites.org/) [23]. Full length open reading frames (ORF) of *abx*AOR (tandem combination of ORFs of *abx*A, *abx*O, *abx*R), *abx*PKSCD (tandem combination of ORFs of *abx*P, *abx*K, *abx*S, *abx*C, *abx*D), and *abx*HM (tandem combination of ORFs of *abx*H and *abx*M) with ribosome binding site (RBS) region (5'-AAGGAG-3') and restriction cloning sites (*abx*AOR: Bgl II (5') and Spe I (3'); *abx*PKSCD: SpeI (5') and Xho I (3'); *abx*HM: Xho I (5') and Hind III (3')) were chemical synthesized by Beijing Invitrogen Biotechnology Co., Ltd (Beijing, China) and were inserted into pGEM-T easy cloning vector (Promega, Beijing, China). Different combinations of ORFs were then excised by restriction enzymes and purified with DNA Gel Extraction kit (Axygen, Hangzhou, Zhejiang, China) followed by inserted into expression plasmid pET32a (Novagen, Madison, Wisconsin, USA) and named as pabxHM, pabxAOR, pabxPKSCD, pabxAORPKS, pabxPKSCDHM, and pabxAORPKSCDHM, respectively, in order to acquire the recombinant expression plasmids (Figure 2), and then, transformed into *E. coli* JM109 competent cells. A sterile micropipette tip was used to transfer a few cells from each colony to a corresponding polymerase chain reaction (PCR) tube. The recombinant expression plasmids were confirmed by colony PCR (Axygen, Hangzhou, Zhejiang, China) with gene specific primers (Table 1), and SapphireAmp Fast PCR Master Mix (Takara, Dalian, Liaoning, China). Briefly, one colony was picked up by using a sterile toothpick and mixed with 0.25 µL of Ex Taq

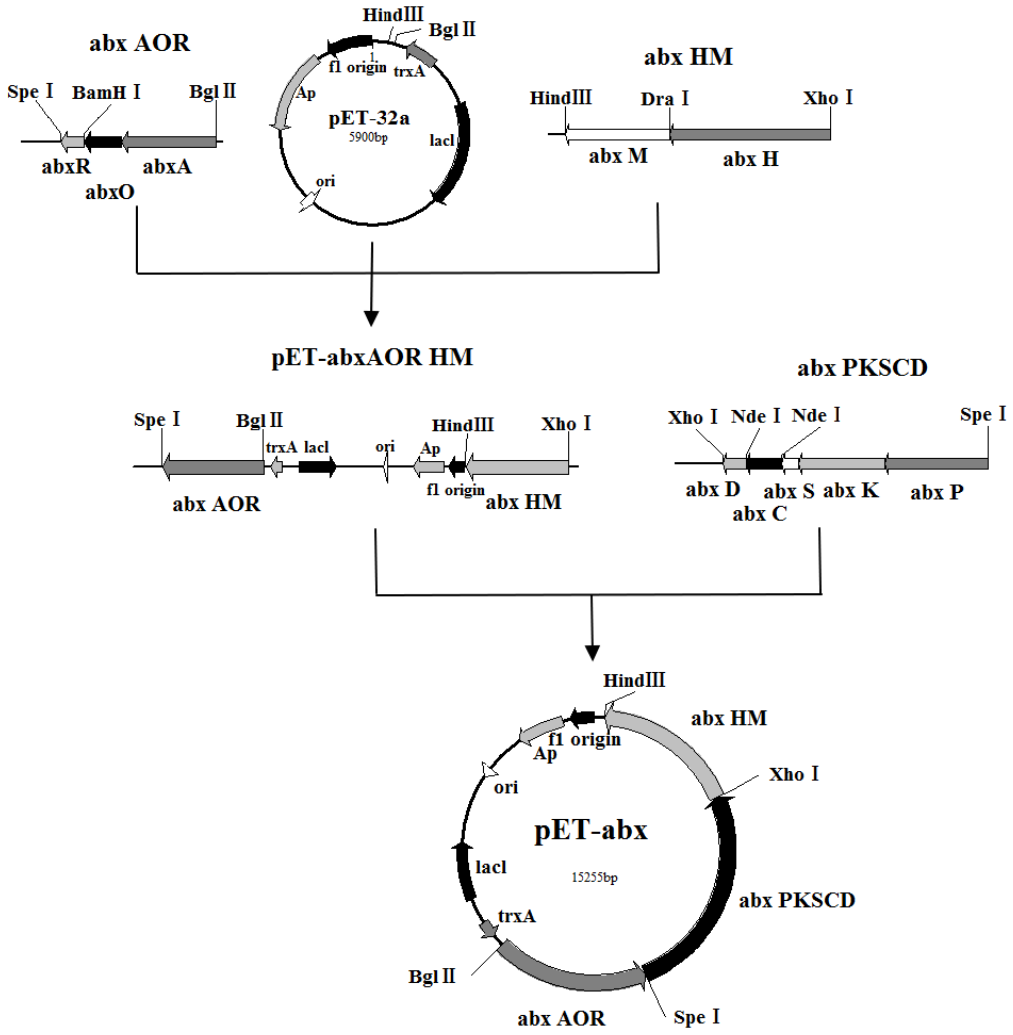

Figure 2. Construction strategies of tandem expression plasmids.

DNA Polymerase (5 units/μL) and 20 pmol of PCR primers in 25 μL reaction mixture. The reaction was performed by using C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the program as 94°C for 2 mins followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and finally 72°C for 5 mins. PCR products were detected on 1.5% agarose gel. The positive clone was double confirmed by digestion with restriction enzymes (Takara, Dalian, Liaoning, China) (Table 2) and then DNA sequencing (Beijing Invitrogen Biotechnology, Beijing, China).

Biosynthesis, isolation, and chemical identification

Plasmid pET32a and the recombinant expression plasmids pabxHM, pabxAOR, pabxPKSCD, pabxAORPKS, pabxPKSCDHM, and pabxAORPKSCDHM were transformed into *E. coli* Rosetta (DE3) competent cells, respectively, and the transformants were named as pabxHMRo, pabxAORRo, pabxPKSCDRo, pabxAORPKSRo, pabxPKSCDHMRo, and pabxAORPKSCDHMRo, respectively. The engineered strain was inoculated into 100 mL of LB liquid medium containing 1% glucose and 100 μg/mL of ampicillin and grew at 28℃ with shaking at 110 rpm. Cells were harvested at OD_{600} = 0.3 by centrifugation at 4,000 rpm for 5 min followed by washing the pellets five times with 10 mL of LB

Table 2. Restriction sites used for DNA fragments cloning.

3' restriction sites	DNA fragments name	5' restriction sites
Spe I	*abx*AOR	Bgl II
BamH I	*abx*AO	Bgl II
Hind III	*abx*HM	Xho I
Dra I	*abx*H	Xho I
Hind III	*abx*M	Dra I
Xho I	*abx*PKSCD	Spe I
Xho I	*abx*D	Nde I
Nde I	*abx*D	Nde I
Nde I	*abx*PKS	Spe I
Xho I	*abx*AORPKSCD	Bgl II
Hind III	pabxAORPKSCDHM	Bgl II

medium. The precipitates were then resuspended in 100 mL of LB medium containing 100 μg/mL ampicillin and shook at 18℃ to induce the target protein expression. For the detection of targe protein expression, precipitates of 1 mL culture broth were harvested by centrifugation at 12,000 rpm for 5 min after fermentation for 3 h and subjected to SDS-PAGE electrophoresis. Engineered strains and pET32aRo were fermented and analyzed in parallel.

After product detection, engineered strain pabxAORPKSCDHMRo was cultured by using 500 mL flask containing 150 mL of LB with 100 μg/mL ampicillin and shook at 18℃ overnight for the full expression and correct folding of the target protein to ensure the full synthesis of the target compound. The culture was mashed and extracted three times with 150 mL ethyl acetate after cultivation. The organic portion was then concentrated with Strike 300 *in vacuo* (SteroGlass, Perugia (PG), Italy) to remove the solvent. The crude extract was applied to silica gel column chromatography using the CHCl3/MeOH (Sigma-Aldrich, St. Louis, MO, USA) gradient to obtain the crude products. Further purification was conducted by using Sephadex LH-20 (GE Healthcare, Tokyo, Japan) MeOH column and Reversed-phase High Performance Liquid Chromatography (RP-HPLC) (Shimadzu SPD-M20A with Xbridge ODS 10 mm × 150 mm column). Compounds were identified with a High-resolution Mass Spectrometry (HRESI-MS) (Waters Xevo G2 QTOF mass spectrometer, Waters corporation, Milford, MA, USA) and analyzed by using a Bruker AV 600 MHz nuclear magnetic resonance (NMR) (Bruker Corporation, Karlsruhe, Germany).

Results

Construction of engineering strains and biosynthesis in flask cultures

After the complete coding region with RBS region and restriction sites for ABX synthesis and modified genes were obtained, *abx*A, *abx*O, *abx*R, *abx*P, *abx*K, *abx*S, *abx*C, *abx*D, *abx*H, and *abx*M were inserted into expression plasmid pET32a with different gene combinations. Six recombinant plasmids were constructed. The recombinant plasmids were then transformed into *E. coli* Rosetta (DE3) competent cells, and twelve engineering strains named pabxHMRo, pabxAORRo, pabxPKSCDRo, pabxAORPKSRo, pabxPKSCDHMRo, pabxAORPKSCDHMRo were identified by colony PCR and DNA sequencing. After fermented, the yielded products were extracted and analyzed by HPLC. The results showed that, among the twelve engineering strains, a unique peak was detected in pabxAORPKSCDHMRo while no analogues were found in the other eleven strains, which indicated that a novel product was biosynthesized by strain pabxAORPKSCDHMRo (Figure 2).

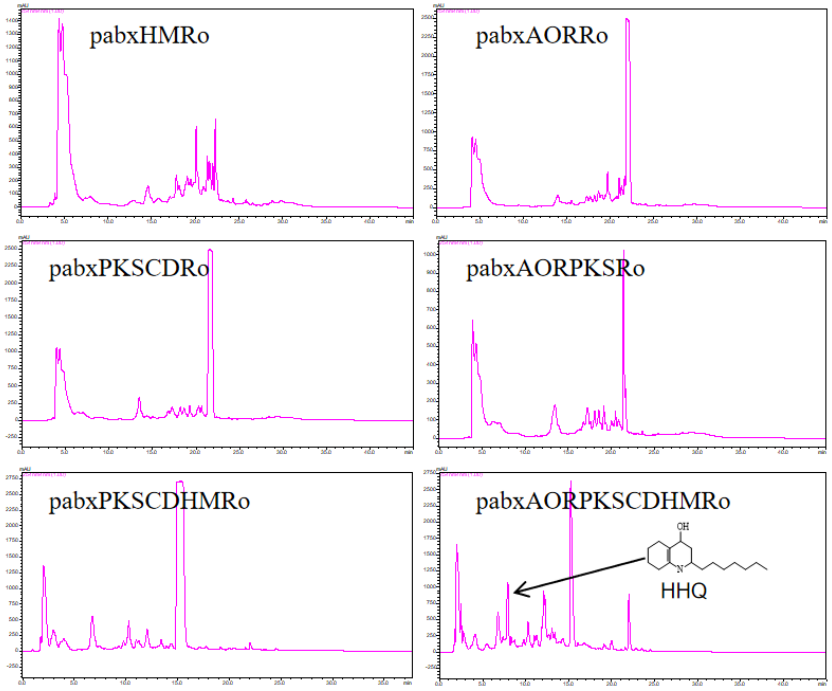

Figure 3. Biosynthesis of HHQ.

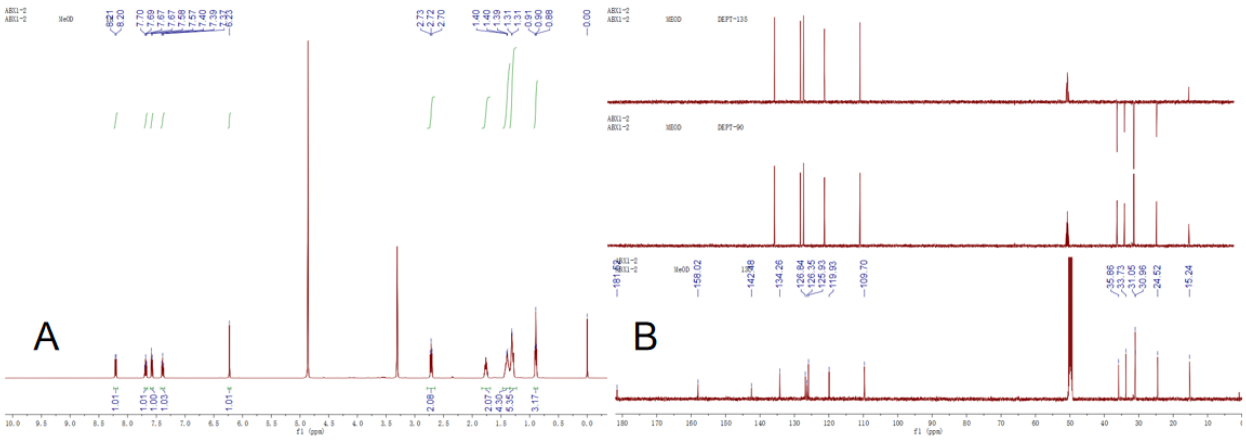

Figure 4. 1H-NMR spectra (A) and 13C-NMR spectra (B) of HHQ.

Chemical identification of HHQ

After chromatography, 100 mg of compound was obtained from strain pabxAORPKSCDHMRo. The NMR data of the two compounds were obtained in MeOD solvent (Figure 4). Compound was obtained as a pale white powder. The 13C-NMR spectra of compound displayed signals for sixteen aromatic carbons at δC ^{13}C NMR (100 MHz, $CDCl_3$). The values of δ_C were 179.1 (C-4), 155.5 (C-2), 140.9 (C-8a), 132.0 (C-5), 125.5 (C-6), 125.2 (C-4a), 123.8 (C-7), 118.8 (C-8), 108.3 (C-3), 34.6 (C-1′), 31.8 (C-5′), 29.4 (C-2′), 29.3 (C-3′), 29.2 (C-4′), 22.8 (C-6′), and 14.2 (C-7′). In addition, the 1H-NMR spectra of compound displayed signals at 12.32 (1H, brs, OH-4), 8.35 (1H, t, *J*=7.6 Hz, H-5), 7.58 (1H, t, *J*=6.8 Hz, H-6, H-

8), 7.32 (1H, t, *J*=7.6 Hz, H-7), 6.24 (1H, brs, H-3), 2.68 (2H, brs, H-1′), 1.69 (4H, d, *J*=6.4 Hz, H-2′, H 6′), 1.23 (6H, m, H-3′, H-4′, H-5′), and 0.80 (3H, t, *J*=7.2 Hz, H-7′). The results demonstrated that the constituent was HHQ [24].

Discussion

Several previous studies reported that HHQ and its derivatives were biosynthesized by the pqsABCDE operon in the opportunistic pathogen *Pseudomonas aeruginosa* and anthranilic acid was identified as the precursor of HHQ [25]. However, there is no report about HHQ compounds biosynthesized by genetic engineered *E. coli* bacteria. In this study, we report, for the first time, the production of HHQ by heterologous tandem expression of the full length *abx* gene cluster from a *Streptomycete* strain in *E. coli*. In order to elucidate the biological mechanism of HHQ in engineered strain, six recombinant expression plasmids were constructed and transformed into *E. coli* BL21 (DE3) plysS or *E. coli* Rosetta (DE3) and fermented under the same conditions, respectively. The HPLC analytical results of the product crude extracts suggested that *E. coli* Rosetta (DE3) was the better host bacteria for HHQ biosynthesis than that of *E. coli* BL21 (DE3) plysS. The coenzyme A ligase from *E. coli* Rosetta (DE3) may play an important role for converting substrate into AA-CoA since there is no anthranilate-coenzyme A ligase or its homologues responsible for the generation of anthraniloyl-CoA in recombinant expression plasmids. The *abx* gene cluster which encodes anthrabenzoxocinone biosynthetic proteins has been confirmed to play an important role in the biosynthesis of natural antimicrobial products in prokaryotes. From the results of this study, at least four enzymes from *abx* gene cluster were believed involving in the biosynthesis of HHQ. Among them, AbxS may involve in synthesis of AA-ACP followed by condensed with malonyl-CoA to 2-aminobenzoylacetyl-Enz (2-ABA-Enz) under the enzymes of AbxP and AbxK before additional condensed with octanoyl-CoA by AbxP and AbxK. In a final step, under the synergy of AbxO, AbxP, and AbxK, intra-molecular witting reaction were performed to form HHQ (Figure 5).

Quinoline and its derivatives are chemicals with rich pharmacological activities [26, 27]. The applications of various quinoline compounds containing substituents in medicine, biology, food, and other fields are more and more recognized by people [28, 29]. Therefore, whether it is used as a kind of drug synthesis intermediates or as its own, the research on its synthesis method will attract the interest of researchers [30]. There are many synthetic methods for 4-hydroxyquinoline compounds, and many new compounds with high activity and high selectivity that can be used for pharmaceutical development have been identified [31]. The synthesis of quinoline derivatives containing various substituents and the industrialization of those synthetic methods are both the directions of the synthetic technique developers [32]. At present, the harm of various diseases to human beings is becoming more and more serious all over the world. Bacterial infection, tumor, and some incurable diseases are serious threats to human health [33]. New drug development with broad-spectrum, high activity, high safety, low toxicity, and environmentally friend to provide continuous prevention and treatment for human diseases is the main task of pharmaceutical scientists [34]. It is believed that people will find new methods and technologies to synthesize 4-hydroxyquinoline chemical compounds, optimize the production process, obtain target compounds more efficiently, enrich the content of organic synthesis methodology, and apply 4-hydroxyquinoline compounds in a wider range of fields [35].

This study is the first-time report about fermentation of microorganism to produce HHQ. Although it still needs to investigate what is behind the phenomenon of heterologous expression of tandem abxAORPKSCDHM evoking the form of HHQ in *E. coli* Rosetta (DE3), the engineered strain fermentation strategy will be

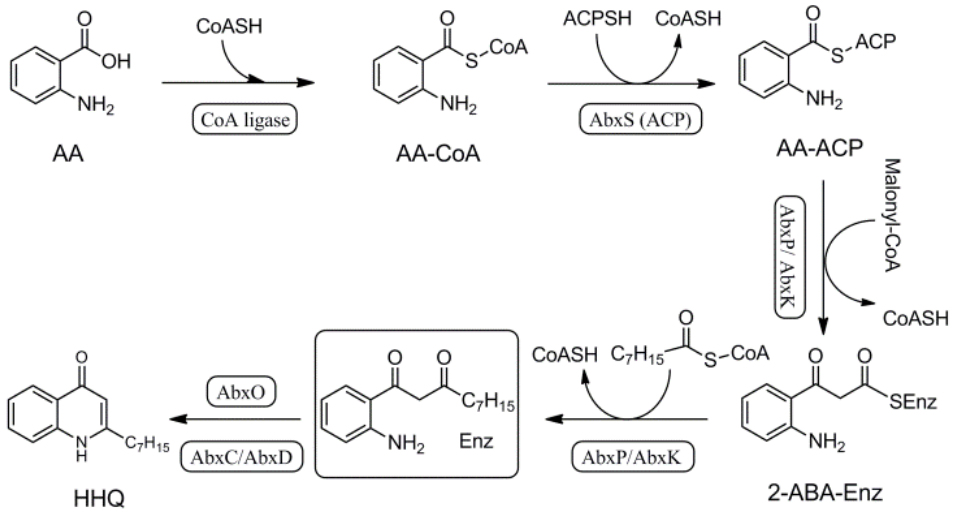

Figure 5. Proposed pathway for the biosynthesis of HHQ in engineering strains.

useful in the development of bio-based production of HHQ due to increasing concerns about global warming and depletion of fossil. Further research works such as optimizing the media and fermentation conditions to reduce the cost and increase the production of HHQ are needed and the strategies in this study will be useful for the development of engineered strain for "green" production of HHQ.

Acknowledgments

This work was partially supported by The National Nature Science Foundation of China (Grant No. 81860653, 82060654), The Science and Technology Foundation of Shaanxi Province (2020JM-550, 2020JM-545, 2020JM-549, and 2021JM-416), Open Project Foundation of Key Laboratory of Noncoding RNA and Drugs in Universities of Sichuan Province (FB19-01 and FB20-02), Open Project Shaanxi Engineering and Technological Research Center for Conversation and Utilization of Regional Biological Resources (sxgczx-2019-02), Initial Scientific Research Fund of Yan'an University (YDBK201850 and YDBK2018-43), Research Program of Yan'an University (YDZ2019-09 and YDZ2020-17), Open Project Key Laboratory of Microbial Resources and Drug Development Characteristics of Ordinary Colleges and Universities in Guizhou Province (No. GZMRD-2016-001), The Research Start Up Foundation of Zunyi Medical University (No. F-905). Zunyi Science and Technology Department Projects [2018(29)].

We would like to thank Dr. Liu Ning of Institute of Microbiology, Chinese Academy of Sciences for helping us complete the spectral analysis of compounds.

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