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# Production of quinolone derivatives in *Escherichia coli*

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**Abstract**

Alkyl-4-quinolones (AQs) are natural compounds synthesized by bacteria. Members of this group are known quorum-sensing molecules. Other biological functions, such as anti-bacterial, anti-algal, antifungal, and anti-malaria activities have also been reported. The synthetic pathways of AQs have been validated in *Pseudomonas aeruginosa*. Five genes (*pqsA–E*) are involved in the synthesis of 2-heptyl-4(1H)-quinolone (HHQ). To synthesize HHQ in a microbial system, *pqsA–E* genes were introduced into *Escherichia coli* and HHQ and 2-methyl-4(1H)-quinolone (MHQ) were synthesized. After the copy number, construct promoters, and substrate supplements were optimized, 141.3 mg/L MHQ and 242.8 mg/L HHQ were synthesized.

**Keywords:** 2-Heptyl-4(1H)-quinolone (HHQ), Metabolic engineering, 2-Methyl-4(1H)-quinolone (MHQ)

## Introduction

Alkyl-4-quinolones (AQs) are 4-quinolone derivatives that are produced mainly by two genera of bacteria: *Pseudomonas* and *Bukholderia* [1]. *Pseudomonas* sp. produces over 55 AQs, and *Burkholderia* sp. synthesizes two [2, 3]. Even though AQs are known quorum-sensing molecules, the culture extracts of *P. aurugìnsa* have also been used as anti-bacterial agents [4]. The main components of the extracts were AQs, which have anti-bacterial, anti-algal, and antifungal activities [5]. Some AQs also exhibit anti-malarial activity [6, 7]. 2-Heptyl-4(1H)-quinolone (HHQ) and other AQs also exhibit anti-asthmatic activity [8]. Like many other small compounds from bacteria, AQs might have other unknown functions in humans, which need to be explored.

AQs have been studied in *Pseudomonas aeruginosa* as a quorum sensing system [9]. *P. aeruginosa* uses two AQs (2-heptyl-3-hydroxy-4(1H)-quinolone and HHQ) as quorum-sensing signaling molecules [10]. HHQ is synthesized by the *pqsABCDE* genes [11]. *pqsA* encodes an enzyme that binds coenzyme A to anthranilate [12]. pqsD uses anthraniloyl-CoA and malonyl-CoA to form 2-aminobenzoylacetyl-CoA, which can spontaneously form either 2, 4-dihydroxyquinoline (DHQ) or HHQ with the help of pqsBCE [13]. When 2-aminobenzoylacetyl-CoA enters the HHQ synthetic pathway, CoA is detached by pqsE, turning it into 2-aminobenzoylacetate [14]. PqsE acts as a pathway-specific thioesterase in the biosynthesis of alkylquinolone signalling molecules [14]. PqsC with the help of pqsB carries an octanoate group and pqsC links the octanoate moiety to 2-aminobenzoylacetate via decarboxylation to form HHQ [15]. PQS is synthesized from HHQ using pqsH, a flavin-dependent monooxygenase [16].

Although *P. aeruginosa* synthesizes diverse AQs, its applications as an AQ producer are hindered by its pathogenic properties [17]. An alternative method to synthesize HHQs is to use a well-characterized microbial system. We transferred the HHQ synthesis pathway from *P. aeruginosa* to *Escherichia coli* and attempted HHQ synthesis (Fig. 1). We optimized the constructs for synthesis of HHQ and engineered *E. coli* to increase synthesis of the anthranilate substrate. Using this engineered *E. coli* strain, both HHQ and an unexpected product, MHQ (2-methyl-4(1H)-quinolone), were synthesized.

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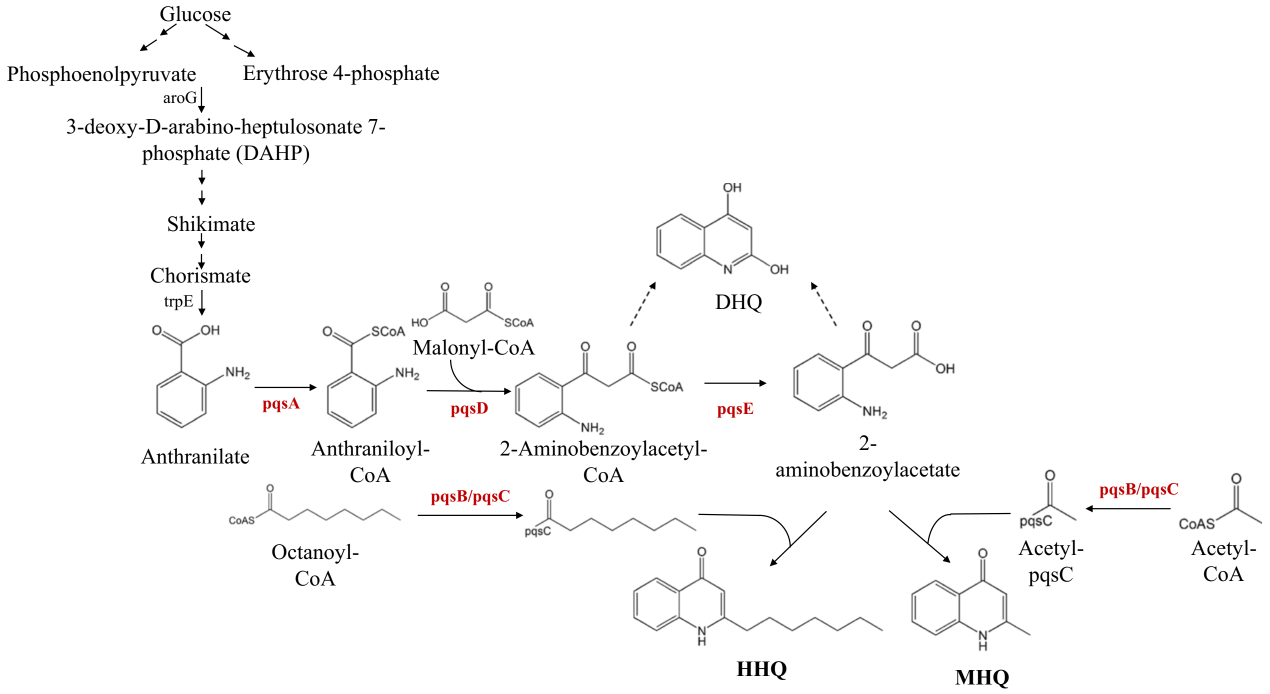

**Fig. 1** Pathway for the synthesis of HHQ and MHQ in *E. coli*. Red coloring indicates introduced genes

## Materials and methods

### Constructs

pC-pqsD-pqsA and pA-pqsD-pqsA were constructed previously [18]. *TrpE*, *aroG*, and *aroG*$^{f}$ have been previously cloned [18, 19]. These genes were subcloned into the pColaDuet-1 vector (Novagen). The modified T7 promoter sequences (H10 and C4) were synthesized based on a previously published sequence [20]. The two T7 promoters from pETDuet-1 were replaced with C4 promoters and those from pACYCDuet-1 were replaced with H10 promoters. The resulting vectors were called pETDuet-1-C and pACYCDuet-1-H, respectively. *pqsD* and *pdsA* were subcloned into the pACYCDuet-1-H vector (pA-H-pqsD-pdsA) (Table 1).

*PsqB* (Gene ID: 883098; aaacatATGTTGATTCAGGCTGTGGG as a forward primer, and aaagatctTTATGCATGAGCTTCTCCCG as a reverse primer; NdeI and BglII sites are indicated by lowercase letters), *pqsC* (Gene ID, 880660; aaagatct<u>aaggagatatacca</u>ATGCATAAGGTCAAACTGGCA as a forward primer and aagatatcTCAGCACACCAGCACCTC as a reverse primer; BglII and EcoRV sites are indicated by lowercase letters; the ribosome binding site (RBS) is underlined.), and *pqsE* (Gene ID, 880721; aaggatccaATGTTGAGGCTTTCGGCTC as a forward primer and aaaagcttTCAGTCCAGAGGCAGCG as the reverse primer; BamHI and HindIII sites are indicated with lowercase letters) were cloned by polymerase chain reaction (PCR) using *P. aeruginosa* genomic DNA as a template. *PsqE* was cloned into the BamHI/HindIII sites of pETDuet-1 (pE-pqsE). *PsqB* was cloned into the NdeI/BglII sites of pE-pqsE (pE-pqsE-pqsB). The pqsC-containing RBS was cloned into the BglII/EcoRV site of pE-pqsE-pqsB. The resulting construct was named pE-pqsE-pqsBC. pE-C-pqsE-pqsBC, which has two modified T7 promoters, was also constructed.

An *E. coli* trpD deletion mutant was generated previously [18] and the kanamycin resistance gene cassette was removed in this *E. coli* strain using a Quick & Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany) as per the manufacture's manual.

### Synthesis and analysis of reaction products

For the synthesis of HHQ and MHQ from anthranilic acid, an overnight culture of an *E. coli* BL21 (DE3) transformant containing pC-pqsD-pqsA and pE-pqsE-pqsBC was grown in Luria-Bertani (LB) broth with 50 µg/mL spectinomycin and ampicillin overnight at 37 °C. The culture was inoculated into fresh LB medium and incubated at 37 °C until the $OD_{600}$ reached 1.0, after which isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to a final concentration of 1 mM before the cells were incubated at 18 °C overnight. The cells were resuspended in 1 mL M9 medium containing 1% yeast

**Table 1** Plasmids and strains used in the present study

| Plasmids or *E. coli* strain | Relevant properties or genetic marker | Source or reference |
|---|---|---|
| Plasmids | | |
| pACYCDuet-1 | P15A ori, Cm$^{r}$ | Novagen |
| pACYCDuet-1-H | Both T7 promoter of pACYCDuet-1 were replaced by promoter H10 | This study |
| pCDFDuet-1 | CloDE13 ori, Str$^{r}$ | Novagen |
| pETDuet-1 | Both T7 promoter of pETDuet-1 were replaced by promoter C4 | Novagen |
| pETDuet-1-C | f1 ori, Amp$^{r}$ | Novagen |
| pColaDuet-1 | ColA ori, Kana$^{r}$ | Novagen |
| pA-pqsD-pqsA | pACYCDuet + *pqsD* and *pqsA* from *Pseudomonas aeruginosa* | [18] |
| pC-pqsD-pqsA | pCDFDuet + *pqsD* and *pqsA* from *Pseudomonas aeruginosa* | [18] |
| pA-H-pqsD-pqsA | pACYCDuet-H + *pqsD* and *pqsA* from *Pseudomonas aeruginosa* | This study |
| pE-pqsE-pqsBC | pETDuet + *pqsE, pqsB* and *pqsC* from *Pseudomonas aeruginosa* | This study |
| pE-C-pqsE-pqsBC | pETDuet-C + *pqsE, pqsB* and *pqsC* from *Pseudomonas aeruginosa* | This study |
| pCol-trpE | pColaDuet + *trpE* from *E. coli* | This study |
| pCol-aroG-trpE | pCDFDuet + *aroG* from *Escherichia coli* in the first multiple cloning site (MCS1) + *trpE* from *E. coli* in the second MCS (MCS2) | This study |
| pCol-aroG$^{f}$-trpE | pCDFDuet + *aroG$^{f}$* from *E. coli* in MCS1 + *trpE* in MCS2 | This study |
| Strains | | |
| DH5α | F– φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1 | Novagen |
| BL21 (DE3) | F$^{-}$ *ompT hsdS$_{B}$*(r$_{B}^{-}$ m$_{B}^{-}$) *gal dcm lon* (DE3) | Novagen |
| B-trpD | *E. coli* BL21 (DE3) deleted in anthranilate phosphoribosyl transferase domain of *trpD* | [18] |
| B-H1 | *E. coli* BL21 (DE3) harboring pC-pqsD-pqsA and pE-C-pqsE-pqsBC | This study |
| B-H2 | *E. coli* BL21 (DE3) harboring pA-H-pqsD-pqsA and pE-C-pqsE-pqsBC | This study |
| B-H3 | *E. coli* BL21 (DE3) harboring pA-pqsD-pqsA and pE-pqsE-pqsBC | This study |
| B-H4 | *E. coli* BL21 (DE3) harboring pA-pqsD-pqsA and pE-C-pqsE-pqsBC | This study |
| B-H5 | *E. coli* BL21 (DE3) harboring pC-pqsD-pqsA, pE-C-pqsE-pqsBC, pCol-trpE | This study |
| B-H6 | B-trpD harboring pC-pqsD-pqsA, pE-C-pqsE-pqsBC, pCol-trpE | This study |
| B-H7 | B-trpD harboring pC-pqsD-pqsA, pE-C-pqsE-pqsBC, pCol-aroG-trpE | This study |
| B-H8 | B-trpD harboring pC-pqsD-pqsA, pE-C-pqsE-pqsBC, pCol-aroG$^{f}$-trpE | This study |

extract, 2% glucose, 50 µg/mL spectinomycin, ampicillin, and 1 mM IPTG at an of $OD_{600}=3$. Anthranilate was also added to the medium at a final concentration of 200 µM, and the culture was incubated at 30 °C for 24 h. The reaction product was extracted using ethyl acetate and vacuum-dried. The dried sample was dissolved in dimethyl sulfoxide (DMSO) and analyzed using high-performance liquid chromatography (HPLC) [18]. The molecular masses of the synthesized compounds were determined as previously described [21].

The reaction products were purified using thin layer chromatography (TLC; TLC silica gel 60 F254; Millipore, Burlington, MA, USA). A mixture of ethyl acetate and hexane (8:1) was used as the developing solvent. The structure was determined using NMR [22]; $^{1}$H NMR of HHQ (DMSO-d6, 500 MHz) δ:0.85 (3H, t, J = 7.0 Hz, CH3), 1.19 ~ 1.36 (10H, overlapped, (CH2)5), 2.57 (2H, t, J = 7.8 Hz, CCH2), 5.90 (1H, s, H3), 7.23 (1H, m, H6), 7.55 ~ 7.56 (2H, overlapped, H7 and H8), 8.02 (1H, d, J = 8.0 Hz, H5). $^{1}$H NMR of MHQ (DMSO-d6, 500 MHz) δ: 2.33 (3H, s, CH3), 5.89 (1H, s, H3), 7.24 (1H, ddd, J = 8.1, 7.6, 1.0 Hz, H6), 7.51 (1H, dd, J = 8.1, 1.0 Hz, H8), 7.57 (1H, ddd, J = 8.1, 7.6, 1.3 Hz, H7), 8.02 (1H, dd, J = 8.1, 1.3 Hz, H5).

## Results and discussion

### Optimization of constructs for synthesis of HHQ and MHQ in *E. coli*

At least five genes (*pqsA*, *B*, *C*, *D*, and *E*) are involved in the synthesis of HHQ from anthranilate (Fig. 1). These genes were divided into two constructs (pA-pqsD-pqsA and pE-pqsE-pqsBC). *E. coli* transformants harboring the two constructs were grown in the presence of anthranilate. The analysis of the culture filtrate using HPLC revealed at least three peaks (Fig. 2). A peak at 6.6 min was DHQ, as determined by comparison with pure DHQ. The identity of the peaks at 4.6 and 11.6 min is unknown. However, the peak at 11.6 min was

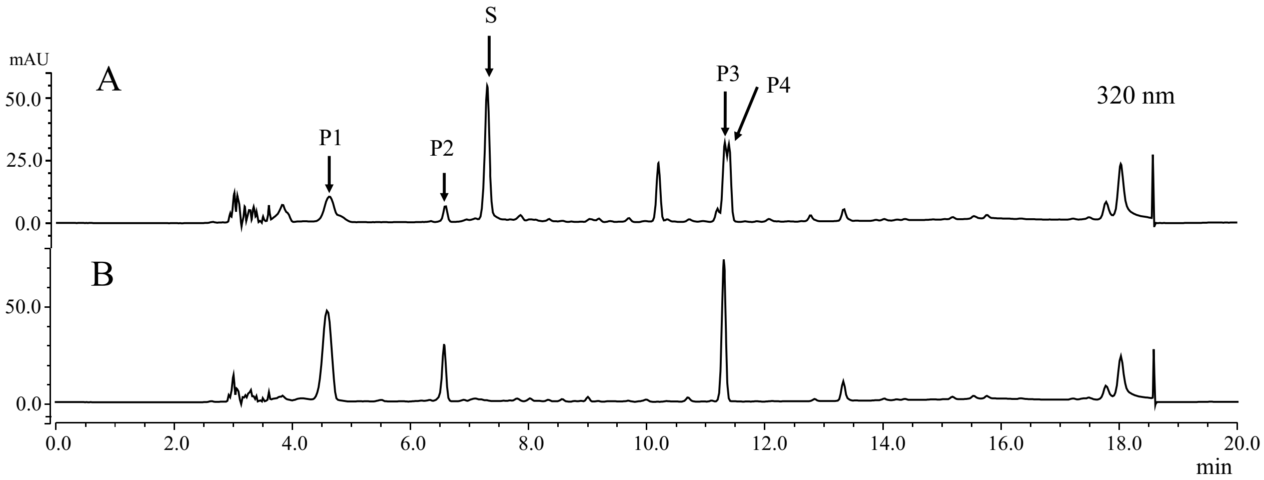

**Fig. 2** **A** HPLC profile of the reaction products from *E. coli* harboring pA-pqsD-pqsA and pE-pE-pqsE-pqsBC; **B** HPLC profile from *E. coli* harboring pC-pqsD-pqsA and pE-pE-pqsE-pqsBC. Anthranilate (200 µM) was supplied to the culture medium. S, anthranilate; P1, P2, and P3 are MHQ, DHQ, and HHQ, respectively. P4 is likely a metabolite derived from acetylation of *E. coli* metabolites by chloramphenicol acetyltransferase encoded by pACYCDuet-1 vector. When pCDEDuet-1 vector instead of pACYCDuet-1 was used, the P4 was not observed (**B**)

close to the other peak (P4 in Fig. 2A) derived from the pACYCDuet-1 vector. This peak was always observed when the pACYCDuet-1 vector was used. This suggests that this product was an acetylated compound generated from an *E. coli* metabolite via chloramphenicol acetyltransferase of pACYCDuet-1. Use of other vectors, such as pCDFDuet-1 instead of pACYCDuet-1, led to the disappearance of this metabolite (Fig. 2B). The molecular masses of each reaction product (P1 and P2 in Fig. 2A) were 243.17 and 159.18-Da, respectively. We purified these two compounds, and their structures were determined using proton NMR (refer to “Materials and methods”). The peak (P3 in Fig. 2A) at 11.6 min corresponds to HHQ, which was the expected reaction product. The peak at 4.6 min (P1 in Fig. 2) A corresponded to MHQ, which was an unexpected product. The structure of each compound was consistent with its measured molecular mass. pqsC attached an octyl group to 2-aminobenzoylacetate (2-ABA), which resulted in the formation of HHQ after decarboxylation [15]. Endogenous octanoic acid in *E. coli* is likely used to synthesize HHQ. An acetate group is required for synthesis of MHQ. It was not clear whether pqsC could carry and attach acetate to 2-ABA. However, our results suggest that MHQ can be synthesized when the five *pqs* genes were introduced into *E. coli*. To determine the function of pqsC during the synthesis of HHQ or MHQ, only four of the *pqs* genes (*pqsA, B, D,* and *E*) were introduced into *E. coli* and the resulting transformant was tested for synthesis of HHQ or MHQ. The resulting transformant synthesized only DHQ but did not synthesize HHQ or HMG. This result indicated that pqsC carried not only an octyl group, but also an acetyl group.

Next, we tested different copy numbers of each construct and the strength of the promoters. *pqsD* and *pqsA* were subcloned into pACYCDuet-1, pACYCDuet-1-H, and pCDFDuet-1. *pqsE*, *pqsB*, and *pqsC* were sub-cloned into pETDuet-1 and pETDuet-1-C. The genes (*pqsE*, *pqsB*, and *pqsC*) downstream of the pathway were cloned into high-copy-number plasmids, and those (*pqsD* and *pqsA*) upstream were cloned into low-copy-number plasmids. Four transformants (BH1–4) were tested for HHQ and MHQ synthesis after addition of 200 µM anthranilate. The productivities of HHQ and MHQ synthesis were clearly different depending on the constructs used. Strain B-H1 harboring pC-pqsA-pqsD and pE-C-pqsE-pqsBC showed the highest HHQ (47.5 mg/L) and MHQ (51.1 mg/L) production, followed by B-H3, B-H4, and B-H2 (Fig. 3). The strains that harbored higher plasmid copy numbers synthesized more HHQ and MHQ. Based on these results, two constructs, pC-pqsA-pqsD and pE-C-pqsE-pqsBC, were used to synthesize HHQ and MHQ, respectively.

### Synthesis of HHQ and MHQ without feeding anthranilate

Anthranilate is a substrate for HHQ and MHQ synthesis and is also an intermediate of tryptophan biosynthesis via the chorismate pathway [23]. To enhance anthranilate synthesis in *E. coli*, we overexpressed selected genes in the chorismate pathway. The first step in the chorismate pathway is catalyzed by aroG. aroG is subject to feedback inhibition. The feedback inhibition-free version of

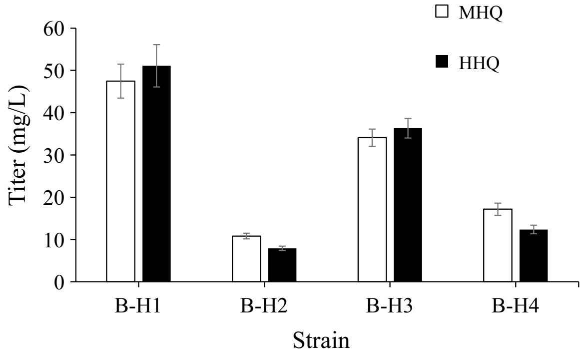

**Fig. 3** Optimization of constructs for MHQ and HHQ synthesis in *E. coli*. Anthranilate (200 μM) was provided and the mixture was incubated at 30 °C for 24 h. The constructs which each strain harbored are shown in Table 1

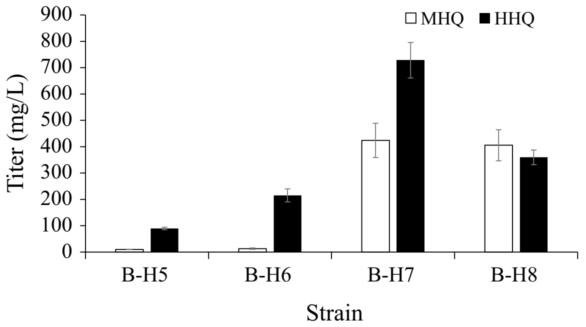

**Fig. 4** Synthesis of MHQ and HHQ from glucose in *E. coli*. The characterization of each strain is listed in Table 1

aroG is aroG$^{f}$, in which the aspartic acid at position 146 is mutated to asparagine [24]. Overexpression of either *aroG* or its feedback inhibition-free version increases levels of chorismate and aromatic amino acids [25]. Chorismate is converted to anthranilate by anthranilate synthase (trpE), which is used for tryptophan synthesis. Deletion of *trpD*, which encodes anthranilate phosphoribosyl transferase which converts anthranilate into *N*-(5′-phopsphoribosyl) anthranilate, resulted in accumulation of anthranilate. Therefore, overexpression of *aroG* and *trpE* and deletion of *trpD* resulted increased anthranilate supply. First, we tested the possibility of synthesizing HHQ and MHQ without supplementation of anthranilate. We overexpressed *trpE* in strain B-H1 and found that both MHQ and HHQ were synthesized. To increase the titer of MHQ and HHQ in *E. coli*, constructs containing a combination of *trpE, aroG,* and *aroG*$^{f}$ were overexpressed and the *trpD* deletion mutant was used. We generated three more *E. coli* transformants harboring a combination of *aroG*, *aroG*$^{f}$, and *trpE* using a *trpD* deletion mutant (B-H6–B-H8). We monitored synthesis of HHQ and MHG, and the remaining anthranilate. As shown in Fig. 4, the strain B-H7 had the highest titer of MHQ (141.3 mg/L; 887.6 μM) and HHQ (242.8 mg/L; 997.7 μM). Strain B-H8 also synthesized comparable amounts of both compounds. However, this strain accumulated approximately 478.2 mg/L of anthranilate, indicating that metabolic balance is critical to increase the final titer of product and that unreacted anthranilate might interfere with the synthesis of HHQ and MHQ.

We synthesized two AQs by introducing a pathway in *P. aeruginosa*. HHQ was previously synthesized in *E. coli* with a titer of 8.0 mg/L. However, the report investigated the transcription factors that regulate *pgs* genes, and did not optimize the entire pathway [26]. The synthesis of MHQ has not been reported previously. The octanoyl group was linked to pqsC using pqsB [15]. However, it was not previously clear whether pqsC plays a role in attaching and delivering the acetyl group to 2-aminobenzoylacetate to form MHQ. We showed that pqsC is involved in the synthesis of both HHQ and MHQ. When *pqsC* was not introduced into *E. coli*, only DHQ was synthesized.

We synthesized DHQ with a titer of 753.7 mg/L, which is approximately 4676.7 μM [18]. The constructs for the synthesis of DHQ contained different genes from the shikimate pathway. Five genes (*aroL* encoding shikimate kinase, *aroG*$^{f}$, *ppsA* encoding phosphoenolpyruvate synthase, *tktA* encoding transketolase A, and *trpE*) were overexpressed and *E. coli* mutants in which *trpD* and *tyrA* were deleted were used. This increased anthranilate synthesis and appeared to increase DHQ synthesis. In the current study, we found that overexpression of *aroG*$^{f}$ and *trpE* resulted in accumulation of high amounts of anthranilate without further synthesis of HHQ or MHQ and a greater amount of DHQ synthesis. Taken together, these results indicate that a high amount of anthranilate drives the pathway towards DHQ synthesis. When *aroG* instead of *aroG*$^{f}$ was expressed, only a small amount of DHQ was synthesized, relative to HHQ or MHQ synthesis. These results suggest that modulation of substrate production is critical for maximizing the final titer(s) of desired product(s).

In conclusion, two AQs, MHQ and HHQ were synthesized in *E. coli*. The synthetic pathway genes (pqsA–E) were introduced, and the expression of these genes were optimized for the maximal synthesis of two AQs. The results presented in here showed the possibility to synthesize diverse AQs, which have diverse biological activities to be discovered.

**Abbreviations**

AQ: Alkyl-4-quinolone; DHQ: Dihydroxyquinoline; DMSO: Dimethyl sulfoxide; HHQ: 2-Heptyl-4(1H)-quinolone; HPLC: High-performance liquid chromatography; IPTG: β-D-1-thiogalactopyranoside; LB: Luria-Bertani; MHQ: 2-Methyl-4(1H)-quinolone; PCR: Polymerase chain reaction; RBS: Ribosome-binding site; TLC: Thin layer chromatography.

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**Author contributions**

GSC and JHA designed the experiments. YJP, GSC, and SWL performed the experiments. YJP, GSC, and JHA analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

**Availability of data and materials**

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

## Declarations

**Competing interests**

The authors declare that they have no competing interests.

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