Efficient degradation of hydroquinone by a metabolically engineered *Pseudarthrobacter sulfonivorans* strain

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Received: 6 August 2022 / Revised: 18 August 2022 / Accepted: 22 August 2022
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Abstract

*Pseudarthrobacter sulfonivorans* strain Ar51 can degrade crude oil and multi-substituted benzene compounds efficiently at low temperatures. However, it cannot degrade hydroquinone, which is a key intermediate in the degradation of several other compounds of environmental importance, such as 4-nitrophenol, g-hexachlorocyclohexane, 4-hydroxyacetophenone and 4-aminophenol. Here we co-expressed the two subunits of hydroquinone dioxygenase from *Sphingomonas sp.* strain TTNP3 with different promoters in the strain Ar51. The strain with 2 *hdnO* promoters exhibited the strongest hydroquinone catabolic activity. However, in the absence of antibiotic selection this ability to degrade hydroquinone was lost due to plasmid instability. Consequently, we constructed a *hisD* knockout strain, which was unable to synthesise histidine. By introducing the *hisD* gene onto the plasmid, the ability to degrade hydroquinone in the absence of antibiotic selection was stabilised. In addition, to make the strain more stable for industrial applications, we knocked out the *recA* gene and integrated the hydroquinone dioxygenase genes at this chromosomal locus. This strain exhibited the strongest activity in catabolizing hydroquinone, up to 470 mg/L in 16 h without antibiotic selection. In addition, this activity was shown to be stable when the strain has cultured in medium without antibiotic selection after 20 passages.

Keywords Pseudarthrobacter · Hydroquinone · Degradation

Introduction

Hydroquinone (HQ) is a key intermediate in the catabolism of several compounds of environmental importance, such as 4-nitrophenol, g-hexachlorocyclohexane, 4-hydroxyacetophenone and 4-aminophenol. There are two established pathways in the literature for the degradation of hydroquinone. One involves direct ring cleavage of hydroquinone by dioxygenases containing Fe(II) in their active center, resulting in the formation of 4-hydroxymuconic acid semialdehyde (HMSA) (Miyauchi et al. 1999; Chauhan et al. 2000; Moonen et al. 2008a). The second pathway requires the hydroxylation of hydroquinone to benzene-1,2,4-triol which is then cleaved to yield maleylactic acid by dioxygenases containing Fe(III) in their active center (Rieble et al. 1994; Travkin et al. 1997; Eppink et al. 2000; Ferraroni et al. 2005). Of these enzymes, the hydroquinone dioxygenase from *Sphingomonas sp.* strain TTNP3 has the higher reported hydroquinone degrading activity (Kolvenbach et al. 2011).

*Pseudarthrobacter sulfonivorans* strain Ar51, a psychrotrophic bacterium isolated from the Tibetan permafrost of China, can degrade crude oil and multi-substituted benzene compounds efficiently at low temperatures. There are several genes or gene clusters in its genome responsible for the catabolism of organic compounds including genes for alkane, biphenyl and benzoate biodegradation. In addition, this strain can tolerate cold shock, osmotic stress and...
oxidative stress (Zhang et al. 2016). Consequently, this strain is a good candidate for sewage treatment, although it has no ability to degrade hydroquinone.

Here we describe the co-expression of the two subunits of hydroquinone dioxygenase from Sphingomonas sp. strain TTNP3 in strain Ar51, conferring the ability to degrade hydroquinone. In addition, we communicate how the resulting hydroquinone catabolic activity can be stably inherited in the recombinant strain in the absence of antibiotic selection.

Materials and methods

Bacterial strains and plasmid construction

E. coli DH5α was used for general cloning purposes and all Pseudarthrobacter strains used in this study are derived from the Pseudarthrobacter sulfonivorans strain Ar51. Electro-competent cells of Pseudarthrobacter were prepared as described (Chen et al. 2017). E. coli strains were grown aerobically in Luria–Bertani (LB) broth at 37 °C. Pseudarthrobacter strains were grown at 20 °C in LB or minimal medium (Gauze’s synthetic medium no.1). For E. coli, antibiotics were used at the following concentrations (μg/ml): kanamycin 50, hygromycin 100, apramycin 50. For Pseudarthrobacter kanamycin was used at 140 μg/ml and hygromycin 200 μg/ml, apramycin 50 μg/ml.

The plasmids used in this work are listed in Table 1. Plasmid pART2 was used for protein expression in Pseudarthrobacter. pIJ8600 contains the apramycin resistance gene, which can work in the Pseudarthrobacter as a selectable marker. pSH152 contains the hygromycin resistance gene, which can work in Pseudarthrobacter as a selectable marker.

The primers used to construct the different plasmids are listed in Table 2. Primers P1, P2 were used to amplify hqdB and insert this gene in pART2 (BamHI and XbaI) to construct pART2-hqdB. Primers P3, P4 were used to amplify hqdB and insert this gene in pART2 (BamHI and XbaI) to construct pART2-hqdA. Primers P1, P5 were used to amplify hqdB-P_hnoR from pART2-hqdB and P6 withP4 were used to amplify P_hnoR-hqdB from the pART2-hqdB. The two DNA fragments were fused by fusion PCR and inserted into pART2 (BamHI and XbaI) to construct plasmid HQ1. Primers P1, P7 were used to amplify from pART2-hqdB and primers P8 with P4 were used to amplify rbs-hqdB from pART2-hqdB. The two DNA fragments were fused by fusion PCR and inserted into pART2 (BamHI and XbaI) to construct plasmid HQ2. Primers P9, P10, P11 and P2 were used to amplify GAPDH promoter and hqdB. The fusion DNA was inserted into pART2 (NcoI and XbaI) to construct pART2-P_GAPDH-hqdB. Primers P9, P12, P13 and P4 were used to amplify GAPDH promoter and hqdB. The fusion DNA was inserted into pART2 (NcoI and XbaI) to construct pART2-P_GAPDH-hqdB. Primers P9, P14, P15 and P4 were used to amplify P_GAPDH-hqdB and P_GAPDH-hqdB. The fusion DNA was inserted into pART2 (NcoI and XbaI) to construct plasmid HQ3. Primers P9, P16, P17 and P4 were used to amplify P_GAPDH-hqdB and rbs-hqdB. The fusion DNA was inserted into pART2 (NcoI and XbaI) to construct plasmid HQ4. Primers P18, P19, P20, P21, P22 and P23 were used to amplify the recombinant 1 fragment, P-hygromycin resistance gene and recombinant 2 fragment. The fusion DNA of the three fragments is HQ5. Primers P24 and P25 were used to amplify the hisD gene with its promoter. This DNA fragment was inserted into HQ1 (HpaI). Primers P26, P27, P28, P29, P30, P31, P32 and P33 were used to amplify the recombinant 1 fragment, P_hnoR-hqdB-P_hnoR-hqdB, P-apramycin resistance gene and recombinant 2 fragment. The fusion DNA of the four fragments is HQ7.

The strains used in this work are listed in Table 3. Plasmids were transferred to Pseudarthrobacter and recombinant strains verified by PCR amplification. The integration of suicide vectors by double cross-over homologous

| Name | Relevant description | References |
|------|----------------------|------------|
| pART2 | Medium copy number plasmid carrying the strong hdnO promoter (P_hdnO), KanR | Sandu et al. (2005) |
| pIJ8600 | Integration plasmid carrying the apramycin resistance gene as selectable marker | Sun et al. (1999) |
| pSH152 | Integration plasmid carrying the hqdB under control of P_hdnO for each gene | Aldridge et al. (2013) |
| HQ1 | pART2 carrying hqdB under control of P_hdnO in one operon | This work |
| HQ2 | pART2 carrying hqdB under control of P_hdnO in one operon | This work |
| HQ3 | pART2 carrying hqdB under control of P_gapDH in one operon | This work |
| HQ4 | pART2 carrying hqdB under control of P_gapDH in one operon | This work |
| HQ5 | Integrative suicide vector based on pSH152 to knockout the hisD gene of strain Ar51 | This work |
| HQ6 | Plasmid of AHQ1 carrying the complementing hisD gene | This work |
| HQ7 | Integrative suicide vector based on pIJ8600 to knock out the recA gene of strain Ar51 and carrying hqdB under control of P_hdnO for each gene | This work |
recombination was subsequently verified by PCR amplification of integrated sequences.

**Hydroquinone concentration analysis**

Hydroquinone concentration was analyzed by the HPLC as described (Moldovan et al. 2017). Briefly, HPLC with an ODS column (25 cm × 4.6 mm) was used with a UV detector set at 295 nm having. Temperature was adjusted at 35 ± 1 °C. The mobile phase was a mixture of water and ethanol (45:55) with a flow rate of 1.5 ml/ min. 20 μl of each sample solution was injected and chromatogram was recorded. Peak areas for each sample were measured and comparisons made between reference and sample solutions peaks.
Results and discussion

Previous studies showed that several aerobic bacteria are capable of utilizing hydroquinone derived from catabolism of several xenobiotics, involving a hydroquinone 1,2-dioxygenase to convert hydroquinone into 4-hydroxymuconic semialdehyde (Ferraroni et al. 2017). *Arthrobacter ureafaciens* CPR706 can degrade 4-chlorophenol via the hydroquinone pathway (Bae et al. 1996). Degradation of para-nitrophenol by *Moraxella* sp., *Pseudomonas* sp. strain WBC-3, and *Pseudomonas* sp. 1–7 also involves the hydroquinone pathway (Spain and Gibson 1991; Zhang et al. 2012; Wang et al. 2017). In the hydroquinone degradation pathway, hydroquinone 1,2-dioxygenases, which play a key role in phenol bioremediation, catalyze the direct phenolic ring fission using 1,4-dihydroxyphenols as substrates and constitute a large family of aromatic ring-fission enzymes (Enguita and Leitao 2013). Hydroquinone dioxygenases can be divided into two subtypes that have few similarities. Members of type I are monomeric nonheme iron containing single chain enzymes (Eltis and Bolin 1996). Another family of hydroquinone 1,2-dioxygenases are composed of two subunits encoded by adjacent genes in genomic clusters involved in the degradation of substituted aromatic compounds. In *Sphingomonas* sp. these genes are designated as *hdqA* and *hdqB*, and the corresponding homologs in *P. fluorescens* are *hapC* and *hapD*. Of these hydroquinone 1,2-dioxygenases, the Hdq holoenzyme has the highest hydroquinone catabolic activity (Moonen et al. 2008a, b).

To express the Hdq holoenzyme in *Pseudoarthrobacter* we used the plasmid pART2 (Sandu et al. 2005) as the backbone, together with the constitutive gapdh promoter and the *hdnO* promoter. Four plasmids were constructed: plasmid AHQ1 in which both genes were expressed individually under the control of separate *hdnO* promoters; plasmid AHQ2 in which both genes were expressed as an operon under control of a single *hdnO* promoter; plasmid AHQ3 in which both genes were expressed under the control of separate *gapdh* promoters; and plasmid AHQ4 in which both genes were expressed as an operon under control of a single *gapdh* promoter (Fig. S1A). Strains were cultured in growth medium with antibiotic selection and analysis of hydroquinone catabolism showed that the strain AHQ1 had the highest activity, degrading almost all of the hydroquinone in only 12 h (Fig. S1B). Strains AHQ2 or AHQ3 had similar hydroquinone catabolic activities, permitting degradation of almost all of the hydroquinone in 24 h. Strain AHQ4 exhibited the weakest activity in degrading hydroquinone, being unable to degrade all of the hydroquinone in 24 h. These data confirm that employing the *hdnO* promoter, a strong promoter in *Arthrobacter*, correlates with elevated hydroquinone catabolic activity.

![Fig. 1](image.png)

**Fig. 1** Complementation of histidine auxotrophy confers plasmid stability without antibiotic selection. **A** Map of the disrupted *hisD* gene from *Pseudoarthrobacter sulfonivorans* strain Ar51 genome in plasmid HQ5 used to generate the auxotrophic mutant; and the complementing plasmid HQ6. **B** Catabolism of HQ by strain AHQ6. **C** Stability of HQ catabolism after serial passaging of strain AHQ6.
The biggest defect and impracticality of genetically engineered strains developed for bioremediation lies in a lack of genetic stability. Previous studies showed that a strain can lose a recombinant plasmid during cell division even with the antibiotic selection (Summers and Sherratt 1984). We cultured the AHQ1 strain in LB medium without selective antibiotic for 20 passages. After 5 passages, the strain was observed to have a much-reduced ability to catabolise hydroquinone and this reduced further after additional passaging (Fig. S2A). Then we analyzed the proportion of antibiotic-resistant bacteria at each passage as a measure of plasmid retention in the absence of selection. As indicated in Fig. S2B, the strain rapidly lost the plasmid when cultured in medium without antibiotic selection. Therefore, plasmid instability is a key factor that could limit the application of the strain for waste-water treatment. There are two strategies to deal with this instability. The first one is to insert an essential gene in the plasmid and knock out this gene from the genome. The second is to insert the functional genes into the bacterial chromosome. Here we use both strategies to stabilize the expression of hdqA and hdqB in the P. sulfonivorans strain Ar51.

Histidine is an essential amino acid for the strain. The hisD gene is the last gene in the histidine biosynthetic pathway (Kulis-Horn et al. 2014). First, we constructed a hisD knock out strain, which can only grow on minimal medium with histidine addition. Then we inserted the hisD gene with its native promoter in the plasmid AQH1 to create plasmid AQH6 (Fig. 1A). After transfer of plasmid AQH6 into the hisD knock out stain, the complementation strain exhibited a high hydroquinone catabolic activity (Fig. 1B). Importantly, the addition of the complementing hisD gene effectively stabilised the plasmid and the ability to degrade hydroquinone when the strain was cultured in the absence of antibiotic selection (Fig. 1C).

With two directly repeated copies of the hdnO promoter flanking the hdqA gene in plasmid AQH6, there exists the possibility that the hdqA gene could be lost due to recombination. Previous research showed the recA gene is essential for homologous recombination (Dixon and Kowalczykowski 1995). Consequently, we used an apramycin-resistance gene as the selective marker to knock-out the recA gene and insert a copy of both the hdqA and hdqB genes in this site (Fig. 2A). This recombinant strain exhibited high hydroquinone catabolic activity without antibiotic selection.
degrading 500 mg/L hydroquinone to just 18.18 mg/L in only 16 h (Fig. 2B). In addition, this activity proved to be stable when the strain was grown in the absence of antibiotic selection (Fig. 2C).

Typical industrial wastewater not only contains hydroquinone, but may also have many complex organic compounds. *Pseudarthrobacter sulfonivorans* strain Ar51 is a psychrotrophic bacterium, isolated from the Tibet permafrost of China, that can degrade crude oil and multi benzene compounds efficiently at low temperatures. Compared with other *Arthrobacter* strain, this strain not only has a very large chromosome but also a large natural plasmid. Its genome contains many different alkane degrading genes or gene clusters. Here we have demonstrated addition of further relevant functional activity, namely, by stable expression of hydroquinone 1,2-dioxygenase to degrade hydroquinone to 4-hydroxymuconic semialdehyde, which in turn can be utilized by the β-ketoacyl diapate pathway of this bacterium.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03214-z.

**Funding** Provincial Key Laboratory Open Fund Project (EEMRE201802), National Natural Science Foundation of China (EEMRE201802), Gansu Natural Science Foundation Project (615-4RA207), National Natural Science Foundation of China (31860176).

**Author contributions** HS, XT, YC, TG, WZ are doing the experiments. XC designed the experiment. GL and PD wrote this paper. All authors reviewed the manuscript.

**Declarations**

**Competing interests** The authors declare no competing interests.

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