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Improved heterologous expression of the membrane-bound quinoprotein quinate dehydrogenase from *Gluconobacter oxydans*

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ABSTRACT

Gluconobacter oxydans produces 3-dehydroquinate by oxidation of quinate through a reaction catalyzed by the quinate dehydrogenase (QDH), membrane-bound, pyrroloquinoline quinone (PQQ)-dependent dehydrogenase. We previously reported the nucleotide and deduced amino acid sequence of QDH and constructed a heterologous expression system of QDH in *Pseudomonas* sp. (A.S. Vangnai, W. Promden, W. De-Eknamkul, K. Matsushita, H. Toyama, Biochemistry (Moscow) 75:452–459, 2010). Through this study, we aim to update the sequences of QDH and improve the heterologous expression of QDH in *Gluconobacter* strains using a broad-host-range plasmid. Expression of QDH using a plasmid containing a long 5′-UTR was higher than that using a plasmid with a short 5′-UTR. In addition, the usage of the putative promoter region of the membrane-bound, alcohol dehydrogenase (ADH) of *Gluconobacter* resulted in higher expression levels compared to the usage of the *lacZ* promoter. Base substitution experiments allowed to identify the correct TTG initiation codon between two possibilities, and the result of these experiments were consistent with the N-terminal amino acid sequence of the expressed QDH. However, change of the TTG codon to ATG did not increase QDH expression. Therefore, the optimal plasmid for QDH expression included the structural gene with a long 5′-UTR and the ADH promoter. Cell membrane of the recombinant *Gluconobacter* strain presented approximately 10-times higher specific QDH activity than that observed in the wild-type strain.

1. Introduction

Acetic acid bacteria inhabit the surface of flowers, fruits, and fermented products such as vinegar, wine, or beer, and they have the ability to oxidize various alcohols, sugars, and sugar alcohols. They are gram-negative, strictly aerobic bacteria of the Acetobacteraceae family, which includes the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter*, and others. Among them, *Gluconobacter* sp. has a strong ability to oxidize sugars and sugar alcohols into the corresponding acids or ketones. *Gluconobacter oxydans* strain NBRC3244 (formerly IFO3244) presents a unique enzyme: the membrane-bound, pyrroloquinoline quinone (PQQ)-dependent quinate dehydrogenase (QDH), localized on the periplasmic side of the cytoplasmic membrane [1]. This enzyme can only be found in some strains of *G. oxydans*, particularly, in the strains NBRC3244, NBRC3292 (formerly IFO3292, also known as *G. oxydans* DSM3504), and NBRC3294 (formerly *G. rubisinosus* IFO3294). In this study, we found that *G. oxydans* strain NBRC3293 has the gene for QDH and, indeed, it shows the enzyme activity. Homologous enzymes have not been found in other species of *Gluconobacter*, or other genera of the Acetobacteraceae family, but they have been found in *Acinetobacter* sp. [2], and in *Pseudomonas putida* [3]. QDH reduces ubiquinone in the membrane upon oxidation of quinate, which connects its function to the respiratory chain that reduces molecular oxygen to water. Thus, quinate oxidation produces energy via the respiratory chain, which is likely to constitute an advantage for the microorganism in plant-associated habitats.

We previously developed a shikimate production system consisting

Abbreviations: PQQ, pyrroloquinoline quinone; QDH, membrane-bound PQQ-dependent quinate dehydrogenase; UTR, untranslated region; DDM, n-dodecyl-β-D-maltoside

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of three enzymes [4,5]: QDH, that oxidizes quinate to 3-dehydroquinate (3-DHQ); 3-DHQ dehydratase, that dehydrates 3-DHQ to 3-dehydroshikimate (3-DHS); and shikimate dehydrogenase, that reduces 3-DHS to shikimate using NADPH. The overall reaction of the system is: quinate $+1/2O_2$ + NADPH + H^+ → shikimate + $2H_2O$ + $NADP^+$, and its efficiency can be enhanced using a NADPH-regeneration system composed of glucose and $NADP^+$-dependent glucose dehydrogenase [4]. This ultimately results in a system that converts quinate to shikimate with nearly 100% efficiency. In addition, we previously reported a biotransformation system using growing *Gluconobacter* cells to produce 3-DHS from quinate [6]. Furthermore, we showed that waste from food industries, such as coffee pulp waste, can be used as a source of chlorogenate, which is hydrolyzed by the fungal enzyme chlorogenate hydrolase to produce caffeate and quinate [7]. Therefore, using these enzymatic systems, a valuable compound such as shikimate can be produced from unused wastes.

QDH is a member of the membrane-bound quinoprotein dehydrogenases, that use quinone as cofactor [8]. No crystal structure for any enzyme of this family has been reported so far, even though the membrane-bound, PQQ-dependent glucose dehydrogenase (mGDH) is well characterized biochemically and is a common member of this family found in a wide variety of acetic acid bacteria and even in Gammaproteobacteria such as *Escherichia coli* [9]. Substrate specificity of mGDH is rather broad [10], while that of QDH is relatively narrow [11]. The biochemical properties of QDH have not been systematically studied yet, and even its purification is still challenging [11] (see the Results section). The levels of QDH produced using plasmid-mediated heterologous expression are not much higher than those found in wild-type *Gluconobacter* [12]. Besides, its purification is complicated by the presence of biological contaminants. For instance, the membrane-bound alcohol dehydrogenase (ADH), a major membrane protein in *Gluconobacter*, interferes with the purification of membrane-bound enzymes such as QDH [1]. Therefore, removal of ADH is a key step in membrane-protein purification in *Gluconobacter* [11]. In this regard, we had previously constructed ADH-deficient variants of *G. oxydans* NBRC12528 [13] and *G. frateurii* CHM43 strains (TY and KM, manuscript in preparation). Because these variant strains do not have genes for QDH, they were used as host strains for the heterologous expression of QDH in this study.

Recently, the complete genome sequence of *G. oxydans* strain DSM3504, corresponding to that of *G. oxydans* strain NBRC3292, was reported [14]. As QDH activity had been described in NBRC3292, in the present work, we retrieved the nucleotide sequence for QDH from the DSM3504 genome data, and compared it with the QDH sequence from *G. oxydans* NBRC3244 that had been previously reported [12]. We found differences between the amino acid sequences of the two QDHs. However, as the nucleotide sequence identity was higher than the amino acid sequence identity, we suspected that our initial sequencing data contained some errors. Therefore, here we present an updated nucleotide sequence for the QDH gene based on the draft genome sequence of *G. oxydans* NBRC3244 and *G. oxydans* NBRC3293 that we have recently determined (MM and KM, unpublished). Furthermore, we have constructed a recombinant *G. frateurii* strain to improve the heterologous expression of QDH.

2. Materials and methods

2.1. Chemicals

Quinic acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). It was dissolved in distilled and deionized water and neutralized with NaOH. Shikimic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). n-Dodecyl-β-D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Yeast extract was kindly provided by Oriental Yeast (Osaka, Japan). The endonucleases and genetic engineering kits were kind gifts from Toyobo (Osaka, Japan). All other chemicals used in this study were commercial products of guaranteed reagent grade.

2.2. Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was used for plasmid construction and triparental mating. *G. frateurii* cells were grown in YPS medium consisting of 50 g of D-sorbitol, 3 g of yeast extract (Oriental Yeast, Osaka, Japan), and 3 g of Polypepton (Nihon Pharmaceuticals, Osaka, Japan) per liter. *G. oxydans* cells were grown in ΔP medium consisting of 20 g of glycerol, 5 g of D-glucose, 10 g of yeast extract, and 10 g of Polypepton per liter. *E. coli* cells were grown in LB medium consisting of 5 g of yeast extract, 10 g of Polypepton, 5 g of NaCl, per liter, pH 7.0 adjusted with NaOH. Ampicillin and kanamycin were used at the final concentration of 50 μg/mL for *E. coli*. Ampicillin was used at the final concentrations of 250 μg/mL and 500 μg/mL for *G. oxydans* and *G. frateurii*, respectively.

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Description	Source or reference
Escherichia coli		
DH5α	F^- *endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1* λ⁻ *recAl gyrA96 relA1 deoR* Δ(*lacZYA-argF*) U169 f80d*lacZ*ΔM15	[40]
HB101	F^- *thi-1 hsdS20* (r_B, m_B) *supE44 recA13 ara14 leuB6 proA2 lacY1 galK2 rpsL20* (str^r *xyl-5 mtl-1* λ^-)	[41]
Gluconobacter oxydans		
NBRC3244	Wild type	NBRC
	Wild type	NBRC
NBRC12528	Wild type	NBRC
NBRC12528 Δ*adhA*	NBRC12528 Δ*adhA*::Km^R	[13]
Gluconobacter frateurii		
CHM43	Wild type, also known as NBRC101659	[42]
SEI46	CHM43 Δ*adhAB*	TY and KM, manuscript in preparation
Plasmid		
pBBR1MCS-4	A broad host range plasmid, Ap^R, *mob rep lacZ*α	[39]
pSHO8	pBBR1MCS-4, P_{adhAB}	[16]
pRK2013	Mediates plasmid transfer, Km^R	[17]
pKK5	pBBR1MCS-4, long *quiA*^{3293}	This study
pKK6	pSHO8, long *quiA*^{3293}	This study
pKK7	pBBR1MCS-4, short *quiA*^{3293}	This study
pKK8	pSHO8, short *quiA*^{3293}	This study
pKK13	pBBR1MCS-4, long *quiA*^{3244}	This study
pKK14	pBBR1MCS-4, short *quiA*^{3244}	This study
pKK15	pSHO8, long *quiA*^{3244}	This study
pKK16	pSHO8, short *quiA*^{3244}	This study
pKK21	pSHO8, ATG^{1st}-*quiA*^{3293}	This study
pKK22	pSHO8, CTC^{1st}-*quiA*^{3293}	This study
pKK23	pSHO8, ATG^{2nd}-*quiA*^{3293}	This study
pKK24	pSHO8, CTC^{2nd}-*quiA*^{3293}	This study
pKK25	pSHO8, ATG^{1st}-*quiA*^{3244}	This study
pKK26	pSHO8, CTC^{1st}-*quiA*^{3244}	This study
pKK27	pSHO8, ATG^{2nd}-*quiA*^{3244}	This study
pKK28	pSHO8, CTC^{2nd}-*quiA*^{3244}	This study

The URL address of the NBRC is "http://www.nbrc.nite.go.jp/".
Abbreviations: long *quiA*^{3293}, NBRC3293 *quiA* with long 5′-UTR; short *quiA*^{3293}, NBRC3293 *quiA* with short 5′-UTR; long *quiA*^{3244}, NBRC3244 *quiA* with long 5′-UTR; short *quiA*^{3244}, NBRC3244 *quiA* with short 5′-UTR; ATG^{1st}-*quiA*, *quiA* with the first TTG codon changed to ATG; CTC^{1st}-*quiA*, *quiA* with first TTG codon changed to CTC; ATG^{2nd}-*quiA*, *quiA* with second TTG codon changed to ATG; CTC^{2nd}-*quiA*, *quiA* with second TTG codon changed to CTC.