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Overexpression of pyrroloquinoline quinone biosynthetic genes affects L-sorbose production in *Gluconobacter oxydans* WSH-003

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ABSTRACT

Pyrroloquinoline quinone (PQQ) is a cofactor of various membrane-bound dehydrogenases. The amount of endogenous PQQ is generally regarded as a bottleneck to achieving higher catalytic efficiency of PQQ-dependent dehydrogenases. Proteins that biosynthesize PQQ in *Gluconobacter oxydans* WSH-003 are encoded by the *pqqABCDE* gene cluster and the *tldD* gene. In this study, PQQ overproduction in *G. oxydans* was attempted by overexpressing PQQ biosynthetic genes using the promoter of *pqqA* and the elongation factor TU (*tufB*). Overexpression of each single gene could enhance PQQ biosynthesis except for the *tldD* gene. Overexpression of *pqqA*, *pqqB*, *pqqC*, *pqqD* and *pqqE* with the *pqqA* promoter enhanced the extracellular PQQ concentration by 38.5%, 68.4%, 19.9%, 30.3% and 8.2%, respectively, whereas production was enhanced by 59.4%, 85.7%, 30.9%, 42.2% and 19.3%, respectively, using the *tufB* promoter. In addition, the results show that PQQ biosynthesis could be enhanced by overexpressing some of the individual genes in the gene cluster in *G. oxydans* and the PQQ levels were positively correlated with the efficiency of conversion of D-sorbitol to L-sorbose. The results demonstrated that cofactor engineering of PQQ in *G. oxydans* is beneficial for enhancing the production of quinoprotein-related products.

1. Introduction

Gluconobacter oxydans is an obligate aerobic Gram-negative bacterium [1,2]. It can incompletely oxidize various alcohols and sugars by dehydrogenases, many of which employ pyrroloquinoline quinone (PQQ) as a cofactor and are called as quinoproteins [3]. Several oxidation reactions carried out by *G. oxydans* quinoproteins are applied in industrial processes, e.g., the conversion of D-glucose to 1-deoxynojirimycin, a precursor for the production of miglitol, and the conversion of D-sorbitol to L-sorbose, an intermediate in the synthesis of vitamin C (L-ascorbic acid) [4,5]. Besides its vital functions in the production of many important bulk chemicals [3], PQQ has attracted considerable attention due to its positive physiological effects on growth and development of mammals [6,7]. PQQ has also been recognized as a neuro-protective factor in a rodent model of hypoxic brain injury [8,9], and therefore qualifies as a newcomer to the B group of vitamins [10].

About twenty years ago, Gupta et al. found that the deficiency of *pqqE* on the genome of *G. oxydans* IFO 3293 coincided with a deficiency in quinoprotein glucose dehydrogenase activity [11]. This resulted in the identification of the *pqqABCDE* cluster in *G. oxydans* ATCC 9937 [12]. Further Tn5 transposon mutagenesis showed that a gene in *G. oxydans* 621H with high sequence identity to the *tldD* gene, which is involved in PQQ biosynthesis of *Escherichia coli* [13]. It was suggested that it may have a similar function to that of the *pqqF* genes present in other PQQ-synthesizing bacteria, such as *Klebsiella pneumoniae* and *Methylobacterium extorquens* [14]. The co-transcription of the *pqqAB* genes indicated that the *pqqA* promoter represents the only promoter within the *pqqABCDE* cluster of *G. oxydans* [13].

Balanced co-expression of both the key enzyme and related cofactors is a useful strategy to enhance catalytic processes [15]. The conversion of D-sorbitol to L-sorbose are catalyzed by a PQQ-dependent or an FAD-dependent D-sorbitol dehydrogenase in *G. oxydans* strains [16]. The D-sorbitol dehydrogenase of *G. oxydans* WSH-003 consisted of D-sorbitol dehydrogenase subunits SldA and SldB, is a PQQ-dependent dehydrogenase [17]. No FAD-dependent

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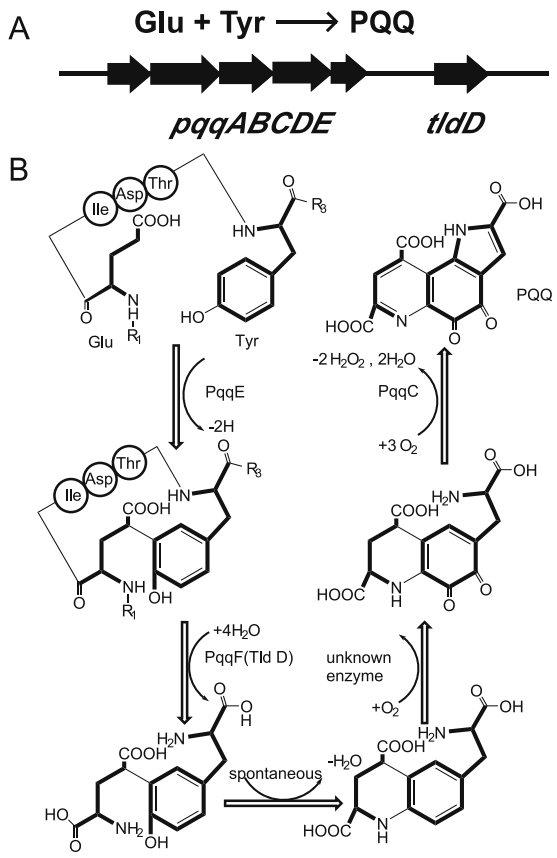

Fig. 1. PQQ biosynthetic genes and the proposed biosynthetic pathway of PQQ in *G. oxydans* WSH-003.
(A) There are six genes involved in PQQ biosynthesis: *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE* and *tldD*. (B) It takes five steps to generate PQQ. Step 1 is catalyzed by PqqE, linking the C atoms from the glutamate and tyrosine to become C9 and C9a of the final product PQQ, enabling recognition and acceptance of the modified PqqA by PqqF. Step 2 is catalyzed by PqqF, deleting the linked amino acids of the PQQ framework. Step 3 is a spontaneous Schiff base reaction. Step 4 is a dioxygenation catalyzed by an unknown enzyme. The last step is a cyclization and oxidation catalyzed by PqqC.

D-sorbitol dehydrogenase is found in *G. oxydans* WSH-003. Genes involved in PQQ synthesis have been characterized in several bacteria, including *M. extorquens* AM1 [14], *K. pneumoniae* and *Acinetobacter calcoaceticus* [18,19]. The genome sequence of *G. oxydans* WSH-003 revealed that this strain carries a *pqqABCDE* operon and a *tldD* gene, which display 78% and 77% sequence identity to the *pqqABCDE* operon and the *tldD* gene from *G. oxydans* 621H, respectively [17]. However, the details of PQQ biosynthesis pathway have not yet been clearly described. Only some of the proteins involved have been functionally characterized.

The *pqqA* genes from different species encode small peptides of 23–29 amino acids with conserved glutamate and tyrosine residues [20], and the PQQ backbone is probably derived from the PqqA peptide [21]. The PqqB protein takes part in transporting PQQ across the membrane [22]. PqqC catalyzes the final step in PQQ formation [23]. PqqE contains a conserved cysteine motif that is present in proteins known as radical S-adenosylmethionine (SAM) enzymes [24]. PqqE may play a role in linking of the amino acid side chains of glutamate and tyrosine in PqqA. PqqD interacts specifically with PqqE, causing a perturbation in the electronic environment around the $[4Fe–4S]^{+}$ clusters [25]. The role of PqqD may be to orient the active site of PqqE, effectively positioning the 5′-deoxyadenosyl radical to attract a hydrogen atom from PqqA. The PqqF and PqqG proteins show similarity to a family of metalloendopeptidases that cleave small peptides [14], and thus have been inferred to be involved in processing the peptide precursor of PQQ (Fig. 1).

Although several recombinant strains were developed to enhance the production of PQQ in *G. oxydans* by overexpression of the *pqqA*, *tldD* genes and *pqqABCDE* operon [13], other genes, *pqqB*, *pqqC*, *pqqD* and *pqqE*, have not been investigated. Herein, all of the PQQ biosynthetic genes, *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE* and *tldD*, were independently overexpressed to determine their impacts on PQQ biosynthesis and L-sorbose production. The results showed that overexpression of each individual gene enhances the production of PQQ in *G. oxydans* except the *tldD* gene. Through analysis of extracellular and intracellular PQQ production of wild-type and recombinant strains, it was confirmed that the PqqB protein takes part in transporting PQQ across the membrane. PqqB protein was also hypothesized to take part in other important steps in PQQ biosynthesis. Moreover, PQQ levels in *G. oxydans* were found to be positively correlated to the efficiency of conversion of D-sorbitol to L-sorbose. The results show that overexpression of PQQ biosynthetic genes is an efficient strategy for enhancing the production of quinoprotein-related products.

2. Materials and methods

2.1. Strains and plasmids

The *G. oxydans* WSH-003 strain, which had been sequenced in our previous study (GenBank Accession No. AHKI00000000.1) [17], was obtained from Jiangshan Pharmaceutical Co., Ltd. (Jingjiang, China). *G. oxydans* 621H was purchased from the American Type Culture Collection (ATCC). *E. coli* JM109, *E. coli* BL21 (DE3) and pET-28a(+) vector were purchased from Novagen (Darmstadt, Germany). The plasmid pBBR1MCS-2 is a broad-host vector that was proven to be effective in *G. oxydans* [26]. The relevant characteristics and references for the plasmids used in this work are listed in Table 1.

2.2. Media and culture conditions

All *E. coli* JM109 strains were cultivated in Luria-Bertani (Oxoid, Basingstoke, UK) medium at 37 °C and 200 rpm. All *E. coli* BL21 (DE3) strains were cultivated in a Terrific broth (Oxoid) mediums at 30 °C and 200 rpm. The *G. oxydans* strains were activated on agar plates that were prepared by adding 2% agar to a sorbitol medium containing 5% D-sorbitol and 0.6% yeast extract. The pre-culture was aerobically cultivated in 25 mL of growth medium (5% D-sorbitol and 0.6% yeast extract) in 250-mL flasks at 30 °C and 200 rpm, and transferred to 50 mL of fermentation medium (15% D-sorbitol and 1% yeast extract) in 500-mL flasks. Ampicillin (100 μg/mL), kanamycin (50 μg/mL) and/or cephalosporins (50 μg/mL) were added when necessary.

2.3. Genetic operations

Genomic DNAs were extracted from *G. oxydans* WSH-003 and *G. oxydans* 621H by FastPure DNA kit (Sangon, Shanghai, China). The open reading frames (ORFs) of *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE*, *tldD*, the *pqqA* promoter from *G. oxydans* WSH-003 and the *tufB* promoter [27] from *G. oxydans* 621H were amplified by PCR with the primers given in Table 2. The *tufB* promoter was maturely used to express recombination proteins in *G. oxydans*. Both of the natural *pqqA* and *tufB* are strong promoters and proper for PQQ synthetic genes expression. In our work, the *tufB* promoter was ligated with *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE* and *tldD*, and the *pqqA* promoter was ligated with *pqqB*, *pqqC*, *pqqD* and *pqqE*, respectively, through fusion PCR [28]. The plasmids constructed to bear various fragments of PQQ biosynthetic genes between multiple cloning sites (MCS) of pBBR1MCS-2 are shown in Fig. 2.

ID	Title	Pages
2708	Overexpression of pyrroloquinoline quinone biosynthetic genes affects l-sorbose production in Gluconobacter oxydans WSH-003	8