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ORIGINAL PAPER

Pyrroloquinoline quinone biosynthesis in *Escherichia coli* through expression of the *Gluconobacter oxydans pqqABCDE* gene cluster

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Abstract We have expressed the *pqqABCDE* gene cluster from *Gluconobacter oxydans*, which is involved in pyrroloquinoline quinone (PQQ) biosynthesis, in *Escherichia coli*, resulting in PQQ accumulation in the medium. Since the gene cluster does not include the *tldD* gene needed for PQQ production, this result suggests that the *E. coli tldD* gene, which shows high homology to the *G. oxydans tldD* gene, carries out that function. The synthesis of PQQ activated *d*-glucose dehydrogenase in *E. coli* and the growth of the recombinant was improved. In an attempt to increase the production of PQQ, which acts as a vitamin or growth factor, we transformed *E. coli* with various recombinant plasmids, resulting in the overproduction of the PQQ synthesis enzymes and, consequently, PQQ accumulation—up to 6 mM—in the medium. This yield is 21.5-fold higher than that obtained in previous studies.

Keywords Biosynthesis · Expression · Gene cluster · Pyrroloquinoline quinone

Introduction

Pyrroloquinoline quinone (PQQ), a redox cofactor, has been reported to occur in dehydrogenases, oxidases, oxygenases, hydratases, and decarboxylases [1]. As PQQ has an orthoquinone structure that is directly responsible for oxidoreduction, the role of these quinoproteins is to catalyze the primary oxidation step of non-phosphorylated substrates, such as alcohols, aldehydes, or aldoses. Consequently, PQQ is considered to the third type of coenzyme, after pyridine nucleotides and flavins, in biological oxidoreduction [2].

PQQ has been found in both prokaryotic and eukaryotic organisms, such as *Klebsiella pneumoniae*, *Methylobacterium extorquens*, *Pseudomonas aeruginosa*, *Polyporus versicolor*, and *Rhus vernicifera* [3]. Interestingly, the enteric bacterium, *Escherichia coli*, is unable to produce PQQ [4]. The *pqq* genes involved in PQQ synthesis have been identified in several bacterial species. In *K. pneumoniae*, the PQQ biosynthetic genes are clustered in the *pqqABCDEF* operon [5]. *M. extorquens* AM1 contains a *pqqABC/DE* operon in which the *pqqC* and *pqqD* genes are fused, while the *pqqFG* genes form an operon with three other genes [6–8]. In *P. aeruginosa*, the *pqqABCDE* operon is separated from the *pqqF* operon [9]. The corresponding *pqq* genes are highly conserved among the various species, with the exception of *K. pneumoniae pqqF*.

The *pqq* cluster of *Gluconobacter oxydans* ATCC 9937 was cloned and sequenced in 2000 [10]. It has five genes, namely, *pqqABCDE*. In 2005, the sequencing of the whole genome of *G. oxydans* 621H revealed that it has a *pqqABCDE* operon that shares high sequence similarity with the *pqqABCDE* operon from *G. oxydans* ATCC 9937 [11]. Holscher more recently reported that, in addition to the *pqqABCDE* cluster, a gene showing high homology to the *E. coli tldD* gene is also essential for PQQ biosynthesis in *G. oxydans* 621H. This gene, *tldD,* from *G. oxydans* 621H, may have the same function as the *pqqF* genes found in other PQQ-synthesizing bacteria [12].

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PQQ biosynthesis in *E. coli* has been successfully achieved through expression of the *pqq* gene clusters of *Acinetobacter calcoaceticus* and *K. pneumoniae* [13, 14]. In the former case, PQQ production was distinctly low, but in the latter case, 280 nM PQQ was accumulated in the medium. The most likely explanation is that PQQ biosynthesis takes place in the cytoplasm, and the PQQ is then released into the medium.

We report here the cloning of the *pqqABCDE* gene cluster from *G. oxydans* M5. *E. coli* strains harboring various plasmids containing the *pqqABCDE* gene cluster were found to accumulate PQQ in large amounts in the medium. We also found that the growth rate of the recombinants was significantly improved in minimal glucose medium.

Materials and methods

Materials

Phenazine methosulfate (PMS), PQQ, and 2,6-dichlorophenolindophenol (DCIP) were purchased from Sigma–Aldrich (St. Louis, MO). LA *Taq* polymerase and restriction and modification enzymes were purchased from TaKaRa (Dalian, China). D-sorbitol, D-mannitol, D-arabitol, and other sugar alcohols were obtained from Amresco (Shanghai Genebase Co., Shanghai, China). Other chemicals were obtained commercially and were of reagent grade.

Bacterial strains, plasmids and cultivation

G. oxydans M5 was selected in our laboratory to produce L-sorbose from D-sorbitol [15]. The pMD19-T kit was purchased from TaKaRa. *G. oxydans* M5 was grown in D-sorbitol medium consisting of 20 g D-sorbitol, 3 g yeast extract, 10 g polypeptone, 1 g KH_2PO_4, and 0.2 g $MgSO_4$–$7H_2O$ in 1 l deionized water. *E. coli* (JM109, BL21) and recombinant strains were grown in Luria–Bertani (LB) medium at 37°C. For the analysis of D-glucose dehydrogenase (GDH) activity, the strains were pre-cultured in LB medium. The pre-culture cells were washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer at the same concentration as in the original pre-culture. The suspension (inoculum size 5%, v/v) was then transferred to 100 ml glucose minimal medium (5 g D-glucose, 2 g citrate, 10 g potassium phosphate dibasic, and 3.5 g sodium ammonium phosphate in 1 l tap water). The pH was adjusted to 7.0. The cultures were grown at 37°C under vigorous agitation, and cells were harvested at the late-exponential phase after about 25 h of incubation. The antibiotics (Sigma–Aldrich) were used in the medium at the following concentrations: kanamycin, 25 μg/ml; ampicillin, 100 μg/ml.

Preparation of membrane fractions

Cells were collected by centrifugation, washed with 50 mM potassium phosphate buffer (pH 7), and resuspended in the same buffer at a concentration of 0.2 g/ml wet cells. Following the addition of a few grains of DNase powder, the suspension was passed twice through a high-pressure laboratory homogenizer (ATS, Italy) and centrifuged at 12,000 *g* for 20 min to remove intact cells and cell debris. The membranes were precipitated by centrifugation at 120,000 *g* for 60 min and homogenized with 50 mM potassium phosphate buffer (pH 7).

D-glucose dehydrogenase assay

GDH activities were determined photometrically at 600 nm in a dye-linked system containing DCIP and PMS at 30°C before or after holo-enzyme formation. The reaction mixture contained enzyme solution, phosphate buffer pH 6.4, 200 mM substrate, 0.67 mM PMS, 0.1 mM DCIP, and 4 mM sodium azide. One unit of dehydrogenase activity was defined as the reduction of 1 μmol/min DCIP, corresponding to the oxidation of 1 μmol/min substrate. The millimolar extinction coefficient of DCIP is 12.6 mM^{-1} cm^{-1} at pH 6.4.

Protein concentrations were determined as described by Bradford [16], using bovine serum albumin as the standard.

PQQ determination

The presence of PQQ in culture supernatants was determined using crude membranes from *E. coli* containing apo-glucose dehydrogenase [17]. Holo-enzyme was prepared by incubating 250 μl of membrane fractions (approximately 0.4 mg protein) at 30°C for 30 min in 50 mM potassium phosphate buffer (pH 6.4) containing 250 μl of sample or a specific amount of PQQ standard (0–10 ng) and 10 mM $MgSO_4$, resulting in 500 μl of enzyme solution. GDH activity was measured as described above after a 5-min incubation in 3 ml of a reaction mixture containing 100 μl solution of enzyme and 0.1% Triton X-100. The reaction was started by the addition of D-glucose substrate. The concentration of PQQ was determined from a standard curve prepared with 0–10 ng PQQ in the reaction mixture.

Construction of expression vectors of the *pqqABCDE* gene cluster

DNA manipulations were performed according to standard protocols [18]. Genomic DNA isolated from *G. oxydans* M5 was used as a template for the PCR analysis. The *pqqABCDE* gene cluster operon sequence information in the whole genome sequence of *G. oxydans* 621H (accession number CP000009) was used to design the primers

Table 1 Sequences of primers used in this study

Primer	Sequence (5′–3′)[a]
pqqF	GCACATGTCGCGGATGTTCAGGTGTTC
pqqR	GTCTTCTACCGGAGAGGACCCTTCT
pqqPLF	GTACGAATTCGCGGATGTTCAGGTGTTTCGC
pqqPLR	ACCGCTGCAGAGAAGATGGCCTCTCCTGGG
PqqPT7F	A CATATGGCCTGGAACACACCGAAAG
PqqPT7R	TGAATTC TTA CGT ATA ACG CCT GTA GAAC

[a] Restriction sites used for subcloning are underlined

according to the methods of Hölscher [12]. The primer sequences and restriction enzymes used in this study are listed in Table 1. Amplification of these gene fragments was performed by PCR using LA DNA polymerase (TaKaRa) as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of 45 s at 95°C, 45 s at 55°C, and 240 s at 72°C. A 3.7-kb DNA fragment of *G. oxydans* M5 bearing the *pqqABCDE* operon and parts of the upstream and downstream open reading frames (ORFs) was amplified using the primers of pqqF and pqqR and then cloned with T-A vector of pMD19-T, resulting in plasmid pMD19-PQQ. For expression of the *pqqABCDE* gene cluster with different promoters, the open reading fragment of the *pqqABCDE* gene cluster was amplified from pMD19/PQQ using two pairs of primers (pqqPLF and pqqPLR; pqqPT7F and pqqPT7R), respectively, and subcloned into the plasmid pUC18 between the *EcoR*I–*Pst*I sites downstream of the *LacZ* promoter, resulting in the plasmid pUC18-PLPQQ, and into pET28a between the *Nde*I–*EcoR*I sites downstream of T7 promoter, resulting in pET28a-T7PQQ, respectively. The insert fragments were sequenced (Invitrogen, Shanghai, China).

Results and discussion

Cloning and sequencing of the *pqqABCDE* gene cluster

The full-length gene cluster of *pqqABCDE* (3.7 kb) was amplified from the chromosome of *G. oxydans* M5 by PCR. The amplified fragment was cloned in plasmid pMD19-T, resulting in pMD19-PQQ. Sequencing of the fragment revealed that it contained five ORFs that are 100% identical with the genome sequence of *G. oxydans* 621H [11]. This 100% sequence identity between *G. oxydans* M5 and *G. oxydans* 621H was unexpected as the *G. oxydans* 621H strain had never been grown in our laboratory and, therefore, could not have resulted from contamination with the DNA from the 621H strain. The five ORFs were named A, B, C, D, E and consisted of 81, 915, 720, 291, and 1,077 bp, encoding 27, 305, 240, 97, and 359 amino acids, respectively.

Overexpression of the gene cluster in *E. coli* using different vectors

To improve PQQ synthesis in *E. coli*, we used standard procedures to construct two expression vectors containing the gene cluster under the control of the *LacZ* and T_7 promoters, respectively. Following the transformation of *E. coli* with the two vectors, colonies having an ampicillin-resistant phenotype were selected.

When the cultures were grown initially at 37°C, recombinant proteins appeared to be mainly present in inclusion bodies. We therefore reduced both the incubation temperature and the isopropyl-beta-thio galactopyranoside (IPTG) concentration. At a culture temperature of 30°C and 0.5 mM IPTG, we detected a large amount of soluble expressed protein. The predicted protein products of the gene cluster, PqqA, PqqB, PqqC, PqqD, and PqqE, have calculated molecular weights of 2.9, 32.9, 25.9, 10.5, 38.77 kDa, respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the soluble fraction resulted in two distinct protein bands with molecular masses of about 40 and 32 kDa, which approximate the calculated molecular weights of PqqB and PqqE (Fig. 1). Under these expression conditions, the presence of the predicted smaller molecular weights proteins could not be detected by SDS–PAGE.

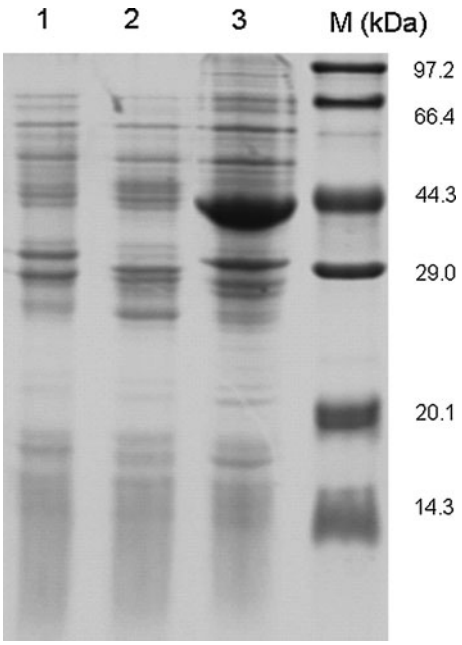

Fig. 1 Expression of *pqqABCDE* in *Escherichia coli*. *Lanes*: *1* Supernatant of cell-free extracts of BL21, *2* supernatant of cell-free extracts of recombinant BL21 (DE3) containing pET28a, *3* supernatant of cell-free extracts of recombinant pET28a-T7PQQ, *M*, molecular mass standard

Restoration of *E. coli* GDH activity by expression of the *pqqABCDE* gene cluster

Glucose dehydrogenase (EC 1.1.99.17) is a widespread quinoprotein present in many species, including members of the *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter*. However, in *E. coli,* which is unable to synthesize PQQ, GDH is present as apo-GDH [4]. In this study, the GHD activity of *E. coli* was restored by expression of the *pqqABCDE* gene cluster. The recombinant strains JM109/pUC18-PLPQQ, JM109/pMD19-PQQ, and BL21/pET28a-T7PQQ produced small clear zones surrounding colonies grown on glucose-calcium carbonate agar; in comparison, there were no such zones surrounding colonies of the wild-type strain. In the former,, the glucose in the medium was oxidized to yield gluconate, which can produce a clear zone on a glucose-calcium carbonate agar plate. To quantitatively assess these results, we further assayed GDH activity using membrane fractions prepared as described in the Materials and Methods. These strains were cultured in minimal glucose medium in order to avoid interference from pre-formed PQQ. As shown in Table 2, the recombinants showed higher PQQ–GDH activities than their wild-type counterpart, indicating that PQQ was synthesized in *E. coli* through the expression of the *pqqABCDE* gene cluster alone. In an earlier study, Hölscher reported that a gene showing high homology to the *E. coli tldD* gene, in addition to the *pqqABCDE* cluster, is also involved in PQQ biosynthesis in *G. oxydans* 621H [12]. Therefore, this result suggests that the *E. coli tldD* gene carries out the same function as that of the *tldD* of *G. oxydans.*

Effect of the *pqqABCDE* gene cluster expression in *E. coli* on growth

The *pqqABCDE* gene cluster expression in *E. coli* had a significant effect on growth. When *E. coli* JM109 and recombinant *E. coli* JM109/pUC18-PLPQQ or JM109/pMD19-PQQ containing the *pqqABCDE* gene cluster plasmid were incubated on LB agar plates at 37°C for 14 h, the recombinant colonies were larger than those of the wild type (Fig. 2). Furthermore, when these stains were cultured in minimal D-glucose medium, the length of the lag phase was only 5 h for both of the recombinant stains; in contrast, the lag phase was 10 h for the wild type. This shows that the length of the lag phase in the recombinant lines was markedly reduced compared with the wild type. Similar effects were found for BL21 and its derivatives controlled by the T7 promoter.

These results indicate that PQQ production resulting from the *pqqABCDE* gene cluster can improve the growth rate of recombinants and reduce the length of the lag phase. We therefore suggest that the oxidative metabolism of glucose in *E. coli* via PQQ–PDH was activated as a result of PQQ synthesis in recombinants and that gluconate, the product of glucose oxidation, is used as a growth substrate. As such, this provides an alternative route for glucose utilization via the Entner–Doudoroff pathway, in addition to the PTS (glucose phosphotransferase system). The results are comparable to those obtained following the addition of PQQ in vitro [19, 20]. The nutritional value of PQQ as a vitamin or growth factor for eukaryotic cells has been also pointed out [21].

PQQ biosynthesis in *E. coli* using different expression vectors

To further investigate the effects of *pqqABCDE* gene cluster expression on PQQ synthesis, we monitored PQQ

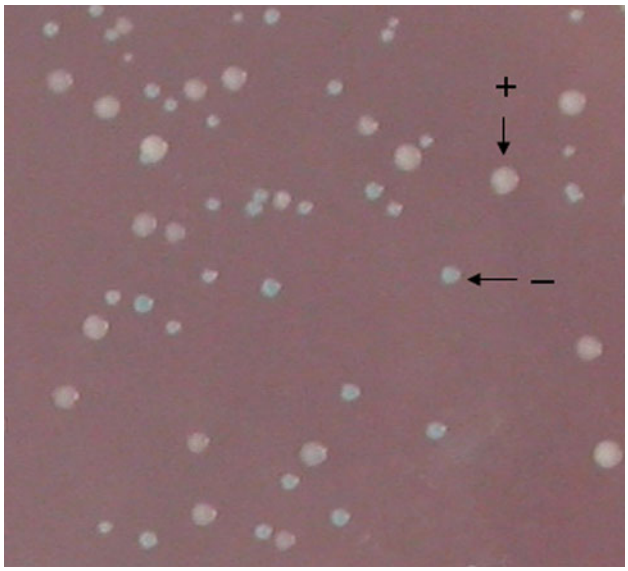

Fig. 2 Colonial morphology of recombinant *E. coli* strains JM109/pUC18-PLPQQ(*white color*) and JM109/pUC18 (*blue color*). The cells were aerobically grown on LB agar plates at 37°C for 14 h

Table 2 Enzyme activities involved in glucose oxidation

Glucose dehydrogenase activity (U/mg)				
E. coli JM109	*Escherichia coli* BL21	JM09/ pMD19-P_{n}-*pqq*	JM109/ pUC18-P_{L}-*pqq*	BL21/ pET28a-P_{T7}-*pqq*
0.003	0.002	2.52	2.98	3.00

Values are the average values of three replicates

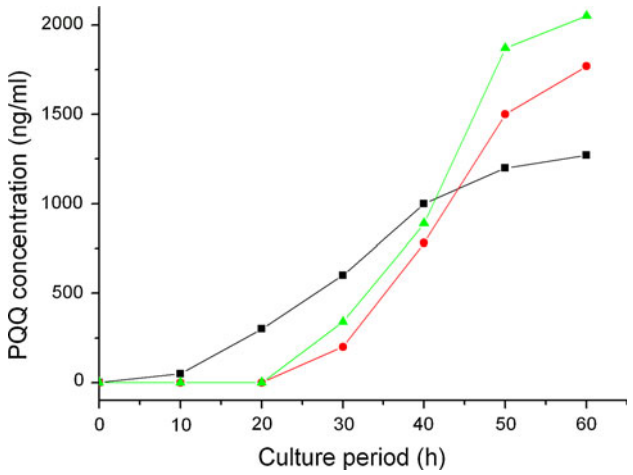

Fig. 3 PQQ synthesis using the *pqqABCDE* gene cluster overexpression strains. The time-course of pyrroloquinoline quinone accumulation (PQQ) in the culture supernatant when JM109/pMD19-PQQ (*filled square*), JM109/pUC18-PLPQQ (*filled circle*), and BL21/pET28a-T7PQQ (*filled triangle*) were grown in minimal glucose medium was monitored. Average values from three replicates are shown

concentration during the growth of the three recombinants using wild-type *E. coli* crude membrane fractions as a source of apo-GDH. A vigorously growing exponential culture (inoculum size 1%, v/v) was inoculated into fresh minimal glucose medium, samples were taken at intervals, and the samples were analyzed for PQQ. The results are summarized in Fig. 3. These findings parallel the results obtained during the growth analysis: the wild-type *E. coli* strain was not able to synthesize PQQ. In contrast, the *E. coli* JM109/pMD19-PQQ recombinant strain was able to produce PQQ up to 1,100 ng/ml. Even larger amounts of PQQ were found in cultures of the *E. coli* JM109/pUC18-PLPQQ or BL21/pET28a-T7PQQ recombinant strains. Using these two latter strains, nearly 2,000 ng/ml (6 mM) PQQ had accumulated 38 h after the start of induction by IPTG. This amount of synthesized PQQ is 21.5-fold higher than that observed in previous studies (280 nM, i.e., 92.4 ng/ml) [14] and demonstrates that the rather elaborate biosynthesis of PQQ can be enhanced significantly through the overexpression of the *pqqABCDE* gene cluster.

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