Investigation of biosynthesis of intracellular and extracellular pyrroloquinoline quinone by the *Erwinia* sp. 34-1 and *Gluconobacter* sp. 33

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Biosynthesis of pyrroloquinoline quinone (PQQ) in *Erwinia* sp. 34-1 and *Gluconobacter* sp. 33 and its alcohol dehydrogenase deficient mutant was investigated. Both the synthesis of cofactor and PQQ-dependent enzymes correlated with the growth of the cells and began at the early logarithmic phase and stopped at the early stationary phase. All strains were able to excrete PQQ into the culture liquid, but at different levels (5–170 pmol/ml). The dependence of biosynthesis of extra-, intracellular PQQ and quinoenzymes on carbon sources was studied. Synthesis of quinoenzymes and intracellular PQQ did not strictly depend on the carbon source used. The presence of substrates of PQQ-dependent enzymes was not necessary for the production of appropriate quinoenzymes.

Key words: pyrroloquinoline quinone, quinoenzymes, glucose dehydrogenase, alcohol dehydrogenase, glycerol dehydrogenase

INTRODUCTION

Pyrroloquinoline quinone (PQQ) is a cofactor for so-called quinoproteins, which usually transfer redox equivalents from the substrates to the respiratory chain. PQQ was detected in the membrane-bound and soluble enzymes produced by a large number of gram-negative, gram-positive bacteria and in animal tissues [1–3]. A PQQ-dependent enzyme may be produced either in active holo form or in the form of apoenzyme (without PQQ) [4–6]. Many bacteria not only synthesise PQQ to provide their quinoprotein apoenzymes with PQQ, but also excrete the compound into their culture medium [1, 7]. Many strains of *Pseudomonas, Acinetobacter, Nocardia, Hyphomicrobium, Methylobacillus* and *Methylobacterium* produced different amounts of free PQQ (5–8000 pmol/ml) in the culture medium [1, 8], but the correlation between biosynthesis of quinoproteins and free PQQ is not yet clear. Moreover, there are no data on the biosynthesis and/or secretion of PQQ in *Erwinia* or *Gluconobacter* strains notwithstanding that the PQQ biosynthesis encoding genes were cloned from the latter organism [9].

There are some methods for detection of PQQ in the enzymes or biological samples. A very sensitive method for detection of PQQ in picomole-range is reduction of nitro blue tetrazolium to its formazan by PQQ in the cycling reaction with sodium glycinate at alkaline pH [10]. However, this reaction was not restricted only to the PQQ, but appeared to be a general property of quinones, so this method for detection of PQQ might be used only for purified enzymes or free PQQ. PQQ in purified enzymes may be also detected by analysis of ultraviolet-visible absorption spectra [11], but the detection of cofactor in the crude extracts or cultural liquids is not proper, because many compounds might absorb in the same region of the spectrum. The most sensitive and useful method for detection of PQQ is a biological assay which is based on reconstitution of the apoenzyme into the active holoenzyme by adding a cofactor in the presence of Mg^{2+} or Ca^{2+} [5, 12, 13]. This method is very specific for PQQ but not for PQQ adducts or other quinonoid compounds. Different apoenzymes (glucose dehydrogenase (GDH) from *Acinetobacter calcoaceticus* [14], alcohol dehydrogenase (ADH) from *Comamonas testosteroni* [15] or crude membranes from *Escherichia coli* [16]) have been used for this purpose.

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PQQ-dependent glucose dehydrogenase from *Erwinia* sp. 34-1, PQQ-dependent alcohol and glycerol (GlycDH) dehydrogenases from *Gluconobacter* sp. 33 were purified to homogeneity and characterized previously [17–19]. In spite of a detailed investigation of PQQ-dependent enzymes, biosynthesis of extracellular and intracellular PQQ and its correlation with the enzyme biosynthesis in these microorganisms were not investigated.

This study has been intended to examine the production of the PQQ cofactor in *Gluconobacter* sp. 33 and its alcohol dehydrogenase deficient mutant (ADH^-) and *Erwinia* sp. 34-1 producing the PQQ-dependent enzymes with the aim to elucidate the influence of cultivation conditions on PQQ biosynthesis and a correlation between biosynthesis of PQQ and the quinoenzymes.

MATERIALS AND METHODS

Materials. All chemicals used in the study were commercial products of guaranteed grade.

Strains and cultivation. The bacterial strains *Erwinia* sp. 34-1, *Gluconobacter* sp. 33 and *Gluconobacter* sp. 33 ADH^- mutant used for this work were described previously and cultivated according to [17–19].

Extraction of PQQ. PQQ was extracted according to a modified method described in [20]. Cells were collected by centrifugation, washed twice with 0.9% NaCl and diluted with distilled water to the optical density 100 at 600 nm. 2 ml of cell suspension was lyophilised, PQQ was extracted with 2 ml of methanol. The precipitate was separated by centrifugation, the supernatant was collected and methanol evaporated. The precipitate containing extracted PQQ was dissolved in 100 µl of 50 mM Tris/HCl buffer, pH 7.5. For determination of extracellular PQQ, 2 ml of culture liquid were lyophilised, PQQ was extracted by 1 ml methanol and collected.

Preparation of the GDH apoenzyme. Glucose dehydrogenase from *Erwinia* sp. 34-1 was purified as described [17]. PQQ was eluted from the purified enzyme by dialysis for 5 days against 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM EDTA and following dialysis against the same buffer without EDTA for two additional days.

Reconstitution of apoenzyme of GDH. Reactivation of apoenzyme to the active holoenzyme was made by a modified method described in [11]. Reaction of reconstitution was performed in 50 µl of 50 mM Tris/HCl buffer, pH 7.5, containing 4 mM $CaCl_2$, 4 mM $MgSO_4$ (buffer A), 5 µl of GDH apoenzyme and 5–10 µl of PQQ solution (commercial, extracted from cells or cultural liquid). The reaction mixture was incubated at room temperature for 30–40 min.

Determination of PQQ amount. A calibration curve of the dependence of the activity of GDH on PQQ amount in the reaction mixture was drawn. The linear interval of the calibration curve was 0–25 pmol of PQQ in the reaction mixture.

Enzyme assay. Activity of GDH, ADH and GlycDH was measured with phenazine methosulfate (PMS) and dichlorophenol indophenol (DCPIP) as electron acceptors as described previously [17–19].

RESULTS AND DISCUSSION

Determination of PQQ. In order to detect even small amounts of PQQ, the cofactor was concentrated by extraction of it from lyophilized samples of culture liquid and cells with methanol, evaporation of solvent and dissolving precipitate in the minimal volume of buffer A. Such extraction of PQQ and the following reconstitution of PQQ-dependent GDH apoenzyme as described in Materials and Methods allowed to detect even very small quantities of PQQ: 3–5 pmoles/ml of cultural liquid and ≥ 0.06 pmoles in cells. In all cases when PQQ was assayed in cell the biomass was collected from 1 ml of culture. It should be stressed that the real concentration of PQQ might be higher, since part of PQQ could form adducts with the amino acids [1, 14] not detected by the biological assay method.

Dependence of PQQ biosynthesis on growth of cells. The cells of *Erwinia* sp. 34-1 and *Gluconobacter* sp. 33 (both wild type and ADH^- strains) were cultivated in the nutrient media containing glycerol and D-mannitol, respectively. The samples were taken every 3 hours and examined for the presence

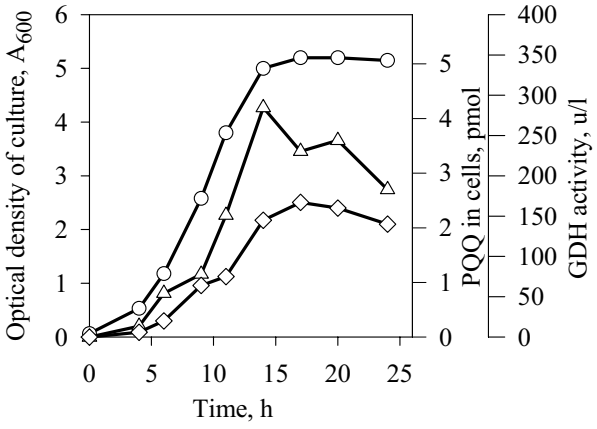

Fig. 1. Dependence of biosynthesis of intracellular PQQ and PQQ-dependent glucose dehydrogenase on cultivation time. Cells of *Erwinia* sp. 34-1 were cultivated and PQQ was assayed as described in Materials and Methods. Circles – optical density of culture, triangles – intracellular PQQ extracted from the cells collected from 1 ml of culture, diamonds – activity of PQQ-dependent glucose dehydrogenase

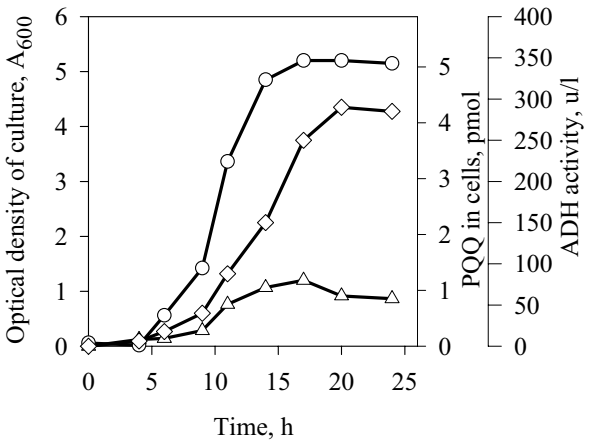

Fig. 2. Dependence of biosynthesis of intracellular PQQ and PQQ-dependent alcohol dehydrogenase on cultivation time. Cells of *Gluconobacter* sp. 33 were cultivated and PQQ was assayed as described in Materials and Methods. Circles – optical density of culture, triangles – intracellular PQQ extracted from the cells collected from 1 ml of culture, diamonds – activity of PQQ-dependent alcohol dehydrogenase

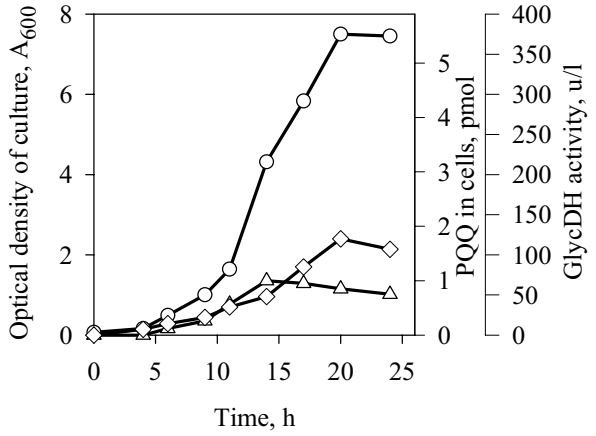

Fig. 3. Dependence of biosynthesis of intracellular PQQ and PQQ-dependent glycerol dehydrogenase on cultivation time. Cells of *Gluconobacter* sp. 33 were cultivated and PQQ was assayed as described in Materials and Methods. Circles – optical density of culture, triangles – intracellular PQQ extracted from the cells collected from 1 ml of culture, diamonds – activity of PQQ-dependent glycerol dehydrogenase.

of PQQ and activity of an appropriate enzyme. Results are shown in Fig. 1, 2 and 3. Biosynthesis of intracellular PQQ began in the early logarithmic phase and lasted up to the early stationary phase and directly correlated with the biosynthesis of PQQ-dependent enzymes in all three strains. The increase of intracellular concentration of PQQ was stopped when growth of the cells was ended.

The content of PQQ in the cells of *Erwinia* sp. 34-1 was about four times higher than in the case of the strain *Gluconobacter* sp. 33 (Figs. 1 and 2). The wild strain and ADH$^-$ mutant of *Gluconobacter* sp. 33 produced the same quantities of intracellular PQQ (Figs. 1 and 2) in spite of a difference in produced enzymes. A decrease of intracellular PQQ in the late stationary phase might be caused by the lysis of the cells and a release of PQQ (bound to the quinoenzymes) into the medium.

The biosynthesis of extracellular PQQ was quite different in all three strains (Fig. 4). The cells of *Erwinia* sp. 34-1 secreted relatively small quantities of PQQ into the culture liquid (≈ 5.5 pmoles/ml). The same level of the cofactor was detected in the cells (4.2 pmoles). On the contrary, the concentration of extracellular PQQ reached 140–170 pmoles/ml at the beginning of stationary phase, and only 0.7% of total PQQ produced by *Gluconobacter* sp. 33 strain was found in the biomass. Almost the same or slightly decreased amount of PQQ remained in the medium during the further cultivation of the strain. The ADH$^-$ mutant of *Gluconobacter* sp. 33 secreted PQQ half as much as the wild strain. It seems that the mutation which prevented biosynthesis of PQQ-dependent ADH might have influenced the biosynthesis of PQQ or changed its transport. It was previously detected that PQQ excretion into the culture medium depended on the growth phase of the cells and had been observed only in the late exponential and stationary phase of the growth of methylotrophic bacteria [1, 21].

Influence of carbon source on biosynthesis of PQQ. The dependence of the biosynthesis of both intracellular and extracellular PQQ on various carbon sources was also investigated in *Erwinia* sp. 34-1 and *Gluconobacter* sp. 33 and its ADH$^-$ mutant. The strains were

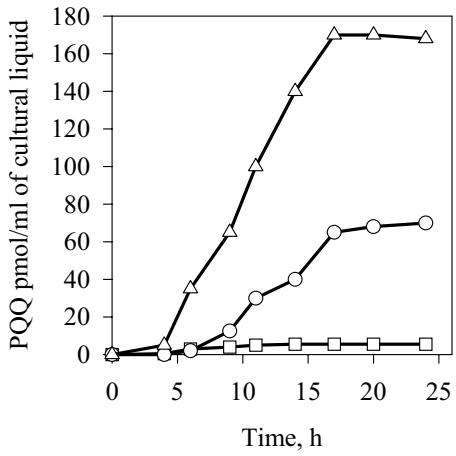

Fig. 4. Dependence of biosynthesis of extracellular PQQ on cultivation time by *Erwinia* sp. 34-1, *Gluconobacter* sp. 33 and *Gluconobacter* sp. 33 (ADH$^-$) mutant. Cells were cultivated and PQQ was assayed as described in Materials and methods. Squares – *Erwinia* sp. 34-1. Circles – *Gluconobacter* sp. 33 ADH$^-$ mutant, triangles – *Gluconobacter* sp. 33

cultivated until the early stationary phase in liquid nutrient media containing different carbon sources. The results are shown in Tables 1, 2 and 3. *Erwinia* sp. 34-1 cultivated on different carbon sources produced a relatively similar amount of PQQ intracellularly – 0.11–0.36 pmol in cells from 1 ml of culture. Almost the same amount of intracellular PQQ was found when the cells were cultivated without an additional carbon source (Table 1). Only glycerol induced the biosynthesis of PQQ in the cells four times.

The amount of intracellular PQQ produced by *Gluconobacter* sp. 33 varied from 0.1 pmol in the control media up to 1.18 pmol when bacteria were cultivated in the presence of D-mannitol (Table 2). The level of intracellular PQQ was only slightly lower (0.06–0.95 pmol) in the ADH-deficient mutant. A exception was D-glucose, which did not induce any biosynthesis of PQQ (Table 3).

The biosynthesis of extracellular PQQ by *Erwinia* sp. 34-1 was even more influenced by cultivation conditions. D-glucose and D-fructose induced the biosynthesis of the cofactor remarkably (up to 70 pmol/ml in culture liquid), but D-mannitol and D-sorbitol and the absence of additional carbon source almost completely suppressed it (Table 1). The extracellular biosynthesis of PQQ by the cells of *Gluconobacter* sp. 33 strain was induced by glycerol, D-glucose, D-fructose and D-mannitol (105–170 pmol/ml in culture liquid) (Table 2). A less significant induction (70–118 pmol/ml) was observed in the case of the ADH-deficient strain. Moreover, the mutant did not secrete PQQ into the culture medium when it was cultivated without additional carbohydrate or in the presence of D-sorbitol (Table 3).

It is known that many factors affect the biosynthesis of extracellular PQQ. These are the initial pH of the medium, the type and concentration of the carbon source, ammonia ions [8, 21], but usually the substrates of appropriate PQQ-dependent enzymes are the best inducers of synthesis of extracellular cofactor. So many strains of *Pseudomonas* pro-

Table 1. **Influence of carbon sources on the biosynthesis of PQQ and PQQ-dependent GDH by *Erwinia* sp. 34-1 strain**

Carbon source*	Optical density of culture, A_{600}	PQQ in cultural liquid**, pmol/ml	PQQ in cells***, pmol	GDH activity, u/l of culture
Glycerol	5.2	5.5	4.2	216.5
D-glucose	1.16	70	0.12	100
D-fructose	2.5	70	0.36	307.16
D-mannitol	0.72	Trace	0.13	8.55
D-sorbitol	0.15	Trace	0.11	5.7
None	0.2	Trace	0.13	4.8

* The strain was cultivated in liquid medium of the following composition (g/l): yeast extract – 0.1, NH_4Cl – 1.0, Na_2HPO_4 – 2.8, KH_2PO_4 – 2.77, $MgSO_4 \times 7H_2O$ – 2.0 and 10 of an appropriate carbon source, pH 7.2, at 30 °C for 18 hours.
** PQQ in culture liquid is the amount of PQQ extracted from 1 ml of culture after separation of the cells.
*** PQQ in cells is calculated as an amount of cofactor extracted from the cells collected from 1 ml of culture.

Table 2. **Influence of carbon sources on the biosynthesis of PQQ and PQQ-dependent enzymes by *Gluconobacter* sp. 33 strain**

Carbon source*	Optical density of culture, A_{600}	PQQ in cultural liquid, pmol/ml	PQQ in cells, pmol/ml	ADH activity, u/l of culture	GDH activity, u/l of culture	GlycDH activity, u/l of culture
Glycerol	4.86	105.0	1.1	122.2	152.0	24.0
D-glucose	3.8	120.0	0.44	0.0	185.0	trace
D-fructose	6.2	100.0	0.95	103.0	175.0	19.4
D-mannitol	6.8	170.0	1.18	253.0	265.0	trace
D-sorbitol	2.75	trace	0.52	173.0	173.0	37.0
none	0.2	0.0	0.1.	1.9	0.0	0.0

* The strain was cultivated in liquid media of the following composition (g/l): yeast extract – 5.0, $(NH_4)_2HPO_4$ – 1.0, $MgSO_4 \times 7H_2O$ – 2.0 and 10 of an appropriate carbon source, pH 5.5, at 30 °C for 18 hours. Other experimental conditions were the same as in Table 1

Table 3. **Influence of carbon sources on biosynthesis of PQQ and PQQ-dependent enzymes by *Gluconobacter* sp. 33 ADH⁻ mutant. Experimental conditions were the same as in Table 2**

Carbon source	Optical density of culture, A_{600}	PQQ in cultural liquid, pmol/ml	PQQ in cells, pmol	GDH activity, u/l of culture	GlycDH activity, u/l of culture
Glycerol	6.6	100	0.2	128.5	116.6
D-glucose	0.84	118	0.06	3.9	trace
D-fructose	5.2	80	0.54	180.3	132.8
D-mannitol	5.8	70	0.95	224.2	112.5
D-sorbitol	1.85	trace	0.37	5.6	84.4
none	0.2	0.0	0.07	1.15	0.26

ducing ADH secreted a huge amount of free PQQ into the culture medium (500–4000 pmol/ml) when they were cultivated on alcohols as a carbon source. Even more of extracellular PQQ was secreted by *Hyphomicrobium* X (3000–6000 pmol/ml) and *Methylobacterium organophylum* (2000–8000 pmol/ml) during cultivation on methanol [1]. It should be noted that both strains are producers of PQQ-dependent methanol dehydrogenases. On the contrary, *Acinetobacter calcoaceticus*, a good producer of PQQ-dependent GDH, secreted only 5–200 pmol/ml of free PQQ during cultivation in the medium containing succinate, quinate or *p*-hydroxybenzoate [1]. Results of this study show that there is no strong correlation between the carbon source and the biosynthesis of extracellular PQQ.

Dependence of biosynthesis of quinoenzymes on carbon source. Data on the influence of carbon source in the cultivation medium on the biosynthesis of PQQ-dependent enzymes in *Erwinia* sp. 34-1, *Gluconobacter* sp. 33 and its ADH⁻ mutant are shown in Tables 1, 2 and 3. As can be seen, the yield of enzymes varied very much, so D-fructose, D-mannitol and D-glucose induced synthesis of GDH in the *Erwinia* sp. 34-1 strain. Almost all carbohydrates induced biosynthesis of GDH in *Gluconobacter* sp. 33, but D-glucose did not show any effect on GDH production in the ADH-deficient mutant of *Gluconobacter* sp. 33. The best yield of GlycDH was obtain when the mutant was cultivated on glycerol, D-fructose and D-mannitol. The ADH biosynthesis in *Gluconobacter* sp. 33 was induced by all the carbon sources tested, but not D-glucose. In the control media (without additional carbon source) a very small but detectable amount of the enzymes was synthesized, too. It can be concluded that a correlation between the carbon source, the strain and the production of PQQ-dependent enzymes was not detected. However, the better yields of quinoenzymes and intracellular PQQ were obtained when the carbon source supported the growth of the cells. A similar effect (that the biosynthesis of quinoenzymes is not induced) has been previously detected by many authors [1, 3, 22]. Schie and coworkers [22] have determined that synthesis of GDH apoenzyme was constitutive in the *Acinetobacter lwoffi* strain, because it occurred during growth in mineral media in the presence of variety of substrates (acetate, succinate, malate and ethanol). *A. calcoaceticus* was able to produce high levels of holoGDH independently on cultivation conditions, too [22]. It was shown that the presence of substrates of PQQ-dependent enzymes was not necessary for synthesis of quinoenzymes and PQQ [1]. It was also suggested that the biosynthesis of free and intracellular cofactors proceeded via different routes, because the discrepancy between the synthesis of free PQQ and of PQQ-dependent enzymes was remarkable [8, 23].

The results of this study allow the following conclusions:

i) PQQ-dependent enzymes and intracellular PQQ were synthesized constitutively and did not depend strictly on cultivation conditions (carbon source in the culture media);

ii) the presence of substrates of PQQ-dependent enzymes is not necessary for the production of the appropriate quinoenzymes;

iii) in contrast, secretion of PQQ into the medium is induced by different carbon sources, but in different manner in *Erwinia* and *Gluconobacter* strains.

ACKNOWLEDGMENTS

We thank R. Meškienė, R. Semėnaitė, V. Dzekevičienė and A. Krutkis for their assistance. This work was supported by the Lithuanian State Science and Studies Foundation grant No. T-518.

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VIDULĄSTELINIO IR NELĄSTELINIO PIROLOCHINOLINCHINONO BIOSINTEZĖS TYRIMAS *ERWINIA* SP. 34-1 IR *GLUCONOBACTER* SP. 33 KAMIENUOSE

S a n t r a u k a

Viduląstelinio ir neląstelinio PQQ biosintezė buvo tirta *Erwinia* sp. 34-1 ir *Gluconobacter* sp. 33 ir jo ADH^- mutanto kamienuose. Modifikuota PQQ ekstrakcijos iš ląstelių ir kultūrinio skysčio metodika leido nustatyti net labai mažus kofaktoriaus (PQQ) kiekius – nuo 0,06 pmolių 1 ml kultūrinio skysčio arba ląstelėse iš 1ml kultūrinio skysčio. PQQ ir nuo PQQ priklausomų fermentų biosintezė tiesiogiai koreliavo su ląstelių augimu, prasidėdavo ankstyvoje logaritminėje fazėje ir baigdavosi ankstyvoje stacionarinėje fazėje. Visi tirti kamienai į kultūrinę terpę sekretavo skirtingus kiekius laisvo PQQ (5–170 pmol/ml). Buvo tirta laisvo ir viduląstelinio PQQ bei PQQ priklausomų fementų biosintezės priklausomybė nuo anglies šaltinio kultivavimo terpėje. Fermentų bei viduląstelinio PQQ biosintezei anglies šaltinis didelės įtakos neturėjo, o nuo PQQ priklausomų fermentų substratų buvimas kultūrinėje terpėje nebuvo būtinas atitinkamų fermentų sintezei. Nustatyta, kad įvairūs anglies šaltiniai skirtingai indukavo neląstelinio PQQ biosintezę.