***Escherichia coli* is unable to produce pyrroloquinoline quinone (PQQ)**

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Many bacteria can synthesize the cofactor pyrroloquinoline quinone (PQQ), a cofactor of several dehydrogenases, including glucose dehydrogenase (GCD). Among the enteric bacteria, *Klebsiella pneumoniae* has been shown to contain the genes required for PQQ biosynthesis. *Escherichia coli* and *Salmonella typhimurium* were thought to be unable to synthesize PQQ but it has been reported that strain EF260, a derivative of *E. coli* FB8, can synthesize PQQ after mutation and can oxidize glucose to gluconate via the GCD/PQQ pathway (F. Biville, E. Turlin & F. Gasser, 1991, *J Gen Microbiol* 137, 1775–1782). We have re-investigated this claim and conclude that it is most likely erroneous. (i) Strain EF260, isolated originally by Biville and coworkers, was unable to synthesize a holo-enzyme GCD unless PQQ was supplied to the growth medium. No GCD activity could be detected in membrane fractions. (ii) The amount of PQQ detected in the growth medium of EF260 was very low and not very different from that found in a medium with its parent strain or in a medium containing no cells. (iii) EF260 cells were unable to produce gluconate from glucose via the PQQ/GCD pathway. (iv) Introduction of a *gcd::Cm* deletion in EF260, eliminating GCD, did not affect glucose metabolism. This suggested a pathway for glucose metabolism other than the PQQ/GCD pathway. (v) Glucose uptake and metabolism in EF260 involved a low-affinity transport system of unknown identity, followed most likely by phosphorylation via glucokinase. It is concluded that *E. coli* cannot synthesize PQQ and that it lacks genes required for PQQ biosynthesis.

Keywords: glucose dehydrogenase, PQQ, *Escherichia coli*, quinoprotein, glucose metabolism

INTRODUCTION

Pyrroloquinoline quinone (PQQ) is an important cofactor of bacterial dehydrogenases, linking the oxidation of many different compounds to the respiratory chain. PQQ was the first of the class of quinone cofactors that have been discovered in the last 18 years and make up the prosthetic group of quinoproteins (for reviews, see Duine, 1991; Klinman, 1996; Klinman & Mu, 1994).

The quinoprotein glucose dehydrogenase (GCD) has been demonstrated in a number of micro-organisms including the enteric bacteria *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella typhimurium*, and in

Acinetobacter, *Pseudomonas*, *Agrobacterium* and *Gluconobacter* species. In most cases, GCD is a membrane-bound enzyme which oxidizes glucose to gluconate in the periplasmic space (for a review, see Duine, 1991). The formed gluconate is subsequently taken up in a number of these organisms via a specific transport system and further metabolized. The reducing equivalents from glucose are donated via PQQ to the respiratory chain. Surprisingly, in some of the organisms mentioned only apo-GCD is found. Thus, *K. pneumoniae* synthesizes a holo-GCD (Neijssel *et al.*, 1983) but the closely related *E. coli* and *S. typhimurium* can synthesize only apo-GCD but not PQQ (Hommes *et al.*, 1986, 1984). However, apo-GCDs in these and other organisms can be converted into active holo-GCDs by the addition of extracellular PQQ (Ameyama *et al.*, 1986; Hommes *et al.*, 1986, 1984; Van Schie *et al.*, 1987).

Abbreviations: GCD, glucose dehydrogenase; PQQ, pyrroloquinoline quinone.

It has been concluded that organisms like *E. coli* and *S. typhimurium* lack the genes to synthesize PQQ and it remains unclear what the role of such apo-GCDs could be. It has been suggested that organisms that are unable to synthesize PQQ could scavenge it from their surroundings and activate their apo-quinoproteins (Matsushita & Adachi, 1993).

Genes involved in PQQ biosynthesis have been cloned, sequenced and characterized from a number of microorganisms. For instance, six *pqq* genes, *pqqABCDEF*, have been found and sequenced in *K. pneumoniae* (Meulenberg *et al.*, 1992). *pqq* genes have also been found in *Acinetobacter calcoaceticus* (Goosen *et al.*, 1989), *Methylobacterium extorquens* (Morris *et al.*, 1994; Springer *et al.*, 1996; Toyama *et al.*, 1997), *Pseudomonas fluorescens* (Schnider *et al.*, 1995) and *Methylobacillus* sp. (GenBank L78064). The enzymes encoded by the corresponding *pqq* genes are highly conserved between the various species but only in one case, *K. pneumoniae* PqqF (see below) and the comparable *M. extorquens* protein, has extensive homology with other proteins in the database been found.

The biosynthetic route of PQQ has not been elucidated yet, but it has been proposed that glutamate and tyrosine are precursors of PQQ (Houck *et al.*, 1988, 1991; Van Kleef & Duine, 1988). In all cases studied, the *pqq* operon contains a small gene; in the case of *K. pneumoniae*, this is *pqqA* which can encode a small peptide of 23–29 aa containing a glutamate and tyrosine residue at conserved positions. Synthesis of the small peptide has been demonstrated (Velterop, 1995) and it is thought to be the precursor of PQQ (Goosen *et al.*, 1989, 1992; Meulenberg *et al.*, 1992). The *K. pneumoniae* *pqqF* gene product shows similarity to proteases like *E. coli* protease III (or pitrilysin) and insulinases and it has been proposed that the PqqF enzyme is involved in cleavage of the PqqA precursor (Meulenberg *et al.*, 1992).

Expression of the six *K. pneumoniae* *pqq* genes in *E. coli* results in PQQ synthesis and an active GCD (Meulenberg *et al.*, 1990, 1992). PQQ biosynthesis most likely takes place in the cytoplasm and PQQ can be released into the medium. Since the PQQ-binding site of GCD faces the periplasm (Yamada *et al.*, 1993b), it is clear that both complementation of *E. coli* with the *K. pneumoniae* *pqq* genes as well as addition of PQQ can result in an active GCD. From growth studies with *E. coli* *ptsHI* mutants complemented with the complete set of *K. pneumoniae* *pqq* genes or with plasmids lacking one of the six *pqq* genes and from measurement of PQQ biosynthesis in the growth medium, it was concluded that each of the six genes is required for PQQ biosynthesis (Velterop *et al.*, 1995). These results support the conclusion that *E. coli* as well as *S. typhimurium* lack the *pqq* genes.

Unexpectedly, Biville *et al.* (1991) isolated a mutant of *E. coli* that seemed to have acquired holo-GCD activity. Starting with a strain that was derived from *E. coli* FB8 by introducing a *ptsHI-crr* deletion and a *galP::Tn10*

mutation (to eliminate glucose uptake and metabolism via known pathways), a mutant was isolated, EF260, which regained growth on glucose. Presence of PQQ in bacterial cell extracts and conversion of glucose to gluconate was claimed. Although in principle a cryptic *pqq* operon could be present in *E. coli*, isolation of similar mutants from other *E. coli* strains has proved negative (P. W. Postma, unpublished). We have re-examined this putative PQQ⁺ *E. coli* strain with respect to its capacity to synthesize PQQ and an active holo-GCD and conclude that at present there is no evidence that *E. coli* can synthesize PQQ, even after mutagenesis and that it most likely does not contain *pqq* genes.

METHODS

Bacterial strains. The following *E. coli* strains were used: FB8 (prototrophic *E. coli* K-12 strain); Aptsal [Δ (*ptsHI-crr*):Km *galP::Tn10*, derived from FB8 and renamed PPA322]; EF260 (Glc⁺, derived from Δ ptsal) (isolated and described by Biville *et al.*, 1991); PPA533 (*gcd::Cm*, derived from EF260); and CT690 (*recB21 recC22 thi-1 thr-1 leu-6 lacY1 mtl-1 xyl-1 ara-14 galK2 his-4 proA2 arg-63 rpsL31 tsx-33 sup-37 sbcB15*, from Dr M. Tsuda, Okayama University, Japan).

Bacterial growth. Strains were pre-cultured in LB medium (w/v: 1% bactotryptone, 0.5% yeast extract, 0.5% NaCl). The pre-culture was washed twice with 10 mM potassium phosphate buffer (pH 7.5) and resuspended in the same buffer at the same concentration as the original pre-culture. The suspension (inoculum size 5%, v/v) was then transferred to 100 ml glucose minimal medium (Biville *et al.*, 1991) containing 0.4% glucose and 21 or 100 mM potassium/sodium phosphate buffer (pH 6.8) and incubated on a rotary shaker (200 r.p.m.) at 30 °C. In a number of cases, cells were grown in a lactate minimal medium containing 100 mM potassium phosphate buffer (Matsushita *et al.*, 1984) as a control. Both PPA322 and EF260 strains were cultivated in the presence of 10 μ g tetracycline ml⁻¹ and 25 μ g kanamycin ml⁻¹.

Construction of a genomic *gcd* disruption mutant. The *HincII* fragment encoding the chloramphenicol acetyltransferase gene from pMC1 (Close & Rodriguez, 1982) was inserted into the *SmaI* site of the *gcd* gene of pUCGCD1 (Yamada *et al.*, 1993a). The resulting plasmid, pUCGDCM, was digested with *SalI* and *PstI* and introduced into CT690. The cells were grown on LB agar containing 10 μ g chloramphenicol ml⁻¹. Absence of GCD activity in the chloramphenicol-resistant colonies was tested on glucose-citrate minimal agar plates [28 mM glucose, 9.5 mM citrate, 25 mM Na(NH₄)HPO₄, 57 mM K₂HPO₄, 4 mM KH₂PO₄, 1.6 mM MgSO₄, 0.06 mM FeSO₄, 0.06 mM MnSO₄, 0.2 mM NaCl] containing 0.1% bromothymol blue and 0.1 mM PQQ. Putative genomic *gcd::Cm* mutants, which showed a weak yellow colour on the indicator plate, were subjected to measurement of GCD activity and genomic analysis by PCR. PCR was performed as described previously (Yamada *et al.*, 1993b). The *gcd::Cm* mutation was transferred into EF260 by P1 transduction, resulting in PPA533. The gene disruption was also confirmed by Western blot analysis (Matsudaira, 1987) using an antibody against the *E. coli* GCD.

Preparation of membrane fractions. Cells were collected by centrifugation, washed with 50 mM potassium phosphate buffer (pH 7.5) and resuspended in the same buffer at a concentration of 1 g wet cells per 5 ml. After adding a few grains of DNase powder, the suspension was passed twice

through a French pressure cell at 110.4 MPa and centrifuged at 12000 *g* for 20 min to remove intact cells and cell debris. The membranes were precipitated by centrifugation at 120000 *g* for 90 min and homogenized with 50 mM potassium phosphate buffer (pH 7.5).

Enzyme assays. GCD activity was measured spectrophotometrically at 25 °C using phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCIP) as electron acceptors as described by Matsushita *et al.* (1980) before or after holo-enzyme formation. Holo-enzyme was prepared by incubating the apo-enzyme at 25 °C for 30 min in 50 mM potassium phosphate buffer (pH 6.5) containing 1 µM PQQ and 10 mM MgSO₄. The assay was performed in 50 mM potassium phosphate buffer (pH 6.5) containing 8 mM sodium azide and enzyme activity was calculated using an extinction coefficient of 13.2 mM⁻¹ cm⁻¹ for DCIP. To determine the *K_m* of GCD for PQQ, membranes or purified GCD were incubated in a medium containing 40 mM potassium phosphate buffer, 30 mM glucose, 6 mM NaN₃, 10 mM MgSO₄, 0.4 mM PMS and 0.2 mM DCIP, final pH 6.5. The reaction was started by the addition of various concentrations of PQQ. The reaction rate increased gradually and the activity was calculated from the final steady-state rate.

PQQ determination. PQQ in the culture medium was concentrated by a Sep-Pak Cartridge (C18, Waters) as follows. After the pH was adjusted with concentrated HCl to 2.0, the medium supernatant (90 ml) was separated from the cells by centrifugation and was applied to the cartridge, pre-washed with 10 mM HCl. After washing the cartridge with 10 mM HCl, PQQ was eluted with 50% (v/v) methanol. To concentrate PQQ 100–200-fold, the eluted fraction was evaporated to about 0.5 ml. PQQ was determined with the use of apo-sGCD (the soluble form of GCD) prepared from an *A. calcoaceticus* PQQ-deficient mutant (Matsushita *et al.*, 1995). GCD activity was measured as described above after incubating for 5 min in 0.1 ml of a reaction mixture that contained per 0.25 ml: 50 mM PIPES/NaOH buffer (pH 6.5), apo-sGCD (approximately 2 ng protein), 3 mM CaCl₂, 0.1% Triton X-100 and the sample or a PQQ standard (0–0.2 ng). The concentration of PQQ was determined from a standard curve prepared with 0–0.2 ng PQQ in the reaction mixture.

Determination of gluconate content. Gluconate content in the culture medium was determined using gluconate dehydrogenase, purified from *Pseudomonas aeruginosa* (Matsushita *et al.*, 1979), which has a high specificity for gluconate (Matsushita *et al.*, 1982). Gluconate dehydrogenase activity was measured spectrophotometrically at 25 °C in a reaction mixture which contained 50 mM PIPES/NaOH buffer (pH 6.5), gluconate dehydrogenase (approximately 0.1 unit), 0.4 mM PMS, 0.2 mM DCIP and the sample, obtained from the medium after centrifugation, or a standard gluconate solution (0–10 mM).

Transport and oxidation studies. Cells were grown overnight at 37 °C on a rotary shaker in minimal medium A [1 g (NH₄)₂SO₄, 10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 1 g MgSO₄ per litre demineralized water] with 0.4% D,L-lactate as carbon source and 1 mM D-fucose as indicated, diluted tenfold and grown in the same medium and harvested when the OD₆₀₀ was approximately 0.5 (Hitachi model 100-50 spectrophotometer). Cells were harvested and washed and transport of ¹⁴C-carbohydrates was measured as described elsewhere (Postma, 1977). Oxygen consumption was measured with a Clark-type electrode in medium A and expressed as nanoatoms of O consumed min⁻¹ (mg dry wt)⁻¹ at 25 °C.

Other procedures. SDS-PAGE, protein determinations, prep-

aration of antibodies and immunoblotting analysis were performed as described by Matsushita *et al.* (1989). *E. coli* GCD was purified as described by Yamada *et al.* (1993b).

Materials. PQQ was obtained from Ube Industries. The immunoblotting kit and pre-stained marker proteins were obtained from Bio-Rad. PVDF microporous membrane was purchased from Millipore. D-[U-¹⁴C]Glucose (10.5 GBq mmol⁻¹) was obtained from The Radiochemical Centre; [¹⁴C]methyl-β-galactopyranoside (1.4 GBq mmol⁻¹) and D-[U-¹⁴C]galactose (13.2 GBq mmol⁻¹) were purchased from Dupont NEN Research Products.

RESULTS AND DISCUSSION

Growth behaviour on various media

To investigate the possibility whether mutations could be isolated in *E. coli* that resulted in the synthesis of PQQ, Biville *et al.* (1991) started with a strain that was unable to grow on glucose due to mutations in the phosphoenolpyruvate:carbohydrate phosphotransferase system and in galactose permease [$\Delta(ptsHI-crr)galP::Tn10$]. This strain, originally named Δ ptsgal and renamed PPA322 by us, was derived from the parent strain FB8. EF260 was isolated as a Glc⁺ derivative of PPA322 by Biville *et al.* (1991) and further analysis led them to conclude that EF260 can synthesize PQQ.

To characterize in more detail the mutation in *E. coli* EF260 that restored growth on glucose, growth of FB8, PPA322 and EF260 was compared in glucose-minimal medium, containing 0.4% glucose and 100 mM potassium phosphate buffer. As shown in Fig. 1, EF260 grew faster than its parent, PPA322, but slower than the wild-type strain, FB8. EF260 also showed a prolonged

Fig. 1. Growth of *E. coli* FB8, PPA322 and EF260 in the presence and absence of PQQ. Cells were grown in glucose minimal medium containing 0.4% glucose and 100 mM potassium/sodium phosphate buffer (as described in Methods). □, FB8; ■, FB8 + 10 nM PQQ; ○, EF260; ●, EF260 + 10 nM PQQ; △, PPA322; ▲, PPA322 + 10 nM PQQ.

Table 1. GCD activities in membranes of *E. coli* FB8, PPA322 and EF260

Growth medium	GCD activity [units (mg protein) ⁻¹]					
	FB8		PPA322		EF260	
	-PQQ	+PQQ*	-PQQ	+PQQ*	-PQQ	+PQQ*
Lactate medium/100 mM P _i	0	0.032	0.002	0.142	0.002	0.089
Glucose medium/21 mM P _i	0.002	1.09	0.003	0.234	0.002	0.347
Glucose medium/100 mM P _i	0.003	0.684	0.001	0.274	0	0.156
Glucose medium/100 mM P _i + 10 nM PQQ	0.003	0.622	0.014	1.12	0.005	0.569

* Membranes were pre-incubated with 1 µM PQQ and 10 mM MgSO₄ before the GCD activity was determined.

lag time. Addition of PQQ increased the growth rate of PPA322 but also of EF260. Qualitatively the same results were obtained when these growth studies were performed with a medium containing 21 mM potassium phosphate buffer, similar to the medium used by Biville *et al.* (1991). However, in this medium the extent of growth was much smaller in the presence of PQQ, most likely because of acid production (the pH of these culture media decreased below pH 4.0 under these growth conditions) (data not shown). All three strains grew rapidly and at the same rate in lactate-containing medium (100 mM P_i) (data not shown).

The comparison of the growth properties of PPA322 and EF260 suggests that the addition of PQQ produced an active GCD, even in strain EF260, resulting in the production of gluconic acid and thus in a decreased medium pH, unless a high concentration of buffer was added to the medium. From these data, it can be speculated that neither EF260 nor PPA322 could produce PQQ.

GCD apo-enzyme and holo-enzyme

Biville *et al.* (1991) detected a low holo-enzyme GCD activity [0.1–0.3 units (mg protein)⁻¹] in the glucose-utilizing strain EF260 and proposed that growth on glucose was due to a PQQ-dependent GCD activity. In the present study GCD activity was determined in the membrane fraction of the three strains, grown in three different media: lactate + 100 mM P_i, glucose + 21 mM P_i and glucose + 100 mM P_i. As shown in Table 1, no enzyme activity [<0.01 units (mg protein)⁻¹] could be detected in any of the three strains grown in three different media. Presence of apo-GCD was detected, however, in the three strains after pre-incubation with cofactor PQQ. The activities of reconstituted holo-enzyme GCD were higher in membranes prepared from cells grown on glucose than in the membrane fraction of lactate-grown cells. The enzyme activity of FB8 was relatively high compared to that of PPA322 and EF260, the latter two strains showing almost the same activity. GCD activity was not increased by the addition of Mg²⁺ in the absence of PQQ (data not shown). Interestingly, addition of PQQ to the culture medium seemed to

stimulate the expression of GCD in PPA322 and EF260. This was tested only in the medium containing 100 mM P_i because acid production disturbed the growth at lower phosphate concentrations, as mentioned above. However, even under these conditions, i.e. growth of cells in a medium containing PQQ, enzyme activity could not be detected. This is probably because high phosphate concentrations disturbed the tight binding of PQQ to the apo-enzyme under the growth conditions. Apparently, GCD apo-enzyme pre-incubated with PQQ is less sensitive to the presence of P_i. It should be realized, however, that reconstitution of apo-enzyme took place in 50 mM P_i with 1 µM PQQ, whereas growth occurred in 100 mM P_i containing 10 nM PQQ. Although the PQQ concentration in the culture is below the *K_m* of GCD for PQQ, a low level of holo-enzyme GCD might be sufficient for growth.

We considered the possibility that GCD in EF260 had altered properties, i.e. increased affinity for PQQ which would allow this strain to scavenge PQQ more efficiently from the medium rather than to synthesize it. The protein level of GCD in the membrane fractions was examined by immunoblotting and paralleled the enzyme activity in the membranes (data not shown). In addition, the mobility of the enzyme was identical in the three strains. Furthermore, the affinity of GCD for PQQ was examined with membranes prepared from the various strains. GCDs in the membranes from the three different types of cells showed the same apparent *K_m* values as those of the purified enzyme (Table 2). Thus, both the structure and the function of GCD in the mutant strains, *E. coli* PPA322 and EF260, did not seem to be changed compared to those of the wild-type strain and an increased capacity to scavenge PQQ seemed unlikely.

PQQ and gluconate production in the various *E. coli* strains

To establish whether or not *E. coli* strains, including the mutants, were able to produce PQQ and whether, as a result, holo-GCD occurred in these cells, the PQQ and gluconate concentration were determined in the culture medium. To determine the PQQ content, a highly sensitive PQQ assay system was developed using apo-

Table 2. Affinity of GCD for PQQ in membranes

GCD fraction	Growth condition	K_m (PQQ) (nM)
Purified GCD*	—	44 and 111
Membranes FB8	Lactate medium/100 mM P_i	40 and 100
Membranes FB8	Glucose medium/21 mM P_i	40 and 100
Membranes PPA322	Lactate medium/100 mM P_i	40 and 100
Membranes EF260	Lactate medium/100 mM P_i	42 and 111
Membranes EF260	Glucose medium/21 mM P_i	33 and 109
Membranes EF260	Glucose medium/100 mM P_i	33 and 86

* *E. coli* GCD was purified as described in Methods.

Table 3. PQQ concentration in culture media of *E. coli* FB8, PPA322 and EF260

Cells were grown to early stationary phase. At the time indicated, the cultures were centrifuged to remove the cell pellets. The supernatant was used for measuring the PQQ content. PPA322 was also grown up with the prolonged lag time as shown originally by Biville *et al.* (1991). Unless indicated otherwise, values represent the mean of at least two independent determinations ($\pm$ SEM).

Strain	Medium condition	Cultivation time (h)	PQQ (nM)
Control	100 mM P_i	—	0.021 $\pm$ 0.005
FB8	100 mM P_i	27	0.025 $\pm$ 0.009
FB8	100 mM P_i /10 nM PQQ	27	8.3 ($n = 1$)
PPA322	100 mM P_i	286	0.018 ($n = 1$)
EF260	100 mM P_i	95	0.021 $\pm$ 0.002

GCD (soluble form) purified from an *A. calcoaceticus* PQQ-deficient mutant (Matsushita *et al.*, 1995). The apo-enzyme was able to detect 0.01–0.4 ng of PQQ in the assay system, as described in Methods. Thus, we could detect PQQ in the culture medium at the level of 1 pM. As shown in Table 3, the original culture medium without any bacterial cells added, contained 0.021 nM PQQ. After growth of the cells, the culture medium contained only slightly higher concentrations of PQQ in all cases. Only in the case of EF260 growing in a medium containing 20 mM P_i was a somewhat higher PQQ concentration found, for unknown reasons, but even in this case the level was very low. Recovery of most PQQ added to the growth medium with FB8 shows that PQQ could be detected in this medium. We conclude that none of the three *E. coli* strains, including EF260, could produce PQQ.

If glucose metabolism in EF260 utilized a PQQ-dependent GCD pathway, gluconate production would be expected. The gluconate content of the culture medium was determined. In the absence of PQQ, all three strains produced only 0.015–0.020 mM gluconate in the culture medium containing 20 mM P_i or no gluconate at all in a medium containing 100 mM P_i . Thus, EF260 was unable to produce gluconate, even though glucose metabolism is supposed to involve the PQQ-dependent GCD pathway in this strain. The effect of the addition of PQQ was tested to verify that in the presence of genuine PQQ gluconate was indeed produced, although the strains did

not grow well in a medium containing 20 mM P_i and PQQ (because of acid production, see above). Both PPA322 and EF260 produced 1.4–2.4 mM gluconate in the PQQ-containing medium. On the other hand, in the cultures containing 100 mM P_i , no gluconate production could be detected (only 1–3 μ M) even in the presence of PQQ, despite the fact that growth under these conditions was normal. This may be caused by the slow production and relatively fast utilization of gluconate in the cultures that contain a high P_i and PQQ concentration.

Since both the buffer capacity and the phosphate concentration were expected to be important for growth and the formation of holo-enzyme, gluconate production was examined in more detail with EF260 in the absence and presence of PQQ in a glucose minimal medium containing either 32 mM P_i (Fig. 2a) or 32 mM P_i + 50 mM MOPS buffer (Fig. 2b). Gluconate accumulated to high levels at the early to mid-exponential phase in the cultures containing PQQ, ranging from 10 to 15 mM. However, in the absence of PQQ, almost no gluconate formation (5–60 μ M) was observed.

It is concluded that *E. coli* strains, even the EF260 strain, were unable to produce PQQ in their culture medium. Although the reason is unclear, high phosphate levels in the growth medium may prevent the interaction between apo-GCD and PQQ. As a consequence, these strains may not have any holo-GCD, required for the production of gluconate, even when PQQ was added to the medium.

Fig. 2. Gluconate production by *E. coli* EF260 in the presence and absence of PQQ. EF260 was grown in glucose-minimal medium containing 32 mM potassium/sodium phosphate buffer (a) or the same phosphate buffer plus 50 mM MOPS, pH 7.0 (b). □, ■, growth curves; ○, ●, gluconate concentration in the culture media; □, ○, with 10 nM PQQ; ■, ●, without PQQ.

Fig. 3. Western blot analysis of the *gcd::Cm* mutant PPA533 and its parent EF260. Membrane fractions (20 µg protein) of PPA533 (lane 1) and EF260 (lane 2) were analysed by 8% SDS-PAGE, blotted on PVDF membrane and detected with a polyclonal antibody against GCD.

Role of GCD in the phenotype of *E. coli* EF260

Although after growth on glucose no GCD holo-enzyme activity could be detected in EF260, nor gluconate formation, we have investigated whether deletion of the *gcd* gene would affect the Glc⁺ phenotype of EF260, as predicted if the PQQ/GCD pathway was active. Fig. 3 shows that introduction of the *gcd::Cm* mutation in EF260 eliminated GCD in PPA533 completely. Growth of PPA533 on glucose was not impaired, however, compared to that of EF260. It is concluded that growth of EF260 on glucose is not dependent on the PQQ/GCD pathway.

Uptake and oxidation of glucose

EF260 was originally selected as a strain able to grow on glucose. Its parent, PPA322 [$\Delta(ptsHI-crr)$ *galP*] is lacking known glucose transporters and cannot grow on glucose. Since we have demonstrated that, in contrast to earlier conclusions, the GCD/PQQ pathway is not

active in EF260, the question arises how glucose is taken up and metabolized. Possibly, the *galP* mutation had reverted and the galactose permease, GalP, had become constitutive. Alternatively, the β -methylgalactoside permease (MglP) might have become active. Both transport systems, when expressed constitutively, can catalyse glucose uptake.

Table 4 shows that uptake of glucose, galactose or β -methylgalactoside was very low in PPA322, as expected since the strain lacks both the phosphoenolpyruvate: carbohydrate phosphotransferase system as well as the GalP. In addition, it has been shown previously that synthesis of MglP is prevented in $\Delta ptsHI$ strains due to the low cAMP level (Postma *et al.*, 1981). Uptake of the three substrates, including glucose, was not increased in the Glc⁺ strain, EF260. Thus, no constitutive glucose transport system had evolved in EF260. Growth in the presence of the gratuitous inducer D-fucose resulted in some induction of the MglP system in both strains. The absence of GalP could also be concluded from the observation that the strains were still resistant to 2-deoxygalactose (Henderson & Giddens, 1977; Nagelkerke & Postma, 1978).

The transport studies showed that none of the known glucose transporters was active in EF260. These transporters have quite high apparent affinity for glucose, ranging from 1 to 100 µM. Conceivably, a low-affinity glucose uptake system could be present. By measuring glucose oxidation with isolated cells grown in LB in the presence of 1% glucose, an apparent affinity for glucose of 15 mM could be determined. Cells grown in LB or a minimal salts medium containing lactate, displayed an apparent K_m in the same range. The V_{max} of oxygen uptake was 174 and 78 nanoatoms O taken up min⁻¹ (mg dry wt)⁻¹ for cells grown on LB and LB + glucose, respectively. Thus, no evidence could be found for a high-affinity glucose uptake system.

Conclusions

The conclusion by Biville *et al.* (1991) that *E. coli* can synthesize PQQ is most likely erroneous. The following observations with *E. coli* EF260 support this statement. (i) *E. coli* EF260 was unable to synthesize a holo-enzyme GCD unless PQQ was supplied to the growth medium. No GCD activity could be detected in membrane fractions. (ii) The amount of PQQ detected in EF260 was not very different from that of its parent and from the amount found in the growth medium in the absence of cells. (iii) EF260 was unable to produce gluconate via the PQQ/GCD pathway. (iv) Introduction of a *gcd::Cm* deletion, eliminating GCD, did not affect glucose metabolism in EF260. This suggested a pathway for glucose metabolism other than the PQQ/GCD pathway. (v) Since EF260 was isolated from its parent (PPA322) as a mutant that regained growth on glucose, an alternative glucose-metabolizing pathway must have been created which was different from the GCD/PQQ pathway. Glucose uptake and metabolism in EF260 involved a low-affinity transport system of unknown identity,

Table 4. Uptake of labelled carbohydrates

Strains were grown in minimal medium containing lactate and, where indicated, 1 mM D-fucose. Cells were harvested and uptake of labelled substrates was measured as described in Methods using 0.5 mM [14 C]glucose, 0.5 mM [14 C]galactose or 0.5 mM β -[14 C]methylgalactoside.

Strain	D-Fucose	Rate of uptake*		
		Galactose	β -Methylgalactoside	Glucose
PPA322	–	0.2	0.3	0.1
PPA322	+	1.9	4.1	1.5
EF260	–	0.3	0.3	0.7
EF260	+	0.6	1.1	0.7

* Activity is expressed as nmol carbohydrate taken up min^{-1} (mg dry wt) $^{-1}$ at 20 °C.

followed most likely by phosphorylation via glucokinase.

Two other observations have to be explained. It was recently reported that an *E. coli* DNA fragment could complement *pqqE/F* mutants of *Methylobacterium organophilum* (Turlin *et al.*, 1996). The ORF responsible, *orf106*, most likely encodes a *K. pneumoniae* PqqF-like protease that can complement the defect in *M. organophilum*. A similar suggestion has been made concerning the complementation of a defective *K. pneumoniae* *pqqF* gene product by an *E. coli* protease (Velterop *et al.*, 1995). The finding that an *Erwinia herbicola* DNA fragment, claimed to contain a *pqqE*-like gene but no other PQQ genes, was sufficient for gluconate production from glucose adds weight to the argument that other *pqq* genes might be present in *E. coli* (Liu *et al.*, 1992). However, the cloned fragment contained a number of *pqq* genes rather than only the *pqqE*-like gene (P. W. Postma & A. H. Goldstein, unpublished observations). Thus, no evidence is available for the presence of *pqq* genes in *E. coli*. Our conclusion that *E. coli* is unable to produce PQQ is supported by the fact that the recently completed nucleotide sequence of the *E. coli* genome contained no ORFs that could encode proteins with similarity to other PQQ proteins.

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