Mutants of *Escherichia coli* producing pyrroloquinoline quinone

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In glucose minimal medium a PTS⁻ strain of *Escherichia coli* [$\Delta(ptsH\ ptsI\ crr)$] could grow slowly (doubling time, $d = 10$ h). When the population reached 5×10^6 to 2×10^7 cells ml⁻¹, mutants growing rapidly ($d = 1.5$ h) appeared and rapidly outgrew the initial population. These mutants (EF mutants) do not use a constitutive galactose permease for glucose translocation. They synthesize sufficient pyrroloquinoline quinone (PQQ) to yield a specific activity of glucose dehydrogenase (GDH) equivalent to that found in the parent strain grown in glucose minimal medium supplemented with 1 nM-PQQ. Membrane preparations containing an active GDH oxidized glucose to gluconic acid, which was also present in the culture supernatant of EF strains in glucose minimal medium. Glucose utilization is the only phenotypic trait distinguishing EF mutants from the parent strain. Glucose utilization by EF mutants was strictly aerobic as expected from a PQQ-dependent catabolism. The regulation of PQQ production by *E. coli* is discussed.

Introduction

Pyrroloquinoline quinone (PQQ) is now recognized as the third important redox cofactor in bacterial dehydrogenases beside NAD(P) and flavins (Duine & Jongejan, 1990). In Gram-negative bacteria, several membrane or periplasmic dehydrogenases used for the primary oxidation step of non-phosphorylated substrates are quinoproteins, i.e. PQQ-containing enzymes (Duine & Jongejan, 1989). The most-studied quinoproteins are methanol dehydrogenase from methylotrophs and glucose dehydrogenase (GDH, EC 1.1.99.17) from Gram-negative heterotrophic bacteria. The latter enzyme has been studied mainly in *Acinetobacter calcoaceticus*, which in fact contains two distinct GDHs: a soluble periplasmic one composed of two identical subunits of 48–54 kDa (Dokter *et al.*, 1986; Geiger & Görisch, 1986) and a membrane-bound monomeric GDH of 83 kDa (Matsushita *et al.*, 1986, 1988; Cleton-Jansen *et al.*, 1988).

GDH is widespread among the *Enterobacteriaceae* (Bouvet & Grimont, 1988; Bouvet *et al.*, 1989) but only some species such as *Klebsiella aerogenes* (*K. pneumoniae*) contain an active holoenzyme (Neijssel *et al.*, 1983; Neijssel, 1987). Other species, including *Escherichia coli*, are seemingly unable to synthesize PQQ but they can

produce the protein moiety, the apoenzyme, of GDH (Hommes *et al.*, 1984). The apoGDH of *E. coli* has been purified and characterized by Ameyama *et al.* (1986): it is a membrane-bound protein of 88 kDa which shows immunological relationships with the membrane-bound GDH of *A. calcoaceticus* and of *Pseudomonas aeruginosa* (Matsushita *et al.*, 1986).

Reconstitution of the active holoenzyme of *E. coli* was obtained *in vitro* after incubation of PQQ with cell-free extracts (Hommes *et al.*, 1984) or with membrane fraction or purified apoGDH (Ameyama *et al.*, 1986). *In vivo*, holoenzyme reconstitution can be shown in *E. coli* PTS⁻ mutants. In these mutants the utilization of several carbohydrates, including glucose, is impaired by the deterioration of the phosphotransferase (PTS) system of concomitant phosphorylation and penetration of the carbohydrate substrate through the cytoplasmic membrane (reviewed by Postma *et al.*, 1989; Erni, 1989). As a consequence, glucose catabolism in PTS⁻ mutants cannot proceed via the usual Embden–Meyerhof pathway. However, their growth on glucose can be restored by addition of PQQ to the culture medium (Hommes *et al.*, 1984). In this situation, the reconstituted holoGDH oxidizes glucose into gluconic acid which is, in turn, presumably catabolized via the Entner–Doudoroff pathway (Gottschalk, 1985), thus providing an alternative route of glucose utilization.

Hommes *et al.* (1985) have shown that in *K. aerogenes*

Abbreviations: PQQ, pyrroloquinoline quinone; GDH, glucose dehydrogenase; PTS, *pts*, phosphotransferase.

Table 1. *Bacterial strains*

Strain	Characteristics	Source or reference
<i>E. coli</i>		
TP2111	F ⁻ <i>xyl argH1 lacX74 aroB ilvA</i>	Roy <i>et al.</i> (1983)
TP2811	Derived from TP2111; $\Delta(ptsH ptsI crr)$ Km ^r	Lévy <i>et al.</i> (1990)
EF15	A TP2811 derivative able to grow on glucose	This work
FB8	F ⁻ ; wild-type	Bruni <i>et al.</i> (1977)
FB8 Δ pts	A $\Delta(ptsH ptsI crr)$ transductant from FB8 $\times$ P1 TP2811	This work
CGSC 6902	F ⁻ <i>his leu ilvA Δlac mglP galP::Tn10</i>	B. J. Bachmann, <i>E. coli</i> Genetic Stock Center, Yale University
FB8 Δ pts <i>galP</i>	A <i>galP::Tn10</i> transductant from FB8 Δ pts $\times$ P1 CGSC 6902	This work
EF16, EF17, EF18, EF19, EF20	FB8 Δ pts derivatives able to grow on glucose	This work
EF260, EF261, EF262, EF263, EF264	FB8 Δ pts <i>galP</i> derivatives able to grow on glucose	This work
<i>Acinetobacter calcoaceticus</i>		
LMD79.42	Wild-type	Dokter <i>et al.</i> (1986)

(*K. pneumoniae*), the oxidation of glucose by GDH is an auxiliary energy-generating pathway under conditions of energy stress, mainly phosphate or potassium limitation. This finding might perhaps be generalized to other PQQ-producing members of the *Enterobacteriaceae*. However, for the species which apparently do not produce PQQ, the question arises whether they have the genetic complement required for PQQ biosynthesis. This question is not limited to *E. coli* and to *Enterobacteriaceae* containing an apoGDH. GDH has been found in many other bacterial genera, either as holoenzyme or as apoenzyme as in the *Enterobacteriaceae*. ApoGDH has been detected in species of *Acinetobacter*, *Agrobacterium*, *Azotobacter*, *Rhizobium* and *Pseudomonas* (Van Schie *et al.*, 1985, 1987; Linton *et al.*, 1987). Moreover, enzymes other than GDH may also be concerned; for example, *Pseudomonas testosteroni* contains a PQQ-dependent apoethanol dehydrogenase which is used for ethanol utilization only in the presence of added PQQ (Groen *et al.*, 1986).

In this paper we describe the isolation and some characteristics of spontaneous mutants that arose from *E. coli* strains lacking *pts* genes, which recovered the ability to utilize glucose as growth substrate, with concomitant appearance of a functional holoGDH containing PQQ.

Methods

Media and growth conditions. The complex media were McConkey agar base (Difco), supplemented with a carbon source (1%, w/v), and LB medium (Miller, 1972). The minimal medium was medium A (MA) of MacLennan *et al.* (1971) supplemented with vitamin B1 (1 μ g ml⁻¹),

a filter-sterilized carbon source at 0.4% for liquid media or 1% for solid media and, if required, PQQ (Fluka) (1 nM) or amino acids (1 mM) to complement auxotrophic requirements. Antibiotics, when added, were used at a final concentration of (μ g ml⁻¹): kanamycin, 25; tetracycline, 10. Solid minimal media contained Difco agar: 1.5% for Petri dishes or 0.6% for deep agar tubes (10 ml medium in 16/160 mm tubes). Before use, the latter were degassed for 30 min in a boiling water-bath, equilibrated at 50 °C, evenly inoculated with 2–3 $\times$ 10² bacterial cells and immediately cooled to solidify the medium before incubation. Anaerobic cultures on the surface of agar plates were incubated in jars containing the GasPak (BBL) oxygen-absorbing mixture. Inocula for growth experiments in liquid minimal media were prepared from overnight cultures in LB medium, washed twice in MA medium without carbon source and adequately diluted. All the liquid cultures were strongly shaken with reciprocal shakers. Carbon source utilization tests (auxanograms) were done in the Unité des Entérobactéries, Institut Pasteur, by using experimental API strips which contained pure carbon sources inoculated with a bacterial suspension in a mineral base (Grimont *et al.*, 1988). One hundred distinct carbon sources were tested. All the cultures were incubated at 37 °C.

Strains. These are listed in Table 1. *E. coli* TP2111 and FB8 had the typical substrate utilization profile of *E. coli* strains as recognized by API test auxanograms using defined culture media (Grimont *et al.*, 1988). Using the same technique the PTS phenotype of *E. coli* TP2811 and of FB8 Δ pts was checked: no growth was obtained in minimal medium containing glucose, fructose, trehalose, mannose, mannitol, sorbitol, *N*-acetylglucosamine or methyl α -D-glucopyranoside.

A phage P1 lysate obtained with TP2811 was used to transduce the *pts* deletion into FB8: on McConkey-glucose plates, the transductants FB8 Δ pts gave white colonies. To obtain FB8 Δ pts *galP*, a P1 lysate from *E. coli* CGSC 6902 was used to transduce FB8 Δ pts. The tetracycline-resistant colonies obtained were checked for their inability to grow on galactose MA medium.

Biochemical techniques. The different steps of preparation and solubilization of the membrane fraction containing GDH were essentially those of Ameyama *et al.* (1986) with minor modifications. Bacterial cells were suspended in Tris/HCl buffer (50 mM, pH 7) containing MgSO₄ (2.5 mM) and passed twice through a French pressure cell at 110 MPa. Broken cell debris was removed by

centrifugation at 6000 *g* for 10 min and the supernatant (crude extract) was ultracentrifuged at 80000 *g* for 90 min. The resulting pellet was suspended in 1–2 ml potassium phosphate buffer (5 mM, pH 7) containing MgSO₄ (2.5 mM) and Triton X-100 (1%). This suspension was stirred for 2 h at 4 °C, centrifuged at 80000 *g* for 90 min and the supernatant containing the solubilized membrane fraction was used for GDH assay and PQQ determination. GDH was assayed by the method of Ameyama *et al.* (1986) either without added PQQ to determine holoenzyme activity or with PQQ at a saturating concentration (45 µM) for apoenzyme determination. Specific activity was expressed as µmol dichlorophenol indophenol reduced min⁻¹ (mg protein)⁻¹.

For PQQ determinations, samples of membrane preparation containing active GDH were heated for 5 min in a boiling water-bath (Biville *et al.*, 1988). Free PQQ was assayed by a method using the apoethanol dehydrogenase purified from *Pseudomonas testosteroni* according to Groen *et al.* (1986). The reconstituted ethanol dehydrogenase activity was proportional to the amount of PQQ added from 0.1 to 5 pmol in a total volume of 1 ml. All the GDH determinations and PQQ assays were achieved in triplicate with each membrane preparation.

Gluconic acid was determined with enzyme kits (Boehringer 428191) containing gluconate kinase and a NADP-dependent 6-phosphogluconate dehydrogenase. Production of gluconic acid by membrane preparations containing active GDH was achieved in a spectrophotometric cuvette containing, in a total volume of 1 ml: membrane preparation (50 µg protein); Tris/HCl, 50 mM; MgSO₄, 2.5 mM; glucose, 20 mM; and Würster's Blue, 10 µM, as the primary electron acceptor. Potassium ferricyanide, the final electron acceptor, was added as 2 µl fractions of a 10 mM solution in water. Complete reduction of each ferricyanide fraction was followed at 420 nm before addition of the next fraction. The system of electron acceptors PQQ–Würster's Blue–ferricyanide was that used by Frank *et al.* (1989). To check that potassium ferrocyanide, accumulated during gluconic acid production in the system described above, did not interfere with gluconic acid determination, the membrane preparation was replaced by a sample of crude extract from *Acinetobacter calcoaceticus* and glucose was replaced by arabinose. *A. calcoaceticus* extracts contain a high level of GDH (Dokter *et al.*, 1986) which can oxidize several aldoses including arabinose (Goosen *et al.*, 1987). Accumulation of potassium ferrocyanide to a concentration of 1.5 mM did not alter the blank controls of the gluconate assay system, nor the determination of known amounts of added gluconate.

Results

Growth kinetics of *E. coli pts* mutants in glucose minimal medium

The initial observation was a residual growth of *E. coli* PTS⁻ strains on glucose MA plates. The strains used were TP2811 and FB8Δ*pts*, both of which are deleted of *ptsH*, *ptsI* and *corr*, three genes coding for essential enzymes of the PTS system, Enzyme I, HPr and Enzyme III^{Glc}. To examine this observation more thoroughly, the growth of an inoculum of 10⁵ bacteria ml⁻¹ in glucose MA medium was followed by plate counts on LB medium. The growth curve obtained (Fig. 1, curve A) showed a first phase of slow growth (doubling time, *d* = 10–12 h) lasting about 3 d, followed by a phase of rapid growth (*d* ≈ 1.4 h). Samples taken at the end of the latter phase and plated on glucose MA medium gave rise

Fig. 1. Growth kinetics of *E. coli* FB8Δ*pts* (curve A) and of FB8Δ*pts galP* (curve B) in glucose MA medium. The population size, *N* (c.f.u. ml⁻¹), of curves A and B was estimated by plating samples on LB medium. The differential count of EF mutants (curve C) emerging from the population of FB8Δ*pts galP* was achieved on glucose minimal plates. The differential count of EF mutants obtained from FB8Δ*pts* is not represented (see text). Curve D: *E. coli* FB8Δ*pts* in minimal medium without glucose.

to colonies growing overnight in numbers comparable to those obtained on LB plates. From such glucose MA plates, strain EF15 was isolated from a culture of TP2811, and strains EF16 to EF20 were isolated from separate growth experiments with FB8Δ*pts*. All the experiments reproduced the growth curve A of Fig. 1. The phenotypes of EF strains and of their respective parental strains TP2811 or FB8Δ*pts* studied with API test auxanograms differed only in the ability to utilize glucose.

Saier *et al.* (1973), using a strain of *Salmonella typhimurium* deleted of essential phosphotransferase genes like our *E. coli* PTS⁻ strains, also obtained mutants growing rapidly in glucose minimal medium. These mutants were found to constitutively synthesize galactose permease. The broad specificity of this permease allowed glucose and some other PTS substrates to cross the cytoplasmic membrane. It was assumed that, after further phosphorylation by hexokinase, glucose was catabolized by the Embden–Meyerhof pathway. To check the possibility that our EF mutants were galactose permease constitutive mutants, the wild-type *galP* gene of FB8Δ*pts* was replaced, by P1 transduction, by a *galP* gene inactivated by a Tn10 insertion. The resulting strain, FB8Δ*pts galP*, was used for growth experiments in liquid MA medium as described above. Fig. 1 shows the similar shapes of the growth curves of the cultures of FB8Δ*pts* (curve A) and of FB8Δ*pts galP* (curve B). The growth of the latter, however, was slower during the first growth phase (*d* = 20 h). As observed with FB8Δ*pts*, cells of FB8Δ*pts galP* growing rapidly in glucose MA medium

Table 2. *GDH activities and PQQ concentrations in membrane preparations of E. coli strains FB8 (wild-type), FB8Δpts and EF mutants*

Strain	Growth conditions	GDH activity*		PQQ concn†
		- PQQ	+ PQQ	
FB8 (w.t.)	Glucose	<0.02	0.2-0.4	<0.5
FB8Δpts	Glucose + 1 nM-PQQ	0.1-0.3	0.2-0.6	5-10
	Glucose + 100 nM-PQQ	1.2	1.3	30
EF 16, 17, 18, 19, 20	Glucose	0.1-0.3	0.2-0.6	5-15
EF 260, 261, 262, 263, 264	Glucose	0.1-0.3	0.2-0.6	5-15

* Units: μmol dichlorophenol indophenol reduced min^{-1} (mg protein^{-1}).† Units: pmol PQQ (mg protein^{-1}).

took over the whole culture during the second growth phase and they could be easily counted by plating on glucose MA medium, on which they produced colonies overnight. From separate growth experiments with FB8Δpts galP, strains EF260 to EF264, growing rapidly in glucose MA medium, were isolated and their identity was checked as for strains EF16 to EF20.

From several experiments with FB8Δpts galP growing in liquid MA medium, it could be deduced, by extrapolation to log 0 (i.e. one cell) of the straight line representing the EF cell population (Fig. 1), that the first EF cell appeared when the population reached approximately 5×10^6 – 2×10^7 cells ml^{-1} .

Direct plating of 2 – 4×10^8 cells of FB8Δpts onto glucose MA medium produced a heavy background of 'residual' growth over which colonies of rapidly growing EF cells could not be easily distinguished. Under the same experimental conditions, cells of FB8Δpts galP produced practically no background and isolated colonies of EF cells were fully developed in 48 h. The frequency of appearance of EF cells, 2×10^{-7} – 5×10^{-8} , which was confirmed in this way, suggested that a single mutational event was involved in the appearance of EF cells in a pts population of *E. coli*.

GDH determination and PQQ assays in membrane preparations

Since the EF mutants were not galactose permease constitutive mutants, their selection under our experimental conditions could have resulted from the emergence of PQQ-producing cells in the PTS⁻ population, making possible the oxidation of glucose by a reconstituted GDH.

GDH activities in crude extract were too low to be correctly assayed, although non-specific dye reduction (i.e. before addition of glucose to the spectrophotometric cuvette) was very low. Supernatants after ultracentrifugation

did not show any GDH activity: it was found only in the pellets solubilized by Triton X-100 treatment. Table 2 reports the results of apo- and holo-GDH activities and of PQQ assays in membrane preparations of *E. coli* FB8 (wild-type), FB8Δpts and EF mutants. As expected, the membrane preparations of *E. coli* FB8 contained apo-GDH. Its specific activity was comparable to that found by Ameyama *et al.* (1986). Both holo-GDH activity and PQQ concentration were below the limit of detection of the methods used.

The growth of FB8Δpts in glucose MA medium required PQQ at the minimal concentration of 1 nM to yield a development similar to EF strains both in growth rate and in final optical density. In these conditions, about half of the GDH was in the apo form. Addition of higher PQQ concentrations to the culture medium did not modify the growth parameters but GDH activity was increased and there was no free apoenzyme in the membrane preparation. An excess of PQQ, saturating all the available apoenzyme, perhaps had a stabilizing effect, explaining the larger amount of GDH in these preparations.

GDH activities and PQQ contents in the different preparations of all the EF mutants were found to be similar, as shown in Table 2. Comparison of GDH assays and PQQ concentrations in the different strains reported in Table 2 shows clearly that glucose utilization by EF strains was related to a synthesis of PQQ by these strains.

EF mutants derived from *E. coli* TP2811, such as EF15, could not be used for biochemical studies because the apoGDH was not stable during the procedure of membrane solubilization with Triton X-100.

Gluconic acid production by EF mutants

Confirmation of the identity of the product of glucose oxidation by membrane preparations as gluconic acid was given by the coupled specificity of the two enzymes

Fig. 2. Growth kinetics (●) of the PQQ⁺ strain of *E. coli* EF16 in glucose MA medium and gluconate concentrations in the culture supernatant (▲).

used for the assay: gluconate kinase and NADP-dependent 6-phosphogluconate dehydrogenase (Möllering & Bergmeyer, 1984). No gluconic acid was produced in the presence of glucose 6-phosphate, indicating the absence of glucose-6-phosphate dehydrogenase in the samples used for assays.

In vitro, membrane preparations of EF cells grown in glucose MA medium catalysed stoichiometric oxidation of glucose into gluconic acid; in several experiments carried out as described in Methods, the reduction of 200 nmol ferricyanide led to the production of 98 nmol gluconic acid. Under the same experimental conditions, no gluconic acid was found when glucose was omitted from the production mixture nor when membranes without GDH activity were used.

In vivo, gluconic acid was detected in the supernatant of EF cells during the exponential phase of cultures in glucose MA medium (Fig. 2). The concentration of gluconic acid increased as the growth developed. Relative to the cell mass, this concentration remained practically constant (40 μM per OD₆₀₀ unit).

It was checked that in the supernatants of the overnight cultures in glycerol MA medium used as inoculum, no gluconic acid was detectable (≤ 0.4 μM). The fact that gluconate did not accumulate in the culture medium suggests that it appeared as an intermediate of glucose catabolism rather than an end-product. In *E. coli* gluconate is catabolized by the Entner–Doudoroff pathway. Presumably, EF cells can dissimilate glucose

by the Entner–Doudoroff pathway via gluconate formation, although we have no formal evidence for the existence of this catabolic route.

Phenotype of EF mutants and relationships with oxygen

All the EF strains isolated retained the whole phenotype of their parent strains except for the ability to utilize glucose in minimal medium. In the case of EF mutants obtained from FB8Δ*pts galP*, a weak restoration of galactose utilization was observed, producing a slow and poor growth on galactose MA medium plates. This result is in accordance with the observation of Hommes *et al.* (1986), who described a PQQ-dependent galactose utilization by *E. coli gal* mutants.

In *E. coli* the reoxidation of reduced PQQ is linked to the respiratory chain (Van Schie *et al.*, 1985; Matsushita *et al.*, 1987). As a consequence, PTS[−] strains in minimal medium supplemented with PQQ can grow only in the presence of oxygen. All the EF mutants utilized glucose only aerobically, as evidenced by the fact that in deep agar tubes containing glucose MA medium they grew only in the upper centimetre of the agar. Gluconate under the same conditions produced growth in the anaerobic as well as in the aerobic zone of deep agar tubes. These results demonstrated that only the step of glucose oxidation into gluconate was oxygen dependent, in accordance with the presence of a PQQ-dependent GDH in EF cells. Other non-PTS substrates tested allowed facultatively aerobic growth of EF strains as in the wild-type *E. coli*. All these results were confirmed in anaerobic jars.

The ability to use glucose only aerobically was used as a marker to check whether glucose-utilizing mutants other than EF cells were present in cultures of FB8Δ*pts* in experiments like that shown in Fig. 1. Two hundred colonies from LB count plates obtained from samples of different experiments taken at the beginning and at the end of the rapid growth phase were patched on glucose and gluconate MA media and incubated in air or in anaerobic jars. Patches of *E. coli* FB8 (wild-type) were added as a positive growth control on each plate. All the patches on gluconate plates grew with or without air, whereas on glucose plates all grew in the presence of air and none in anaerobiosis. This result showed that if glucose-utilizing mutants other than EF were present in this type of experiment, their proportion was always lower than 0.5%.

Discussion

The EF mutants described in this work were PTS[−] PQQ⁺ strains of *E. coli* able to grow rapidly in glucose minimal

medium. PQQ was synthesized in sufficient amount to promote a GDH specific activity similar to that found in *E. coli* Δpts grown in glucose minimal medium supplemented with 1 nM-PQQ. The EF mutants could utilize glucose only aerobically, as expected from a PQQ-dependent glucose catabolism. The product of glucose oxidation by GDH, gluconate, was presumably catabolized via the Entner–Doudoroff pathway. These characteristics clearly distinguished EF mutants from the mutants of PTS^- strains of *Salmonella typhimurium* described by Saier *et al.* (1973), which translocated glucose via a constitutive galactose permease. The fact that EF mutants could be obtained from *E. coli* Δpts *galP* eliminates the possibility of confusing the two types of mutants. Moreover, additional substrate utilization by EF mutants was limited to glucose whereas galactose permease constitutive mutants could grow with several PTS substrates (Saier *et al.*, 1973). The reason why we did not find *galPc* mutants can only be a matter for speculation (difference of organisms, lower frequency of spontaneous mutation, mode of mutagenesis and of selection, etc.). The *S. typhimurium* mutants were selected after NTG mutagenesis, which was apparently without effect on the frequency of appearance of EF mutants in *E. coli* (unpublished results). Under our experimental conditions, spontaneous mutants producing measurable amounts of PQQ appeared at a frequency of 2×10^{-7} – 5×10^{-8} . They were selected by the strong selective pressure imposed on the population of *E. coli* Δpts cells by the glucose minimal medium.

Recently six *pqq* genes involved in PQQ biosynthesis were cloned from *Methylobacterium organophilum* (Biville *et al.*, 1989) and the cloned genes were used as hybridization probes in an attempt to identify similar genes in *E. coli*, but without success (F. Biville, unpublished results). Nevertheless, the experimental data presented here demonstrates that *E. coli* contains all the genetic information required for PQQ biosynthesis. If we consider EF mutants as regulatory mutants in which PQQ biosynthesis is derepressed, the target locus of the mutation was probably a genetic element involved in the level of expression of *pqq* genes. We presently have no information on the nature of this locus. Attempts to alter a repressor gene by *Tn10* chromosomal insertion in a *pts* strain did not yield EF strains (unpublished experiment).

Another possibility is the presence of a cryptic operon, expression of which might be triggered by an IS or *Tn* insertion upstream from *pqq* genes. This type of regulation, which appears as the emergence of spontaneous mutations insensitive to chemical mutagens, has been described in several instances (for a review, see Discussion in Aronson *et al.*, 1989).

Before any mutation affecting the expression of *pqq*

genes, the slow growth of *E. coli* PTS^- in glucose minimal medium (Fig. 1) was caused by two distinct mechanisms. The difference in doubling time between the cultures of FB8 Δpts ($d = 10$ h) and of FB8 Δpts *galP* ($d = 20$ h) indicates a large participation of the galactose permease in the translocation of glucose through the cytoplasmic membrane, in accordance with the fact that about 10% of the fully induced permease activity was still present in uninduced cells, as was observed by Saier *et al.* (1973) for *S. typhimurium*. The nature of the growth observed after the *galP* inactivation is still unknown. Besides the possibility of an unknown system of glucose diffusion through the cytoplasmic membrane, a low level of PQQ production, not detectable by our assay method, is also possible; this could be demonstrated only with the use of a more sensitive means of PQQ detection.

The production of PQQ by *E. coli* was first claimed by Ameyama *et al.* (1984), who found a growth factor in culture supernatants which turned out to be PQQ. With our experimental conditions and assay method we did not confirm this result, since we never found any trace of PQQ (≤ 1 nM) in the culture supernatants. It is possible, however, that other strains or other experimental conditions might at least partially deregulate PQQ biosynthesis in *E. coli*.

In our work, PQQ was characterized by its activity as the apoenzyme of GDH from *E. coli* and of ethanol dehydrogenase from *P. testosteroni* used in the assay method. Duine *et al.* (1980) have shown that any modification of the PQQ molecule abolishes its activity as enzymic cofactor. This narrow specificity offered a good guarantee that the enzymic activities detected in this work were indeed caused by PQQ.

During the course of PQQ assays, we never experienced problems of unwanted PQQ in minimal culture media nor contaminant PQQ sticking to glassware as claimed by Van Kleef *et al.* (1987). The large number of our negative assays, the negative control and the everyday practice of PQQ assay in our laboratory attested the absence of contamination with correctly cleaned glassware. Moreover, the perfect reproducibility of the growth curves of FB8 Δpts *galP* would have been impossible in contaminated vessels.

Klebsiella pneumoniae is an enteric species that produces PQQ. Recently Meulenberg *et al.* (1990) have shown that a PTS^- strain of this species can utilize glucose as growth substrate. This ability is lost in PQQ- PTS^- mutants and restored after addition of PQQ to the culture medium. This situation is worth comparing with what we observed in *E. coli*. PQQ production in *E. coli* appeared only after a mutation allowing a PTS^- strain to grow rapidly in glucose minimal medium. We have not yet selected PQQ- mutants of *E. coli*. In *E. coli*, as in *K. pneumoniae*, growth in glucose minimal medium of *pts*

strains is associated with a PQQ-dependent oxidation of glucose into gluconate. How the gluconate resulting from this glucose oxidation is catabolized has not yet been analysed in *K. pneumoniae* or in *E. coli*. The most probable hypothesis is a dissimilation by the Entner–Doudoroff pathway as is the case for *E. coli* cultivated with gluconate as growth substrate (Gottschalk, 1985).

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