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PQQ-Dependent Production of Gluconic Acid by *Acinetobacter*, *Agrobacterium* and *Rhizobium* Species

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Acinetobacter lwoffi, Azotobacter vinelandii, Agrobacterium and *Rhizobium* species contain quinoprotein glucose dehydrogenase apoenzyme (EC 1.1.99.17). Addition to whole cells of pyrrolo-quinoline quinone (PQQ), the prosthetic group of this enzyme, resulted in the production of gluconic acid from glucose. The *in vivo* reconstitution of apo-glucose dehydrogenase with PQQ was dependent on the presence of Ca^{2+} or Mg^{2+}. Optimal conditions for reconstitution allowed maximal glucose dehydrogenase activity in the presence of 1–10 nmol PQQ l^{-1}. Synthesis of the apoenzyme of glucose dehydrogenase was not dependent on glucose in the growth media. The physiological significance of the synthesis of apo-glucose dehydrogenase, as found in a variety of bacteria, is discussed.

INTRODUCTION

The bacterial metabolism of sugars may proceed via different catabolic routes. Differences in sugar metabolism may also be found in the mode of entrance of sugars into these routes. For example in *Escherichia coli* phosphorylation is the first step in aldose metabolism whereas *Pseudomonas* spp. can catalyse the initial oxidation of the phosphorylated or non-phosphorylated sugar to the corresponding (phosphorylated) aldonic acid, which may transiently accumulate in the medium. The importance of the direct oxidation pathway relative to the phosphorylative route has been intensively studied (Dawes, 1981; Lessie & Phibbs, 1984). In *Pseudomonas* spp. the direct oxidation pathway is obligatory for growth on aldopentoses (Doelle, 1975) but during growth on aldohexoses this route may be bypassed depending on environmental conditions (Lessie & Phibbs, 1984).

In all cases examined so far the accumulation of aldonic acids by bacteria is associated with the presence of a membrane-bound aldose dehydrogenase (known as 'glucose dehydrogenase', GDH) which contains pyrrolo-quinoline quinone (PQQ) as a prosthetic group (Duine *et al.*, 1979). The enzyme is probably located on the periplasmic side of the cell membrane (Dawes, 1981) and donates its electrons to the electron transport chain at the level of cytochrome *b* (Hauge, 1960; Beardmore-Gray & Anthony, 1986). A peculiar example of GDH synthesis in bacteria can be found in *Acinetobacter calcoaceticus*, most strains of which can oxidize glucose to gluconic acid but do not grow at all on either of the two compounds (Juni, 1978).

Bacteria unable to oxidize glucose to gluconic acid are not necessarily devoid of GDH protein. We have recently shown that *Acinetobacter lwoffi* constitutively synthesizes apo-GDH which can be reconstituted to active enzyme upon addition of nanomolar quantities of PQQ (van Schie *et al.*, 1984). As a result, PQQ supplemented cultures of *Ac. lwoffi* can oxidize glucose instantaneously to gluconic acid at a rate similar to that of *Ac. calcoaceticus* (van Schie *et al.*, 1984). Like *Ac. lwoffi*, various *E. coli* strains also contain apo-GDH (Hommes *et al.*, 1984; van

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Abbreviations: GDH, glucose dehydrogenase; PQQ, pyrrolo-quinoline quinone; PES, phenazine ethosulphate.

Schie *et al.*, 1985), the synthesis of which is constitutive. Existence of the synthesis of non-functional apo-enzyme is not restricted to glucose dehydrogenase; PQQ-dependent alcohol dehydrogenase (Groen *et al.*, 1986) and polyvinyl alcohol dehydrogenase (Shimao *et al.*, 1986) were reported recently to exist as apo-enzymes.

Glucose appeared to be a very effective energy source for driving secondary solute transport in PQQ preincubated membrane vesicles of *E. coli* (van Schie *et al.*, 1985) and in membrane vesicles of *Ac. calcoaceticus* (Pronk *et al.*, 1986). This establishes a role for GDH in energy metabolism in these bacteria.

In view of the phenomena discussed above the following questions are considered in this paper. (1) Are bacteria which produce apo-GDH auxotrophic for PQQ, or is the synthesis of this cofactor dependent on environmental conditions in these organisms? (2) Is the synthesis of apo-GDH a widespread phenomenon?

METHODS

Organisms. The following strains were obtained from the Laboratory of Microbiology Delft culture collection: *Acinetobacter calcoaceticus* LMD 79.41, *Acinetobacter lwoffi* LMD 73.1, *Escherichia coli* B/r/l. *Agrobacterium tumefaciens* C58 (see Zambryski *et al.*, 1983), *Agrobacterium radiobacter* NCIB 11883, *Pseudomonas* sp. NCIB 49592, *Xanthomonas campestris* pathovars – *begoniae* (NCPPB 2266), *hyacinthii* (NCPPB 599), *juglandis* (NCPPB 411), *nakataecorchori* (NCPPB 1337) and *phaseoli* (NCPPB 1811) – and *Xanthomonas campestris* NCIB 11803 were obtained from Shell Research Laboratories, Sittingbourne. *Agrobacterium tumefaciens* LBA 201 was provided by Dr P.J.J. Hooykaas, and *Rhizobium leguminosarum* 248, *R. leguminosarum* biovar *trifolii* LPR 5001 and *Rhizobium meliloti* 1126 were a gift from Dr C. Wijffelman (both of the Department of Plant Molecular Biology, State University of Leiden, The Netherlands).

Growth media and culture conditions. The *Acinetobacter* strains and *Ag. tumefaciens* LBA 201 were grown in an acetate- (30 mmol l^{-1}) or glycerol- (15 mmol l^{-1}) limited continuous culture as described previously (van Schie *et al.*, 1984). The mineral salts medium contained, per litre: $(NH_4)_2SO_4$, 4·0 g; K_2HPO_4, 1·4 g; KH_2PO_4, 0·8 g; $MgSO_4.7H_2O$, 0·2 g; EDTA, 15 mg; $ZnSO_4.7H_2O$, 4·5 mg; $CoCl_2.6H_2O$, 0·3 mg; $MnCl_2.4H_2O$, 1 mg; $CuSO_4.5H_2O$, 0·3 mg; $CaCl_2.2H_2O$, 4·5 mg; $FeSO_4.7H_2O$, 3 mg; $NaMoO_4.2H_2O$, 0·04 mg; H_3BO_3, 1 mg; KI, 0·1 mg. *R. leguminosarum* 248 was grown in batch cultures on glucose (10 g l^{-1}) or grown in a glucose-limited continuous culture on a mineral medium (de Vries, 1980).

During continuous cultivation, dissolved oxygen was measured with a galvanic oxygen electrode, and controlled at the desired value by the stirring rate. Cultures were sparged with mixtures of air and pure oxygen to obtain partial oxygen pressures above air saturation.

The *Xanthomonas* strains, *Ag. tumefaciens* C58, *Ag. radiobacter* and *Pseudomonas* sp. were grown in batch cultures on a mineral medium containing, per litre: glucose 10·0 g; $(NH_4)_2SO_4$, 3·0 g; Na_2HPO_4, 3·0 g; KH_2PO_4, 3·0 g; $MgSO_4.7H_2O$, 0·2 g; $CaCl_2$, 0·015 g; $FeCl_3$, 0·065 g; 2 ml of a trace element solution (Porter *et al.*, 1983), pH 6·8. PQQ was added as indicated in the text. Lyophilized cells of *E. coli* strain W (ATCC 9637) and *Azotobacter vinelandii* ATCC 12518 were obtained from Sigma.

Isolation of bacteroids. R. leguminosarum bacteroids were isolated from the nodules of 21-d-old *Pisum sativum* L. cv 'Rondo' (Cebuco, Rotterdam). Inoculation and cultivation of plants, and preparation of bacteroids were as described by Planqué & Brussel (1979).

Measurement of aldose-dependent oxygen consumption. PQQ-dependent glucose or xylose oxidation by whole cells and bacteroids was assayed by following the rate of oxygen consumption with a Clark type oxygen electrode. Xylose was used to test the aldose dehydrogenase (GDH) activity in case cells were able to metabolize glucose through other pathways than that via GDH. The reaction was started by the addition of sugar to a final concentration of 20 mmol l^{-1}. For *in vivo* reconstitution of GDH, cells were suspended in a potassium phosphate buffer (50 mmol l^{-1}) containing 10 mmol $MgSO_4$ l^{-1} and preincubated for 20 min in the presence of 4 μM-PQQ unless stated otherwise. Suspensions not preincubated with PQQ served as a blank. In the case of experiments with lyophilized cells of *Az. vinelandii* the assay medium in addition contained 0·2 mmol phenazine ethosulphate (PES) l^{-1} to mediate electron transfer between the dehydrogenase and oxygen. All experiments were carried out at least in duplicate with two independently grown cell batches. The duplicate respiration activities never differed by more than 10%.

Total organic carbon measurements. A Beckman model 915B Tocamaster total organic carbon analyser was used to determine the carbon content of whole cultures or culture supernatants, the carbon content of bacteria being obtained from the difference. Reproducibility was about 1%. Bacterial dry weight was calculated assuming a carbon content of 50%.

Analytical assays. Glucose was measured by the GOD-PAP method and gluconate with gluconate kinase/6-phosphogluconate dehydrogenase (testkits, Boehringer Mannheim).

Chemicals. PQQ [2,7,9-tricarboxy-1H-pyrrolo(2,3-f)quinoline-4,5-dione] was kindly provided by J.A. Duine, from the Department of Microbiology and Enzymology, Delft University of Technology. (PQQ is commercially available from Fluka.) Xylose was of the purest reagent grade and contained less than 0·02% glucose.

RESULTS

In vivo reconstitution of GDH activity in Ac. lwoffi

Apo-GDH of *Ac. lwoffi* could be reconstituted *in vivo* to active GDH by addition of trace amounts of PQQ (2 nmol l^{-1}) to the culture liquid (van Schie *et al.*, 1984). However, washing of cells with a phosphate buffer or distilled water prior to reconstitution resulted in an almost complete loss of their ability to oxidize glucose even in the presence of 200 nmol PQQ l^{-1}. In contrast, washing of suspensions of holo-GDH- containing *Ac. calcoaceticus* in phosphate buffer had no effect on the rate of glucose oxidation in this organism (Table 1). When *Ac. lwoffi* was grown in standard medium in the presence of 200 nmol PQQ l^{-1}, washing of the suspensions with distilled water had no effect: glucose oxidation was instantaneous in the absence of PQQ (Table 1).

Both in suspensions of *Ac. calcoaceticus* and in those of *Ac. lwoffi* glucose oxidation was accompanied by the formation of stoichiometric amounts of gluconic acid. A systematic analysis of the factors involved in the PQQ-mediated oxidation of glucose to gluconic acid in *Ac. lwoffi* revealed that Ca^{2+} or Mg^{2+} were required for reconstitution of apo-GDH with its prosthetic group PQQ. The rate of PQQ-dependent glucose oxidation by washed cells was strongly dependent on the concentration of these ions between 0·01 and 1 mmol l^{-1} (Fig. 1). Only above this concentration did the rate of glucose oxidation equal that of untreated cells. This explains the marked differences of the effects of the type of water used for the preparation of the washing buffer, since the tap water used contained 1·2 mmol Ca^{2+} l^{-1} and 0·3 mmol Mg^{2+} l^{-1}. In the presence of excess Ca^{2+} or Mg^{2+} only nanomolar quantities of PQQ were required for a maximal rate of glucose oxidation (Fig. 2*a*). Without the addition of Ca^{2+} and Mg^{2+} reconstitution could be accomplished by addition of high concentrations (10–50 μmol l^{-1}) of PQQ although activity was never more than 75% of the maximal rate (Fig 2*b*). Both the rate and extent of reconstitution were also affected by the pH of the suspension, with an optimum of pH 6·0 (results not shown). Similar Ca^{2+} dependent reconstitution of apo-GDH with PQQ was found with suspensions of *E. coli*. In the presence of 4 mmol $Ca(NO_3)_2$ l^{-1}, only 2 nmol PQQ l^{-1} was necessary to obtain maximal GDH activity (Fig. 2*c*). Washing of the reconstituted suspensions

Table 1. *Effect of washing of Ac. calcoaceticus or Ac. lwoffi cells on PQQ-dependent glucose oxidation*

Cell suspensions of acetate-limited chemostat cultures (0·35 g l^{-1}) were centrifuged and resuspended to the same density in various media (pH 6·0). In the case of *Ac. lwoffi*, the cell suspensions thus obtained were preincubated for 20 min with 200 nmol PQQ l^{-1}. Without this preincubation the rate of glucose oxidation was negligible.

Washing liquid	Glucose oxidation [nmol O_2 min^{-1} (mg dry wt)$^{-1}$]	
	Ac. calcoaceticus	*Ac. lwoffi*
Demi* or distilled water	170	0
Potassium phosphate buffer (2 mmol l^{-1}) in demi water	232	41
Filtered culture fluid	239	225
Potassium phosphate buffer (2 mmol l^{-1}) in tap water	200	215
Potassium phosphate buffer (2 mmol l^{-1}) in demi water + $Ca(NO_3)_2$ (10 mmol l^{-1})	ND	234
Potassium phosphate buffer (2 mmol l^{-1}) in demi water + $MgSO_4$ (10 mmol l^{-1})	213	191

* 'Demi water' is water demineralized by ion exchange chromatography
ND, Not determined.

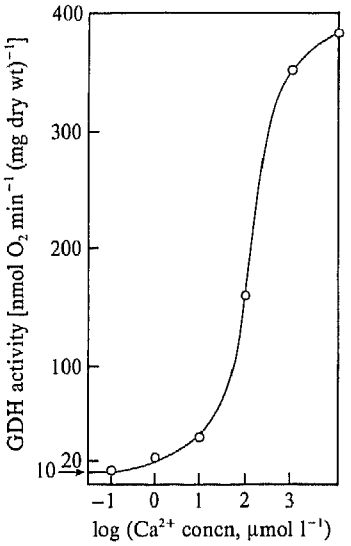

Fig. 1. Effect of Ca^{2+} on the specific rate of glucose-dependent oxygen consumption by *Ac. lwoffi* cells as a function of the logarithm of the Ca^{2+} concentration; calcium was added as $Ca(NO_3)_2$. Cells (0·3 g dry weight l^{-1}) obtained from an acetate limited chemostat culture were resuspended in potassium phosphate buffer (2 mmol l^{-1}, pH 6·0) with 200 nmol PQQ l^{-1}. Cells were preincubated with the appropriate Ca^{2+} concentration for 20 min prior to addition of glucose (20 mmol l^{-1}).

with buffer without Ca^{2+} and PQQ did not affect GDH activity, indicating that once the *in vivo* reconstitution has taken place, PQQ remains firmly bound to the enzyme.

Effect of cultivation conditions on the glucose oxidizing capacity of Ac. lwoffi

In order to test whether the synthesis of PQQ might be inducible in *Ac. lwoffi* various cultivation conditions were examined for their effect on the capacity of cells to oxidize glucose. In all cases, however, glucose oxidation was strictly dependent on the addition of PQQ. Growth on various substrates such as malate, acetate, succinate, ethanol, histidine or peptone in batch or carbon-limited chemostat cultures and in the absence or presence of those aldose sugars which are typical substrates for GDH (glucose, xylose and ribose) did not result in sugar consumption, neither *in situ* nor in washed cell suspensions. Other parameters which have been shown to affect the level of active (holo) GDH in *Pseudomonas* spp. (Lessie & Phibbs, 1984) and thus might have an influence on PQQ synthesis, were tested as well. Growth at a lower temperature (15 instead of 30 °C) and variations in the dissolved oxygen tension (50% or 300% air saturation) or culture pH (6·0–8·0) had no effect on the aldose oxidizing capacity: glucose oxidation was insignificant in the absence of exogenous PQQ [i.e. less than 0·005 μmol min^{-1}(mg cells)$^{-1}$] but amounted to 0·2–0·4 μmol min^{-1} (mg cells)$^{-1}$ in the presence of the cofactor.

Apo-glucose dehydrogenase in other bacteria

Various *E. coli* strains have also been reported to synthesize apo-GDH constitutively. As with *Ac. lwoffi*, we have not found, so far, any cultivation condition which resulted in the formation of active (holo) enzyme in *E. coli* B/r/1. Carbon-limited chemostat cultures of this organism growing on acetate or glucose at various pH (6–8) and temperature (15–37 °C) values, at different levels of dissolved oxygen tension (50% or 300% air saturation) oxidized xylose only in the presence of PQQ.

In a further investigation a number of species among the *Rhizobiaceae* were tested for the presence of apo-GDH. For example *Rhizobium leguminosarum* grown in batch cultures on tryptone/yeast extract exhibited a PQQ-dependent oxidation of glucose and xylose (Table 2).

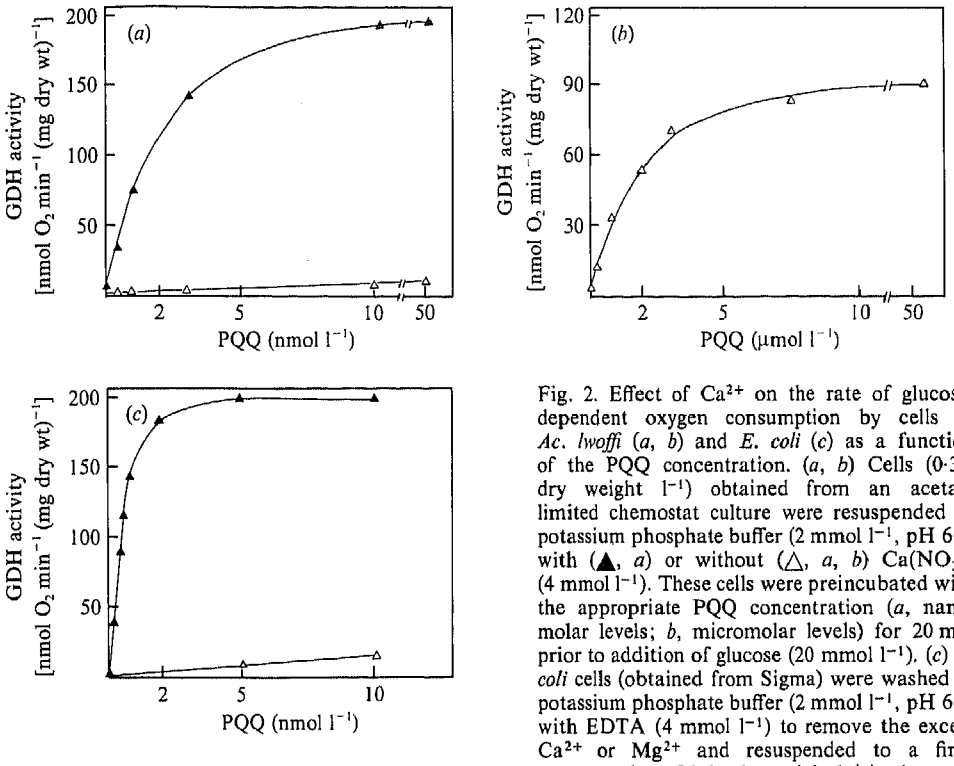

Fig. 2. Effect of Ca^{2+} on the rate of glucose-dependent oxygen consumption by cells of *Ac. lwoffi* (*a*, *b*) and *E. coli* (*c*) as a function of the PQQ concentration. (*a*, *b*) Cells (0·3 g dry weight l^{-1}) obtained from an acetate limited chemostat culture were resuspended in potassium phosphate buffer (2 mmol l^{-1}, pH 6·0) with (▲, *a*) or without (△, *a*, *b*) $Ca(NO_3)_2$ (4 mmol l^{-1}). These cells were preincubated with the appropriate PQQ concentration (*a*, nanomolar levels; *b*, micromolar levels) for 20 min prior to addition of glucose (20 mmol l^{-1}). (*c*) *E. coli* cells (obtained from Sigma) were washed in potassium phosphate buffer (2 mmol l^{-1}, pH 6·0) with EDTA (4 mmol l^{-1}) to remove the excess Ca^{2+} or Mg^{2+} and resuspended to a final concentration of 1·6 g dry weight l^{-1} in the same buffer supplemented with (▲) or without (△) $Ca(NO_3)_2$ (4 mmol l^{-1}).

Table 2. *PQQ-dependent aldose oxidation by whole cells or bacteroids of R. leguminosarum 248*

Cells from the stationary phase of batch cultures grown on TY medium were directly assayed for PQQ-dependent aldose oxidation. Bacteroids were isolated as described in Methods. Measurements with the bacteroids were made in two independent preparations, *a* and *b*.

	Oxygen consumption [nmoles min^{-1}(mg dry wt)$^{-1}$]		
	Cells	Bacteroids	
Substrate		*a*	*b*
Xylose	4·5	ND	ND
Xylose + PQQ	66·3	ND	ND
Glucose	5·2	4·1	0
Glucose + PQQ	72·5	6·1	2·4
Succinate	ND	45·0	25·0

ND, Not determined.

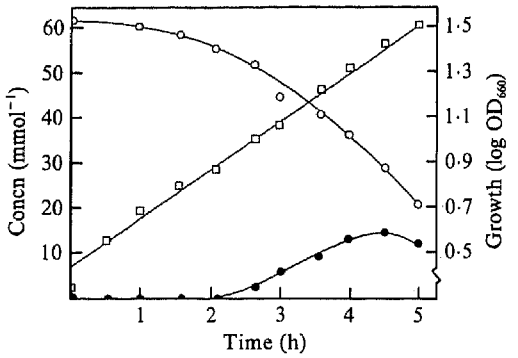

Fig. 3. PQQ-dependent glucose consumption and gluconate production by *Ag. tumefaciens* LBA 201. The organism was pregrown on glycerol in a carbon-limited chemostat pH 6·5, at a cell density of 0·85 g dry weight l^{-1}. The medium supply was turned off 30 min before the experiment, and CO_2 production from glycerol stopped immediately, indicating that glycerol was depleted. At zero time glucose was added at a final concentration of 60 mmol l^{-1}. After 2 h, PQQ (final concentration of 4 μmol l^{-1}) was added to the fermenter. ○, Glucose; ●, gluconate; □, growth.

Table 3. *PQQ-dependent gluconic acid production by several Gram-negative bacteria*

Organisms were grown in batch cultures as described in Methods on 10 g glucose l^{-1} with or without 4 μmol PQQ l^{-1}. Gluconic acid formation was measured after depletion of glucose in the medium.

Organism	PQQ	μ_{max} (h^{-1})	Gluconic acid (mmol l^{-1})
Agrobacterium radiobacter NCIB 11883	−	0·41	0
	+	0·41	23·5
Agrobacterium tumefaciens C58	−	0·23	0
	+	0·26	15·3
Pseudomonas sp. NCIB 49592	−	0·35	0·3
	+	0·36	21·1
Rhizobium leguminosarum 248	−	0·096	0
	+	0·091	14·4

High concentrations of gluconic acid were found in cultures of *R. leguminosarum* grown in the presence of PQQ (Table 3). The presence of apo-GDH in two other *Rhizobium* species, *R. leguminosarum* biovar *trifolii* and *R. meliloti*, grown on tryptone/yeast extract, was evident from the PQQ-dependent glucose and xylose oxidation (results not shown). The influence of cultivation conditions on the synthesis of apo-GDH in these organisms has not yet been systematically investigated. However, in bacteroids of *R. leguminosarum* isolated from root nodules of *Pisum sativum* the rate of PQQ-dependent aldose oxidation was very low compared to that of free-living cells (Table 2).

Various *Agrobacterium* strains were also found to synthesize apo-GDH. Apo-enzyme was detected in *Ag. radiobacter* (NCIB 11883) and *Ag. tumefaciens* LBA 201 when grown in batch cultures (Table 3). Production of gluconic acid accounted for up to 40% of the initial amount of glucose and did not significantly influence the growth rate. Similarly, when glucose was added to cells of *Ag. tumefaciens* LBA 201 pregrown in a glycerol-limited chemostat, gluconic acid was produced instantaneously after the addition of PQQ (Fig. 3). Again, the growth rate did not change during the production of gluconic acid.

The *in vivo* activity of apo-GDH as visualized by PQQ-dependent gluconic acid production was quite stable. Cells of *Ag. tumefaciens* LBA 201 starved for 2 weeks in mineral medium without carbon source still showed PQQ dependent formation of gluconic acid from glucose (results not shown). Suspensions of lyophilized cells of *Az. vinelandii* (ATCC 12518) obtained from a commercial source also exhibited PQQ-dependent oxidation of xylose, in the presence of

PES (to optimize electron transfer from the GDH to oxygen), of 70 nmol xylose oxidized min^{-1} (mg cells)$^{-1}$.

Non-coordinated synthesis of PQQ and apo-GDH protein has been reported to occur in *Pseudomonas aeruginosa* during anaerobic growth on glucose with nitrate (van Schie *et al.*, 1984). Table 3 shows that also in aerobic cultures of *Pseudomonas* sp. NCIB 49592, substantial apo-GDH can be demonstrated in addition to holo-GDH.

In view of their taxonomic relationship to the genus *Pseudomonas*, various *Xanthomonas* strains (see Methods) were tested for the presence of apo-GDH. However, all strains tested failed to catalyse PQQ dependent gluconate production, suggesting the absence of apo-GDH in this genus.

DISCUSSION

The recent finding (van Schie *et al.*, 1984, 1985; Hommes *et al.*, 1984) that organisms such as *Ac. lwoffi* and *E. coli* contain exclusively apo-enzyme GDH, and no holo-enzyme activity, has prompted the investigation of various aspects of this phenomenon in more detail. The present study has shown that asynchronous or even independent synthesis of apo-GDH and its coenzyme PQQ can be found in strains of *Ac. lwoffi* and *E. coli*, and also in *Pseudomonas*, *Azotobacter*, *Rhizobium* and *Agrobacterium* species. Thus, the 'cryptic' presence of GDH is widespread in nature. The amounts of PQQ required for the reconstitution of GDH under optimal conditions are in the nanomolar range and it may well be that such concentrations are available in the environment since a number of bacteria excrete micromolar quantities of PQQ (Ameyama *et al.*, 1984*a*; Duine *et al.*, 1985). The possibility that PQQ might, in fact, be considered as a vitamin (van Schie *et al.*, 1984) is supported by the recent observation (Shimao *et al.*, 1986) that polyvinyl degrading *Pseudomonas* spp. require PQQ for growth on this compound.

An important experimental aspect of the *in vivo* reconstitution of GDH is the requirement for Ca^{2+} or Mg^{2+}. In the presence of Ca^{2+}, only 2 nmol PQQ l^{-1} was necessary for the reconstitution of GDH in both *Ac. lwoffi* and *E. coli* strain W, whereas micromolar quantities were required in its absence (Fig 2). Experiments with washed bacteria containing either native holo-GDH or reconstituted GDH showed that once active GDH is present, these cations are no longer needed. These observations show that earlier reports on the stimulatory effect of Ca^{2+} or Mg^{2+} on dye-linked glucose oxidation (Dalby & Blackwood, 1955; Hauge, 1961) and on the reconstitution of GDH activity (Ameyama *et al.*, 1985) in cell-free extracts are also relevant for the *in vivo* situation.

In view of the very low amounts of PQQ required for the reconstitution of GDH, glassware or media contaminated with traces of PQQ might produce false positive tests for the presence of holo-GDH in a culture (PQQ is very stable and tends to stick to glass: M.A.G. van Kleef, P. Dokter & J.A. Duine, personal communication). In addition, it is possible that deionized water might be contaminated with PQQ, as ion exchange resins often contain hyphomicrobia (J.B.M. Meiberg & W. Harder, personal communication) which produce and excrete PQQ. *Hyphomicrobium* spp. are also well-known inhabitants of water pipes (Harder & Attwood, 1978) and therefore tap water may also contain PQQ. Recently it has been reported that *E. coli* K12, and *Ag. radiobacter*, grown in media prepared with tap water contained low holo-GDH activity (Ameyama *et al.*, 1985), and that another *E. coli* strain excreted low amounts of PQQ (0·2 nmol l^{-1}) into the growth medium (Ameyama *et al.*, 1984*b*). It is our experience that the presence of low GDH activity in, for example *E. coli*, can be traced back to scavenging of the cofactor from the medium rather than from biosynthesis. Unequivocal evidence for PQQ production requires the demonstration of an increase in the total PQQ present in a closed system as a function of metabolic activity at various time intervals. Thus far, this remains to be demonstrated for *Ac. lwoffi* and *E. coli*.

In *Pseudomonas* spp. the activity of GDH may be regulated by the synthesis of PQQ (Table 3, van Schie *et al.*, 1984). It thus remained possible that species such as *Ac. lwoffi* and *E. coli* which synthesize apo-enzyme exclusively might synthesize PQQ under unknown growth conditions. Both organisms were therefore grown under conditions which might favour PQQ synthesis, such

as energy stress (van Schie *et al.*, 1985) and high temperature or osmotic values (Lessie & Phibbs, 1984). All attempts to detect PQQ synthesis have failed and therefore the possibility that PQQ is a vitamin seems the most logical alternative.

Even if PQQ was available to the organism as a vitamin, its physiological function is only partially understood. In organisms such as *Acinetobacter* spp. and *E. coli* GDH may play a role in energy metabolism, for example as an (auxiliary) energy generating system (van Schie *et al.*, 1985).

The role of GDH in the *Rhizobium* and *Agrobacterium* species tested is not understood. It is known that in the *Rhizobiaceae* glucose can be metabolized through the Entner–Doudoroff and pentose phosphate pathways (Arthur *et al.*, 1973; Glenn *et al.*, 1984), but the direct oxidation pathway was considered to be absent (Vardanis *et al.*, 1961; Glenn *et al.*, 1984). However there are clear indications for a direct oxidation of L-arabinose (Pedrosa & Zancan, 1974; Duncan, 1979) and for direct oxidation of glucose to 2-ketogluconic acid (Courtois *et al.*, 1979). As this probably proceeds via the production of gluconate by GDH, it is an indication that *Rhizobium* species may sometimes contain active GDH. These data, combined with the finding by Trinchant *et al.* (1981) that bacteroids may show glucose-dependent nitrogenase activity *in vitro*, and our observation that bacteroids of *R. leguminosarum* possess low but significant GDH activities, provide a basis for a reinvestigation of the glucose metabolism in *Rhizobiaceae*.

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