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Factors Relevant in Bacterial Pyrroloquinoline Quinone Production

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Quinoprotein content and levels of external pyrroloquinoline quinone (PQQ) were determined for several bacteria under a variety of growth conditions. From these data and those from the literature, a number of factors can be indicated which are relevant for PQQ production. Synthesis of PQQ is only started if synthesis of a quinoprotein occurs, but quinoprotein synthesis does not depend on PQQ synthesis. The presence of quinoprotein substrates is not necessary for quinoprotein and PQQ syntheses. Although the extent of PQQ production was determined by the type of organism and quinoprotein produced, coordination between quinoprotein and PQQ syntheses is loose, since underproduction and overproduction of PQQ with respect to quinoprotein were observed. The results can be interpreted to indicate that quinoprotein synthesis depends on the growth rate whereas PQQ synthesis does not. In that view, the highest PQQ production can be achieved under limiting growth conditions, as was shown indeed by the much higher levels of PQQ produced in fed-batch cultures compared with those produced in batch experiments. The presence of nucleophiles, especially amino acids, in culture media may cause losses of PQQ due to transformation into biologically inactive compounds. Some organisms continued to synthesize PQQ de novo when this cofactor was administered exogenously. Most probably PQQ cannot be taken up by either passive diffusion or active transport mechanisms and is therefore not able to exert feedback regulation on its biosynthesis in these organisms.

From current knowledge on the occurrence of pyrroloquinoline quinone (PQQ), it can be concluded that its distribution ranges from microbes to humans. PQQ is the cofactor of the so-called quinoproteins (14). To develop this novel branch of enzymology, one of the important aspects to investigate is the production of the cofactor. Knowledge of this aspect is relevant not only for the performance of commercial PQQ fermentation processes but also for obtaining insight into biosynthesis and the possible role of the cofactor as a vitamin. Since the mechanisms of induction and regulation are completely unknown, as a first attempt a search was made for the factors that are relevant in bacterial PQQ production.

Quite different situations appear to exist with respect to bacterial PQQ production. Gram-negative methylotrophs not only synthesize PQQ to provide their quinoprotein apoenzymes with PQQ but also excrete the compound (amounting to the micromolar level) into their culture media (13). It is therefore not surprising that such an organism is mentioned in all the recent patent applications on the fermentative production of PQQ (3; M. Ameyama and O. Adachi, Jpn. Kokai Tokkyo Koho JP 62,126,988 [87,126,988], 1987; S. Uragami, Jpn. Kokai Tokkyo Koho JP 61,247,397 [86,247,397], 1986; T. Urakami, European patent application EP 206,471, 1987). The same phenomenon is found for some bacteria growing on ethanol, e.g., *Pseudomonas* spp. and acetic acid bacteria (responsible for the substantial amounts of PQQ in vinegar) (14). However, it appears that the provision with PQQ is not always adequate in these cases. Thus, it was reported (6, 7) that the lag time in growth of *Acetobacter* species can be shortened by supplementation of the culture medium with PQQ. Whether this effect is related to uncoordinated synthesis of PQQ and apoquinoprotein or to removal of PQQ from the holoenzyme during manipulation of the organism is not clear. The latter explanation is supported by the fact that detectability of the effect required repeated washings of the cells (8). On the other hand, several bacteria have a variable ratio of apoquinoprotein enzyme to holoquinoprotein enzyme (14, 25, 39).

A very curious phenomenon, shown by many bacteria and unexplained so far, is the production of apoquinoprotein enzyme but not PQQ. This effect was found originally for glucose dehydrogenase in *Acinetobacter lwoffii* (37) and later on in many *Escherichia coli* strains (24). When *A. lwoffii* is provided with PQQ, it appears that aldose sugars can be used as an auxillary energy source (38). In *E. coli*, the reconstituted glucose dehydrogenase provides an additional route for sugar metabolism (24). A more direct role is obvious in the case of quinoprotein alcohol dehydrogenase of *Pseudomonas testosteroni*, produced in the apo form. On supplementing the medium with PQQ, growth rates on alcohols were stimulated significantly (22). A crucial role of PQQ has been observed in polyvinyl alcohol degradation by a *Pseudomonas* species that is only able to grow on this substrate when provided with PQQ (31). In all of these cases, the question can be posed of why the organisms do not produce the holoenzyme. The answer could be that (part of) the genetic information for PQQ biosynthesis is lacking. This view is supported by the recent finding that transfer of four genes of PQQ biosynthesis, cloned from *Acinetobacter calcoaceticus*, led to glucose dehydrogenase holoenzyme production in *A. lwoffii* (20) and in *E. coli* (N. Goosen, personal communication).

From the foregoing it is clear that PQQ synthesis by bacteria is not a straightforward process. Based on the present work and data from the literature, factors are indicated which are relevant for the production of the cofactor.

MATERIALS AND METHODS

Cultivation. *A. calcoaceticus* LMD 79.41 was cultivated on an inorganic medium (35). *Pseudomonas putida* biovar B LMD 84.56, *P. stutzeri* LMD 26.48, and *P. aureofaciens* ATCC 15926 (obtained from F. Lingens [30]) were cultivated

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on an inorganic medium containing the following (per liter): 4.6 g of KH_2PO_4, 11.5 g of K_2HPO_4, 2.5 g of NH_4Cl, and 0.2 g of $MgSO_4 \cdot 7H_2O$ and calcium, iron, and spore solutions as described previously (16). *Hyphomicrobium* sp. strain X was grown on methanol (16) and *Methylobacterium organophilum* XX was grown as described previously (9). In batch experiments, mineral media were supplemented with carbon and energy sources as indicated, and cells were grown to the stationary phase. In fed-batch experiments, strains were precultured on 20 ml of mineral medium supplemented with a carbon source (0.2%) as indicated. When good growth was obtained (optical density at 610 nm of >1.0), the culture was diluted 10 times with fresh mineral medium, and feeding was started with the carbon source (0.8%) in mineral medium at a flow rate of 10 ml/h. All organisms were grown at 30°C on a rotary shaker (200 rpm).

Enzyme assays. Cell extracts of *A. calcoaceticus* and *P. putida* (15) and *Hyphomicrobium* sp. strain X (18) were prepared as described previously.

Glucose dehydrogenase (GDH) activity was measured as described previously (15) by measuring the reduction rate of Wurster's blue in 0.1 M Tris hydrochloride buffer (pH 7.0). Quinate dehydrogenase (QDH) was assayed as described previously (36) by measuring the reduction rate of Wurster's blue in 0.1 M Tris hydrochloride buffer (pH 7.5). Methanol dehydrogenase was assayed in a reaction mixture containing the following (in a total volume of 1 ml): 0.06 M borate plus 0.06 M NH_4Cl (pH 9.0), 100 μM Wurster's blue, 5 mM methanol, and cell extract. All measurements were performed at room temperature. One unit of enzyme activity was defined as the amount of enzyme required to convert 1 μmol of substrate per min under the assay conditions.

PQQ assay. PQQ was assayed enzymically by using PQQ-dependent alcohol dehydrogenase apoenzyme from *P. testosteroni* (22).

RESULTS

Induction of PQQ synthesis. In Table 1, a compilation is given of the production of quinoproteins and PQQ in the culture medium under a variety of conditions and with different bacteria. The following points are relevant with respect to induction: (i) in the absence of quinoprotein synthesis, there is no production of extracellular PQQ (data not shown); and (ii) production of holoquinoprotein, and thus of PQQ, can occur in the absence of the quinoprotein substrate. The latter applies for instance to *A. calcoaceticus* and several *Pseudomonas* species showing constitutive glucose dehydrogenase holoenzyme production and to *M. organophilum* XX synthesizing appreciable amounts of methanol dehydrogenase with succinate as the sole carbon and energy source. Gratuitous induction also occurred. For instance, growth on *p*-hydroxybenzoate induced QDH in *A. calcoaceticus*. This carbon source is not degraded via quinate but via protocatechuic acid, which has been shown to be the inducer of all the enzymes of both the β-ketoadipate and hydroaromatic pathways, including QDH in *A. calcoaceticus* (26, 32). In all cases where quinoprotein synthesis was observed in the absence of its substrate, biosynthesis of PQQ could be demonstrated (Table 1), either directly when the cofactor was excreted in the culture fluid or indirectly by measuring quinoprotein holoenzyme activity.

Factors relevant to the production rate. On the basis of numerous batch and fed-batch experiments in which growth and PQQ production was followed simultaneously, the rate of external PQQ synthesis in several organisms was estimated. Some of the most relevant data are presented in Table 2. It appears that substantial differences exist that are related to the organism and carbon source used. It should be noted that the rate of PQQ synthesis was very low on carbon sources that do not induce quinoproteins in these organisms.

Coordination between quinoprotein and PQQ synthesis. The fact that several organisms produced apoquinoprotein but not PQQ indicates that the first process does not depend on the second. The absence of a tight coupling of the processes is also apparent from the overproduction of PQQ with respect to quinoprotein synthesis, resulting in excretion of PQQ into the medium. Reversibly, situations exist in which PQQ is underproduced. In experiments with fast exponentially growing batch cultures of *A. calcoaceticus* (e.g., on ethanol), no PQQ could be detected in the culture fluid, and only 25% of the GDH in the cell extract was in the holoenzyme form. In view of the very low rates of PQQ biosynthesis in *A. calcoaceticus* grown on carbon sources on which QDH is not induced (Table 2) and the appreciable activities of GDH formed under these conditions (Table 1), apo-GDH detection is not surprising. The same observation was made for *P. putida* biovar B grown on glucose in batch culture; only 25% of the GDH in the cell extract was in the holoenzyme form.

Regulation of PQQ synthesis. Some bacteria that excrete large amounts of PQQ when grown on alcohols were used to study the influence of exogenously added PQQ on its biosynthesis. Thus, *Hyphomicrobium* sp. strain X was precultured in the presence of methanol, inducing quinoprotein and PQQ synthesis, and subcultured (1 ml/50 ml) on the same medium to which known amounts of PQQ were added. Before and after growth, PQQ concentrations in the culture fluid were measured, and the amount of newly formed PQQ was calculated. Synthesis of PQQ by *Hyphomicrobium* sp. strain X was unimpaired by the additions; the amount of PQQ biosynthesized was about 4.0 μmol $liter^{-1}$ in all cases (Table 3). The same results were obtained with *P. putida* grown on ethanol and, in one experiment, with *A. calcoaceticus* grown in fed-batch culture of quinate (results not shown). However, *P. stutzeri* LMD 26.48 behaved differently, since on growth with ethanol a significant decrease of synthesis occurred on supplementation of the media with PQQ (results not shown).

Effect of amino acids in culture media. When amino acids or amino-acid-containing carbon and energy sources (e.g., peptone, yeast extract) were added to media on which quinoproteins (and PQQ) are induced, lower levels of PQQ in the medium were observed; the extent of decrease was related to the incubation time and to the composition and pH of the medium. For example, when *Pseudomonas* spp. were grown on ethanol in the presence of 1% peptone, less than 10% of the expected amount of PQQ was detected. Most probably, the lower level results from degradation, since incubation of PQQ with the sterile medium led to its disappearance. This is caused by the reactivity of PQQ toward nucleophilic amino acids, resulting in (biologically inactive) oxazoles (M. A. G. van Kleef, J. A. Jongejan, and J. A. Duine, submitted for publication).

DISCUSSION

The concentration of PQQ in culture medium is governed by (i) the rate of PQQ biosynthesis, (ii) excretion of PQQ from the cells into the culture medium, (iii) degradation of periplasmic quinoproteins, and (iv) losses of PQQ due to

TABLE 1. Bacterial quinoprotein and PQQ production

Culture type	Organism	Carbon source	Quinoprotein(s) synthesized		Extracellular PQQ production (nmol/l)
			Type[a]	Amt (U/g of wet cells)	
Batch cultures	*A. calcoaceticus*	Succinate	GDH	10–20	<5
		Quinate	GDH	20–30	
			QDH	5–10	50–200
		p-OH benzoate	GDH	20–30	
			QDH	5–10	50–200
	A. lwoffii RAG-1[b]	Peptone	Apo-GDH	2–10	0
		Quinate	Apo-GDH	2–10	0
			Apo-QDH	0.27	0
	E. coli[c]		Apo-GDH	1–2	0
	Agrobacterium spp.[d]	Glucose	Apo-GDH	NP[e]	0
	R. leguminosarum[d]	Glucose	Apo-GDH	NP	0
	Acetobacter aceti	Alcohols	ADH[f]	195	
			AldDH[g]	250	NP
	Gluconobacter suboxydans	Glucose	GDH[h]	140	NP
		Alcohols	ADH[f]	375	
			AldDH[g]	160	NP
	P. aeruginosa	Gluconate	GDH	NP	<5
		Alcohols	ADH	40[i]	500–3,000
	P. fluorescens[j]	Glucose	GDH	70	<5
	P. testosteroni[k]	Alcohols	Apo-ADH	34	0
	P. stutzeri	Alcohols	ADH	NP	2,000
	P. putida biovar B	Alcohols	ADH	NP	2,000–3,000
		Glucose	GDH	2	<5
		Quinate	QDH	1–2	<5
	P. aureofaciens	Benzoate	GDH	NP	<5
		Quinate	QDH	3	<5
	Hyphomicrobium X	Methanol	MDH	48	3,000–6,000
	M. organophilum XX	Methanol	MDH	2.2[l]	2,000–8,000
		Succinate	MDH	2.6[l]	400
	Pseudomonas sp. strain BB1[m]	Methanol	MDH	32	2,000–4,000
	Nocardia sp. strain 239[n]	Methanol	MDH		2,000
	Arthrobacter sp. strain P1[o]	Methylamine	MeAO	3.7	0
	Thiobacillus versutus[p]	Methylamine	MADH	6	3,000–6,000
Fed-batch cultures	*A. calcoaceticus*	Succinate	GDH	20–60	50–150
		Quinate	GDH	20–80	
			QDH	5–10	50–700
	P. stutzeri	Alcohols	ADH	NP	4,000–10,000
	P. putida biovar B	Alcohols	ADH	NP	3,000–6,000
	Nocardia sp. strain 239[n]	Methanol	MDH		6,000

[a] ADH, Alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; MDH, Methanol dehydrogenase; MADH, Methylamine dehydrogenase; MeAO, Methylamine oxidase.
[b] From reference 36.
[c] From reference 24.
[d] From reference 39.
[e] NP, Not performed.
[f] From reference 1.
[g] From reference 2.
[h] From reference 5.
[i] From reference 21.
[j] From reference 5.
[k] From reference 22.
[l] From reference 9.
[m] M. Dijkstra, personal communication.
[n] From reference 23 and P. van Ophem, personal communication.
[o] From reference 34 and R. van der Meer, personal communication.
[p] J. Frank and J. van Wielink, personal communication.

reaction with nucleophilic compounds, especially amino acids.

From the preliminary work described here, it appears that PQQ synthesis is only induced if quinoprotein synthesis is initiated. However, this relationship does not hold for the reverse case; quinoprotein synthesis can proceed in the absence of PQQ synthesis. Although the events that trigger the synthesis are presently unknown, the presence of a quinoprotein substrate is not essential for induction.

The rate of PQQ biosynthesis seems to be connected with the type of quinoprotein induced. Although no linear relationship exists, it is evident (Table 2) that quinoproteins having a low specific activity but constituting 10 to 20% of the total cellular protein (so that they will be synthesized at a relatively high rate, e.g., methanol dehydrogenase in *Hyphomicrobium* sp. strain X) give rise to high PQQ production rates, whereas the rate of PQQ synthesis with quinoproteins having a high specific activity but occurring in

TABLE 2. Comparison between quinoprotein synthesized and rate of PQQ biosynthesis

Organism	Carbon source	Quinoprotein synthesized			Rate of PQQ synthesis[b]
		Type[a]	Amt (U/g of wet cells)	Sp act (U/mg of protein)	
A. calcoaceticus	Succinate	GDH	10–60	635[c]	0.5–1.0
	Quinate	GDH	20–80	635[c]	
		QDH	5–10		2.5–3.0
P. fluorescens	Glucose	GDH	70[d]	386[d]	<2.0
P. aeruginosa	Ethanol	ADH	40[e]	35[e]	50
P. stutzeri	Ethanol	ADH	NP[f]		55
Hyphomicrobium sp. strain X	Methanol	MDH	48	8[g]	70

[a] See footnote *a* of Table 1.
[b] Expressed as nanomoles of excreted PQQ per g (wet weight) of cells per h.
[c] From reference 12.
[d] From reference 5.
[e] From reference 21.
[f] NP, Not performed.
[g] From reference 16.

low amounts (e.g., GDH, usually constituting only 0.01 to 0.05% of the total cellular protein in *A. calcoaceticus*) is low. On the other hand, quinoprotein and PQQ syntheses are not well coordinated, since overproduction and underproduction of PQQ occur. For *Hyphomicrobium* sp. strain X it can be calculated from Table 2 (M_r of methanol dehydrogenase, 120,000 [16]; one methanol dehydrogenase molecule contains two PQQs [17]) that a 10-fold excess of PQQ is excreted into the culture medium. However, the presence of GDH apoenzyme in ethanol-grown batch cultures of *A. calcoaceticus* and glucose-grown batch cultures of *P. putida* shows that underproduction of PQQ occurs as well. The data in Table 1 can therefore be explained by assuming that quinoprotein and PQQ syntheses proceed at different rates and are differently influenced by cellular growth rate. In addition, PQQ and quinoprotein synthesis rates can be influenced by certain limiting growth conditions (25, 39).

It should be realized that external PQQ production might not reflect overall PQQ production. Excretion of PQQ into the culture medium occurring only in the late-exponential and stationary phase of growth has been observed with methylotrophic bacteria (4, 28; F. Gasser, personal communication) and *Nocardia* sp. strain 239 (23). Measurements of cellular PQQ concentrations in the exponential phase of growth suggest, however, that PQQ is already present but is excreted only at the end of the growth phase (F. Gasser, personal communication). On the other hand, with *Acinetobacter* and *Pseudomonas* species PQQ is excreted gradually during growth (results not shown). Finally, it should be mentioned in this context that the determination of PQQ in culture fluids is severely biased under conditions in which nucleophiles occur, because the products formed from PQQ are undetectable with the common procedures of PQQ analysis (35).

TABLE 3. Effects of exogenously added PQQ on PQQ biosynthesis in *Hyphomicrobium* sp. strain X[a]

PQQ (μM)		
Added before growth	Determined after growth	Synthesized by the organism
0.2	3.8	3.6
1.5	5.9	4.4
3.9	8.0	4.1
8.1	12.1	4.0
9.3	12.8	3.5
12.0	15.4	3.4

[a] *Hyphomicrobium* sp. strain X was precultured on 0.3% (vol/vol) methanol and subcultured on the same medium to which various concentrations of PQQ were added.

The fact that quinoprotein dehydrogenases occur in gram-negative bacteria and that several of them (if not all) are located in the periplasm could explain excretion of PQQ into the medium. If it is assumed that assemblage of the quinoproteins occurs in the periplasm, holoenzyme formation might simply proceed by recombination with PQQ transported from the cytoplasm to the periplasm, from which it easily escapes to the medium (this process would be more complicated for methylamine dehydrogenase, since PQQ is covalently bound to the protein chain in this case [33]). This is in accordance with the observation that the gram-positive bacterium *Arthrobacter* sp. strain P1 (lacking a periplasm, the quinoprotein methylamine oxidase being bound to the membrane at the cytoplasmic site [27] and with PQQ covalently bound [34]) did not excrete PQQ (Table 1). On the other hand, degradation of periplasmic proteins could also contribute to excretion of PQQ into the medium.

In cases in which quinoprotein apoenzymes are produced, it has been found that exogenously added PQQ is able to effectively reconstitute quinoprotein apoenzymes in vivo: with GDH in *A. lwoffii* (37), *A. calcoaceticus* PQQ^- mutants (35), and *E. coli* (24); with QDH in *A. lwoffii*, *A. calcoaceticus*, and *P. aureofaciens* PQQ^- mutants (36); with alcohol dehydrogenase in *P. testosteroni* (22); and with methanol dehydrogenase in a PQQ^- mutant from *M. organophilum* XX (9). The absence of feedback inhibition on PQQ synthesis for several organisms (*P. putida* biovar B, *Hyphomicrobium* sp. strain X, and probably *A. calcoaceticus* but not *P. stutzeri*), as revealed by the experiments in which PQQ was added to the medium, suggests that there is no uptake mechanism for PQQ in these cases. Other cofactors behave differently. For instance, it has been shown (11) that after exogenous addition of either pyridoxal or pyridoxol (600 nM) to an *E. coli* B culture, de novo pyridoxine synthesis stops within 20 s. Also synthesis of the enzymes of NAD (19) and FAD (10) biosynthesis are subject to repression mechanisms triggered by exogenous cofactors. Further work, especially with bacteria containing cytoplasmic quinoproteins, is necessary to prove that PQQ has a real unique position among cofactors with respect to this aspect.

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