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Factors Affecting the Production of Pyrroloquinoline Quinone by the Methylotrophic Bacterium W3A1

WILLIAM S. McINTIRE* AND WALTER WEYLER

Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121

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Two variants of the methylotrophic bacterium W3A1, designated W3A1-S (slimy) and W3A1-NS (nonslimy), were compared with respect to their ability to grow in batch culture on the C_1 substrates methylamine, methanol, and trimethylamine. Substrate utilization, cell density, pH, cellular and soluble polysaccharide production, and concentrations of the enzymes methylamine dehydrogenase, trimethylamine dehydrogenase, and methanol dehydrogenase produced were measured as a function of growth. The ability of the two bacterial variants to excrete the redox cofactor pyrroloquinoline quinone into the growth medium was also investigated. The two variants were similar with respect to all properties measured, except that W3A1-S produced significantly more capsular polysaccharides than variant W3A1-NS. Pyrroloquinoline quinone was excreted when either variant was grown on any of the C_1 substrates investigated but was maximally produced when the methylamine concentration was 0.45% (wt/vol). This cofactor is excreted only as bacterial growth enters the stationary phase, a time when the levels of trimethylamine dehydrogenase and the quinoproteins methanol dehydrogenase and methylamine dehydrogenase begin to decline. It is not known whether the pyrroloquinoline quinone found in the medium is made de novo for excretion, derived from the quinoprotein pool, or both. Pyrroloquinoline quinone excretion has been observed with other methylotrophs, but this is the first instance where the excretion was observed with substrates other than methanol.

In recent years methylotrophic bacteria grown on methanol have been used for the production of several important natural products including single cell proteins, vitamins and coenzymes, amino acids, carboxylic acids, and polysaccharides (5, 11, 12, 20, 22, 25). Recently it was found that a number of methylotrophic bacteria excrete substantial quantities of the novel enzyme cofactor pyrroloquinoline quinone (PQQ), also known as methoxatin (1; M. Ameyama and O. Adachi, Chem. Abstr. **104**:107920t, 1986). PQQ is of importance for these organisms because it is the cofactor for methanol dehydrogenase (MDH) and methylamine dehydrogenase (MADH). These are among the enzymes involved in the initial assimilation of C_1 substrates. Other enzymes from nonmethylotrophic bacteria contain PQQ (9), and this quinone seems to be the cofactor for several eucaryotic copper-containing amine oxidases and the mitochondrial choline dehydrogenase (1, 4, 10, 16, 23, 24). Moreover, this cofactor in its pure form stimulates the growth of nonmethylotrophic bacteria (3), as well as plant and animal cells in culture (Ameyama and Adachi, Chem. Abstr. **104**: 205512f, 1986).

Since PQQ is the cofactor for a number of enzymes from bacterium W3A1 we were interested in determining whether this organism would excrete PQQ into the medium and whether supplementation of the growth medium with PQQ would stimulate growth. These studies led to the discovery of the first instance where substrates other than methanol (MeOH) induce the excretion of free PQQ from a microorganism. We have shown elsewhere that the excreted PQQ is identical to synthetic 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid (2,7,9-tricarboxy-PQQ) by mass spectral analysis (17).

During this work we were able to isolate a phenotypic variant of W3A1 that does not produce a polysaccharide capsule (slime). This phenotype was characterized with respect to growth properties in batch culture under a variety of growth conditions and compared with the usual slimy variety.

(Bacterium W3A1 has recently been classified in the taxon *Methylophilus*; however, this is not a recognized genus name [13]. For the time being we will continue to call this organism W3A1 until it is classified in an accepted genus.)

MATERIALS AND METHODS

Materials. Bacterium W3A1 was obtained from the National Collection of Industrial and Marine Bacteria Ltd., Scotland, as NCIB 11348. Methylamine (MA) hydrochloride, MA-free base, and trimethylamine (TMA) hydrochloride were purchased from Sigma Chemical Co. Dehydrated nutrient broth was purchased from Difco Laboratories. 2,7,9-Tricarboxy-PQQ was purchased from Fluka Chemical Corp. The high-pressure liquid chromatography columns used were Hypersil C_{18} reverse phase (3-μm particle size, 50 by 4.6 mm) obtained from Phenomenex, and Cyclobond I (type B [β-cyclodextrin], 250 by 4.6 mm) obtained from Advanced Separation Technologies, Inc. DEAE-cellulose, DE-52, was from Whatman Inc. The C_{18} reverse-phase silica gel (40 μm) for flash chromatography was from J. T. Baker Chemical Co.

Growth of bacteria. Bacterium W3A1 was grown aerobically in either 50 or 250 ml of sterile medium in 250-ml and 1-liter Erlenmeyer flasks, respectively, on a mechanical shaker at 30°C. Growth was also carried out in 12-liter FS-614 fermentation jars (New Brunswick Scientific Co., Inc.) at 30°C. The bacteria were grown at pH 6.7 in mineral base E of Owens and Keddie (18), supplemented with $(NH_4)_2SO_4$ where indicated below. Solutions of MA-hydrochloride and TMA-hydrochloride (30%, wt/vol), were filter sterilized (0.2-μm filters) before addition to the cooled autoclaved growth media. Absolute MeOH was added without sterilization. The final concentration of the substrates were varied from 0.3 to 1.2% (wt/vol). To investigate the

* Corresponding author.

effect of pH on the growth, the pH was controlled with K_2HPO_4 or MA-free base.

Isolation of the variant forms of bacterium W3A1. Bacteria that were obtained from the Culture Collection grew slowly and unpredictably on MA. After selected colonies were transferred from agar plates containing 0.3% MA to plates containing various growth substrates, some colonies grew well only on MA, TMA, dimethylamine, and MeOH, whereas others grew on these compounds, on citrate, glutamate, glucose, nutrient broth, tetramethylammonium chloride, and many other compounds. According to Colby and Zatman (6), bacterium W3A1 is a restricted facultative methylotroph since it grows rapidly on C_1 compounds and only poorly on glucose. The W3A1 culture was purified by alternately streaking cells on MA plates and transferring growing colonies to liquid growth medium containing 0.3% MA. This process produced a culture which was slimy and had growth requirements conforming to those defined for W3A1 (6). For preliminary studies investigating PQQ production we used this culture of bacterium W3A1.

After bacteria were subcultured 15 to 20 times in liquid medium (0.3% MA), close inspection indicated that some colonies of bacteria subsequently grown on MA agar plates were raised and opaque, whereas others were flat and transparent. Several of each were isolated and transferred to liquid medium containing 0.3% MA. Bacteria derived from the opaque colonies grew in clumps and on centrifugation produced a large, flocculent, pale yellow pellet, as previously observed, suggesting the presence of a polysaccharide capsule. This organism was designated W3A1-S (slimy). Bacteria derived from the transparent colonies grew in a homogeneous and well-dispersed manner and centrifuged as a tight pink pellet. This bacterium was designated W3A1-NS (nonslimy). Both of these variants possessed the characteristic nutrient requirements of W3A1 (6). Each variant was then obtained from liquid culture growth medium (0.3% MA) in the mid-log phase and stored as a viable culture in 15% (vol/vol) glycerol at −70°C.

Cell density and pH of the medium. The cell density was measured at 600 nm, with a spectral bandwidth of 2 nm, in a 3-ml cuvette with a Uvicon 810 spectrophotometer (Kontron Instruments). The pH of the medium was measured with a combination electrode after the bacteria were removed by centrifugation. Total proton release correlated better with cell growth than with pH because at the higher pHs the buffering due to 11.5 mM phosphate distorted the pH data.

Polysaccharide determinations. Polysaccharides were determined by a reaction with anthrone as described in the literature (21). A standard curve was constructed by using 0, 10, 20, and 40 μg of glucose. The level of polysaccharide was measured on 250 μl of the growth medium before and after centrifugation. This allowed the determination of both the soluble and bacterium-associated carbohydrate.

Analysis of the MA concentration. MA was oxidized by purified MADH (14) coupled to the redox dyes phenazine ethosulfate and 2,6-dichlorophenolindophenol. The assay mixture contained 420 μM phenazine ethosulfate, 89.5 μM 2,6-dichlorophenolindophenol, and 7 to 18 μg of enzyme in 3.0 ml of 90 mM potassium phosphate (pH 7). Diluted samples of centrifuged growth medium containing MA were added to start the reaction. The change in A_{600} due to the reduction of 2,6-dichlorophenolindophenol (ε = 21.7 mM^{-1} cm^{-1}) was used to calculate the concentration of MA.

Analysis of PQQ concentration in growth medium. Samples (20 μl) of centrifuged culture medium or standard PQQ solutions were injected onto the reverse-phase column (see above) and eluted at a flow rate of 0.5 ml/min with a 70:30 mixture of 56 mM phosphoric acid (pH 2)–MeOH. The high-pressure liquid chromatography analyses were performed with a dual pump system from Beckman Instruments, Inc., and a Shimazu C-RIA integrator. Eluted PQQ was detected with a Spectra/Glow fluorometer (Gilson Medical Electronics, Inc.) with excitation and emissions filters of 360 and 455 nm, respectively. PQQ concentrations were determined by relating the peak areas of unknowns to the area produced by a 430 nM aqueous solution of authentic PQQ.

PQQ isolated from the culture medium (see below) was characterized by comparison of retention times to authentic PQQ on the reverse-phase column and on the Cyclobond I column. The latter column was eluted with a 40:60 mixture of 10 mM potassium phosphate (pH 6.7)–MeOH at a flow rate of 0.8 ml/min. PQQ was detected by fluorescence as described above or by absorbance measurement at 436, 254, and 214 nm with an Altex 160 absorbance detector (Beckman). PQQ was further characterized by mass spectral analysis as reported elsewhere (17). PQQ was stable in the culture medium but degraded slowly when the phosphate concentration exceeded 25 mM at pH 7 and 37°C.

Purification of PQQ from the growth medium. To 36 liters of centrifuged cell culture supernatant was added 200 ml of settled DE-52 ion exchanger initially suspended in water. The mixture was stirred for about 30 min, and the DEAE-cellulose was collected by filtration and washed with 10 volumes of cold water. Two such batches were combined and transferred to a 30-by-6.5-cm column and eluted with a 2-liter gradient of 0 to 1 M NaCl in 20 mM potassium phosphate (pH 7.0) followed by an additional liter of 1 M NaCl in buffer. The fractions containing PQQ were pooled and adjusted to pH 2 by the addition of 50 mM H_3PO_4. For flash chromatography, the acidified solution was passed through a 20-by-0.9-cm column packed with C_{18} reverse-phase silica gel which had previously been equilibrated with 10 mM HCl. The column was washed with 200 ml of 10 mM HCl and eluted with 8:2 H_2O-MeOH to afford PQQ in greater than 95% purity as judged by high-pressure liquid chromatography analysis.

Quantitation of the dehydrogenases. At various times during growth, 1-ml samples of culture medium were centrifuged. The pellets were solubilized with gel sample buffer; after the addition of carbonic anhydrase as an internal standard, a sample containing 1 μg of this enzyme was analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis method of Laemmli (15) modified by the addition of urea to the buffer (8). The gels were 10% acrylamide and 1.5 mm thick. External standards (1 μg each) of MDH, MADH, and carbonic anhydrase were run in separate lanes. Gels were stained with Coomassie brilliant blue R-250, and staining density was determined with a Bio-Rad model 1650 transmittance-reflectance scanning densitometer in the transmittance mode. Areas of the peaks of interest were integrated and normalized to the average integrated value of the internal standard carbonic anhydrase (16 to 18 lanes per gel for the internal standard). The concentration of MADH, MDH, and TMA dehydrogenase (TMADH) were estimated by comparison to the external or internal standards.

RESULTS

Comparison of growth properties of variants W3A1-S and W3A1-NS. As described in Materials and Methods, two

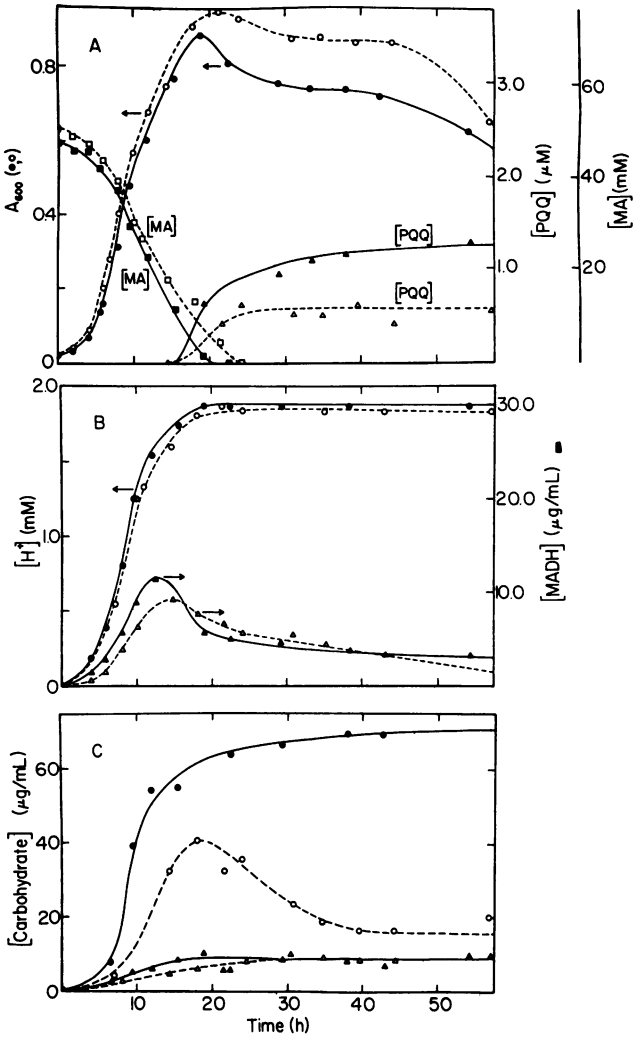

FIG. 1. Comparison of growth parameters for bacterium W3A1-S (———) and bacterium W3A1-NS (– – – –) raised on methylamine. Bacteria were cultured and sampled as described in Materials and Methods. (A) Symbols: (●, ○) cell density; (■, □) substrate concentration; (▲, △) PQQ concentration. (B) Symbols: (●, ○) hydrogen ion concentration; (▲, △) enzyme concentration. (C) Symbols: (●, ○) total carbohydrate concentration; (▲, △) soluble carbohydrate concentration (supernatant). The arrows point to the axes defining the curves.

variants of bacterium W3A1 were isolated from the initial culture. We designated these bacteria W3A1-S and W3A1-NS for the variants with and without the polysaccharide capsule, respectively. Detailed comparison of these two organisms under a variety of growth conditions showed relatively large differences for carbohydrate production and smaller differences in other properties. The results with the substrates MA, TMA, and MeOH are presented in Fig. 1 through 4.

Estimation of concentrations for MADH, TMADH, and MDH, were made by optical densitometry of Coomassie brilliant blue R-250 stained sodium dodecyl sulfate-polyacrylamide gels similar to the one shown in Fig. 4. Measurement of Coomassie stain was chosen over activity measurements because there are components in crude homogenates which affect the reliability of the latter, and bacterium W3A1 (particularly when grown on TMA) is difficult to rupture on a small scale, resulting in poor release or denaturation of the proteins, thus causing concern that the activity measurement would be unreliable. It has been observed in this laboratory (unpublished results) and reported by others (19) that MDH is only partially active or inactive, although it contains the requisite amount of PQQ for full activity. For this reason, we concluded that activity assays would also contribute to a misleading account of holo-MDH.

There is a remarkable similarity for the protein band patterns on Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels among the variants grown on all substrates (Fig. 4). However, bands corresponding to the M_r of MADH and MDH were absent, and only a minor band corresponding to TMADH was seen when cells were grown on MeOH, on MA or TMA, and on MA or MeOH, respectively. Additionally, the amounts of MADH and TMADH found during large-scale purifications correlated well with the amounts predicted from the gel experiments. These

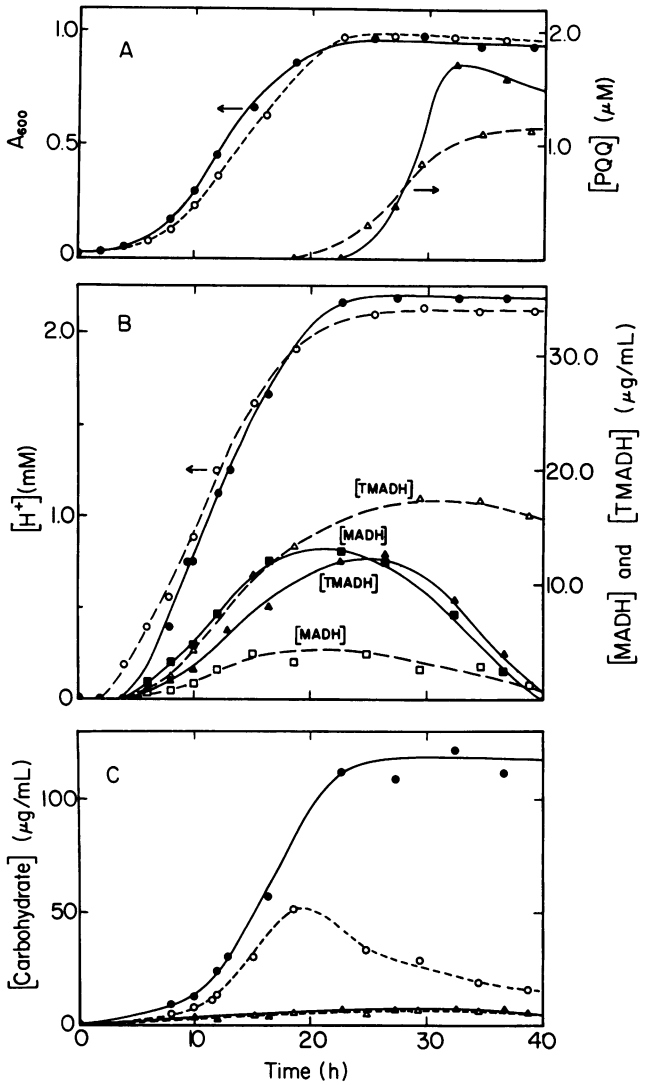

FIG. 2. Comparison of growth parameters for bacteria W3A1-S and W3A1-NS raised on trimethylamine. Bacteria were cultured and sampled as described in Materials and Methods. Symbols are as in Fig. 1, but two enzymes are shown in panel B: (▲, △) TMADH; (■, □) MADH. The arrows point to the axes defining the curves.

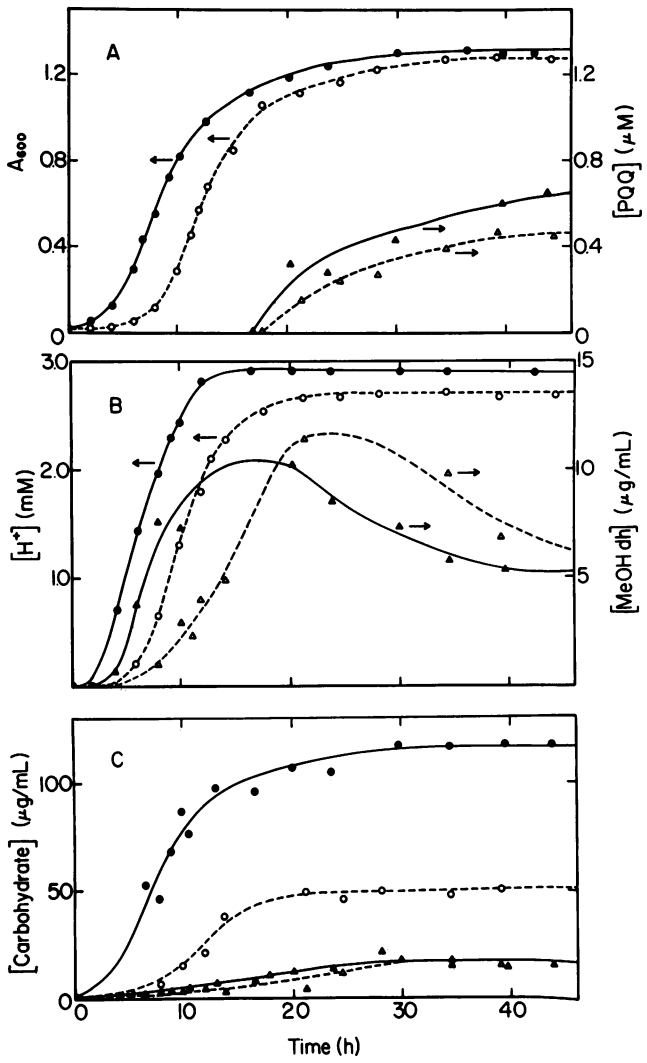

FIG. 3. Comparison of growth parameters for bacteria W3A1-S and W3A1-NS raised on methanol. Bacteria were cultured and sampled as described in Materials and Methods. Symbols are as in Fig. 1. The arrows point to the axes defining the curves. The longer lag period seen for W3A1-NS results from the inoculum having fewer bacteria than that for W3A1-S.

observations gave us further confidence that Coomassie blue staining of the gels was the method of choice to measure intact MADH, TMADH, and MDH in these experiments. The results from the densitometric measurements are summarized in Fig. 1B, 2B, and 3B.

PQQ production in W3A1. Ameyama and co-workers (1, 2; Ameyama and Adachi, Chem. Abstr. **104**:107920t, 1986) reported levels of excreted PQQ (10 to 300 μM) in culture medium of a number of methylotrophs when these were grown on MeOH. Since bacterium W3A1 produces a number of quinoproteins, it was of interest to learn whether this bacterium would be a source for PQQ when cells were grown on MeOH or other C_1 compounds such as MA or TMA and whether growth medium composition would affect PQQ production. Initial studies were carried out with a culture of the bacteria of the phenotype of W3A1-S but in fact may have contained a very small amount of W3A1-NS. As mentioned above, the properties of these two variants are quite similar, suggesting that the results from these initial studies can be extrapolated to the purified cultures.

With MeOH, MA, or TMA as a substrate, PQQ was excreted into the growth medium of W3A1 cultures (Table 1). The amount of PQQ excreted was moderately affected by the concentration of the substrate used but was not significantly affected by a threefold change (3.8 to 11.4 mM) in NH_4^+ concentration, except when the cells were grown on 1.0% (wt/vol) MeOH. Growth in the absence of NH_4^+ and in the presence of 0.3% MA lowered PQQ production by half when compared with the experiment with added NH_4^+. Of the three substrates studied at a level of 0.3%, TMA was the best inducer of PQQ and MA was the worst. However, in a more detailed experiment (data not shown) with both W3A1-NS and W3A1-S, optimum growth of cells and production of PQQ and MADH occurred at 0.45% (wt/vol) MA. The extracellular concentration of PQQ achieved was 3.83 μM, which is about 2 to 3 times higher than yields observed with MeOH.

When the initial concentration of MA was 0.3% (wt/vol), growth paralleled the level of MA (Fig. 5A). However, when MA concentration was 0.6%, growth leveled off well before the substrate was consumed (Fig. 5A). We noted in all experiments that the pH of the culture medium decreased approximately linearly as a function of the increase in cell density (A_{600}) and that this drop was independent of the substrate used. It seems likely that at lower substrate concentrations both the drop in pH and substrate depletion contribute to the cessation of bacterial growth.

PQQ production depended on the growth phase of the cells (Fig. 1 through 3). Excretion commenced only after the A_{600} approached its maximum and rapidly reached its own maximal value after the cells were in the stationary phase.

In attempts to find factors that affect PQQ production, we investigated variations in substrate concentration, buffer strength, and pH of the medium. For example, W3A1 cultures initially at 0.3% MA were replenished with an additional 0.23% MA-hydrochloride after 24 h. This addition caused a slight increase of the maximum cell density (A_{600}) and increased PQQ excretion 2.5-fold. An analogous experiment with MeOH did not change either of these parameters. Further, delayed addition of MeOH to cultures growing on 0.3% MA had no effect on PQQ production. Growth on 0.6% MA gave about a 2.4-fold increase of PQQ compared with growth on 0.3% MA (Fig. 5A).

Changing the phosphate concentration from 11.5 to 46 mM at pH 7 increased the time to reach the stationary phase from 48 to 96 h but had no apparent effect on maximal PQQ production. A similar trend was noted when the starting pH was 7.5 (stationary phase was reached at 145 h, and there was no effect on PQQ concentration).

A few experiments were carried out in 12-liter fermentors with the variant W3A1-NS at different starting pH values. Bacteria were grown in standard medium with 0.3% MA or medium which was raised to pH 7.2 or 7.5 with a trace of MA-free base. In contrast to experiments at pH 6.7, at pH 7.2 cell density and MADH concentration increased by a factor of 1.5 but PQQ levels were not significantly affected. At pH 7.5 cell growth was slow, and the experiment was not carried out to conclusion. A sixfold increase of PQQ concentration and a doubling of cell density were observed with variant W3A1-NS when the medium contained 0.3% MA and pH was 7.2 at the start of the growth experiment and when the MA concentration and the pH were boosted back to the original values after the A_{600} reached 1.1 with a combination of MA-hydrochloride and MA-free base.

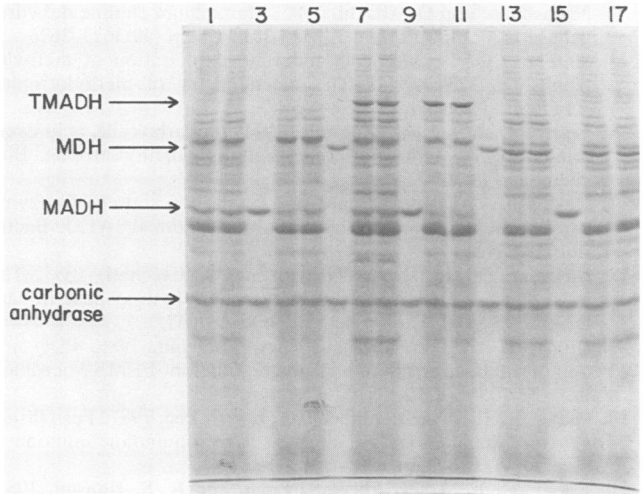

FIG. 4. Sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis of bacteria W3A1-S and W3A1-NS grown on different C_1 compounds. Lanes 1 and 2, 7 and 8, and 13 and 14 represent W3A1-S grown on MA, TMA, and MeOH, respectively. Lanes 4 and 5, 10 and 11, and 16 and 17 represent W3A1-NS grown on MA, TMA, MeOH, respectively. One microgram of MADH was applied to lanes 3, 9, and 15 as a standard. One microgram of MDH was applied to lanes 6 and 12 as a standard. Samples applied to all lanes also contained 1 μg of carbonic anhydrase as an internal standard used to normalize the data. In all cases 0.3% (wt/vol) of the C_1 compounds were present at the start of growth, and the cells were removed for analysis in the late log phase.

Finally, it has been reported that the addition of PQQ to the growth medium of a number of bacteria stimulates their growth (3). However, the addition of 1 μM PQQ to the medium of bacterium W3A1 at zero time had no effect on the rate of growth or production of PQQ with the carbon source MA, TMA, or MeOH.

DISCUSSION

Preliminary studies on the production of PQQ by the bacterium W3A1 led to the observation that two phenotypes of this bacterium could be isolated which were distinguished by the production of a polysaccharide capsule for one but not the other. We designated these variants W3A1-S and W3A1-NS, respectively. Growth characteristics of the two strains under a variety of conditions indicate that these variants were otherwise similar. An observation of practical application was that bacterium W3A1-NS appeared to be more readily broken by sonication or with a French pressure cell than bacterium W3A1-S. This is probably due to the lowered viscosity of the variant without the polysaccharide capsule. Repeated passage of a pure colony of W3A1-S in liquid culture again produced W3A1-NS. The converse was not found. These observations suggest that W3A1-NS is derived from W3A1-S. A report on slime production by bacterium W3A1 has been published (7). Some of our results are not in agreement with the previously reported observations, but this may be due to the mixed population of bacteria (see Materials and Methods) used in the earlier work.

Since W3A1 contains a number of enzymes that use PQQ for their cofactor and since a number of methylotrophic organisms have been reported to produce PQQ when grown on MeOH, we were interested to see whether W3A1 also excretes this cofactor into the growth medium. Our results indicate that PQQ is excreted when the cells are grown on MeOH, MA, and TMA. Although the amounts of PQQ observed in our experiments were modest when compared with those for other organisms, it is clear that substrates

TABLE 1. Growth of bacterium W3A1 at various concentrations of C_1 substrates[a]

Substrate, concn (%, wt/vol)	PQQ concn[b] (μM)	A_{600}[c] (time, h)	pH[d]
MA			
0.3	1.67	0.758 (24)	5.7
0.6	3.83	0.884 (48)	5.3
0.9	2.29	0.680 (70)	5.7
MeOH			
0.3	1.38	1.291 (48)	4.5
1.0[e]	1.10	1.259 (48)	ND[f]
1.0[g]	1.96	0.632 (48)	ND
TMA			
0.3	3.51	0.989 (70)	5.1
0.4	2.69	1.08 (70)	4.9
0.5	1.48	ND	ND
0.7	1.67	ND	ND
0.9	1.63	ND	ND

[a] For cells grown on MA, the concentration of NH_4^+ was varied from 3.8 to 11.4 mM with little effect on growth or the level of PQQ produced. When TMA was used the NH_4^+ concentration was 3.8 mM.
[b] Levels after 48 to 70 h for MA and MeOH and 92 h for TMA.
[c] Maximum levels found at the time listed in parentheses.
[d] pH at the end of the growth period.
[e] NH_4^+ concentration was 3.8 mM.
[f] ND, Not determined.
[g] NH_4^+ concentration was 11.4 mM.

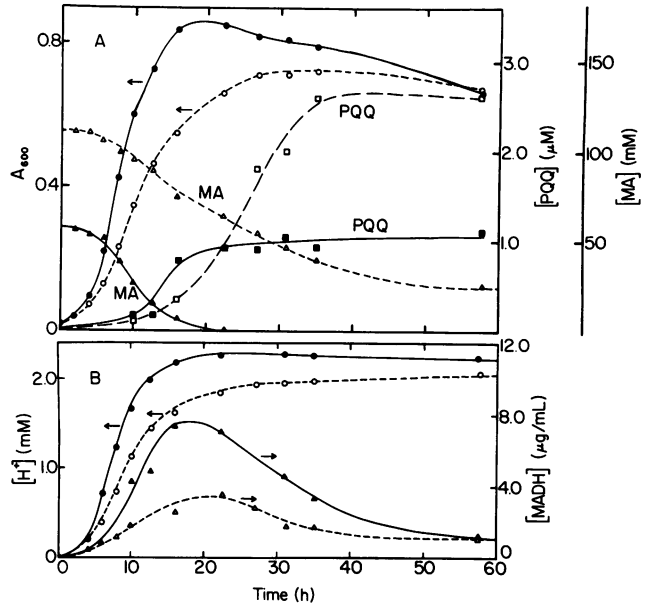

FIG. 5. Comparison of growth parameters for bacterium W3A1-S at two different substrate concentrations. Bacteria were cultured and sampled as described in Materials and Methods. Lines: (———) 0.3% methylamine; (– – – –) 0.6% methylamine (initial concentrations). (A) Symbols: (●, ○) cell density; (▲, △) substrate concentration; (■, □) PQQ concentration. (B) Symbols: (●, ○) hydrogen ion concentration; (▲, △) for enzyme concentration. The arrows point to the axes defining the curves.

other than MeOH are effective in eliciting the excretion of this cofactor. A number of factors appear to be involved in the excretion of PQQ. All parameters tested, e.g., initial pH, initial substrate concentration, type of substrate, and ammonia ions had some effect on the quantity of PQQ produced. It is important to note that optimal concentration of the various enzymes monitored did not coincide with optimal PQQ production. PQQ is made only after cells have reached the maximum density (Fig. 1 through 3). An important outcome of these studies is that very simple modifications of the growth medium significantly increase the yield of bacteria, the enzymes of interest, and excreted PQQ.

PQQ is excreted from W3A1 in amounts exceeding the endogenous known quinoprotein pool by a factor up to 10 times or more. Although 2,7,9-tricarboxy-PQQ is primarily excreted in the stationary phase at a time when enzyme levels are decreasing, the amount of PQQ excreted cannot easily be accounted for by the release of PQQ from the known quinoprotein pool. Only MDH uses the form of PQQ that is excreted into the medium. It is interesting that 2,7,9-tricarboxy-PQQ is excreted by cells grown on MA and TMA. The only known quinoprotein synthesized by bacterium W3A1 under these conditions is MADH. The PQQ bound to MADH is covalently attached via two distant amino acid residues of the polypeptide chain corresponding to the small subunit of this enzyme. The modified cofactor does not contain any of the carboxyl groups (either as the carboxylates or as amides or esters) characteristic of the excreted form of PQQ (17). It is extremely unlikely that MADH could be a source of 2,7,9-tricarboxy-PQQ, although the latter is the likely precursor for the MADH cofactor.

Several hypotheses can be offered to account for the excretion of 2,7,9-tricarboxy-PQQ: (i) the PQQ found in the medium is synthesized de novo for the express purpose of excretion; (ii) there are quinoproteins in addition to MDH which are degraded; and (iii) these quinoproteins undergo rapid turnover which results in the release of 2,7,9-tricarboxy-PQQ in the stationary phase. A corollary to points ii and iii is that PQQ released from the proteins is not reassimilated and finds its way into the bulk medium.

The observation that PQQ stimulates the growth of a large number of cultured cells suggests that excretion of PQQ may be a symbiotic adaptation which finds its payoff in the thriving of organisms that produce nutrients for bacterium W3A1 and other methylotrophs. If this is so then the PQQ released to the medium could be better described as a secretion.

ACKNOWLEDGMENTS

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