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Dijken, J.P. van; Veenhuis, M.; Harder, W.

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Arthrobacter P 1, a Fast Growing Versatile Methylotroph with Amine Oxidase as a Key Enzyme in the Metabolism of Methylated Amines

P. R. Levering, J. P. van Dijken[1], M. Veenhuis, and W. Harder

Department of Microbiology and Laboratory for Electron Microscopy, Biological Centre, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Abstract. A facultative methylotrophic bacterium was isolated from enrichment cultures containing methylamine as the sole carbon source. It was tentatively identified as an *Arthrobacter* species. Extracts of cells grown on methylamine or ethylamine contained high levels of amine oxidase (E.C. 1.4.3) activity. Glucose- or choline-grown cells lacked this enzyme. Oxidation of primary amines by the enzyme resulted in the formation of H_2O_2; as a consequence high levels of catalase were present in methylamine- and ethylamine-grown cells. The significance of catalase in vivo was demonstrated by addition of 20 mM aminotriazole (a catalase inhibitor) to exponentially growing cells. This completely blocked growth on methylamine whereas growth on glucose was hardly affected. Cytochemical studies showed that methylamine-dependent H_2O_2 production mainly occurred on invaginations of the cytoplasmic membrane. Assimilation of formaldehyde which is generated during methylamine oxidation was by the FBP variant of the RuMP cycle of formaldehyde fixation. The absence of NAD-dependent formaldehyde and formate dehydrogenases indicated the operation of a non-linear oxidation sequence for formaldehyde via hexulose phosphate synthase. Enzyme profiles of the organism grown on various substrates suggested that the synthesis of amine oxidase, catalase and the enzymes of the RuMP cycle is not under coordinate control.

Key words: *Arthrobacter* – Facultative methylotroph – Amine oxidase – Catalase – RuMP cycle of formaldehyde fixation – Ultrastructure

Aerobic growth of microorganisms on one carbon compounds generally involves the operation of a cyclic assimilation pathway. Sofar, 4 different routes have been encountered namely the Calvin cycle, the serine pathway, the ribulose monophosphate (RuMP) cycle of formaldehyde fixation (in bacteria) and the dihydroxyacetone pathway (in yeasts) (Quayle 1980). Although studies on the regulation of the Calvin cycle are now well under way, comparatively little is known about the regulation of the other 3 pathways of C_1-assimilation. This is especially true for the RuMP cycle.

Recently we initiated a study on the regulation of the synthesis of assimilatory and dissimilatory pathways of C_1 metabolism in facultative methylotrophs. A screening of available organisms showed that the facultative methylotrophs with the RuMP cycle described in the literature were unsatisfactory mainly because of poor growth rates in mineral media with C_1 compounds and/or "heterotrophic" substrates. It was therefore decided to isolate such organisms by enrichment using C_1 compounds as the carbon source and select for fast growing versatile methylotrophs. The present paper describes a physiological and biochemical analysis of such an organism which, on the basis of the results presented, seems to be a peculiar yet ideal model organism for a study of the regulation of C_1 metabolism.

Materials and Methods

Medium. For enrichment and isolation of methylotrophic organisms a mineral salts medium was used, which contained per litre of deionized water: $NaH_2PO_4 \cdot H_2O$, 0.5 g; K_2HPO_4, 1.55 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7\,H_2O$, 0.2 g; trace element solution according to Vishniac and Santer (1957), 0.2 ml. After heat sterilization and cooling 1 ml vitamin solution (filter-sterilized) was added together with either methylammonium chloride (0.3% w/v, heat-sterilized) or trimethylammonium chloride (0.3% w/v, filter sterilized) as the sole carbon and energy source. The pH of this medium was 7.0. The vitamin solution contained (per litre): thiamin hydrochloride, 100 mg; riboflavin, 100 mg; pyridoxal hydrochloride, 100 mg; pantothenate, 100 mg; nicotinic acid, 100 mg; p-aminobenzoic acid, 20 mg; folic acid, 1 mg; biotin, 1 mg. For growth experiments with *Arthrobacter* P 1 the same medium was used, except that the vitamin mixture was omitted, since this organism did not show any vitamin requirement. Carbon sources were added to a final concentration of 0.2% (w/v).

Enrichment and Isolation. Samples of garden soil from 20 different sources from a subtropical greenhouse (University of Groningen, Haren, The Netherlands) were taken as an inoculum for batch culture enrichments in both methylamine and trimethylamine medium. 100 ml Erlenmeyer flasks containing 30 ml medium were inoculated with 1 g of soil and incubated at 30° C for 5–7 days on a rotary shaker. The cultures were then transferred to flasks containing the same medium and incubated for an additional 2–5 days after which the cultures were plated on methylamine medium solidified with 1.5% (w/v) Bacto-agar. Pure cultures were obtained using conventional techniques.

Maintenance. Arthrobacter P 1 was maintained on slopes containing the above mineral salts medium containing 0.3% (w/v) methylammonium chloride and 1.5% (w/v) Bacto-agar. Stock cultures were grown at 30° C, stored at 4° C and transferred monthly. The same procedure was adopted for the other isolates, except that the medium was supplemented with vitamins.

[1] Present address: Department of Microbiology, Delft University of Technology, Julianalaan 67A, 2628 BC Delft, The Netherlands
Offprint requests to: W. Harder

Abbreviations: DAB = 3,3′-diaminobenzidine, FBP = fructose-1,6-bisphosphate, GSH = reduced glutathione, KDPG = 2-keto-3-deoxy-6-phosphogluconate, PMS = phenazine methosulphate, RuBP = ribulose-1,5-bisphosphate, RuMP = ribulose monophosphate, TCA = tricarboxylic acid

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Screening for Facultative Methylotrophs with the RuMP Cycle. All isolates were tested for growth on plates containing nutrient broth medium. This medium contained (per litre deionized water): nutrient broth, 4 g; glucose, 0.5 g; succinate, 0.5 g; malate, 0.1 g; acetate, 0.1 g; yeast extract, 4 g; trace element solution (Vishniac and Santer 1957), 0.2 ml; Bacto-agar, 15 g. The pH was adjusted to 7.2 with NaOH prior to sterilization. Those isolates which formed visible colonies within 3 days of incubation at 30° C were assumed to be facultative methylotrophs and were subjected to further physiological studies.

The growth rates of these isolates in media with glucose or methylamine were determined subsequently as follows: organisms were pregrown in glucose or methylamine medium supplemented with vitamins on a rotary shaker at 30° C and then transferred to the same medium. Growth was monitored by measuring the absorbance of the cultures at 433 nm in a Vitatron 280 colorimeter (Vitatron, Dieren, The Netherlands). When the doubling times of the isolate under study on both glucose and methylamine were less than 4 h, hydroxypyruvate reductase activity was assayed in extracts of methylamine-grown cells. Those isolates exhibiting hydroxypyruvate reductase activities of less than 0.1 μmol/min · mg protein were assayed for hexulose phosphate synthase activity using the radioactive assay described below.

Continuous Cultivation of Arthrobacter P 1. The organism was grown in carbon-limited chemostat cultures in a fermenter (working volume of 1 l) of the type described by Harder et al. (1974). The temperature was controlled at 30° C and the pH was controlled at 7.0 by automatic adjustment with 1 N NaOH. The medium contained (per l): KH_2PO_4, 1.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; trace element solution (Vishniac and Santer 1957), 0.2 ml and carbon source, 0.3 % (w/v).

Batch Cultivation of Arthrobacter P 1. Growth rates of the organism in media supplemented with various carbon-substrates were determined in Erlenmeyer flasks on a darkened rotary shaker as described above. For enzyme studies the organism was grown in a stirred 2-l fermenter (Harder et al. 1974), filled with 1 l mineral salts medium containing either methylammonium chloride, ethylammonium chloride, choline chloride or glucose to a final concentration of 0.2 % (w/v). The temperature was controlled at 30° C. Cells were harvested at the end of the exponential growth phase by centrifugation at 6000 *g* for 10 min at 4° C, washed once with 50 mM potassium phosphate buffer pH 7.0 + 5 mM $MgSO_4$ and resuspended in the same buffer to a concentration of 5 – 10 mg dry weight/ml. The suspensions were either used directly for enzyme assays or stored at −20° C. When used within 2 months, extracts of frozen cells exhibited enzyme activities within the range of those obtained with extracts of freshly harvested cells (i.e. ± 10 %).

Preparation of Cell-Free Extracts. Suspensions were sonified at 20 kHz for 2.5 min at 0° C with a MSE 100 W ultrasonic desintegrator (MSE Ltd., Crawley, Sussex, U.K.). This procedure resulted in an almost quantitative desintegration of the cells. Unbroken cells and debris were removed by centrifugation at 40,000 *g* for 15 min at 4° C. The supernatant which contained 3 – 7 mg protein/ml was used as cell-free extract for enzyme assays, unless stated otherwise.

Unmasking of Enzyme Activity by Benzene Treatment of Whole Cells. When enzyme activities were unexpectedly low or zero, the activity of the enzyme in cell-free extracts was compared with that in whole cells permeabilized with benzene. Permeabilized suspensions were prepared as follows: to 1 ml suspension (approx. 10 mg dry weight/ml) 1 ml benzene was added and the mixture was shaken on a Vortex mixer for 3 periods of 1 min with 5 min intervals. After separation of the two phases for 30 min the water phase was used for enzyme assays. Minimal enzyme activities were calculated on the basis of an assumed 100 % permeabilization, using a dry weight to protein content ratio of the cells of 2.

Enzyme Assays. All assays were performed at 30° C. The observed rate was linear for at least 2 min and was proportional to the amount of extract added. Spectrophotometric assays were performed with a Perkin-Elmer 124 spectrophotometer, the reaction volumes were 1 ml. Oxidase activities were determined polarographically using a Clarktype oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., Yellow Springs, Ohio, USA). Enzyme activities are expressed as units/mg protein. One unit of activity is defined as that amount of enzyme catalyzing the transformation of 1 μmol of substrate or the formation of 1 μmol of product in 1 min. For the oxidases 1 unit corresponds to 0.5 μmol of oxygen consumed/min. Catalase units are expressed as ΔE_{240}/min. For most enzymes published assay procedures could not be adopted without extensive modification. These assays have therefore been described in detail below.

Amine Oxidase (E.C. 1.4.3). The reaction mixture (3 ml) contained: potassium phosphate buffer pH 8.0, 150 μmol; catalase, 20 μg; and extract. After air saturation and temperature equilibration the rate of endogenous oxygen consumption was recorded for 2 min. The reaction was then started by the addition of 10 μmol methylammonium chloride. The initial rate of oxygen consumption was corrected for endogenous respiration.

Choline Oxidase. Conditions were the same as for amine oxidase, except that the reaction was started by the addition of 10 μmol choline chloride.

Hydroxypyruvate Reductase (E.C. 1.1.1.29). (NADH- or NADPH dependent) was assayed according to Blackmore and Quayle (1970) except that 50 mM potassium phosphate pH 7.0 was used as a buffer.

Glucose-6-Phosphate Dehydrogenase (NAD- or NADP-dependent) (E.C. 1.1.1.49). This enzyme was very unstable in cell-free extracts. Over 50 % of the activity was lost within 1 h. It was not stabilized by the inclusion of dithiothreitol or mercaptoethanol in the sonication buffer. Furthermore, it had a pH optimum of 6.5, which is remarkably low for a glucose-6-phosphate dehydrogenase. The activity of this enzyme was assayed immediately after sonification in an uncentrifuged extract. The reaction mixture (1 ml) contained: sodium phosphate buffer pH 6.5, 50 μmol; NAD or NADP, 0.4 μmol; magnesium chloride, 5 μmol; and extract. The reaction was started by the addition of 5 μmol glucose-6-phosphate.

6-Phosphogluconate Dehydrogenase (NAD- or NADP-dependent) (E.C. 1.1.1.44) was assayed according to van Dijken and Quayle (1977) except that 50 mM Tris-maleate pH 8.0 was used as a buffer.

6-Phosphofructokinase (E.C. 2.7.1.11) was assayed according to van Dijken and Quayle (1977). The activity of this enzyme could only be established in assays with cells which were permeabilized with benzene, similar to the observations of Ratledge and Botham (1977) for the yeasts *Rhodotorula graminis* and *Candida* 107.

Fructose-1,6-Bisphosphatase (E.C. 3.1.3.11) was assayed according to van Dijken and Quayle (1977) except that 50 mM Tris maleate pH 9.0 was used as a buffer.

Fructose-1,6-Bisphosphate Aldolase (E.C. 4.1.2.13) was assayed according to van Dijken et al. (1978) except that 50 mM Tris-maleate pH 7.5 was used as a buffer. The activity of this enzyme was markedly stimulated by KCl; a 3.3-fold stimulation was observed upon addition of 0.1 M KCl.

Hexulose Phosphate Synthase. This enzyme was assayed by two methods: a) Radioactive assay. The activity was determined by establishing the pentose phosphate-dependent fixation of ^{14}C-formaldehyde into sugar phosphates as follows. A reaction mixture (0.2 ml) containing potassium phosphate buffer pH 7.0, 10 μmol; magnesium chloride, 1 μmol; ribose-5-phosphate isomerase, 0.5 units and ribose-5-phosphate, 1 μmol was incubated at 30° C for 5 min. Then 0.4 μmol ^{14}C-formaldehyde (0.2 mCi/mmol) was added. After 1 min of further incubation the reaction was started by the addition of extract. The reaction was allowed to proceed for various periods of time (0 – 5 min), after which it was stopped by the addition of 1 ml ice-cold absolute ethanol. Sugar phosphates were then precipitated by the addition of 0.1 ml 5 % barium acetate. The mixtures were kept on ice for 10 min, then filtered through a 0.45 μm millipore filter and washed once with 1 ml ice-cold absolute ethanol. The filters were dried for 20 min at 110° C. Radioactivity was measured in a liquid scintillation counter (Beckman LS 250) in 5 ml scintillation fluid (toluene/Triton 2:1, v/v, containing 0.5 % w/v PPO).

b) Spectrophotometric assay. In this assay the formaldehyde- and pentose phosphate-dependent rate of hexulose phosphate formation was determined. The reaction mixture (1 ml) contained: potassium phosphate buffer pH 7.0, 50 μmol; magnesium chloride, 5 μmol; glucose-6-phosphate dehydrogenase, 1 unit; glucose-6-phosphate isomerase, 1 unit; NADP, 0.4 μmol; phosphoriboisomerase, 2.5 units; partially

purified hexulose phosphate isomerase from *Methylophilus methylotrophus*, 17 µg and extract. After temperature equilibration 5 µmol ribose-5-phosphate was added. After 1 min of further preincubation the reaction was started by the addition of 5 µmol formaldehyde. The reaction velocity was linearly proportional to the amount of extract added up to an activity of 0.03 units/assay.

NAD(P)H Oxidase. The reaction mixture (1 ml) contained: Tris-maleate buffer pH 8.0, 50 µmol; magnesium chloride, 5 µmol; NADH or NADPH, 0.15 µmol. The reaction was started by the addition of extract.

Transhydrogenase, E.C. 1.6.1.1. Formation of NADH from NADPH was followed by coupling the NADH formation to the endogenous NADH oxidase activity, which was possible because of the negligible NADPH oxidase activity. The reaction mixture (1 ml) contained: Tris-maleate buffer pH 7.0, 50 µmol; magnesium chloride, 5 µmol; NADPH, 0.15 µmol; and extract. After 3 min of incubation to establish any NADPH oxidase activity the reaction was started by the addition of a catalytic amount of NAD (0.04 µmol).

The following enzymes were assayed according to published procedures: catalase (Lück 1963). Combined activity of 6-phosphogluconate dehydratase, E.C. 4.2.1.12 and KDPG aldolase, E.C. 4.1.2.14, according to van Dijken and Quayle (1977). NAD- and GSH-dependent formaldehyde dehydrogenase, E.C. 1.2.1.1, according to van Dijken et al. (1976). NAD-dependent formate dehydrogenase, E.C. 1.2.1.2, according to Johnson et al. (1964). Isocitrate lyase, E.C. 4.1.3.1, according to Dixon and Kornberg (1959).

Partial Purification of Hexulose Phosphate Isomerase from Methylophilus methylotrophus. In order to obtain a preparation of hexulose phosphate isomerase with negligible hexulose phosphate synthase activity, a procedure was adopted which is based on the irreversible inactivation of hexulose phosphate synthase in the absence of magnesium ions (Ferenci et al. 1974). *Methylophilus methylotrophus*, kindly supplied by Dr. A. J. Beardsmore, I.C.I., Billingham, U.K., was grown in the mineral salts medium described above which contained 1% (v/v) methanol. Incubation was at 37° C and the 20 l growth vessel was sparged with air. Cells were harvested at the end of the exponential growth phase, washed twice with 50 mM potassium phosphate buffer containing 5 mM EDTA and then resuspended in the same buffer to a concentration of ca. 0.1 g wet weight/ml. 350 ml of this cell suspension was disrupted in a Bühler Fibrogen Zell Mühle (Edmund Bühler, Melsungen, Federal Republic of Germany). Unbroken cells and debris were removed by centrifugation at 40,000 *g* for 20 min. The supernatant was subjected to ammonium sulphate precipitation; protein precipitating between 50 and 90% saturation was collected by centrifugation, dissolved in 50 ml potassium phosphate buffer pH 7.0 containing 1 mM EDTA and dialyzed against 400 volumes of this buffer. The dialyzed enzyme solution which contained 8.5 mg protein/ml was used in the spectrophotometric assay for hexulose phosphate synthase. The amount (17 µg) of dialyzed enzyme required in this assay to couple the reaction did contain negligible hexulose phosphate synthase activity and was sufficient for detecting an activity of up to 0.03 units.

Protein and Dry Weight Determinations

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Dry weight of bacterial suspensions was determined with a carbon analyzer (Beckman, model 915 A), connected to an infrared analyzer (Beckman, model 865). Carbon contents were multiplied by a factor of 2 to obtain dry weight.

Electron Microscopy

Negative Staining. Negative staining procedures were performed on cells prefixed with 3% glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.0, for 15 min at 0° C. After washing the cells with distilled water, they were negatively stained with 0.5% uranyl acetate.

Cytochemical Staining Experiments. Cytochemical staining experiments were performed on cells, prefixed with 3% glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.0, for 30 min at 0° C. Amine oxidase activity was demonstrated with the cerium technique (Veenhuis et al. 1976). Glutaraldehyde-fixed cells were washed twice with 0.1 M Na-cacodylate buffer pH 6.6, preincubated in the same buffer supplemented with 5 mM $CeCl_3$ and 50 mM aminotriazole for 90 min and subsequently incubated for 30 min in the same mixture supplemented with 10 mM methylamine under continuous aeration at 30° C. Enzyme activity was also demonstrated during aerobic incubations with DAB in the presence of an oxidase substrate (van Dijken et al. 1975; Veenhuis et al. 1976). This method involved incubation of the glutaraldehyde-fixed cells in 5 ml of 0.1 M Na-cacodylate buffer pH 6.6, supplemented with 10 mg DAB and 10 mM methylamine for 60 min at 30° C. Controls were performed in the absence of substrate, under anaerobic conditions, or by inclusion of acetonitrile which, in enzyme studies, proved to be a potent inhibitor of the oxidase. Catalase activity was demonstrated with the conventional DAB-technique (Graham and Karnovsky 1966). Glutaraldehyde-fixed cells were incubated in 5 ml 0.1 M Na-cacodylate buffer pH 6.6, supplemented with 10 mg DAB and 0.06% H_2O_2 for 90 min at 30° C. Controls were performed in the absence of H_2O_2 or by inclusion of aminotriazole (50 mM) in the incubation mixture.

Fixation Procedures. For routine fixation the cells were prefixed with 3% glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.0 for 60 min at room temperature. Postfixation – also after cytochemical experiments – was performed in a mixture of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in the same buffer for 45 min. After washing with distilled water the cells were poststained in 1% uranyl acetate for 60 min. After dehydration in a alcohol series, the cells were embedded in Epon. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

Biochemicals and Enzymes

^{14}C-formaldehyde was obtained from the Radiochemical Centre, Amersham, U.K. 3-Amino-1,2,4-triazole and phosphoriboisomerase were obtained from Sigma Chemical Co. (St. Louis, USA). All other biochemicals and enzymes were obtained from Boehringer, Mannheim, FRG.

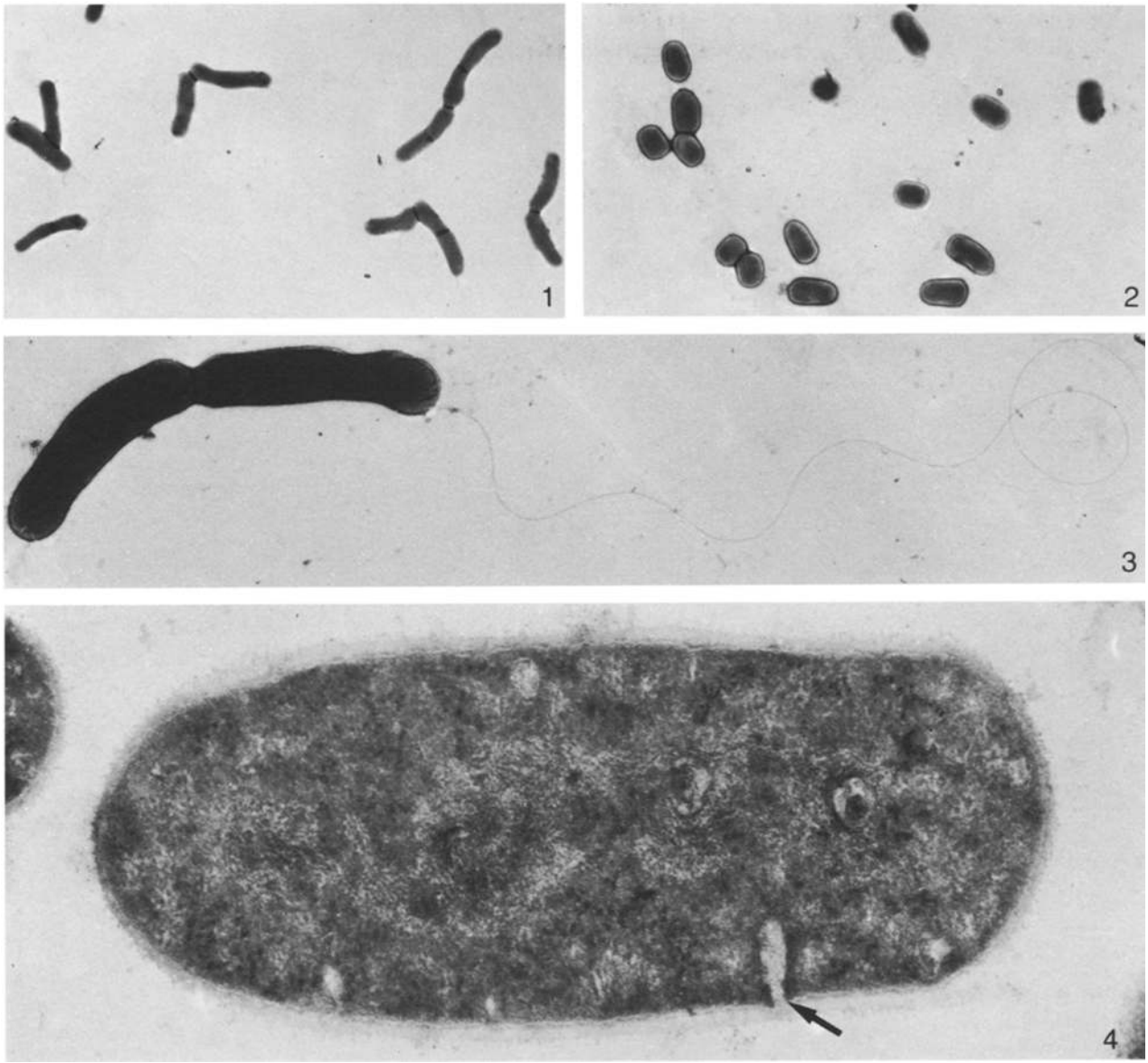
Results

Isolation of Facultative Methylotrophs

Enrichments using mineral media containing methylamine or trimethylamine as the sole carbon source were invariably successful: 27 out of 33 isolates which were obtained in pure culture were facultative methylotrophs. Of these organisms 11 grew rapidly on methylamine (doubling times of less than 4 h) and were screened for their pathway of formaldehyde assimilation. Only one isolate (a *Hyphomicrobium* species) contained high levels of hydroxypyruvate reductase, indicating the presence of the serine pathway. The other organisms (mostly Coryneform bacteria) turned out to be RuMP cycle organisms. One of them (coded P 1) was chosen for further studies. The organism has been deposited in the National Collection of Industrial Bacteria at Torry Research Station, Aberdeen, U.K., under number NCIB 11625.

Characterization of Isolate P 1

Isolate P 1 was tentatively identified as an *Arthrobacter* species on the basis of morphological characteristics, cell wall composition and total fatty acid spectra. The organism showed the complete cycle of development, which is characteristic for many *Arthrobacter* species (Veldkamp 1970). Cells from the exponential growth phase were pleomorphic rods which completely transformed into coccoid forms at the end of the growth phase (Figs. 1 and 2). This phenomenon was independent of the growth substrate. After transfer of the coccoid-shaped cells into fresh medium they turned again into pleomorphic rods. V-forms were rather common indicating



Figs. 1—3. Unfixed cells of *Arthrobacter* P 1, negatively stained with uranyl acetate, demonstrating the rod-shaped cells characteristic for the logarithmic growth phase (Fig. 1, magn. ×4,200) in contrast to the coccoid cells from the stationary phase of growth (Fig. 2, ×6,900). Figure 3 shows the presence of a subpolar flagellum (×11,000)

Fig. 4. Thin section of a cell demonstrating its ultrastructure after conventional fixation techniques. Several invaginations of the cell membrane (*arrow*) are observed (×39,500)

snapping movement after cell division or fusion of two separate cells (Veldkamp 1970). Slow tumbling motility of cells was observed during growth on "fast growth substrates" such as glucose, succinate or yeast extract. Cells grown on methylated amines were invariably non-motile. Electron microscopy of *Arthrobacter* P 1 growing exponentially on glucose revealed the presence of one subpolar or lateral flagellum (Fig. 3).

Arthrobacter P 1 was very versatile with respect to its growth substrates. Doubling times on methylated amines and some other carbon sources are listed in Table 1. The organism was also able to grow on: fructose, xylose, galactose, lactose, mannose, saccharose, cellobiose, raffinose, melibiose, maltose, rhamnose, mannitol, sorbitol, glyoxylate, glycerate, pyruvate, lactate, propionate, mesaconate, fumarate, tartrate, 4-hydroxybenzoate, ascorbate, N-methylglutamate, γ-aminobutyrate, aspartate, asparagine, serine, homoserine, histidine, arginine, proline, lysine, tryptophan, threonine, valine, glutamine, trimethylamine-N-oxide, diethylamine, N,N-ethyldimethylamine, N,N-diethylmethylamine, urate, xanthine, acetylcholine, betaine, sarcosine, glucosamine, citrullin, creatine, yeast extract and nutrient broth. Substrates which did not support growth were: methanol, formate, dimethylsulfide, dimethylsulfoxide, tetramethylamine, acetamide, triethanolamine, triethylamine, ethanolamine, 1,5-diaminopentane, phenylalanine, leucine, isoleucine, cysteine, methionine, hydroxyproline, acetone, uracil, glycollate, oxalate, oxamate, caproate, butyrate, crotonate, itaconate, malonate, ribose, arabinose, sorbose, adonitol, erythritol and ornithine.

Table 1. Doubling times (h) of *Arthrobacter* P 1 during growth on various substrates

Substrate	Doubling time (h)
Methylamine	2.6
Dimethylamine	4.1
Trimethylamine	3.7
Ethylamine	2.4
Choline	2.2
Glycine	1.7
α-Methylglucoside	4.3
Glucose	1.2
Gluconate	1.3
Glycerol	1.8
Alanine	2.5
Ethanol	6.5
Acetate	2.2
Citrate	4.6
α-Ketoglutarate	1.6
Glutamate	3.3
Succinate	1.3
Malate	1.5

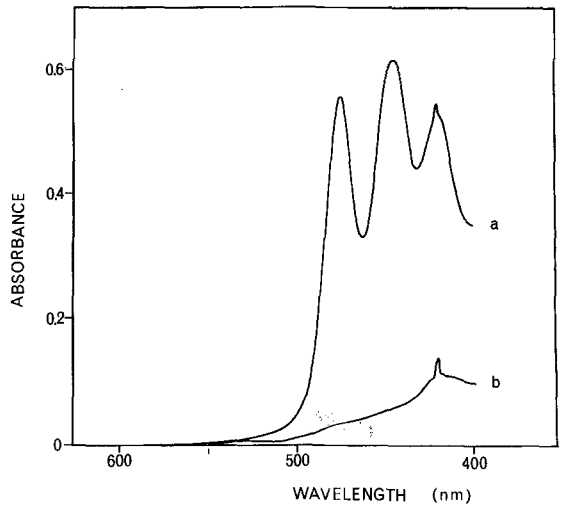


Fig. 5. Absorption spectrum of cell-free extracts (3 mg protein/ml) of *Arthrobacter* P 1 grown in glucose-limited chemostat cultures in the dark (*b*) or in the light (*a*), (5000 lux)

Table 2. Enzyme profiles of *Arthrobacter* P 1 during growth on various substrates (activities are expressed as munits/mg protein, except for catalase which is expressed as ΔE_{240}/min × mg protein)

Enzyme	Growth substrate			
	Methylamine	Ethylamine	Glucose	Choline
Amine oxidase	740	780	1	1
Choline oxidase	1	–	–	224
Catalase	139	200	32	139
Hexulose phosphate synthase[a]	592 (742[b])	47	1	197
Glucose-6-phosphate dehydrogenase (NAD)	1	–	1	–
(NADP)	264	72	198	97
6-Phosphogluconate dehydrogenase (NAD)	1	–	1	–
(NADP)	216	166	270	141
6-Phosphofructokinase	58	16	64	–
Fructose-1,6-bisphosphate aldolase	410	145	402	250
6-Phosphogluconate dehydratase + KDPG aldolase	1	–	1	–
Fructose-1,6-bisphosphatase	23	38	30	–
Formate dehydrogenase (NAD)	1	–	–	–
Formaldehyde dehydrogenase (GSH/NAD)	1	–	–	–
NADH oxidase	249	228	384	257
NADPH oxidase	3	–	–	–
Transhydrogenase	10	–	–	–
Isocitrate lyase	7	173	3	4

– = not determined
[a] radioactive assay
[b] spectrophotometric assay

Cells of *Arthrobacter* P 1 exposed to light formed a yellow pigment, a phenomenon which was independent of the growth substrate. After a high-speed centrifugation of cell-free extracts most of the pigment was sedimented. The absorption spectrum (Fig. 5) suggests a carotenoid-like structure. Exposure of cells to light had a pronounced effect on the cell yields in that growth yields of methylamine- or glucose-limited chemostat cultures were 10 – 20 % higher when grown in the dark.

Oxidation of Primary Amines

Extracts of methylamine- and ethylamine-grown cells catalyzed the oxidation of primary amines with oxygen as the electron acceptor. The activity was not detected in extracts of glucose- or choline-grown cells (Table 2). The rate of oxidation was not affected by the addition of PMS or dialysis of the extract. After a high-speed centrifugation (150,000 *g* for 2 h) of a French press cell extract, the activity was quantitatively recovered in the supernatant, whereas 75 % of the NADH oxidase activity was sedimented. The relative activity of the amine oxidase towards different primary amines was almost identical in extracts of methylamine- and ethylamine-grown cells, suggesting the presence of the same enzyme in both extracts. High levels of the enzyme were also present in trimethylamine- and dimethylamine-grown cells, suggesting a key role in the oxidation of these compounds.

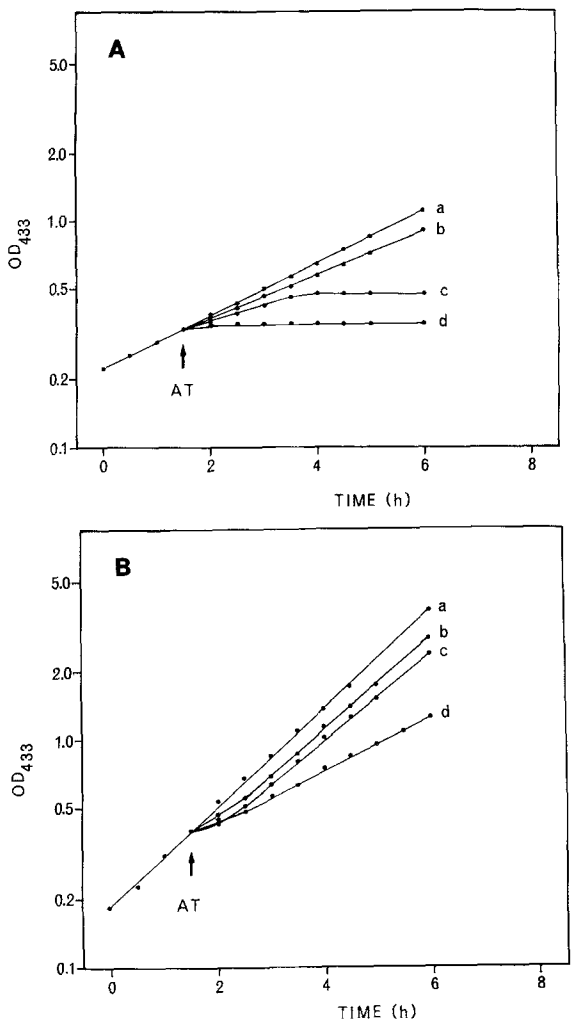


Fig. 6. Effect of addition of aminotriazole (*AT*) (*arrow*) to a culture of *Arthrobacter* P 1 growing on methylamine (**A**) or glucose (**B**). *a* no addition; *b* 5 mM AT; *c* 20 mM AT; *d* 50 mM AT

Since the in vitro experiments described above suggested that in vivo oxidation of methylamine and ethylamine by *Arthrobacter* P 1 proceeds with oxygen as the primary electron acceptor, amine-grown cells were assayed for catalase activity. High levels of this enzyme in methylamine- and ethylamine-grown cells as compared to glucose-grown cells (Table 2) suggest that hydrogen peroxide is formed in the oxidation of these substrates. A high catalase activity in choline-grown cells confirmed the observation that also the oxidation of choline was mediated by an oxidase (Table 2). As with amine oxidase, catalase activity was not sedimentable in the crude extract after high-speed centrifugation.

Confirmation of the important role of catalase in the in vivo oxidation of methylamine by *Arthrobacter* P 1 was obtained in inhibition studies with whole cells. The addition of aminotriazole, which is known to inhibit catalase irreversibly (Cohen and Somerson 1969) to exponentially growing cells had a profound inhibitory effect on growth on methylamine, whereas growth on glucose was hardly affected (Fig. 6A and B). On the basis of these observations it is suggested that in *Arthrobacter* P 1 methylamine is oxidized according to

$$CH_3-NH_2 + H_2O + O_2 \rightarrow H_2C = O + NH_3 + H_2O_2.$$

Cell Ultrastructure and Cytochemical Localization of Amine Oxidase and Catalase

A survey of a glutaraldehyde-OsO_4/$K_2Cr_2O_7$ fixed cell of methylamine-grown *Arthrobacter* P 1 is shown in Fig. 4. In the cell a number of membraneous structures were observed, both in the cytoplasm as well as in the nucleoid. Serial sectioning showed that these membranes were invaginations of the cell-membrane.

In order to study the subcellular localization of the amine oxidase and catalase, cytochemical staining experiments were performed. Aerobic incubations of glutaraldehyde fixed cells with $CeCl_3$ and methylamine indicated that H_2O_2 production mainly occurred on invaginations of the cell-membrane (Fig. 7). Staining deposits, albeit to a lesser extent, were also observed on the cell membrane. Similar results were obtained after aerobic incubations with DAB and methylamine (Figs. 9 and 10), indicating that amine oxidase activity was mainly located on the membraneous invaginations. Controls, in the absence of substrate, under anaerobic conditions or in the presence of acetonitrile as an amine oxidase inhibitor, gave negative results (Figs. 8 and 12).

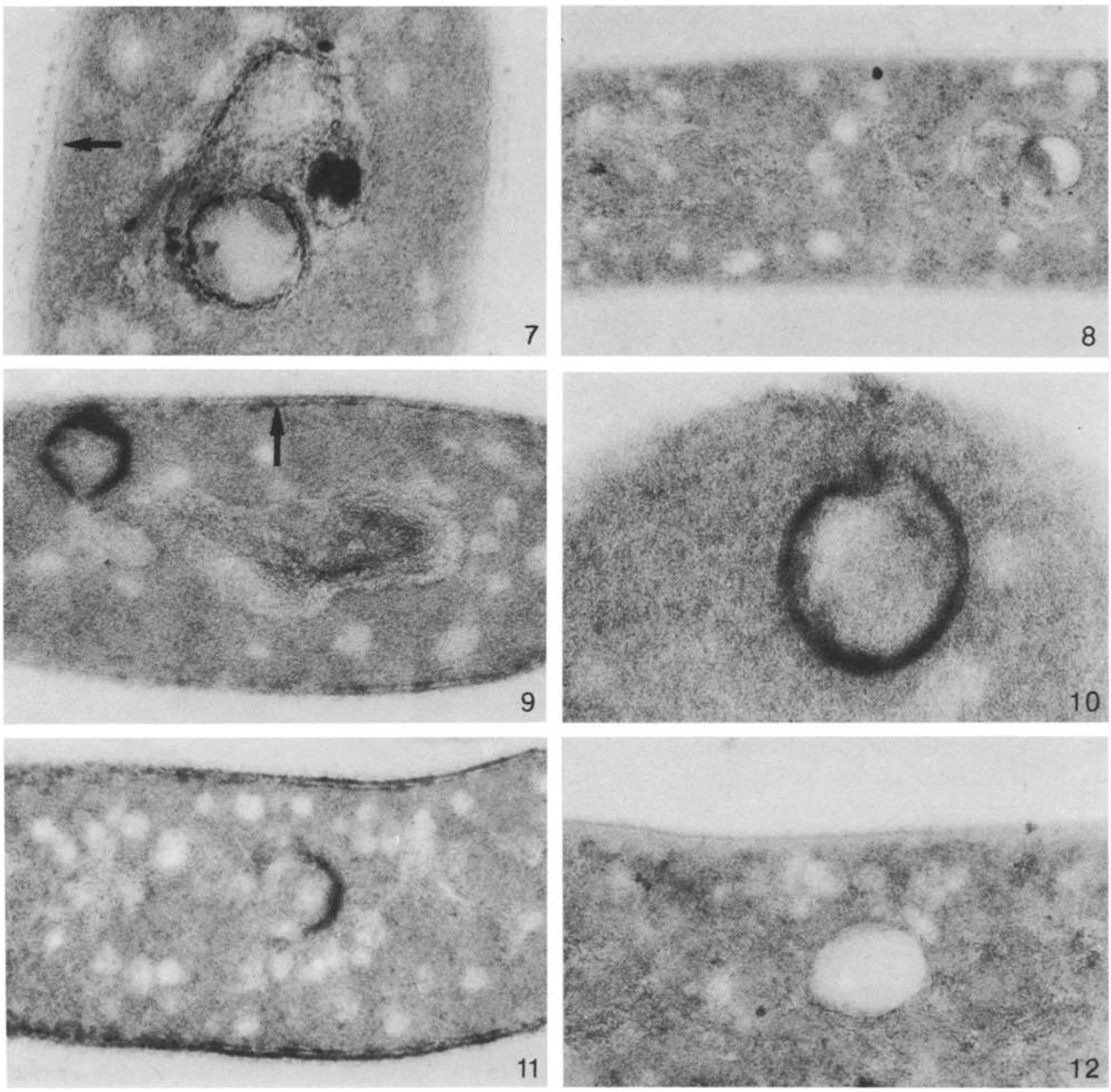
Incubations with DAB in the presence of H_2O_2 showed, that the cell membrane and its invaginations, as well as the cell wall contained catalase activity (Fig. 11). Preincubation with 50 mM aminotriazole greatly reduced staining of these cell components. Incubations without hydrogen peroxide gave negative results (Fig. 12).

Oxidation of Formaldehyde

Extracts of methylamine-grown cells did not contain NAD-dependent formaldehyde or formate dehydrogenase activities (Table 2). Furthermore, whole cells did not oxidize formate. These observations suggest that formaldehyde is not oxidized to CO_2 via a linear reaction sequence. The formaldehyde generated from methylamine may, however, be oxidized via enzymes of the RuMP cycle and glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, as in other methylotrophs with the RuMP cycle (Quayle 1980; Colby et al. 1979). These enzymes were present in methylamine-grown *Arthrobacter* P 1 at sufficiently high levels; they were strictly NADP-dependent, although high NADH oxidase and negligible NADPH oxidase activities (Table 2) indicate that NADH is the preferred electron donor for the electron transport chain in this organism.

Assimilation of Formaldehyde

A peculiar problem was encountered in the initial attempts to establish the nature of the assimilation pathway of formaldehyde during growth of *Arthrobacter* P 1 and some other isolates (mainly *Arthrobacter*-like organisms) on methylamine. Using the standard spectrophotometric assay for hexulose phosphate synthase (Dahl et al. 1972), a high rate of pentose phosphate-dependent formation of fructose-6-phosphate was observed. This reaction started after a lag of 3–5 min, but was not stimulated upon addition of formaldehyde, indicating the absence of either hexulose phosphate synthase or hexulose phosphate isomerase or both. However, the results of radioactive assays clearly showed the presence of the synthase in methylamine-grown cells, but not in glucose-grown *Arthrobacter* P 1. These results are most



Figs. 7–12. Thin sections of *Arthrobacter* P1 cells showing the results of cytochemical staining experiments

Fig. 7. Section of a cell after incubation with $CeCl_3$ and methylamine. The reaction products are mainly located on the invagination of the cell membrane, but some staining of the cell membrane is also observed (*arrow*) (× 122,500)

Fig. 8. Micrograph of cells from a control with $CeCl_3$ and methylamine in the presence of acetonitrile to inhibit amine oxidase activity (× 79,000)

Fig. 9. Section of a cell after incubation with DAB and methylamine. Reaction products are observed mainly on membrane invaginations, but some staining of the cell membrane is also observed (*arrow*) (× 102,500)

Fig. 10. Section of a cell at high magnification demonstrating the distribution of the reaction product on membrane invaginations after incubation with DAB and methylamine (× 194,500)

Fig. 11. Section of a cell demonstrating the localization of catalase activity after incubation with DAB and H_2O_2. Reaction products are observed on the cell membrane and its invaginations and, in addition, on the cell wall (× 77,500)

Fig. 12. Micrograph of cells from a control experiment demonstrating the absence of staining after incubation with DAB in the absence of any substrate (× 105,000)

probably explained by an instability of the organism's hexulose phosphate isomerase under the conditions of the spectrophotometric assay, since an almost immediate stimulation of the rate of fructose-6-phosphate formation in this assay was observed, when a partially purified hexulose phosphate isomerase from *Methylophilus methylotrophus* (see Materials and Methods) was added to the complete assay mixture. It seems therefore likely that hexulose phosphate isomerase of *Arthrobacter* P 1 is not active under the conditions used for the preparation of the extract and/or the spectrophotometric assay.

Arthrobacter P 1 uses the Embden-Meyerhof pathway for the generation of C_3 compounds during growth on methylamine and glucose. The enzymes of the Entner-Doudoroff pathway were absent, whereas high activities of 6-phosphofructokinase and FBP aldolase were detected in methylamine- and glucose-grown cells as compared to ethylamine-grown cells (Table 2).

Discussion

The results of our enrichment studies confirm the observations of Colby and Zatman (1973, 1975) that facultative methylotrophs are easily enriched from their natural habitat in media containing either trimethylamine or methylamine. The score of facultative methylotrophs from enrichments with these substrates was similar: 4 out of 5 enrichments were successful. Generally, facultative methylotrophs thus obtained do not grow on methanol. Although this has been reported for *Arthrobacter* 2B2 (Colby and Zatman 1973), we were not able to grow this organism in the presence of methanol. The organism which was selected for further studies, isolate P 1, is a versatile methylotroph which is able to grow fast on both methylamine and a variety of "heterotrophic" substrates. The organism is probably an *Arthrobacter* species on the basis of morphological and chemical criteria. In contrast to the majority of Arthrobacters, but similar to *Arthrobacter simplum* and *Arthrobacter atrocyaneus* it is motile during growth on certain substrates (Clark and Carr 1951; Kuhn and Starr 1960; Veldkamp 1970). The ability of the organism to utilize uric acid, glycine, creatine, betaine and sarcosine indicates that it may be a strain of *Arthrobacter globiformis* (Keddie 1974). A characteristic property of *Arthrobacter* P 1 is the formation of a light-induced carotenoid-like pigment. The production of the pigment has significant physiological consequences since the molar cell yields on both methylamine and glucose were 10–20% lower in the light.

The most striking feature of methylamine metabolism by *Arthrobacter* P 1 is the involvement of an amine oxidase. Low activities of amine oxidase during growth on methylamine have been previously detected in cell-free extracts of *Arthrobacter globiformis* B-175 (Loginova and Trotsenko 1976). However, umambiquous evidence with respect to the oxidase character of the enzyme (i.e. in vivo production of H_2O_2) has not been presented before. In *Arthrobacter* P 1 the available evidence strongly suggests that both in vitro and in vivo oxidation of methylamine by the enzyme results in the formation of hydrogen peroxide (Fig. 6) which therefore necessitates the presence of catalase (Table 2). These enzymes probably play a key role in the metabolism of ethylamine as well (Table 2). The results of cytochemical staining experiments show that both the amine oxidase and catalase are membrane bound enzymes in *Arthrobacter* P 1, although a significant activity of the latter enzyme was also observed in the cell wall. Since after French press treatment or sonication of whole cells these enzymes were quantitatively recovered in the high speed supernatant it must be concluded that they are easily solubilized.

Arthrobacter P 1 utilizes the RuMP cycle for the fixation of formaldehyde. The key enzyme of this cycle was readily detectable using a radioactive assay. However, in the spectrophotometric assay, which relies on the activity of both hexulose phosphate synthase and hexulose phosphate isomerase, no formaldehyde-dependent conversion of pentose phosphate into fructose-6-phosphate could be detected, unless a partially purified preparation of hexulose phosphate isomerase from another source was present. Similar results were obtained by Cox and Zatman (1974) with *Arthrobacter* 2B2. These findings show that a negative result with the spectrophotometrical assay for hexulose phosphate synthase should be interpreted with care and warrant further comparative biochemical studies on hexulose phosphate isomerase of the *Arthrobacters* mentioned above and that of other RuMP cycle organisms.

Hexulose phosphate synthase and isomerase are not only typical assimilatory enzymes in *Arthrobacter* P 1. The absence of NAD-dependent formaldehyde and formate dehydrogenases indicate that the key enzymes of the RuMP cycle are also indispensable for formaldehyde oxidation. However, a cyclic oxidation of formaldehyde via the RuMP cycle enzymes with glucose-6-phosphate and 6-phosphogluconate dehydrogenase in *Arthrobacter* P 1 is rather hypothetical at present, since the latter two enzymes were strictly NADP-dependent. Since, moreover, high NADH oxidase activities and only a very low apparent transhydrogenase activity were detected in methylamine-grown cells (Table 2), an exclusive catabolic role of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase is dubious.

Arthrobacter P 1 strongly regulated its "methylotrophic" enzymes (Table 2). However, the synthesis of amine oxidase and catalase is probably not under coordinate control. This was not unexpected, since catalase is not necessarily a unique enzyme in the metabolism of methylamine and ethylamine. Whenever a hydrogen peroxide producing oxidase is functioning – as probably in choline oxidation – the enzyme must be synthesized. Interestingly, also the synthesis of amine oxidase and hexulose phosphate synthase seems to be independently regulated. The significantly higher levels of hexulose phosphate synthase in choline-grown cells as compared to ethylamine-grown cells indicates that the enzyme may have an important role in formaldehyde metabolism during growth on choline.

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References

Blackmore MA, Quayle JR (1970) Microbial growth on oxalate by a route not involving glyoxylate carboligase. Biochem J 118:53–59

Clark FE, Carr PH (1951) Motility and flagellation of the soil corynebacteria. J Bacteriol 62:1–7

Cohen G, Somerson NL (1969) Catalase-aminotriazole method for measuring secretion of hydrogen peroxide by microorganisms. J Bacteriol 98:543–546

Colby J, Zatman LJ (1973) Trimethylamine metabolism in obligate and facultative methylotrophs. Biochem J 132:101–112

Colby J, Zatman LJ (1975) Tricarboxylic acid-cycle and related enzymes in restricted facultative methylotrophs. Biochem J 148:505–511

Colby J, Dalton H, Whittenbury R (1979) Biological and biochemical aspects of microbial growth on C_1 compounds. In: Starr MP, Ingraham JL, Raffels S (eds) Ann Rev Microbiol, Vol 33. Palo Alto, California: Annual Reviews Inc, pp 481–517

Cox RB, Zatman LJ (1974) Hexose phosphate synthase in trimethylamine-grown *Bacterium* 2B2, a facultative methylotroph. Biochem J 141:605–608

Dahl JS, Mehta RJ, Hoare DS (1972) New obligate methylotroph. J Bacteriol 109:916–921

van Dijken JP, Veenhuis M, Vermeulen CA, Harder W (1975) Cytochemical localization of catalase activity in methanol-grown *Hansenula polymorpha*. Arch Microbiol 105:261–267

van Dijken JP, Oostra-Demkes GJ, Otto R, Harder W (1976) S-Formylglutathione: the substrate for formate dehydrogenase in methanol-utilizing yeasts. Arch Microbiol 111:77–84

van Dijken JP, Quayle JR (1977) Fructose metabolism in four *Pseudomonas* species. Arch Microbiol 114:281–286

van Dijken JP, Harder W, Beardsmore AJ, Quayle JR (1978) Dihydroxyacetone: an intermediate in the assimilation of methanol by yeasts? FEMS Microbiol Lett 4:97–102

Dixon GH, Kornberg HL (1959) Assay methods for key enzymes of the glyoxylate cycle. Biochem J 72:3p

Ferenci T, Strøm T, Quayle JR (1974) Purification and properties of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase from *Methylococcus capsulatus*. Biochem J 144:477–486

Graham RC, Karnovsky MJ (1966) The early stages of absorption of injected horse-radish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302

Harder W, Visser K, Kuenen JG (1974) Laboratory fermenter with an improved magnetic drive. Lab Practice 23:644–645

Johnson PA, Jones-Mortimer MC, Quayle JR (1972) Use of a purified bacterial formate dehydrogenase for the micro-estimation of formate. Biochim Biophys Acta 89:351–353

Keddie RM (1974) *Arthrobacter*. In: Buchanan and Gibbons (eds) Bergey's Manual of determinative bacteriology, 8th ed. The Williams and Wilkins Company, Baltimore, pp 618–625

Kuhn DA, Starr MP (1960) *Arthrobacter atrocyaneus* n.sp. and its blue pigment. Arch Microbiol 36:175–181

Loginova NV, Trotsenko YuA (1976) Pathways of oxidation and assimilation of methylated amines in *Arthrobacter globiformis*. Microbiology 45:196–201

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lück H (1963) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York London, pp 885–894

Quayle JR (1980) Microbial assimilation of C_1 compounds. Biochem Soc Transact 8:1–10

Ratledge C, Botham PA (1977) Pathways of glucose metabolism in *Candida 107*, a lipid-accumulating yeast. J Gen Microbiol 102:391–395

Veenhuis M, van Dijken JP, Harder W (1976) Cytochemical studies on the localization of methanol oxidase and other oxidases in peroxisomes of methanol-grown *Hansenula polymorpha*. Arch Microbiol 111:123–135

Veldkamp H (1970) Saprophytic coryneform bacteria. Ann Rev Microbiol 24:209–233

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195–213

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