The Isolation of Pleiotropic Mutants of *Pseudomonas aminovorans* Deficient in the Ability to Grow on Methylamine and an Examination of their Enzymic Constitution

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Mutants deficient in several enzymes of methylamine oxidation and assimilation have been obtained by treating *Pseudomonas aminovorans* with ultraviolet light. One of these mutants was studied in detail and was shown to lack the enzymes of trimethylamine and dimethylamine oxidation. In addition, activities of the following enzymes, all postulated as being involved in C₁ metabolism, were lost: hydroxypyruvate reductase, serine-glyoxylate aminotransferase, isocitrate lyase, phosphoenolpyruvate carboxylase, *N*-methylglutamate dehydrogenase, γ -glutamylmethylamide synthetase, formate dehydrogenase and dye-linked formaldehyde dehydrogenase. In contrast, three enzymes – NAD⁺, glutathione-linked formaldehyde dehydrogenase, *N*-methylalanine dehydrogenase and *N*-methylglutamate synthase – were retained. This phenotype suggests either a lesion in a regulatory gene, a deletion in an early structural gene of an operon or the loss of a plasmid. Results obtained indicated that *P. aminovorans* possesses an isocitrate lyase-serine pathway.

INTRODUCTION

It has been suggested (Jarman & Large, 1972; Boulton & Large, 1977) that *Pseudomonas aminovorans* grows on trimethylamine by the successive oxidation of each of the carbon atoms to formaldehyde by a series of enzymes: trimethylamine monooxygenase (EC 1.14.13.8) (Boulton *et al.*, 1974); trimethylamine *N*-oxide aldolase (EC 4.1.1.2.–) (Large, 1971); dimethylamine monooxygenase (EC 1.14.99.–) (Eady *et al.*, 1971); *N*-methylglutamate synthase (EC 2.1.1.21) (Jarman, 1973; Bamforth, 1976); and *N*-methylglutamate dehydrogenase (EC 1.5.99.5) (Bamforth & Large, 1975). The product formaldehyde can then either be further oxidized by means of formaldehyde dehydrogenase and formate dehydrogenase (EC 1.2.1.2) to carbon dioxide (Eady, 1970) or assimilated by the enzymes of the serine pathway (Large & Carter, 1973). The enzymes of trimethylamine catabolism are induced by transfer to media containing trimethylamine as sole carbon source (Boulton & Large, 1977), and the serine pathway enzymes are induced by growth on methylamine (Large & Carter, 1973). It is likely that the enzymes of C₁ dissimilation and assimilation are coordinately expressed in *P. aminovorans* in a manner comparable to other methylotrophs (Dunstan *et al.*, 1972; O'Connor & Hanson, 1978).

However, one of the enzymes purported to be involved in the oxidation of methylamine in *P. aminovorans*, *N*-methylglutamate dehydrogenase, has too low a specific activity in cell-free extracts to account for the growth rate of the organism on methylamine (Bamforth

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& Large, 1977). In an attempt to ascertain whether this enzyme was specifically involved in growth on methylamine we tried to isolate mutants of *P. aminovorans* deficient in the ability to grow on methylamine and to ascertain whether these mutants were specifically lacking in *N*-methylglutamate dehydrogenase activity.

We report in this paper the isolation of mutants of *P. aminovorans* which were unable to grow on methylamine, but these were deficient in a number of enzymes which have been postulated as being functionaries in C_1 metabolism. One of the mutants was studied in detail, with additional investigation of its inability to grow on dimethylamine and trimethylamine hydrochlorides.

Several unanswered and important questions concerning the growth of *P. aminovorans* on methylamine (and other methylamines) are considered. (i) Of what significance is the presence of two separate formaldehyde dehydrogenases in *P. aminovorans*, namely one catalysing an NAD^+ , glutathione-dependent reaction (EC 1.2.1.1) and the other dye-linked (EC 1.2.99.-) (Boulton & Large, 1977)? (ii) Is the low specific activity of isocitrate lyase (Large & Carter, 1973) specifically due to this enzyme or is it a function of isocitrate dehydrogenase (Attwood & Harder, 1977)? (iii) Are the enzymes γ -glutamyl-methylamide synthetase (EC 6.3.4.12) (Kung & Wagner, 1969) and *N*-methylalanine dehydrogenase (EC 1.5.1.-) (Lin & Wagner, 1975), which have been considered as possible functionaries in the oxidation of methylamine by *Pseudomonas* MS, of any importance to growth of *P. aminovorans* on C_1 substrates?

METHODS

Organism. *Pseudomonas aminovorans* (NCIB 9039) was obtained from Dr P. J. Large, Department of Biochemistry, University of Hull. The organism was maintained and grown as described by Eady *et al.* (1971).

Preparation of mutant organisms. Mutants unable to grow on methylamine were isolated using ultraviolet light mutagenesis essentially by the method of O'Connor *et al.* (1977). Ultraviolet light exposure was at a distance of 40 cm for 2.5 min. Permissive medium contained 0.2% (w/v) sodium succinate as carbon source; non-permissive medium contained 0.5% (w/v) methylamine hydrochloride as carbon source. Two rounds of penicillin (330 units ml^{-1}) selection were performed and mutant selection was by replica-plating successively on to methylamine and succinate plates.

Determination of protein. Protein was estimated by the method of Lowry *et al.* (1951) using dried bovine serum albumin as standard.

Enzyme assays. Enzymes were assayed by unmodified published methods unless otherwise indicated: hydroxypyruvate reductase (EC 1.1.1.29) (Large & Quayle, 1963) assayed at pH 5.0; serine-glyoxylate aminotransferase (EC 2.6.1.45) (Blackmore & Quayle, 1970) using an extract of the wild-type organism (14 μg protein) as source of hydroxypyruvate reductase; isocitrate lyase (EC 4.1.3.1) (Dixon & Kornberg, 1959); phosphoenolpyruvate carboxylase (EC 4.1.1.31) (spectrophotometric method of Large *et al.*, 1962) assayed at pH 7.3 (see Large & Carter, 1973); trimethylamine monooxygenase (Boulton *et al.*, 1974); trimethylamine *N*-oxide aldolase (Large, 1971) using the method of Chrastil & Wilson (1975) for formaldehyde estimation; dimethylamine monooxygenase (spectrophotometric method of Eady *et al.*, 1971); *N*-methylglutamate synthase (radiochemical assay of Pollock & Hersh, 1971); *N*-methylglutamate dehydrogenase (Bamforth & Large, 1977); *N*-methylalanine dehydrogenase (Lin & Wagner, 1975); dye-linked formaldehyde dehydrogenase (Johnson & Quayle, 1964); NAD^+ , glutathione-linked formaldehyde dehydrogenase (van Dijken, 1976); formate dehydrogenase (Johnson & Quayle, 1964); $NADP^+$ -linked isocitrate dehydrogenase (Kornberg, 1955).

γ -Glutamylmethylamide synthetase was assayed essentially as described by Levitch (1977) except that samples of reaction mixture were made alkaline in scintillation vials and dried in a vacuum oven at 25 °C. Liquid scintillator NE213 (5 ml; Nuclear Enterprises, Edinburgh 11) was then added to each vial and radioactivity was counted in an Isocap 300 scintillation counter.

Enzyme units. One milliunit (munit) of enzyme was the amount required to catalyse the formation of 1 nmol product (or the disappearance of 1 nmol substrate) min^{-1} .

Preparation of cell-free extracts. Wild-type and methylamine-negative organisms were grown in separate 1-litre batches of succinate medium for 24 h, harvested aseptically and resuspended in 1-litre batches of methylamine- or trimethylamine-medium. Cultures were incubated for 48 h at 30 °C on a gyratory shaker before harvesting, washing once in distilled water and resuspending in approx. 5 vol. 50 mM-potassium

phosphate, pH 7.0. (For assay of *N*-methylglutamate synthase the extracting buffer was 100 mM-Tris/HCl, pH 8.5, containing 60 mM-KCl, 50 mM-L-glutamate, 1 mM-EDTA and 50 μ M-FMN; for assay of γ -glutamyl-methylamide synthetase the extracting buffer was 10 mM-potassium phosphate, pH 7.0, containing 1 mM-methylamine hydrochloride, 1 mM-L-glutamate, 1 mM-MnCl₂, 0.25 mM-ATP and 1 mM-dithiothreitol.) Bacteria adapted to growth on methylamine were disrupted with an MSE ultrasonic disintegrator (model 150 W) at full power for 4 $\times$ 0.5 min intervals interspersed by cooling in ice. Debris was removed by centrifuging at 10000g for 10 min at 4 °C. Bacteria adapted to growth on trimethylamine were broken by passing once through a French pressure cell, pre-cooled to 0 °C, at 103 MPa. Debris was removed as above.

Enzymes other than *N*-methylglutamate dehydrogenase were assayed using the supernatant from crude extracts centrifuged at 100000g for 1 h. *N*-Methylglutamate dehydrogenase activity was estimated in the resulting pellet.

Determination of the product of isocitrate metabolism. The product of isocitrate metabolism was determined by analysis of dinitrophenylhydrazone derivatives as described by Attwood & Harder (1977).

RESULTS

Isolation and screening of mutants unable to grow on methylamine

A large number of mutants unable to grow on methylamine but capable of growth on succinate were isolated. Approximately 75% of the culture obtained was estimated to be of this phenotype.

Four mutants (M₁, M₂, M₄ and M₅) possessing the above phenotype were selected and obtained in pure culture for detailed study. On permissive substrates the mutants produced a characteristic soluble yellow pigment which was indistinguishable from that produced by the wild-type organism. There were no noticeable morphological differences between the mutant and wild-type species when cells stained with methylene blue were examined microscopically.

Mutant and wild-type organisms were examined for a number of enzymes believed to be involved in growth on methylamine (Anthony, 1975; Loginova & Trotsenko, 1977) (Table 1). Each of the mutant organisms had lost the activities of serine-glyoxylate aminotransferase, *N*-methylglutamate dehydrogenase, dye-linked formaldehyde dehydrogenase and formate dehydrogenase. [Although limited by the amount of hydroxypyruvate reductase added by way of wild-type crude extract, sufficient extract was added to allow a rate for a potential serine-glyoxylate aminotransferase reaction of 3.6 munits (mg protein)⁻¹.] Mutant M₁ retained a very much reduced level of hydroxypyruvate reductase, whereas this enzyme was absent from the other mutants. Mutant M₄ was the only one to retain phosphoenolpyruvate carboxylase, albeit at a four-fold lower specific activity than the wild-type species. Mutants M₁, and to a lesser extent M₂, retained isocitrate lyase unlike the other two mutants. All the mutants retained *N*-methylalanine dehydrogenase and NAD⁺, glutathione-linked formaldehyde dehydrogenase.

Mutant M₅

Mutant M₅ was completely devoid of the enzymes referred to above, and so was selected for further work. It was able to grow on succinate, glucose, pyruvate, glutamate and citrate, in common with the wild-type organism (Bamforth, 1976), but not on methylamine, dimethylamine or trimethylamine. Although ethanol was not a substrate for either the wild-type or M₅, acetate supported very weak growth of both strains.

Mutant M₅ was unable to synthesize γ -glutamylmethylamide synthetase but retained the ability to produce *N*-methylglutamate synthase (Table 1). Following exposure to trimethylamine the mutant did not produce trimethylamine monooxygenase, trimethylamine *N*-oxide aldolase or dimethylamine monooxygenase, which were detectable in the wild-type at specific activities of 8.3, 0.8 and 5.6 munits (mg protein)⁻¹, respectively. These specific activities, in addition to several quoted in Table 1, are low compared with those reported by Large & Carter (1973) and Boulton & Large (1977); this difference is probably due to extracts being prepared from cells harvested well into stationary phase of growth.

Table 1. *Enzyme activities in wild-type and mutant strains of P. aminovorans*

Cells were grown on succinate medium before harvesting and resuspending in medium containing 0.5% methylamine hydrochloride as sole carbon source. Cells were harvested and washed after 48 h and extracts were prepared as described in Methods.

Enzyme	Specific activity [munits (mg protein) ⁻¹]				
	Wild-type	M ₁	M ₂	M ₄	M ₅
Hydroxypyruvate reductase	256	32	0	0	0*
Serine-glyoxylate aminotransferase	27.4	0	0	0	0
Isocitrate lyase	15.3	13.6	4.7	0	0
Phosphoenolpyruvate carboxylase	32.7	0	0	8.1	0*
N-Methylglutamate synthase	5.4	—	—	—	3.0
N-Methylglutamate dehydrogenase	1.24	0	0	0	0
γ-Glutamylmethylamide synthetase	12.3	—	—	—	0
N-Methylalanine dehydrogenase	16.3	13.8	19.4	19.1	23.8
Dye-linked formaldehyde dehydrogenase	1.5	0	0	0	0
NAD ⁺ , glutathione-linked formaldehyde dehydrogenase	1630	1810	1150	1570	1710
Formate dehydrogenase	3.2	0	0	0	0
NADP ⁺ -linked isocitrate dehydrogenase	17.2	—	—	—	16.5

0, Not detected; —, not tested.

* Upon completion of the assay with mutant extract, addition of extract of wild-type organism started the reaction, indicating that the absence of activity in the mutant was not due to an inhibitor in the mutant preparation.

Isocitrate lyase activity was not detected in M₅ whereas the enzyme had a specific activity of 15.3 munits (mg protein)⁻¹ in crude extracts of wild-type organism grown on methylamine. The presence of isocitrate dehydrogenase in wild-type and M₅ extracts suggests that the measured isocitrate lyase activity is not an artefact caused by the activity of isocitrate dehydrogenase (see Attwood & Harder, 1977). Glyoxylate was detected as its dinitrophenylhydrazone from analysis of terminated isocitrate lyase reaction mixtures, whereas there was no evidence for the formation of 2-oxoglutarate.

Attempted isolation of revertants of mutant M₅

Attempts using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to isolate revertants of M₅ capable of growth on methylamine were unsuccessful.

DISCUSSION

We have isolated pleiotropic mutants of *P. aminovorans* unable to metabolize methylamines. These mutants, with the possible exception of M₁, seem to be fundamentally similar in having lost many of the enzymes which have been postulated as being involved in the oxidation of methylamine and the allied biosynthetic pathways in *P. aminovorans*. Simultaneous loss of several related functions is generally indicative of common regulatory systems for the genes involved. Pleiotropy of this sort in other bacteria has been found to result from deletion, polar nonsense or frameshift mutations in an operon, or from mutations in regulatory genes (Goldberger & Berberich, 1966; Yanofsky & Ito, 1966; Cohn & Monod, 1953). Any of these kinds of mutations could explain the phenotypes of the mutants described here, although the first three require the affected genes to be physically linked. Linkage of some genes for C₁-related functions has been reported in the methylotroph *Methylobacterium organophilum* (O'Connor & Hanson, 1978), but no linkage data is available for *P. aminovorans*.

An alternative explanation for the phenotype of mutant M_5 would be the loss of a plasmid coding for the affected genes. The presence of low but detectable levels of some C_1 enzymes in mutants M_1 , M_2 and M_4 makes this unlikely unless these mutants contained lesions on the plasmid, while mutant M_5 has lost the plasmid completely. Several plasmids coding for catabolic pathway genes have been described in *Pseudomonas* spp. (Williams & Worsey, 1976). Further work must centre on attempts to isolate such a structure, as has been done, for example, with *P. putida* (Palchaudhuri & Chakrabarty, 1976). Bacterial strains have typically been 'cured' of plasmids by treatment with mitomycin C, an inhibitor of DNA synthesis (Iyer & Szybalski, 1963), and it would be interesting to discover whether the ability to metabolize methylamines could be lost from *P. aminovorans* by treatment with this mutagen.

Loss of a plasmid would account for our lack of success in isolating revertants of the *P. aminovorans* mutants capable of growth on methylamine. Alternative explanations are that the ultraviolet light-induced mutation is either a deletion of a base pair or is due to multiple lesions. The probability of obtaining a mutant with several single lesions is essentially negligible.

The presence of *N*-methylalanine dehydrogenase, *N*-methylglutamate synthase and NAD^+ ,glutathione-linked formaldehyde dehydrogenase activities in the mutants suggests that they are regulated independently from the other enzymes postulated as being involved in C_1 metabolism. *N*-Methylalanine dehydrogenase (Boulton & Large, 1977) and *N*-methylglutamate synthase both appear to be constitutive enzymes in *P. aminovorans* and it seems that they may have a more general role in the economy of the cell. It is conceivable that they are functionally related. The demonstration of γ -glutamylmethylamide synthetase in the wild-type is the first report of this activity in *P. aminovorans*. Both this enzyme and *N*-methylglutamate dehydrogenase are absent from mutant M_5 . Loginova *et al.* (1976) reported that when trimethylamine-grown *Hyphomicrobium vulgare* ZV was pulsed with [^{14}C]methylamine or [^{14}C]trimethylamine the label appeared earliest in γ -glutamylmethylamide, with *N*-methylglutamate being next labelled. They concluded that methylamine entered metabolism via this route with an unidentified enzyme catalysing the interconversion of these compounds. Studies with *Hyphomicrobium* x supported this conclusion (Meiberg & Harder, 1978). Whether γ -glutamylmethylamide and *N*-methylglutamate are intermediates on a common pathway in *P. aminovorans* also, or are separate ports of entry for methylamine into metabolism, is as yet unknown.

From the results of this study, no definite conclusions can be drawn concerning the role of the formaldehyde dehydrogenases present in *P. aminovorans* (i.e. which is involved in amine metabolism, or whether both take part). However, it would appear that it is the dye-linked activity which is regulated with the other enzymes of C_1 metabolism suggesting that its role may be specifically linked to growth on C_1 compounds. The nicotinamide nucleotide-linked activity may have a less specific role; the latter enzyme certainly seems to be regulated independently from the C_1 -specific enzymes. Both enzymes are induced during growth of *P. aminovorans* on trimethylamine (Boulton & Large, 1977). [It should be noted that the use of non-physiological electron acceptors is unlikely to provide true activity values – this has been discussed previously with regard to *N*-methylglutamate dehydrogenase (Bamforth & Large, 1977) – and therefore the two formaldehyde dehydrogenases may in fact not be so disproportionate in activity.] The possibility of a plasmid in *P. aminovorans* coding for C_1 functions might offer an explanation for the presence of two formaldehyde dehydrogenases in this organism. If methylotrophy in *P. aminovorans* were the result of the receipt of a ' C_1 plasmid' from an unknown donor, then it is conceivable that the dye-linked formaldehyde dehydrogenase was acquired via this plasmid, and the NAD^+ ,glutathione-linked activity was already present as a chromosomal gene.

The demonstration of glyoxylate but not 2-oxoglutarate as product in the isocitrate lyase reaction mixture seems to confirm that the serine pathway of carbon assimilation in

P. aminovorans is of the icl⁺ variety (Large & Carter, 1973). Quayle (1975) has calculated that a specific activity for isocitrate lyase of 115 munits (mg protein)⁻¹ is required to support a 5 h doubling time for an icl-serine pathway organism. Considering that the doubling time of *P. aminovorans* growing on methylamine is about 6 h (Bamforth & Large, 1977) our reported activities are low, as were those of Large & Carter (1973).

In view of the apparent loss of phosphoenolpyruvate carboxylase activity in mutant M₅, its ability to grow using glucose and pyruvate as carbon sources raises the question of the nature of the anaplerotic enzyme(s) replenishing the tricarboxylic acid cycle. It will be interesting to investigate whether *P. aminovorans* can synthesize two different phosphoenolpyruvate carboxylase enzymes as in *Pseudomonas* MA (Newaz & Hersh, 1975).

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