The Metabolism of Pyruvate by the Facultative Methylotroph *Pseudomonas* AM1

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Thirteen mutants unable to grow on pyruvate have been isolated from the facultative methylotroph *Pseudomonas* AM1. Five of these lacked 2-oxoglutarate dehydrogenase and were thus obligate methylotrophs; one lacked the E1 (decarboxylase) component and the others probably lacked the E2 (transsuccinylase) component, but this could not be confirmed because transacylases could not be measured in the oxo-acid dehydrogenases of *Pseudomonas* AM1. Six mutants lacked pyruvate dehydrogenase (probably the E2, transacetylase, component) and one lacked both oxo-acid dehydrogenases. Although unable to oxidize pyruvate, one of the pyruvate dehydrogenase mutants was able to catalyse a coenzyme A-independent decarboxylation of pyruvate. The growth properties of the pyruvate dehydrogenase mutants confirmed that the loss of this enzyme is sufficient to confer on a previously facultative methylotroph the properties of a restricted facultative methylotroph similar to the hyphomicrobia. One mutant was only able to grow on C₁, C₂ and C₃ compounds when a supplement of glyoxylate was added but was able to grow on other multicarbon compounds such as succinate. This confirms that there is a common reaction in the pathways for assimilation of C₁, C₂ and C₃ compounds in this organism.

INTRODUCTION

Pseudomonas AM1 is a pink facultative methylotroph which grows on methanol and other C₁ compounds by way of the serine pathway (Quayle, 1972; Anthony, 1975). The usual routes for assimilation of ethanol, 3-hydroxybutyrate and pyruvate cannot operate in this bacterium because it lacks isocitrate lyase, pyruvate carboxylase, phosphoenolpyruvate synthetase and pyruvate, orthophosphate dikinase. Acetyl-CoA, produced from ethanol, 3-hydroxybutyrate or pyruvate has been thought to be assimilated by way of an unconventional route (Dunstan *et al.*, 1972*a*; Dunstan & Anthony, 1973; Salem *et al.*, 1973; Anthony, 1975), which was originally called the malate synthase pathway because this enzyme was thought to catalyse condensation of acetyl-CoA and glyoxylate produced by oxidation of acetyl-CoA. However, it has now been shown that 'malate synthase' activity in *Pseudomonas* AM1 is due to the sequential action of malyl-CoA lyase and malyl-CoA hydrolase (Cox & Quayle, 1976; Taylor & Anthony, 1976*a*), and the growth on ethanol, 3-hydroxybutyrate and pyruvate of mutant PCT57, lacking malyl-CoA lyase, also argues against the existence of this pathway.

Because of the growth properties of this mutant (Dunstan *et al.*, 1972*b*; Salem *et al.*, 1974) all discussions of assimilation of C₂ and C₃ compounds have had to include the possibility of alternative (unknown) routes for assimilation of these compounds (Cox & Quayle, 1976; Taylor & Anthony, 1976*a*).

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In order to explore this problem further, we set out to isolate mutants lacking pyruvate dehydrogenase in the hope that a study of assimilation of [^{14}C]pyruvate in such a mutant might provide information on the existence or otherwise of these postulated alternative routes in *Pseudomonas* AM1. This paper describes the isolation and characterization of such mutants and of mutants lacking 2-oxoglutarate dehydrogenase. The properties of the pyruvate dehydrogenase mutants confirm that lack of this enzyme is sufficient to confer on a previously facultative methylotroph the acquired property of restricted facultative methylotrophy as observed in the hyphomicrobia.

METHODS

Growth media and maintenance of cultures. The growth medium was based on that of MacLennan *et al.* (1971). It contained 3.6 g $(\text{NH}_4)_2\text{SO}_4$ l^{-1} ; to this was added concentrated phosphoric acid to a final concentration of 20 mM. Stock trace elements solution was added at 5 ml l^{-1} , and the pH was adjusted to 7.0 with NaOH/KOH. The trace elements solution contained the following (g l^{-1}): $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.06; MnSO_4 , 0.04; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.008; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.008; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.008; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.008; H_3BO_3 , 0.006; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.80. The growth medium was sterilized by autoclaving at 121 °C for 20 min and sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution was added to give 0.9 g l^{-1} . Concentrated stock solutions of carbon sources were adjusted to pH 7.0 when necessary and sterilized; they were then added, aseptically, to the sterile salts medium to give a final concentration of 0.2 % (w/v) except for methanol and ethanol (0.5 %, v/v) and methylamine (0.4 %, w/v). Methanol, methylamine, ethanol and succinate were sterilized by autoclaving. All other carbon sources were sterilized by passage through a Millipore filter (type HA, pore size 0.45 μm).

Stock cultures were maintained on methylamine- or succinate-agar slopes at 4 °C and were subcultured every 2 months.

Growth and harvesting of cultures. Cultures (500 ml) were grown in shaken 2 l baffled flasks at 30 °C from a 5 % inoculum of bacteria grown on the same carbon source. Growth responses of mutants were checked before harvesting (using liquid or solid media) to ensure that wild-type contaminants or spontaneous revertants were absent. Bacteria were harvested in the late-exponential phase of growth by centrifugation at 4000 g for 15 min. Before use, the bacteria were washed twice in 20 mM-sodium/potassium phosphate buffer, pH 7.0.

Isolation of mutants. Mutants were isolated by a method, based on that of Heptinstall & Quayle (1970), designed to select for mutants unable to grow on pyruvate but able to grow on methylamine, 3-hydroxybutyrate or succinate. Bacteria treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were grown for gene expression in appropriate permissive growth medium and then treated three times with benzylpenicillin (1000 i.u. ml^{-1} medium) in the presence of pyruvate. Revertants were isolated on pyruvate-agar plates from streptomycin-resistant derivatives of the mutants by the method described by Taylor & Anthony (1976a). The growth characteristics of mutant bacteria were determined in liquid media as described by Taylor & Anthony (1976b).

Measurement of oxygen uptake by bacterial suspensions. O_2 uptake by suspensions of washed bacteria was measured using a Rank O_2 electrode at 30 °C. The incubation vessel contained, in 2 ml, 40 μmol sodium/potassium phosphate buffer, pH 7.0 and sufficient bacteria to give an O_2 uptake rate of 0.5 to 1.5 $\mu\text{l min}^{-1}$ in the presence of substrate. The endogenous rate of O_2 uptake was followed for 5 to 6 min before substrate was injected. Substrates were used at a final concentration of 7.5 mM except for formate (40 mM). Rates of O_2 uptake were calculated by assuming that 0.45 $\mu\text{mol O}_2$ is dissolved in 2 ml buffer at 30 °C.

Preparation of cell-free extracts. Washed bacteria were suspended in cold 20 mM-potassium phosphate buffer, pH 7.0 to about 0.3 g wet wt ml^{-1} and disrupted at 2 °C for 3 $\times$ 2 min periods (separated by 3 min cooling periods) in a 100 W MSE ultrasonic disintegrator at 20 kHz using a probe tip of 1.9 cm diam. The sonicate was centrifuged at 2 °C at 3000 g for 15 min to remove unbroken bacteria before centrifuging at 40000 g for 1 h. The pellet from the second centrifugation was resuspended in a portion of buffer equal in volume to the original volume of the extract; this membrane-containing preparation was used for assays of succinate dehydrogenase.

Enzyme assays. All spectrophotometric assays were done in a Pye Unicam SP8000 recording spectrophotometer at 25 °C. The following enzymes were assayed by published procedures: citrate synthase, EC 4.1.3.7 (Srere, 1969); aconitate hydratase, EC 4.2.1.3, and fumarate hydratase, EC 4.2.1.2 (Bergmeyer, 1974); isocitrate dehydrogenase (NADP^+), EC 1.1.1.42 (Ochoa, 1955a); malate dehydrogenase EC 1.1.1.37 (Ochoa, 1955b). Succinyl-CoA synthetase, EC 6.2.1.5, was assayed by the hydroxamate

method of Kaufman (1955) and membrane-bound succinate dehydrogenase, EC 1.3.99.1, was assayed according to the method of Veeger *et al.* (1969). Malate synthase was assayed by the method of Dixon & Kornberg (1959). Overall 2-oxoglutarate and pyruvate dehydrogenase activities were assayed by an adaptation of the method of Guest & Creaghan (1973). The reaction mixture contained (μmol , in 1 ml final volume): Tris/HCl pH 8.5, 100; L-cysteine.HCl, 3.0; coenzyme A, 0.1; thiamin pyrophosphate, 0.2; NAD^+ , 0.8; sodium pyruvate or sodium 2-oxoglutarate, 7.5; extract. After incubating for 2 to 3 min, the reaction was started by adding oxo-acid and the absorbance at 340 nm was followed. A similar method was used to test for reconstitution of overall 2-oxoglutarate or pyruvate dehydrogenase activity from different sources of components. The two sources of components (about 2 mg total protein) were incubated in the reaction mixture for 5 to 10 min before adding the oxo-acid. Either NAD^+ or acetylpyridine adenine dinucleotide (APAD), both at 1.5 mM, was used in reconstitution assays (reduction of APAD was followed at 366 nm).

The decarboxylase (E1) components of the 2-oxo-acid dehydrogenase complexes were assayed spectrophotometrically at 420 nm with ferricyanide as the electron acceptor (method modified from Hager & Kornberg, 1961). The reaction mixture contained (μmol , in 1 ml final volume): potassium phosphate, pH 6.5, 100; thiamin pyrophosphate, 0.2; potassium ferricyanide, 2.5; magnesium sulphate, 5; sodium 2-oxoglutarate or sodium pyruvate, 20; extract, up to 2 mg protein. After incubation for 2 to 3 min, the reaction was started by adding oxo-acid.

The lipoamide dehydrogenase (E3) component was assayed by recording the lipoamide-dependent oxidation of NADH at 340 nm by a modified assay of Massey (1966). The reaction mixture contained (μmol , in 1 ml final volume): potassium phosphate, pH 6.5, 350; EDTA, 1; NADH, 2; NAD^+ , 2; lipoamide (oxidized form), 1; bovine serum albumin was included to a concentration of 0.1 % (w/v). Endogenous activity was monitored for 2 to 3 min before starting the reaction by adding lipoamide. Assays for the lipoate transacetylase and lipoate transsuccinylase (E2) components of the pyruvate and 2-oxoglutarate dehydrogenases were performed using the method of Knight & Gunsalus (1962).

Reconstitution experiments. Assays were performed as described above; mutants of *Escherichia coli* were maintained and grown, and extracts were prepared as described by Guest & Creaghan (1973). These mutants were kindly supplied by Dr J. R. Guest. Mutant *lpd1* lacked lipoamide dehydrogenase, *aceE2* lacked the E1 component of pyruvate dehydrogenase and *aceF10* lacked the transacetylase (E2) component of pyruvate dehydrogenase. Before using the extracts in mixed reconstitution experiments with extracts of *Pseudomonas* AM1, it was shown that pyruvate dehydrogenase activity could be reconstituted using these *E. coli* extracts.

Analysis of growth medium. Amino acids present in the growth medium of bacterial cultures were analysed on a JEOL (model JLC 6AH) amino acid analyser with the help of Mr P. Vincent and Dr M. Gore (Southampton University). Culture samples were centrifuged at 15000 *g* for 15 min and the supernatant was filtered to remove any remaining bacteria. Samples of 2.5 ml were analysed after adjusting the pH to 2.0. Norleucine was used as an internal standard. Lysine was assayed separately by the spectrophotometric method of Vogel & Shimura (1971).

The concentration of methanol in cultures was measured by gas-liquid chromatography with the help of Dr D. L. Corina (Southampton University). Samples were centrifuged as described above and the supernatants were assayed using a Pye Unicam 104 gas chromatograph. Methanol was quantified using a column of 10 % (w/w) Carbowax 1540 on Teflon 6 at a temperature of 80 °C.

The distribution of 2-oxo-acid dehydrogenase components after ultracentrifugation. Cultures (500 ml) of wild-type *Pseudomonas* AM1 and mutants JAB10, JAB21 and JAB30 were grown using methanol (0.5 %, v/v) plus pyruvate (10 mM) as carbon sources. The bacteria were harvested and washed and sonic extracts were prepared as described above. These were centrifuged at 40000 *g* for 1 h at 2 °C. The supernatants obtained were carefully decanted and 8 ml of each was then centrifuged at 400000 *g* for 1.5 h in an MSE Superspeed 65 ultracentrifuge with the rotor temperature maintained at 2 °C. After ultracentrifugation, the top 4 ml of each supernatant and the bottom portion were decanted into separate vials and the pellets were resuspended in 8 ml of cold 20 mM-potassium phosphate buffer, pH 7.0. The supernatant from the initial centrifugation and the ultracentrifuged fractions were then assayed for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase and also for the E1 and E3 components of these enzymes. Attempts to assay the E2 components failed.

Measurements of radioactivity. Radioactivity was measured using an Intertechnique SL30 liquid scintillation spectrometer programmed for automatic quench correction. Radioactivity in liquid samples was determined by placing the sample (up to 1 ml) in a scintillation vial containing 1 ml methanol and 10 ml 'Tritoscint' scintillation fluid (see below). Radioactivity in bacteria was determined by filtering samples (usually 3 ml) through a 25 mm diam. Millipore filter (type HA, pore size 0.45 μm) previously washed with 2 ml 20 mM-pyruvate. The bacteria on the filter were washed with 10 ml distilled water, 2 ml formic acid (5 %, v/v) to remove dissolved [^{14}C]bicarbonate and finally with a further 5 ml distilled water.

The filter was then transferred to a scintillation vial containing 0.5 ml hyamine hydroxide (1 M solution in methanol) to solubilize the bacteria and incubated at 40 °C for 30 min. After this period, 0.5 ml glacial acetic acid and 10 ml 'Tritoscint' scintillation fluid were added. Background values were estimated by using a portion of the final water-washing to soak a filter which was then treated in the same way as described above. Radioactivity of K_2CO_3 on filter paper was determined by transferring the filter papers to scintillation vials containing 1 ml distilled water and leaving for 15 min to equilibrate before adding 1 ml methanol and 10 ml 'Tritoscint' scintillation fluid.

'Tritoscint' scintillation fluid. This contained, in 1 l: 670 ml xylene, 330 ml Symperonic NXP (phenyl-ethoxylate non-ionic detergent), 4 g PPO (2,5-diphenyloxazole), 0.3 g dimethyl POPOP [1,4-di-2-(4-methyl-5-phenyloxazolyl)benzene].

$^{14}CO_2$ evolution from [^{14}C]pyruvate catalysed by wild-type and mutant bacteria. Wild-type *Pseudomonas* AM1 and mutant JAB21 (lacking pyruvate dehydrogenase activity) were grown on methanol (0.5 %, v/v) plus pyruvate (10 mM), harvested in the late-exponential phase, washed twice and resuspended in salts medium. Wild-type and mutant bacteria (10 mg dry wt) were incubated with either [$1-^{14}C$]pyruvate or [$3-^{14}C$]pyruvate (20 mM; 1.25 μ Ci, 46 kBq) and evolution of $^{14}CO_2$ and incorporation of ^{14}C into bacteria were measured. Incubations were carried out in salts medium (2.2 ml) in the main compartment of a Warburg manometer flask. The centre well contained 0.2 ml 10 % (w/v) KOH on a filter paper to absorb CO_2 . Concentrated sulphuric acid (50 μ l) was added from a side-arm at the end of the experiment to release dissolved CO_2 .

$^{14}CO_2$ evolution from [$1-^{14}C$]pyruvate catalysed by cell-free systems from wild-type and mutant bacteria. Cell-free systems of wild-type *Pseudomonas* AM1 and mutant JAB21 (lacking pyruvate dehydrogenase) were prepared by resuspension of pellets obtained by ultracentrifugation in 20 mM-potassium phosphate buffer, pH 7.0. The bacteria were grown on methanol plus pyruvate (10 mM). Evolution of $^{14}CO_2$ was measured as described above. The reaction mixture contained (μ mol, in 1.5 ml final volume): Tris/HCl, pH 8.5, 150; potassium phosphate, pH 8.5, 20; cysteine.HCl, 6; coenzyme A, 0.25; thiamin pyrophosphate, 0.4; NAD^+ , 0.75; [$1-^{14}C$]pyruvate (0.5 μ Ci, 19 kBq), pH 8.5, 40; lactate dehydrogenase (pig heart), 3.5 units; phospho-transacetylase (Clostridium), 7.5 units; extract, 5 mg protein.

RESULTS

Mutants of Pseudomonas AM1 unable to grow on pyruvate

Thirteen mutants were selected for their failure to grow on pyruvate. Five of these lacked 2-oxoglutarate dehydrogenase, six lacked pyruvate dehydrogenase, one lacked both dehydrogenases and one lacked an enzyme in the unknown assimilatory pathway.

Mutants lacking 2-oxoglutarate dehydrogenase. Four of these mutants (JAB10, 11, 12 and 13) were isolated using methylamine in the permissive growth medium; in most respects these were similar to mutant ICT41 (Taylor & Anthony, 1976b) which was selected as being capable of growth on methanol but not on 3-hydroxybutyrate; they were obligate methylotrophs except for very slight growth on ethanol. Supplements of succinate (5 mM) failed to promote growth on pyruvate or 3-hydroxybutyrate.

Most substrates were oxidized at similar rates to those in wild-type bacteria, but endogenous substrate, 3-hydroxybutyrate, pyruvate and lactate were oxidized at diminished rates (Table 1). Of all the enzymes tested, only 2-oxoglutarate dehydrogenase was absent (Table 2). Like mutant ICT41, mutants JAB10 to 13 lacked overall 2-oxoglutarate dehydrogenase activity but had wild-type levels of the E1 and E3 components (Table 3). The activity of the E2 component was negligible in crude extracts of wild-type bacteria and so the characterization of these mutants as lacking the E2 (transsuccinylase) component can only be tentative; the reason for the difficulty of assaying this component is not known. Revertants were obtained from all four mutants and these had regained 2-oxoglutarate dehydrogenase concomitant with ability to grow on the same substrates as wild-type bacteria.

Mutant JAB14 also lacked overall 2-oxoglutarate dehydrogenase activity; it was similar in all respects to mutants JAB10 to 13 except that no revertant could be obtained and that it lacked an active E1 (decarboxylase) component of the 2-oxoglutarate dehydrogenase. This mutant had similar levels to wild-type bacteria of the E1 component of pyruvate dehydrogenase (Table 3) confirming that in *Pseudomonas* AM1 the E1 components of

Table 1. Comparison of rates of oxygen uptake by suspensions of wild-type *Pseudomonas* AM1 and derived mutants

Bacteria were grown on methanol (0.5 %, v/v) plus succinate (0.1 %, w/v). The results are expressed as a percentage of the rate obtained with wild-type bacteria and are average values for the mutants indicated (all values were within 20 % of the average value quoted). Figures in parentheses are absolute values of oxygen uptake [$\mu\text{l O}_2$ (mg dry wt) $^{-1}$ h $^{-1}$]. All values have been corrected for endogenous respiration.

Substrate for oxidation	Rate of oxygen uptake (%)			
	Wild-type	JAB10-14	JAB30	JAB20-25
Methanol	100 (220)	97	100	99
Formaldehyde	100 (230)	97	98	85
Formate	100 (92)	116	100	110
Ethanol	100 (214)	99	95	96
Succinate	100 (50)	135	105	110
Malate	100 (98)	91	98	96
3-Hydroxybutyrate	100 (20)	34	25	100
Malonate	100 (25)	0	0	22
Pyruvate	100 (30)	21	0	0
Lactate	100 (33)	28	0	0
Endogenous substrate	100 (15)	50	25	80

Table 2. Specific activities of the tricarboxylic acid cycle enzymes, pyruvate dehydrogenase and 'malate synthase' in extracts of wild-type *Pseudomonas* AM1 and derived mutants

Bacteria were grown on methanol (0.5 %, v/v) plus succinate (0.1 %, w/v). After growth, sonic extracts of washed bacteria were prepared and assayed for the enzymes listed below. The specific activities given are the average of a number of determinations using different crude extracts; all values were within 10 % of the average value quoted.

Enzyme	Specific activity [nmol min $^{-1}$ (mg protein) $^{-1}$]			
	Wild-type	JAB10-14	JAB30	JAB20-25
Citrate synthase	41	25	50	36
Aconitate hydratase	100	95	115	94
Isocitrate dehydrogenase	180	150	200	180
Succinyl-CoA synthetase	60	60	55	54
Succinate dehydrogenase	95	84	120	97
Fumarate hydratase	253	220	321	350
Malate dehydrogenase	1512	2009	2021	2200
'Malate synthase' activity	102	92	100	90
Pyruvate dehydrogenase	30	20	0	0
2-Oxoglutarate dehydrogenase	24	0	0	26

2-oxoglutarate and pyruvate dehydrogenases are separate components, as found for these enzymes in *E. coli*.

Mutants lacking pyruvate dehydrogenase (mutants JAB20 to 25). Mutant JAB20 was isolated as being able to grow on methylamine but not on pyruvate while for the other five mutants of this type (JAB21 to 25) the permissive medium contained 3-hydroxybutyrate. These mutants were similar in all respects; they all lacked overall pyruvate dehydrogenase activity but had wild-type levels of all other enzymes tested (Table 2). They oxidized malonate at diminished rates and pyruvate and lactate not at all, while oxidation of other substrates was unimpaired (Table 1). All six mutants had similar activities of the E1 (decarboxylase) and E3 (lipoamide dehydrogenase) components of pyruvate dehydrogenase but they lacked overall activity (Table 3). This suggests that they all lacked an active E2 (transacetylase) component, but, as mentioned above, this could not be tested directly.

Table 3. *Specific activities of the components of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase in crude extracts of wild-type Pseudomonas AM1 and derived mutants*

Extracts of bacteria grown on methanol (0.5 %, v/v) plus succinate (0.1 %, w/v) were prepared and assayed as described in Methods. The specific activities given are the average of a number of determinations using different crude extracts; all values were within 10 % of the average value quoted.

Enzyme component	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]				
	Wild-type	JAB10-13	JAB14	JAB30	JAB20-25
2-Oxoglutarate dehydrogenase	24	0	0	0	26
E1 component of 2-oxoglutarate dehydrogenase	12	9.6	0	15	12
Pyruvate dehydrogenase	30	20	21	0	0
E1 component of pyruvate dehydrogenase	7	5	4.2	5.3	5.3
Lipoamide dehydrogenase (E3)	45	44	30	80	42

A mutant lacking 2-oxoglutarate and pyruvate dehydrogenases (mutant JAB30). Mutant JAB30 was isolated as being able to grow on methylamine but not on pyruvate. It was an obligate methylotroph being able to grow only on C₁ compounds and on oxalate (which is assimilated by the same serine pathway as are C₁ compounds). Supplements of acetate (5 mM) did not promote growth on succinate, and supplements of succinate (5 mM) plus acetate (5 mM) did not promote growth on pyruvate. This mutant lacked overall pyruvate and 2-oxoglutarate dehydrogenases but had wild-type levels of all other enzymes tested (Table 2). As expected, the oxidative properties of this mutant were the same as those of other mutants lacking 2-oxoglutarate dehydrogenase but it also failed to oxidize pyruvate and lactate. It might be expected that a mutant lacking both dehydrogenases would lack the E3 (lipoamide dehydrogenase) component because in *E. coli* this component is common to both 2-oxoglutarate and pyruvate dehydrogenases (Guest & Creaghan, 1973). However, mutant JAB30 had undiminished activities of the lipoamide dehydrogenase and of the E1 (decarboxylase) components of the 2-oxoglutarate and pyruvate dehydrogenase complexes (Table 3). This would suggest that mutant JAB30 is a double mutant lacking both E2 components (transacetylase and transsuccinylase). It is unlikely that there is a single E2 component for both complexes because mutants JAB10 to 13 all appear to lack the E2 component of the 2-oxoglutarate dehydrogenase and yet have active pyruvate dehydrogenase; similarly, mutants JAB20 to 25 all appear to lack the E2 component of pyruvate dehydrogenase and yet have an active 2-oxoglutarate dehydrogenase. That mutant JAB30 is a double mutant is also suggested by the failure to obtain revertants of this mutant on either pyruvate or 3-hydroxybutyrate; an alternative possibility is that it has a single lesion leading to an auxotrophic requirement common to the E2 components of both dehydrogenases. However, supplements of DL-lipoate, pantothenate and thiamin (all at 0.1 µg ml⁻¹) did not allow growth of this mutant on pyruvate.

A mutant unable to convert C₃ compounds to C₄ compounds (mutant JAB40). This mutant was isolated as being able to grow on succinate but not on pyruvate. It was unable to grow on any C₁, C₂ or C₃ compound but a supplement of succinate (5 mM), glyoxylate (5 mM) or glycollate (5 mM) was sufficient to restore growth on all carbon sources. This mutant is similar to mutant PCT48 (Dunstan *et al.*, 1972*a, b*; Dunstan & Anthony, 1973) and to mutant C5 (Salem *et al.*, 1973) in its failure to grow on C₁ or C₂ compounds in the absence of glyoxylate, but it differs from them in its failure to grow on pyruvate. Revertants of none of these mutants have been obtained and so discussion is complicated by the possibility that they may be double mutants.

Amino acid excretion by mutants lacking pyruvate dehydrogenase (JAB21), 2-oxoglutarate dehydrogenase (JAB10) or both dehydrogenases (JAB30)

During growth of wild-type bacteria on methanol the only amino acid accumulating was 2-aminoadipate ($5\ \mu\text{M}$), the amount increasing when succinate (0.1 %) was included in the growth medium. This accumulation was similar in mutants JAB10 and 21 but it was doubled during growth of mutant JAB30. All three mutants accumulated alanine, valine and leucine (5 to $35\ \mu\text{M}$) during growth on methanol plus succinate and mutants lacking 2-oxoglutarate dehydrogenase (JAB10 and 30) also accumulated glutamate, glutamine and aspartate (10 to $40\ \mu\text{M}$). The mutant lacking both dehydrogenases (JAB30) also accumulated small amounts (about $5\ \mu\text{M}$) of lysine, threonine, methionine and isoleucine during growth on methanol plus succinate.

Washed suspensions of the mutant lacking pyruvate dehydrogenase (JAB21) accumulated alanine ($150\ \mu\text{M}$), valine ($20\ \mu\text{M}$) and leucine ($10\ \mu\text{M}$) when incubated for 1.5 h in an aerated suspension with pyruvate (the suspension contained 30 mM-pyruvate and 30 mg dry wt bacteria). In a similar experiment with wild-type bacteria, 2-aminoadipate was the only amino acid excreted; this did not accumulate during incubation of the mutant with pyruvate.

Most of these accumulations are consistent with the lesions proposed in the mutants (above) but the accumulation by wild-type and mutants of 2-aminoadipate was unexpected, this acid being an intermediate in the pathway for lysine biosynthesis in eukaryotes but not in bacteria.

Homocitrate and homoisocitrate are precursors of 2-aminoadipate in the eukaryotic pathway for lysine biosynthesis and they are also intermediates in the proposed homoisocitrate-glyoxylate pathway for oxidation of acetyl-CoA to glyoxylate in *Pseudomonas* AM1 (Kortstee, 1980). The accumulation of 2-aminoadipate may reflect high concentrations of homocitrate and homoisocitrate in this organism and may thus support the conclusions of Kortstee (1980). Indeed, observation of aminoadipate accumulation in this organism has led to an informal proposal for the homoisocitrate lyase route for this oxidation previously (Taylor, 1975; Bolbot, 1979), but the low activities of homocitrate synthase appeared to rule this out. If this enzyme does operate in *Pseudomonas* AM1 then higher concentrations of one of its substrates (2-oxoglutarate), likely in a mutant (JAB30) lacking 2-oxoglutarate dehydrogenase, might be expected to lead to the observed accumulation of 2-aminoadipate.

The distribution of 2-oxo-acid dehydrogenase after ultracentrifugation

It was concluded that, because many of the mutants had wild-type activities of the E1 and E3 components, they probably lack an active transacylase (E2) component of the dehydrogenase complex. An alternative explanation is that active components might be altered such that aggregation of the complex no longer occurs thus giving an inactive enzyme. To test this possibility the distribution of each component was determined after centrifugation of crude extracts at $400\,000\ g$ for 1.5 h. The results in Table 4 show that in extracts of wild-type bacteria and mutants JAB10, 21 and 30, the distribution was similar. As expected, when present, the dehydrogenase complexes and the lipoamide (E3) component sedimented together. The decarboxylation components (E1) appeared to sediment together with the complex but no E1 activity could be detected in the resuspended pellet from wild-type or mutants. A similar result was obtained with pyruvate dehydrogenase from *E. coli* (Dietrich & Henning, 1970). These workers found that addition of the supernatant to the resuspended pellet led to reactivation of the enzyme in the ferricyanide assay for the E1 component of the complex and they presumed that an unknown factor was involved. Addition of supernatant to the resuspended pellet obtained from wild-type *Pseudomonas* AM1 did not result in a reactivation of either activity, the rate measured being the same as that obtained in

Table 4. *The distribution of 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase and their components after ultracentrifugation of extracts of Pseudomonas AM1*

The results are expressed as percentages of the total activity present in the crude extract before ultracentrifugation. Experimental procedures are described in detail in Methods. After ultracentrifugation, the distribution of pyruvate and 2-oxoglutarate dehydrogenases in extracts from mutants JAB10 and JAB21, respectively, were similar to those observed in extracts of wild-type bacteria.

Enzyme component	Activity (%) in fractions of ultracentrifuged extract		
	Top 4 ml	Bottom 3.5 ml	Resuspended pellet
Wild-type			
2-Oxoglutarate dehydrogenase	5	35	44
E1 component of 2-oxoglutarate dehydrogenase	12	55	ND
Pyruvate dehydrogenase	3	16	66
E1 component of pyruvate dehydrogenase	13	53	ND
Lipoamide dehydrogenase (E3)	7	27	55
Mutant JAB10			
2-Oxoglutarate dehydrogenase	ND	—	ND
E1 component of 2-oxoglutarate dehydrogenase	10	57	—
Lipoamide dehydrogenase (E3)	10	30	51
Mutant JAB30			
2-Oxoglutarate dehydrogenase	ND	—	ND
E1 component of 2-oxoglutarate dehydrogenase	11	50	—
Pyruvate dehydrogenase	ND	—	ND
E1 component of pyruvate dehydrogenase	8	54	—
Lipoamide dehydrogenase (E3)	9	36	45
Mutant JAB21			
Pyruvate dehydrogenase	ND	—	ND
E1 component of pyruvate dehydrogenase	15	53	ND
Lipoamide dehydrogenase (E3)	9	30	56

ND, Not detected; —, not measured.

the absence of resuspended pellet. It is difficult to explain this result because large proportions of both dehydrogenase activities were recovered from the ultracentrifuged pellet. It should be noted that the E1 component must be present in the complex even when it cannot be assayed with the usual ferricyanide assay system.

These results suggest that the lesions in the mutants described here produce inactive components rather than components markedly altered in their ability to aggregate into active complexes.

Failure to reconstitute active dehydrogenase by mixing extracts from mutants ('in vitro' complementation)

In the absence of a suitable assay for the E2 (transacylase) components it was hoped to demonstrate its absence from some mutants by mixing extracts of these with other mutants having this component but lacking another of the components of the same dehydrogenase complex; such reconstitution experiments have been successful with pyruvate dehydrogenase mutants of *E. coli* (Guest & Creaghan, 1973). However, no reconstitution could be demonstrated when extracts of JAB14 (lacking in E1 component of 2-oxoglutarate dehydrogenase) were mixed with extracts of other 2-oxoglutarate dehydrogenase mutants in which the E2 might be lacking. Parallel reconstitution experiments could not be done with the pyruvate dehydrogenase mutants because these all seemed to have the same lesion (no E2 component). No reconstitution of activity could be measured with extracts of these mutants when mixed with extracts of mutants of *E. coli* lacking either the E1, E2 or E3 complexes of pyruvate dehydrogenase. These experiments thus failed to provide evidence for or against the conclusion that the pyruvate dehydrogenase mutants all lack an active E2 component.

Table 5. *Specific activities of 2-oxoglutarate and pyruvate dehydrogenases in wild-type Pseudomonas AM1 grown on various carbon sources*

Bacteria were grown to the end of the exponential phase in 500 ml batch cultures. After harvesting, crude extracts of suspensions of the washed cells were prepared and assayed for 2-oxoglutarate and pyruvate dehydrogenase activities as described in Methods.

Growth substrate	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	2-Oxoglutarate dehydrogenase	Pyruvate dehydrogenase
Methanol	12	4
3-Hydroxybutyrate	60	22
Pyruvate	90	120
Succinate	110	50
Methanol + succinate	24	30

Oxidation of 2-oxobutyrate

Although unable to grow on 2-oxobutyrate, this substrate was oxidized by whole bacteria at about 30 % of the rate measured with pyruvate. Crude extracts catalysed a coenzyme A-dependent reduction of NAD⁺ when 2-oxobutyrate replaced pyruvate in the standard pyruvate dehydrogenase assay system, the rate being about 30 % of that measured with pyruvate. Mutants (whole bacteria and extracts) lacking pyruvate dehydrogenase (JAB20 to 25 and JAB30) did not oxidize 2-oxobutyrate, whereas the oxidation of this substrate was unaffected in mutants lacking only 2-oxoglutarate dehydrogenase (JAB10 to 14). These results suggest that the pyruvate dehydrogenase of *Pseudomonas* AM1 is similar to that in *E. coli* in being able to oxidize 2-oxobutyrate (Dietrich & Henning, 1970).

Activity of pyruvate and 2-oxoglutarate dehydrogenases in Pseudomonas AM1 grown on various carbon sources

The results in Table 5 support the conclusion that pyruvate and 2-oxoglutarate dehydrogenases are relatively unimportant during growth on methanol compared with growth on multicarbon compounds, the activities of these enzymes on methanol being less than 20 % of the activity on any other growth substrate tested.

Decarboxylation of [¹⁴C]pyruvate by mutant and wild-type bacteria

It was hoped that pyruvate dehydrogenase mutants isolated in this work might be used to study pyruvate assimilation into cell material in a system where no assimilation after decarboxylation could occur. However, preliminary experiments with mutant JAB21 indicated that this would not be possible because so long as methanol was provided as an energy source during growth, [1-¹⁴C]- and [3-¹⁴C]pyruvate were assimilated in a similar manner in mutant and wild-type bacteria, the specific activities of the bacteria in both cases being greater with [3-¹⁴C]pyruvate than with [1-¹⁴C]pyruvate. Similar proportions were obtained in shorter experiments in which metabolism by washed cell suspensions in the absence of methanol was measured.

These results indicated that although the mutant lacked overall pyruvate dehydrogenase activity, it was able to decarboxylate pyruvate and to assimilate the decarboxylation product. This is confirmed in Table 6. The rate of decarboxylation and assimilation of [1-¹⁴C]pyruvate in the mutant was about half that in the wild-type but the proportion of [1-¹⁴C]pyruvate metabolized to CO₂ compared with that assimilated into cell material was similar. In contrast, the rate of decarboxylation and assimilation of [3-¹⁴C]pyruvate by the mutant was about 20 % of that in wild-type bacteria; again, the relative amounts assimilated

Table 6. $^{14}\text{CO}_2$ evolution from $[^{14}\text{C}]$ pyruvate by wild-type *Pseudomonas* AM1 and a mutant lacking pyruvate dehydrogenase

Wild-type *Pseudomonas* AM1 and mutant JAB21 (lacking pyruvate dehydrogenase) (10 mg dry wt) were incubated with either $[1-^{14}\text{C}]$ pyruvate or $[3-^{14}\text{C}]$ pyruvate (20 mM; 1.25 μCi); the $^{14}\text{CO}_2$ evolved (measured as $\text{K}_2^{14}\text{CO}_3$) and the incorporation of ^{14}C into bacteria were measured after incubation at 30 °C for 1.5 h. The values given are percentages of the total radioactivity present at the start of the incubation. In parentheses, these values are expressed as a percentage of the total pyruvate metabolized. The experimental procedure is described in Methods. The inability of the mutant suspension to oxidize pyruvate was confirmed using an oxygen electrode; the wild-type QO_2 for pyruvate was 24.0 $\mu\text{l O}_2$ (mg dry wt) $^{-1}$.

	Wild-type bacteria		Mutant JAB21	
	$[1-^{14}\text{C}]$ - pyruvate	$[3-^{14}\text{C}]$ - pyruvate	$[1-^{14}\text{C}]$ - pyruvate	$[3-^{14}\text{C}]$ - pyruvate
$^{14}\text{CO}_2$ evolved	55 (81)	16 (25)	28 (78)	3 (25)
^{14}C in bacteria	13 (19)	48 (75)	8 (22)	9 (75)

and decarboxylated were similar in wild-type and mutant. The main difference between wild-type and mutant was the much lower rate of CO_2 production from $[3-^{14}\text{C}]$ pyruvate in the mutant compared with wild-type bacteria; this was presumably because initial decarboxylation of pyruvate in the mutant gave a different C_2 product (possibly acetaldehyde) which could only slowly be decarboxylated.

Measurements of $^{14}\text{CO}_2$ evolution from $[1-^{14}\text{C}]$ pyruvate catalysed by cell-free extracts were consistent with this possibility. While the rates of CO_2 evolution were similar using an extract of wild-type or mutant bacteria the decarboxylation process in the mutant did not require coenzyme A. Thus, the rate catalysed by extracts of wild-type bacteria in the absence of coenzyme A was only 9 % of that in its presence whereas omission of coenzyme A only decreased the rate catalysed by extracts of mutant bacteria to 83 % of that in its presence. The rate catalysed by extracts of mutant bacteria in the complete system was 82 % of that catalysed by extracts of wild-type bacteria in the complete system.

The results are all consistent with the suggestion (above) that mutant JAB21 lacks overall pyruvate dehydrogenase activity because it has an altered E2 (transacetylase) component; the E1 (decarboxylase) component is able to catalyse the decarboxylation of pyruvate to a C_2 compound which cannot be transferred by an E2 component to coenzyme A to give acetyl-CoA.

DISCUSSION

The characteristics of the pyruvate dehydrogenase of *Pseudomonas* AM1 described above show that it is not markedly different from the pyruvate dehydrogenases from other sources (see Guest, 1979).

Because the mutant lacking pyruvate dehydrogenase (JAB21) was still able to decarboxylate, but not oxidize, pyruvate it was impossible to use this mutant to study the assimilation of $[^{14}\text{C}]$ pyruvate as had been intended (see Introduction).

The growth properties of the mutants lacking pyruvate dehydrogenase (growth only on C_1 and C_2 compounds and 3-hydroxybutyrate) are similar to those of hyphomicrobia (Harder & Attwood, 1978) confirming the suggestion of Harder *et al.* (1975) that lack of pyruvate dehydrogenase in hyphomicrobia is sufficient to account for their growth properties and hence their categorization as restricted facultative methylotrophs (Colby & Zatman, 1975).

Mutant JAB40 is similar to mutants C5 (Salem *et al.*, 1973) and PCT48 (Dunstan *et al.*, 1972b); none of these mutants grows on C_1 or C_2 compounds unless supplemented with

succinate, glyoxylate or glycollate. The isolation of mutant JAB40 as a mutant unable to grow on lactate, and the observation that it has also lost the ability to grow on C_1 and C_2 compounds, supports the previous evidence that there is a common step in the assimilation pathway for these compounds, this common step probably being the oxidation of acetyl-CoA to glyoxylate (Dunstan *et al.*, 1972*a, b*; Dunstan & Anthony, 1973; Salem *et al.*, 1973; Anthony, 1975; Taylor & Anthony, 1976*b*). It has recently been proposed (Kortstee, 1980) that this oxidation proceeds by way of a homoisocitrate-glyoxylate cycle. It will be of considerable interest to determine whether mutants PCT48, C5 and JAB40 lack any of the proposed enzymes of this pathway. If this pathway is confirmed then the problem of how mutants lacking malyl-CoA lyase and malyl-CoA hydrolase are able to grow on pyruvate and lactate remains unsolved (see Taylor & Anthony, 1976*b*; Cox & Quayle, 1976). A pathway involving a hypothetical oxidation of acetoacetyl-CoA to malyl-CoA (Bolbot, 1979) would avoid part of this problem but there is no direct evidence for this pathway.

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