Isolation of a *Citrobacter* species able to grow on malonate under strictly anaerobic conditions

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An anaerobic enrichment from lake mud yielded a pure culture of a facultatively anaerobic bacterium able to grow on malonate under strictly anaerobic conditions. Strain 16mall was identified as a member of the family *Enterobacteriaceae*, and assigned to the genus *Citrobacter* on the basis of morphological, metabolic and biochemical characteristics. Malonate was fermented under strictly anaerobic (sulphide-reduced) conditions to acetate and CO₂ concomitant with growth. A maximum growth rate of 1.88 generations h⁻¹ ($\mu = 1.30$ h⁻¹) was measured. The dry weight yield of cells from malonate was estimated at 2.5 g mol⁻¹. Yeast extract was required for growth on malonate: other additives, or a vitamin solution, could not replace this requirement. Other dicarboxylic acids were not degraded in the absence or presence of malonate. Malonate was degraded under anaerobic, but not aerobic conditions. Malonate-decarboxylating activity was inducible by malonate under both anaerobic and aerobic conditions, and was not expressed in glucose- or citrate-grown anaerobic cultures. Monensin had no effect on malonate degradation, while 2,4-dinitrophenol decreased the rate of malonate degradation. This, with the lack of a sodium requirement for anaerobic growth on malonate, suggested that ATP generation may not be mediated by a sodium-pumping mechanism.

Introduction

Malonic acid is an aliphatic dicarboxylic acid of three carbon atoms. Under aerobic conditions malonate has been shown to be degraded by some members of the family *Enterobacteriaceae* (Brenner, 1984); this is used as a diagnostic test for identification of species within the family. Malonate is used by some members of the family *Rhodospirillaceae* under anaerobic conditions in the light (Trüper & Pfennig, 1981). Organisms able to grow on malonate under dark anaerobic conditions have been reported. *Sporomusa termitida* degraded malonate with poor but consistent growth (Breznak *et al.*, 1988). *Malonomonas rubra* (Dehning & Schink, 1989) and *Sporomusa malonica* (Dehning *et al.*, 1989) are able to grow on malonate, decarboxylating it to acetate with low growth yields of 1.9 to 2.1 g dry weight of cell material (mol malonate)⁻¹. In this study we report the isolation of a *Citrobacter* sp. able to grow under strictly anaerobic conditions by the decarboxylation of malonate.

Methods

Sites and enrichments. Sediment samples from six sites were collected in sealed glass bottles and stored at 4 °C until required. The six sites were all in New Zealand: site 9, oxidation pond for meat works

effluent, Cambridge; site 10, tidal mud flats, Whangarei Harbour, near Oakleigh; site 12, tidal mud flats, Firth of Thames, near Thames; site 16, duck pond, University of Waikato campus, Hamilton; site 17, tidal mud flats, Mangaora Creek, Kawhia Harbour; site 19, effluent pond from anaerobic solids digester, Water Pollution Control Laboratory, Hamilton. Sites 9, 16 and 19 were 'freshwater' in nature, while the other three were estuarine.

Media and growth conditions. The freshwater medium was made up as follows: distilled H₂O, 1000 ml; MgCl₂·6H₂O, 0.4 g; KCl, 0.5 g; Na₂SO₄, 0.1 g; NaCl, 1.0 g; NH₄Cl, 0.3 g; Na₂HPO₄·12H₂O, 0.7 g; CaCl₂·2H₂O, 0.01 g; resazurin, 0.0001 g; Fe(III)citrate, 0.005 g; modified SL-7 trace elements, 1 ml; selenite/tungstate solution (Tschech & Pfennig, 1984), 1 ml. The estuarine medium was the same as the freshwater except that the NaCl and MgCl₂·6H₂O concentrations were increased to 15.0 and 2.0 g l⁻¹, respectively. Modified SL-7 trace elements consisted of: distilled H₂O, 1000 ml; 1 M-HCl, 3 ml; ZnCl₂, 70 mg; MnCl₂·4H₂O, 100 mg; H₃BO₃, 60 mg; CoCl₂·6H₂O, 200 mg; CuCl₂·2H₂O, 20 mg; NiCl₂·6H₂O, 20 mg; Na₂MoO₄·2H₂O, 40 mg.

The pH of the medium was adjusted with NaOH or HCl prior to dispensing so that the final pH was 6.8 to 7.2, unless noted otherwise. After boiling to remove O₂ and cooling under an N₂ gas stream, 2.0 g NaHCO₃ was added and the medium was dispensed under an N₂ headspace by the method of Patel *et al.* (1985a) except that the medium was reduced after sterilization. The cysteine/sulphide reductant for enrichment cultures consisted of: distilled H₂O (boiled and then cooled under an N₂ stream), 100 ml; L-cysteine·HCl, 2.5 g; Na₂S·9H₂O, 2.5 g; stored sealed under an N₂ atmosphere. This reductant was added at a concentration 0.1 ml per 10 ml of medium. For all other work sulphide reductant was employed, containing distilled H₂O, 100 ml and Na₂S·9H₂O, 3.6 g; the pH was adjusted to 7.0 with 5 M-HCl. This was

prepared and used in the same manner as the cysteine/sulphide reductant.

Vitamins were added from filter-sterilized stock solutions to the following final concentrations (per litre of culture medium): d-biotin, 1 µg; *p*-aminobenzoic acid, 10 µg; nicotinic acid, 100 µg; DL-pantothenate (hemicalcium salt), 50 µg; pyridoxamine.(HCl)₂, 50 µg; thiamin.HCl, 100 µg; cyanocobalamin, 20 µg; DL-6,8-thioctic acid, 10 µg; folic acid, 30 µg; riboflavin, 100 µg.

KCN medium consisted of nutrient broth (BBL) with KCN added at a concentration of 75 mg l⁻¹. TYEG medium consisted of the freshwater medium plus trypticase peptone, yeast extract and glucose, each at 2 g l⁻¹. The agar shake technique was used as described by Pfennig (1978), adding 0.75 g purified agar (1 basal medium)⁻¹, with 48 mM-malonate and 0.2 g yeast extract l⁻¹.

Inoculations were made and samples taken using disposable plastic syringes and 25-gauge hypodermic needles. Carbon sources and other medium amendments were added from 100 mM to 3 M autoclaved or filter-sterilized stock solutions. All incubations were at 34 °C and the pH was between 6.8 and 7.2, unless otherwise indicated. All experiments were done in duplicate or triplicate.

Cellular characterization. Gram-staining was by the Hucker method (Doetsch, 1981). Detection of aminopeptidase activity was modified from the method of Cerny (1978): to 1 ml of liquid culture was added 0.1 ml of 1% (w/v) L-alanine-*p*-nitroanilide (Sigma) in Tris/maleate buffer.

To produce spectrophotometric scans of crude cell-free extracts, approximately 200 ml of late-exponential phase/early-stationary-phase culture was harvested by centrifugation, washed twice in sodium phosphate buffer (100 mM, pH 7.0) and resuspended in 1 to 2 ml of the sodium phosphate buffer. The cell suspension was sonicated for 2 to 3 min with a micro-ultrasonic cell disruptor (Kontes), at 10 W, keeping the sample in a small plastic tube in an ice-water bath. The sample was then centrifuged in a Runne microcentrifuge (H. Rehm) at maximum speed for 5 min. The supernatant was examined for cytochromes. Oxidized versus reduced spectra were produced by vigorously shaking a volume of the supernatant for the sample cell (oxidized), and adding a few grains of sodium dithionite to another volume of the supernatant for the reference cell (reduced). Scans were done using a Shimadzu UV-250 UV-visible recording spectrophotometer with a Shimadzu PR-1 graphic printer.

Negatively stained whole cells and thin sections were prepared and examined as described by Patel *et al.* (1985b).

DNA was isolated by the method of Owen & Lapage (1976). The melting temperature (*T*_m) of the DNA was determined by the method described by Patel *et al.* (1985b).

Biochemical characterization. Oxidase activity was determined by the 1-naphthol/*N,N*-dimethyl-*p*-phenylenediamine method (Smibert & Krieg, 1981). The methods used for measuring catalase activity and indole production from 5 mM-L-tryptophan using Ehrlich's reagent were described by Smibert & Krieg (1981). Urea hydrolysis was tested by adding urea and phenol red at 2 g l⁻¹ and 1 mg l⁻¹, respectively. Aesculin hydrolysis was tested by observing the disappearance of characteristic fluorescence at 366 nm of a culture containing 0.1 g aesculin l⁻¹. Sulphide production from 10 mM-L-cysteine was measured using the method of Cord-Ruwisch (1985). These tests were all carried out in TYEG medium. Hydrogenase was assayed using a qualitative adaptation of the method described by Badziong & Thauer (1980).

Growth determinations. Optical densities of growing cultures were measured at 660 nm in Hungate tubes inserted directly into a Spectronic 20 single-beam spectrophotometer (Bausch and Lomb) fitted with a suitable sample compartment. The instrument was zeroed with uninoculated medium.

To determine dry weight yields, cultures were harvested by centrifugation and washed with sodium phosphate buffer (5 mM,

pH 7.0). The pellets were resuspended in a minimal amount of distilled water and transferred to pre-weighed aluminium foil cups. Samples were then dried to constant weight at 90 °C, and the final weight determined. Cultures were grown in 160 ml serum vials and samples were taken for HPLC analysis at the times of inoculation and harvesting to determine substrate transformation. Quantitative measurements of organic acids were made by HPLC (Patel *et al.*, 1987). Data were collected via a 760 series interface (Nelson Analytical) to an Exzel XT computer (Computer Imports) using 3000 series chromatography data software (Nelson Analytical).

Induction experiments. Cultures were pre-grown as indicated on freshwater medium with 1.0 g yeast extract l⁻¹, and one of: 48 mM-malonate, 20 mM-citrate or 10 mM-glucose. Experiments were inoculated to an OD₆₆₀ of 0.2 in 30 ml freshwater medium in serum vials with 48 mM-malonate and 1.0 g yeast extract l⁻¹. Chloramphenicol (0.25 mM), an inhibitor of protein synthesis, was added to some vials prior to inoculation. Degradation of malonate was followed by its disappearance and the accumulation of acetate.

Results and Discussion

Enrichment cultures and isolation of strain 16mall

Enrichments were set up with freshwater and estuarine media containing 0.2 g yeast extract l⁻¹ and 48 mM-malonate with cysteine/sulphide reductant, and incubated for 7 d at 34 °C. The enrichments were subcultured three times into the same medium. Complete degradation of malonate was observed in the third transfer for all three estuarine sites using estuarine medium. The freshwater enrichments originating from the three freshwater sites showed greater ability to degrade malonate in the third transfer (25% to 100% of the malonate supplied was degraded in 7 d) than when inoculated into estuarine medium (2% to 13%).

This suggested that malonate was not degraded by a mechanism similar to that of succinate degradation by *Propionigenium modestum*. This latter organism has a requirement for at least 1% (w/v) NaCl (Schink & Pfennig, 1982) and couples the decarboxylation of succinate to the generation of a transmembrane sodium gradient (Hilpert *et al.*, 1984). The malonate-degrading anaerobe *Malonomonas rubra* also displays a similar sodium requirement, and may conserve energy by a similar mechanism (Dehning & Schink, 1989).

Agar shake cultures were set up from the subcultures of the enrichments on malonate. From these two pure cultures were obtained: 10mall and 16mall. Strain 10mall was able to grow on malonate for two transfers then apparently lost its ability to grow on this substrate. Strain 16mall could be repeatedly and reproducibly grown on malonate, forming greyish-green colonies in agar shakes. This strain was characterized to identify its taxonomic position. The culture was found to grow better in freshwater medium with 1.0 g yeast extract l⁻¹. This medium was used in further experimental work

presented in this paper unless otherwise indicated. This medium, with 48 mM-malonate, was also used as the maintenance medium for strain 16mall. For all further work the cysteine/sulphide reductant was replaced by sulphide reductant.

Morphological characteristics

Cells of strain 16mall were round-ended rods occurring singly. The cells were 0.45 µm wide by 1.0 µm long. Motility was never observed. No flagella were seen in negatively-stained preparations examined by electron microscopy. Spores were never observed, not even in cooked meat medium (Difco). Neither a 1-d-old nor a 5-d-old culture produced viable subcultures after heating at 80 °C for 5 min.

Cells of strain 16mall stained Gram-negative. The aminopeptidase reaction was positive. Ultra-thin sections of strain 16mall showed that the cell wall was of a typical Gram-negative type. Cytochromes were not detected in cell-free crude extracts of cultures of 16mall grown anaerobically on malonate yeast extract medium. Cultures grown aerobically on 10 mM-glucose did contain cytochromes. A mol% G + C value of 56 was determined by thermal denaturation.

Nutritional and biochemical characteristics

Strain 16mall was able to grow anaerobically on a range of carbohydrates, and on serine, cysteine, pyruvate, citrate, malonate and yeast extract. No growth was possible on H₂ plus CO₂ (80:20, v/v), on 60 mM-formate or on a wide range of other organic acids, alcohols, amino acids and aromatic compounds. Glucose was fermented to lactate, formate, acetate, ethanol and 2,3-butanediol (H₂ and CO₂ were not tested for).

Strain 16mall was able to grow aerobically on glucose, citrate and acetate in the absence of added yeast extract or vitamins. Malonate was not degraded under aerobic conditions in the presence or absence of yeast extract. Similarly, under anaerobic conditions growth was possible on glucose and citrate when yeast extract and vitamins were omitted. However, on malonate under anaerobic conditions there was a requirement for yeast extract which could not be replaced by a vitamin mix, vitamin-free Casamino acids or trypticase peptone.

Sulphate and thiosulphate were not used as terminal electron acceptors with malonate as the carbon source. Nitrate was reduced to nitrite on TYEG medium with no production of N₂ gas. Succinate was produced from 20 mM-fumarate with 20 mM-malonate but not with 20 mM-formate.

Using the API 10 test strip (Montalieu), strain 16mall was positive for the following tests: β-galactosidase, glucose fermentation, arabinose fermentation, ornithine decarboxylase, Simmon's citrate, tryptophan deaminase and nitrate reduction; the following tests were negative: lysine decarboxylase, H₂S production, urease, indole production and oxidase. In addition the following tests were found to be positive: aerobic growth in KCN medium, anaerobic growth in KCN medium and sulphide production from cysteine. Negative results were obtained for these tests: oxidase, catalase, aesculin hydrolysis, urea hydrolysis and indole formation from L-tryptophan.

No hydrogenase activity was detected in cell-free crude extracts of strain 16mall grown anaerobically on malonate under an atmosphere of H₂ plus CO₂ (80:20) or of N₂.

Identification of strain 16mall

Strain 16mall was a facultatively anaerobic Gram-negative rod. This places it in section 5 of Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984). Based on its size, its straight rod morphology, lack of motility and negative oxidase test it belonged to the family *Enterobacteriaceae* rather than the families *Vibrionaceae* or *Pasteurellaceae*. Using the API 10 test strip, the isolate fell within the group of *Citrobacter* spp., but could not be assigned to any one species of the genus. The species in this genus are not clearly distinguished (Brenner, 1984). Comparison of differential characteristics of the genus *Citrobacter* and biochemically similar genera (Sakazaki, 1984) reveals that strain 16mall could also belong to the genus *Enterobacter*. Strain 16mall was tentatively assigned to the genus *Citrobacter* on the basis of the API 10 test-strip identification. The characteristic of most interest in this strain was its ability to grow on malonate under strictly anaerobic conditions.

Growth on malonate

When strain 16mall was grown under strictly anaerobic conditions in freshwater medium containing 48 mM-malonate and 1.0 g yeast extract l⁻¹ the following growth parameters were determined. The temperature optimum was around 41 to 43 °C ($\mu = 1.30 \text{ h}^{-1}$), with no growth at 47 °C. At 34 °C, the normal growth temperature, the maximal growth rate was 1.18 generations h⁻¹ ($\mu = 0.82 \text{ h}^{-1}$). Growth at 5 °C was very slow. A broad pH optimum of 5.5 to 6.5 was found, with no growth at either 4.5 or 8.0. There was a distinct optimum NaCl concentration of 5 g l⁻¹ ($\mu = 0.77 \text{ h}^{-1}$ at 34 °C) with no

Fig. 1. Growth curve of strain 16mall grown on 70 mM-malonate in the presence of 1.0 g yeast extract l⁻¹ in freshwater medium. ○, Malonate concentration; △, acetate concentration; ●, culture density (OD₆₆₀).

growth occurring at 80 g NaCl l⁻¹. Good growth ($\mu = 0.48 \text{ h}^{-1}$ at 34 °C) occurred at a sodium concentration equivalent to 0.2 g NaCl l⁻¹, the lowest tested.

When strain 16mall was grown anaerobically in freshwater medium containing 70 mM-malonate and 1.0 g yeast extract l⁻¹, growth occurred concomitant with the degradation of malonate to acetate (Fig. 1). Increasing the yeast extract concentration resulted in increased growth in the presence and absence of malonate; however growth in the presence of malonate was consistently greater (by the same amount) than growth on the yeast extract alone.

Strain 16mall was tested for the ability to degrade other dicarboxylic acids (succinate, glutarate, adipate and pimelate). These were added in the presence and absence of malonate to give a total substrate concentration of 50 mM, that is 50 mM of a single substrate or 25 mM each of malonate and another dicarboxylic acid. Malonate was degraded to acetate in all cases and was responsible for the growth observed. The other dicarboxylic acids were not degraded, either alone or in the presence of malonate. Succinate, glutarate, adipate or pimelate did not inhibit malonate utilization.

Strain 16mall was grown on a variety of substrates and heavily inoculated into anaerobic medium with 48 mM-malonate as the substrate. Chloramphenicol was added to some cultures as an inhibitor of protein synthesis. When cells were pre-grown with malonate under anaerobic or aerobic conditions, malonate degradation began immediately on transfer to fresh medium, regardless of whether or not chloramphenicol was present. When cells were pre-grown anaerobically on glucose or

on citrate, malonate degradation did not begin for about 40 min in the absence of chloramphenicol, and did not occur at all if chloramphenicol was present. Malonate-decarboxylating activity thus was not constitutive, but was induced by malonate under aerobic and anaerobic conditions.

Dry weight yield and fermentation balances were carried out on strain 16mall grown on malonate in the presence of 0.1 g yeast extract l⁻¹ (Table 1). One mol malonate was decarboxylated to 1 mol acetate plus one mol CO₂, with a growth yield of 2.5 g dry weight per mol of substrate. Given that the yeast extract may have in fact improved the yield, this is similar to the yields of 1.9 to 2.1 g mol⁻¹ found for *Malonomonas rubra* (Dehning & Schink, 1989) and *Sporomusa malonica* (Dehning *et al.*, 1989). The fermentation balance agreed with the following equation for malonate fermentation (Dehning & Schink, 1989):

The low growth yield obtained is in line with the small free-energy change associated with this reaction. The free-energy change is too low to allow ATP synthesis via substrate-level phosphorylation. The mechanism of ATP formation coupled to malonate decarboxylation in strain 16mall has not yet been elucidated. To give an indication of the role of sodium gradients in the degradation of malonate by strain 16mall, the effect of gradient-abolishing ionophores was investigated (Table 2).

Anaerobic glucose metabolism was not affected by either 2,4-dinitrophenol or monensin. This was expected, since glucose fermentation yields ATP by substrate-level phosphorylation. Citrate degradation was markedly inhibited by monensin, a sodium ionophore, while 2,4-dinitrophenol, a proton ionophore, had little effect. The role of sodium in citrate metabolism of enterobacteria has been shown in the decarboxylation of oxaloacetate generated from citrate (Stern, 1967; O'Brien & Stern, 1969; Dimroth, 1982a, b). This decarboxylation step generates a sodium gradient. In the present study, 2,4-dinitrophenol inhibited the degradation of malonate, while monensin had no effect. This suggests a role for proton gradients in malonate fermentation by strain 16mall. Whether 2,4-dinitrophenol interferes with an energy-generating mechanism or a transport process remains to be elucidated. The involvement of a sodium-pumping mechanism seems to be excluded when these results are considered with the ability of the organism to grow on malonate at a very low sodium concentration. Further studies on malonate decarboxylation by strain 16mall and other strains of enterobacteria are being carried out.

Table 1. *Malonate utilization stoichiometry and growth yields of strain 16mall*

The yield on malonate was calculated by difference from the yields on 0.1 g yeast extract l⁻¹ and on malonate plus 1.0 g yeast extract l⁻¹. The mean results of three determinations are shown. The dry weight yield was converted to a mmol l⁻¹ value using C₆H_{10.84}N_{1.4}O_{3.07} as the empirical formula for cell material (Stouthamer, 1979). The carbon recovery was calculated assuming that 1 mol of CO₂ was produced per mol of malonate degraded.

Substrate	Malonate degraded (mmol l ⁻¹)	Acetate produced (mmol l ⁻¹)	Dry weight (mg l ⁻¹)	Dry weight (mmol l ⁻¹)	Carbon recovery (%)	Yield (g mol ⁻¹)
Yeast extract	—	1.1	16.5	—	—	—
Malonate + yeast extract	11.7	13.5	46.3	—	—	—
Malonate	11.7	12.4	29.8	0.2	107	2.5

Table 2. *Effect of various ionophores on Citrobacter sp. strain 16 mall grown under anaerobic conditions on various substrates*

Pre-cultures were grown in freshwater medium containing 1.0 g yeast extract l⁻¹ plus the substrate to be tested, then used to inoculate (to an OD₆₆₀ of 0.2) serum vials containing 30 ml freshwater medium plus 1.0 g yeast extract l⁻¹ and 50 mM-malonate, 20 mM-citrate or 10 mM-glucose, under anaerobic (sulphide-reduced) conditions. Ionophores, from stock solutions dissolved in ethanol, were added at 100 µl to 30 ml culture to give desired final concentration. The rate of malonate degradation was compared to that of a control with only 100 µl ethanol added.

Substrate	Ionophore	Ionophore concn (µM)	Rate of substrate utilization as percentage of control
Malonate	2,4-Dinitrophenol	76	45
	Monensin	50	98
Citrate	2,4-Dinitrophenol	76	87
	Monensin	10	25
	Monensin	50	11
Glucose	2,4-Dinitrophenol	76	96
	Monensin	50	106

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