The Tricarboxylic Acid Cycle of Heterogeneous and Swarmer Cell Populations of *Rhodomicrobium vannielii* Rm5

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Studies on the tricarboxylic acid cycle of *Rhodomicrobium vannielii* Rm5 demonstrated that, unlike other *Rhodospirillaceae*, this organism has a functionally incomplete cycle, broken at 2-oxoglutarate dehydrogenase, under photoheterotrophic conditions. This enzyme was, however, synthesized when *Rm. vannielii* was grown under microaerophilic chemoheterotrophic conditions. The citrate synthase exhibited responses to inhibitors characteristic of Gram-negative organisms but, unlike many microbes exhibiting an incomplete cycle, was not inhibited by 2-oxoglutarate. Both NAD- and NADP-linked isocitrate dehydrogenase activity was detectable but the functional roles of these enzymes are unclear. No significant differences in enzyme activities or inhibitor sensitivities of enzymes were detected between heterogeneous cultures and synchronous swarmer cell populations.

INTRODUCTION

Rhodomicrobium vannielii (Duchow & Douglas, 1949) is a member of the *Rhodospirillaceae* in which cell growth is polar and cell division asymmetric (Whittenbury & Dow, 1977; Kelly & Dow, 1984). Batch cultures of this photoheterotroph contain several morphologically distinct cell types with the characteristic cellular expression consisting of multicellular arrays of cells linked by prosthecae from the tips of which ovoid, peritrichously flagellated swarmer cells are produced (Whittenbury & Dow, 1977). A particularly interesting feature of this microbe is the variety of alternative cellular expressions possible under different environmental conditions. For example, high partial pressures of CO₂ coupled with low light intensities during growth can induce a simplified vegetative cell cycle where swarmer cells are produced constitutively (Dow & France, 1980). Alternatively, exhaustion of nutrients in batch cultures or growth on poor carbon sources can lead to the production of heat-resistant, angular exospores (Whittenbury & Dow, 1977; Potts & Dow, 1979).

As part of our investigations into cellular morphogenesis in *Rm. vannielii* we have focused attention upon the nature of the swarmer cell and the control of differentiation. Synchronized swarmer cell populations can be obtained quickly and in quantity by filtration of a heterogeneous, asynchronous batch culture through glass wool (Whittenbury & Dow, 1977) and, when incubated anaerobically in the light, these cells undergo an obligate sequence of events leading to the formation of a daughter cell. These events have been well characterized and include nucleoid relaxation, flagella loss and prostheca synthesis as the major morphological 'landmarks' in addition to a temporal programme of protein synthesis (Whittenbury & Dow, 1977; Dow *et al.*, 1983).

A necessary prerequisite for a greater understanding of the swarmer cell is a knowledge of the pathways of intermediary metabolism. There is little information in the literature concerning the metabolism of swarmer cells and we therefore chose to study the role of the tricarboxylic acid (TCA) cycle in the provision of cellular intermediates. This is of particular interest in view of the apparent metabolic quiescence of the swarmer cell with regard to transcription and translation.

Abbreviation: TCA, tricarboxylic acid.

METHODS

Organisms and culture conditions. *Rhodomicrobium vannielii* Rm5 was grown photoheterotrophically at 30 °C in pyruvate/malate/mineral salts (PM) medium (Whittenbury & Dow, 1977) under an atmosphere of oxygen-free nitrogen and with a light intensity of 1000 lx. Cultures were continuously stirred. Microaerophilic cultures were grown in 2-litre volumes of PM medium in 2.5-litre conical flasks in darkness at 30 °C on a rotary shaker (175 r.p.m.). *Escherichia coli* C and *Pseudomonas putida* were grown aerobically at 30 °C in M9 mineral salts medium (Lark *et al.*, 1963) containing 0.02% (w/v) glucose or sodium acetate. Growth was followed by measuring optical density at 600 nm and by total cell counts obtained using a Coulter counter model ZBI.

Radiolabelling experiments. *Rm. vannielii* was grown as described above in 100 ml batches of PM medium containing 1 µCi sodium [U-¹⁴C]acetate ml⁻¹ (specific activity 59 mCi mmol⁻¹; 2183 MBq mmol⁻¹).

Synchronization of *Rm. vannielii*. Cultures (10 litres) of *Rm. vannielii* in the late exponential phase (OD₆₀₀ 1.2) of growth were synchronized by passage through glass wool filled columns as described by Whittenbury & Dow (1977). The degree of synchrony was determined by light microscopy and by particle volume distribution analysis using a Coulter counter ZBI-C1000 channelyzer system linked to an Acorn BBC model B microcomputer. The effluent from the columns was maintained in darkness to prevent cellular differentiation and concentrated to a volume of approximately 500 ml by a Millipore Pellicon ultrafiltration system.

Hydrolysis of cellular protein. Portions of radiolabelled late exponential phase cultures of *Rm. vannielii* were added to equal volumes of 10% (w/v) trichloroacetic acid and incubated at 4 °C for 30 min. Trichloroacetic acid precipitable material was pelleted by centrifugation at 15000 g and 4 °C for 15 min. The pellet was resuspended in 70% (v/v) ethanol, incubated at 45 °C for 30 min and the precipitate pelleted as described above. The pellet was resuspended in diethyl ether/ethanol (1:1, v/v), incubated at 30 °C for 45 min and centrifuged as described above. The pellet was resuspended in 5% (w/v) trichloroacetic acid and incubated at 95 °C for 30 min. After centrifugation as described above, the precipitate was washed three times with ethanol and resuspended in 5 ml 6 M-HCl. The suspension was sealed in hydrolysis tubes and incubated at 105 °C for 18 h.

Disruption of cells. Cells were harvested from 5-litre (*Rm. vannielii*) or 1-litre (*E. coli* and *P. putida*) cultures, or from synchronous *Rm. vannielii* swarmer cell concentrates, by centrifugation at 14000 g and 4 °C for 10 min. Cell pellets were washed twice in sterile medium and resuspended in approximately 20 ml sterile 10 mM-Tris/HCl buffer (pH 7.0). Two methods of disruption were used: ultrasonication using a MSE 12/76 mark II ultrasonicator with a peak wavelength of 10–12 µm in 15 s pulses (cumulative sonication time 150 s) with the sample cooled in a bath of methanol-ice; disruption by French pressure cell involving two passages at 4 °C at a pressure of 137 MPa. Unbroken cells and cell debris were removed by centrifugation at 18000 g and 4 °C for 45 min.

Two-dimensional chromatography. Samples (10 and 25 µl, 10⁴–2 × 10⁵ c.p.m.) of *Rm. vannielii* protein hydrolysates were separated by two-dimensional paper chromatography on Whatman no. 1 chromatography paper. The solvent system for the first dimension was butanol/acetic acid/water (180:45:75, by vol.; run-time 16 h) and for the second dimension 80% (w/v) aqueous phenol/aqueous ammonia (300:1:5, v/v; run-time 15 h). Chromatograms were air dried and developed by spraying with 0.25% (w/v) ninhydrin dissolved in acetone followed by heating at 100 °C. Amino acid spots were identified by comparison with chromatograms of known amino acid standards.

Enzyme assay methods. Citrate synthase [citrate oxaloacetate-lyase (CoA acetylating); EC 4.1.3.7], isocitrate dehydrogenase [L₃-isocitrate:NAD⁺ oxidoreductase (decarboxylating); EC 1.1.1.42 and L₃-isocitrate:NAD⁺ oxidoreductase (decarboxylating); EC 1.1.1.41], malate dehydrogenase (L-malate:NAD⁺ oxidoreductase; EC 1.1.1.37) and fumarate (L-malate hydro-lyase; EC 4.2.1.2) were assayed spectrophotometrically according to the methods of Reeves *et al.* (1971). Inhibitors of citrate synthase were added directly to the assay system which contained (µmol in 3 ml): Tris/HCl (pH 8.1), 600; potassium oxaloacetate, 1.5; acetyl-CoA, 0.3; 5,5'-dithiobis(2-nitrobenzoate), 0.6.

The 2-oxoglutarate dehydrogenase enzyme system was assayed by a modification of the method of Reeves *et al.* (1971). The system contained (µmol in 3 ml): Tris/HCl (pH 8.5), 500; L-cysteine, 7.8; potassium 2-oxoglutarate, 25; Coenzyme A, 0.25; NAD, 6; potassium cyanide, 10; plus a variable volume of cell lysate. The reaction was started by the addition of NAD⁺ and followed by measuring the 2-oxoglutarate-dependent reduction of NAD⁺ at 340 nm.

Aconitase [citrate(isocitrate) hydro-lyase; EC 4.2.1.3] was assayed spectrophotometrically in a system containing (µmol in 3 ml): manganese chloride, 6; Tris/HCl (pH 7.5), 300; sodium *cis*-aconitate, 15; NADP⁺ 1.5; isocitrate dehydrogenase, 1 unit; plus a variable volume of cell lysate. The reaction was started by the addition of *cis*-aconitate and followed at 340 nm, by measuring the reduction of NADP⁺ by the action of isocitrate dehydrogenase utilizing isocitrate produced from *cis*-aconitate by the action of aconitase.

Succinate thiokinase [succinate-CoA ligase (ADP-forming); EC 6.2.1.5] was assayed by coupling the reverse reaction of the enzyme through pyruvate kinase and lactate dehydrogenase as described by Cha (1969).

Succinate dehydrogenase [succinate:(acceptor) oxidoreductase; EC 1.3.99.1] was assayed by spectrophotometrically measuring at 600 nm the reduction of 2,6-dichlorophenolindophenol mediated by phenazine

methosulphate using a method modified from that of King (1963). The system contained (μmol in 3 ml): potassium phosphate buffer (pH 7.6), 150; 2,6-dichlorophenolindophenol, 0.18; phenazine methosulphate, 1.2; sodium succinate, 20; potassium cyanide, 7; plus a variable volume of cell lysate. The reaction was started by the addition of succinate.

Protein concentrations were assayed by the Lowry method as described by Herbert *et al.* (1971) using bovine serum albumin standards.

Conversion of 2-oxoglutarate by cell-free extracts. Cell-free extracts of *Rm. vannielii* and *E. coli* were incubated at 30 °C for up to 2 h in the presence of the reagents described above for the 2-oxoglutarate dehydrogenase assay (omitting potassium cyanide) plus 0.1 μCi sodium 2-oxo[5- ^{14}C]glutarate ml^{-1} . Samples were taken at regular intervals and the reaction was stopped by freezing in liquid nitrogen. Separation of organic acids, after thawing of the frozen samples on ice, was by one-dimensional paper chromatography (Whatman no. 1 paper) of 50 μl samples using a solvent system of butanol/acetic acid/water (60:15:25, by vol.) and a run-time of 5 h. Standard organic acids were visualized by immersing the paper in 0.1% (w/v) bromophenol blue + 0.1% (w/v) methyl red in 95% (v/v) ethanol and appeared as yellow spots on a blue background.

Determination of radioactivity. Ninhydrin-stained amino acid spots from two-dimensional chromatograms and sequential 1 cm bands from one-dimensional chromatograms were cut out and placed in Beckman EP scintillation fluid. Liquid scintillation counting was done with an LKB 1212 Minibeta counter.

Autoradiography. Kodak Kodirex X-ray film was exposed to two-dimensional chromatograms for 2 weeks and developed using Kodak LX-24 developer followed by Kodak FX-40 fixer. Exposed areas of the film were cut out and quantified by the silver grain elution technique described by Suissa (1983). The film areas were incubated for 2 h in 1 M-NaOH and the absorbance of the supernatants at 500 nm was measured against that from a piece of developed but unexposed film.

Reagents. Sodium [^{14}C]acetate and 2-oxo[5- ^{14}C]glutarate (sodium salt) were obtained from Amersham. Enzymes and fine biochemicals were obtained from Sigma. All other reagents were of Analar standard or the highest purity commercially available.

RESULTS

Incorporation of ^{14}C into amino acids. Two-dimensional paper chromatography of ^{14}C -labelled protein hydrolysates of *Rm. vannielii* yielded clear separation of the amino acid residues which, with the exception of one spot, could be identified by comparison with the standard amino acid mixtures. Visual examination of autoradiographs of chromatograms loaded with hydrolysates from photoheterotrophically grown cells revealed that the label predominated in the leucine group, glutamine, proline and arginine with relatively little incorporation of label elsewhere. These observations were confirmed and quantified by both liquid scintillation counting and spectrophotometric evaluation of the silver grains eluted from autoradiograms (Table 1), the aforementioned amino acids accounting for about 70% of the total recoverable radioactivity or total silver grain absorbance.

Table 1. *Incorporation of radiolabel from sodium [^{14}C]acetate into amino acids of *Rm. vannielii* during photoheterotrophic or chemoheterotrophic growth*

Protein hydrolysate (10 μl , 2×10^5 c.p.m.) was applied to the origin and chromatographed as described in Methods. Quantification was achieved after autoradiography by spectrophotometry of the eluted silver grains. Comparisons of this method with data from liquid scintillation counting of ninhydrin-stained spots on the chromatograms always showed excellent agreement.

Amino acid	Percentage of total silver grain absorbance		Amino acid	Percentage of total silver grain absorbance	
	Photo-heterotrophic	Chemo-heterotrophic		Photo-heterotrophic	Chemo-heterotrophic
Aspartate	7.1	10.0	Leucine + isoleucine +	24.1	17.6
Glutamine	18.5	14.7	phenylalanine		
Cysteine + cystine	0.4	1.0	Proline	11.5	8.3
Serine	1.8	1.8	Arginine	15.2	10.3
Glycine	1.9	2.6	Methionine	0.9	0.2
Threonine	3.9	4.7	Lysine + histidine	4.9	4.6
Alanine	1.8	2.7	Ornithine	0.7	0.2
Tyrosine	2.6	2.4	Unknown	2.5	16.5
Valine	1.6	2.4			

Table 2. *Activities of enzymes of the TCA cycle in photoheterotrophically grown heterogeneous cultures and synchronous swarmer cell populations of Rm. vannielii*

Activities (nmol substrate converted per min by 1 mg protein) are the means of between 3 and 7 independent experiments with at least 3 assays done in each experiment. 2-Oxoglutarate dehydrogenase activity [$43 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was detectable in microaerophilically grown cells. ND, Not determined. For details of assay procedures see text.

Enzyme	Activity [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]	
	Heterogeneous culture	Swarmer cells
Citrate synthase	37.1	45.2
Aconitase	43.3	40.0
Isocitrate dehydrogenase		
NADP-linked	242.1	229.6
NAD-linked	74.5	59.2
2-Oxoglutarate dehydrogenase	< 0.1	< 0.1
Succinate thiokinase	23.6	25.3
Succinate dehydrogenase	75.7	ND
Fumarase	10.8	ND
Malate dehydrogenase	1835.0	1821.0

Table 3. *Effects of inhibitors on citrate synthase from heterogeneous cultures and synchronous swarmer cell populations of photoheterotrophically grown Rm. vannielii*

For details of the assay system see Methods. Activities are expressed as a percentage of the control system: 100% activity corresponds to $37.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. ND, Not determined.

Inhibitor	Concn (mM)	Activity (percentage of control)	
		Heterogeneous culture	Swarmer cells
ATP	1.0	98.4	ND
	2.0	92.3	94.3
	5.0	75.0	77.6
	10.0	56.7	53.0
ADP	2.0	98.1	ND
	5.0	97.3	ND
	10.0	88.7	89.6
AMP	10.0	99.0	99.8
NAD	5.0	100.1	100.3
NADH	0.1	92.4	92.4
	0.5	53.7	55.6
	1.0	15.9	15.7
	2.0	10.8	11.6
NADH + AMP	2 + 1	95.3	ND
	1 + 1	98.4	97.4
2-Oxoglutarate	10	101.7	103.4

When *Rm. vannielii* was grown under microaerophilic chemoheterotrophic conditions the percentage distribution of acetate-derived carbon in the amino acids of protein hydrolysates was significantly altered (Table 1). Compared to photoheterotrophically grown cells, less label was observed in glutamine, the leucine group, proline and arginine while more label was incorporated into aspartate and threonine. In addition, a large increase in the labelling of an unidentified ninhydrin-staining spot was a feature of growth under chemoheterotrophic conditions.

TCA cycle enzymes of heterogeneous and synchronous populations of Rm. vannielii. Values for enzyme activity in both late exponential phase photoheterotrophic heterogeneous and

Fig. 1. Incorporation of radioactivity from 2-oxo[5-¹⁴C]glutarate into other organic acids. One-dimensional paper chromatograms were run as described in the text and cut into 1 cm strips. The results are liquid scintillation count data along individual tracks corrected for background activity. The positions of standard organic acids are marked: OG, 2-oxoglutarate; S, succinate. (a) *E. coli* lysate (7.7 mg ml⁻¹ protein), 30 min incubation period (●); *Rm. vanielii* photoheterotrophic lysate (7.9 mg ml⁻¹ protein), 30 min incubation period (■). (b) *Rm. vanielii* chemoheterotrophic lysate (4 mg ml⁻¹ protein) with samples taken at 0 h (●) and after 2 h incubation (■).

synchronous swarmer cell populations are given in Table 2. At no time was there any detectable difference in activity between cell-free extracts prepared by sonication and the French pressure cell. 2-Oxoglutarate dehydrogenase activity was virtually undetectable in such cultures but significant activity [about 43 nmol min⁻¹ (mg protein)⁻¹] was found in extracts of microaerophilically grown cells, suggesting the existence of a complete cycle under such conditions. In experiments where 2-oxoglutarate dehydrogenase activity was not detectable in *Rm. vanielii*, lysates of aerobically grown *E. coli* C and *P. putida* contained demonstrable activity and served as positive controls.

Properties of the citrate synthase of *Rm. vanielii*. The effects of exogenous compounds on the activity of citrate synthase are summarized in Table 3. Significant inhibition by ATP and NADH was observed and the latter inhibition could be reversed by the addition of AMP. No significant reduction in activity was observed in the presence of 10 mM-2-oxoglutarate.

Utilization of 2-oxo[5-¹⁴C]glutarate by cell-free extracts. To complement the enzyme activity data, an assay for the conversion of radiolabelled 2-oxoglutarate into succinate was used. The distribution of radioactivity along the chromatograms is illustrated in Fig. 1. No significant conversion of 2-oxoglutarate into succinate could be demonstrated in photoheterotrophic cell lysates of *Rm. vanielii* but this was observed with lysates from *E. coli*. However, with extracts derived from chemoheterotrophically grown cells of *Rm. vanielii*, a radioactive peak corresponding with the position of succinate (run on the same chromatogram) was detected. The relative position of succinate to 2-oxoglutarate varied somewhat in different separations, but the radioactive peaks always correlated with the position of standard acids when run on the same chromatograms.

DISCUSSION

The predominance of acetate-derived carbon in the leucine, glutamine, proline and arginine residues of protein hydrolysates of photoheterotrophically grown *Rm. vannielii* (Table 1) is suggestive of the existence of an incomplete TCA cycle under such conditions. Similar labelling patterns have been obtained in a variety of phototrophic, lithotrophic and methylotrophic bacteria (Hoare *et al.*, 1967; Pearce *et al.*, 1969; Smith *et al.*, 1967; Smith & Hoare, 1977) and in these cases are a result of the operation of an incomplete TCA cycle lacking 2-oxoglutarate dehydrogenase or one exhibiting very low levels of activity of this enzyme. Direct enzyme assays demonstrated the presence of all other enzymes of the TCA cycle but an extremely low level of 2-oxoglutarate dehydrogenase (Table 2). The absence of the latter enzyme was confirmed (Fig. 1) by the negligible amount of 2-oxo[5-¹⁴C]glutarate converted to succinate by cell-free extracts of *Rm. vannielii*. These results all suggest the existence of a functionally incomplete TCA cycle in this organism under anaerobic conditions.

Under aerobic conditions the presence of 2-oxoglutarate dehydrogenase suggests the operation of a complete TCA cycle. This observation is similar to those made for other members of the *Rhodospirillaceae* and also for *E. coli* under variable growth conditions (Amarasingham & Davis, 1965). However, in the other *Rhodospirillaceae* even under anaerobic photoheterotrophic growth conditions a low but significant level of 2-oxoglutarate dehydrogenase activity is detectable (Beatty & Gest, 1981*a, b*; Eley *et al.*, 1979; Cox *et al.*, 1983). The various lines of evidence suggest that this is not the case in *Rm. vannielii* and that repression of 2-oxoglutarate dehydrogenase synthesis under anaerobic conditions is more complete. However, its response to increasing oxygen levels, the production of higher levels of the enzyme, is identical to that observed in other *Rhodospirillaceae* (Eley *et al.*, 1979; Cox *et al.*, 1983). Under anaerobic conditions the role of the TCA cycle of *Rm. vannielii* appears solely biosynthetic in function, as is the case with many autotrophs (Pearce *et al.*, 1969; Weitzman, 1982).

The pattern of inhibitor effects on the *Rm. vannielii* citrate synthase (Table 3) is similar to those reported for other members of the *Rhodospirillaceae* (Flechtner & Hanson, 1970; Weitzman, 1982) and is typical of the results obtained in Gram-negative organisms (ATP and NADH sensitive: Weitzman & Jones, 1968). However, the absence of inhibition by 2-oxoglutarate was considered surprising. It has been proposed (Weitzman & Dunmore, 1969) that such an inhibition may represent a feedback control process in an incomplete TCA cycle and subsequent observations in autotrophic thiobacilli (Taylor, 1970), Gram-positive facultative anaerobes (Tanaka & Hanson, 1975) and strict anaerobes (Gottschalk & Dittbrenner, 1970) support this hypothesis. *Rm. vannielii* would appear to be an exception to this pattern of behaviour.

Rm. vannielii is also unusual in its possession of both NAD- and NADP-linked isocitrate dehydrogenase activity. The vast majority of bacteria exhibit only NADP-linked activity, NAD-linked activity having been detected in a restricted yet disparate group of organisms, for example *Xanthomonas pruni* (Ragland *et al.*, 1966), *Acetobacter aceti*, *Gluconobacter suboxydans* (Greenfield & Claus, 1969), *Rhizobium* sp. (Moustafa & Leong, 1975), some thiobacilli (Matin & Rittenberg, 1971) and a number of methylotrophs (Colby & Zatman, 1975). The functional role of the two systems is as yet unclear, for although Tabita & Lundgren (1971) demonstrated that *Thiobacillus ferrooxidans* utilized NADP-linked isocitrate dehydrogenase during aerobic, heterotrophic growth and NAD-linked activity under anaerobic conditions in an incomplete TCA cycle, such an explanation does not follow for *Rm. vannielii* which possesses both enzymes under all growth conditions (D. J. Kelly, P. Morgan & C. S. Dow, unpublished) nor for many of the other organisms cited above.

The absence of significant differences in enzyme activity and inhibitor patterns between heterogeneous and swarmer cell populations of *Rm. vannielii* suggests that the 'shut down' nature of the swarmer cell (Dow *et al.*, 1983) does not involve direct alteration of enzyme activities of the TCA cycle. Whether such effects *in vivo* are undetectable in crude assays of activity and whether such effects would be detectable in organisms possessing a complete TCA cycle is unclear. In the former case it is certainly possible for *in vivo* levels of inhibitors to have an effect which is undetectable in these experiments. It is possible that significant effects on such

important routes of intermediary metabolism as the TCA cycle in the different cell types of prosthecate bacteria would be counter-productive if, as we have proposed, the physiological and ecological roles of swarmer cells (Dow *et al.*, 1983; Morgan & Dow, 1986) *in situ* necessitate a rapid response to changing environmental conditions.

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