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Acetate Metabolism in *Rhodopseudomonas gelatinosa* and Several Other Rhodospirillaceae

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Abstract. When *Rhodopseudomonas gelatinosa* was grown on acetate aerobically in the dark both enzymes of the glyoxylate bypass, isocitrate lyase and malate synthase, could be detected. However, under anaerobic conditions in the light only isocitrate lyase, but not malate synthase, could be found.

The reactions, which bypass the malate synthase reaction are those catalyzed by alanine glyoxylate aminotransferase and the enzymes of the serine pathway.

Other Rhodospirillaceae were tested for isocitrate lyase and malate synthase activity after growth with acetate; they could be divided into three groups: 1. organisms possessing both enzymes; 2. organisms containing malate synthase only; 3. *R. gelatinosa* containing only isocitrate lyase when grown anaerobically in the light.

Key words: *Rhodopseudomonas gelatinosa* – Rhodospirillaceae – Isocitrate lyase – Malate synthase – Alanine glyoxylate aminotransferase – Serine pathway.

During phototrophic growth of *Rhodopseudomonas gelatinosa* on citrate, large amounts of acetate are excreted which serve as growth substrate after exhaustion of citrate (Schaab et al., 1972). As some Rhodospirillaceae have been found to lack isocitrate lyase during growth on acetate (Kornberg and Lascelles, 1960), a study was undertaken to determine whether the glyoxylate cycle only, or perhaps other anaplerotic sequences, play a significant role in acetate metabolism by *R. gelatinosa*.

MATERIALS AND METHODS

Organisms. *Rhodopseudomonas gelatinosa* strain 2150, DSM 149; *R. capsulata* strain Kb 1, DSM 155; *R. palustris*, DSM 124; *R. sphaeroides*, DSM 158; *Rhodomicrobium vannielii* strain 7256, DSM 166; *Rhodospirillum fulvum* strain 1360, DSM 113; *Rsp. rubrum* strain Ha, DSM 107; *Rsp. tenue* strain 2761, and 3761, DSM 109 and 111; *R. viridis* strain 2750 and R.v.F., DSM 135 and 133; *Rsp. molischianum* strain NTHC 131, DSM 120; *Escherichia coli* strain K12, DSM 498; *Paracoccus denitrificans* strain Morris, DSM 413.

Growth and Storage of Bacteria. All phototrophic organisms were grown in the following medium: KH_2PO_4, 1 g; NH_4Cl, 0.4 g; $MgSO_4 \cdot 7\,H_2O$, 0.4 g; $CaCl_2 \cdot 2\,H_2O$, 0.05 g; NaCl, 0.4 g; yeast extract, 0.2 g; trace element solution of Pfennig and Lippert (1966), 10 ml; sodium acetate, 1.64 g and water to 1000 ml. The pH was adjusted to 6.8. Cells were grown in screw-cap bottles (50 or 500 ml) or in 10 l carboys at 1500–2000 lux. For aerobic growth in the dark Erlenmeyer flasks were filled up to 10% with growth medium. The flasks were covered with aluminum foil to exclude light. The incubation temperature was 28°C. The cells were harvested by centrifugation at 38500 g for 20 min and were stored at −18°C.

The optical density of the cultures was measured at 650 nm in a Zeiss PM4 spectrophotometer in cuvettes of 1 cm light path against a medium blank.

Determination of Protein. Protein was determined according to Beisenherz et al. (1953) after extraction of the pigments with 4 ml of acetone.

Preparation of Cell Extract. Approx. 1 g of cells (wet weight) was suspended in 8 ml of 50 mM potassium phosphate buffer, pH 7.2. The suspension was passed through a French pressure cell twice at 4°C and a pressure of 8 kg/cm^2 and then centrifuged at 30000 × g for 30 min.

Enzyme Assays. Spectrophotometric results were obtained with a Zeiss PM4 spectrophotometer. One unit of enzyme is defined as that amount of enzyme catalyzing the conversion of 1 µmole of substrate per minute under assay conditions. Tests were run at room temperature, if not otherwise stated.

a) Isocitrate lyase (EC 4.1.3.1) was tested according to Dixon and Kornberg (1959).

ba) Malate synthase (EC 4.1.3.2) activity was measured according to the method of Dixon and Kornberg (1959).

bb) Malate synthase measurements were also made using the method of Srere et al. (1963). In some organisms (*R. gelatinosa* and *R. palustris*) the enzyme was inhibited by the assay component 5,5′dithio-bis-nitrobenzoate (DTNB), and the method was accordingly modified as follows:

1. The complete mixture was allowed to react for 2 min. The reaction was then stopped with DTNB and the final extinction read at 412 nm.

2. The same reaction mixture minus glyoxylate was run for 2 min. Again it was stopped with DTNB and the extinction read at

412 nm. This value represented acetyl-CoA deacetylase activity, for which value 1 had to be corrected [$E_{412} = 14.14 \cdot 10^3$ according to Collier (1973)].

c) β-Hydroxyaspartate aldolase (EC 4.1.3.14) was assayed according to Gibbs and Morris (1964).

d) Hydroxyaspartate dehydratase (EC 4.2.1.38). This enzyme was assayed by the method described by Kornberg and Morris (1962).

e) Glyoxylate carboligase (EC 4.1.1.47) was assayed by measuring CO_2 evolution in Warburg vessels at 30°C. The reaction mixture contained in a final volume of 2.5 ml: potassium phosphate buffer, pH 6.4, 24 mM; thiaminepyrophosphate, 0.2 mM; $MgCl_2$, 2 mM; sodium glyoxylate, 60 mM and 1.4–5.6 mg extract.

f) Serine hydroxymethyltransferase activity (EC 2.1.2.1) was determined using the method of Taylor and Weissbach (1965).

g) Aminotransferase activities:

1. Glyoxylate aspartate aminotransferase (EC 2.6.1.35) was measured according to Gibbs and Morris (1970).

2. Alanine glyoxylate aminotransferase (EC 2.6.1.122) was determined using a modification of the method of Richardson and Thompson (1970). The reaction mixture contained in a final volume of 2.5 ml: sodium glyoxylate, 25 mM; pyridoxalphosphate, 25 µM; equimolar mixture of boric acid and KCl, adjusted to pH 8.4 with NaOH, 80 mM; alanine, 25 mM and 1.7–2.5 mg extract. The reaction mixture was incubated at 37° C for 5 min and then stopped with 0.3 ml of 3 M TCA. After neutralization with 5 N NaOH, 1/10 of the reaction mixture was added to 100 mM Tris buffer, pH 8.3, containing 1 µg lactate dehydrogenase and 1 mM $NADH_2$ in a final volume of 3 ml; the reduction of pyruvate was followed at 340 nm. It was of great importance to determine the pyruvate concentration in Tris-buffer, otherwise remaining glyoxylate could also react with lactate dehydrogenase.

3. Serine glyoxylate aminotransferase (EC 2.6.1.–) was determined using thin layer chromatography. The reaction mixture was incubated for 4 h at 30° C. 6 µl samples were spotted on cellulose thin layer plates. After development in *n*-butanol-acetone-diethylamine-water (30:30:6:15) the plates were dried and the amino acids detected by spraying with ninhydrin. L-Serine pyruvate aminotransferase (EC 2.6.1.51) was determined by the same method.

4. The serine oxaloacetate aminotransferase activity (EC 2.6.1.–) was measured according to Cripps and Noble (1973).

h) Hydroxypyruvate reductase (EC 1.1.1.81). This enzyme was assayed by following the oxidation of $NAD(P)H_2$ spectrophotometrically at 340 nm. The reaction mixture contained in a final volume of 1 ml: potassium phosphate buffer, pH 6.2, 4 mM; $NAD(P)H_2$, 0.25 mM; lithium hydroxypyruvate, 10 mM and 0.5–1.0 mg extract.

i) Glyoxylate reductase (EC 1.1.1.26 and 1.1.1.79) measurements were made as for hydroxypyruvate reductase, except that hydroxypyruvate was replaced by the same amount of glyoxylate.

k) ATP-D-Glycerate phosphotransferase (glycerate kinase EC 2.7.1.31) was assayed by spectrophotometrically measuring $NADH_2$ oxidation at 340 nm. The complete assay system consisted of a total volume of 1 ml: potassium phosphate buffer, pH 7.3, 50 mM; EDTA, 1 mM; ATP, 0.5 mM; $MgCl_2$, 10 mM; DL-glycerate, 1 mM; $NADH_2$, 0.3 mM; phosphoglyceromutase, 500 µg; enolase, 200 µg; pyruvate kinase, 1 µg; lactate dehydrogenase, 500 µg and 0.6 mg extract. The rate of $NADH_2$ oxidase was first measured in the absence of glycerate and total activity was corrected for this amount.

l) L-Serine dehydratase (EC 4.2.1.13) was assayed according to the method of Bellion and Hersh (1972).

m) The method of Henning and Seubert (1964) was used for measuring pyruvate carboxylase (EC 6.4.1.1). The citrate formed was measured according to Dagley (1969).

n) PEP synthase was assayed according to the method of Cooper and Kornberg (1969).

o) PEP carboxylase (EC 4.1.1.32). The reaction mixture contained in a total volume of 1 ml: Tris-HCl, pH 7.2, 100 mM; $NADH_2$, 0.25 mM; malate dehydrogenase, 10 µg; $KHCO_3$, 50 mM; $MnCl_2$, 1 mM; PEP, 5 mM and 2.15–8.6 mg extract. $NADH_2$ oxidation was followed spectrophotometrically at 340 nm.

Chemicals. Enzymes and biochemicals were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Threo-β- and erythro-β-hydroxyaspartate were from Calbiochem (Los Angeles, California) and hydroxypyruvate from NBC (Nutritional Biochemical Corporation, Cleveland, Ohio). All other chemicals used were from Merck (Darmstadt, Germany). Acetyl-CoA was prepared as described by Simon and Shemin (1953) and tetrahydrofolic acid according to Jones et al. (1961).

RESULTS

Determination of Isocitrate Lyase and Malate Synthase in Rhodopseudomonas gelatinosa Grown with 20 mM Acetate

Growth on acetate requires anaplerotic sequences as the glyoxylate cycle. Therefore, acetate-grown cells of *Rhodopseudomonas gelatinosa* were assayed for isocitrate lyase and malate synthase activity.

When grown anaerobically in the light *R. gelatinosa* contained isocitrate lyase, but no malate synthase (Table 1). However, in cells grown aerobically in the dark both isocitrate lyase and malate synthase could be demonstrated.

The results show that malate synthase was present under one condition but not another, while isocitrate lyase activity was present under both conditions.

Comparative Studies on Nine Other Rhodospirillaceae

That a change in growth conditions affects the activity of enzymes of the glyoxylate cycle has, to our knowledge, never been described. Therefore, nine other Rhodospirillaceae were tested for isocitrate lyase and malate synthase activity after phototrophic growth and after aerobic growth in the dark. Unlike, *R. gelatinosa* drastic changes in enzyme activity could not be detected as shown in Table 2.

Rhodomicrobium vannielii, *Rhodopseudomonas palustris* and *Rhodospirillum tenue* contained both en-

Table 1. Enzymes of the glyoxylate cycle in *Rhodopseudomonas gelatinosa* grown with 20 mM acetate

Growth conditions	Spec. activity (U/g protein)	
	Isocitrate lyase	Malate synthase
Aerobic, dark	115.2	149.0
Anaerobic, light	84.6	<2.0

Isocitrate lyase and malate synthase were assayed as described in "Materials and Methods"

Table 2. Specific activity of isocitrate lyase and malate synthase after either phototrophic growth or aerobic growth in the dark

Organism	Specific activity (U/g protein)					
	Anaerobic light			Aerobic dark		
	ICL	MS	Exp	ICL	MS	Exp
Rhodomicrobium vannielii strain 7256	35.9	17.1	3	18.3	34.9	2
Rhodopseudomonas palustris strain 17002	29.5	50.3	3	47.0	49.0	2
Rhodospirillum tenue strain 3761	40.0	14.8	3	17.6	25.6	1
Rhodospirillum tenue strain 2761	42.0	29.0	2	n.g.	n.g.	–
Rhodopseudomonas gelatinosa strain 2150	84.6	<2.0	10	115.0	149.0	10
Rhodopseudomonas sphaeroides strains 17023	<1.2	50.8	3	1.2	82.6	2
Rhodospirillum rubrum strain Ha	<1.4	66.1	4	1.7	45.2	2
Rhodospirillum fulvum strain 1360	<0.45	16.8	2	n.g.	n.g.	–
Rhodospirillum molischianum strain N 131	<4.4	12.6	1	n.g.	n.g.	–
Rhodopseudomonas viridis strain 2750	<1.7	8.5	2	n.g.	n.g.	–
Rhodopseudomonas viridis strain R.v.F.	<0.3	22.0	2	n.g.	n.g.	–
Rhodopseudomonas capsulata strain Kb 1	<2.0	192.1	4	<0.35	102.1	3

Organisms were grown on 20 mM acetate as described in "Materials and Methods". 1 ml of 5% ascorbic acid solution was added to 100 ml growth medium of *Rsp. fulvum* and *Rsp. molischianum*. ICL = isocitrate lyase; MS = malate synthase; Exp = number of experiments; n.g. = no growth

zymes, independent of the conditions for growth. A large group of Rhodospirillaceae including *Rsp. rubrum* and *R. sphaeroides* lacked isocitrate lyase under anaerobic conditions; as far as these organisms grew with acetate under aerobic conditions this enzyme was also not detectable in the cells.

Enzymes of the β-Hydroxyaspartate and Glycerate Pathway

As *R. gelatinosa* did not contain a detectable malate synthase under anaerobic conditions in the light, it was necessary to look for other enzymes that might "replace" this activity. The first to be investigated were the enzymes of the β-hydroxyaspartate and glycerate pathway.

Erythro-β-hydroxyaspartate dehydratase and erythro-β-hydroxyaspartate aldolase were not present in sonic extracts of acetate-grown cells of *R. gelatinosa*. When extracts of *Paracoccus denitrificans* were assayed, both enzymes could be identified.

In addition, the key enzyme of the glycerate pathway, glyoxylate carboligase, was not present in crude extracts of acetate or glycolate-grown cells of *R. gelatinosa*. Comparative studies with glycolate-grown cells of *Escherichia coli* showed high activities in this organism (3030 U/g protein).

The absence of β-hydroxyaspartate dehydratase and glyoxylate carboligase precluded the operation of these known routes for the assimilation of C_2-compounds.

Aminotransferase Activities

It was then tested, whether aminotransferase reactions might be involved in glyoxylate metabolism. Thus, *R. gelatinosa* was assayed for alanine glyoxylate aminotransferase and aspartate glyoxylate aminotransferase. Aspartate glyoxylate aminotransferase activity could not be detected, however, alanine glyoxylate aminotransferase was present. If the assumption was correct that alanine glyoxylate aminotransferase was one of the reactions which replaced malate synthase, one should expect that the aminotransferase would show higher activity when malate synthase activity was decreasing. This was indeed the case as shown in Figure 1 in which aerobically grown cells were used as inoculum for a growth experiment under anaerobic conditions in the light. The decrease of aminotransferase activity in the late growth phase was unexpected and cannot be explained yet.

Enzymes of the Serine Pathway

Large et al. (1962) proposed a scheme for the formation of C_3- and C_4-compounds in *Pseudomonas* AM1 by a pathway involving a) the hydroxymethylation of glycine to serine and b) the carboxylation of a C_3-compound derived from serine to give a C_4-dicarboxylic acid such as oxaloacetate or malate. This sequence of reactions is known as "serine pathway" (Heptinstall and Quayle, 1970).

Since alanine glyoxylate aminotransferase was present in cells of *R. gelatinosa* grown with acetate anaerobically in the light the glycine formed from

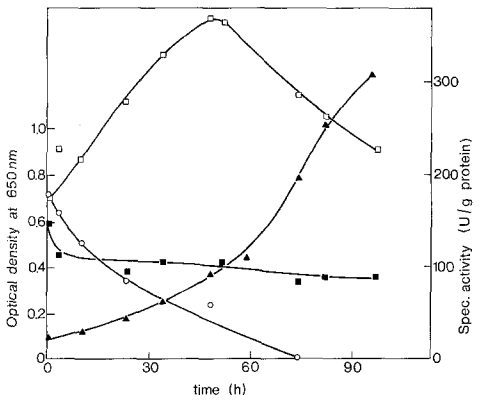

Fig. 1. Increase of alanine glyoxylate aminotransferase activity and decrease of malate synthase activity when changing growth conditions from aerobic dark to anaerobic light. Cells of *Rhodopseudomonas gelatinosa* which had been grown with acetate aerobically in the dark were used as inoculum for a 10-l culture under anaerobic conditions in light. Samples were withdrawn and enzyme activities were determined in the cell extracts prepared. ▲——▲ optical density; ■——■ isocitrate lyase activity; ○——○ malate synthase activity; □——□ alanine glyoxylate aminotransferase activity

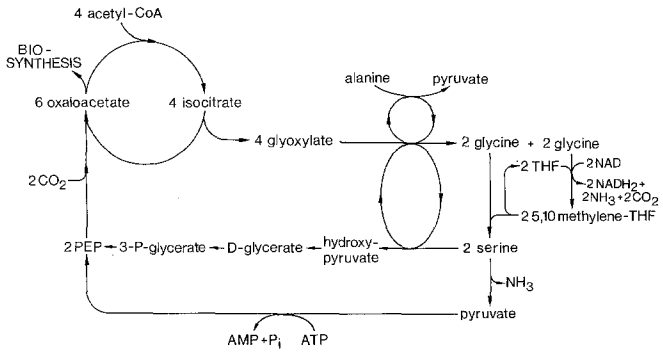

Fig. 2. Possible anaplerotic sequences employed by *Rhodopseudomonas gelatinosa* during phototrophic growth on acetate. Acetyl-CoA is oxidized to glyoxylate by the reactions of the tricarboxylic acid cycle plus isocitrate lyase. Transamination with either alanine or serine as NH_2-donor yields glycine which is converted to serine. Two routes have been found for the formation of PEP from serine, via pyruvate and via D-glycerate. Finally, oxaloacetate is formed by the action of PEP carboxylase. THF, tetrahydrofolate

Table 3. Presence of the enzymes of the serine pathway in extracts of *R. gelatinosa*

Enzyme	Spec. activity (U/g protein)
Serine hydroxymethyltransferase	2.2
Serine dehydratase	17.9
Serine glyoxylate aminotransferase	+
Serine ketoglutarate aminotransferase	−
Serine pyruvate aminotransferase	−
Hydroxypyruvate reductase (NADP-spec.)	20.7
Hydroxypyruvate reductase (NAD-spec.)	<1.0
Glycerate kinase	15.5
PEP synthase	38.2
PEP carboxylase	12.3
Pyruvate carboxylase	<1.5

Enzymes were assayed as described under "Materials and Methods"

glyoxylate by this enzyme could serve as starting material for the synthesis of C_4-dicarboxylic acids via the serine pathway. Therefore, cell extracts were assayed for the individual enzymes of the pathway. The results are summarized in Table 3. Serine hydroxymethyltransferase could be detected in low activity using the radioactive assay of Taylor and Weissbach (1965). Serine dehydratase, PEP synthase and PEP carboxylase were present in high activity so that serine could be converted into oxaloacetate. The data of Table 3 also indicate that an alternate route existed in *R. gelatinosa* for the further metabolism of serine. Activity of an aminotransferase, which converts serine to hydroxypyruvate could be qualitatively demonstrated by thin layer chromatography; furthermore, a NADP specific hydroxypyruvate reductase and glycerate kinase were present in the extracts investigated.

DISCUSSION

It was first shown by Kornberg and Lascelles (1960) that some Rhodospirillaceae lack isocitrate lyase during growth on acetate. *Rhodopseudomonas palustris* and *R. capsulata* were found by these authors to contain both, isocitrate lyase and malate synthase, whereas in *Rhodospirillum rubrum* and *R. sphaeroides* only malate synthase was detectable. The operation of the glyoxylate cycle during growth of *R. palustris* on acetate was further supported by the labelling experiments of Chernyad'ev et al. (1970). Payne and Morris (1969) reported that a mutant strain of *R. sphaeroides* devoid of pyruvate carboxylase was still able to grow on acetate plus CO_2, but unable to grow on pyruvate or on glucose; the authors concluded that this organism can synthesize C_4-dicarboxylic acids from acetate and CO_2 by a pathway which does not involve pyruvate carboxylase.

We have extended the work on anaplerotic enzyme systems involved in acetate metabolism of phototrophic bacteria with special reference to *R. gelatinosa*. This organism contains isocitrate lyase but lacks malate synthase during phototrophic growth on acetate, and, in this respect, it is unique among Rhodospirillaceae we have investigated. *R. gelatinosa* was found to contain an enzyme sequence for the synthesis of phosphoenolpyruvate and C_4-dicarboxylic acids from glyoxylate which does not involve malate synthase: alanine glyoxylate aminotransferase, the en-

zymes of the serine pathway, PEP synthase and PEP carboxylase. These reactions are summarized in Figure 2. The enzyme serine transhydroxymethylase showed only low activity. This was perhaps so because enzyme activity was estimated with the help of tetrahydropteroyl monoglutamate. In the case of *Pseudomonas* AM1 the triglutamate form of the coenzyme had a much greater affinity for the substrate than the monoglutamate (Large and Quayle, 1963).

The serine pathway is responsible for the assimilation of C_1-compounds by a number of microorganisms (Quayle, 1972). To our knowledge this pathway has never been found so far to be involved in the assimilation of acetate.

The majority of the species investigated lacked isocitrate lyase during growth on acetate. What kind of anaplerotic sequence these species employ is not known. Porter and Merrett (1970) provided evidence for the formation of glyoxylate and propionate from α-hydroxyglutarate in *Rsp. rubrum*. Recently, Geissler and Kindl (1975) showed that isocitrate lyase from microbodies can be activated by succinylation, and therefore, isocitrate lyase might be present in extracts of some of the phototrophic bacteria in an inactive form. However, preliminary experiments to activate an isocitrate lyase possibly present in extracts of *Rsp. rubrum* by succinylation were unsuccessful.

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