Formate and Oxalate Metabolism in *Alcaligenes eutrophus*

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Alcaligenes eutrophus strain H16 when grown on formate or oxalate as the sole source of carbon and energy had doubling times between 3.5 and 4.5 h. The respective molar growth yields (Y_m) were 2.35 and 3.9. During growth on formate or oxalate both a soluble and a membrane-bound formate dehydrogenase were formed. The key enzymes of autotrophic CO₂ fixation, ribulose-5-phosphate kinase and ribulosebisphosphate carboxylase, were formed during growth on formate but not on oxalate. Oxalate induced the synthesis of the enzymes of the glycerate pathway. Mutants impaired in autotrophic CO₂ fixation but unaffected in the synthesis of the formate dehydrogenases lost their ability to grow on formate but not to grow on oxalate, giving further evidence that formate was assimilated via CO₂.

INTRODUCTION

Growth on one-carbon (C₁) compounds and their metabolism has been intensively investigated with facultative methylotrophic micro-organisms. The ability to utilize C₁ compounds such as methanol, methylamine or formate is distributed over a wide variety of micro-organisms belonging to different taxonomic groups as reviewed by Quayle (1972) and Anthony (1975). Among the phototrophic bacteria, detailed work has been done with *Rhodospseudomonas palustris* (Stokes & Hoare, 1969) and *Rhodospseudomonas acidophila* (Sahm *et al.*, 1976) which grow anaerobically with methanol or formate in the light, and with some Rhodospirillaceae able to grow on methanol (Quayle & Pfennig, 1975). The information available on the utilization of C₁ compounds by aerobic facultative chemolithotrophic bacteria has so far been limited to *Thiobacillus novellus* (Chandra & Shethna, 1977), *Thiobacillus* A2 (Gottschal & Kuenen, 1977), *Paracoccus denitrificans* (Cox & Quayle, 1975) and *Hydrogenomonas eutropha* Z-1 (Namsaraev *et al.*, 1971). Among anaerobic micro-organisms, some methanogenic bacteria can utilize formate or oxalate for methanogenesis (Dawson & Allison, 1977; Sineriz & Wolin, 1977). Micro-organisms able to grow autotrophically on formate can often also grow on oxalate. This is especially true for a large number of the so called 'hydrogen' bacteria (C. G. Friedrich, unpublished results) and other facultative autotrophic micro-organisms. Micro-organisms utilizing C₁ compounds have developed different methods of carbon assimilation (Quayle & Ferenci, 1978). *Methylomonas methanolica*, *Pseudomonas* C (Goldberg *et al.*, 1976) and several other strains use the ribulose monophosphate cycle (Quayle, 1972), whereas *Pseudomonas* AM1 (Quayle, 1972) and *Hyphomicrobium* X (Harder *et al.*, 1973) use the serine pathway. *Thiobacillus novellus* (Chandra & Shethna, 1977) and *Pseudomonas oxalaticus* oxidize formate to CO₂, as *Paracoccus denitrificans* does with methanol, the CO₂ then being assimilated via the Calvin cycle. This type of C₁ utilization is therefore designated as autotrophic growth on an

organic carbon source (Cox & Quayle, 1975). Among the facultative chemolithoautotrophic micro-organisms, the hydrogen bacteria have not been investigated in detail with respect to their ability to grow on a variety of organic C_1 compounds or oxalate. It is, however, of interest to know whether such bacteria can grow on these compounds and, if so, whether they use the enzymes of the Calvin cycle for CO_2 fixation or have developed another assimilatory system. The present study was done with one of the best characterized representatives of the hydrogen bacteria, *Alcaligenes eutrophus* strain H16, to examine these points.

METHODS

Organisms. *Alcaligenes eutrophus* strain H16 (ATCC 17699, DSM 428) and mutants derived from it were used throughout this study.

Isolation of mutants. Mutants unable to grow autotrophically with a gas mixture of $H_2/O_2/CO_2$ (80:10:10, by vol.) were isolated as follows. Cells were mutagenized with sodium nitrite as described by Friedrich & Schlegel (1975). Since the enrichment of mutants with colistine sulphate usually employed for this organism is ineffective under autotrophic growth conditions, an enrichment procedure with cycloserine was developed that allowed the killing of autotrophically as well as heterotrophically growing cells. Mutagenized cells, grown in a fructose-containing minimal medium for phenotypic expression, were resuspended in minimal medium and incubated overnight under $H_2/O_2/CO_2$. Cycloserine was then added at 1 mg ml^{-1} and the culture was shaken for 4.5 h. The cells were washed to remove the drug and plated on minimal medium containing 0.004% (w/v) sodium pyruvate. Plates were incubated autotrophically. Mutants were identified as pin-point colonies and further characterized by replica-plating and enzyme analysis.

Media and growth conditions. The medium of Schlegel *et al.* (1961) was used for autotrophic and heterotrophic growth in volumes up to 300 ml in 1 l baffled Erlenmeyer flasks. Inocula were grown on the same carbon source as the main culture at 30 °C. For enzyme studies, cells were grown in a medium containing one-fifth of the original buffer concentration and 2 g NH_4Cl in 1 l. The cells were cultivated in a 12 l fermenter (B. Braun, Melsungen, FRG) with a working volume of 10 l at 600 rev. min^{-1} and an aeration rate of 4 l min^{-1} . The pH was kept constant at 7.0 by automatic addition of 5 M-NaOH or 1.7 M- H_3PO_4 . When organic acids were used as carbon sources, cells were grown in extended culture using 50% (v/v) formic acid or 10% (w/v) of other organic acids as titrant for pH adjustment. For autotrophic growth, the fermenter was gassed with $H_2/O_2/CO_2$ (80:10:10, by vol.) at 850 ml min^{-1} . The growth yield (Y_s) or the molar growth yield (Y_m) obtained from the different carbon sources was calculated using the equation Y_s or $Y_m = (X_2 - X_1)/(S_2 - S_1)$, where X was the cell concentration (g dry wt) produced and S the amount of substrate (g or mol) used within a time interval $t_2 - t_1$. Growth was followed turbidimetrically at 436 nm in a Zeiss PM4 spectrophotometer; 1 g dry wt l^{-1} corresponded to an A_{436} of 4.8. The substrate consumption of organic acids was calculated from the integrated cycles of a calibrated piston pump coupled to the pH-stat of the fermenter and the concentration of the respective acid.

Substrate shift experiments were conducted in a fermenter with 10 l mineral medium containing 0.4% (w/v) sodium pyruvate, and the pH controlled by adding 1.7 M- H_3PO_4 . At an A_{436} of 5.8 pyruvate was exhausted. After another 30 min, a concentrated sodium formate solution was added to the culture to give a final concentration of 20 mM, and the pH was then kept constant at 7.0 by adding 50% (v/v) formic acid. For a substrate change to oxalate, 10% (w/v) oxalic acid was added dropwise to the medium which was simultaneously neutralized with a 5 M-NaOH until a final concentration of 20 mM-sodium oxalate was obtained. During the subsequent oxalate consumption, the pH was adjusted to 7.0 by adding 10% (w/v) oxalic acid. For sampling, cells from 200 ml suspension were collected by centrifuging (yielding about 1 g wet wt), washed once with 0.9% (w/v) sodium chloride and stored at -20 °C.

Preparation of cell free extracts. Cells (about 1 g wet wt) were suspended in 5 ml 50 mM-Tris/HCl buffer, pH 7.6; 0.5 mg deoxyribonuclease I was then added and the cells were disrupted in a pre-cooled French press at 140 MPa. Cell debris was removed by centrifuging at 120000 g for 60 min at 4 °C. The supernatant is referred to as the soluble fraction. To stabilize the soluble hydrogenase, a portion of the soluble fraction was treated with ferricyanide at a final concentration of 0.5 mM. The membranes sedimented above the cell debris were scraped off and resuspended in 2.5 ml 50 mM-potassium phosphate buffer, pH 7.0, and homogenized with a Potter-Elvehjem homogenizer. All extracts were kept at 0 °C.

Enzyme assays. Enzyme activities were determined at 30 °C. Where appropriate, a Zeiss PM4 spectrophotometer fitted with a transmission converter and recorder was used.

The soluble formate dehydrogenase (EC 1.2.1.2) was assayed in 1 ml cuvettes (path length 1 cm) containing (final vol. 1 ml) 50 mM-potassium phosphate buffer, pH 7.5, 1.2 mM-NAD⁺ and 10 mM-sodium formate. The reaction was initiated by adding the extract and the formation of NADH was recorded at 340 nm.

The particulate formate dehydrogenase was assayed anaerobically under N_2 . All solutions except the extract were kept under N_2 . The reaction mixture contained (final vol. 3.0 ml) 50 mM-potassium phosphate buffer, pH 7.0, 0.17 mM-methylene blue and 5 to 50 μ l of the membrane fraction. The reaction was initiated by adding 50 μ l 200 mM-sodium formate and was followed by the decrease in A_{570} . The molar extinction coefficient of methylene blue was $13.1 \text{ l mmol}^{-1} \text{ cm}^{-1}$.

Glyoxylate dehydrogenase was determined according to the procedure of Quayle (1966) with modifications. The reaction mixture contained (final vol. 1 ml) 50 mM-sodium pyrophosphate buffer, pH 9.5, 2 mM-cysteine, 5 mM-sodium glyoxylate, 0.02 mM-coenzyme A and 0.2 mM-NAD⁺. The reaction was started by adding the enzyme.

Glyoxylate carboligase (EC 4.1.1.47) was assayed in a total volume of 1 ml according to Kaltwasser (1968).

Ribulosebisphosphate carboxylase (EC 4.1.1.39) activity was measured by ribulose bisphosphate-dependent incorporation of $^{14}\text{CO}_2$ into acid-stable product as described by Bowien *et al.* (1976). The activity of ribulose-5-phosphate kinase (EC 2.7.1.19) was determined in a two-stage assay by the incorporation of $^{14}\text{CO}_2$ into acid-stable product when the ribulose-5-phosphate kinase was coupled to ribulosebisphosphate carboxylase purified from *A. eutrophus* (Bowien *et al.*, 1976). The ribulosebisphosphate carboxylase preparation was free of ribulose-5-phosphate kinase activity. The primary reaction mixture contained (in 0.25 ml) 25 μ mol Tris/HCl buffer, pH 8.0, 2.5 μ mol MgCl_2 , 1.25 μ mol ATP, 0.125 μ mol NADH, 0.375 μ mol ribulose 5-phosphate and 5 to 20 μ l of the soluble fraction. After 5 min incubation at 30 °C, the reaction was initiated by adding the ribulose 5-phosphate, and 2 min later it was terminated by heating in a boiling water bath for 1 min. The mixture was then incubated at 30 °C with 0.1 ml 0.1 M-Tris/HCl buffer, pH 8.0, containing 1 μ mol MgCl_2 , 12.5 μ mol $\text{NaH}^{14}\text{CO}_3$ (0.05 Ci mol^{-1}) and 0.02 units of ribulose bisphosphate carboxylase. After 15 min, 0.15 ml 60% (w/v) trichloroacetic acid was added and the sample was shaken for 60 min at room temperature to expel excess $^{14}\text{CO}_2$. Acid-stable radioactivity was measured in a Packard liquid scintillation spectrometer.

The soluble hydrogenase (EC 1.12.1.2) was assayed as described by Schneider & Schlegel (1976); the membrane-bound hydrogenase was measured according to Schink & Schlegel (1978), but with glucose, glucose oxidase and catalase omitted.

One unit of enzyme activity is defined as 1 μ mol substrate transformed or product formed min^{-1} . Protein was determined by Lowry's method.

RESULTS

Growth studies

Alcaligenes eutrophus grew aerobically with formate, oxalate, glyoxylate, glycollate or acetate (in a mineral salts medium) as the sole source of carbon and energy (Table 1). It did not grow on methanol, formaldehyde, methylamine, formamide or ethanol as carbon source. The mean doubling time on formate and oxalate was about 4 h; this was high compared with autotrophic growth on H_2/CO_2 (3.2 h) and heterotrophic growth on fructose (2.0 h) or pyruvate (1.5 h) (Table 1).

Although cells were grown with formate or oxalate in extended culture, it was not possible to obtain such high cell concentrations as were obtained under autotrophic conditions with $\text{H}_2/\text{O}_2/\text{CO}_2$ or heterotrophic conditions with pyruvate or fructose. Growth ceased at cell concentrations of 0.8 to 1.2 g dry wt l^{-1} . This was thought to be due to O_2 starvation. To yield the same cell mass, growth on, e.g., formate requires six to eight times more O_2 than growth on fructose. The yields obtained with formate or oxalate were about 50 mg dry wt (g substrate) $^{-1}$. (Low values were expected from such highly oxidized substrates.) However, the molar yields were distinctly different, being 2.35 g dry wt mol^{-1} for formate and 3.9 for oxalate (Table 1). This was surprising since the energy yield of both substrates is identical and 1 mol of each substrate yields 1 mol NADH (Blackmore *et al.*, 1968). Formate was utilized with almost 50% greater efficiency by *Paracoccus denitrificans* (Y_m 3.6) (van Verseveld & Stouthamer, 1978), by *Thiobacillus* A2 (Y_m 3.5) (Gottschal & Kuenen, 1977) or by *Pseudomonas oxalaticus* OX1 (Y_m 3.4); this last strain grew with a similar molar yield on oxalate (Y_m 3.8) (Dijkhuizen *et al.*, 1977) to that of *A. eutrophus*.

Table 1. *Growth rates and yields of A. eutrophus*

Growth yields [Y_s , expressed as g dry wt (g substrate) $^{-1}$, and Y_m , expressed as g dry wt (mol substrate) $^{-1}$] were determined as described in Methods and are calculated on the basis of free acids.

Substrate	Doubling time (d)	Y_s	Y_m
Formate	4.0	0.051	2.35
Oxalate	4.0	0.044	3.9
Pyruvate	1.5	0.38	33.7
Fructose	2.0	0.44	79.2
H ₂ /CO ₂	3.2	ND	ND
Glyoxylate	6 to 9	0.083	6.12
Glycollate	3.3	0.146	11.10

ND, Not determined.

Table 2. *Activities of energy-generating enzymes of A. eutrophus during growth on different substrates*

Cells were grown in a fermenter with a 10 l working volume, different sample volumes were taken to yield about 1 g wet wt cells and enzymes were determined as described in Methods. There was no change in the specific activity of all enzymes measured during the exponential and early-stationary phases caused by nitrogen depletion in the medium. The data presented were obtained from samples of mid-exponential growth.

Substrate	Specific activity [units (mg protein) $^{-1}$]			
	Formate dehydrogenase		Hydrogenase	
	Soluble	Particulate	Soluble	Particulate
Formate	0.245	0.128	0.524	0.424
Oxalate	0.247	0.216	0.008	0.025
H ₂ /CO ₂	0.007	0.231	0.419	0.918
Fructose	0.003	0.028	0.108	0.101
Pyruvate	0.002	0.018	0.003	0.003

Enzyme studies

To elucidate the mode of formate and oxalate utilization, the enzymes of energy generation and carbon assimilation were studied. *Alcaligenes eutrophus* formed two formate dehydrogenases during growth on formate and oxalate – a soluble formate:NAD⁺ oxidoreductase and a membrane-bound formate:acceptor oxidoreductase. A membrane-bound formate oxidase was reported by Chandra & Shethna (1975) from *Alcaligenes LOX* grown on succinate plus formate. The membrane-bound formate dehydrogenase from *A. eutrophus* was routinely assayed anaerobically with methylene blue as electron acceptor. Under the same conditions of growth, both soluble and membrane-bound hydrogenases were formed (Table 2). The hydrogenases apparently resemble the formate dehydrogenases with respect to location and acceptor specificity. It was possible that the formate dehydrogenase activities were identical with the hydrogenase proteins but with a lack of substrate specificity. However, highly purified soluble as well as particulate hydrogenase did not function with formate as substrate. Furthermore, some enzymic properties of the formate dehydrogenases differed significantly from those of the hydrogenases (Y. Aharonowitz & C. G. Friedrich, unpublished results).

Growth on various carbon and energy sources revealed that the synthesis of the soluble formate dehydrogenase was regulated by induction (Table 2). Both enzymes were formed at a high activity during growth on formate or oxalate. The soluble enzyme was almost absent when fructose, pyruvate or H₂/CO₂ served as energy and carbon sources. Also, the expression of the particulate enzyme was markedly decreased except under chemolitho-

Table 3. Activities of carbon-assimilating enzymes of *A. eutrophus* during growth on different substrates

Conditions were as described in Table 2.

Substrate	Specific activity [units (mg protein) ⁻¹]			
	Glyoxylate carboligase	Glyoxylate dehydrogenase	Ribulose-bisphosphate carboxylase	Ribulose-5-phosphate kinase
Formate	0.030	0.004	0.221	0.113
Oxalate	0.454	0.175	0.023	0.001
H ₂ /CO ₂	0.018	0.002	0.263	0.211
Fructose	0.015	0.001	0.053	< 0.001
Pyruvate	0.009	0.004	< 0.001	< 0.001

Fig. 1. Formation of energy-generating and carbon-assimilating enzymes of *A. eutrophus* after a substrate shift from pyruvate to formate. The substrate shift and the determination of the enzyme activity were done as described in Methods. Formate was added at zero time. (a) ○, Membrane-bound formate dehydrogenase; ●, soluble formate dehydrogenase. (b) ○, Ribulose-5-phosphate kinase; ●, ribulose-bisphosphate carboxylase. □ and ■ represent chloramphenicol controls for enzymes with open and closed symbols, respectively.

autotrophic conditions. There was no correlation between the formation of hydrogenases and formate dehydrogenases.

Of the various possible carbon-assimilating reactions during growth on formate or oxalate, we decided to follow the activities of ribulose-5-phosphate kinase and ribulose-bisphosphate carboxylase and of glyoxylate dehydrogenase and glyoxylate carboligase. Only ribulose-5-phosphate kinase and ribulose-bisphosphate carboxylase were formed during growth on formate and, as expected, under autotrophic conditions with hydrogen as electron donor. Ribulose-bisphosphate carboxylase, however, was also formed with oxalate as carbon source at up to 10% of the autotrophic level and with fructose at up to 20%. During heterotrophic growth, ribulose-5-phosphate kinase was absent. Glyoxylate dehydrogenase and glyoxylate carboligase were formed exclusively during growth on oxalate (Table 3). Glyoxylate dehydrogenase reacted only with NAD⁺ and not with NADP⁺ as described for the enzyme from *Ps. oxalaticus* (Quayle, 1966).

Fig. 2. Formation of energy-generating and carbon-assimilating enzymes of *A. eutrophus* after a substrate shift from pyruvate to oxalate. The substrate shift and enzyme determination were done as described in Methods. Oxalate was added at zero time. (a) ○, Membrane-bound formate dehydrogenase; ●, soluble formate dehydrogenase. (b) ○, Ribulose-5-phosphate kinase; ●, ribulosebiphosphate carboxylase; △, glyoxylate carbolygase. □ and ■ represent chloramphenicol controls for the enzymes with open and closed symbols, respectively, and ▲ the control for glyoxylate carbolygase.

Table 4. Activities of formate dehydrogenases and CO₂-assimilating enzymes in mutants of *A. eutrophus*

Cells were grown in 400 ml minimal medium containing 0.4% (w/v) sodium pyruvate stirred magnetically at 30 °C. The pH was kept constant at 7.0 by adding 1.7 M-H₃PO₄. After growth had ceased, 200 ml of the culture was centrifuged as described in Methods, and sodium formate was added to the remainder to give 20 mM. Incubation was continued for another 4 h while the pH was kept at 7.0 by adding 5 M-formic acid.

Strain	Relevant phenotype*	Carbon source	Specific activity [units (mg protein) ⁻¹]			
			Formate dehydrogenase		Ribulose-bisphosphate carboxylase	Ribulose-5-phosphate kinase
			Soluble	Particulate		
H16	Aut ⁺ Hyd ⁺	Pyruvate	0.002	0.008	< 0.001	< 0.001
		Formate	0.447	0.244	0.243	0.311
HF18	Aut ⁺ Hyd ⁻	Pyruvate	0.002	0.010	< 0.001	< 0.001
		Formate	0.294	0.066	0.232	0.307
HF17	Aut ⁻ Hyd ⁺	Pyruvate	0.002	0.013	< 0.001	< 0.001
		Formate	0.047	0.011	< 0.001	< 0.001

* Aut⁻, Inability to assimilate CO₂ via the Calvin cycle; Hyd⁻, lack of soluble and membrane-bound hydrogenase activity.

Substrate shift and enzyme formation

From Tables 2 and 3 it is evident that neither the specific energy-generating nor the specific carbon-assimilating enzymes involved in the metabolism of oxalate and formate were present during growth on pyruvate. Bacteria grown on pyruvate were therefore suitable for following enzyme formation after exhaustion of pyruvate in the medium and exposure of the culture to formate or oxalate. A shift from pyruvate to formate caused the immediate formation of ribulose-5-phosphate kinase and ribulosebiphosphate carboxylase. One hour after adding formate, increases in the activity of the soluble and membrane-bound formate

dehydrogenases were detected. The maximum rates of increase in the specific activities of the enzymes were almost identical (Fig. 1).

During a shift from pyruvate to oxalate, the activities of the same enzymes were followed and, in addition, glyoxylate carboligase was assayed as a specific enzyme of the glycerate pathway. The soluble formate dehydrogenase [the only enzyme for energy generation during growth on oxalate (Blackmore *et al.*, 1968)] and the membrane-bound formate dehydrogenase increased about 1 h after the substrate change and were synthesized during the following 4 h (Fig. 2). Glyoxylate carboligase was also formed at a constant rate starting about 1 h after the introduction of oxalate. As expected, the key enzymes for CO₂ fixation (ribulose-5-phosphate kinase and ribulosebispophosphate carboxylase) remained at very low specific activities (Fig. 2*b*). The increase in enzyme activities after the shifts was due to *de novo* synthesis since no increase was observed in the presence of chloramphenicol.

Investigation with mutants

The biochemical data suggested that autotrophic growth on formate was occurring since the key enzymes of CO₂ fixation were present at a high activity. This evidence was further supported by experimental results with mutants that had lost the ability to grow autotrophically with H₂ as energy source and CO₂ as carbon source. Two classes of mutants were characterized: (i) strains lacking the key enzymes of the Calvin cycle (Aut⁻) and (ii) strains lacking the soluble as well as the membrane-bound hydrogenase (Hyd⁻). Aut⁻ mutants were unable to grow on formate but grew well on oxalate. Hyd⁻ mutants were able to grow on both substrates. Both types of mutants still formed formate dehydrogenases after shifting the cells from pyruvate to formate (Table 4). However, strain HF17 (Aut⁻) did not reveal any activity of ribulosebispophosphate carboxylase or ribulose-5-phosphate kinase, which obviously resulted in the failure to grow on formate.

DISCUSSION

Alcaligenes eutrophus strain H16 grew well on formate or oxalate as sole source of carbon and energy. From the data presented here, it is clear that *A. eutrophus* did not assimilate formate directly but first oxidized it to CO₂ by the action of two formate dehydrogenases. The soluble enzyme was specifically formed when formate was introduced into the medium and, being linked to NAD⁺, it enabled the cell to derive energy from formate. The particulate enzyme was unspecifically formed and appeared under different conditions of growth at certain levels; thus its physiological function as well as its physiological electron acceptors remain to be elucidated. That the CO₂ derived from formate was fixed via the Calvin cycle is indicated by two results: (i) the appearance of ribulose-5-phosphate kinase and ribulosebispophosphate carboxylase during growth on formate and also on the introduction of formate into the medium; and (ii) mutants unable to fix CO₂ via the Calvin cycle were also unable to grow on formate.

The multivalent use of the term 'autotrophy' has led to a generally accepted definition which restricts its use to the carbon source regardless of where the energy comes from: an autotrophic organism is able to derive cellular carbon from CO₂ as the major carbon source. Since *A. eutrophus* derived its carbon from CO₂ during growth on formate, by this definition it grows autotrophically on this substrate.

Growth on oxalate was heterotrophic since mutants unable to grow autotrophically grew on oxalate as well as the wild-type. Furthermore, the key enzymes for CO₂ fixation could be detected neither in oxalate-grown cells nor in those from the induction experiment with oxalate.

Alcaligenes eutrophus therefore utilized formate like a number of other bacteria, e.g. *Ps. oxalaticus* (Quayle & Keech, 1959), *Pc. denitrificans* (Cox & Quayle, 1975), *Thiobacillus* A2 (Gottschal & Kuenen, 1977) and *T. novellus* (Chandra & Shethna, 1977), another facultative chemolithoautotrophic organism that can also grow on oxalate. A wide variety of

bacteria can utilize formate and two mechanisms of utilization have been described (Quayle, 1972; Quayle & Ferenci, 1978): (i) the reduction of formate and subsequent assimilation of formaldehyde via the serine pathway and (ii) the oxidation of formate and assimilation of CO₂. With a few exceptions, bacteria oxidize formate to CO₂ and, except for the methanogenic bacteria, assimilate CO₂ via the Calvin cycle. Among these organisms only *A. eutrophus*, *Pc. denitrificans*, *T. novellus* and *Thiobacillus* A2 are facultative chemolithoautotrophs. The questions of whether the ability to utilize formate was gained by autotrophic microorganisms and whether the ability to use another energy source like hydrogen or thiosulphate was lost by certain organisms during evolution remain to be answered.

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