Biosynthetic pathways in Oxalobacter formigenes

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Biosynthetic pathways in *Oxalobacter formigenes*

by

Nancy A. Cornick

A Dissertation Submitted to the
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Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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Major: Microbiology

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For the Graduate College

Iowa State University
Ames, Iowa

1995
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Oxalate is an organic, dicarboxylic acid which is present in a variety of foods consumed by man and other animals. Spinach, rhubarb, chocolate, tea, and coffee contain significant amounts of oxalate. A normal western diet, that includes milk products, contains enough calcium to bind much of the oxalate in diets and thus may inhibit its absorption from the gastrointestinal tract (Hodgkinson 1977).

In western countries, about 2% of the population suffer from urinary tract calculi, the majority of which are composed of calcium oxalate. People who form "idiopathic" calcium oxalate stones tend to have increased urinary excretion of both calcium and oxalate. In addition, they may have decreased levels of inhibitors of calcium oxalate crystal formation in their urine. This combination of factors probably leads to the formation of stones. A diet low in both calcium and oxalate can reduce the recurrence rate of stone formation (Hodgkinson 1977).

Oxalic acid is poisonous for animals. Cattle and sheep become intoxicated by grazing oxalate-rich plants such as halogeton, sourisob or greasewood. Halogeton is commonly found in the arid and semi-arid areas of the western US. It is a palatable plant and animals will consume it even when other forage is available (James 1972). The severity of poisoning depends on the overall nutritional status of the animal, the amount of calcium in the diet, and the amount of oxalate ingested. A healthy well-fed animal can tolerate a much larger dose of oxalate than can a hungry, dehydrated animal (Hodgkinson 1977).

Oxalate is degraded in the rumen by the resident microbial population (James...
1972). Sheep can become adapted to dietary levels of halogeton that would intoxicate a naive animal (Allison and Reddy 1984; James 1972). Rumen microbial populations from adapted animals degrade oxalate faster than unadapted populations (Allison et al. 1977; Daniel et al. 1989), and this is probably what protects the animal from acute poisoning. However, increasing amounts of oxalate will eventually overwhelm the rumen microbes and the animal becomes intoxicated (Allison et al. 1977).

The metabolism of oxalate by aerobic bacteria has been well documented (Hodgkinson 1977). Several genera of bacteria are able to utilize oxalate as the sole source of carbon and energy. The metabolic pathways used by these bacteria involve the activation of oxalate to oxalyl-CoA and then decarboxylation to formyl-CoA and CO₂. Formate is oxidized to CO₂ which appears to be the main energy generating mechanism. Oxalate is assimilated into cell biomass via the glycerate pathway or by a variation of the serine pathway. However, aerobic bacteria are not likely agents in the rumen or other anaerobic habitats. *Oxalobacter formigenes* is believed to be the organism that is responsible for oxalate degradation in the rumen and other gastrointestinal habitats.

The isolation of *O. formigenes* represented the first well described characterization of an anaerobic oxalate-degrading bacterium (Allison et al. 1985; Dawson et al. 1980). Subsequent studies revealed how *O. formigenes* coupled the transport and decarboxylation of oxalate to the generation of ATP (Anantharam et al. 1989; Ruan et al. 1992). Several other anaerobic oxalate-degrading bacteria have now been described, but nothing is known about how these bacteria obtain energy from oxalate or assimilate carbon from oxalate into cellular material (Daniel and Drake 1993;
Dehning and Schink 1989; Postgate 1963). This dissertation addresses the assimilation of oxalate, acetate and CO₂ into cellular material and is an attempt to predict the biosynthetic pathways that are operative in O. formigenes.

Dissertation Organization

This dissertation contains three manuscripts. The first manuscript is to be submitted to Archives of Microbiology and the second and third manuscripts are to be submitted to the Canadian Journal of Microbiology. The manuscripts are preceded by a literature review and followed by a general summary and an appendix to the second manuscript. The candidate, Nancy A. Cornick, was the principal investigator for the studies. The data in Table 1 in the first manuscript was provided by the coauthor of the manuscript, M. J. Allison.

References


Oxalate-degrading Bacteria

Both aerobic and anaerobic bacteria are capable of utilizing oxalate as a principal carbon and energy source. Most of these organisms also utilize other sources of carbon but several are specialists and grow only at the expense of oxalate. The currently known oxalate-degrading bacteria are listed in Table 1.

Aerobic bacteria

*Pseudomonas oxalaticus* is a strictly aerobic gram negative rod that was isolated from earthworms (Kambata and Bhat 1953). *P. oxalaticus* grows on a variety of substrates in addition to oxalate. Formate, lactate, succinate, glyoxylate, glycolate, malonate, acetate and glycerol all support growth. Formate is oxidized to CO$_2$ and assimilated via the Calvin-Benson cycle (Blackmore et al. 1968). Labeling studies with $^{14}$C-formate indicate that at least 94% of the formate that was assimilated into cell biomass passed through the carbonate pool (Quayle and Keech 1959a). Growth on substrates other than formate is heterotrophic.

Cells grown on oxalate do not form ribulosebisphosphate carboxylase (RuBisCO) (Quayle and Keech 1959b, c). Membrane vesicles made from cells grown on oxalate actively transport oxalate in the presence of the artificial electron donor, ascorbate-phenazine methosulfate (Dijkhuizen et al. 1977). The uptake of oxalate was also observed with NADH, but at a lower rate. Other potential electron donors such as formate and succinate did not stimulate oxalate uptake. Vesicle preparations made from cells grown on formate or lactate did not accumulate oxalate. Dijkhuizen et al.
Table 1. Oxalate-degrading bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Habitat</th>
<th>Reference</th>
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<tr>
<td><strong>Aerobic bacteria</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Pseudomonas oxalaticus</em></td>
<td>soil</td>
<td>Khambata and Bhat 1953</td>
</tr>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>water, soil</td>
<td>Friedrich et al. 1979</td>
</tr>
<tr>
<td><em>Methylobacterium extorquens</em></td>
<td>soil</td>
<td>Blackmore and Quayle 1970</td>
</tr>
<tr>
<td><em>Thiobacillus novellus</em></td>
<td>soil</td>
<td>Chandra and Shethna 1977</td>
</tr>
<tr>
<td><em>Xanthobacter</em> species</td>
<td>water, soil</td>
<td>Jenni et al. 1987</td>
</tr>
<tr>
<td><em>Bacillus oxalophilus</em></td>
<td>soil</td>
<td>Zaitsev et al. 1993</td>
</tr>
<tr>
<td><em>Carbophilus carboxidus</em></td>
<td>soil</td>
<td>Meyer et al. 1993</td>
</tr>
<tr>
<td><em>Oligotropha carboxidivorans</em></td>
<td>soil, sewage</td>
<td>Meyer et al. 1993</td>
</tr>
<tr>
<td><em>Pseudomonas carboxydohydrogena</em></td>
<td>soil</td>
<td>Palleroni 1984</td>
</tr>
<tr>
<td><strong>Anaerobic bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Oxalobacter formigenes</em></td>
<td>gut, sediment</td>
<td>Allison et al. 1985</td>
</tr>
<tr>
<td><em>Oxalobacter vibriiformis</em></td>
<td>sediment</td>
<td>Dehning and Schink 1989</td>
</tr>
<tr>
<td><em>Oxalophilus oxalaticus</em></td>
<td>sediment</td>
<td>Dehning and Schink 1989</td>
</tr>
<tr>
<td><em>Desulfovibrio vulgaris ssp. oxamicus</em></td>
<td>sediment</td>
<td>Postgate 1963</td>
</tr>
<tr>
<td><em>Clostridium thermoaceticum</em></td>
<td>manure</td>
<td>Daniel and Drake 1993</td>
</tr>
<tr>
<td><em>Clostridium thermoautotrophicum</em></td>
<td>hot spring</td>
<td>Daniel and Drake 1993</td>
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°This organism was originally placed in the genus *Clostridium*, however after comparisons of 16S rRNA sequences it was assigned to a new genus (Collins et al. 1994)
(1977) concluded that oxalate was taken up by an inducible active transport system and that this system had both a high affinity and a high specificity for oxalate.

Upon entering the cells, oxalate is activated to oxalyl-CoA by a CoA transferase (Quayle 1963b). Oxalyl-CoA is decarboxylated to formyl-CoA by the enzyme oxalyl-CoA decarboxylase (Quayle 1963b; Quayle et al. 1961). Cell-free extracts decarboxylate oxalate in the presence of formyl-CoA. Formyl-CoA is an intermediary metabolite between oxalate and formate and does not participate in any other reaction in the cells (Quayle 1963b). Oxalyl-CoA decarboxylase was purified by Quayle et al. (1961). Thiamine pyrophosphate (TPP) was required for enzymatic activity and Mg\(^{2+}\) or Mn\(^{2+}\) ions enhanced that activity. Oxalyl-CoA decarboxylase was only produced when cells were grown on oxalate.

The formate produced by the decarboxylation of oxalyl-CoA is oxidized to CO\(_2\) and reductant by formate dehydrogenase. The oxidation of formate is the main energy-yielding reaction of cells grown on oxalate (Quayle et al. 1961). *P. oxalaticus* produces both a soluble, NAD-dependent formate dehydrogenase (Quayle et al. 1961) and a membrane-bound NAD-independent formate dehydrogenase (Dijkhuizen et al. 1979). Dijkhuizen et al. (1979) concluded that the membrane-bound enzyme did not react directly with O\(_2\), but transferred electrons through a membrane-bound electron transport system. Both the soluble and membrane-bound enzyme compete equally for substrate.

*P. oxalaticus* was incorrectly named by Khambata and Bhat (1953). Jenni et al. (1988) reviewed the relationship between *P. oxalaticus* and *Alcaligenes eutrophus*. Phenotypically both organisms were very similar although *P. oxalaticus* does not grow
autotrophically on H$_2$ plus CO$_2$. The two bacteria share 50% DNA/DNA homology with each other (Jenni et al. 1988). A DNA/DNA homology of this level suggests that these species are closely related to one another but are distinct species (Johnson 1984).

Bacteria in the genus *Alcaligenes* are a group of strict aerobes which are capable of using a variety of organic acids and amino acids as carbon sources (Kersters and De Ley 1984). *A. eutrophus* strain H16 utilizes oxalate in addition to formate, H$_2$ plus CO$_2$, organic acids, fructose and glucose. Growth on formate is considered autotrophic since formate is oxidized to CO$_2$ and then assimilated using the Calvin-Benson cycle. RuBisCO and ribulose-5-phospho kinase, the key enzymes of the Calvin cycle, are not formed when cells are grown on oxalate (Friedrich et al. 1979). Oxalate is decarboxylated to formate and CO$_2$ and formate is oxidized by both a soluble, NAD-dependent formate dehydrogenase and a membrane-bound formate dehydrogenase.

Although *A. eutrophus* is the validly published name for this group of organisms, they are listed as *species incertae sedis* in the 9th edition of Bergey's Manual (Kersters and De Ley 1984), because of phenotypic and phylogenetic differences from the type strain of the genus, *A. faecalis*. DNA/rRNA homology studies indicate that *A. eutrophus* is more closely related to *Pseudomonas solanacearum* then to *A. faecalis* (Kersters and De Ley 1984). Other strains of *Pseudomonas* (RJ1, KOx, M27, OD1) and *Alcaligenes* LOx have been reported to grow on oxalate (Chandra and Shethna 1975a, b; Hodgkinson 1977). Currently, the taxonomic status of these strains is uncertain.

The pink-pigmented facultative methylotrophic bacteria are a group of organisms that utilize C$_1$ compounds in addition to organic compounds as substrates. A number of these organisms also utilize oxalate as a substrate. Oxalate is decarboxylated to
formate and CO\(_2\) by oxalyl-CoA decarboxylase. Formate is oxidized by a NAD-dependent formate dehydrogenase (Blackmore and Quayle 1970). This group of bacteria were placed in various genera (*Pseudomonas, Protaminobacter*) (Hodgkinson 1977) until Green and Bousfield (1985, 1982) proposed they be reclassified as *Methylobacterium extorquens*.

*Thiobacillus* sp. are a group of bacteria that oxidize reduced or partially reduced sulfur compounds to sulfate (Kelly and Harrison 1989). Some species are obligate autotrophs and others utilize organic substrates. Oxalate and formate support the growth of *T. novellus* (Chandra and Shethna 1977). Cells grown on oxalate did not oxidize thiosulfate whereas those grown on formate did. High levels of RuBisCO are induced in cells grown on formate but not in cells grown on oxalate. Formate is oxidized by an NAD-dependent formate dehydrogenase.

*Xanthobacter* sp. are obligately aerobic bacteria that oxidize H\(_2\), grow autotrophically on CO\(_2\) via the Calvin-Benson cycle, and fix N\(_2\). Most species also grow heterotrophically on alcohols and organic acids (Jenni et al. 1987; Wiegel and Schlegel 1984). *X. autotrophicum* and *X. flavus* utilize oxalate as a carbon and energy source (Jenni et al. 1987).

*Bacillus oxalophilus* is a recently described bacterium that was isolated from the rhizosphere of sorrel (Zaitsev et al. 1993). Unlike other aerobic oxalate-degrading bacteria, oxalate is the only individual substrate that supports growth. Zaitsev et al. (1993) reported "poor growth" on mixtures of formate/glyoxylate, formate/glycerate, and methanol/glyoxylate. Other substrates are not utilized. Oxalate is decarboxylated by oxalyl-CoA decarboxylase to formate and CO\(_2\). Formate is oxidized by formate
dehydrogenase (phenazine methosulfate dependent).

*Carboxiphilus carboxidus, Oligotropha carboxidovorans,* and *Pseudomonas carboxydohydrogena* have the ability to utilize CO as a sole carbon and energy source for autotrophic growth. These aerobic bacteria also utilize a variety of substrates for heterotrophic growth, including oxalate and glyoxylate (Meyer et al. 1993; Palleroni 1984).

**Anaerobic bacteria**

*Oxalobacter formigenes* was the first anaerobic oxalate-degrading bacterium to be described in detail. It was isolated by Dawson et al. (1980) from an enrichment culture inoculated with ovine rumen contents. *O. formigenes* has also been isolated from the gastrointestinal contents of cattle, pigs, (Allison et al. 1985) humans (Allison et al. 1986), wild rats, (Daniel et al. 1987b) and lake sediments (Smith et al. 1985). Horses, rabbits (Allison and Cook 1981), hamsters, pack rats and sand rats (Shirley and Schmidt-Nielsen 1967) are probably colonized as well. When the cecal contents of these animals was incubated with $^{14}$C-oxalate, $^{14}$CO$_2$ was produced.

Daniel et al. (1987b) found only one colony of laboratory rats from five commercial breeders that was colonized with *O. formigenes*. When an isolate from this colony (OxCR6) was inoculated into laboratory rats that did not initially harbor *O. formigenes*, the organism persisted only when the rats were fed a high oxalate diet (4.5% oxalate). When the dietary oxalate was below 3%, *O. formigenes* could not be detected (Daniel et al. 1987a). A similar effect was found between dietary oxalate levels and colonization in sheep (Daniel et al. 1989). Other strains of *O. formigenes* isolated from monogastric animals also colonized laboratory rats fed the high oxalate
diet. However, a rumen strain, OxB, did not colonize the rats under any of the conditions tried, suggesting some type of host specificity (Daniel et al. 1987a). Daniel et al. (1987b) concluded that the procedures used by commercial breeders, primarily cesarean section and isolation, limited the establishment of oxalate-degrading bacteria in the normal flora of laboratory rats.

The growth of *O. formigenes* is dependent on the presence of oxalate (Allison et al. 1985). Other substrates are not utilized. Oxalate is decarboxylated to formate and CO₂ in nearly equimolar amounts (44% and 49%, respectively) (Dawson et al. 1980). The degradation of one mole of oxalate results in the loss of one mole of H⁺ and an increase in the pH of the medium. Low levels of acetate (0.5-1 mM) are required for growth in defined medium (Allison et al. 1985). Since acetate alone does not support growth it is probably used for biosynthetic reactions.

*Oxalobacter vibrioformis* and *Oxalophagus oxalicus* (*Clostridium oxamicus*), two organisms isolated from fresh water sediment, are similar to *O. formigenes* in that oxalate is the sole substrate that supports growth (Dehning and Schink 1989; Collins et al. 1994). Both organisms decarboxylate oxalate to formate plus CO₂ and require acetate for growth in defined medium. *O. vibrioformis* is a gram negative, vibroid-shaped rod, and *O. oxalicus* (*C. oxamicus*) is a gram positive, spore-forming rod. The mechanism by which these organisms produce ATP from oxalate is not known, but could be similar to energy conservation mechanism utilized by *O. formigenes* (Dehning and Schink 1989).

Metabolically, *Desulfovibrio vulgaris* ssp. *oxamicus* is quite different from the previously described anaerobic oxalate-degrading bacteria (Postgate 1963). Oxalate,
oxamate, lactate or pyruvate are utilized as carbon sources. Proton motive force is generated by an anaerobic electron transport system that utilizes sulfur as the final electron acceptor. The type strain was isolated from stream sediment. The stability of the oxalate-degrading capability of this organism is now in doubt since Dehning and Schink (1989) were unable to verify oxalate degradation by a strain that had been maintained on lactate for a number of years.

Acetogens are a group of bacteria which reduce CO$_2$ and oxidize H$_2$ to produce acetate. As a group, they are metabolically diverse and most ferment a number of organic compounds to acetate. *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* utilize oxalate and glyoxylate as substrates for growth (Daniel and Drake 1993). Other dicarboxylic acids do not support growth. Acetate was the major end product recovered from cultures grown on oxalate or glyoxylate. When *C. thermoaceticum* was grown with $^{14}$C-oxalate, $^{14}$CO$_2$ was the major $^{14}$C-labeled product recovered. Only a small amount of $^{14}$C was found in acetate or biomass. Daniel and Drake (1993) proposed that oxalyl-CoA and formyl-CoA were potential intermediates of oxalate metabolism. Formate arising from formyl-CoA could then be oxidized by a NADP-dependent formate dehydrogenase (Li et al. 1966). However, later studies indicated that oxalate degradation by *C. thermoaceticum* was dependent upon the presence of an electron acceptor (viologen dye) and that the addition of CoA to cell-free extracts did not enhance oxalate-degrading activity (Daniel and Wagner 1994). These results suggest that CoA intermediates may not be involved in oxalate degradation in this organism. B cytochromes, menaquinone and other components of an electron transport system have been isolated from *C. thermoaceticum* and *C.*
thermoautotrophicum (Gottwald et al. 1975; Hugenholtz and Ljungdahl 1990). Energy is also conserved by substrate level phosphorylation (Gottwald et al. 1975).

**Energy Conservation in *Oxalobacter formigenes***

The majority of oxalate-degrading bacteria oxidize formate, produced by the decarboxylation of oxalate, to CO₂ and reductant. The cofactors associated with formate dehydrogenase are reoxidized via an electron transport system and thus, the oxidation of formate is directly linked to the generation of ATP.

*O. formigenes* produces formate as an end product of oxalate metabolism indicating the oxidation of formate is not involved in the generation of energy (Allison et al. 1985). Substrate level phosphorylation linked to oxalate decarboxylation is also unlikely since the free energy change associated with the decarboxylation reaction (-26.7 kJ/mole) was less than the energy required to synthesize one mole of ATP (Thauer et al. 1977).

Ananthram et al. (1989) proposed that *O. formigenes* generates ATP in conjunction with the transport of oxalate across the cell membrane (Fig. 1). Oxalate enters the cell through an electrogenic oxalate⁻-formate⁻ antiport. Oxalate is activated to oxalyl-CoA by the transfer of CoA from formyl-CoA. The oxalyl-CoA is decarboxylated to formyl-CoA and CoA is transferred to another molecule of oxalate. Formate exits the cell through the antiport. The decarboxylation of oxalyl-CoA to formyl-CoA consumes a proton. This cycle of influx, decarboxylation and efflux generates a membrane potential which is used to drive a H⁺-dependent ATPase.

Oxalyl-CoA decarboxylase (EC 4.1.1.8) and formyl-CoA transferase (EC 2.8.3.4)
Figure 1. Proposed model of ATP generation in *O. formigenes* (courtesy of M. J. Allison)
have been purified from *O. formigenes* (Baetz and Allison 1989, 1990). Both enzymes were located in the cytoplasmic fraction. Only 2.4% and 0.4% of the decarboxylase and transferase respectively, were contained in the membrane fraction (Baetz and Allison 1992). The model for the generation of ATP by *O. formigenes* was further substantiated by the isolation and purification of the antiport protein OxIT (Ruan et al. 1992). The antiporter was shown to be a single integral membrane protein that mediated anion exchange without any additional input of energy. The transfer of oxalate was estimated to be extremely rapid, 1000/s, and OxIT represented 5-10% of the total inner membrane protein (Ruan et al. 1992).

**Anabolic Pathways of Oxalate Metabolism**

Oxalate is a highly oxidized molecule and its incorporation into cell material must involve reductive reactions. Little is known about the anabolic use of oxalate by anaerobic bacteria. Two pathways are utilized by aerobic bacteria for the reduction of oxalate, the glycerate pathway and a variant of the serine pathway.

**Glycerate pathway**

The left branch of Fig. 2 outlines reactions of the glycerate pathway for reduction of oxalate by the aerobic bacteria *P. oxalaticus* (Quayle and Keech 1959d; Quayle et al. 1961), *A. eutrophus* (Friedrich et al. 1979) and *T. novello* (Chandra and Shethna 1977). Oxalate is transported into the cell and activated to oxalyl-CoA by the transfer of CoA from formyl-CoA (Quayle 1963b; Quayle et al. 1961). Oxalyl-CoA is reduced to glyoxylate by oxalyl-CoA reductase using NAD(P)H as an electron donor (Quayle 1963a; Quayle and Taylor 1961; Quayle et al. 1961). Two molecules of
Figure 2. Pathways used by aerobic bacteria to reduce oxalate. Left branch-glycerate pathway, right branch-serine pathway.
glyoxylate are condensed by glyoxylate carboligase (EC 4.1.1.47) to form the C₃ compound tartronic semialdehyde plus CO₂ (Quayle and Keech 1959d). Tartronic semialdehyde is reduced to glycerate by tartronic semialdehyde reductase (EC 1.1.1.60) using NADH as an electron donor. Glycerate is then phosphorylated by glycerate kinase (EC 2.7.1.31) and incorporated into cell components using common biosynthetic pathways.

The glycerate pathway is not unique to oxalate metabolism. This pathway is also utilized by *Escherichia coli* (Krakow and Barkulis 1956) and *Pseudomonas ovalis* (Kornberg and Gotto 1959) growing on glycolate. Glycolate is oxidized to glyoxylate and then incorporated via the glycerate pathway. Dagley et al. (1961) proposed that the glycerate pathway was also utilized by a strain of *Pseudomonas* growing on glycine. The deamination of glycine yielded glyoxylate, the first common intermediate of the pathway. The key enzymes of the pathway, glyoxylate carboligase and tartronic semialdehyde reductase have been purified. The glyoxylate carboligase purified from *E. coli* contains FAD and requires TPP and Mg²⁺ ions for maximum activity (Gupta and Vennesland 1964). For each μmole of glyoxylate consumed, 0.5 μmole of CO₂ is produced. The tartronic semialdehyde reductase purified from *P. ovalis* oxidized both NADH and NADPH (Gotto and Kornberg 1961). Hydroxypyruvate, the tautomer of tartronic semialdehyde, was also reduced by the purified enzyme. However, the affinity for hydroxypyruvate was much lower than the affinity for tartronic semialdehyde.
Serine Pathway

The other pathway that has been described for the reduction of oxalate is a variant of the serine pathway (Fig. 2, right branch). This pathway is utilized by *M. extorquens* (Blackmore and Quayle 1970) and *B. oxalophilus* (Zaitsev et al. 1993) to reduce oxalate. Oxalate is activated to oxalyl-CoA after entering the cell. Oxalyl-CoA is reduced to glyoxylate by oxalyl-CoA reductase using NAD(P)H as an electron donor. A C$_1$ group is transferred from serine to glyoxylate by serine-glyoxylate aminotransferase (EC 2.6.1.45) to form the C$_3$ compound hydroxypyruvate plus glycine. Hydroxypyruvate is reduced to glycerate by hydroxypyruvate reductase (EC 1.1.1.81) using NADH as an electron donor. Glycerate is phosphorylated by glycerate kinase to 3-phosphoglycerate (3P-glycerate) and incorporated via common biosynthetic pathways. Overall, the glycerate pathway yields one molecule of glycerate from two molecules of oxalate and the serine pathway yields one molecule of glycerate from one molecule of glyoxylate (Blackmore and Quayle 1970).

One of the major enzymes of the serine pathway, hydroxypyruvate reductase, has been purified from *M. extorquens* AM1 (Chistoserdova and Lidstrom 1991). The enzyme contains two subunits of 37 kD each and has an isoelectric point of 4.75. Maximum activity requires NADH as a cofactor but the enzyme also oxidizes NADPH. Chistoserdova and Lidstrom (1992) isolated a second hydroxypyruvate reducing enzyme from *M. extorquens* AM1. This enzyme has an isoelectric point of 6.5 and also catalyzes the interconversion of glyoxylate and glycolate. The oxidation of glycolate to glyoxylate is necessary for growth on C$_2$ substrates other than oxalate.
Assimilation of Acetate by Bacteria

Acetate is an important precursor in many biosynthetic pathways. It is a building block for lipids, amino acids and nucleic acids. The majority of bacteria produce acetate from pyruvate. Acetogens have the unique ability to condense CO₂ and H₂ to form acetate via the acetyl-CoA pathway (Wood 1991). Some organisms, including \textit{O. formigenes}, cannot synthesize acetate and it must be provided for growth to occur in a defined medium. The incorporation of acetate into major cell fractions of various bacteria is shown in Table 2.

Oxidative tricarboxcyclic acid cycle

\textit{E. coli} grown on glucose and 1-\textsuperscript{14}C acetate incorporates \textsuperscript{14}C into intermediates of the Krebs cycle and the amino acids derived from pyruvate, \(\alpha\)-ketoglutarate, and oxaloacetate (Kornberg et al. 1960; Roberts et al. 1963). The same pattern of incorporation is found with 1-\textsuperscript{14}C and 2-\textsuperscript{14}C acetate indicating that acetate is incorporated directly and is not split. The C2 and C3 of aspartate are labeled from 2C-acetate and the carboxyl groups are labeled by 1C-acetate and CO₂. The C4 of glutamate is labeled by 2C-acetate and the C1 carboxyl is labeled by CO₂ (Roberts et al. 1963). Pulse-labeling studies indicate that \textsuperscript{14}C-acetate is incorporated into citrate and malate before it is incorporated into succinate and fumarate. Isotope from \textsuperscript{14}CO₃ was incorporated into cells at only 2% of the rate of acetate incorporation (Kornberg et al. 1960). This labeling pattern is consistent with the oxidative tricarboxylic acid cycle (TCA) and the glyoxylate bypass. Roberts et al. (1963) estimated that \textit{E. coli} grown on glucose and acetate obtained about 20% of the cell carbon from acetate.
Table 2. Incorporation of $^{14}$C-acetate and $^{14}$CO$_2$ into major cell components of various bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Compound</th>
<th>Percent of isotope incorporated into fraction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inter.$^a$    Lipids  DNA/RNA  Proteins</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>UC-acetate</td>
<td>5     43    4      40</td>
<td>Roberts et al. 1963</td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>1C-acetate</td>
<td>6     9      13     72</td>
<td>Weimer and Zeikus 1978</td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>2C-acetate</td>
<td>8     8      10     74</td>
<td>Weimer and Zeikus 1978</td>
</tr>
<tr>
<td><em>Chlorobium thiosulphilum</em></td>
<td>1C-acetate</td>
<td>6     24     20      51</td>
<td>Hoare and Gibson 1964</td>
</tr>
<tr>
<td><em>Chlorobium thiosulphilum</em></td>
<td>2C-acetate</td>
<td>4     22     13      61</td>
<td>Hoare and Gibson 1964</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>CO$_2$</td>
<td>4     11     33      45</td>
<td>Roberts et al. 1963</td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>CO$_2$</td>
<td>4     7      20      70</td>
<td>Weimer and Zeikus 1978</td>
</tr>
</tbody>
</table>

$^a$metabolic intermediates
Reductive TCA cycle

Many anaerobic bacteria assimilate acetate and CO$_2$ into amino acids using the reactions of the reductive TCA cycle or a horseshoe type of TCA pathway (Fig. 3). *Methanobacterium thermoautotrophicum* grows autotrophically on CO$_2$ and H$_2$. However, when $^{14}$C acetate is included in culture medium, its uptake is dependent on the concentration of acetate in the medium (Fuchs et al. 1978). At a concentration of 1 mM acetate, approximately 10% of the cell carbon is derived from acetate. At 20 mM acetate, almost 65% of the cell carbon is derived from acetate. Alanine, glutamate, and aspartate isolated from cells grown with $^{14}$C-labeled acetate all have similar specific activities (Fuchs et al. 1978). Degradation studies indicate that the carboxyl groups of alanine and aspartate were derived from CO$_2$ and that the C2 and C3 carbons were derived from acetate. The C3 and C4 carbons of glutamate were derived from acetate. This labeling pattern is consistent with the reductive TCA cycle whereby acetate is carboxylated to pyruvate, PEP is carboxylated to oxaloacetate and succinyl-CoA is carboxylated to $\alpha$-ketoglutarate (Fuchs et al. 1978). Additional experiments growing *M. thermoautotrophicum* in the presence of $^{14}$C-succinate found that succinate was incorporated into glutamate but not into alanine or aspartate. This led Fuchs and Stupperich (1978) to conclude that *M. thermoautotrophicum* did not utilize a complete reductive TCA cycle.

Ekiel et al. (1983) studied the biosynthetic pathways utilized by *Methanospirillum hungatei* using $^{13}$C nuclear magnetic resonance (NMR). Bacteria were grown in either labeled acetate or labeled CO$_2$, and the cells were chemically fractionated into the major cell constituents. Labeling patterns for the amino acids alanine, aspartate and
Figure 3. Reactions of the reductive TCA pathway (solid line) and the horseshoe TCA pathway (dashed line).
glutamate were consistent with the reductive carboxylation of acetate, pyruvate, and succinyl-CoA. Other amino acids were synthesized using typical pathways except for isoleucine. The labeling pattern of isoleucine suggested that it was formed from α-ketobutyrate via citramalate rather than from threonine.

**Horseshoe TCA pathway**

*Methanosarcina barkeri* grows heterotrophically on methanol, methylamine or acetate in addition to autotrophic growth on H₂ plus CO₂. When acetate is added to medium containing other substrates, approximately 60% of the cell carbon is derived from acetate (Weimer and Zeikus 1978, 1979). The majority of the acetate is incorporated into the protein fraction of the cells rather than the lipid fraction (Table 2) (Weimer and Zeikus 1978). Alanine, aspartate, and glutamate from cells grown with ¹⁴C-acetate were degraded to determine the position of the label (Weimer and Zeikus 1979). Acetate was not incorporated into the carboxyl carbons of alanine or aspartate. Acetate was also not incorporated into the C1 carboxyl of glutamate but was incorporated into the C5 carboxyl. Glutamate from cells grown on either 1-¹⁴C or 2-¹⁴C labeled acetate had a specific activity that was double that of aspartate. Cell-free extracts of *M. barkeri* contain the following enzymatic activities: pyruvate synthase, citrate synthase, aconitase, NADP-linked isocitrate dehydrogenase and NAD-linked malate dehydrogenase. These results led Weimer and Zeikus (1979) to conclude that acetate was assimilated into glutamate via citrate, isocitrate and α-ketoglutarate. Acetate was assimilated into alanine and aspartate from pyruvate and oxaloacetate by the reductive carboxylation of acetate and PEP.

The assimilation of acetate and CO₂ by *Methanothrix concilii* (Ekiel et al. 1985)
and *Thermoproteus neutrophilus* (Schafer et al. 1989) was investigated using $^{13}$C-NMR. Glutamate was labeled at the C2 and the C4 positions by 2C acetate and at the C3 position by 1C acetate. Alanine and aspartate were labeled at the C2 carbon by 1C acetate and at the C3 carbon by 2C acetate. This pattern was consistent with $\alpha$-ketoglutarate being formed through citrate in the oxidative direction of the TCA pathway and alanine and aspartate being formed from pyruvate and oxaloacetate in the reductive direction of the TCA pathway. Labeling of *M. conciliii* also showed a high degree of scrambling between the carboxyl group of acetate and CO$_3$. Ekiel et al. (1985) concluded that 25% of the label from CO$_3$ went into positions that would be expected to be labeled by the carboxyl of acetate. *Clostridium kluyveri* (Tomlinson and Barker 1954), *Desulfovibrio vulgaris* (Badziong et al. 1979) and *Chlorobium thiosulphatophilum* (Hoare and Gibson 1964) also assimilate acetate and CO$_2$ via a horseshoe type of TCA pathway.

**Assimilation of Carbon Dioxide by Heterotrophic Bacteria**

Autotrophic bacteria, by definition, assimilate all of their the cellular components from CO$_2$. Many heterotrophic bacteria also assimilate CO$_2$ into cell material (Wood 1985). *E. coli* (Roberts et al. 1963) and *M. barkeri* (Weimer and Zeikus 1978) incorporate $^{14}$CO$_3$ into all fractions of cell material (Table 2). Heterotrophic assimilation of CO$_2$ occurs by three general enzymatic reactions: (i) reductive carboxylation; (ii) carboxylation of PEP; (iii) carboxylation by biotin-containing enzymes (Wood and Utter 1965).
Reductive carboxylation

The two key reactions of the reductive TCA cycle, pyruvate synthase and 2-oxoglutarate synthase, are reductive carboxylations of acetyl-CoA and succinyl-CoA, respectively. In addition to these well known reactions, there are several more reductive carboxylation reactions that are potentially important routes of CO₂ assimilation. *Peptostreptococcus elsdenii* and *Prevotella ruminicola* carboxylate isobutyrate to form valine (Allison and Peel 1971). When cell-free extracts were incubated with glutamine, CO₂, ATP, and a low-potential electron carrier, ¹⁴C-isobutyrate was incorporated into valine. ¹⁴CO₃ was incorporated into valine when extracts were incubated with isobutyrate, ATP, CoA, glutamine and ferredoxin. When isovalerate or 2-methylbutyrate were substituted for isobutyrate, ¹⁴CO₃ was incorporated into leucine and isoleucine (Allison and Peel 1971). Several other species of bacteria found in the rumen reductively carboxylate branched chain volatile fatty acids to form amino acids (Robinson and Allison 1969).

The enzyme α-ketobutyrate synthase catalyzes a ferredoxin-dependent carboxylation of propionyl-CoA to form α-ketobutyrate. This reaction provides an alternate pathway for the formation of isoleucine that does not involve threonine as a precursor. Enzymatic activity for this reaction has been found in cell-free extracts of *Chromatium*, *Clostridium pasteurianum*, and *Desulfovibrio desulfuricans* (Buchanan 1969, 1973). The carboxylation of phenylacetyl-CoA to phenylpyruvate was first proposed as an alternate pathway for phenylalanine biosynthesis in *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* (Allison 1965). Gehring and Arnon (1971) incubated cell-free extracts of *Chromatium* with ¹⁴C-bicarbonate, phenylacetate,
ferredoxin, TPP, ATP, CoA and Mg$^{2+}$. The $^{14}$C was incorporated into phenylalanine. $^{14}$C-phenylacetate was also incorporated into phenylalanine under the same conditions (Gerhing and Arnon 1971). Cell-free extracts of the photosynthetic bacteria *Chlorobium thiosulfatophilum* and *Chloropseudomonas ethylicum* also carboxylate phenylacetate to phenylpyruvate (Gerhing and Arnon 1971). *Ruminococcus albus* incorporated $^{14}$C-indole-3-acetic acid into tryptophan suggesting that the carboxylation of indole-3-acetic acid is an alternate pathway for the biosynthesis of tryptophan (Allison and Robinson 1967). All known reductive carboxylation reactions require an anaerobic environment and a low potential electron carrier such as ferredoxin (Buchanan 1972).

**Carboxylation of phosphoenolpyruvate**

In heterotrophic bacteria, the main role for the carboxylation of PEP is anaplerotic and allows for the continuation of the TCA cycle when intermediates are removed for biosynthesis (Utter and Kolenbrander 1972). Two enzymes catalyze the carboxylation of PEP to oxaloacetate, PEP carboxylase (EC 4.1.1.31), and PEP carboxytransphosphorylase (EC 4.1.1.38) (Wood and Utter 1965). The phosphate acceptors for these reactions are H$_2$O, and inorganic phosphate (Pi), respectively. PEP carboxytransphosphorylase reaction is reversible while the PEP carboxylase reaction is not (Utter and Kolenbrander 1972).

PEP carboxylase has been purified from *E. coli* (Canovas and Kornberg 1969), *M. thermoautotrophicum* (Kenealy and Zeikus 1982) and *M. extorquens* (Quayle 1969). All three enzymes are specific for PEP and are dependent upon the presence of divalent cations (Mg$^{2+}$ or Mn$^{2+}$). PEP carboxylase activity has also been found in other genera.
of bacteria and is probably widespread (Utter and Kolenbrander 1972). In *M. thermoautotrophicum*, PEP carboxylase is important in CO₂ assimilation since the reductive TCA cycle is incomplete (Fuchs and Stupperich 1978). The carboxylation of PEP to oxaloacetate is the main reaction in the formation of C₄ compounds from C₃ compounds (Kenealy and Zeikus 1982). The carboxylation of PEP also has a biosynthetic role in the aerobic chemoautotrophs belonging to the genus *Thiobacillus* (Howden et al. 1972).

PEP carboxytransphorylase has been purified from *Propionibacterium shermanii* (Wood et al. 1969). This enzyme catalyzes a PPi-dependent exchange of ^14^CO₂ into oxaloacetate. It also catalyzes the formation of PPi and pyruvate from PEP and Pi.

When cell-free extracts of *P. oxalaticus* grown on oxalate were incubated with ^14^CO₃, there was rapid incorporation of isotope into malate, aspartate and other TCA intermediates. Since these cells contained very low levels of malate synthase activity, Quayle et al. (1961) suggested that CO₂ was fixed by the carboxylation of PEP or pyruvate.

**Biotin-containing enzymes**

Biotin-containing enzymes catalyze an ATP-dependent carboxylation reaction using a two-step process. The first step is the formation of a carboxybiotin-enzyme complex and the second step is the transfer of the carboxyl group to the acceptor molecule (Alberts and Vagelos 1972). Four biotin-dependent carboxylations have been described in bacteria, acetyl-CoA carboxylase (EC 6.4.1.2), propionyl-CoA carboxylase (EC 6.4.1.3), β-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) and pyruvate carboxylase (EC 6.4.1.1).
Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. This reaction is the first committed step of fatty acid synthesis. Acetyl-CoA carboxylase is widely distributed in microorganisms and has been extensively studied in *E. coli* (Alberts and Vagelos 1972). The *E. coli* enzyme is composed of three separate units: biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase (Alberts and Vagelos 1972).

Propionic acid is a by-product of fatty acid metabolism and is also formed from the degradation of branched-chain amino acids. It can be further metabolized by activation to propionyl-CoA and carboxylation to methylmalonyl-CoA by propionyl-CoA carboxylase (Alberts and Vagelos 1972). This enzyme has been purified from *Mycobacterium smegatis* and *Rhodospirillum rubrum* and crude preparations from many other bacteria contain propionyl-CoA carboxylase activity. The purified enzyme from *R. rubrum* also carboxylates acetyl-CoA and butyryl-CoA but at rates that were slower than when propionyl-CoA was the substrate. Both acetyl-CoA carboxylase and propionyl-CoA carboxylase are key enzymes in a newly described CO₂ fixation pathway, the 3-hydroxypropionate cycle of *Chloroflexus aurantiacus* (Strauss and Fuchs 1993).

β-methylcrotonyl-CoA carboxylase catalyzes the carboxylation of β-methylcrotonyl-CoA to form β-methylglutaconyl-CoA. This enzyme has been purified from *Mycobacterium*, *Achromobacter*, and *Pseudomonas* (Alberts and Vagelos 1972). β-methylcrotonyl-CoA is an intermediary metabolite of leucine degradation.

Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate. This enzyme has been found in a variety of bacteria including *Pseudomonas aeruginosa,*
P. citronellolis, Arthrobacter globiformis, Rhodopseudomonas sphaeroides and Acinetobacter calcoaceticus (Seubert and Weicker 1969). In general, pyruvate carboxylases require monovalent and divalent cations for activation, although enzymes from different organisms require different cations (Scrutton and Young 1972). The enzyme from Bacillus stearothermophilus also requires acetyl-CoA for activation while the enzyme from Pseudomonas citronellolis is inhibited by oxalate (Seubert and Weicker 1969).

References


Allison, M.J., and Robinson, I.M. 1967. Tryptophan biosynthesis from indole-3-acetic acid by anaerobic bacteria from the rumen. Biochem. J. 102:36P-37P.


ANABOLIC REDUCTION OF OXALATE BY *OXALOBACTER FORMIGENES*

A paper to be submitted to Archives of Microbiology

Nancy A. Cornick and Milton J. Allison

Abstract

Oxalate is the only substrate that supports the growth of *Oxalobacter formigenes*, an anaerobic, gram negative rod that inhabits the intestinal tract of many mammals. Oxalate is decarboxylated to formate and CO$_2$ in a nearly 1:1 ratio but some carbon from oxalate is also used for cell synthesis. Two pathways for assimilation of oxalate (the glycerate pathway and the serine pathway) have been described in aerobic bacteria. We found that cell-free lysates of *O. formigenes* contained the following enzymatic activities: oxalyl-CoA reductase, glyoxylate carboligase, tartronic semialdehyde reductase, glycerate kinase, hydroxypyruvate reductase and glyoxylate dehydrogenase. No evidence for serine-glyoxylate aminotransferase found. These results support the concept that *O. formigenes* assimilates carbon from oxalate using the glycerate pathway and that the serine pathway is not functional.

Introduction

*Oxalobacter formigenes* is a gram negative anaerobic bacterium that inhabits the gastrointestinal tracts of many warm-blooded animals (Allison et al. 1985; Daniel et al. 1987). Oxalate is the only substrate that supports the growth of this organism and it is
decarboxylated to formate and CO$_2$ (Dawson et al. 1980). Since oxalate is a highly oxidized compound, incorporation of oxalate carbon into cell material by *O. formigenes* must involve reductive reactions. Several other anaerobic oxalate-degrading bacteria have been described (Daniel and Drake 1993; Dehning and Schink 1989; Postgate 1963; Smith 1985), but there is no information regarding the mechanisms used for the reduction of oxalate by these organisms.

Two pathways are utilized by aerobic bacteria to reduce oxalate to 3-phosphoglycerate (3P-glycerate) (Fig. 1). *Pseudomonas oxalaticus* (Quayle and Keech 1959; Quayle et al. 1961), *Alcaligenes eutrophus* H16 (Friedrich et al. 1979), and *Thiobacillus novellus* (Chandra and Shethna 1977) utilize the glycerate pathway to reduce oxalate. The key enzymes of this pathway are glyoxylate carboligase and tartronic semialdehyde reductase. *Methylobacterium extorquens* (Blackmore and Quayle 1970) and *Bacillus oxalophilus* (Zaitsev et al. 1993), reduce oxalate to 3P-glycerate using a variation of the serine pathway. Serine-glyoxylate aminotransferase is the key enzyme of this pathway. Zaitsev et al. (1993) suggested that *B. oxalophilus* also incorporates oxalate via the glycerate pathway, but they did not report any data for glyoxylate carboligase or tartronic semialdehyde reductase.

The first step of both the serine and glycerate pathways, is the activation of oxalate to oxalyl-CoA. Studies with *O. formigenes* indicated oxalate was converted to oxalyl-CoA before decarboxylation to formyl-CoA (Allison et al. 1985). The enzymes responsible for these reactions, oxalyl-CoA decarboxylase and formyl-CoA transferase have been purified (Baetz and Allison 1989, 1990). We assayed cell-free lysates of
Figure 1. Pathways of oxalate assimilation in aerobic bacteria. Left branch-glycerate pathway, right branch-serine pathway.
O. formigenes for key enzymes in both the serine and glycerate pathways to determine if oxalate was reduced by either of these routes.

A preliminary report of these results was presented at the Annual Meeting of the American Society for Microbiology, 1994, K5.

Materials and Methods

Bacteria

O. formigenes strain OxB (ATCC 35274) was grown in a 12 L fermenter in medium B with 100 mM oxalate under CO₂ (Allison et al. 1985). The pH of the medium was maintained at 6.8 by the infusion of 0.8 M oxalic acid. The bacteria were harvested using a continuous flow centrifuge (Sharples Corp., Philadelphia, PA). Cell-free lysates were prepared by suspending cells in anaerobic phosphate buffer (100 mM, pH 6.7) containing 1 mM dithiothreitol and passing the bacteria through a French pressure cell at 20,000 lb/in² (American Instr. Co., Silver Spring, MD). After three centrifugations to remove cell debris (12,000 x g, 10 min), the supernate was centrifuged at high speed to remove the cell membranes (62,000 x g for 1 h). The supernate from this step was used for enzyme assays and is referred to as cell-free lysate. All of the steps in the preparation of cell-free extracts were done under N₂. Two different batches of cell lysate were passed through a DEAE-cellulose column (Allison and Peel 1971). Cell lysates, both with membranes (27 mg protein/ml) and without membranes (20 mg protein/ml), were scanned for chromophores using a dual wavelength spectrophotometer (Aminco DW-2000). Lysates were chemically reduced by adding dithionite and resting versus reduced spectra were measured. Protein was
measured by a modification of the Lowry assay using bovine serum albumin as the standard (Peterson 1977).

Growth in $^{14}\text{C}$ oxalate

Bacteria were grown in medium B with 100 mM oxalate under CO$_2$. $^{14}\text{C}$-oxalate, (80 kBq) was added to 3 ml of growing cultures for 0-22 h, 16-18 h (log phase), or 21-22 h (stationary phase) of growth. $^{14}\text{C}$-oxalate and $^{14}\text{C}$-formate were separated by HPLC and collected with a fraction collector (Daniel and Drake 1993). The $^{14}\text{C}$ in the formate and oxalate fractions was counted in a model LS-7800 liquid scintillation counter (Beckman Instr., San Ramon, CA) with 10 ml of Ecoscint (National Diagnostics, Manville, NJ). Efficiency was monitored by H number. $^{14}\text{CO}_3^-$ was measured after diffusion with acid (Conway 1962) and trapping in phenethylamine (Allison et al. 1977). $^{14}\text{C}$-oxalic acid (137 MBq/mmol) and $^{14}\text{C}$ Na formate (1.59 GBq/mmol) were purchased from New England Nuclear (Boston, MA).

Enzyme assays

Assays for enzymatic activity were done aerobically and anaerobically to check for O$_2$ sensitivity. Data reported is that collected from aerobic assays unless otherwise noted. The data reported from positive assays was an average of triplicate assays from at least two different preparations of cell lysate. Spectrophotometric measurements were determined using a Gilford Response II spectrophotometer (Clia Corning Diagnostic Corp., Oberlin OH). Controls were identical to reaction mixtures except that substrates were deleted.

Oxalyl-CoA reductase was assayed using the method of Quayle (1963). Oxalyl-CoA was synthesized from thiocresol oxalate and coenzyme A (Quayle 1962).
Glyoxylate dehydrogenase (EC 1.2.1.17) was assayed under argon using a previously described method (Quayle and Taylor 1961). Tartronic semialdehyde reductase (EC 1.1.1.60) was measured using the method of Kornberg and Gotto (1965). Tartronic semialdehyde was prepared enzymatically using glyoxylate carboligase purified from *Escherichia coli* by the method of Gupta and Vennesland (1965). The glyoxylate carboligase reaction was stopped with 200 µl of 50% trichloroacetic acid.

Hydroxypyruvate reductase (EC 1.1.1.29) was assayed spectrophotometrically using previously described methods (Hepinstall and Quayle 1970) and by HPLC. Serine-glyoxylate aminotransferase (EC 2.1.6.45) was assayed by the procedure of Goodwin (1990) except that the disappearance of serine and formation of glycine were measured by HPLC. Amino acids were detected as the dabsylated derivatives (Anderson et al. 1993). Glyoxylate carboligase (EC 4.1.1.47) was measured under N₂ or argon using the method of Gupta and Vennesland (1965). The pressure in the headspace was calculated by measuring the volume of displaced gas with a glass syringe. CO₂ was measured using a gas chromatograph (Gow Mac, Bridgewater, NJ) equipped with a thermal conductivity detector (Cornick et al. 1994). Glycerate kinase (EC 2.7.1.31) was assayed by coupling the formation of ADP to the oxidation of NADH by adding phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase to the reaction mixture (Hepinstall and Quayle 1970). Formate dehydrogenase (EC 1.2.1.2) was measured under N₂, argon, or a mixture of N₂:CO₂:H₂ (80:5:10) using a methyl viologen dye assay (Diekert and Thauer 1978) and by HPLC. The molar extinction coefficient used for methyl viologen was 9.7 x 10³ at 578 nm (Diekert and Thauer 1978). Formate dehydrogenase was also assayed by adding ¹⁴C-formate (18.5 kBq/3
ml) to the reaction mixture above. Reactions were stopped after 20 min by injecting 1 ml of 1 N NaOH through the rubber stoppers of the tubes. $^{14}$C trapped as carbonate was measured by acid diffusion as described above.

**HPLC**

The spectrophotometric assays for hydroxypyruvate reductase and formate dehydrogenase were verified by measuring the disappearance of substrates and the appearance of products using HPLC (System Gold, Beckman Instr.). Reactions were stopped at 0, 15, 30 and 60 min with 500 $\mu$l of 4 N $\text{H}_2\text{SO}_4$. Samples were centrifuged at 2,000 x g for 10 min and the supernate was filtered through a 22 $\mu$m filter. Organic acids were separated on an Aminex HPX-87H column (Bio Rad Laboratories, Richmond, CA) using 0.008 N $\text{H}_2\text{SO}_4$ (0.7 ml/min) as the mobile phase (Daniel and Drake 1993). Absorbance at 210 nm was measured using a diode array spectrophotometer. Retention times (min) were: oxalate, 6.1; hydroxypyruvate, 7.5; pyruvate, 8.5; glyoxylate, 8.7; glycerate, 9.9; glycolaldehyde, 10.8; glycolate, 11.3; formate, 12.7; and acetate, 13.7. Tartronic semialdehyde was not detected under these conditions.

**Results**

**Metabolism of $^{14}$C oxalate**

Cells of *O. formigenes* in the exponential phase of growth degraded oxalate (100%) to formate (44.8%) and $\text{CO}_2$ (54.5%), (Table 1). Cells in the stationary phase of growth degraded oxalate (100%) to almost equal proportions of formate (49%) and $\text{CO}_2$ (50.4%). The differences in the ratios of end products (formate and $\text{CO}_2$) between growing and resting cells indicated that more carbon from oxalate was
Table 1. Effect of growth stage on the metabolic fate of oxalate carbon

<table>
<thead>
<tr>
<th></th>
<th>Exposure interval with $^{14}$C-oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-22 h</td>
</tr>
<tr>
<td>$\text{CO}_2^a$</td>
<td>43.0 (54.9)$^b$</td>
</tr>
<tr>
<td>Formate</td>
<td>34.6 (44.2)</td>
</tr>
<tr>
<td>Cells</td>
<td>0.73 (0.9)</td>
</tr>
<tr>
<td>Oxalate</td>
<td>1.88</td>
</tr>
<tr>
<td>Total $^{14}$C recovered</td>
<td>79.6</td>
</tr>
</tbody>
</table>

$^a$Measured after diffusion from acid  
$^b$kBq recovered (% of product)

oxidized to $\text{CO}_2$ when the cells were actively growing. Nearly 1% of the oxalate carbon metabolized during the growth of $O. \text{formigenes}$ was incorporated into cellular material.

**Enzyme assays**

Cell-free extracts of $O. \text{formigenes}$ contained oxalyl-CoA reductase, glyoxylate carboligase, tartronic semialdehyde reductase, glycerate kinase and glyoxylate dehydrogenase activities (Table 2). These are the enzymes of the glycerate pathway. Hydroxypyruvate reductase activity was also detected but the specific activity was ten times less than the rate observed for tartronic semialdehyde reductase. We did not detect serine-glyoxylate aminotransferase activity.
Table 2. Activity of biosynthetic enzymes in cell-free lysates of *O. formigenes*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay Measurement</th>
<th>Specific Activity[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalyl-CoA reductase</td>
<td>NADH oxidation</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>NADPH oxidation</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>Glyoxylate dehydrogenase</td>
<td>NAD reduction</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td></td>
<td>NADP reduction</td>
<td>221.0</td>
</tr>
<tr>
<td>Glyoxylate carboligase</td>
<td>CO₂ production</td>
<td>32.1</td>
</tr>
<tr>
<td>Tartronic semialdehyde reductase</td>
<td>NADH oxidation</td>
<td>71.0</td>
</tr>
<tr>
<td></td>
<td>NADPH oxidation</td>
<td>62.0</td>
</tr>
<tr>
<td>Glycerate kinase</td>
<td>NADH oxidation[^b]</td>
<td>2.3</td>
</tr>
<tr>
<td>Hydroxypyruvate reductase</td>
<td>NADH oxidation</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>NADPH oxidation</td>
<td>3.8</td>
</tr>
<tr>
<td>Hydroxypyruvate reductase</td>
<td>Loss of hydroxypyruvate[^c]</td>
<td>1.6</td>
</tr>
<tr>
<td>Serine-glyoxylate aminotransferase</td>
<td>Loss of serine[^c]</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

[^a]: nmol/min/mg protein
[^b]: Assay coupled to lactate dehydrogenase
[^c]: Substrate loss measured by HPLC

**Formate dehydrogenase**

Formate dehydrogenase activity was assayed by three different methods (Table 3). No activity was detected when NAD or NADP were supplied as electron acceptors in the spectrophotometric assay. However, when methyl or benzyl viologen dye was added, cell-free extracts reduced the dye. Viologen dye was not reduced when formate was not included in the assay, or when the lysate was boiled. Formyl-CoA, oxalate, acetate, carbon monoxide, formaldehyde, methanol or glyoxylate did not serve as...
Table 3. Comparison of formate dehydrogenase activity by different methods

<table>
<thead>
<tr>
<th>Assay Method</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viologen dye reduction</td>
<td>1900</td>
</tr>
<tr>
<td>CO₂ production</td>
<td>4</td>
</tr>
<tr>
<td>HPLC-formate loss</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

See materials and methods

Substrates for viologen dye reduction in place of formate. The reduction of viologen dye was proportional to the concentration of formate up to 1.6 mM. The lag phase of the reaction was shorter when dithionite was added to the assay mixture, but dithionite was not required for reduction of the dye. DEAE-cellulose treated lysates did not reduce viologen dye when formate was added. The addition of either Clostridium ferredoxin or spinach ferredoxin did not restore the activity of the DEAE-cellulose treated lysates.

When \(^{14}\text{C}\)-formate (17 mM, 6.15 kBq/ml) was added to the assay mixture, less than 1% of the \(^{14}\text{C}\) was recovered as \(^{14}\text{CO}_2\). This indicated that only nanomolar amounts of formate had been converted to CO₂. The specific activity of formate dehydrogenase based on amounts of \(^{14}\text{CO}_2\) formed was 500-fold less than the specific activity calculated by the viologen dye assay. When reaction mixtures were analyzed by HPLC, no detectable change in the concentration of formate was observed during 40 min. The concentration of formate in the reaction mixture (46.5 μM) was a limiting amount based on the viologen dye assay.
Optical spectra

The optical spectra of cell-free extracts of *O. formigenes* did not show any evidence of chromophores. Resting minus reduced spectra of lysates containing membranes did not suggest the presence of cytochromes or other components of an electron transport system.

Discussion

Our results indicate that the key metabolic enzymes of the glycerate pathway, but not those of the serine pathway, were present in cell-free extracts of *O. formigenes*. The glycerate pathway is also utilized by *P. oxalaticus* (Quayle and Keech 1959; Quayle et al. 1961), *A. eutrophus* H16 (Friedrich et al. 1979) and *T. novellus* (Chandra and Shethna 1977) to reduce oxalate to 3P-glycerate. *O. formigenes* utilized NADH as a cofactor in the reduction of oxalyl-CoA to glyoxylate as does *A. eutrophus* (Friedrich et al. 1979). However, *P. oxalaticus* required NADPH for this reaction (Quayle 1963; Quayle et al. 1961). In *P. oxalaticus* this reaction is reversible through the enzyme glyoxylate dehydrogenase (glyoxylate + NADP → oxalyl-CoA + NADPH). When the reverse reaction was assayed in *O. formigenes*, activity was detected with NADP but not with NAD. Since we did not measure the formation of oxalyl-CoA, we do not know that it was the product of this reaction and it is possible that the activity observed is an alternative reaction that utilizes glyoxylate as a substrate.

Both *P. oxalaticus* and *M. extorquens* produce NAD-linked formate dehydrogenase (Hodgkinson 1977). Formate dehydrogenase is the main energy-producing reaction in the metabolism of oxalate by these aerobic bacteria. The formation of NADH provides
reducing equivalents for the cell and is also involved in ATP synthesis via an electron transport system. However, since formate is a major end product of *O. formigenes*, some other energy generation mechanism must function. The synthesis of ATP by *O. formigenes* has been shown to be linked to the transport of oxalate by an electrogenic oxalate$^{2-}$-formate$^{1-}$ antiporter (Ananthram et al. 1989).

*O. formigenes* probably does not produce formate dehydrogenase. Although an enzymatic reaction that reduced viologen dye in the presence of formate occurred, we were unable to measure the loss of formate by HPLC. We were also unable to measure appropriate amounts of $^{14}$CO$_2$ produced from $^{14}$C-formate under conditions used for the viologen dye assay. *Oxalobacter vibrioformis* and *Oxalophilus oxa/icus* (*Clostridium oxalicum*) also produce formate as an end product of oxalate metabolism (Dehning and Schink 1989). Permeabilized cell suspensions of both of the above organisms reduced viologen dye in the presence of formate. However, the loss of formate and the production of CO$_2$ was not documented (Dehning and Schink 1989), so we do not know whether the reduction of viologen dye represents formate dehydrogenase or a reaction that is similar to the one in *O. formigenes*.

The difference in the ratio of CO$_2$:formate produced from oxalate between growing and resting cells, is evidence for the oxidation of oxalate, or a metabolite of oxalate, during growth when a reductant is needed for biosynthetic reactions. The condensation of glyoxylate to tartronic semialdehyde plus CO$_2$ is one reaction that could contribute to the increase in the ratio of CO$_2$:formate. However, this reaction does not involve a net gain or loss of electrons and we still do not know how *O. formigenes* obtains reduced NADH to supply the reactions of the glycerate pathway.
Several species of *Clostridium* transfer reducing power to NAD(P) through ferredoxin using the enzyme NAD(P)-ferredoxin oxidoreductase (Jungerman et al. 1973; Petitdemange et al. 1976). Our attempts to isolate or detect ferredoxin from *O. formigenes* have been unsuccessful. *Archaeoglobus fulgidus* (Kunow et al. 1993) and several methanogens (Dimarco et al. 1990) produce a F₄₂₀-dependent NADP reductase which links anabolic and catabolic pathways. We did not detect any chromophores or other components of an electron transport system in *O. formigenes*. Even if the ferredoxin oxidoreductase (or other chromophore) was found the more basic question of what was oxidized to reduce the ferredoxin (or chromophore) would still remain. The fundamental reductant must be something that eventually ends up as CO₂ (Table 1). It is possible that the "glyoxylate dehydrogenase" or "formate dehydrogenase" activities we detected are somehow involved in the generation of reducing power in *O. formigenes*.

**Acknowledgements**

We thank Alan A. DiSpirito for the absorption spectroscopy and acknowledge the expert technical assistance of Herb Cook.

**References**


Heptinstall J, Quayle JR. (1970) Pathways leading to and from serine during growth of *Pseudomonas* AM1 on C1 compounds or succinate. Biochem. J. 117:563-572


ASSIMILATION OF OXALATE, ACETATE AND CO₂ BY *OXALOBACTER FORMIGENES*

A paper to be submitted to the Canadian Journal of Microbiology

N. A. Cornick and M. J. Allison

Abstract

*Oxalobacter formigenes* is the only well-documented oxalate-degrading bacterium of gastrointestinal tracts of animals. Oxalate is the essential substrate for the growth of this organism but a small amount (0.5-1 mM) of acetate is also needed. We grew *O. formigenes* in ^14^C-labeled oxalate, CO₃, acetate or formate and calculated the contribution of each of these carbon sources to the total cell biomass. Measurements of the specific activities of total cell carbon after growth with different ^14^C-labeled precursors indicated that at least 54% of the total cell carbon was derived from oxalate and at least 7% was derived from acetate. The only other known carbon source was CO₃. Formate was not a significant source of cell carbon. In addition, we determined the distribution of the carbons from these sources in major cell fractions and in amino acids in cellular protein. Carbon skeletons of amino acids derived from pyruvate, oxaloacetate, α-ketoglutarate, 3P-glycerate and the aromatic amino acids were labeled by ^14^C-oxalate and ^14^CO₃. Carbon from ^14^C-acetate was found mainly in amino acids derived from α-ketoglutarate, oxaloacetate and pyruvate. Our evidence that cell-free extracts contained citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase activities fits with the labeling patterns observed with ^14^C-acetate and
supports the concept that acetate carbon entered into amino acid biosynthesis via well known pathways.

Introduction

Most oxalate-degrading bacteria utilize a variety of substrates in addition to oxalate (Blackmore and Quayle 1970; Chandra and Shethna 1977; Daniel and Drake 1993; Friedrich et al. 1979; Khambata and Bhat 1953; Postgate 1963). The majority of these bacteria are aerobes that decarboxylate oxalate to formate plus CO₂ and then derive energy by oxidizing the formate. Only three bacteria have been described which exclusively utilize oxalate as a substrate. *Oxalobacter formigenes* (Allison et al. 1985), *Oxalobacter vibrioformis* and *Oxalophagus oxalicus* (*Clostridium oxalicum*) (Collins et al. 1994; Dehning and Schink 1989) are strictly anaerobic bacteria that decarboxylate oxalate to formate and CO₂. Unlike aerobic oxalate-degrading bacteria, formate is not oxidized by these organisms, but is produced as an end product. *O. formigenes* has been isolated from the rumen of cattle and sheep (Dawson et al. 1980), the large bowel of pigs (Allison et al. 1985), humans (Allison et al. 1986) and rats (Daniel et al. 1987), and from lake sediment (Smith et al. 1985). *O. vibrioformis* and *O. oxalicus* (*C. oxalicum*) were isolated from anoxic sediment (Dehning and Schink 1989). All three bacteria require acetate for growth. This absolute requirement for acetate suggests that oxalate is not reduced to acetate by these organisms, and that acetate is utilized as a source of cell carbon. Information regarding the incorporation of oxalate and acetate into cell constituents by *O. vibrioformis* and *O. oxalicus* (*C. oxalicum*) is not available (Dehning and Schink 1989). Previous data from our laboratory suggest that
O. formigenes reduced oxalate to 3-phosphoglycerate (3P-glycerate) a precursor for biosynthetic pathways (Cornick and Allison 1995).

When O. formigenes is grown on defined medium there are four potential sources of carbon for cell synthesis; oxalate, acetate, CO\textsubscript{3}, and formate. We grew O. formigenes in the presence of each of these \(^{14}\text{C}\) labeled compounds to determine the relative contributions of each precursor to cell biomass. In addition, we determined which amino acids were labeled with \(^{14}\text{C}\) from each carbon source and assayed cell-free lysates for key enzymes in the Krebs cycle, the reverse TCA cycle and the acetyl-CoA (Wood) pathway.

A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology, 1994, K-5.

Materials and Methods

Culture medium

O. formigenes (ATCC 35274) was grown in medium 188-129 or medium 188-102. Medium 188-129 contained (g/L): Na 2-N-morpholinoethanesulfonic acid (MES), 0.54; MES, 0.49; K\textsubscript{2}HPO\textsubscript{4}, 0.25; KH\textsubscript{2}PO\textsubscript{4}, 0.25; (NH\textsubscript{4}SO\textsubscript{4})\textsubscript{2}, 0.5; MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O, 0.025; trace metals solution (Pfennig and Lippert 1966), 20 ml; cysteine HCl \cdot H\textsubscript{2}O, 0.5; resazurin, 0.001. The pH was adjusted to 6.0 and the medium was boiled and cooled under N\textsubscript{2}. Sterile, anaerobic sodium oxalate and sodium acetate were added to a final concentration of 100 mM and 1 mM, respectively. Medium 188-102 was the same as 188-129 except the MES buffer was replaced with Na\textsubscript{2}CO\textsubscript{3} (4 g/L). The pH was adjusted to 6.8 and the medium was boiled and cooled under CO\textsubscript{2}. CO\textsubscript{2}-free water
was prepared by boiling and cooling distilled water under N₂. The water was stored in a stoppered bottle connected to a trap filled with soda lime.

**Growth of bacteria in ¹⁴C compounds**

The growth of the bacteria was followed by measuring the absorbance (A₆₀₀) using a Spectronic 70 (Bausch and Lomb, Rochester NY) with 18 mm cuvettes. Bacteria were grown in medium 188-102 (oxalate, acetate, and formate) or 188-129 (CO₃) to early log phase (A₆₀₀ 0.1-0.25). Cells were centrifuged (12,000 x g), washed once and resuspended in medium containing 40 mM oxalate, 1 mM acetate and either ¹⁴C-oxalate (100 µCi), ¹⁴C or 2¹⁴C-acetate (100 µCi), ¹⁴C-formate (10 µCi) or Na¹⁴CO₃ (100 µCi). All steps were carried out under CO₂ or N₂. The bacteria were grown for another 0.5-1 generation and then collected by centrifugation. Cells were washed twice and fractionated using the methods of Roberts et al. (1963). The cold 5% trichloroacetic acid soluble fraction has been shown to contain low molecular weight intermediates and was designated as the intermediate fraction, the ethyl-ether soluble fraction was designated as the lipid fraction, the hot trichloroacetic acid soluble fraction was designated as the nucleic acid fraction and the remaining pellet was considered cell protein. The cell protein was hydrolyzed in 6 N HCl at 105°C for 20 h under N₂ and the HCl was removed under vacuum.

**HPLC**

Amino acids were analyzed as the dabsylated derivatives using HPLC with a diode array spectrophotometer (436 nm) (System Gold, Beckman Instr., San Ramon, CA) (Anderson et al. 1993). Radioactivity in the column eluate was measured using a flow-through scintillation counter (Radiomatic Instr., Meriden, CT) (Figure 1). Scintillation
Figure 1. HPLC tracings of amino acids from protein hydrolysate from cells grown in $1^{14}C$ acetate. A) UV detector, 436 nm (eg. Asp 13.4 min) B) Flow through scintillation counter (eg. Asp 14.7 min)
fluid (FloScint IV, Radiomatic) was added in a 2:1 ratio with the mobile phase from the HPLC. Samples containing 10,000 to 40,000 cpm were injected onto the column. Norleucine was used as an internal standard and individual amino acids were used as external standards. Valine and proline were not separated using this method. Arginine and isoleucine were also not separated.

Radioactivity measurements

Samples (10 µl) from major cell fractions were added to 10 ml of scintillation fluid (Ecoscint, National Diagnostics, Atlanta, GA) and counted using a LS-7800 liquid scintillation counter (Beckman Instr.). Efficiency was monitored by H number and 14C-toluene was used as the internal standard. The percent of isotope incorporated was calculated from measurement of radioactivity in washed cells from a known volume of medium. Na14CO3 (56 mCi/mmol) was purchased from ICN (Irvine, CA). 1-14C acetic acid (18.6 mCi/mmol) was purchased from Sigma (St. Louis, MO). 2-14C Na acetate (50 mCi/mmol) and (22.5 mCi/mmol) was purchased from Schwartz/Mann (Orangeburg, NY) and CalBiochem (San Diego, CA), respectively. 14C-oxalic acid (3.4 mCi/mmol) and 14C Na formate (43 mCi/mmol) were purchased from New England Nuclear (Boston, MA).

Enzyme assays

Cell-free extracts were prepared as previously described (Cornick and Allison 1995). Pyruvate synthase (EC 1.2.7.1) was assayed using the method of Buchanan et al. (1964). Glutamine-pyruvate transaminase was added to the reaction mixture to convert the pyruvate to alanine, which was then measured by HPLC. Pyruvate synthase and 2-oxoglutarate synthase (EC 1.2.7.3) were also assayed in the reverse
direction using methyl viologen as the electron acceptor (Odom and Peck 1981) and by measuring the production of CO₂. Assays for CO₂ production were stopped in a 70°C water bath. HCl (500 µl, 4 N) was injected through the rubber stopper to acidify the mixture and CO₂ in the headspace gas was measured by gas chromatography (Cornick et al. 1994). Pyruvate dehydrogenase (EC 1.5.1.12) and α-ketoglutarate dehydrogenase complex were assayed by following the reduction of NAD using a spectrophotometer (Gilford Response II, Ciba Corning, Oberlin, OH) (Reed and Mukherjee 1969). Malate dehydrogenase (EC 1.1.1.37) and isocitrate dehydrogenase (EC 1.1.1.41) were assayed using previously described methods (Reeves et al. 1971). Citrate synthase (EC 4.1.3.7) and malate synthase (EC 4.1.3.2) were assayed by following the loss of absorbance at 232 nm of the thioester bond of acetyl-CoA (Reeves et al. 1971; Stadtman 1957). CO dehydrogenase activity was measured using methyl viologen dye as the electron acceptor (Diekert and Thauer 1978). Protein was measured using a modification of the Lowry assay (Peterson 1977). Bovine serum albumin was the standard.

Determination of the specific activity of cellular carbon

Bacteria were grown in medium 188-102 or 188-129 (1 expt. each) with 100 mM sodium oxalate and 1 mM sodium acetate. Culture volume was 100 ml or 500 ml (1 expt. each) and an inoculum size of 0.6% was used. ¹⁴C-oxalate (40 µCi), ¹⁴C-acetate (2 or 4 µCi) or ¹⁴CO₃ (20 µCi) were added to the medium at inoculation. At the end of the log phase of growth, cells were harvested by centrifugation and washed. The cell pellets were frozen in an acetone/dry ice bath and lyophilized (Freezemobile, Vitris Instr., Mt. Prospect, IL). The cells were burned in a combustion oven (Biological
Material Oxidizer, RJ Harvey Instr. Co., Hillsdale, NJ) at 900° C. The resulting \(^{14}\)CO\(_2\) was trapped in 15 ml of 0.25 N NaOH (prepared from CO\(_2\)-free water). Mannitol (25 mg) was burned between each sample to eliminate any carryover of \(^{14}\)CO\(_2\) between samples. The \(^{14}\)CO\(_3\) was precipitated with 2 ml of 1 M BaCl\(_2\) (CO\(_2\)-free). The Ba\(^{14}\)CO\(_3\) was collected by filtration, washed with distilled water and ethanol and dried at 125° C. After cooling, the Ba\(^{14}\)CO\(_3\) was ground to a powder and divided in two. Each portion of BaCO\(_3\) was weighed and suspended in scintillation fluid (Ecoscint) containing fumed silica (Cab-o-sil, Packard Instr., Downers Grove, IL) (Hash 1972; Turner 1969). The counting efficiency was determined from measurements of Ba\(^{14}\)CO\(_3\) that had a known specific activity. The contribution of each precursor to total cell carbon was calculated by dividing the specific activity of the \(^{14}\)C obtained as Ba\(^{14}\)CO\(_3\) by the initial specific activity of the \(^{14}\)C of the precursor.

**Results and Discussion**

**Sources of cell carbon**

Measurements of specific activities of carbon from cells labeled with either \(^{14}\)C-oxalate or \(^{14}\)C-acetate indicated that at least 54% of the cell carbon was derived from oxalate and at least 7% of the cell carbon was derived from acetate (Table 1). Due to the dilution of the specific activity of CO\(_3\) by the decarboxylation of oxalate during growth, we were unable to estimate the contribution of CO\(_3\) to the cell carbon pool.
Table 1. Specific activity of cell carbon of *O. formigenes* grown in $^{14}$C oxalate or $^{14}$C-acetate

<table>
<thead>
<tr>
<th>Labeled Substrate</th>
<th>Experiment</th>
<th>Substrate</th>
<th>Cell Carbon</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>A</td>
<td>$2.5 \times 10^5$</td>
<td>$1.3 \times 10^5$</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>$5.4 \times 10^4$</td>
<td>$3.1 \times 10^4$</td>
<td>57</td>
</tr>
<tr>
<td>Acetate</td>
<td>A</td>
<td>$3.3 \times 10^6$</td>
<td>$2.0 \times 10^6$</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>$1.3 \times 10^6$</td>
<td>$1.3 \times 10^5$</td>
<td>10</td>
</tr>
</tbody>
</table>

$^a$dpm/mg of C

Distribution of label in cell fractions

*O. formigenes* incorporated approximately 1% of the available carbon from oxalate and carbonate and 12% of the available carbon from acetate into cell biomass. This is consistent with previous data which indicated that 99% of the oxalate utilized by *O. formigenes* was decarboxylated to formate and CO$_2$ (Cornick and Allison 1995; Dawson et al. 1980). Carbon from oxalate, acetate and CO$_3$ was distributed into all of the major cell fractions of *O. formigenes* (Table 2). Radioactive carbon from acetate was incorporated primarily into the lipid and protein fractions. The distribution of radioactivity from 1C and 2C labeled acetate in cell fractions was similar.

When *Escherichia coli* is grown in glucose and U$^{14}$C-acetate, 43% of the acetate is incorporated into lipid and another 43% is incorporated into protein (Roberts et al. 1963). *Methanosarcina barkeri* (Weimer and Zeikus 1978) and *Chlorobium thiosulfatum* (Hoare and Gibson 1964) incorporated a greater percentage of acetate into cell protein (50-70%) than did *O. formigenes*. When cells of *O. formigenes* were grown in $^{14}$C-formate only 0.08% of the label was incorporated into cell biomass. Since the
Table 2. Relative distribution of $^{14}$C in fractions of *O. formigenes* cells after growth in labeled compounds

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Oxalate</th>
<th>CO$_3$</th>
<th>1C-Acetate</th>
<th>2C-Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of $^{14}$C incorporated</td>
<td>0.7</td>
<td>1.3</td>
<td>14.8</td>
<td>9</td>
</tr>
<tr>
<td>% Recovery in Fractions</td>
<td>70</td>
<td>102</td>
<td>60</td>
<td>67</td>
</tr>
<tr>
<td>% of $^{14}$C recovered$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediates</td>
<td>29</td>
<td>9</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Lipid</td>
<td>17</td>
<td>21</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Nucleic Acid</td>
<td>29</td>
<td>34</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Protein</td>
<td>27</td>
<td>36</td>
<td>28</td>
<td>27</td>
</tr>
</tbody>
</table>

$^a$Mean of 2 experiments, cells were fractionated using the method of Roberts et al. (1963)

Specific activities of $^{14}$C-formate and $^{14}$CO$_3$ would be diluted to the same extent by the decarboxylation of oxalate during growth, the calculated contribution of formate carbon to cell biomass is clearly much less than the contribution of carbon from carbonate.

Relative specific activity of amino acids

$^{14}$C carbon from oxalate was incorporated into amino acids derived from $\alpha$-ketoglutarate, oxaloacetate, pyruvate, 3P-glycerate and in the aromatic amino acids (Table 3). This supports previous enzymatic data which indicated that oxalate was reduced to 3P-glycerate before entering central metabolic pathways (Cornick and Allison 1995). Carbon from $^{14}$CO$_3$ was also detected in amino acids derived from $\alpha$-ketoglutarate, oxaloacetate, pyruvate, 3P-glycerate and in the aromatic amino acids.
Table 3: Relative specific activity of amino acids labeled by $^{14}$C-oxalate, $^{14}$C-acetate and $^{14}$CO$_3$

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Relative Specific Activity$^a$</th>
<th>$^{14}$C-Oxalate</th>
<th>$^{14}$CO$_3$</th>
<th>$^{114}$C-acetate</th>
<th>$^{2^{14}}$C-acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td></td>
<td>9</td>
<td>65</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>Proline/Valine</td>
<td></td>
<td>16</td>
<td>42</td>
<td>64</td>
<td>73</td>
</tr>
<tr>
<td>Arginine/Isoleucine</td>
<td></td>
<td>66</td>
<td>100</td>
<td>81</td>
<td>37</td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
<td>48</td>
<td>_$^b$</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td>-</td>
<td>58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td>9</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td>50</td>
<td>38</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td>16</td>
<td>2</td>
<td>77</td>
<td>100</td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td>11</td>
<td>-</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Serine</td>
<td></td>
<td>17</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>7</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td></td>
<td>100</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Based on cpm/nmol of amino acid, mean of 2 experiments

The apparent lack of $^{14}$C incorporation into histidine probably reflects our limited ability to measure $^{14}$C in histidine due to the relatively low concentration of histidine in the protein hydrolysates studied (data not shown).

Label from acetate was incorporated into the amino acids derived from pyruvate, oxaloacetate and $\alpha$-ketoglutarate. When the relative specific activities of glutamate,
aspartate and alanine from cells grown with 1C or 2C-labeled acetate were compared, the specific activity of aspartate was approximately half the specific activity of glutamate. Alanine derived from 2C-acetate had the same relative specific activity as glutamate but the alanine derived from 1C-acetate had only 33% of the relative specific activity of glutamate. Label from 2^{14}C-acetate was also incorporated into lysine but label from 1^{14}C-acetate was not (Table 3).

* Methanobacterium thermoautotrophicum* assimilates acetate by the reverse TCA cycle via pyruvate synthase and 2-oxoglutarate synthase. The specific activities of glutamate, aspartate and alanine from cells grown in U-^{14}C acetate are all similar (Fuchs et al. 1978). *Methanosarcina barkeri* assimilates acetate using a horseshoe type of TCA pathway. Acetyl-CoA is condensed with oxaloacetate via citrate synthase in the oxidative direction of the TCA pathway and acetate is also carboxylated by pyruvate synthase in the reductive direction. This results in the specific activity of glutamate being double that of both alanine and aspartate (Weimer and Zeikus 1979). In *M. barkeri* the specific activity of these amino acids are the same whether the cells are grown in 1-^{14}C or 2-^{14}C acetate. This is in contrast to our data from *O. formigenes* in which the specific activity of alanine derived from 1-^{14}C and 2-^{14}C acetate was different. Our results suggest that acetate was not always incorporated as a C_{2} unit into amino acids.

### Enzymatic activity

We detected citrate synthase, isocitrate dehydrogenase and malate dehydrogenase activities in cell-free extracts of *O. formigenes* (Table 4). The presence of citrate synthase and isocitrate dehydrogenase activities suggests that the reactions of the TCA
Table 4. Enzymatic activities in cell-free extracts of *O. formigenes*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay Measurement</th>
<th>Specific Activity^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase</td>
<td>thioester bond reduction</td>
<td>23</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>NAD reduction</td>
<td>8.5</td>
</tr>
<tr>
<td>α-ketoglutarate dehydrogenase</td>
<td>NAD reduction</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>NAD reduction</td>
<td>2.4</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>NAD reduction</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Malate synthase</td>
<td>thioester bond reduction</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Pyruvate synthase</td>
<td>^14C-alanine produced</td>
<td>NA^b</td>
</tr>
<tr>
<td>(reverse reaction)</td>
<td>CO₂ production</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(reverse reaction)</td>
<td>methyl viologen reduction</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>2-oxoglutarate synthase</td>
<td>CO₂ uptake</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>(reverse reaction)</td>
<td>CO₂ production</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(reverse reaction)</td>
<td>methyl viologen reduction</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Carbon monoxide dehydrogenase</td>
<td>methyl viologen reduction</td>
<td>&lt; 20</td>
</tr>
</tbody>
</table>

^a^nmol/min/mg protein

^b^NA, no activity detected

Pathway operate in the oxidative direction as far as α-ketoglutarate. The lack of α-ketoglutarate dehydrogenase activity was not surprising since this enzyme is not produced by strictly anaerobic bacteria. We assayed cell extracts for malate synthase because it catalyzes the condensation of glyoxylate and acetyl-CoA to form malate. This reaction functions in an anaplerotic role in many aerobic bacteria to replace C₄
units siphoned off into biosynthetic pathways (Gottschalk 1979). Although oxalate is reduced to glyoxylate by \textit{O. formigenes} (Cornick and Allison 1995) and other oxalate-degrading bacteria, malate synthase activity has not been detected in these organisms (Quayle et al. 1961; Zaitsev et al. 1993). The lack of pyruvate synthase, 2-oxoglutarate synthase and carbon monoxide dehydrogenase activities imply that the reverse TCA cycle and the acetyl-CoA (Wood) pathway do not function in \textit{O. formigenes}.

Acknowledgements

We thank Dr. Mike Stahr for the use of the combustion oven and Herb Cook for technical assistance.

References


BIOSYNTHESIS OF AMINO ACIDS BY *OXALOBACTER FORMIGENES*: ANALYSIS USING $^{13}$C NMR

A paper to be submitted to the Canadian Journal of Microbiology

Nancy A. Cornick, Bin Yan, Shelton Bank, and Milton J. Allison

Abstract

The gram-negative anaerobe, *Oxalobacter formigenes*, grows with oxalate as the principal energy and carbon source, but some acetate is required for biosynthetic reactions. We grew *O. formigenes* in $^{13}$C-oxalate, $^{13}$C-acetate or $^{13}$CO$_3$ and determined the distribution and position of labeling in cellular amino acids using $^{13}$C-NMR. The labeling pattern of all of the amino acids was consistent with their formation through common biosynthetic pathways. Our results support previous findings which indicated that oxalate enters biosynthetic pathways after conversion to a C$_3$ unit via the glycerate pathway. The majority of the carbons in the amino acids derived from pyruvate, oxaloacetate, $\alpha$-ketoglutarate, 3P-glycerate and the aromatic amino acids were labeled by oxalate. $^{13}$CO$_3$ was assimilated primarily into amino acids derived from aspartate and $\alpha$-ketoglutarate. Approximately 60% of the acetate that was assimilated into amino acids was incorporated as a C$_2$ unit into proline, arginine, glutamate and leucine. The pattern of labeling in glutamate, arginine and proline was consistent with the formation of $\alpha$-ketoglutarate via (S)-citrate synthase and the first
third of the TCA pathway. The labeling of leucine suggests that it was formed via isopropylmalate. Acetate was also assimilated into other amino acids as a single carbon. Methyl carbon from acetate labeled alanine, valine, leucine, threonine, lysine, isoleucine, methionine, aspartate, tyrosine and phenylalanine. Carboxyl carbon from acetate labeled isoleucine, aspartate, valine and alanine. Based on these findings, cell-lysates were assayed for several key biosynthetic enzymes. Lysates of *O. formigenes* contained glutamate dehydrogenase (NADPH and NADH-dependent), phosphoenolpyruvate carboxylase and pyruvate carboxylase activities.

**Introduction**

*Oxalobacter formigenes* is an anaerobic gram negative rod that utilizes oxalate as the sole substrate for growth. It has been isolated from the gastrointestinal tract of many warm-blooded animals (Allison et al. 1985; Daniel et al. 1987), including humans (Allison et al. 1986) and from anoxic sediments (Smith et al. 1985). Approximately 99% of the oxalate metabolized is decarboxylated to formate and CO$_2$ in a nearly 1:1 ratio (Allison et al. 1985). The remaining oxalate (1%) is utilized for cell biosynthesis. Cell-free extracts of *O. formigenes* contain enzymatic activity for the key enzymes of the glycerate pathway, whereby oxalate is reduced to 3-phosphoglycerate (3P-glycerate) before entering central metabolic pathways (Cornick and Allison 1995a).

Although acetate alone does not support the growth of *O. formigenes*, a small amount (0.5 mM-1 mM) is required for growth (Allison et al. 1985). Anaerobic bacteria generally assimilate acetate and CO$_2$ into cell protein using the reverse tricarboxylic acid (TCA) cycle or a horseshoe type of TCA pathway. The key enzymes of the
reverse TCA cycle are pyruvate synthase, which catalyzes the reductive carboxylation of acetate to pyruvate, and 2-oxoglutarate synthase, which catalyzes the reductive carboxylation of succinyl-CoA to α-ketoglutarate. The key enzymes for a horseshoe type of TCA pathway are pyruvate synthase and citrate synthase. CO₂ is also assimilated by heterotrophic, anaerobic bacteria by pyruvate carboxylase, phosphoenolpyruvate (PEP) carboxylase (Seubert and Weicker 1969) and a variety of reductive carboxylations of short chain fatty acids (Buchanan 1972). We were unable to detect pyruvate synthase or 2-oxoglutarate synthase activities in cell-free extracts of *O. formigenes*, but we did detect citrate synthase activity (Cornick and Allison 1995b).

When *O. formigenes* was grown in 1⁻¹⁴C or 2⁻¹⁴C-acetate, lysine was labeled by the methyl carbon of acetate but not by the carboxyl carbon. This suggested to us that acetate was not always assimilated as a two carbon unit (Cornick and Allison 1995b). In order to gain additional insight into the metabolism of *O. formigenes* we grew the organism in ¹³C-oxalate, ¹³C-acetate and ¹³CO₃ and determined the extent and position of the ¹³C label in amino acids. We also assayed cell-free extracts for the following enzymatic activities: pyruvate carboxylase, PEP carboxylase, glutamate dehydrogenase and β-hydroxyaspartate dehydratase.

**Materials and Methods**

**Bacteria**

*O. formigenes* (ATCC 35274) was grown in 1 liter batches (0.5% inoculum) in a defined, synthetic medium 188-129 (Cornick and Allison 1995b) with 100 mM oxalate and either 1 mM ¹³C, ₂⁻¹³C, or U⁻¹³C acetate or 24 mM Na¹³CO₃. The flask
containing $^{13}\text{CO}_3$ was adjusted to pH 5.8 using filter sterilized 1 N HCl. Cells labeled with oxalate were grown under CO₂ and in a carbonate buffer system (medium 188-102, Cornick and Allison 1995b) to minimize the contribution of carbonate arising from decarboxylated $^{13}\text{C}$-oxalate to cell biomass. The final concentration of $^{13}\text{C}$-oxalate (26% enriched) was 70 mM rather than 100 mM to minimize dilution of the label. Sterile Na acetate was added to a final concentration of 1 mM to flasks containing labeled oxalate or CO₃.

**Fractionation of cells**

Cells were fractionated using the method of Roberts et al. (1963). Cell protein was hydrolyzed in a sealed tube under N₂ in 6 N HCl for 20 h at 105°C. The HCl was removed under vacuum.

**NMR spectra**

The spectra of all of the protein hydrolysates except the hydrolysate from cells grown in $^{13}\text{C}$-oxalate were acquired on a Varian XL-300 multinuclear spectrometer at 75.43 MHz with a 5 mm probe, using broadband proton decoupling, a 20 kHz spectral width, and 20 032 data points. The pulse width was set at 10 µs (CO₃ sample, 6 µs), corresponding to 45 degree flip angles. The recycle time was 2.5 s, including a 2 s delay time (CO₃ sample, 1 s) and a 0.5 s acquisition time. A signal/noise ratio of high quality was obtained by using 6 000-33 000 transients.

The spectrum from the protein hydrolysate of cells grown on $^{13}\text{C}$-oxalate was acquired on a Varian 500 MHz UNITY spectrometer at 125.7 MHz with a 5 mm probe. The delay time was 1 s and the acquisition time was 0.5 s. The pulse width was 8 µs and 52 656 transients were collected.
Chemical shifts were referenced using dioxane as an external standard. The identity of the signals was based on previously published values (Miller et al. 1995). When there was a question regarding the identity of the signals, unlabeled amino acid was added to the sample to enhance the signal. The spectra from the oxalate-enriched sample contained approximately 20 peaks in the 170-174 ppm range which precluded the identification of individual carboxyl carbons. D$_2$O was the solvent and locking material for all of the samples. Due to the distinct nuclear Overhauser effect (NOE) for different carbons, a coefficient ratio of approximately 3:1 of the carboxyl peaks to other protonated peaks was applied to each sample in order to determine the semi-quantitative enrichment distribution on each labeled position. The 3:1 ratio was determined by running standard amino acids under the same conditions used for the enriched samples.

**Enzyme assays**

Cell-free extracts were prepared as previously described (Cornick and Allison 1995a). Glutamate dehydrogenase (EC 1.4.1.4) was assayed using the method of Joyner and Baldwin (1966). Pyruvate carboxylase (EC 6.4.1.1) and PEP carboxylase (EC 4.1.1.31) were assayed by coupling the formation of oxaloacetate to the formation of malate by malate dehydrogenase (Scrutton 1971). β-hydroxyaspartate dehydratase (EC 4.2.1.38) was also assayed by linking the formation of oxaloacetate to malate (Gibbs and Morris 1970). Since cell-free lysates of *O. formigenes* contain a negligible amount of NADH oxidase, NADH-linked malate dehydrogenase was used (rather than NADPH-linked). Malate dehydrogenase was purchased from Sigma Chemical Co. (St. Louis, MO). Protein was measured using a modified Lowry assay (Peterson 1977) with
bovine serum albumin as the standard.

**13C labeled compounds**

1-13C acetic acid (99% enriched), Na13CO3 (99% enriched) and U-13C Na acetate (99% enriched) were purchased from Cambridge Isotope Laboratories (Andover, MA).

2-13C Na acetate (92% enriched) and 13C2 oxalic acid (90% and 99% enriched) were purchased from MSD Isotopes (Montreal, Canada). 13C2 oxalic acid (99% enriched) was also purchased from Isotech (Miamisburg, OH).

**Results**

The major labeling patterns of the acid-stable amino acids derived from 13C-labeled oxalate, acetate, and CO3 are shown in Fig. 1.

**Aspartate**

Aspartate was labeled by oxalate in positions C1, C2 and C3. The C4 position was labeled by oxalate and CO3. This labeling is consistent with the carboxylation of pyruvate or PEP (which was derived from oxalate via the glycerate pathway) to form oxaloacetate. 13CO3 arising from the decarboxylation of 13C-oxalate would result in the labeling of the C4 position by oxalate.

**Lysine, threonine and methionine**

The labeling of these amino acids was consistent with their derivation from aspartate. The labeling of lysine indicated that biosynthesis proceeded via diaminopimelic acid which is the common pathway used by bacteria. If lysine had been synthesized via the aminoadipic acid pathway, the C1 and C6 carbons of lysine would be derived from the carboxyl carbon of acetate and the C2 and C5 carbons
Figure 1. Major labeling patterns of acid stable amino acids synthesized by *O. formigenes*. ▲, oxalate; △, estimated oxalate; ▲ =, oxalate (doublet); ○, 2C-acetate; *, 1C-acetate; +, CO$_3$. 
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamate</td>
<td><img src="image" alt="L-glutamate" /></td>
</tr>
<tr>
<td>L-arginine</td>
<td><img src="image" alt="L-arginine" /></td>
</tr>
<tr>
<td>L-proline</td>
<td><img src="image" alt="L-proline" /></td>
</tr>
<tr>
<td>L-aspartate</td>
<td><img src="image" alt="L-aspartate" /></td>
</tr>
<tr>
<td>L-isoleucine</td>
<td><img src="image" alt="L-isoleucine" /></td>
</tr>
<tr>
<td>L-lysine</td>
<td><img src="image" alt="L-lysine" /></td>
</tr>
<tr>
<td>L-threonine</td>
<td><img src="image" alt="L-threonine" /></td>
</tr>
<tr>
<td>L-methionine</td>
<td><img src="image" alt="L-methionine" /></td>
</tr>
<tr>
<td>L-alanine</td>
<td><img src="image" alt="L-alanine" /></td>
</tr>
<tr>
<td>L-valine</td>
<td><img src="image" alt="L-valine" /></td>
</tr>
<tr>
<td>L-leucine</td>
<td><img src="image" alt="L-leucine" /></td>
</tr>
<tr>
<td>L-serine</td>
<td><img src="image" alt="L-serine" /></td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td><img src="image" alt="L-phenylalanine" /></td>
</tr>
<tr>
<td>L-tyrosine</td>
<td><img src="image" alt="L-tyrosine" /></td>
</tr>
</tbody>
</table>

* denotes the presence of a functional group.
would be labeled by the methyl carbon of acetate.

Isoleucine

The C1 of isoleucine was labeled by both oxalate and CO₃. The remaining carbon skeleton was derived from oxalate. This labeling indicates that isoleucine was synthesized from threonine by the common biosynthetic pathway whereby threonine is converted to α-ketobutyrate and then condensed with pyruvate to form α-acetoα-hydroxybutyrate. The labeling of the C1 position of isoleucine by CO₃ indicates that O. formigenes must synthesize isoleucine by a second pathway, perhaps by the carboxylation of propionate to α-ketobutyrate or the carboxylation of 2-methylbutyrate followed by transamination to isoleucine.

Glutamate, arginine and proline

These amino acids were labeled by oxalate in C1, C2 and C3 positions. The C4 and C5 positions were labeled by the methyl and carboxyl carbons of acetate, respectively. The C1 position was also labeled by CO₃. This labeling pattern is consistent with the formation of α-ketoglutarate from oxaloacetate plus acetate by (S)-citrate synthase.

Alanine, valine and leucine

All of the carbons from alanine and valine were labeled by oxalate. This is consistent with the usual biosynthetic pathways whereby these amino acids are derived from pyruvate (oxalate). Leucine was labeled by acetate at the C1 and C2 positions. This is consistent with its formation through the isopropylmalate pathway.
Glycine and serine

The labeling of serine was consistent with its formation from 3P-glycerate which was derived from oxalate via the glycerate pathway. The labeling of glycine is as expected if it was formed from serine.

Phenylalanine and tyrosine

The labeling of phenylalanine and tyrosine was consistent with known pathways via skimate and chorismate with erythrose-4-P and PEP being derived from oxalate.

We did not detect $^{13}$C in histidine. This was probably due to the small amount of histidine in the cells.

Minor labeling of amino acids by acetate

The spectra obtained from cells grown with $1^{-13}$C acetate, $2^{-13}$C acetate and U-$^{13}$C acetate are shown in Fig. 2. The majority of acetate incorporated into amino acids (60%) was assimilated as a C$_2$ unit into proline, arginine, glutamate and leucine. This was confirmed by the spectra of the cells grown with U-$^{13}$C acetate. Only the signals from these four amino acids show the effect of C$_{13}$-C$_{13}$ spin-spin coupling whereby the signal is split due to having adjacent carbons labeled. In other amino acids acetate was not incorporated as a unit but as a single carbon. The methyl carbon of acetate was incorporated into alanine (C2 and C3), valine (C2, C4 and C4$_1$), leucine (C5 and C5$_1$), threonine (C3), lysine (C3 and C6), isoleucine (C3$_1$ and C5), methionine (C2), aspartate (C3), tyrosine (C3) and phenylalanine (C3). The distribution of the methyl carbon of acetate ranged from 0.1% for tyrosine (C3) to 5.3% for alanine (C3). The carboxyl carbon of acetate was incorporated into the C1 position of isoleucine, aspartate, valine, and alanine. The distribution of the carboxyl carbon of acetate in these amino acids
Figure 2. NMR spectra of amino acids labeled by A) 1-$^{13}$C acetate, B) 2-$^{13}$C acetate, C) U-$^{13}$C acetate. Identification of peaks: 1, C5 glutamate; 2, C1 leucine; 3, C5 proline; 4, C5 arginine; 5, C2 leucine; 6, C4 glutamate; 7, C4 proline; 8, C4 arginine.
was 1.1%, 1.5%, 0.8%, and 0.9%, respectively. By comparison, the distribution of the eight carbons labeled by intact acetate ranged from 12.2% for the C4 of arginine to 25.2% for the C4 of glutamate.

**Enzyme activity**

Cell-free extracts of *O. formigenes* contained both NADPH-dependent and NADH-dependent glutamate dehydrogenase, pyruvate carboxylase, and PEP carboxylase activities (Table 1). PEP carboxylase activity was enhanced by acetyl-CoA but was not dependent on it. Pyruvate carboxylase was not activated by acetyl-CoA. β-hydroxyaspartate dehydratase activity was not detected.

**Table 1. Enzyme activities in cell-free extracts of *O. formigenes***

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay method</th>
<th>Specific activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>NADPH oxidation</td>
<td>286</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>NADH oxidation</td>
<td>37</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>NAD reductionb</td>
<td>25</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>NAD reductionb</td>
<td>32</td>
</tr>
<tr>
<td>β-hydroxyaspartate dehydratase</td>
<td>NAD reductionb</td>
<td>NAc</td>
</tr>
</tbody>
</table>

a nmol/min/mg protein  
bThese assays were coupled to malate dehydrogenase activity  
cNA, no activity detected
Discussion

The labeling of all of the amino acids indicates that they were synthesized by conventional pathways. This supports previous enzymatic data which indicated: (i) the glycerate pathway operates to synthesize 3P-glycerate and thus to incorporate carbon from oxalate into a C₃ unit for utilization in biosynthetic pathways; (ii) acetate is incorporated into proteins via citrate synthase and the first third of the TCA pathway; (iii) the reverse TCA cycle does not function in *O. formigenes*. The majority of the carbon (54%) that *O. formigenes* assimilates is derived from oxalate, while only 7% is derived from acetate (Cornick and Allison 1995b). The labeling of the majority of the carbons in all of the amino acids by oxalate supports the concept that oxalate is the major source of cellular carbon for this organism.

The main role of PEP carboxylase and pyruvate carboxylase in heterotrophic bacteria is anaplerotic. The formation of C₄ units allows for the continuation of the Krebs cycle when intermediates are removed for biosynthetic reactions (Utter and Kolenbrander 1972). The major route for the formation of C₄ compounds from C₃ compounds in *O. formigenes* is probably through PEP carboxylase and pyruvate carboxylase. PEP carboxylase is also primarily a biosynthetic enzyme in *Methanobacterium thermoautotrophicum* (Kenealy and Zeikus 1982) and *Thiobacillus thiooxidans* (Howden et al. 1972). An alternate pathway to form C₄ compounds has been noted with *Micrococcus denitrificans* (Gibbs and Morris 1964, 1970). When this organism is grown on glycolate, the enzymes of the β-hydroxyaspartate pathway catalyze the formation of β-hydroxyaspartate from glyoxylate plus glycine and the deamination of β-hydroxyaspartate to oxaloacetate. Since aspartate from *O.*
formigenes was labeled in all four carbons by oxalate this was a potential route for the formation of oxaloacetate. Our failure to detect β-hydroxyaspartate dehydratase activity in cell-free extracts indicates this pathway probably does not function in O. formigenes.

The labeling of glutamate in the C1 position by CO$_3$ and the C4 and C5 positions by acetate indicates that O. formigenes produces a (S)-citrate synthase (Gottschalk and Barker 1966; Tomlinson 1954). A (R)-citrate synthase would have resulted in the C1 and C2 positions of glutamate being labeled by the carboxyl and methyl carbons of acetate and the C5 position of glutamate being labeled by CO$_3$. Acetate was incorporated as a two-carbon unit into glutamate, arginine, proline and leucine. Although these four amino acids account for 60% of the acetate assimilated into protein, the other 40% was split and assimilated as single carbons. All of the positions that were labeled by either the carboxyl or methyl carbons of acetate were also labeled by oxalate. This suggests some scrambling of label between oxalate and acetate. Preliminary data from cells grown in $^{14}$C-oxalate or $^{14}$C-acetate indicated there may be additional unidentified metabolites that are labeled by both oxalate and acetate but not by CO$_3$ (see appendix).

Although the metabolism of other oxalate-degrading bacteria has not been examined using $^{13}$C NMR, scrambling of carbons has been described in several other genera of bacteria. Methanococcus jannaschii scrambles $^{13}$C label among the three carbons of pyruvate (Sprott et al. 1993). When the bacteria are grown in 1-$^{13}$C pyruvate, some $^{13}$C is recovered in the C2 and C3 positions of pyruvate. Sprott et al. (1993) suggested this could occur by the formation of a one-carbon intermediate
formed from the carboxyl of pyruvate and CoA. After dilution by unlabeled precursors and the release of some carboxyl as $^{13}$CO$_2$, the labeled CoA intermediate could combine with methyltetrahydrofolate to form acetyl-CoA. The reductive carboxylation of acetyl-CoA would then result in pyruvate labeled at the 2C and 3C positions.

A high degree of scrambling between the carboxyl of acetate and CO$_2$ is also found in *Methanothrix concilli* (Ekiel et al. 1985). Approximately 25% of $^{13}$CO$_2$ goes to positions that are expected to be labeled by the carbon of acetate carboxyl. A small amount of $^{13}$CO$_2$ (5%) is also found in positions that should be labeled by the methyl carbon of acetate. *M. concilli* also incorporates some acetate carboxyl into positions that should be labeled by CO$_2$. *Methanosarcina barkeri* is another organism that exchanges the carboxyl of acetate with CO$_2$ but does not exchange carbons between formate and acetate or between formate and CO$_2$. However, formate and CO$_2$ are rapidly exchanged by *Desulfovibrio baarsii* (Jansen et al. 1984). We have preliminary data that suggests *O. formigenes* exchanges formate and CO$_2$ but does not exchange the carboxyl of acetate with CO$_2$ (see appendix).

Our labeling data suggests that *O. formigenes* is capable of synthesizing all of the amino acids from oxalate, CO$_3$ and intact acetate. The splitting of acetate and scrambling with oxalate must therefore occur for reasons beyond amino acid biosynthesis. We propose this scrambling occurs at the level of pyruvate or another intermediate rather than in a direct interaction between oxalate and acetate.

The requirement for acetate may well be related to its role in the citrate synthase reaction and the demand for its products for the subsequent synthesis of the carbon skeletons of amino acids in the glutamate family. Results of previous experiments have
provided evidence that carbon from $^{14}$C labeled acetate is incorporated mainly into the lipid and protein fractions of $O. formigenes$ cells (Cornick and Allison 1995b). The extent of incorporation of carbon from oxalate and carbonate into the cellular lipid fraction (Cornick and Allison 1995b) suggests that some acetate carbon may be synthesized \textit{de novo} by $O. formigenes$ but we as yet have no conclusive evidence for this. Even if such synthesis occurs, the absolute requirement of a supply of exogenous acetate for growth indicates that endogenous synthesis cannot provide enough acetate for cellular metabolism. As acetate is invariably present at relatively high concentrations in the rumen and in other gastrointestinal habitats for $O. formigenes$, the maintenance of an efficient acetate synthesis capacity (if indeed this exists) might well be lost because it would probably have little survival value.

\section*{Acknowledgements}

We appreciate the technical assistance of Herb Cook.

\section*{References}


GENERAL CONCLUSIONS

Oxalate is degraded by *O. formigenes* to formate and CO$_2$. Since oxalate is the only substrate that supports the growth of this organism, it is reasonable to expect that some oxalate carbon is incorporated into cell biomass. Results from the first manuscript in this dissertation indicate that oxalate is reduced to 3P-glycerate before entering common biosynthetic pathways. Aerobic oxalate-degrading bacteria also reduce oxalate to 3P-glycerate prior to assimilating it into cell material.

The second manuscript in the dissertation addresses the potential sources of carbon for cell biomass that are available to *O. formigenes*. In addition to oxalate other potential sources of cell carbon for *O. formigenes* are formate, CO$_2$ and acetate. Results indicate that oxalate, acetate and CO$_2$ are utilized for cell synthesis and that the majority of cellular carbon is derived from oxalate. Formate is not incorporated to a significant extent. Acetate is assimilated into amino acids via citrate synthase and the first third of the TCA pathway. Many strict anaerobic bacteria also assimilate acetate and CO$_2$ using the reverse TCA pathway. Our results from both the second and third manuscripts confirm that this pathway does not function in *O. formigenes*.

The third manuscript of this dissertation examines the flow of carbon into amino acids in greater detail using $^{13}$C-NMR. This technique allows for discrimination of the labeling of individual carbons. If the position of labeled carbons in a given amino acid is known, biosynthetic pathways can be analyzed retrospectively. Our results indicate that *O. formigenes* utilizes common routes for the synthesis of amino acids and confirms that oxalate is the major source of cell carbon. The majority of acetate
assimilated into amino acids enters as a C₂ unit into glutamate, arginine, proline and leucine. Our results also indicate that some acetate incorporated into amino acids enters as a C₁ unit. This is not typical of most bacteria which assimilate acetate as a complete molecule. Our data shows some scrambling between the methyl carbon of acetate and oxalate which we propose occurs at the level of pyruvate or another intermediate compound rather than a direct interaction between oxalate and acetate. Our data regarding this interaction is still too preliminary to draw conclusions from.

A better understanding of the basic metabolism of *O. formigenes* may be important if this bacterium is ever genetically manipulated or used as a therapeutic agent for calcium oxalate urolithiasis. The results of this dissertation have increased our knowledge regarding the anabolic metabolism of *O. formigenes*. 
I thank my major professor Milton J. Allison for his oversight of this project and for making a major contribution to my development as a scientist. His critical and thought-provoking questions always served to broaden my outlook on a problem. He has been a fine example of a scientist, a mentor and a person. I hope I can pass along some of what he has taught me.

I also thank the NADC for the financial support throughout the course of this study. The facilities and equipment available greatly aided me in completing my research in a timely manner. Everyone in the Physiopathology unit, and particularly B-1, was generous with their advice, criticism and humor. Even on a "bad science day" there was something to smile about.

I appreciate the encouragement I received early in my career from Sherwood Gorbach. His support reinforced my desire to pursue a graduate degree.

Lastly, I thank my family, Anson, Andy and Chris for their unfailing love, support and understanding.
Materials and Methods

Bacteria

The bacteria used for the experiments in this section were the same samples described in the second manuscript under "growth of bacteria in ^14C compounds".

Measurement of ^14CO2

Supernate from cultures was acidified with citrate buffer in a stoppered flask. ^14CO2 was trapped in phenethylamine and counted by liquid scintillation.

HPLC

Organic acids were separated using an Aminex HPX-87H column (Biorad) and isocratic 0.008 N H2SO4 as the solvent. A diode array detector (210 nm) and a flow through scintillation counter (Radiomatic) were used for detection. FloScint II cocktail was added in a 3:1 ratio with the HPLC solvent.

Results

Carboxyl exchange reaction

When bacteria were grown with ^14C formate, 5% of the radioactivity was recovered in ^14CO2 (Table 1). When the bacteria were grown with ^14CO3, 6.6% of the radioactivity was recovered in ^14C-formate. This data suggests that there is an exchange reaction between the carboxyl carbon of formate and CO3. Bacteria grown with 1^14-C acetate or 2^14-C acetate oxidized 3.8% and 1.8% of the labeled acetate to ^14CO2, respectively.
Table 1. Recovery of $^{14}$C products from cells grown on $^{14}$C carbon sources

<table>
<thead>
<tr>
<th>$^{14}$C carbon source</th>
<th>CO$_2$</th>
<th>Formate</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>5</td>
<td>-</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>CO$_3$</td>
<td>-</td>
<td>6.6</td>
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$^a$% of initial $^{14}$C recovered as $^{14}$C product

$^b$NA, not assayed

$^c$ND, none detected

$^{14}$C-oxalate and acetate

HPLC data from bacteria grown in $^{14}$C precursors is given in Table 2. $^{14}$C labeled peaks with retention times of 6.2, 9.5 and 11.5 min were detected in the culture supernates of bacteria grown with $^{14}$C-oxalate, 1-$^{14}$C acetate, and 2-$^{14}$C acetate. The retention times of these peaks did not match any of the standards run with this experiment. The retention time of 9.5 min is close to the retention times of pyruvate and glyoxylate (9.2 and 9.4 min, respectively). The retention time of 11.5 min matches the retention time of succinate (11.5 min). The minor labeling pattern of the amino acids from cells grown in both 1-$^{13}$C acetate and 2-$^{13}$C acetate in positions that are also labeled by oxalate suggests there is some scrambling between the carbons of oxalate and acetate (manuscript 3). This HPLC data could be additional evidence of a common intermediate(s) between oxalate and acetate. Supernate from bacteria grown in $^{14}$CO$_3$ did not contain these labeled peaks.
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<th>14C-precursor</th>
<th>A_800</th>
<th>Retention time (min)</th>
<th>cpm/peak^a</th>
<th>% cpm^b</th>
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^a cpm per peak
^b % of cpm per sample