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Fermentation of Fumarate and L-Malate by *Clostridium formicoaceticum*

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The fermentation of fumarate and L-malate by *Clostridium formicoaceticum* was investigated. Growing and nongrowing cells degraded fumarate by dismutation to succinate, acetate, and CO_2; on the other hand, only small amounts of succinate were detected when the organism was grown on L-malate. This dicarboxylic acid was mainly converted to acetate and CO_2. The fermentation balances were modified if bicarbonate or formate were present in the medium. When *C. formicoaceticum* was grown in the presence of both dicarboxylic acids, fumarate was consumed before L-malate. The latter was mainly converted to acetate, whereas fumarate was fermented to acetate and succinate. Molar growth yields were determined to be 6 g of dry weight per mol of fumarate and 8 g of dry weight per mol of L-malate fermented.

Clostridium formicoaceticum has been shown to grow on fructose, several hexonic and hexuronic acids, glycerol, pyruvate, lactate, and glutamate as well as fumarate and L-malate (2, 9). Cells growing on fructose form acetate as the main fermentation product (2). Acetate is synthesized (i) from pyruvate via acetyl-coenzyme A (acetyl-CoA) and (ii) from CO_2 via the tetrahydrofolate- and corrinoid-dependent pathway (19, 27).

Since there are only a few reports on the fermentation of C_4-dicarboxylic acids by clostridia (8, 23, 35), it was of interest to study the metabolism of fumarate and L-malate by *C. formicoaceticum*. Several obligate and facultative anaerobes have been reported to couple the electron transfer from H_2, formate, reduced nicotinamide adenine dinucleotide (NADH), lactate, or glycerol-phosphate to fumarate with the phosphorylation of ADP (4, 10, 18, 21, 24, 32). It was, therefore, of special interest whether *C. formicoaceticum* could form succinate in addition to acetate as the fermentation end product. In the present communication, we show that L-malate is fermented by *C. formicoaceticum* to acetate, but fumarate is fermented to acetate and succinate.

MATERIALS AND METHODS

Organism and growth conditions. *C. formicoaceticum* strain A1 ([2], ATCC 27076; DSM 92) was grown in a medium similar to that described by Gottwald et al. (11), except that peptone was omitted and bicarbonate and formate were added only when indicated. The pH of the medium was adjusted to between 8.0 and 8.5. Cells were grown anaerobically at 37°C in 1-liter volumetric flasks or in 3-liter carboys under an atmosphere of oxygen-free nitrogen. Traces of oxygen were removed from N_2 by passing it through a Pyrex column packed with the copper catalyst Aktimet 13 (Doduco Chemie, Pforzheim, West Germany) and heated to approximately 200°C. Samples of the cultures were withdrawn anaerobically. The absorbance of cultures was measured at 600 nm, using cuvettes of 1-cm light path. Samples having an absorbance of more than 0.3 were appropriately diluted with medium to obtain accurate readings. Cells were harvested by centrifugation at 10,000 × *g* for 30 min at 4°C. The clear supernatant fluid was stored at −20°C until substrates and fermentation end products were determined.

Determination of dry weight and protein. Culture samples (20 to 50 ml) were centrifuged at 13,000 × *g* for 15 min at 4°C, and the supernatant fluids were discarded. The cells were washed once with 20 ml of 0.03% (wt/vol) sodium chloride, suspended in 2 to 4 ml of distilled water, quantitatively transferred to small, preweighed test tubes, and dried at 80 to 100°C for 24 to 48 h. After cooling to room temperature (20°C) in a desiccator, the tubes were weighed.

Protein content of whole cells was determined by the method of Stickland (31).

Fermentation experiments with nongrowing cells. Freshly harvested, unwashed cells (1 to 2 g of wet weight) were suspended in anaerobic 57 mM potassium phosphate buffer, pH 8.0, containing 1 mM dithioerythritol (DTE) and 120 mM bicarbonate or formate where indicated. The absorbance of the suspension at 600 nm was adjusted to a value of about 9. Samples (1 ml) were withdrawn at intervals of 1 to 2 h, and after the determination of the absorbance at 600 nm, the cell suspension was centrifuged at 15,000 × *g* for 10 min (4°C). The supernatant fluids were kept

frozen (−20°C) until used for the determinations of substrates and fermentation products.

Fermentation of [1,4-^{14}C]fumarate. The tests were performed in two-armed Warburg vessels closed with serum stoppers. The vessels contained: 1.8 ml of cell suspension (absorbance at 600 nm, 13.0) in 57 mM potassium phosphate buffer, pH 8.0, containing 1 mM DTE, in the main compartment; 0.2 ml of 1 M fumarate (0.5 μCi of [1,4-^{14}C]fumarate) in one side arm; 0.2 ml of 4 N H_3PO_4 in the second side arm; and 0.2 ml of 1.8 N NaOH in the center well. The vessels were gassed with nitrogen from which traces of oxygen were removed, as described above, and shaken for 20 min at 37°C before fumarate was added. In two vessels, the fermentation was stopped by addition of phosphoric acid immediately after tipping in the radioactive fumarate. In two other vessels the fermentation of fumarate was allowed to proceed for 4 h. Phosphoric acid was added, and vessels were shaken for 1 h. The cells were sedimented by centrifugation at 10,000 × *g* for 10 min (4°C), and fumarate, succinate, and acetate were enzymatically determined in the supernatant fluids as described below.

Samples (0.2 ml) of the supernatant fluids and the NaOH solutions (0.2 ml) from the center wells were transferred into scintillation vials containing 10 ml of Aquasol solution. Radioactivity was determined with a Tri-Carb scintillation spectrometer, model 3320 (Packard Instruments, Downers Grove, Ill.). The radioactivity present in acetate was measured after steam distillation of the samples (22).

Determination of organic acids. Fumarate and L-malate concentrations were determined by the method of Kröger (17); acetate was determined according to the following method (Boehringer, Mannheim, personal communication). The assay mixture contained, in a total volume of 1 ml: 81 mM triethanolamine-hydrochloride, pH 7.0, 0.26 mM CoA, 0.23 mM ATP, 1 mM phosphoenolpyruvate, 5 mM KCl, 1.5 mM $MgSO_4$, 0.7 mM NADH, sample or acetate standard solution containing up to 0.05 mM acetate, 10 μg of lactate dehydrogenase (550 U/mg), 10 μg of pyruvate kinase (200 U/mg), and 20 μg of myokinase (360 U/mg). After preincubation of the mixture at 25°C for 15 min, the reaction was started by addition of 0.1 mg of acetyl-CoA synthetase (3 U/mg), and the oxidation of NADH was followed at 365 nm (1-cm light path). Because of the acetate content of the reagents, a blank had to be run in each series of assays.

Succinate was determined by the following method (Boehringer, Mannheim, personal communication). The assay mixture contained, in a final volume of 1 ml: 80 mM glycylglycine, pH 8.4, 5 mM $MgCl_2$, 0.65 mM CoA, 0.55 mM GTP, 0.95 mM phosphoenolpyruvate, 0.7 mM NADH, 20 μg of lactate dehydrogenase (550 U/mg), 60 μg of pyruvate kinase (200 U/mg), and sample or succinate standard solution containing up to 0.1 mM succinate. The mixture was incubated at 25°C for 15 min before the reaction was started by the addition of 50 μg of succinyl-CoA synthetase from pig hearts (10 U/mg). The oxidation of NADH was measured spectrophotometrically at 365 nm. The values were corrected for the succinate content of a reagent blank.

Formate was determined with formyl-tetrahydrofolate synthetase by the method of Rabinowitz and Pricer (29), which was slightly modified. The reaction mixture contained, in a final volume of 0.75 ml: 0.19 M mercaptoethanol, 0.113 M triethanolamine-hydrochloride, pH 8.0, 0.01 M $MgCl_2$, 5 mM ATP, 0.4 mM L-tetrahydrofolic acid, 6.6 mM NH_4Cl, and 50 μg of purified formyl-tetrahydrofolate synthetase (110 U/mg) from *C. thermoaceticum*. The reaction was started by addition of sample or formate standard solution (up to 0.2 mM). The mixtures were incubated at 50°C. After 5, 10, 20, and 30 min, 0.2 ml was withdrawn, and the reaction was immediately stopped in 0.6 ml of 0.36 M HCl. After at least 10 min, the extinction of the acidified samples was measured at 350 nm (1-cm light path) against corresponding blanks without enzyme. The extinction coefficient for 5,10-methenyl-tetrahydrofolate at 350 nm of 24.9 mM^{-1} cm^{-1} (29) was used in the calculations.

Fructose was determined by a modified anthrone method (D. Siebert, Ph.D. Thesis, University of Göttingen, Göttingen, West Germany, 1969). The reagent was prepared as follows: 0.2 g of anthrone (9,10-dihydro-9-oxoanthracene) was mixed with 8 ml of ethanol (96%, vol/vol) and 30 ml of water and kept on ice. A 100-ml amount of concentrated H_2SO_4 was slowly added. A total of 0.5 ml each of the samples or of the fructose standard containing 0.1 to 0.5 μmol of fructose was layered upon 5 ml of ice-cold reagent. After mixing, the test tubes were incubated in a boiling water bath for 7 min and cooled in ice. The extinction was measured at 620 nm (1-cm light path) against a blank.

Materials. Yeast extract and sodium thioglycolate were obtained from Difco Laboratories, Detroit, Mich. Anthrone was purchased from British Drug Houses Ltd., Poole, U.K.; Aquasol came from New England Nuclear, Boston, Mass., and [1,4-^{14}C]fumarate was obtained from The Radiochemical Centre, Amersham, England. Formyl-tetrahydrofolate synthetase from *C. thermoaceticum* and L-tetrahydrofolic acid were gifts from L. G. Ljungdahl, University of Georgia, Athens, which are gratefully acknowledged. The enzymes used in the determinations of the fermentation end products were purchased from Boehringer, Mannheim.

RESULTS

C. formicoaceticum grew with maximal rates in a medium containing 0.5% (wt/vol) yeast extract and 100 mM fumarate or L-malate at initial pH values between 8.0 and 9.5. Surprisingly, an addition of bicarbonate or formate to the medium was not necessary to get growth on fumarate and L-malate (Fig. 1). Growth of *C. formicoaceticum* on fructose, however, is strictly dependent on the presence of bicarbonate or formate in the medium (2).

When bicarbonate was added to an L-malate-containing medium, growth was slower than in the absence of this electron acceptor (Fig. 1B), whereas bicarbonate only slightly retarded growth on fumarate within the first 10 h after inoculation (Fig. 1A). Microscopic examination of cultures of *C. formicoaceticum* growing on

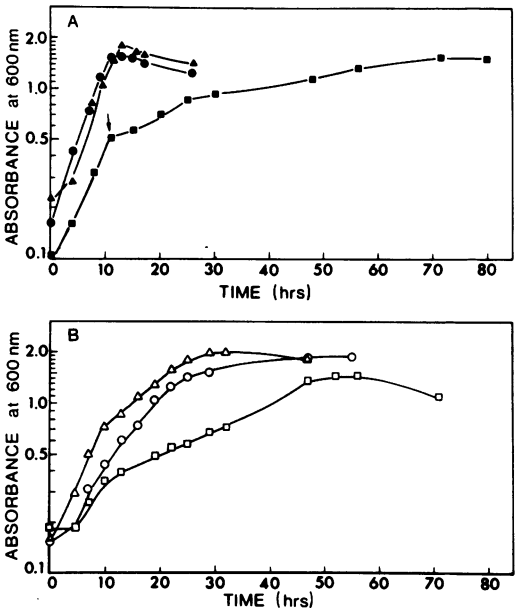

FIG. 1. *Effect of bicarbonate and formate on growth of C. formicoaceticum with fumarate and L-malate. (A) Growth on fumarate; (B) growth on L-malate. Cells were grown at 37°C in 1-liter flasks under an atmosphere of oxygen-free nitrogen. The inoculum was 20 ml of a culture grown with 100 mM fumarate (L-malate). Symbols: (●,○) without additional bicarbonate or formate, (■,□) in the presence of 120 mM bicarbonate, (▲,△) in the presence of 120 mM formate. The arrow in (A) indicates the beginning of sporulation.*

fumarate in the presence of bicarbonate showed that massive sporulation started at an absorbance (600 nm) of about 0.5 (arrow, Fig. 1A). At that time, however, only 20% of the fumarate added to the medium (100 mM) had been consumed by the cells. With the other substrate combinations tested (legend, Fig. 1), spores were only occasionally observed.

The determination of fermentation products formed during growth of *C. formicoaceticum* on fumarate and L-malate indicated important differences in the metabolism of the two substrates. Per 100 mol of fumarate fermented, 66 mol of succinate and 33 mol of acetate were produced (Fig. 2), whereas L-malate was almost completely fermented to acetate (Fig. 3). During fumarate fermentation, usually small amounts of L-malate were transiently excreted into the medium, as shown in Fig. 2.

Molar growth yields (Y_m) of *C. formicoaceticum* were determined with cells grown with fumarate or L-malate either in the presence or in the absence of bicarbonate or formate. Y_m was obtained from the slope of the plot of the increase in dry weight versus the corresponding substrate consumption (Fig. 4). The results are

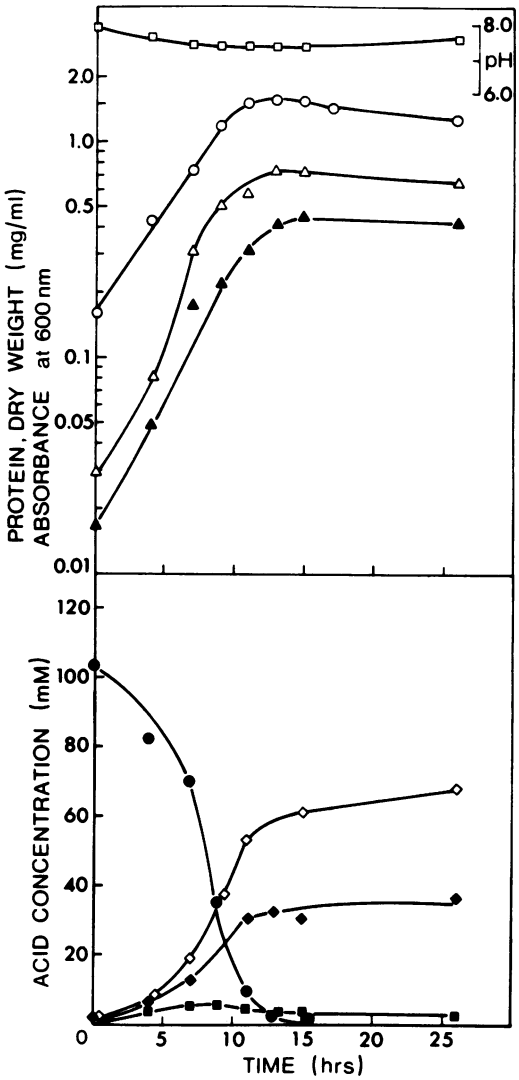

FIG. 2. *Formation of succinate and acetate during growth of C. formicoaceticum on fumarate without additional bicarbonate or formate. Cells were grown as described in Fig. 1. Determinations were done as described in Materials and Methods. Symbols: (○) absorbance at 600 nm, (△) dry weight, (▲) protein, (□) pH, (●) fumarate, (■) L-malate, (◆) acetate, (◇) succinate.*

shown in Table 1. Per 1 mol of fumarate fermented, about 6 g of dry cell mass was formed, whereas 8 g of dry cells was produced from 1 mol of L-malate.

The fermentation of fumarate and L-malate was also studied with nongrowing cells of *C. formicoaceticum*. Cells were harvested at the late exponential growth phase and suspended in anaerobic potassium phosphate buffer, pH 8.0, containing 1 mM DTE. Fumarate and L-malate were readily fermented by nongrowing cells of

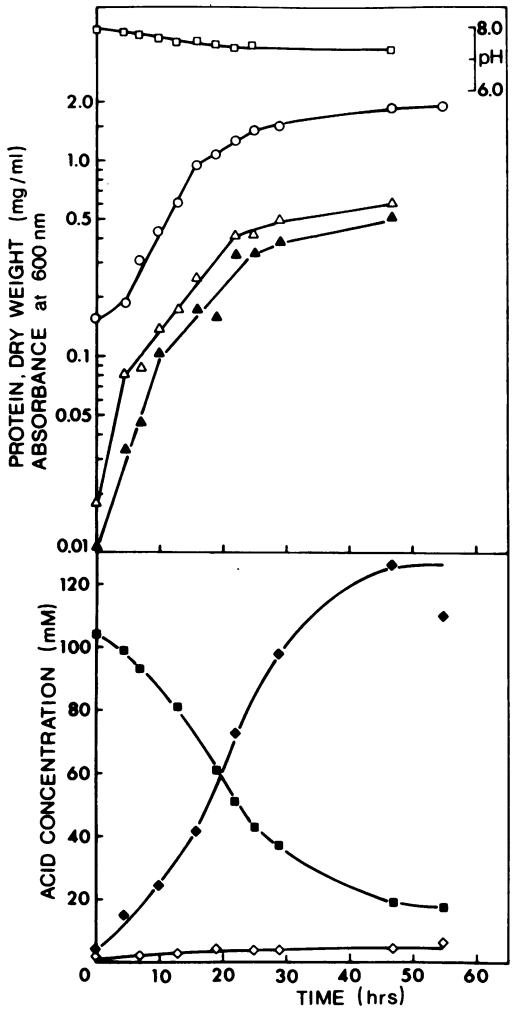

FIG. 3. *Formation of acetate during growth of C. formicoaceticum on L-malate without additional bicarbonate or formate. Symbols:* (○) *absorbance at 600 nm,* (△) *dry weight,* (▲) *protein,* (□) *pH,* (■) *L-malate,* (◆) *acetate,* (◇) *succinate.*

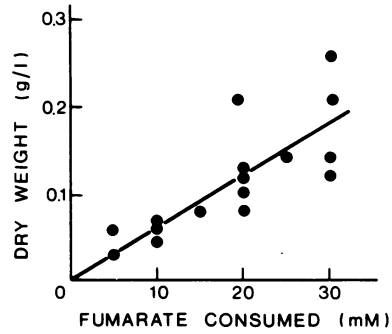

FIG. 4. *Determination of the molar growth yields of C. formicoaceticum. Cells were grown on fumarate without additional bicarbonate or formate (see Fig. 2). The increase of dry weight was plotted against the corresponding fumarate consumption. The concentration of L-malate transiently excreted was subtracted from the fumarate consumed; L-malate taken up again was added to the amount of substrate degraded.*

TABLE 1. *Molar growth yields of C. formicoaceticum with fumarate and L-malate as substrates*

Substrate	Y_m[a]		
	Without additions	With bicarbonate	With formate
Fumarate	6.0	6.0	5.0
L-Malate	8.0	7.0	8.0

[a] Grams of dry weight per mole of substrate. The yields were graphically determined as shown in Fig. 4.

C. formicoaceticum (Table 2). If neither bicarbonate nor formate was added to the fermentation buffer, 3 mol of fumarate converted to 2 mol of succinate, 1 mol of acetate, and 2 mol of CO_2 (Table 2, first column). The dismutation of fumarate to succinate, acetate, and CO_2 was confirmed by additional experiments with [1,4-^{14}C]fumarate (Table 3).

Fumarate fermentation by nongrowing cells of *C. formicoaceticum* was more variable in the presence of bicarbonate or formate than in its absence. In some experiments, fumarate dismutation was not affected by addition of bicarbonate (Table 2, fumarate with bicarbonate, experiment 1); in others, however, acetate formation from fumarate was increased at the expense of succinate production (Table 2, fumarate with bicarbonate, experiment 2). When formate was added, it could be metabolized via different pathways. Sometimes dismutation to CO_2 and acetate occurred (Table 2, fumarate with formate, experiment 1); in other experiments, however, formate was used as electron donor to fumarate, thus increasing the amount of succinate formed (Table 2, fumarate with formate, experiment 2). Since the assay conditions were always the same, these variations must have been connected with the physiological state of the cells used in the respective experiments.

In contrast to growing cells of *C. formicoaceticum* that produced very little succinate from L-malate (Fig. 3), nongrowing cells formed up to 1 mol of succinate per 2 mol of substrate fermented (Table 2, last column). The characteristic feature of the fermentation of L-malate was, however, the formation of large amounts of acetate. Formate that was readily utilized by L-malate-fermenting cells was partly oxidized to CO_2 and partly reduced to acetate, as indicated by the fermentation balance (Table 2, last column).

TABLE 2. *Fermentation of fumarate and L-malate by nongrowing cells of C. formicoaceticum*

Substrate	Formate used (mM)[a]	Products formed[a]			Available H recovered (%)[c]
		Succinate	Acetate	CO_2[b]	
Fumarate					
No additions	—[d]	66	33	66	99
With bicarbonate					
Expt 1	—	61	36	84	95
Expt 2	—	50	63	75	100
With formate					
Expt 1	85	69	45	119	97
Expt 2	92	80	30	112	98
L-Malate					
No additions					
Expt 1	—	15	122	99	99
Expt 2	—	35	89	83	100
With bicarbonate	—	37	85	82	100
With formate	218	45	130	178	102

[a] All concentrations were calculated for the consumption of 100 mM substrate. The fermentation buffer contained 120 mM fumarate or L-malate, respectively, and 120 mM bicarbonate or formate where indicated.

[b] Estimated from the amount of acetate and succinate formed assuming a carbon recovery of 100%

[c] Calculated according to the method of Barker (3).

[d] —, No formate added.

TABLE 3. *Fermentation of [1,4-^{14}C]fumarate by nongrowing cells of C. formicoaceticum*[a]

Product	Concn (mM)	Total cpm	% cpm	cpm/μmol
Fumarate consumed	100	872,000	100	4,360
Succinate formed	66	571,000	66	4,330
Acetate formed	32	37,000	4	580
CO_2 formed	65[b]	235,000	27	1,810

[a] Cells were grown on fumarate without additional bicarbonate. Experimental details are described in Materials and Methods.

[b] The concentration of CO_2 was calculated from the amounts of acetate and succinate formed.

When *C. formicoaceticum* was grown on medium containing 50 mM each fumarate and L-malate, the former seemed to be the preferred substrate (Fig. 5); fumarate was degraded before L-malate. Succinate was only formed during the consumption of fumarate, whereas the subsequent fermentation of L-malate yielded, predominantly, acetate (Fig. 5).

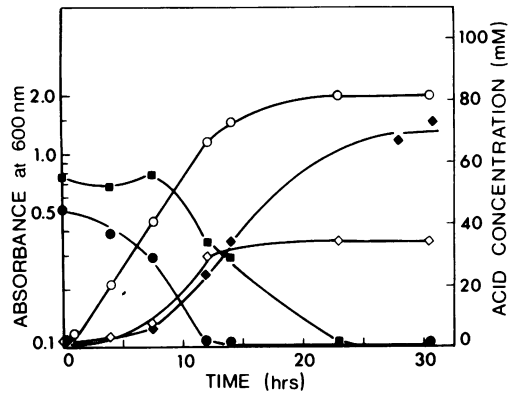

FIG. 5. *Growth of C. formicoaceticum on a medium containing 50 mM fumarate and 50 mM L-malate. The organism was grown anaerobically in a 1-liter flask. The inoculum was 20 ml of a culture grown with 100 mM L-malate. Symbols:* (○) *absorbance at 600 nm,* (●) *fumarate,* (■) *L-malate,* (◆) *acetate,* (◇) *succinate.*

DISCUSSION

Several bacteria such as *Escherichia coli* (28), *Desulfovibrio gigas* (25, 26), *Vibrio succinogenes* (34), and *Streptococcus faecalis* (5) are able to utilize fumarate or L-malate under anaerobic conditions. In most of these organisms, however, a second substrate is required, and the dicarboxylic acids serve as acceptors for electrons that arise from the oxidation of compounds such as formate, molecular hydrogen, glycerol, or glucose (6, 15, 16, 20, 28). Only a few bacteria are known that ferment fumarate without an additional substrate. *Enterobacter* (*Aerobacter*) *aerogenes* produces succinate, acetate, CO_2, ethanol, formate, and sometimes H_2 from fumarate and L-malate (3). *D. gigas* catalyzes the dismutation of fumarate and L-malate to acetate, CO_2, and succinate (12, 13, 25, 26).

Reports about dicarboxylic acid metabolism in clostridia are sparse. Woods and Clifton (35) observed that washed cells of *Clostridium tetanomorphum* fermented fumarate and L-malate. However, the fermentation end products were not identified. Growth of *C. tartarivorum* on fumarate and L-malate depends on the presence of acetate in the medium (23); *C. acetobutylicum* only grows on fumarate if the medium contains glycerol in addition (8).

The dismutation of fumarate to acetate, CO_2, and succinate, which had already been detected with *D. gigas* (26), was also observed with *C. formicoaceticum*. L-Malate, however, was almost completely fermented to acetate and CO_2 by this clostridium. Per 100 mol of L-malate consumed during growth, only about 5 mol of succinate was formed. Hence, the redox balance with L-malate was predominantly equalized by CO_2 reduction to acetate. This pathway also serves to compensate the redox balance during growth of *C. formicoaceticum* on fructose or pryuvate (1, 2, 19).

It is not known so far why *C. formicoaceticum* ferments fumarate and L-malate via different

pathways. *S. faecalis* is not able to oxidize fumarate to pyruvate since this organism lacks fumarase activity (5). However, high fumarase and fumarate reductase activities have been detected in cells of *C. formicoaceticum* grown either on fumarate or on L-malate (M. Dorn, Ph.D. Thesis, University of Göttingen, Göttingen, West Germany, 1976). One reason for the differences between fumarate and L-malate fermentation in this organism might be the location of fumarate reductase on the outside of the cytoplasmic membrane, which renders the enzyme inaccessible to intracellularly produced fumarate (Dorn, Ph.D. Thesis, University of Göttingen, 1976).

Formate may substitute for bicarbonate as an electron acceptor in fructose fermentation by *C. formicoaceticum* (2). When formate was present during the fermentation of fumarate, more succinate was produced than in the absence of formate. Consequently, formate must have been used as an electron donor to fumarate as it was observed to do with *E. coli* (16, 20), *V. succinogenes* (15, 34), *Proteus rettgeri* (17), and *Proteus mirabilis* (33). Since *C. formicoaceticum* obviously could not supply the fumarate-reducing system with fumarate during growth on L-malate, formate failed to increase the succinate production in these cells.

Growing cells of *C. formicoaceticum* fermented fumarate according to the equation:

$$3 \text{ fumarate} + 2H_2O \rightarrow 2 \text{ succinate} + 1 \text{ acetate} + 2 CO_2$$

ATP is produced during acetate formation from acetyl-phosphate by substrate-level phosphorylation. Hence, 0.33 mol of ATP per mol of fumarate was available for cell growth. L-Malate was almost completely fermented to acetate and CO_2 by growing cells of *C. formicoaceticum*:

$$3 \text{ L-malate} \rightarrow 0.16 \text{ succinate} + 4.2 \text{ acetate} + 2.9 CO_2 + 0.08H_2O$$

From 1 mol of L-malate nearly 1 mol of ATP is produced by substrate-level phosphorylation in the acetate kinase reaction. Although the ATP yields calculated from the fermentation equations for fumarate and L-malate differ by a factor of 3, the differences in the molar growth yields were small: 6 g of dry weight was formed per mol of fumarate and 8 g of dry weight per mol of L-malate. This apparent inconsistency might be explained as follows. During growth on fumarate, the cells of *C. formicoaceticum* might have to expend less energy on substrate transport than during growth on L-malate. The latter substrate was completely metabolized inside the cells. The portion of fumarate, however, that was reduced to succinate did not have to be transported into the cell because of the location of the fumarate reductase on the outer face of the cytoplasmic membrane (Dorn, Ph.D. Thesis, University of Göttingen, 1976). Furthermore, an electron-transport chain involved in succinate formation from fumarate might yield additional energy via electron-transport phosphorylation. Unusually high growth yields indicated electron-transport phosphorylation coupled to fumarate reduction in succinate- or propionate-forming bacteria such as *Selenomonas ruminantium* (14), *Propionibacterium freudenreichii* (7), *S. faecalis* (30), *Proteus rettgeri* (17), and *Bacteroides fragilis* (21). A *b*-type cytochrome and menaquinone, which have been detected in *C. formicoaceticum* (11), possibly function as electron carriers in an electron-transport system with fumarate as the terminal acceptor. In many organisms, cytochrome *b* and menaquinone are essential components of the fumarate-reducing electron-transfer chain (32).

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