Cell and ATP Yields of *Citrobacter freundii* Growing with Fumarate and H₂ or Formate in Continuous Culture

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Out of 19 strains belonging to the family *Enterobacteriaceae* only *Escherichia coli* and *Citrobacter* strains fermented fumarate exclusively to succinate. This fermentation was dependent on the presence of molecular hydrogen or formate. The inability of these micro-organisms to convert fumarate to succinate, acetate and CO₂ correlated with their lack, or low activity, of oxaloacetate decarboxylase.

Continuous culture experiments were performed with *Citrobacter freundii* in minimal or complex medium with fumarate + H₂ or formate, and the growth parameters were determined. From the data obtained, a $Y_{\text{fumarate dissimilated}}^{\text{max}}$ value of 10.5 ± 0.8 g dry wt per mol fumarate dissimilated was calculated. This value demonstrates that, per mol fumarate reduced, at least 0.6 ± 0.05 mol ATP is produced and subsequently used for biosynthetic purposes.

INTRODUCTION

It is well established that the reduction of fumarate, as catalysed by the membrane-bound fumarate reductase system, is coupled to energy conservation by electron transport phosphorylation (Thauer *et al.*, 1977; Kröger, 1978; Stouthamer, 1980). Evidence for this comes from *in vitro* studies, from growth yield studies, and also from the finding that some micro-organisms can grow at the expense of succinate formation from fumarate and molecular hydrogen or formate:

Such a fermentation can be carried out by *Vibrio succinogenes* (Wolin *et al.*, 1961) and also by *Escherichia coli* (Macy *et al.*, 1976). Since all the energy for maintenance and growth is derived from the fumarate reductase system, growth yield measurements in continuous culture and the application of the Pirt equation (Pirt, 1965) together with theoretical values calculated by Stouthamer (1973) allow the estimation of the ATP yield of the fumarate reductase reaction. For *E. coli* an ATP yield of 0.43 mol per mol fumarate reduced has been determined in this way (Bernhard & Gottschalk, 1978).

We were interested in testing other enterobacteria for their ability to carry out an H₂- or formate-dependent conversion of fumarate to succinate. Furthermore, we wanted to find a strain which from its growth rates on fumarate + H₂ would be more suitable than *E. coli* for an investigation in continuous culture. In this respect *Citrobacter freundii* seemed advantageous and was studied in detail.

METHODS

Micro-organisms and growth conditions. The strains listed in Table 1 were obtained from the German Collection of Microorganisms (DSM), Göttingen, F.R.G. The minimal medium for growth on L-malate, fumarate or citrate contained (g l⁻¹): KH₂PO₄, 0.45; K₂HPO₄, 0.45; NaCl, 0.9; CaCl₂.2H₂O, 0.02; MgCl₂.6H₂O, 0.02; MnCl₂.4H₂O, 0.01; CoCl₂.6H₂O, 0.01; (NH₄)₂SO₄, 0.9; and resazurin, 0.001. The amounts of SL-4 trace element solution (Pfennig & Lippert, 1966) and Na₂SeO₃.5H₂O present in the medium were 1 ml l⁻¹ and 0.26 µg l⁻¹, respectively. The pH was adjusted to 7.5. Final concentrations of the substrates were: sodium L-malate or

sodium fumarate, 60 mM; sodium formate, 70 mM; sodium citrate, 20 mM; and glucose, 20 mM. The complex medium contained, in addition to the components listed above, 0.1% (w/v) vitamin-free Casamino acids.

Anaerobic growth experiments were performed either in 16 ml Bellco tubes (Bellco Glass, Vineland, N.J., U.S.A.) containing 10 ml medium or in a Braun Biostat fermenter (Braun, Melsungen, F.R.G.) with a 1 l culture vessel. For experiments in Bellco tubes, the media were prepared according to Hungate (1969) under an N_2 atmosphere. When the effect of molecular hydrogen on growth was studied, 6 ml H_2 was injected into each tube, and the tubes were incubated horizontally on a shaker. Traces of O_2 were removed from N_2 and H_2 by passing them through a vertical Pyrex column packed with copper turnings and heated electrically to approximately 350 °C. For growth experiments in the Biostat fermenter, anaerobic conditions were established by gassing the medium with an O_2 -free N_2 stream (200 ml min⁻¹) after heat sterilization.

Continuous culture experiments were performed in a Braun Biostat fermenter with 1 l working volume, connected to a 20 l reservoir. The medium was prepared and filter-sterilized under N_2 . After passing O_2 -free N_2 through it for 5 h, solid $Na_2S_2O_4$ was added to give a final concentration of 35 mg l⁻¹. The reservoir was then connected to the fermenter which contained 1 l medium and which had been heat-sterilized. The inoculum was 50 ml of a culture which had been grown with fumarate + H_2 or formate for 24 h. The pH value of the culture medium was measured with a glass electrode (Ingold, Zürich, Switzerland); during growth it was maintained at pH 7.5 by autotitration with either 0.67 M- H_3PO_4 or 2 M-NaOH. The cultures growing on fumarate + H_2 were stirred at 100 rev. min⁻¹, and H_2 was passed through the medium at a rate of 500 ml min⁻¹. The cultures growing on fumarate + formate were stirred at 50 rev. min⁻¹. In order to avoid the continuous operation of the autotitrator because of formic acid consumption, the medium in the reservoir contained 10 mM-sodium formate + 60 mM-formic acid instead of 70 mM-sodium formate. Its final pH was 5.5. Such cultures were kept anaerobic by passing a stream of N_2 through the reservoir and the vessel at 50 ml h⁻¹.

The dilution rate was regulated by the inflow of fresh medium which was passed through stainless-steel spiral tubing kept at 80 °C to prevent bacterial growth from the culture vessel back into the medium reservoir. The cultures were frequently checked for contaminants using agar plates. For anaerobic growth, nutrient broth (Difco)/glucose plates and nutrient broth/citrate plates were incubated in an anaerobic GasPak vessel (Becton & Dickinson, Heidelberg, F.R.G.) under H_2/CO_2 . In addition, the cultures were checked aerobically on nutrient broth/glucose plates. After each run the maximum growth rate of the organisms was determined. This was done in batch culture by replacing 90% of the suspension in the vessel with fresh medium and monitoring growth.

Analytical methods. The dry weight of the cultures was determined according to Stouthamer (1969) using membrane filters (Sartorius, Göttingen, Germany). The turbidity of the samples at 600 nm was also determined. The ratio of dry weight to turbidity (0.54 g l⁻¹/A₆₀₀) was constant in all continuous culture experiments. In addition, no alterations of the protein content of the cells (0.43 g l⁻¹/A₆₀₀) were detectable.

Fumarate was determined by measuring the absorbance of the supernatant fluid of the cultures at 240 nm in a Zeiss PM4 spectrophotometer ($\epsilon = 2.45$ l mmol⁻¹ cm⁻¹). A 10 mM solution of sodium fumarate served as a standard. Acetate and succinate were determined according to Dorn *et al.* (1978a, b) and formate according to Lang & Lang (1972).

For the determination of oxaloacetate decarboxylase activity (EC 4.1.1.3) the cells were grown in 6 l carboys containing 1.5 l complex medium with 20 mM-glucose + 20 mM-sodium citrate. After 20 h growth under anaerobic conditions the cultures were harvested by centrifugation (20 min, 20000 g, 4 °C). The cells (approx. 2 g wet wt) were resuspended in 10 ml 50 mM-potassium phosphate buffer, pH 7.2 containing 3 mM-MgCl₂ and used immediately for extract preparation. The cell suspension, kept in ice until used, was passed quickly through a French press at a pressure of 78 MPa. Cell debris was then removed by centrifugation (30 min, 20000 g, 4 °C). The protein content of the cell extract was determined according to Beisenherz *et al.* (1953) with crystalline bovine serum albumin as a standard. Oxaloacetate decarboxylase activity was determined according to Herbert (1955). One unit of enzyme activity is defined as the amount catalysing the conversion of 1 μ mol substrate min⁻¹ at 25 °C.

RESULTS

Anaerobic growth on L-malate or citrate and oxaloacetate decarboxylase activity

A number of enterobacterial strains were tested for their ability to grow on L-malate alone or on L-malate + H_2 . In addition, growth on citrate, on citrate + glucose and the activity of oxaloacetate decarboxylase was determined. The latter was assayed because the inability of *E. coli* to grow on L-malate or citrate alone has been shown to be connected to the absence of this enzyme (Lütgens & Gottschalk, 1980). The data summarized in Table 1 indicate that three groups of organisms could be distinguished. *Escherichia coli* strain B and other *E. coli* strains (Macy *et al.*, 1976) and *Citrobacter* strains grew on L-malate only in the presence of H_2 ; these strains showed very low oxaloacetate decarboxylase activity. The second group comprised strains with

Table 1. Growth of various enterobacterial strains on L-malate, L-malate + H₂, citrate and citrate + glucose and their oxaloacetate decarboxylase activity

Growth experiments were performed in Belco tubes containing 10 ml medium (see Methods). The values given represent the turbidity of the cultures in the stationary phase and are the mean values of triplicate experiments. When the effect of molecular hydrogen was tested, tubes containing in addition 6 ml H₂ were incubated horizontally on a shaker.

Micro-organism	Final turbidity at 600 nm				Oxaloacetate decarboxylase activity* [units (g protein) ⁻¹]
	L-Malate	L-Malate + H ₂	Citrate	Citrate + glucose	
<i>Escherichia coli</i> B DSM 500	—	0.26	—	1.5	<5
<i>Citrobacter freundii</i> DSM 30039	—	0.34	0.16	1.5	<5
<i>Citrobacter</i> sp. DSM 30045	—	0.35	—	1.17	<5
<i>Citrobacter</i> sp. DSM 30046	0.12	0.25	—	1.84	<5
<i>Enterobacter cloacae</i> DSM 30056	0.33	0.35	0.61	1.48	193
<i>Erwinia herbicola</i> DSM 30076	0.22	—	0.37	1.13	39
<i>Klebsiella</i> sp. DSM 30108	0.39	0.51	0.60	1.31	108
<i>Enterobacter aerogenes</i> DSM 30053	—	—	0.57	1.38	137
<i>Enterobacter cloacae</i> DSM 30061	—	—	0.65	1.46	69
<i>Erwinia herbicola</i> DSM 30075	—	—	0.62	1.28	81
<i>Hafnia alvei</i> DSM 30101	—	—	—	1.58	16
<i>Proteus mirabilis</i> DSM 30116	—	—	—	—	65
<i>Serratia marcescens</i> DSM 30121	—	—	0.32	0.96	26

—, Final turbidity below 0.004.

* Determined in cells which had been grown in a citrate + glucose medium except strain DSM 30116 which had been grown in a medium containing in addition 0.09% peptone.

high oxaloacetate decarboxylase activities and the ability to grow on L-malate or citrate in the absence of co-substrates. A third group of strains could not grow on L-malate at all in a minimal medium: some of these strains utilized citrate, however, and exhibited high oxaloacetate decarboxylase activities. Nevertheless, among the L-malate utilizers there was a correlation between the ability to form oxaloacetate decarboxylase and the ability to grow on L-malate or citrate alone. Since in addition to *E. coli* only *Citrobacter* strains showed H₂-dependent growth on L-malate, one of these strains, *Citrobacter freundii* DSM 30039, was chosen for further studies. In batch culture it grew reproducibly faster on fumarate + H₂ ($\mu_{\max} = 0.31 \text{ h}^{-1}$) than did *E. coli* ($\mu_{\max} = 0.17 \text{ h}^{-1}$) (Bernhard, 1978) and it appeared to be suitable for continuous culture studies.

Determination of $Y_{\text{fumarate}}^{\max}$ in continuous culture

Citrobacter freundii was grown anaerobically in continuous culture with fumarate as the growth-limiting substrate and H₂ or formate as the hydrogen donor. Limitation by fumarate was confirmed by the finding that when the concentration of all other components of the medium was doubled, no deviations in growth or substrate consumption were observed. The fumarate concentration in the reservoir was 60 mM, and the concentration of fumarate in the outflow was 0.5–1 mM. The growth yields at various growth rates were calculated using the equation $Y_{\text{fumarate}} = x/S_r - S$, where x is the dry weight of the cells (g l⁻¹), S_r is the substrate concentration (mol l⁻¹) of the inflowing medium and S is the substrate concentration (mol l⁻¹) of the outflowing medium. The growth yields at different growth rates were ascertained at steady state, under which conditions the turbidity varied only $\pm 1\%$ around a mean value. For the determination of Y_{fumarate} at a certain growth rate, six to eight measurements of the dry weight, and the substrate and product concentrations in the outflowing medium were performed under steady state conditions.

Four continuous cultures were run using fumarate + H₂ or formate in either minimal or complex medium. Steady state conditions were allowed to become established at five different growth rates and the necessary measurements were made. Double reciprocal plots of the data according to Pirt (1965) are shown in Fig. 1. On the basis of the equation $1/Y_{\text{fumarate}} = m_s(1/\mu) + (1/Y_{\text{fumarate}}^{\max})$, these plots allowed the determination of the maximal growth yields $Y_{\text{fumarate}}^{\max}$ and of

Fig. 1. Double reciprocal plots of molar growth yields against growth rates of *C. freundii* grown on fumarate + H₂ or formate in either minimal medium (○) or complex medium (●). The straight lines were calculated by the method of least squares. (a) The medium contained 60 mM-sodium fumarate and H₂ (500 ml min⁻¹) was passed through the cultures. (b) The medium contained 60 mM-sodium fumarate + 70 mM-sodium formate and N₂ (200 ml min⁻¹) was passed through the cultures.

Table 2. *Molar growth yields and ATP yields of C. freundii growing anaerobically on fumarate + H₂ or formate*

$Y_{\text{fumarate}}^{\text{max}}$ is expressed as g dry wt (mol fumarate consumed)⁻¹; m_{fumarate} as mmol fumarate (g dry wt)⁻¹ h⁻¹; $Y_{\text{fumarate diss.}}^{\text{max}}$ as g dry wt (mol fumarate dissimilated)⁻¹; $(Y_{\text{ATP}}^{\text{max}})_{\text{fumarate}}^{\text{theor.}}$ as g dry wt from fumarate (mol ATP)⁻¹; $q_{\text{ATP, biosyn.}}$ as mol ATP consumed in biosynthesis (mol fumarate dissimilated)⁻¹.

Culture medium	$Y_{\text{fumarate}}^{\text{max}}$	m_{fumarate}	$Y_{\text{fumarate diss.}}^{\text{max}}$	$(Y_{\text{ATP}}^{\text{max}})_{\text{fumarate}}^{\text{theor.}}$	$q_{\text{ATP, biosyn.}}$
Fumarate + H ₂ , minimal medium	9.49	9.3	9.96	15.4	0.65
Fumarate + H ₂ , complex medium	10.64	4.6	11.17	17.6	0.63
Fumarate + formate, minimal medium	9.26	9.9	9.72	15.4	0.63
Fumarate + formate, complex medium	9.52	7.6	10.0	17.6	0.57

Table 3. *Products formed in continuous cultures of C. freundii on fumarate + H₂ or formate*

Product	Amount formed [mol (mol fumarate consumed) ⁻¹]
Succinate	92 ± 0.2
Acetate	2.5 ± 0.2
Formate	1.75 ± 0.2
Fumarate dissimilated	94.5 ± 0.2

the maintenance coefficients. The data obtained are summarized in Table 2. The maintenance coefficient was higher when formate instead of H₂ served as the hydrogen donor. For both hydrogen donors it was lower in complex medium than in minimal medium. The $Y_{\text{fumarate}}^{\text{max}}$ varied between 9.26 and 10.64 g per mol. Before these values could be related to ATP production in the fumarate reaction, the fumarate consumed had to be corrected for the amount of fumarate assimilated. This was done by comparing the amounts of fumarate consumed and products formed (Table 3). The values obtained for succinate, acetate and formate indicated that from

100 mol fumarate consumed 5.5 mol fumarate was assimilated. There was no significant difference between the amounts of products formed in minimal medium and in complex medium.

The sum of succinate and acetate produced was taken as the total fumarate consumed. Since acetate formation was minor and since its formation is also coupled to the phosphorylation of ADP, it is not considered separately in the discussion of the energetics of this process. The correction of the $Y_{\text{fumarate}}^{\text{max}}$ values for the portion of fumarate that was assimilated led to the $Y_{\text{fumarate diss.}}^{\text{max}}$ values in Table 2. Comparison of these values with the amount of cells which can theoretically be made from fumarate at the expense of 1 mol ATP [$(Y_{\text{ATP/fumarate}}^{\text{max}})^{\text{theor.}}$] showed that the cell mass formed accounted for 0.57–0.65 mol ATP produced in the reduction of 1 mol fumarate to succinate.

DISCUSSION

Recently, it has been shown that *E. coli* utilizes citrate under anaerobic conditions in the presence of glucose (Lütgens & Gottschalk, 1980). Citrate alone is not utilized; this has been explained by the absence of detectable amounts of oxaloacetate decarboxylase in *E. coli*. The lack of this enzyme apparently accounts also for the finding that fumarate or L-malate can be converted by *E. coli* only to succinate, not to acetate and CO_2 . Therefore, the fermentation of these C_4 dicarboxylic acids requires the simultaneous utilization of hydrogen donors, such as glycerol, molecular hydrogen or formate (Miki & Lin, 1975; Macy *et al.*, 1976; Guest, 1979). These correlations also apply to *C. freundii* and other *Citrobacter* strains. They lack oxaloacetate decarboxylase activity and are unable to grow on citrate or fumarate alone. Other enterobacterial strains such as *Klebsiella* sp. possess oxaloacetate decarboxylase and can grow on citrate or fumarate without additional substrates.

The dismutation of fumarate by *Enterobacter aerogenes* to yield acetate, CO_2 , formate and succinate was first described by Barker (1936). An analogous fermentation is carried out by *Clostridium formicoaceticum* (Dorn *et al.*, 1978a, b). In *Streptococcus faecalis*, L-malic enzyme is induced during growth on L-malate and the substrate is converted to acetate and ethanol (London & Meyer, 1969). *Proteus rettgeri* and *Campylobacter* sp. display an alternative pathway for the fermentation of fumarate or L-aspartate. They oxidize part of the C_4 dicarboxylic acid via citrate and 2-oxoglutarate to succinate, thus generating reducing power for the reduction of fumarate to succinate (Kröger, 1974; Laanbroek *et al.*, 1978).

That the reduction of fumarate to succinate is coupled to ATP synthesis from ADP and inorganic phosphate has been demonstrated by *in vivo* and *in vitro* studies (see Barton *et al.*, 1970; Kröger, 1978; Thauer *et al.*, 1977; Gottschalk & Andreessen, 1979). The translocation of two protons per one molecule of fumarate reduced has been observed with spheroplasts of *E. coli* (Miki & Wilson, 1978; Jones, 1980). The transmembrane electrochemical potential gradient of protons generated by the fumarate reductase system amounts to -100 mV (Hellingwerf *et al.*, 1981).

Recently, ATP synthesis by membrane vesicles of *Vibrio succinogenes* has been measured and evidence has been obtained that 1 mol ATP is formed per mol fumarate reduced (Kröger & Winkler, 1981). On the basis of this value and the value for $q_{\text{ATP, biosyn.}}$ (0.6 mol ATP per mol fumarate) determined for *C. freundii* in continuous culture, it can be concluded that 60% of the ATP produced in the fumarate reductase system is used in biosynthetic processes. What about the remaining 40%? Their expenditure must be independent of the growth rate. Energy will be required for the transport of macromolecules into or through the cytoplasmic membrane. One also has to consider that the activity of certain enzymes depends on an energized state of the cytoplasmic membrane. Thus, there is energy expenditure in growing cells which has not been taken into account in the calculation of $Y_{\text{ATP}}^{\text{max}}$ values (Stouthamer, 1973, 1977). On the other hand, it is unlikely that the synthesis of cell material from a carbon source and ATP proceeds with an efficiency of 100%. A value around 60%, in agreement with the results of this study, is more reasonable and is supported by the results of other investigations. Farmer & Jones (1976) determined a value for $(Y_{\text{ATP}}^{\text{max}})_{\text{exp.}}$ of 10.1 g dry wt per mol ATP for *E. coli* growing aerobically on fumarate. This value corresponds well with the $Y_{\text{fumarate diss.}}^{\text{max}}$ value found in this study. Values in

the same range were also found for *Campylobacter* sp. growing on aspartate (Laanbroek & Veldkamp, 1979).

In earlier continuous cultures studies with *E. coli* a q_{ATP} value of 0.43 mol ATP used in biosynthesis per mol fumarate reduced was determined (Bernhard & Gottschalk, 1978). This value was not corrected for fumarate assimilated. Even after correction (increase by approximately 0.05) it is somewhat lower than the value for *C. freundii*. Thus, the efficiency of ATP utilization in *C. freundii* seems to be greater than in *E. coli* when these organisms are grown with fumarate + H_2 or formate.

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