Mixotrophic Capabilities of *Alcaligenes eutrophus*

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The facultatively chemolithoautotrophic hydrogen bacterium *Alcaligenes eutrophus* was able to utilize organic and inorganic substrates concomitantly, i.e. to grow mixotrophically. The mixotrophic capabilities were investigated under succinate-limited growth of *A. eutrophus* with molecular hydrogen in a gas atmosphere devoid of carbon dioxide. At a dilution rate (D) of 0.2 h^{-1} , the mixotrophic cellular yield was increased by 135% over the heterotrophic yield with succinate alone. Total carbon analysis revealed that under these conditions 95% of the succinate carbon was converted to cell carbon. The mixotrophic yield decreased only slightly at dilution rates lower than 0.2 h^{-1} but significantly at higher dilution rates and was only 18% above the heterotrophic yield at $D = 0.32\text{ h}^{-1}$. Unlike other facultative chemoautotrophs, mixotrophic growth of *A. eutrophus* required both H_2 oxidation (Hox) and autotrophic CO_2 fixation (Cfx), as evident from mutants defective in either H_2 oxidation (Hox^-) or autotrophic metabolism (Cfx^-), as well as from incorporation studies of radioactive substrates. The cellular yield of a Cfx^- mutant, HF17, increased only slightly (by 14%) upon the addition of H_2 , indicating that the ability of *A. eutrophus* to change the metabolism of a heterotrophic substrate was limited. Hox^- mutants did not increase their cellular yield under identical growth conditions.

INTRODUCTION

Among the aerobic chemolithoautotrophic bacteria only the H_2 -oxidizing bacteria are generally facultative chemolithoautotrophs. These bacteria grow on a variety of organic compounds as carbon and energy source, as well as with H_2 as electron donor and CO_2 as carbon source.

H_2 is activated by hydrogenases and CO_2 is assimilated autotrophically via the Calvin cycle with the initial reactions of phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase (RuBPCase). The majority of the so-called thiobacilli are obligate chemolithoautotrophs (Rittenberg, 1969). However, the nitrifying bacteria have been reported to utilize organic substrates (Steinmüller & Bock, 1976). The concomitant utilization of organic and inorganic substrates as carbon and energy sources has been designated as mixotrophy. This type of metabolism is probably of major ecological significance for the facultative chemolithotrophic bacteria since both substrates are present in their natural environment.

Mixotrophic growth is judged from the increase in cellular yield as compared to growth on one substrate alone. This can be achieved by two different metabolic routes. First, the chemolithotrophic metabolism, rather than the dissimilation of a carbon source, is used to satisfy the energy needs of the bacterium and the carbon source is assimilated instead. Secondly, mixotrophy involves autotrophic CO_2 fixation for which the inorganic electron donor supplies the necessary energy, while the heterotrophic metabolism of the organic substrate remains largely unchanged.

Mixotrophy has been observed in a number of hydrogen bacteria and some thiobacilli, as reviewed by Bowien & Schlegel (1981) and Matin (1978). For *Thiobacillus* A2, the carbon

Abbreviation: RuBPCase, ribulose-1,5-bisphosphate carboxylase.

monoxide oxidizing bacterium *Pseudomonas carboxydoflava*, and the hydrogen bacterium *Aquaspirillum autotrophicum* (Fasnacht & Aragno, 1982) evidence was presented that mixotrophy does not involve autotrophic CO₂ fixation (Smith *et al.*, 1980; Kiessling & Meyer, 1982).

Mixotrophic growth has been reported for *Alcaligenes eutrophus* when pre-cultivated under autotrophic growth conditions (Rittenberg & Goodman, 1969). However, when cells were pre-cultivated on a heterotrophic substrate like pyruvate, a biphasic growth was observed (Stukus & DeCicco, 1970) suggesting subsequent autotrophic growth. With pyruvate the formation of the key enzymes of autotrophic metabolism is repressed in *A. eutrophus* (Stukus & DeCicco, 1970; Friedrich *et al.*, 1981). However, their formation is derepressed during carbon-limited heterotrophic growth in a chemostat (Friedrich, 1982). We therefore used the continuous-culture technique to quantify the mixotrophic capabilities of *A. eutrophus*. We used radioactive substrates and mutants defective in either H₂ oxidation or autotrophic CO₂ fixation to determine the involvement of the Calvin cycle in the mixotrophy in this strain. Here we report that *A. eutrophus* dissimilates the organic carbon source under mixotrophic growth conditions in the same way as it does during heterotrophic growth. With H₂ as additional energy source, the CO₂ derived from the dissimilation is completely refixed via the Calvin cycle.

METHODS

Bacterial strains. *Alcaligenes eutrophus* strain H16 (ATCC 17699; DSM 428) and mutants deficient in hydrogenase activity (Friedrich *et al.*, 1982) or deficient in autotrophic CO₂ fixation (Friedrich *et al.*, 1979) were used.

Media and growth conditions. The media for seed cultures and continuous cultivation were identical to those described by Friedrich (1982), with the exception that the concentration of succinic acid was 2.0 g l⁻¹ and antifoam was used (polypropylene glycol, 50 mg l⁻¹). For heterotrophic and mixotrophic growth a 2 l fermenter (B. Braun, Melsungen, FRG) with pH control was used as previously described (Friedrich, 1982). For heterotrophic growth the culture was aerated (300 ml min⁻¹) by precision piston pumps with artificial air [N₂/O₂ (80:20, v/v)] devoid of CO₂. Mixotrophic growth conditions were given by a gas atmosphere of N₂/O₂/H₂ (60:20:20, by vol.). The dilution rate was 0.05 h⁻¹ unless otherwise stated.

The steady state of a culture had been reached when OD₄₃₆ and the enzyme activities remained constant with time. This required usually three to five volume changes. The strain characteristics of the mutants were examined frequently and found to be constant throughout the duration of the continuous culture.

Dry cell weight and total carbon analysis. Dry cell weight was determined by filtration of 10 ml of medium; cells were washed once with 0.9% (w/v) NaCl and dried overnight at 90 °C. Total organic carbon was determined from culture fluids, supernatants and samples of washed cell suspensions. Organic carbon was oxidized to CO₂ and quantified by an infrared analyser, model 915-B (Beckman Instruments).

Radioactive tracer studies. Cells were grown in batch cultures (20 ml) with isoleucine (0.1%, w/v) under an atmosphere of artificial air [N₂/O₂ (80:20, v/v)] or where N₂ was replaced by H₂. At early exponential growth phase 1 µCi (37 kBq) L-[U-¹⁴C]isoleucine was added and the incorporation of radioactive carbon into the cells was followed. Samples (1 ml) were acidified with 0.1 ml 3.3 M-H₃PO₄, washed once with 1 M-H₃PO₄, resuspended in 50 mM-potassium phosphate buffer pH 7.0 and the radioactivity of a sample was determined.

Enzyme assays. Enzyme activities were measured at 30 °C. Soluble hydrogenase (EC 1.12.1.2) either from whole cells or cell-free extracts was determined by spectrophotometric analysis of NADH as previously described (Friedrich *et al.*, 1982). Membrane-bound hydrogenase was determined from washed membranes as described by Friedrich *et al.* (1979). RuBPCase (EC 4.1.1.39) was determined from whole cells according to Leadbeater *et al.* (1982). One unit (U) of enzyme activity is defined as 1 µmol substrate transformed or product formed min⁻¹. Protein was determined by the Lowry method.

Chemicals. NAD was purchased from Boehringer. Antifoam P-2000 was from Fluka AG (Buchs, Switzerland). Ribulose-1,5-bisphosphate was obtained from G. Whitesides, Harvard University (Cambridge, Mass., USA). L-[U-¹⁴C]isoleucine was purchased from Amersham Buchler (Braunschweig, FRG). All gases were of purissime grade and from Messer Griesheim (Kassel, FRG). Other chemicals were of analytical grade and purchased from Merck.

RESULTS

Yield studies

For determination of the cellular yields (*Y_s*) *A. eutrophus* strain H16 and mutants defective in autotrophic or chemolithotrophic metabolism were grown in a succinate-limited chemostat at a

Fig. 1. Cell mass and enzyme activities of *A. eutrophus* during transition from heterotrophic to mixotrophic growth in a succinate-limited chemostat. The culture conditions and gas atmospheres were as described in Methods. The dilution rate was 0.05 h^{-1} . Arrows indicate the addition or removal of 20% (v/v) H_2 to the gas atmosphere; the changes in gas composition were compensated with N_2 . $\circ$, Optical density at 436 nm; $\bullet$, specific activity of soluble hydrogenase; $\square$, specific activity of RuBPCase.

Table 1. Yields of *Alcaligenes eutrophus* during succinate-limited heterotrophic and mixotrophic growth in a chemostat

The yields were determined from at least five samples withdrawn at various times during the steady state, all giving identical results. Mixotrophic growth conditions were produced in the succinate-limited chemostats by the addition of 20% (v/v) H_2 to the gas phase.

Strain	Relevant phenotype	D (h^{-1})	Cellular yield [g dry wt (g succinic acid) $^{-1}$]	
			$-\text{H}_2$	$+\text{H}_2$
H16	Hox $^{+*}$	0.050	0.35	0.70
HF08	Hop $^{-\dagger}$	0.051	0.33	0.70
HF14	Hos $^{-\ddagger}$	0.053	0.34	0.71
HF18	Hox $^{-}$	0.055	0.36	0.35
HF17	Cfx $^{-\S}$	0.050	0.35	0.40

* Ability to oxidize H_2 .

$\dagger$ Inability to form particulate hydrogenase activity.

$\ddagger$ Inability to form soluble hydrogenase activity.

$\S$ Inability to fix CO_2 via the Calvin cycle.

dilution rate (D) of about 0.05 h^{-1} . During steady state the cellular yield of the wild-type strain H16 was $0.35 \text{ g dry wt (g succinic acid)}^{-1}$.

When mixotrophic growth conditions were produced by the addition of 20% (v/v) H_2 to the gas phase, the Y_s increased by 100% to $0.70 \text{ g dry wt (g succinic acid)}^{-1}$ (Table 1). Identical yields during heterotrophic and mixotrophic growth were observed with mutant strain HF08, deficient in the particulate hydrogenase (Hop $^{-}$), or strain HF14, deficient in the soluble hydrogenase (Hos $^{-}$) activity. As expected, the yield of strain HF18, unable to oxidase H_2 , was identical under heterotrophic and mixotrophic growth conditions. A slight but distinct yield increase of 14% was observed with strain HF17, unable to fix CO_2 via the Calvin cycle (Table 1). The protein content of the strains investigated was identical during heterotrophic or mixotrophic growth and was about $0.7 \text{ g protein (g dry wt)}^{-1}$.

To correlate the cellular yields with the yield of the assimilated carbon (Y_c) the total organic carbon of washed cells was determined. The elemental analysis of the wild type and mutants revealed that the carbon content was about 47% of the dry cell weight from cells grown either

heterotrophically or mixotrophically. The Y_c values paralleled those obtained for the cellular yield Y_s (data not shown). Besides the yields the growth characteristics during transition from heterotrophic to mixotrophic steady state were determined. Upon the addition of hydrogen to the chemostat the cell concentration of strain H16 increased exponentially with a transitional growth rate (μ_t) of 0.047 h^{-1} (Fig. 1). During steady state in a chemostat the dilution rate, D , is equivalent to the specific growth rate, μ . Therefore, during transition from heterotrophic to mixotrophic steady state the overall growth rate (μ) is given by the dilution rate and the transitional growth rate μ_t (equation 1):

$$\mu = D + \mu_t \quad (1)$$

Since for strain H16 the dilution rate was 0.050 h^{-1} (Table 1), the overall growth rate was 0.097 h^{-1} . An identical growth rate (0.098 h^{-1}) was determined with strain HF08 (Hop^-). However, with strain HF14, lacking the soluble hydrogenase activity, a substantially lower rate of 0.074 h^{-1} was observed. Strain HF17 (Cfx^-) grew with an overall rate of 0.058 h^{-1} .

Mixotrophic enzyme activities

The above results suggested the involvement of autotrophic CO_2 fixation as well as H_2 oxidation during mixotrophy. Therefore, the activities of the key enzymes of autotrophic metabolism were determined from cells grown under both conditions.

During succinate-limited heterotrophic growth at a dilution rate of 0.05 h^{-1} *A. eutrophus* formed both the soluble and the particulate hydrogenase at highly derepressed activities of 1.09 and $0.35 \text{ U (mg protein)}^{-1}$, respectively. RuBPCase activity was low [$0.014 \text{ U (mg protein)}^{-1}$] (Table 2). These results confirmed those of Friedrich (1982). Upon the addition of H_2 the activity of the soluble hydrogenase decreased dramatically with first order kinetics and a half life of 1.5 h (Fig. 1) in the wild type and similarly in the Hop^- strain HF08 and the Cfx^- strain HF17. The drop in activity was due to *in vivo* inactivation, as described by Schlesier & Friedrich (1981). The activity of the particulate hydrogenase remained high under both growth conditions in the parent and in the mutant strains able to form particulate hydrogenase.

A 20-fold increase in RuBPCase activity during mixotrophic growth was observed in the wild type (Fig. 1), and the Hop^- and Hos^- strains HF08 and HF14, respectively (Table 2). In these strains RuBPCase was formed in about tenfold excess over the catalytic needs of CO_2 -fixing activity (Cfa) of the CO_2 derived from succinate. This was calculated according to equation (2):

$$\text{Cfa} = \frac{D/60 \text{ } \mu\text{mol C l}^{-1}}{\text{mg protein l}^{-1}} \quad (2)$$

In the Hox^- strain HF18, RuBPCase activity remained low during mixotrophic growth.

Radioactive labelling studies

The inability of the Cfx^- mutant HF17 to increase the yield equivalent to the parent under mixotrophic growth conditions with succinate plus H_2 strongly suggested the involvement of autotrophic CO_2 fixation in mixotrophy in the parent. This line of evidence was further investigated. With succinate, *A. eutrophus* does not form the key enzymes of autotrophic metabolism in batch culture, while these enzymes are well expressed with isoleucine as carbon and energy source (Friedrich & Friedrich, 1983). Therefore, uniformly labelled [^{14}C]isoleucine was used to determine the amount of carbon incorporated into cell mass (as described in Methods). During heterotrophic growth, about 35% of the initial radioactive carbon was incorporated by the wild type but 67% was incorporated under mixotrophic conditions with H_2 . The addition of 3 mM unlabelled bicarbonate decreased the incorporation of the radioactivity to 48% during mixotrophy. Strain HF17 (Cfx^-) did not increase the yield or incorporation of radioactive carbon with isoleucine under mixotrophic growth conditions in batch culture (data not shown).

Table 2. Enzyme activities of autotrophic metabolism during succinate-limited heterotrophic and mixotrophic growth of *A. eutrophus* in a chemostat

The growth conditions with respect to substrate concentration, gas atmosphere, and dilution rate were identical to those of Table 1. Hydrogenase activities were determined in extracts as described in Methods.

		Specific activity [U (mg protein) ⁻¹]					
		Hydrogenase					
		Soluble		Particulate		RuBPCase	
Strain	Relevant phenotype*	- H ₂	+ H ₂	- H ₂	+ H ₂	- H ₂	+ H ₂
H16	Hox ⁺	1.09	0.09	0.35	0.33	0.014	0.254
HF08	Hop ⁻	1.49	0.15	0	0	0.012	0.237
HF14	Hos ⁻	0	0	0.59	0.53	0.014	0.261
HF18	Hox ⁻	0	0	0	0	0.005	0.004
HF17	Cfx ⁻	1.35	0	0.35	0.35	0	0

* The phenotype designation is given in the text and Table 1.

Growth rate dependent mixotrophic metabolism

The yield increase during mixotrophy is determined by the rate of substrate conversion in autotrophic metabolism. This rate depends on two factors: (i) the activities of the enzymes involved, and (ii) the availability of the autotrophic substrates. The mixotrophic capability of *A. eutrophus* was determined by changing the dilution rate in a chemostat. The activities of the key enzymes of autotrophic metabolism as well as the yields were determined from the various steady states.

During heterotrophic growth under succinate limitation the concentration of cell mass (X) of *A. eutrophus* was about 0.7 g dry wt l⁻¹ at a dilution rate of 0.065 h⁻¹, increasing slightly to 0.73 g dry wt l⁻¹ at $D = 0.4$ h⁻¹ (Fig. 2a). Consequently the productivity (P) of the culture given by the product of the cell concentration, X , and the dilution rate, D (see equation 3), increased linearly with increasing dilution rate (Fig. 2b).

$$P \left(\frac{\text{g dry wt}}{\text{l.h}} \right) = X \left(\frac{\text{g dry wt}}{\text{l}} \right) \cdot D \text{ (h}^{-1}\text{)} \quad (3)$$

Under succinate-limited mixotrophic growth with H₂ in the gas atmosphere the cell concentration increased by 100% to 1.4 g dry wt l⁻¹ at a dilution rate of 0.048 h⁻¹ (Fig. 2). This was equivalent to a cellular yield of 0.7 g dry wt (g succinic acid)⁻¹ (Table 1). When the dilution rate was increased to 0.12 h⁻¹ the cell concentration increase was 135% to 1.65 g dry wt l⁻¹. At faster dilution rates the steady state cell concentrations decreased gradually to 0.76 g dry wt l⁻¹ at a dilution rate of 0.4 h⁻¹.

The fraction of the autotrophic metabolism contributing to mixotrophic growth at the various dilution rates can be deduced from these data by the differences in cell concentration (X) or the productivities (P) of mixotrophic and heterotrophic growth (Fig. 2a, b; broken line) according to equation (4):

$$P_{\text{autotrophic}} = P_{\text{mixotrophic}} - P_{\text{heterotrophic}} \quad (4)$$

Under the given growth conditions with 2 g succinic acid l⁻¹ in the feed medium, the productivity of the autotrophic metabolism exhibited a maximum of about 0.15 g dry wt l⁻¹ h⁻¹ at a dilution rate of 0.155 h⁻¹ (Fig. 2b). At this and lower dilution rates the autotrophic metabolism contributed up to 66% of the overall mixotrophic yield and consequently its productivity. Above $D = 0.2$ h⁻¹ the fraction of the autotrophic metabolism declined but was still 18% of the total productivity at $D = 0.32$ h⁻¹.

Fig. 2. Heterotrophic and mixotrophic cellular growth, productivities and enzyme activities of *A. eutrophus* grown in a succinate-limited chemostat. The culture conditions and gas atmospheres were as described in Methods. (a) Optical density of mixotrophic ($\circ$) and heterotrophic growth ($\bullet$); (b) productivity of mixotrophic ($\circ$) and heterotrophic ($\bullet$) growth; (c) specific activities of RuBPCase ($\bullet$) and soluble hydrogenase ($\circ$) under mixotrophic growth conditions. The broken lines indicate the difference between mixotrophic and heterotrophic growth (a) and productivity (b).

To estimate whether or not the activities of the key enzymes of autotrophic metabolism caused the restriction in yield and productivity during mixotrophic growth at the higher dilution rates, the specific activities of soluble hydrogenase and RuBPCase were determined. The maximum specific activity of soluble hydrogenase during mixotrophic growth was $0.18 \text{ U (mg protein)}^{-1}$ at a dilution rate of 0.085 h^{-1} (Fig. 2c). At this dilution rate the hydrogenase activity was only 10 to 20% of that found during heterotrophic growth. At higher dilution rates the specific activity declined drastically and was hardly detectable above $D = 0.3 \text{ h}^{-1}$. The specific activity of RuBPCase appeared at a maximum of about $0.14 \text{ U (mg protein)}^{-1}$ at $D = 0.15 \text{ h}^{-1}$. With increasing dilution rate the specific activity decreased almost continuously to $0.04 \text{ U (mg protein)}^{-1}$ at $D = 0.4 \text{ h}^{-1}$ (Fig. 1c).

DISCUSSION

The addition of H_2 to *A. eutrophus* growing under carbon limitation in a succinate-limited chemostat resulted in mixotrophic growth. The yield increase with succinate plus H_2 of 135% over the heterotrophic yield suggested that 95% of the carbon deriving from succinate was converted to cell carbon under optimal growth conditions. This evidence was supported by the data of total carbon analysis from cells grown under both conditions. Such an enormous yield increase has not been found for the mixotrophic capacities of other chemolithotrophic bacteria grown either in batch or chemostat culture. In batch culture the carbon monoxide oxidizing bacterium *Pseudomonas carboxydoflava* increased the yield by 7% and 35% growing on pyruvate plus CO or H_2 , respectively.

Thiobacillus A2 growing in an acetate-limited chemostat plus thiosulphate as additional energy source increased the yield by 55% compared to growth on acetate alone and about 76% of

the acetate carbon was assimilated (Gottschal & Kuenen, 1980). When *Thiobacillus* A2 grew on a substrate mixture of glucose plus thiosulphate, assimilation of glucose was increased at the expense of the energy derived from thiosulphate oxidation (Smith *et al.*, 1980). The data on mixotrophic yields communicated for other thiobacilli or hydrogen bacteria cannot be compared, since growth was either energy-limited (Leefeldt & Matin, 1980) or CO₂ was included as an additional carbon source (Stikus & DeCicco, 1970; Rittenberg, 1969). Convincing evidence has been obtained that in *Thiobacillus* A2 autotrophic CO₂ fixation is not involved in mixotrophy, as evident from enzymic data of Smith *et al.* (1980) and Gottschal & Kuenen (1980). In *P. carboxydoflava*, mixotrophic growth on pyruvate or glucose plus CO does not require CO₂ fixation (Kiessling & Meyer, 1982), as has also been reported for the hydrogen bacterium *Aquaspirillum autotrophicum* in a pyruvate-limited chemostat plus H₂ (Fasnacht & Aragno, 1982). In contrast, mixotrophic growth of *A. eutrophus* requires both H₂ oxidation and autotrophic CO₂ fixation. This was evident from physiological and biochemical studies with mutants defective either in chemolithotrophic or autotrophic metabolism. Mutants unable to oxidize H₂ did not grow mixotrophically. However, the soluble hydrogenase was distinctly more effective than the particulate enzyme in contributing to mixotrophic growth, as shown by the higher transitional growth rate of the Hop⁻ strain HF08 over the Hos⁻ strain HF14. These data are in agreement with the previous findings of Schlesier & Friedrich (1981).

The mutant HF17, unable to fix CO₂ autotrophically but not impaired in H₂ oxidation, did not increase the cellular yield significantly under mixotrophic growth conditions with succinate. A mixotrophic yield increase excluding autotrophic CO₂ fixation can either derive from an altered metabolism of the heterotrophic substrate which avoids the catabolism to generate reductant for respiration or from heterotrophic CO₂ fixation. For our studies we chose succinate, since the metabolism of this substrate does not allow a heterotrophic CO₂ fixation for anaplerotic reactions or glucogenesis. These examples show that *A. eutrophus* is unable to alter the metabolism of heterotrophic substrate, when an additional energy source such as H₂ is used.

The key enzymes of autotrophic metabolism are highly derepressed under succinate-limited growth (Friedrich, 1982). The addition of H₂, however, decreased the high level of the soluble hydrogenase dramatically, due to inactivation as described previously. The particulate enzyme is not subject to inactivation *in vivo* or *in vitro* (Schlesier & Friedrich, 1981) and consequently remained constant. The decrease in activity of the soluble enzyme, however, did not affect the mixotrophic yield.

The enormous increase of RuBPCase activity indicated that the cells were starved of carbon, since the key enzymes of autotrophic CO₂ fixation are derepressed under this condition (Im & Friedrich, 1983). Very probably this high enzyme level is necessary for the cell to refix effectively the CO₂ generated from succinate catabolism. This is also evident from the growth rate dependent mixotrophic capacity of *A. eutrophus*. The contribution of the autotrophic metabolism to the increase in cellular yield and productivity is decreased above the maximum autotrophic growth rate of 0.21 h⁻¹, where especially the activity of RuBPCase decreased significantly. Since CO₂ is formed at a rate equivalent to the cellular productivity, the restriction in mixotrophic capacity is linked to the rate of synthesis or function of the key enzymes of autotrophic metabolism at faster growth rates.

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