SHORT COMMUNICATIONS

Prototrophic Growth of *Citrobacter freundii* and the Biochemical Basis for its Apparent Growth Requirements in Aerated Media

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INTRODUCTION

Many of the Enterobacteriaceae can grow in simple salts media with a variety of carbohydrates as their only source of carbon and energy. In contrast, other species grow poorly, if at all, unless supplements of amino acids and vitamins are added (Véron & Le Minor, 1975). *Citrobacter freundii* is normally cultured in complex media, but aerobic growth also occurs in a minimal medium supplemented with glutamate, methionine, lysine and thiamin (de Graaf & Stouthamer, 1971). We used this simplified medium for our recent investigation into the regulation of aerobic and anaerobic metabolism in *C. freundii*, but found on analysis of data from carbon balances, that cell yields decreased during the later stages of anaerobic growth, possibly because of the accumulation of an inhibitory metabolite of one of the supplements (Keevil, Hough & Cole 1976). We have therefore investigated the physiological basis for these multiple growth requirements in order to simplify further the growth media for these bacteria.

METHODS

Citrobacter freundii strain NCTC9750 was maintained on minimal agar plus glucose (MAG) supplemented with either 100 μ M-methionine, or with 133 μ M-methionine, 1 mM-sodium glutamate, 110 μ M-lysine and 9 μ M-thiamin (de Graaf & Stouthamer, 1971). Cultures were stored at 4 °C for up to 4 weeks. The minimal salts medium contained (per l distilled water): KH_2PO_4 , 13.7 g; NH_4Cl , 1.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg; and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg. The pH was adjusted to 6.5 with KOH. After sterilization, sterile 40 % (w/v) glucose was added to 1 % (v/v) to give MG. MAG was MG solidified with 1.5 % (w/v) Oxoid L13 agar. MAGS and MGS were MAG and MG supplemented with methionine, glutamate, lysine and thiamin as specified above. Some MGS cultures were wrapped in foil to exclude light and aerated for 16 h with acridine orange (50 to 500 μ g ml⁻¹). All cultures were incubated at 37 °C.

Cell densities were determined spectrophotometrically by measuring the turbidity at 650 nm. To relate culture turbidity to cell dry weight, aerobic and anaerobic batch cultures in 100 ml MGS were harvested by centrifugation at 30000 g for 5 min, resuspended in 100 ml distilled water, recentrifuged and resuspended in 10 ml distilled water. Duplicate 4 ml samples were dried to constant weight at 105 °C. For aerated cultures, 0.25, 1.0 or 2.0 l conical flasks containing 50, 100 or 250 ml medium were shaken vigorously. For anaerobic cultures, unshaken flasks were filled to capacity. During the initial experiments, 10 l of medium was maintained at 37 °C and pH 6.5 in a laboratory fermenter (L. H. Engineering,

Stoke Poges, Buckinghamshire). The gas flow rate and the dissolved oxygen concentration were monitored during growth.

The concentrations of carbon in washed cell suspensions and culture supernatants were determined with a Beckman-RHC model 915 total organic carbon analyser. Aqueous samples (5 μ l) were oxidized quantitatively in a stream of O₂ at 950 °C using a cobaltic oxide catalyst, and the quantity of CO₂ formed was measured with an infrared detector. Each determination was made in triplicate: the variation between replicates was less than 2 %.

RESULTS AND DISCUSSION

Growth characteristics of Citrobacter freundii

This investigation into the physiological basis for the growth requirements of *C. freundii* was stimulated by three unexpected observations made during experiments designed to show the effect of inorganic electron acceptors on the regulation of intermediary metabolism. First, growth rates of anaerobic batch cultures were at least as fast as those of aerated cultures. No growth occurred during the first 12 h of incubation in aerated MG, but bacteria in aerated MGS grew as rapidly as those in nutrient broth. The specific growth rate in aerated MGS was 0.26 h⁻¹ with 1 l O₂ min⁻¹, and 0.495 h⁻¹ with 5 l O₂ min⁻¹. Dissolved oxygen concentrations with the higher rate of aeration were in excess of 150 μ M throughout the growth cycle. The specific growth rate during anaerobic growth in MGS was 0.69 h⁻¹ and 0.495 h⁻¹ with and without nitrate, respectively. Secondly, although the cell yield at the stationary phase was higher when nitrate or oxygen were supplied, glucose was still present in each culture at the end of exponential growth and it was not, therefore, the growth-limiting factor. Thirdly, identical molar growth yields from glucose of 37 ± 1 g were obtained from all three cultures during exponential growth. The yield coefficient decreased when the anaerobic cultures had used approximately 20 % of the available glucose, and a similar decrease occurred when the aerated cultures had used 40 % of the available glucose. It appeared, therefore, that either a product of glucose metabolism, or a product of one or more of the growth supplements was accumulating and inhibiting cell synthesis, especially during anaerobic growth.

Further experiments in which the concentration of each supplement was varied in turn suggested that glutamate, or a product of glutamate metabolism, was responsible for the inhibitory effects. No attempt has been made to identify the inhibitory metabolite, but the increased sensitivity during anaerobic growth is consistent with the possibility that glutamate might be preferentially metabolized to 3-hydroxybutyrate by the Ehrlich pathway under these conditions (Äyräpää, 1965).

Effect of individual supplements on growth

Attempts to simplify the growth medium were based on the realization that succinyl-CoA is implicated, directly or indirectly, in the synthesis of all four of the growth supplements. Glutamate is the precursor of glutamine and of many other biosynthetic products: it is synthesized by the reductive amination of 2-oxoglutarate, the precursor of succinyl-CoA in the tricarboxylic acid (TCA) cycle. Succinyl CoA is required in catalytic or stoichiometric quantities for the synthesis of lysine, thiamin and methionine, so succinate or any of these compounds might be predicted to stimulate aerobic growth. Conversely cysteine, an amino acid that is not synthesized from succinyl-CoA, would not be expected to stimulate growth. Furthermore, if a deficiency in succinyl-CoA is indeed the physiological basis for these growth

Table 1. *Growth rates and carbon balances of C. freundii*

Bacteria were grown in MG with various supplements at 100 μ M for aerated cultures, or 67 μ M for anaerobic cultures.

Supplement	Lag phase (h)	Specific growth rate (h ⁻¹)	% Glucose converted to:	
			Biomass	Soluble fermentation products
Aerated cultures				
None	>6	0.46	27.8	42.4
Cysteine	>6	0.77	41.7	16.6
Methionine	0	1.15	48.2	18.5
Lysine	0	0.99	49.3	15.3
Thiamin	0	0.87	34.8	25.0
Glutamate	0	0.77	51.5	27.1
Threonine	>6	0.41	35.8	35.0
Isoleucine	>6	0.41	35.2	34.9
Citrate	2	0.87	ND	ND
Isocitrate	2	0.87	ND	ND
2-Oxoglutarate	2	0.87	ND	ND
Succinate	0	0.99	45.8	27.8
Oxaloacetate	2	0.87	ND	ND
Fumarate	>6	0.46	ND	ND
Malate	>6	0.46	ND	ND
4-Hydroxybenzoate	2	0.77	36.8	34.0
4-Aminolaevulinate	2	0.69	38.9	18.8
Anaerobic cultures				
None	4	0.28	ND	ND
Cysteine	2	0.35	ND	ND
Methionine	2	0.38	ND	ND

ND, Not determined.

requirements, *C. freundii* should show a prototrophic phenotype during anaerobic growth: under these conditions succinate accumulates as a fermentation product (Keevil *et al.*, 1976).

Bacteria from an aerated, stationary-phase MGS culture were subcultured into MG and incubated either aerobically or anaerobically. Aerobic growth occurred essentially without a lag phase in cultures supplemented with methionine, lysine, glutamate or succinate, and only brief lags were observed with citrate, isocitrate, 2-oxoglutarate, oxaloacetate, 4-hydroxybenzoate or 4-aminolaevulinate (Table 1). No growth occurred for at least 6 h in unsupplemented MG or when cysteine was supplied. The lag phase usually lasted for 12 to 20 h, and in several experiments no growth had occurred after several days of incubation. However, growth was usually complete even in unsupplemented cultures after 48 h, and the final yield was comparable to that from MGS cultures.

In contrast to the aerated cultures, the lag phase in unsupplemented anaerobic cultures was reduced to 4 h, but lags of 2 h were also observed in cultures supplemented with methionine or cysteine (Table 1). When only 6.7 μ M-methionine was supplied, exponential growth at a specific growth rate of 0.38 h^{-1} was followed by a brief lag phase at a cell density of 75 μ g dry wt ml^{-1} : exponential growth then resumed at a specific growth rate of 0.28 h^{-1} which was identical to that of the unsupplemented culture (Table 1).

The duration of the lag phase was partially determined by the age of the inoculum and the medium on which it was propagated. The longest lags were observed with bacteria which had been stored for several weeks on solid media, or with liquid cultures in the

late stationary phase. The shortest lags occurred when the inoculum was growing exponentially in MG plus methionine.

It is also possible that aerobic growth in defined media is limited by the availability of TCA cycle intermediates in general, rather than specifically by succinyl-CoA. However, the lag phase did not decrease, and the specific growth rate did not increase, when aerated MG was supplemented with fumarate, malate, threonine or isoleucine at 0.1 mM. Threonine and isoleucine, like lysine and methionine, are amino acids synthesized from oxaloacetate and aspartate. Aspartate at 0.1 mM, 1.0 mM or 10 mM also failed to stimulate aerobic growth.

The effect of individual supplements on growth on solid media was also investigated. Large colonies formed within 48 h at 37 °C with any of the supplements that stimulated growth in liquid media. Little variation in colony size was observed when MAG was supplemented with methionine, but with other supplements, the sizes of individual colonies were extremely variable. Bacteria from large and small colonies produced both types of colony when replated on to similar media, as did bacteria which had been repeatedly subcultured into various media containing acridine orange. Thus, no evidence was obtained to suggest that prototrophic growth was dependent upon the presence of a curable plasmid (Hirota, 1960).

The effect of various supplements on the cell yield is summarized in Table 1. More glucose was converted into cell mass when cultures were supplemented with glutamate, lysine, methionine or succinate, but unsupplemented cultures preferentially metabolized the glucose to soluble fermentation products.

We conclude that *C. freundii* has no absolute requirement for growth supplements. It is a genetically competent prototroph when grown aerobically with succinate, or a TCA-cycle precursor of succinate, as the carbon source, and during anaerobic growth with a fermentable carbon source. Lag phases, which are especially apparent in aerated media, can be eliminated by adding compounds which require succinyl-CoA for their synthesis. It will be interesting to see how widely this explanation for the apparent growth requirements of *C. freundii* can be applied to other genera.

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