The physiology of *Clostridium sporogenes* NCIB 8053 growing in defined media

R.W. LOVITT, D.B. KELL & J.G. MORRIS *Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23 3DA, Wales, UK*

LOVITT, R.W., KELL, D.B. & MORRIS, J.G. 1987. The physiology of *Clostridium sporogenes* NCIB 8053 growing in defined media. *Journal of Applied Bacteriology* **62**, 81–92

The physiology of *Clostridium sporogenes* was investigated in defined, minimal media. In batch culture, the major end products of glucose dissimilation were acetate, ethanol and formate. When L-proline was present as an electron acceptor, acetate production was strongly enhanced at the expense of ethanol. As judged by assay of the relevant enzymes, glucose was metabolized *via* the Embden-Meyerhof-Parnas pathway. The growth energetics of *Cl. sporogenes* were investigated in glucose- or L-valine-limited chemostat cultures. In the former case, the addition of L-proline to the medium caused a significant increase in the molar growth yield (as calculated by extrapolation to infinite dilution rate). This finding adds weight to the view that the reduction of L-proline by *Cl. sporogenes* is coupled to the conservation of free energy.

Whilst *Clostridium sporogenes* is a proteolytic organism, capable of growth in complex media containing no carbohydrate, the addition of glucose to such media causes a significant enhancement of growth (Smith & Hobbs 1974). Nonetheless, quantitative and even qualitative studies of the physiology of this organism have been severely hindered by the general lack of defined media capable of supporting growth, because the complex media usually employed prevent a quantitative interpretation of the metabolism of glucose since the fermentation of many amino acids leads to the production of the same compounds as those derived from saccharolytic metabolism (Barker 1961, 1981).

Glucose metabolism in proteolytic anaerobes was first examined in the closely related organism *Cl. botulinum*. Clifton (1940) studied the fermentation of glucose in cell suspensions of this organism, and noted that the major end product was ethanol. The enzymology of glucose metabolism in *Cl. botulinum* was studied by Simmonds & Costilow (1962) but, as noted by Golovchenko *et al.* (1983), no data concerning the nature of the end products of this strain

were given.

The effect of glucose on end product formation by *Cl. sporogenes* in complex media has been studied by several workers (Saissac *et al.* 1948; Moore *et al.* 1966; Lewis *et al.* 1967; Brooks & Moore 1969; Anema *et al.* 1973; Holdman & Moore 1975; Turton *et al.* 1983; Montville *et al.* 1985) with similar approaches. These authors found that the addition of glucose led to increases in the amounts of acetate, butyrate, ethanol and butanol relative to control media containing no added carbohydrate. Detailed analysis of the fermentation could not be performed, however, because of the complexity of the growth media employed.

Lovitt *et al.* (1987) describe the development of a defined and minimal medium capable of supporting the growth of several strains of *Cl. sporogenes*. With this medium it is possible to investigate systematically and quantitatively the carbon and energy sources used by this organism; the present paper reports our observations on the kinetics and energetics of the growth of this organism using glucose and other electron donors and acceptors.

Materials and Methods

SOURCE AND MAINTENANCE OF ORGANISMS

These are described in Lovitt *et al.* (1987).

BATCH CULTURES

Two types of batch culture were used. The first was in pressure tubes using the essential amino acid/fatty acid (EAA/FA)/glucose/proline medium described by Lovitt *et al.* (1987); such cultures were used as inocula for both larger batch cultures and chemostat cultures. Small (50 ml) batch cultures were grown in 150 ml Wheaton serum bottles (Phase Separations, Queensferry, Clwyd) sealed with butyl rubber bungs (Bellco Vineland, NJ) under a N_2 headspace. Larger (1·5 l) cultures were grown, harvested and washed once as described (Lovitt *et al.* 1987) except that the washing buffer consisted of 25 mmol/l triethanolamine phosphate pH 6·5 containing 100 mmol/l KCl, 5 mmol/l $MgCl_2$ and 0·1% w/v cysteine, gassed for 1 h with oxygen-free nitrogen. The cell suspension, at a concentration of *ca* 20 mg dry weight/ml, was stored on ice under nitrogen in a serum bottle. Growth was determined turbidimetrically as described by Lovitt *et al.* (1987).

CHEMOSTAT CULTURE

Chemostat culture was performed in an LH Engineering (Stoke Poges, Bucks) fermenter (1 l capacity) equipped with pH and temperature control and using a working volume of 480 ml. Medium flow from the reservoir was controlled with an LKB (Salsdon, Surrey) Microperpex pump. The media were prepared in 20 l batches autoclaved for 45 min at 121°C. EAA/FA medium containing double-strength amino acid supplement (EAA) was used to ensure that the amino acids necessary for anabolism were not limiting. The medium was then further supplemented with glucose, L-valine and/or L-proline as required. The culture vessel was filled with distilled water and autoclaved separately. After autoclaving, the water was tipped out by the weir, then replaced with pre-reduced medium and gassed with carbon dioxide. The pH was controlled at 7·0 (by adding 2 mol/l KOH or 2 mol/l HCl), temperature at 37°C, stirring at 250 rev/min and the medium gassed with CO_2 at 50 ml/min. Studies of the effect of CO_2 concentration on the physiology of this organism will be reported elsewhere.

ANALYSIS OF END PRODUCTS

End products were assayed as described (Lovitt *et al.* 1987), except that formate was estimated using formate dehydrogenase linked *via* phenazine methosulphate to the reduction of iodonitrotetrazolium violet. A 0·2 ml sample was added to 1 ml of 0·1 mol/l Tris HCl pH 8·0 containing 1 U formate dehydrogenase (Boehringer), 2·8 mol NAD, 0·25 µmol phenazine methosulphate and 5·8 µmol iodonitrotetrazolium. Samples were incubated for 30 min and the reaction stopped by the addition of 5 ml 0·1 mol/l HCl. The absorbance was measured at 520 nm with a 1 cm light path and the formate concentration calculated by means of a standard curve obtained in the same way. The assay was linear over a range of 0–200 nmol formate per sample.

ANALYSIS OF ENZYMES INVOLVED IN GLYCOLYSIS AND END PRODUCT FORMATION

The enzymes of glucose metabolism were estimated as described by Lamed & Zeikus (1980), except that permeabilized cells were used rather than a cell-free extract.

PERMEABILIZATION OF CELLS BY TOLUENE

Cell suspensions were prepared as described above. Cells were permeabilized by treatment with 20 µl of a 10:90 (v/v) toluene:ethanol mixture per ml of cell suspension and incubated at 37°C for 10 min. These (optimal) conditions were determined by two methods: firstly by a method (Kell & Walter 1986; Walter *et al.* 1986) which relies upon the fact that glycolytic acid production in the absence of exogenous cofactors is inhibited in permeabilized cells, and secondly by an assessment of the increase of NADH oxidase activity (O'Brien & Morris 1971) as the cells are permeabilized by the toluenization process.

Results

THE EFFECTS OF PROLINE ON CELL GROWTH AND THE FERMENTATION OF GLUCOSE IN BATCH CULTURE

Figure 1 shows the effect of excess L-proline on the time-course of a glucose fermentation of *Cl. sporogenes*. The influence of proline was manifested in a number of different ways. Thus both growth rate and growth yield (per mol glucose) were somewhat increased, and the fermentation, which in the absence of proline was not purely glucose-limited, became so when proline was present. The lack of glucose limitation in the former case was due to the reduction of the essential amino acid L-phenylalanine to a non-utilizable compound, most probably phenyl propionic acid (hydrocinnamic acid) (see Elsden *et al.* 1976; Barker 1981; Bader *et al.* 1982).

The major fermentation end products were acetate, ethanol, formate, CO_2 and H_2. On some occasions, small amounts of lactate could also be detected in the culture lacking proline. A typical fermentation balance in the absence and presence of proline is shown in Table 1. The effect of L-proline was to increase the proportion of acetate production at the expense of the reduced end products, ethanol and hydrogen. The amount of formate produced by this organism did not seem to be influenced by the addition of proline, suggesting that its production is not directly redox-linked but that it arises from pyruvate *via* the action of pyruvate formate lyase (Thauer *et al.* 1972).

THE PATHWAY OF GLUCOSE METABOLISM

To investigate the energetics and kinetics of growth more precisely, and to assess the effects of growth rate and the presence of L-proline on the glucose fermentation under more rigorously controlled conditions, we wished to study chemostat cultures of *Cl. sporogenes*. To make an accurate assessment of growth energetics in terms of Y_{ATP}, not only the products of glucose dissimilation but the pathway of their production must be known. We therefore surveyed the enzymes involved in the flow of carbon from glucose in this organism (Table 2); it may be observed (Table 2) that *Cl. sporogenes* has an enzyme complement typical of organisms performing glycolysis by means of an Embden-Meyerhof-Parnas (EMP) pathway. Glucose- 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were not detectable by the methods employed, whilst a highly active FDP aldolase and glucose-6-phosphate isomerase were present. The alcohol dehydrogenase activity was NAD-dependent. All assays were performed anaerobically because of the high NADH oxidase activity of the permeabilized cells (Table 2).

CHEMOSTAT STUDY OF GLUCOSE METABOLISM AND GROWTH ENERGETICS

Two particular reasons prompted us to investigate the growth of *Cl. sporogenes* in chemostat cultures: (1) no sound data on the growth yields of *Cl. sporogenes* exist, so that an investigation of this organism performing either Stickland
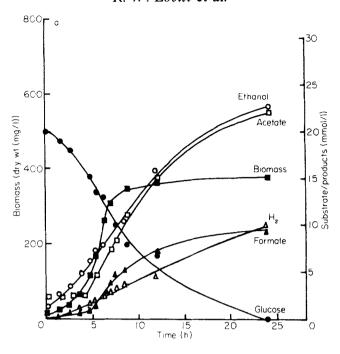
Table 1. Fermentation balances of *Clostridium sporogenes* on glucose with and without proline addition*

| Medium | Final pH | Biomass (mg/l) | Glucose utilized (mmol/l) | Fermentation products† | | | | | O/R | Recovery of glucose carbon (%) |
				Acetate	Ethanol	Formate	CO_2	H_2		
Glucose	6·15	382	20·5 (100)	20·1 (98)	24·0 (117)	8·5 (42)	35·5 (173)	10·1 (49)	0·94	107
Glucose plus L-proline	4·85	870	15·0 (100)	22·2 (146)	2·5 (17)	9·5 (63)	15·2 (100)	2·3 (15)	1·14	82

* *Clostridium sporogenes* was grown in pressure tubes, in the media described in Materials and Methods. Fermentation balances are calculated from the data in Fig. 1. CO_2 was calculated from the amount of acetate and ethanol and formate produced.

† Values as mmol/l and, in parentheses, as mmol/l/100 mmol/l glucose utilized.
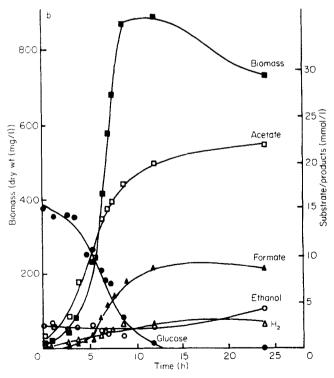
O/R, Oxidation/reduction balance.

Fig. 1. Effect of L-proline on the growth and fermentation products of *Clostridium sporogenes* in batch culture. Cells were grown in Wheaton bottles as described in Materials and Methods. a, No proline addition; b, plus 40 mmol/l L-proline.

Table 2. Enzymes of glucose metabolism in permeabilized cells of *Clostridium sporogenes* grown on glucose-complex medium

Enzyme	Specific activity* (μmol/min/mg dry wt)
Glucose 6P isomerase	1·680
Glucose 6P DH	ND
6P-gluconate DH	ND
FDP-aldolase	0·664
Glyceraldehyde DH	1·850
Pyruvate kinase	0·163
Pyruvate DH	1·430
Acetate kinase	0·160
Alcohol dehydrogenase	
acetaldehyde → ethanol	0·190
ethanol → acetaldehyde	0·011
Hydrogenase (H$_2$ uptake)	0·015
NADH oxidase	0·131

* Specific activities of the enzymes given were measured on toluenized cells grown and prepared as described in Materials and Methods.
ND, Not detectable.
DH, Dehydrogenase.

reactions or 'mixed Stickland' reactions (carbohydrate donor, amino acid acceptor) would otherwise be premature and (2) the effects noted in batch culture could be investigated under more suitably controlled conditions to yield information on the kinetics of the metabolic biotransformations catalysed by this organism. The media used contained either glucose alone, glucose plus L-proline or L-valine plus L-proline as energy sources, and the results obtained are shown in Tables 3–5.

GLUCOSE-LIMITED CHEMOSTAT CULTURE

The steady state criterion for all experiments was that the biomass content was unchanging after seven to nine volume changes following the shift to a new dilution rate, and at each dilution rate studied the end products were determined. With glucose as sole energy source, wash-out occurred at a dilution rate of 0·32/h. The fermentation products are shown in Table 3; in contrast to what was observed in batch cultures (Fig. 1 and Table 1), a significant fraction of glucose carbon was diverted to the production of butyrate and butanol, whilst production of ethanol was decreased. Apart from formate production, which varied monotonically with growth rate, no clear relationships between growth rate and end product formation were discernible.

GLUCOSE-LIMITED, PROLINE-SUPPLEMENTED CHEMOSTAT CULTURE

When *Cl. sporogenes* is grown glucose-limited in the presence of L-proline as an electron acceptor, it is to be assumed that both the growth rate (and hence growth yield) will be increased, and the fermentation products more oxidized, than when proline is absent. An assessment of whether the maximum growth yield is also increased under these conditions is of some importance, since the coupling of L-proline reduction to ATP synthesis has yet to be established (Seto 1980), so that the present physiological approach, complementing our recent demonstration that L-proline reduction is coupled to proton-motive activity (Lovitt *et al.* 1986), could provide crucial evidence on this point. Under the conditions described, wash-out occurred at a dilution rate exceeding 0·5/h and the major fermentation product was acetate (Table 4). However, the biomass yield, as well as the maximum growth yield (see later), was in all cases greater than that observed in the presence of glucose alone, strongly suggesting that the reduction of L-proline contributes significantly to ATP production in this organism (and see later).

VALINE-LIMITED (PLUS PROLINE) CHEMOSTAT CULTURE

Clostridium sporogenes does not grow on L-valine unless a Stickland acceptor such as proline is present. Cultures were therefore grown in an L-valine-limited chemostat in the presence of L-proline; wash-out under these conditions was observed at a dilution rate of only 0·18/h. This may be compared with the maximum specific growth rate in batch culture (in pressure tubes) which was 0·38/h (Lovitt *et al.* 1987). It is likely that the difference is largely ascribable to the different pCO$_2$ values established under the two conditions, since we have found (Dixon *et al.*, unpublished) that the optimal pCO$_2$ for the growth of this organism is approx. 0·45 atm. As indicated in Table 5, the growth yield on L-valine was significantly less than that on glucose. The end products of L-valine metabolism were isobutyrate and CO$_2$, and the small amounts of acetate and ethanol formed, particularly at low growth rates, may be ascribed to a secondary Stickland reaction in

Table 3. The effect of dilution rate on the fermentation of glucose by *Clostridium sporogenes* in glucose-limited chemostat culture*

D (/h)	Biomass (g/l)	$q_{glucose}$ (mmol/(g h))	$Y_{x/s}$† (g/mol)	ATP_{glu} (mol/mol)	Fermentation products‡						Recovery of glucose carbon (%)
					Formate	Acetate	Ethanol	Butyrate	Butanol	CO_2	
0·095	0·262	2·43	39·1	3·7	0·18 (3)	10·1 (150)	0·42 (6)	1·94 (29)	0·56 (8)	15·3	114
0·133	0·268	3·32	40·0	3·6	0·31 (5)	10·2 (152)	0·13 (2)	0·77 (12)	0·29 (4)	12·5	93
0·163	0·307	3·55	45·8	3·8	0·31 (5)	11·0 (160)	0·31 (4)	1·06 (15)	0·44 (6)	14·0	102
0·200	0·323	4·15	48·2	3·6	0·36 (5)	9·8 (146)	0·27 (4)	0·90 (13)	0·35 (5)	12·2	94
0·225	0·329	4·58	49·1	3·8	0·37 (5)	10·7 (159)	0·45 (7)	1·32 (20)	0·55 (8)	14·5	111
0·246	0·339	4·86	50·6	3·8	0·50 (7)	11·1 (166)	0·36 (5)	0·86 (12)	0·33 (5)	13·3	103
0·270	0·342	5·29	51·0	3·6 Mean 3·7	0·81 (12)	9·8 (146)	0·51 (7)	0·72 (11)	0·26 (4)	11·5	92

* The experiments were performed in a chemostat with a total volume of 480 ml. The organisms were grown at pH 7·0, at 37°C in EAA medium containing 6·7 mmol/l glucose and the medium was gassed with CO_2. End products were determined as described in Materials and Methods, except that CO_2 was assessed from the other products and the known patterns of their production.

† $Y_{x/s}$, Specific yield of biomass, i.e. g dry wt of cells produced per mol glucose utilized.

‡ Values are given in mmol/l and, in parentheses, as mmol/l/100 mmol/l glucose utilized.

Table 4. The effect of growth rate on the fermentation of glucose by *Clostridium sporogenes* in the presence of L-proline*

D (/h)	Biomass (g/l)	$q_{glucose}$ (mmol/(g h))	$Y_{x/s}$† (g/mol)	ATP_{glu} (mol/mol)	Fermentation products‡						Recovery of glucose carbon (%)
					Formate	Acetate	Ethanol	Butyrate	Butanol	CO_2	
0·109	0·339	2·35	46·4	3·9	0·06	14·1 (193)	0·30 (2)	ND	ND	14·4	99
0·132	0·410	2·35	56·2	3·7	0·10 (1)	12·4 (169)	0·10 (1)	ND	ND	12·4	86
0·218	0·454	3·50	62·1	3·8	0·18 (2)	13·1 (179)	0·13 (2)	ND	ND	13·1	91
0·240	0·467	3·75	64·0	3·7	0·25 (3)	12·1 (165)	0·10 (1)	ND	ND	12·0	84
0·300	0·512	4·28	70·1	3·8	0·18 (2)	13·3 (182)	0·20 (3)	ND	ND	13·3	92
0·390	0·457	6·23	62·6	3·7	0·50 (6)	12·0 (167)	<0·05	ND	ND	11·5	82
0·493	0·416	8·65	56·9	3·7	0·56 (7)	12·4 (169)	<0·05	ND	ND	11·8	85
				Mean 3·75							

* The organisms were grown at 37°C in EAA medium containing 7·3 mmol/l glucose and 30 mmol/l L-proline, in a chemostat as described in Materials and Methods and in the legend to Table 3.

† $Y_{x/s}$, Specific biomass yield, i.e. g dry wt cells produced per mol glucose utilized.

‡ Values in mmol/l and, in parentheses, as mmol/l/100 mmol/l glucose utilized.

ND, Not detectable.

Table 5. The effect of dilution rate on the fermentation of valine plus proline by *Clostridium sporogenes* in chemostat culture*

D (/h)	Biomass (g/l)	q_{valine} (mmol/(g h))	$Y_{x/s}$† (g/mol)	ATP_{val} (mol/mol)	Fermentation products‡							Recovery of valine carbon (%)
					Formate	Acetate	Ethanol	Butyrate	Butanol	iso-butyrate	CO_2	
0·061	0·160	8·39	7·3	1·03	ND	1·17 (5)	0·25 (1)	ND	ND	22·8 (103)	22·8	103
0·088	0·202	9·58	9·2	1·05	ND	1·89 (8)	0·14 (1)	ND	ND	23·2 (105)	23·2	105
0·108	0·224	10·60	10·2	1·10	ND	1·0 (5)	0·10 (1)	ND	ND	24·2 (110)	24·2	110
0·133	0·230	12·72	10·5	1·05	ND	0·14 (1)	0·11 (1)	ND	ND	23·1 (105)	23·1	105
0·143	0·236	13·33	10·7	0·97	ND	ND	0·05 (1)	ND	ND	21·5 (97)	21·5	97
				Mean 1·04								

* The organisms were grown on EAA medium with 22 mmol/l valine and 40 mmol/l L-proline in a valine-limited chemostat as described in the legend to Table 4 except that 10 mmol/l potassium acetate was also present (Lovitt *et al.* 1986a). The values given for acetate are corrected for that initially added.

† $Y_{x/s}$, Specific biomass yield, i.e. g dry wt cells produced per mol valine utilized.

‡ Values in mmol/l and, in parentheses, as mmol/l/100 mmol/l valine utilized.

ND, Not detectable.

which glycine, an amino acid essential for growth, was reduced to acetate (Lovitt *et al.* 1987).

KINETIC ASPECTS OF GROWTH IN DIFFERENT MEDIA

Figure 2 shows a plot of the specific rate of substrate consumption *vs* the dilution rate, using the data from Tables 3–5. From the intercept it is possible to determine the so-called maintenance requirement (Pirt 1975; Tempest & Neijssel 1984). The energetic and kinetic data are summarized in Table 6, which shows firstly that the maintenance requirement was very much greater for L-valine grown cells than for glucose grown cells. This is at least partly to be expected, since on valine-proline medium the
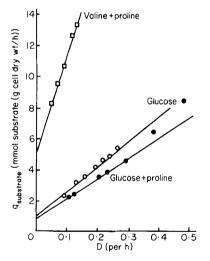
acetate which must be added for anabolic purposes must first be activated. Table 6 also shows the molar growth yield; at 80·8 g cells per mol glucose, that observed on glucose-proline medium was as high as those observed with some aerobic organisms, while that on glucose alone (65·5 g/mol glucose) was somewhat lower and that on valine-proline medium (17·5 g/mol L-valine) significantly less. When the Y_{ATP}^{max} values were compared (on the bases that the reduction of proline does not contribute to ATP synthesis and that 1 mol ATP is formed per mol of valine oxidized), it was found that they were 17·5 g cells/mol ATP on glucose or valine-proline medium whilst on glucose-proline medium this value rose to 21·5 g cells/mol ATP.

Discussion

The development of a defined, minimal medium for the growth of *Cl. sporogenes* NCIB 8053 has allowed us to undertake what we believe to be the first quantitative studies of the physiology of this organism in chemostat culture, although preliminary studies of the growth of *Cl. sporogenes* on complex media in continuous flow culture have been reported (Ashley & Shoesmith 1977).

GLUCOSE METABOLISM OF *Clostridium sporogenes*

As previously described by others, glucose is fermented by *Cl. sporogenes* (see above and Belokopytov *et al.* 1982). The route of glucose dissimilation is *via* the EMP pathway, as indicated by the spectrum of enzyme activities detected in permeabilized cells. The demonstration of this pathway allowed the calculation of the Y_{ATP} values resulting from substrate level phosphorylation.

Fig. 2. Catabolic rates exhibited by *Clostridium sporogenes* growing on various energy sources. Values of $q_{substrate}$ were obtained in chemostat cultures as described in the legends to Tables 3–5.

Table 6. The growth yield and maintenance requirements of *Clostridium sporogenes* grown in glucose- or valine-limited chemostat culture (with excess proline or phenylalanine as noted)

Substrate	$m(q_m)$	m_{ATP}	ATP_{glu}	Y^{max}	Y_{ATP}^{max}
Glucose	1·0	3·70	3·70	65·5	17·5
Glucose-proline	0·9	3·37	3·75	80·0	21·3
Valine-proline	5·0	5·20	1·00	17·5	17·5

Data are derived from Fig. 2 and Tables 3-5. Units are: m (q_m) in mmol/(g cell dry wt h); m_{ATP} in mmol ATP/(g cell dry wt h); Y^{max} g in cell dry wt/(mol substrate); Y_{ATP}^{max} g in cell dry weight/(mol ATP).

The fermentation of glucose of *Cl. sporogenes* is of the acetate-ethanol type, with formate also being produced in significant quantities. Such a fermentation pattern differs from that typical of many saccharolytic clostridia, which normally form butyrate as a major end product. The majority of other workers (Moore *et al.* 1966; Holdman & Moore 1975; Turton *et al.* 1983) who have reported on the end products formed by *Cl. sporogenes*, albeit on complex media, have in fact suggested that butyrate is a major fermentation product. Aside from strain differences, there may be a number of reasons for this: (1) the analyses of end products were performed by gas chromatography of solvent (usually diethyl ether) extracts of the medium; this method permits good extraction and detection of butyrate and butanol whilst acetate and ethanol are only poorly extracted (unpublished observation) and (2) although the growth of *Cl. sporogenes* is rapid in either complex or defined media (see above and Lovitt *et al.* 1987), analyses of the end products were often performed well into the stationary phase of 3- to 7-d-old cultures; under these conditions, butyrate may be formed as a product of the secondary metabolism of glucose or from other medium constituents. Some workers, however, have also reported that ethanol and acetate are the major end products of glucose fermentation in *Cl. sporogenes* (Anema *et al.* 1973) and the related *Cl. botulinum* (Clifton 1940).

Although H_2 production was detectable in the batch cultures it was not observed when cells were growing in energy-limited chemostat cultures; this would strongly suggest that H_2 production is to be observed only under conditions of energy excess and would also indicate that the reactions exerting the greatest control on the maximum specific growth rate are related to electron transport and energy generation; this is certainly true of the control of metabolic flux in cell suspensions (Lovitt *et al.* 1987). It is of interest that hydrogenase (uptake) activity was detectable only at relatively low activities in cell-free extracts, a result that is fairly unusual for clostridia but which was observed for *Cl. sticklandii* by Stadtman (1966). In contrast to other strains of this organism (Hoogerheide & Kocholaty 1938; Bader *et al.* 1982), that used in the present work did not consume H_2 in the presence of electron acceptors (Lovitt *et al.* 1987).

GLUCOSE METABOLISM AND THE INFLUENCE OF PROLINE

The addition of L-proline to the medium affected the growth of the culture in two ways: (1) the maximum growth rate was increased from approx. 0·33 to 0·5/h and (2) the most important, the molar growth yield on glucose was increased even when end product formation was taken into account. Indeed, the molar growth yields of *Cl. sporogenes* are high when compared with most other anaerobes (Bauchop & Elsden 1960; Stouthamer 1979; Thauer & Morris 1984). However, values of Y_{ATP}^{max} (in g cells/mol ATP) approaching those observed here have been obtained in batch cultures of various clostridia, e.g. 17·5 for *Cl. aminobutyricum* growing on γ-hydroxybutyrate (Hardman & Stadtman 1963), 15·4 for *Cl. glycolicum* growing on ethylene glycol (Gaston & Stadtman 1963) and 14·6 for *Cl. perfringens* growing in complex medium (Hasan & Hall 1975). Glycine reduction is also reported to be linked to ATP production, although *via* a substrate level phosphorylation mechanism (Stadtman 1966; Seto 1980). As shown by Lovitt *et al.* (1987), however, good growth of *Cl. sporogenes* NCIB 8053 could not be obtained when glycine was used as the Stickland acceptor.

The growth results obtained here suggest that the reduction of L-proline is linked to free energy conservation. On the assumption that only substrate level phosphorylation contributes to ATP production, values of Y_{ATP}^{max} of 17·5 and 21·5 g cells/mol ATP were found in the absence and presence of L-proline, respectively. We are therefore led to consider the possibility that L-proline reduction is linked to free energy conservation by electron transport phosphorylation. Circumstantial evidence for this is provided by the finding of Stadtman & Elliot (1957) and Seto (1980) that the L-proline reductase system is membrane-bound and requires at least three proteins for electron transport from NADH. Such an electron transport chain could be coupled to vectorial proton ejection and thence to phosphorylation *via* a proton-translocating ATP synthase, and recent work from this laboratory (Lovitt *et al.* 1986) has confirmed the veracity of the former statement.

A related process in which vectorial proton transport seems likely to be coupled to electron flow is *via* the enoate reductase of this organism,

and cinnamate reduction has been shown to be coupled to ATP synthesis in the strain of *Cl. sporogenes* studied by Bader & Simon (1983). Indeed, growth of this organism was possible when the sole source of energy was provided by electron transport from H_2 to cinnamate (Bader & Simon 1983). Such findings enhance the significance of the studies reported here and elsewhere (Lovitt *et al.* 1987) that suggest that not all Stickland acceptors act in the same fashion. Indeed, as studies of many anaerobic microorganisms are showing, not all 'classical' fermentations are confined to the aqueous cytoplasm of the cell (Thauer & Morris 1984).

References

ANEMA, P.J., KOOIMAN, W.J. & GEERS, J.M. 1973 Volatile acid production by *Clostridium sporogenes* under controlled culture conditions. *Journal of Applied Bacteriology* **36**, 683–687.

ASHLEY, N.V. & SHOESMITH, J.G. 1977 Continuous culture of *Clostridium sporogenes* and *Clostridium bifermentans* in the presence of oxygen. *Proceedings of the Society for General Microbiology* **4**, 144.

BADER, J. & SIMON, H. 1983 ATP formation is coupled to the hydrogenation of 2-enoates in *Clostridium sporogenes*. *FEMS Microbiology Letters* **20**, 171–175.

BADER, J., RAUSCHENBACH, P. & SIMON, H. 1982 On a hitherto unknown fermentation pathway of several amino acids by proteolytic clostridia. *FEBS Letters* **140**, 67–72.

BARKER, H.A. 1961 Fermentations of nitrogenous organic compounds. In *The Bacteria, Vol. II. Metabolism* ed. Gunsalus, I.C. & Stanier, R.Y. pp. 151–208. London: Academic Press.

BARKER, H.A. 1981 Amino acid degradation by anaerobic bacteria. *Annual Review of Biochemistry* **50**, 23–40.

BAUCHOP, T. & ELSDEN, S.R. 1960 The growth of microorganisms in relation to their energy supply. *Journal of General Microbiology* **23**, 457–469.

BELOKOPYTOV, B.F., GOLOVCHENKO, N.P., KRAUZOVA, V.I., CHUVILSKAYA, N.A., AKIMENKO, V.K. & LOZINOV, A.B. 1982 A chemically defined medium for cultivation of *Clostridium sporogenes*. *Mikrobiologiya* **51**, 354–361.

BROOKS, J.B. & MOORE, W.E.C. 1969 Gas chromatographic analysis of amines and other compounds produced by several species of *Clostridium*. *Canadian Journal of Microbiology* **15**, 1433–1447.

CLIFTON, C.E. 1940 The utilization of amino acids and of glucose by *Clostridium botulinum*. *Journal of Bac-*

teriology **38**, 485–497.

ELSDEN, S.R., MILTON, M.G. & WALTER, J.M. 1976 The end products of the metabolism of aromatic amino acids by Clostridia. *Archives of Microbiology* **107**, 283–288.

GASTON, L.W. & STADTMAN, E.R. 1963 Fermentation of ethylene glycol by *Clostridium glycolicum* sp. n. *Journal of Bacteriology* **85**, 356–362.

GOLOVCHENKO, N.P., BELOKOPYTOV, B.F. & AKIMENKO, V.K. 1983 Glucose metabolism in the bacteria: *Clostridium sporogenes* and *Clostridium sticklandii*. *Microbiology (USSR)* **52**, 681–686.

HARDMAN, J.K. & STADTMAN, T.C. 1963 Metabolism of ω-amino acids V. Energetics of the γ-aminobutyrate fermentation of *Clostridium aminobutyricum*. *Journal of Bacteriology* **85**, 1326–1333.

HASAN, S.M. & HALL, S.B. 1975 The physiological function of nitrate reduction in *Clostridium perfringens*. *Journal of General Microbiology* **87**, 120–128.

HOLDEMAN, L.V. & MOORE, W.E.C. 1975 *Anaerobe Laboratory Manual*, 3rd edn. Blacksburg: Virginia Polytechnic Institute and State.

HOOGERHEIDE, J.C. & KOCHOLATY, W. 1938 Metabolism of the strict anaerobes (genus *Clostridium*). II. Reduction of amino acids with gaseous hydrogen by suspensions of *Cl. sporogenes*. *Biochemical Journal* **32**, 949–957.

KELL, D.B. & WALTER, R.P. 1986 Organization and control of energy metabolism in anaerobic bacteria. In *Organization of Cell Metabolism* ed. Welch, G.R. & Clegg, J.S. New York: Plenum Press. In press.

LAMED, R. & ZEIKUS, J.G. 1980 Glucose fermentation pathway of *Thermoanaerobium brockii*. *Journal of Bacteriology* **141**, 1251–1257.

LEWIS, V.J., MOSS, C.W. & JONES, W.L. 1967 Determination of volatile acid production of *Clostridium* by gas chromatography. *Canadian Journal of Microbiology* **13**, 1033–1040.

LOVITT, R.W., KELL, D.B. & MORRIS, J.G. 1986 Proline reduction by *Clostridium sporogenes* is coupled to vectorial proton ejection. *FEMS Microbiology Letters* **36**, 269–273.

LOVITT, R.W., MORRIS, J.G. & KELL, D.B. 1987 The growth and nutrition of *Clostridium sporogenes* NCIB 8053 in defined media. *Journal of Applied Bacteriology* **62**, 71–80.

MONTVILLE, T.J., PARRIS, N. & CONWAY, L.K. 1985 Influence of pH on organic acid production by *Clostridium sporogenes* in test tube and fermenter cultures. *Applied and Environmental Microbiology* **49**, 733–736.

MOORE, W.E.C., CATO, E.P. & HOLDEMAN, L.V. 1966 Fermentation patterns of some *Clostridium* species. *International Journal of Systematic Bacteriology* **16**, 383–415.

PIRT, S.J. 1975 *Principles of Microbe and Cell Cultivation* Oxford: Blackwell Scientific Publications.

O'BRIEN, R.W. & MORRIS, J.G. 1971 Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. *Journal of General Microbiology* **68**, 307–318.

SAISSAC, R., RAYNAUD, M. & COHEN, G.N. 1948 Variation du type fermentaire des bacteries anaerobies due groupe de *Cl. sporogenes* sous l'influence du glucose. *Annales de l'Institute Pasteur* **75**, 305–309.

SETO, B. 1980 The Stickland reaction. In *Diversity of Bacterial Respiratory Systems*, Vol. 2. ed. Knowles, C.J. pp. 50–64. Boca Raton: CRC Press.

SIMMONDS, R.J. & COSTILOW, R.N. 1962 Enzymes of glucose and pyruvate catabolism in cells, spores and germinated spores of *Clostridium botulinum*. *Journal of Bacteriology* **84**, 1274–1281.

SMITH, L.D.S. & HOBBS, G. 1974 Genus III Clostridium. In *Bergey's Manual of Determinative Bacteriology*, 7th edn. pp. 551–572. Baltimore: Williams and Wilkins.

STADTMAN, T.C. 1966 Glycine reduction to acetate and ammonia: Identification of ferredoxin and another low molecular weight acidic protein as components of the reduction system. *Archives of Biochemistry and Biophysics* **113**, 9–19.

STADTMAN, T.C. & ELLIOT, P. 1957 Studies on the enzymatic reduction of amino acids. II. Purification and properties of a proline reductase and a proline racemase from *Clostridium sticklandii*. *Journal of Biological Chemistry* **228**, 983–997.

STOUTHAMER, A.H. 1979 The search for correlation between theoretical and experimental growth yields. In *International Review of Biochemistry Vol. 21 Microbial Biochemistry* ed. Quayle, J.R., Kornberg, H.L. & Phillips, D.C. pp. 1–47. Baltimore: University Park Press.

TEMPEST, D.W. & NEIJSSEL, O.M. 1984 The status of Y_{ATP} and maintenance energy as biologically interpretable phenomena. *Annual Review of Microbiology* **38**, 459–486.

THAUER, R.K., KIRCHNIAWY, F.H. & JUNGERMANN, K.A. 1972 Properties and functions of the pyruvate-formate-lyase reaction in clostridia. *European Journal of Biochemistry* **27**, 282–290.

THAUER, R.K. & MORRIS, J.G. 1984 Metabolism of chemotrophic anaerobes: old views and new aspects. In *The Anaerobe 1984 Part II Prokaryotes and Eukaryotes*. Society for General Microbiology Symposium, No. 36 ed. Kelly, D.P. & Carr, N.G. pp. 123–168. Cambridge: Cambridge University Press.

TURTON, L.J., DRUCKER, D.B. & GANGULI, L.I. 1983 Effect of glucose concentration in the growth medium upon neutral and acid fermentation end products of *Clostridium bifermentans*, *Clostridium sporogenes* and *Peptostreptococcus anaerobius*. *Journal of Medical Microbiology* **16**, 61–67.

WALTER, R.P., KELL, D.B. & MORRIS, J.G. 1986 The roles of osmotic stress and water activity in the inhibition of the growth, glucolysis and glucose phosphotransferase system of *Clostridium pasteurianum*. *Journal of General Microbiology*. In press.