Control of Product Formation During Glucose Fermentation by *Bacillus macerans*

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The anaerobic fermentation of glucose by *Bacillus macerans* ATCC 7068 was studied in batch culture with and without pH control. The fermentation was characterized by two distinct metabolic phases. In the primary growth phase, the concentrations of ethanol and acetic acid increased exponentially, and formate was detected as a minor product. The secondary phase was marked by a slowing and eventual cessation of growth, along with the disappearance of formate and acetate, and the appearance of H₂, CO₂, and acetone. Exogenously-added substrates were converted with stoichiometries of 1 formate → 1 H₂ + 1 CO₂, and approximately 2 acetate → 1 acetone. Consumption of >8 g glucose l⁻¹ required exogenous pH control, and acetone formation was strongly pH-dependent. Glucose was fermented by the Embden–Meyerhof pathway. Cell extracts contained pyruvate formate-lyase and formate dehydrogenase activities, but only low pyruvate dehydrogenase activity. A balanced fermentation pathway is presented which is consistent with reaction stoichiometries and [¹⁴C]glucose labelling data in whole cultures, and with enzyme activities in extracts. The pathway is compared with those of other facultative anaerobes and the acetone-producing *Clostridium acetobutylicum*.

INTRODUCTION

The anaerobic fermentation of carbohydrates by *Bacillus macerans* was first described by Schardinger (1905). In contrast to the well-known acetone/butanol fermentation of *Clostridium acetobutylicum*, the *Bacillus* fermentation produces ethanol as the chief reduced product, with acetone, H₂, and CO₂, but not n-butanol, as co-products. Subsequent work by Northrop *et al.* (1919), and by Arzberger *et al.* (1920) on a similar organism, '*B. acetoethylicus*', demonstrated that formate and acetate were transient fermentation products, and that formation of acetone occurred only late in the fermentation and was enhanced by low culture pH. The physiology and biochemistry of acetone-producing *Bacillus* strains have received relatively little attention (Speakman, 1925; Prescott & Dunn, 1959).

The purpose of the present study was to examine in more detail the product formation during glucose fermentation, particularly with regard to the effect of pH control and the involvement of specific enzymes in the catabolic pathway.

METHODS

Materials. All chemicals were reagent grade, obtained from either Sigma or Fisher (Fair Lawn, N.J., U.S.A.). Enzymes used for coupled enzymic assays were obtained from Sigma. Anaerobic grade N₂ was purchased from Union Carbide-Linde Division (Somerset, N.J., U.S.A.). [¹⁴C]Glucose specifically labelled in the C-1, C-3,4 or C-6 positions was obtained from New England Nuclear.

Organism and growth conditions. *Bacillus macerans* ATCC 7068 was obtained from the American Type Culture Collection. According to Gordon *et al.* (1973), this strain is identical to '*B. acetoethylicus*' described by Northrop *et al.* (1919). The organism was grown in CM5 medium which contained (l⁻¹): KH₂PO₄, 0.26 g; Na₂HPO₄·7H₂O, 2.17 g; (NH₄)₂SO₄, 1.98 g; MgCl₂·6H₂O, 0.20 g; CaCl₂, 0.025 g; yeast extract, 2.0 g; mineral solution, 2.0 ml. The mineral solution contained (mg l⁻¹): Na₂EDTA, 400; MnSO₄·4H₂O, 340; (NH₄)₆Mo₇O₂₄·4H₂O, 400; FeSO₄·7H₂O, 800; CoCl₂·6H₂O, 40; NiCl₂·6H₂O, 20; ZnSO₄·7H₂O, 20; CuSO₄, 2.

Unless otherwise indicated, all incubations were carried out at 43 °C, using the anaerobic culture technique of Balch & Wolfe (1976), except that N₂ (1 atm) was used as gas phase, and no reducing agents were added to the medium. Experiments were performed in 18 × 150 mm anaerobic tubes (Bellco Glass, Vineland, N.J., U.S.A.) containing 10 ml of medium, or in a 2 l Multigen fermenter (New Brunswick Scientific Co.). Addition of primary growth substrates or effectors was made using sterile plastic hypodermic syringes.

Analysis of products. Solvents, alcohols, and volatile acids were analysed by GLC. Samples were prepared by adding 1 ml of culture to chilled microcentrifuge tubes, centrifuging for 2 min in a Beckman microcentrifuge, and adding 100–400 µl of supernatant to vials containing 0.33 M-H₃PO₄ to give a total volume of 800 µl. Samples were injected into a Hewlett-Packard 5880A gas chromatograph equipped with a model 7672A automatic liquid sampler. The following chromatographic conditions were used: column, 1.8 m × 0.32 cm (o.d.) Teflon-lined stainless steel packed with 10% SP1200/1% H₃PO₄ on Chromosorb W/AW (Supelco, Bellefonte, Pa., U.S.A.); carrier gas, He at 18 ml min⁻¹; injector temperature, 200 °C; flame ionization detector temperature, 200 °C; oven temperature programme, isothermal at 50 °C for 2.5 min, followed by heating at 15 °C min⁻¹ for 6 min, then holding at 140 °C for 1.5 min. Under these conditions the following retention times were obtained (min): acetaldehyde, 0.75; acetone, 1.3; ethanol, 1.6; iso-propanol, 1.9; methyl ethyl ketone, 3.0; n-propanol, 3.8; n-butanol, 5.6; acetic acid, 6.4; butyric acid, 7.8. Lower limits for detection of these compounds were 0.02–0.06 mM.

H₂ and CO₂ were analysed essentially by the procedure of Nelson & Zeikus (1974). Corrections were made for dissolved gases using Henry's Law; unless otherwise indicated CO₂ is expressed as the total of all species (CO₂, HCO₃⁻, CO₃²⁻), calculated from pH-dependent bicarbonate equilibrium data. Reducing sugars were determined by the dinitrosalicylic acid method of Miller *et al.* (1960). Cell growth was usually monitored turbidimetrically at 525 nm. Cell dry weight was measured by first filtering 20–50 ml of culture through tared 47 mm × 0.4 µm pore size polycarbonate membranes (Nucleopore, Pleasanton, Cal., U.S.A.), and then drying to constant weight at 100 °C. ¹⁴CO₂ was determined by injecting gas phase samples into Oxifluor-CO₂ (New England Nuclear) followed by subsequent counting in a Packard 3255 liquid scintillation spectrometer. Protein was measured by the Lowry method, using bovine serum albumin as a standard.

pH studies. Batch culture pH studies were carried out in a New Brunswick Multigen fermenter. An overnight tube culture (approx. 10 ml) of *B. macerans* was inoculated into 1.5 l N₂-gassed CM5 containing 4.5% (w/v) glucose. After inoculation, N₂-gassing was discontinued and stirring maintained at 100 r.p.m. Gas leaving the fermenter was channelled through a 25 cm condenser mounted vertically on top of the fermenter. The condenser fluid was maintained at 5 °C using a Brinkmann-Lauda (Westbury, N.Y., U.S.A.) RM3 refrigerated circulator. During the fermentation, the lower culture pH was regulated by automatic addition of 5 M-NaOH. Antifoam B (Sigma) was added manually at 8–16 h intervals to reduce the large amounts of foam produced in agitated cultures.

Preparation of cell extracts. Frozen cell paste (1.5 g) was suspended in 3.0 ml N₂-gassed 50 mM-phosphate buffer, pH 7.0, containing 2 mM-DTT. The suspension was passed two or three times through a cold (4 °C) French pressure cell (American Instrument Co., Silver Spring, Md., U.S.A.) at approximately 138 MPa (20000 lbf in⁻²). The resulting mixture was gassed briefly with N₂ and centrifuged at 23000 g for 90 min. The supernatant was transferred via syringe to sealed vials containing a N₂ gas phase. This supernatant ('cell extract') was kept on ice and used immediately for enzyme assays.

Enzyme assays. Spectrophotometric assays were performed using a hybrid Gilford 240/Beckman DU UV-visible spectrophotometer. Assays were done at 43 °C in 1.3 ml cuvettes sealed with soft rubber stoppers. Cuvettes contained 1 ml of reaction mixture and a N₂ gas phase. Various buffers were used, including Tris, pH 8.7; sodium/potassium phosphate, pH 7.0; or MES, pH 5.7.

Formate dehydrogenase (EC 1.2.1.2) was assayed by measuring the reduction of methyl viologen at 578 nm or of NAD or NADP at 340 nm. Cuvettes contained 95 mM-buffer, 10 mM-electron acceptor, 2–30 µg of extract protein and, in the case of the viologen, sufficient Na₂S₂O₄ to render the solution pale blue. The reaction was initiated by addition of 5 mM-sodium formate. Hydrogenase (EC 1.18.3.1) was assayed similarly, except that 0.40 ml of H₂ was used instead of formate to initiate the reaction. Benzyl viologen was also used as electron acceptor in some assays.

Formic hydrogen lyase (EC 1.2.1.1) was assayed by polarographic measurement of H₂ production (Sweet *et al.*, 1980) following addition of 10 mM-sodium formate to reaction mixtures that contained (in 1.5 ml) 100 mM-MES, pH 5.7–6.0, 2 µg extract protein, and various electron carriers, including NAD, methyl viologen, benzyl viologen, or horse heart cytochrome c.

Pyruvate formate-lyase (EC 2.3.1.54) was assayed by coupling the reaction to formate dehydrogenase and measuring the reduction of NAD at 340 nm. Cuvettes contained 90 mM-buffer, 1 mM-NAD, 1 mM-CoA, 7 mM-KAsO₃, 2 units of *Clostridium kluyveri* phosphotransacetylase (EC 2.3.1.8), 5 mM-DTT, 1 unit *Pseudomonas oxalaticus* formate dehydrogenase (EC 1.2.1.2), and 2–30 µg extract protein. The reaction was initiated by addition of 10 mM-sodium pyruvate.

Pyruvate dehydrogenase (EC 1.2.4.1) was assayed as for pyruvate formate-lyase, except that formate dehydrogenase was omitted. Occasionally, methyl viologen replaced NAD or NADP, and the absorbance changes during the reaction were measured at 578 nm.

RESULTS

Product formation during carbohydrate fermentation

Bacillus macerans ATCC 7068 fermented a wide variety of carbohydrates for growth, including monohexoses (glucose, fructose, mannose, galactose), dihexoses (maltose, cellobiose, sucrose, lactose, trehalose, melibiose), and trihexoses (melezitose, raffinose), as well as xylose, mannitol, starch, and xylan. Cellulose, xylitol, and sorbitol were not fermented. Fermentation of glucose (0.5%, w/v) in tube cultures resulted in the following reaction stoichiometry (mean value of three experiments after 6 d incubation): 1 glucose $\rightarrow$ 1.2 ethanol + 0.4 acetone + 0.1 acetic acid + 1.8 H₂ + 2.3 CO₂. The fermentation was balanced with respect to both electrons [O/R index (Wood, 1961) = 0.93] and carbon recovery (102%). An ethanol/acetone ratio of 2.2–3.8 was observed for all carbohydrates tested and varied slightly among substrates. The following compounds were not detected as products of glucose fermentation: methanol, isopropanol, C₃–C₆ volatile acids or alcohols, C₂–C₄ diols, lactic acid, methyl ethyl ketone, diacetyl, or acetoin. n-Butanol was not detected as a product, even when n-butanal or n-butyrate were added as substrates.

Carbohydrate fermentations usually stopped when the culture pH dropped to approximately 4.9, regardless of the initial culture pH within a range of 6.0–8.5. Net carbohydrate consumption in these cultures was always within the range 3–8 g l⁻¹. CaCO₃ increased the extent of glucose utilization and product (especially ethanol) formation (Table 1). NaOH was also effective in stimulating the extent of glucose utilization.

A time course of product formation (Fig. 1) revealed an initial primary metabolic phase during which cell material, ethanol, and acetate concentrations increased at an exponential rate, and pH decreased from 6.8 to a preset lower limit of 5.0. During this phase, formic acid, and occasionally acetaldehyde, were detected transiently in the culture supernatants, reaching maximum concentrations of 5.4 and 0.8 mM, respectively after 20 h incubation. This growth phase was followed by a secondary metabolic phase marked by a slowing and eventual cessation of growth, continued ethanol production, a decrease in formic and acetic acid concentrations, and the appearance of H₂, CO₂, and acetone.

The disappearance of formate during stationary phase, and the late appearance of H₂ and CO₂, suggested a cleavage of a formate intermediate. Addition of 0, 350, or 500 µmol sodium formate to tube cultures (10 ml) growing on 280 µmol glucose plus 100 µmol CaCO₃ resulted in accumulation of 470, 849, or 970 µmol H₂ and 803, 1251, or 1341 µmol CO₂, respectively.

The transient appearance of acetate prior to acetone production suggested that acetate serves as an intermediate in acetone formation. Addition of sodium acetate to tube cultures growing on glucose increased the extent of glucose consumption due to pH buffering, which resulted in a more complete utilization of glucose before the fermentation reached the minimum pH for catabolic activity (pH 4.9–5.1). Addition of sodium acetate also resulted in a preferential

Table 1. *Effect of CaCO₃ and sodium acetate on glucose consumption and solvent formation by B. macerans*

Cultures were grown for 6.8 d on CM5 medium containing 2% glucose and 0.2% yeast extract. Results are expressed as mean values from triplicate cultures.

Effector	Glucose consumed (mM)	Final pH	Product formed		Gas phase CO ₂ (mmol)	Ethanol/acetone
			Acetone (mM)	Ethanol (mM)		
None	31	5.1	12	44	0.7	3.6
CaCO ₃ (100 mM)	93	5.8	22	132	2.4	6.1
Sodium acetate (24 mM)	78	5.7	43	85	1.5	2.0
CaCO ₃ + sodium acetate	104	6.1	40	154	2.1	3.9

Fig. 1. Time course of product formation by *B. macerans* grown in a 12 l fermenter that contained 10 l CM5 medium plus 500 g glucose. Control of pH was achieved by automatic metered addition of NaOH. (a) Cell density (○), pH (●). (b) Residual glucose concentration (○); accumulated concentration of ethanol (●), acetate (□), and acetone (■); instantaneous rate of CO₂ (Δ) and H₂ (▲) production. The transient appearances of formate and acetaldehyde are not indicated.

Table 2. Effect of acetate addition on glucose fermentation by *B. macerans*

Cultures were grown for 5.8 d in CM5 medium containing 2% glucose and 0.2% yeast extract. Results are expressed as mean values of duplicate cultures.

Acetate (mm)		CaCO ₃ (mm) added	Product concentration (mm)		Ethanol/ acetone	Acetate consumed/ acetone formed	Final pH
Added	Consumed*		Acetone	Ethanol			
0	—	100	24	137	5.8	—	5.7
12	10	100	31	125	4.0	1.4	5.8
24	25	100	32	112	3.5	3.0	5.8
48	39	100	36	116	3.2	3.1	5.9
100	40	100	38	108	2.9	2.8	6.1
48	36	—	44	88	1.9	1.8	5.9

* Corrected for acetate concentration produced in control tubes without added acetate.

increase in acetone accumulation, and reversed the preferential ethanol production directed by CaCO₃ (Table 1). The increase in acetone accumulation observed after addition of sodium acetate exhibited a stoichiometry of 2.4 ± 0.8 acetate consumed per acetone formed (Table 2). Although acetate addition stimulated final acetone yield, it did not hasten the onset of acetone synthesis.

Effect of pH control on product formation

The observation that acetone production occurred only late in the fermentation after pH had declined, along with the preferential ethanol production observed during pH control with CaCO₃, suggested that an early event in the fermentation, related to a lowering of culture pH, was required to induce acetone synthesis.

The effect of pH on product formation was further investigated in 1.5 l batch culture containing 4–5% (w/v) glucose. An initial pH of 6.8 (occasionally higher) was used to permit a rapid onset of growth. In each fermentation, pH was controlled at a different preset lower

Fig. 2. Effect of lower limiting culture pH on the conversion of acetate to acetone by *B. macerans* ATCC 7068. Each point represents a separate fermenter run.

Table 3. Production of $^{14}\text{CO}_2$ by *B. macerans* from [^{14}C]glucose labelled in various carbon atoms

Cultures were grown in CM5 medium containing 0.5% glucose and 0.2% yeast extract.

Position of ^{14}C -label in glucose	Radioactivity [d.p.m. ($\mu\text{g-atom C}$) $^{-1}$]*		Ratio†
	Glucose	CO_2	
C-1	12687	404	0.032
C-3,4	3153	2169	0.687
C-6	12295	26	0.002

* d.p.m. ($\mu\text{g-atom C}$) $^{-1}$ for glucose equals d.p.m. ($\mu\text{g-atom C}$) $^{-1}$ for [$1\text{-}^{14}\text{C}$]glucose or [$6\text{-}^{14}\text{C}$]glucose and $0.5 \times$ d.p.m. ($\mu\text{g-atom C}$) $^{-1}$ for [$3,4\text{-}^{14}\text{C}$]glucose.

† Ratio of specific radioactivity of CO_2 /specific radioactivity (C atom glucose) $^{-1}$.

limiting value by automatic addition of NaOH. Samples withdrawn at intervals over a 4 d period were analysed for substrate and product concentrations. Two ratios were calculated to characterize carbon flux in the fermentations. The first ratio

$$[\text{acetone}]/([\text{acetone}] + 0.5[\text{acetic acid}])$$

provided a measure of the extent of acetate conversion to acetone. The second ratio

$$[\text{ethanol}]/([\text{ethanol}] + [\text{acetic acid}] + 2[\text{acetone}])$$

provided a measure of the relative conversion of the presumed intermediate acetyl-CoA to ethanol, versus the total conversion of acetyl-CoA to all products. For each fermentation, both ratios reached equilibrium values after approximately 50–60 h of incubation, and did not change significantly upon further incubation.

The extent of conversion of acetic acid to acetone increased with decreasing lower limiting pH, with no distinct break point in the curve (Fig. 2). At pH values of ≤ 5.2 , no residual acetate remained in the broth.

By contrast, the relative conversion of acetyl-CoA to ethanol was independent of the lower limiting pH value, and varied within the range 0.53–0.73 (mean value $\pm$ S.E.M. = 0.63 ± 0.06). The molar ratio of ethanol produced per glucose fermented was also independent of the lower limiting pH value, and varied within the range 0.95–1.34 (mean value $\pm$ S.E.M. = 1.11 ± 0.15).

Glucose fermentation pathway

Elucidation of the glucose fermentation pathway in *B. macerans* was achieved by determination of specific radioactivity of $^{14}\text{CO}_2$ formed from [^{14}C]glucose labelled in different carbon atoms (Table 3). More than two-thirds of the CO_2 arose from the C-3 and C-4 positions of glucose, while the C-1 and C-6 positions contributed only 3% and 0.2% of the CO_2 , respectively.

Table 4. *Enzyme activities in extracts of B. macerans cells harvested in exponential (16 h) and stationary (46 h) growth phases*

Enzyme	Assay pH	Specific enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		16 h Cultures	46 h Cultures
Formate dehydrogenase	5.7	15	47
	7.0	8	18
Pyruvate formate-lyase	7.0	276	300
Pyruvate dehydrogenase	5.7	0	0
	7.0	3	2

Growth yields

Exponential-phase cultures which had consumed 1.09 and 5.23 g glucose l⁻¹ exhibited an increase in cell dry weight of 147 and 746 mg l⁻¹, respectively. Thus, calculated molar growth yields were 24.3 and 25.7 g cells (mol glucose)⁻¹, respectively.

Effect of gas phase on growth

Growth of *B. macerans* ATCC 7068 in sealed tubes or bottles containing unreduced medium was accompanied by reduction of added resazurin indicator during the first few hours of growth, regardless of initial gas phase (N₂, CO₂, or air). In fermenter culture, this strain grew well in media gassed continuously with N₂, or if previously gassed with N₂ and incubated without gassing. However, no growth was observed during gassing with air. Cells growing under N₂ ceased growth upon initiation of air-sparging. Similarly, cultures streaked on to plates of CM5/ glucose agar grew when incubated under N₂, but grew poorly under air. Addition of reducing agents (0.5 g l⁻¹ each Na₂S.9H₂O and cysteine. HCl) did not alter the rate or extent of growth, substrate consumption, or product formation relative to control cultures without added reducing agent. Similar product balances were obtained with He and N₂ gas phases. However, incubation under 1 atm H₂ resulted in ethanol/acetone ratios of 6.2 ± 0.4, compared to 3.9 ± 0.5 for He and 3.9 ± 0.4 for N₂.

Enzyme activities in cell extracts

Enzyme activities for the metabolism of pyruvate and formate in cell extracts of *B. macerans* are shown in Table 4. Extracts contained an active pyruvate formate-lyase which required reducing conditions (e.g. 5 mM-DTT). Extracts also contained a formate dehydrogenase activity which utilized methyl viologen, but not NAD, NADP or ferredoxin as electron acceptor. Pyruvate dehydrogenase was detected only in trace quantities, and displayed similar responses to different electron carriers as did the formate dehydrogenase activity. The above enzymes displayed similar activities in extracts prepared from cells grown to either exponential or stationary phase. No activity was observed for lactate dehydrogenase (in either direction, using NAD(P) as electron carrier) or hydrogenase (using methyl viologen, benzyl viologen, NAD(P) or ferredoxin as electron acceptors).

DISCUSSION

Glucose fermentation by *B. macerans* ATCC 7068 was marked by two distinct metabolic phases, the primary phase characterized by cell growth and the formation of ethanol, acetic acid, and formic acid, and the secondary phase marked by continued ethanol production, conversion of acetate to acetone, and conversion of formate to H₂ and CO₂ (Fig. 1). The secondary phase appeared to be induced by events related to achievement of a low culture pH, as product ratios were related to the lower limiting culture pH (Fig. 2). Although the preferential production of ethanol in the presence of CaCO₃ (Table 2) may have been due in part to inhibition of acetone formation by increased amounts of CO₂, the observation that similar elevations in the ethanol/

Fig. 3. Glucose fermentation pathway of *B. macerans* growing under anaerobic conditions. Pyruvate formation via the Embden-Meyerhof pathway was determined by use of [^{14}C]glucose labelled in different carbon atoms. Other reactions were inferred from fermentation balances and from enzyme assays in cell extracts. Reactions involved in conversion of acetate to acetone remain to be elucidated, as do the *in vivo* electron carriers involved in oxidoreductase reactions. The stoichiometries are estimates based on attainment of a low culture pH, with subsequent complete conversion of acetate and formate to their respective products. Broken arrows indicate reactions which occur predominantly after exponential cell growth has been completed. Pyruvate dehydrogenase and pyruvate decarboxylase are not considered to be of quantitative importance in this generalized pathway.

acetone ratio were obtained with other pH control agents (e.g. NaOH) suggests that CaCO_3 preferentially enhanced ethanol production as a result of pH control. These data are in general agreement with those of other workers (Northrop *et al.*, 1919; Arzberger *et al.*, 1920; Speakman, 1925).

The product stoichiometries and the fate of specifically labelled [^{14}C]glucose (Table 3) suggest a glucose fermentation pathway for this organism as shown in Fig. 3. Glucose is catabolized primarily by the Embden-Meyerhof pathway to yield pyruvate, which may then be converted via acetyl-CoA to ethanol, acetate, and, ultimately, acetone.

The transient appearance of acetate prior to acetone production, along with the enhanced production of acetone observed upon addition of acetate, suggests that acetate is an intermediate in acetone formation. The fractions of CO_2 derived from the C-1, C-3,4 and C-6 positions of glucose yield a combined total of only 0.72, indicating that a significant portion of the total CO_2 is derived from the C-2 and C-5 positions of glucose and perhaps from yeast extract. The data suggest that acetone synthesis in this organism proceeds in a manner similar to that suggested for *C. acetobutylicum*, via stationary phase condensation of acetate-derived two-carbon units with subsequent decarboxylation to yield acetone (Johnson *et al.*, 1933; Wood *et al.*, 1945). The specific sequence of enzyme reactions which mediate the conversion of acetate to acetone, however, remains to be elucidated.

Production of H_2 from glucose by *B. macerans* apparently proceeded primarily via a formate intermediate. This is suggested firstly by the appearance of formate during exponential growth, and its subsequent disappearance in older cultures as H_2 and CO_2 are evolved; secondly, by the conversion of added formate to stoichiometric amounts of H_2 and CO_2 by cells growing on glucose; and thirdly, by the presence of an active pyruvate formate-lyase but only a very low level of pyruvate dehydrogenase in cell extracts.

Although a methyl viologen-linked formate dehydrogenase was demonstrated in cell extracts of *B. macerans*, attempts to measure H_2 evolution from formate in the presence of a variety of added electron carriers was unsuccessful, even when a sensitive polarographic assay for H_2 was employed. Hamilton & Wolfe (1959) have shown that resting cells of *B. macerans* produce H_2 from pyruvate or formate, but that cell extracts lacked this capacity despite the presence of an active pyruvate-formate exchange reaction. It is likely that a formic hydrogen lyase system similar to that in other facultative anaerobes (i.e., a formate dehydrogenase and hydrogenase complex which utilizes electron carriers other than pyridine nucleotides or ferredoxin) may

operate in *B. macerans* (Gray & Gest, 1968). The inability to detect the formic hydrogen lyase activity, suggested for *B. macerans* by the above physiological data, is probably due to the lack of the proper electron carriers in the diluted assay mixture. Gray & Gest (1968) have proposed that the low-potential *c*-type cytochromes involved in formate metabolism in other facultative anaerobes are not involved in *B. macerans*, due to the apparant inability of this organism to produce cytochromes during anaerobic growth.

The fermentation product ratios observed here indicate that *B. macerans* produces up to 1.3 ethanol per glucose fermented and converts over 50% of its acetyl-CoA to ethanol. These ratios suggest that reducing equivalents for ethanol synthesis are derived not only from oxidation of glucose to pyruvate, but also from metabolism of pyruvate or its subsequent products, formate and H₂. Although exogenous H₂ results in an increased ethanol yield during glucose fermentation in a closed system, this may be the result of end-product inhibition of hydrogenase with a consequent channelling of electrons from oxidation of pyruvate or formate to coenzyme-mediated ethanol production. Pyruvate as a direct source of reducing equivalents in ethanol production would require an active pyruvate dehydrogenase activity. Although cell extracts of *B. macerans* have been shown to catalyse a pyruvate-CO₂ exchange reaction (Hamilton & Wolfe, 1959), the current investigation revealed only traces of methyl viologen-linked pyruvate dehydrogenase, which could be a result of combined activities of pyruvate formate-lyase and formate dehydrogenase. Reducing equivalents for ethanol production might also be obtained from formate via a formate dehydrogenase reaction normally associated with the formic hydrogen lyase complex. Resolution of these possibilities will be aided by elucidation of the currently unidentified *in vivo* electron carriers for the relevant dehydrogenases (ethanol, formate, and possibly pyruvate dehydrogenases) in this organism.

An alternative hypothesis which explains the excess ethanol production is that a small portion of the pyruvate is metabolised via a pyruvate decarboxylase (EC 4.1.1.1) to produce CO₂ and acetaldehyde. The requirements for reducing equivalents to convert acetaldehyde to ethanol could then be met by the reduced pyridine nucleotides generated during conversion of glucose to pyruvate. However, metabolism of pyruvate in this manner proceeds without H₂ formation due to the unfavourable thermodynamics for conversion of reduced pyridine nucleotides (the only available source of electrons) to H₂. In contrast, *B. macerans* produced approximately 1.8 H₂ per glucose fermented, indicating that at least 90% of the pyruvate is catabolized via reactions which yield reduced products suitable for oxidation to H₂ (i.e., mediated by pyruvate formate-lyase and/or pyruvate dehydrogenase). Although a thiamine pyrophosphate-dependent pyruvate decarboxylase was not detected in cell extracts, the possibility of a minor involvement of this enzyme in pyruvate metabolism cannot be excluded by the data presented here.

It is instructive to compare the anaerobic glucose catabolism of *B. macerans* with that of two bacterial types which it most closely resembles, *C. acetobutylicum* and certain facultative anaerobes such as *Escherichia coli*. Both *B. macerans* and *C. acetobutylicum* are endospore-forming rods which produce a reduced, neutral solvent as the chief non-gaseous product, and acetone, H₂, and CO₂ as co-products (Prescott & Dunn, 1959). In both organisms, acetone formation occurs only during secondary metabolism, from acetate produced during the primary growth phase. There are, however, several important differences in the fermentative metabolism of these two organisms. Reducing equivalents generated during carbohydrate fermentation are disposed of as ethanol by *B. macerans*, and primarily as n-butanol and n-butyrate by *C. acetobutylicum*. The inability of *B. macerans* to produce n-butanol or n-butyrate during carbohydrate fermentation, or to reduce exogenous n-butyrate or n-butanol, suggests that this organism lacks the enzymes of the butanol pathway. Both organisms produce acetone only at low pH. However, while induction of acetone and n-butanol synthesis in *C. acetobutylicum* can be achieved by addition of acetate plus n-butyrate (total concentration 10 mM) to cultures maintained at pH 5.0 (Gottschal & Morris, 1981), addition of approximately 60 mM-acetate at pH 4.9–6.0 was not successful in hastening acetone synthesis in *B. macerans*.

Pyruvate metabolism and H₂ production in *B. macerans* resembles that of other facultative anaerobes (Gray & Gest, 1968). Pyruvate is cleaved by a pyruvate formate-lyase reaction to yield formate, which is subsequently oxidized by a formate dehydrogenase. Most of the reducing

equivalents thus generated are used in H_2 production, probably by a hydrogenase associated with a formic hydrogen lyase complex. Some of the reducing equivalents, however, appear to be utilized in other reductive reactions, especially ethanol production (Fig. 3). Pyruvate dehydrogenase appears to be of little importance in this organism. A direct comparison of pyruvate metabolism in *B. macerans* and *C. acetobutylicum* is not possible, owing to a lack of data on the latter system. Most *Clostridium* species catabolize pyruvate via a ferredoxin-linked pyruvate dehydrogenase to yield acetyl-CoA, CO_2 , and reduced ferredoxin, without the involvement of a formate intermediate (Gray & Gest, 1968; Hugo *et al.*, 1972; Thauer *et al.*, 1977). In this case, reduced ferredoxin is either used directly for H_2 production or is oxidized via a NAD/ferredoxin oxidoreductase to generate reduced electron carriers for other reductions (Thauer *et al.*, 1977). However, several *Clostridium* species contain both pyruvate dehydrogenase and pyruvate formate-lyase activities. The function of the latter, freely-reversible enzyme is thought to be either solely anabolic, as in *C. kluyveri* (Thauer *et al.*, 1972), or both anabolic and catabolic, as in *C. butyricum* (Thauer *et al.*, 1970). Pyruvate formate-lyase is also found in *C. butylicum* (Thauer *et al.*, 1972), a species which differs from *C. acetobutylicum* primarily in its ability to further reduce acetone to isopropanol.

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