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Hydrogen Utilization by Clostridia in Sewage Sludge

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A sporeformer morphologically different but physiologically similar to *Clostridium aceticum* Wieringa was isolated from sewage sludge. It used large amounts of H_2 and CO_2, converting them chiefly to acetic acid. Growth occurs anaerobically on yeast extract alone, but after the nutrients in yeast extract are used, growth continues at a reduced rate, supported by the conversion of the gases to acetate.

In 1936 Wieringa (10) isolated from Holland canal mud an anaerobic sporeformer, later named *Clostridium aceticum* (11), forming acetic acid and traces of formic acid in a medium containing H_2 and CO_2 plus mud extract and added minerals. Subsequent attempts to isolate sporeformers converting exogenous H_2-CO_2 to acetate have been generally unsuccessful, though Schoberth and Balch (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I90, p. 131) have grown a coccoid-to-rod-shaped nonsporeformer in continuous culture. *Butyribacterium rettgeri* (3), *Clostridium thermoaceticum* (5) and *Clostridium formico-aceticum* (2, 4) produce acetic acid from CO_2 and reductants generated during fermentation of carbohydrates, but there is no net utilization of H_2 added to the culture.

The conversion of H_2-CO_2 to acetate by *C. aceticum* has been presumed to support growth, but quantitative evidence is lacking.

During the isolation of *Methanobacterium formicicum* from sludge (9), Mylroie observed a rapid uptake of H_2-CO_2 by sludge (Ph.D. thesis, Washington State University, Pullman, 1953) and isolated a mesophilic clostridium that uses H_2-CO_2. Acetic acid but no methane was formed. Clostridia using H_2-CO_2 have been isolated several times from sludge at Davis. The present study was undertaken to test whether use of exogenous H_2-CO_2 increases the amount of growth.

MATERIALS AND METHODS

Cultural procedures. The anaerobic roll-tube technique with syringe injection was used (7). Any O_2 in gases was absorbed by passage over hot copper. Gases were mixed with the aid of flowmeters attached to the respective cylinders or were injected in required amounts via a syringe through the butyl-rubber stoppers closing the tooled-neck culture tubes (16 by 150 mm). Screw-capped culture tubes (15 by 125 mm) with a butyl rubber closure (Bellco Glass Co.) were also used. During preparation of the media, air was displaced with 20% CO_2 plus 80% H_2 or N_2. Cultures were incubated at 37°C in a horizontal position, either stationary on an incubator shelf or agitated on a tube roller or shaker.

The culture medium contained yeast extract (YE) in various concentrations, but usually contained 1 or 2 g/liter of tapwater containing 1 mg of resazurin. After the medium was autoclaved, a solution containing $NaHCO_3$, $Na_2S \cdot 9H_2O$, folic acid, and cobalamin was injected to give final concentrations of 0.8, 0.03, 0.00001, and 0.00001%, respectively. This was the basal medium. Complete medium was basal medium plus 5×10^{-7} M Na_2SeO_3, 10^{-6} M Na_2MoO_4, and 10^{-5} M $FeSO_4$ (1).

Strains were isolated from tubes of basal medium plus agar (1.5%) and peptone (0.5%) inoculated with serial dilutions of sludge from the first anaerobic digestor at the university sewage disposal plant or the comparable digestor at Woodland. Stocks were transferred by inoculating 0.1 ml of a liquid culture into 3 to 10 ml of liquid 0.1% YE basal or complete medium in a culture tube filled with 80% H_2-20% CO_2. Growth in liquid cultures was routinely followed by measuring the optical density (OD) at 600 nm on a Bausch and Lomb Spectronic-20 spectrophotometer. Cultures were examined microscopically for purity, and any contamination was eliminated by inoculating the agar dilution series. Contamination was rare, but in one instance it could be eliminated only by a crude pasteurization procedure consisting of inoculating into the hot medium as it came from the autoclave.

Measurement of gas uptake. At various times during incubation, cultures with H_2-CO_2 were tested for the disappearance of gas and H_2-CO_2 was replenished by injection. The injection procedure was to insert through the rubber closure a 1-inch (ca. 2.54 cm), 20-gauge needle attached to a 10-ml sterile glass syringe, either dry or lubricated with sterile water, containing sterile O_2-free gas. Residual air in the dead space of syringes was displaced by inserting the needle through a butyl rubber cap closing an outlet for the gas source, withdrawing the plunger,

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allowing the gas to flush out through the barrel of the syringe, and then inserting the plunger. The plunger was adjusted to the volume of gas to be added, the needle was withdrawn and immediately inserted through the rubber closure into the culture tube, and the gas was injected. The slight error due to the fact that, after injection, the gas in the dead space of the syringe was at greater than atmospheric pressure, was neglected.

In testing a culture presumed to have utilized gas, the test syringe was first filled with 5 or 10 ml of the sterile O_2-free gas, and the needle was inserted through the rubber closing the culture. Movement of the plunger as the needle tip penetrated the stopper showed the volume deficit or excess over atmospheric pressure. The effect of the plunger weight on the volume of the gas was negligible. In measuring the gas volume left at the conclusion of an experiment, a known volume of N_2 was injected, if necessary, to raise the pressure above atmospheric in order to read the volume at ambient pressure. The volume of gas used in experiments was initially determined by comparison with incubated uninoculated controls, but the volume of gases in the controls changed so little that this precaution was later omitted.

Analysis for CO_2 (including $NaHCO_3$) and H_2. After the culture tubes were brought to room temperature, the residual excess gas (including any added N_2) was measured. A solution of 5 M H_3PO_4 was injected in slight excess to convert the bicarbonate into CO_2, the tube was shaken to equilibrate liquid and gas phases, and the gas volume in the syringe was read. The increase in volume represented the residual bicarbonate. Dissolved CO_2 in the acidified system was calculated from the partial pressure of CO_2 in the gas phase. A solution of 2 M NaOH was then injected, and the tube was shaken to absorb all CO_2. The volume decrease, plus the calculated dissolved CO_2, measured the total CO_2 plus bicarbonate in the culture.

The gas left in the tube after CO_2 absorption was analyzed for H_2 on a thermal conductivity gas chromatograph (Perkin-Elmer 154-B) fitted with a silica gel column and operated at room temperature with N_2 as carrier gas.

Other analytical procedures. To analyze for total nitrogen in the cells, cultures were centrifuged at 13,000 × *g* for 30 min at 0°C, and the supernatant was discarded. The sedimented cells were washed with 70% ethanol twice and analyzed for total nitrogen by the micro-Kjeldahl method.

Formic and acetic acids were separated by vacuum distillation of the acidified culture to dryness at ca. 50°C in a closed system. The distillate was titrated and evaporated to dryness. The dried salts were taken up with 0.1 ml of purified propionic acid containing enough phosphoric acid to convert the salts to the free acids. One- to 6-μl portions were injected into a thermal conductivity gas chromatograph (F & M 700) equipped with a Poropak QS column. The propionic acid served both as a solvent and as an internal standard.

In experiments 4 of Table 3, all of the volatile fatty acids except formic acid were also determined by direct injection of acidified culture supernatant into an F & M 700 flame ionization gas chromatograph provided with an FFAP column with N_2 as the carrier gas.

RESULTS

After a 12-h incubation of the agar dilution series inoculated with sludge, numerous colonies had appeared in the lower dilution tubes, and by 24 h the decrease in gas volume was easily measurable. Gas continued to be used for several days if the H_2-CO_2 was replenished. Many prominent colonies were branched, some of them extensively, with a rhizoid to mycelioid appearance. Examples of the various types were isolated on basal agar medium and tested for gas absorption. Cultures of a number of the branched types took up H_2 in varying amounts, and two of the most active, strains 16 and 22, were isolated. Strain 22 has been more extensively studied.

Vegetative cells are gram-positive rods measuring 5.6 by 1.7 μm, single or in pairs and short chains. Oval spores are subterminal, swelling the cells only slightly. They appear after 24 h and are abundant in old cultures. If H_2-CO_2 remains in the tube, some vegetative cells are present even after weeks of incubation, and a slight absorption of gas continues. The culture counts for branched bacterial colonies in agar dilutions inoculated from various samples of sewage sludge are collected in Table 1. The spore culture counts were from pasteurized inoculum.

Some variations in growth, also encountered by Karlsson et al. (8), were ascribed to variable small amounts of air which gained access to the cultures during gas sampling and injection. One milliliter of air injected into a culture com-

TABLE 1. *Numbers of H_2-utilizing clostridia*

Expt	Investigator	Source of inoculum	Count (10^4 ml^{-1})
1	Ohwaki	Sewage digestor, Davis, Calif.	1.9
2	Saleh	Sewage digestor, Davis, Calif.	30[a] 20[b]
3	Saleh	Sewage digestor, Davis, Calif.	40[a] 35[b]
4	Ohwaki	Sewage digestor, Woodland, Calif.	0.04
5	Mylroie[c]	Sewage digestor, Pullman, Wash.	120 to 700

[a] Total count.
[b] Spore count.
[c] Ph.D. thesis, Washington State Univ., Pullman.

pletely inhibited growth and gas uptake, even though the sulfide in the medium was sufficient to absorb 0.25 ml of O_2. Doubling the size of the inoculum, use of glass instead of plastic syringes, increasing the concentration of YE, and stringent exclusion of air eliminated most of the problem. When duplicates differed, the one having the greater gas uptake almost invariably showed greater growth (OD).

When strain 22 was inoculated into tubes of basal medium with 80% H_2-20% CO_2 adjusted to various pH's by adding different amounts of $NaHCO_3$, there was greater growth and gas uptake at an initial pH of 8.0 than at pH 7.5 or 7.0 (Fig. 1 and experiment 1 of Table 2). In other experiments (Table 2), strain 22 showed more gas uptake at pH 7.8 than at pH 9.0, possibly because of the higher concentration of CO_2 at the lower pH. Provision of CO_2 at pH's more alkaline than 9.0 poses a problem in that the concentration of $NaHCO_3$ must be extremely high in order to have sufficient CO_2 for the reaction shown below.

$$4\ H_2 + CO_2 + NaHCO_3 \rightarrow CH_3COONa + 3\ H_2O$$

When $NaHCO_3$ was included but CO_2 was omitted from the medium, there was no growth.

A stimulation of growth by the trace minerals and by folic acid plus B_{12} could be demonstrated after strain 22 was carried through six transfers in 0.1% YE in distilled water.

Figure 2 shows the growth curves for strain 22 at various concentrations of YE under N_2-CO_2. Maximum OD was reached after 10 to 12 h, followed by an extended period of zero or negative growth. The rate of exponential growth was 1.5/h. During the active growth on YE under N_2, a little H_2 was produced, but almost all of it later disappeared.

During the first 12 h of incubation, cultures on 80% H_2-20% CO_2 always showed a slightly greater OD than did the N_2 control. In the H_2 cultures, there was some utilization of H_2 during this time, and the OD of H_2 cultures was always slightly greater than in those with N_2. After the 12-h period, the OD of cultures on N_2 always decreased, but with H_2 there was increased gas absorption and the OD increased up to 48 to 72 h, though at a greatly decreased rate as compared to the initial 12-h period. Figure 3 and experiments 1 and 4 in Table 3 show the magnitude of the increase, with the H_2 cultures showing a final OD two to three times greater than the maximal OD in the N_2 tubes. Note that in duplicate cultures A and B grown with H_2 (Fig. 3) there is a correlation between growth and gas utilization, a feature observed almost invariably when duplicate measurements of these parameters differed.

Direct microscopic examinations showed more cells in the H_2-CO_2 cultures of the same approximate size as in the N_2 controls. In one experiment, the OD of the H_2-CO_2 culture on 0.1% YE was 0.19 as compared to 0.068 with N_2-CO_2, and the total nitrogen contents were 3.54 and 1.18 μg/ml, respectively.

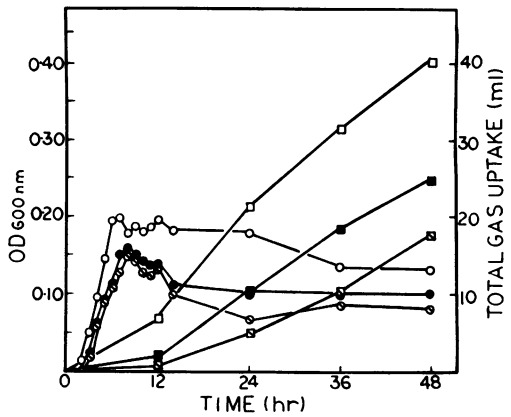

FIG. 1. *Effect of initial pH on growth of strain 22 in shaken cultures of complete medium containing 0.2% YE with 80% H_2-20% CO_2. Symbols: OD, initial pH of 8.0* (○); *gas uptake* (□); *OD, initial pH of 7.5* (●); *gas uptake* (■); *OD, initial pH of 7.0* (◑); *gas uptake* (◪).

TABLE 2. *Effect of pH on utilization of H_2-CO_2*

Expt	Initial pH	Medium	Duration of experiment (h)	Uncorrected vol of gas used[a] (ml)	Gas utilized/ml of culture (ml)	Final pH
1	7.0	0.2% YE broth	48	17.4	1.7	
	7.5	0.2% YE broth	48	24.9	2.5	
	8.0	0.2% YE broth	48	40.2	4.0	
2	7.8	0.2% YE agar, evenly rolled	72	31.5	6.3	7.2
	9.0	0.2% YE agar, evenly rolled	72	30.0	6.0	7.7
3	7.8	0.2% YE agar, evenly rolled	120	70.4	14.1	
	9.0	0.2% YE agar, evenly rolled	120	57.6	11.5	

[a] Average of duplicate cultures.

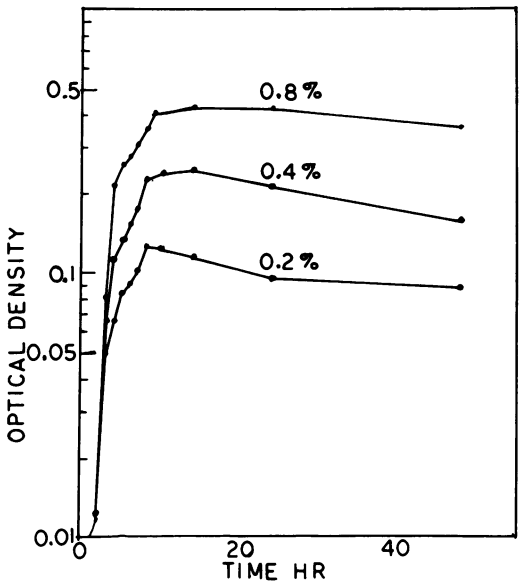

FIG. 2. *OD, plotted logarithmically against time, for cultures of strain 22 in basal medium containing 0.2, 0.4, and 0.8% YE, respectively.*

To test whether utilization of the gases would promote growth more effectively if the nutrients in YE were more continuously supplied, 3.65 ml of melted complete agar medium (experiment 2 of Table 3) was added to a culture tube that was rolled to coat the agar on the wall of the tube. Then 1.5 ml of liquid medium was added. In another experiment, to allow accurate measurement of OD (experiment 3 of Table 3), only the upper part of the tube was coated with the agar medium, with none on the lower 3 cm. Three milliliters of liquid medium was added, and the tubes were inoculated and incubated on the tube roller. Provision of part of the nutrients in the agar in experiments 2 and 3 prolonged the period of growth but did not significantly increase the total growth as compared to broth cultures.

Table 2 shows the results of all analyses for gases absorbed and products formed. The fermentation balances for the experiments of Table 3 are summarized in Table 4. In all of these experiments, traces of higher volatile fatty acids were detected, but there was no significant production of butyric acid. No production of propionic acid was detected in the flame ionization analyses of experiment 4.

The amount of H_2 used cannot be explained by reduction of the organic matter in YE with H_2, since the weight of acetic acid formed was between 1.8 and 6.1 times the total weight of YE in the medium.

DISCUSSION

Most rapid growth of strain 22 occurs between 0 and 12 h, chiefly at the expense of the nutrients in YE but with some augmentation of growth if H_2 is supplied in place of N_2. Growth after 12 h is slower and is supported chiefly by the conversion of H_2 and CO_2 into acetate. The amount of acetate formed is about that expected from the stoichiometry when H_2 and CO_2 are the substrates. The amount of acetate formed exceeds the total quantity of organic substrates provided.

The rhizoid-to-mycelioid type of colony reported by Mylroie (Ph.D. thesis) has correlated so consistently with high H_2 utilization that, together with rapid growth, reduced pressure, and absence of methane, it can tentatively identify strains in sewage sludge capable of using exogenous H_2. Not all our isolates continued to use large amounts of H_2 during continued subculture, but strains 16 and 22 have retained this capacity for over 3 years.

Part of the success in isolating these strains may lie in the omission of preliminary enrichment in liquid medium. Such enrichment changes the proportions in which the various natural strains occur (6) and may eliminate significant components of the population, or so reduce their proportion that they do not occur in the dilutions from which separate colonies can be picked.

Strain 22 possesses many of the physiological characteristics described for *C. aceticum* Wieringa (11), but fortunately it has been possible for Wieringa to examine strain 22, and he re-

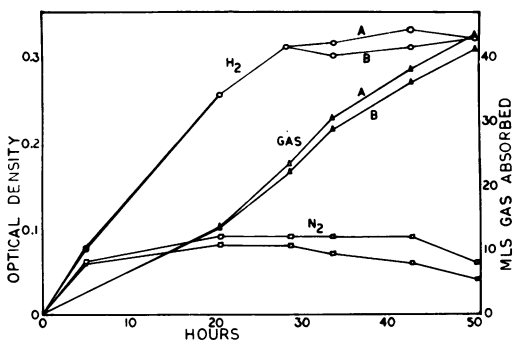

FIG. 3. *Effect of H_2 on growth of strain 22 in 5 ml of 0.2% YE in complete medium, rolled initially at 86 rpm, increased at 29 h to 114 rpm. H_2 cultures were prepared under H_2, and 10 ml of 80% H_2-20% CO_2 was added at the start, with additional H_2-CO_2 added as needed. N_2 cultures were prepared under N_2, and 2 ml of CO_2 was added at the start. Symbols: OD of H_2 cultures (○); OD of N_2 cultures (□); corrected volume of gas used by H_2 cultures (△).*

TABLE 3. *Substrates used and products formed by strain 22*

Expt	Incubation time (h)	Type of medium	Substrate provided (μg/ml)		Maximum increase in OD	Amt of gases used (μmol/ml)		Amt of acids produced (μmol/ml)		
			YE	Gas		H_2	CO_2	Formic	Acetic	5-C
1	50	5 ml of complete broth	1,800	H_2-CO_2	0.33	319	161	19	64 (3,840)[a]	1.0
			1,800	N_2-CO_2	0.11	0.22	26	2	8 (480)	0.11
2	90	3.65 ml of agar evenly rolled, 1.5 ml of broth	1,136	H_2-CO_2	0.50[b]	270	119	5	61 (3,660)	0.31
			1,136	N_2-CO_2	0.11	−0.09	−18	1	4 (240)	0.05
3	120	3.5 ml of agar upper rolled, 3 ml of broth	1,000	H_2-CO_2	0.38	362	133	11	103 (6,180)	0.04
			1,000	N_2-CO_2	0.16	−0.01	4	1	3 (180)	0.02
4	113	5 ml of broth	1,800	H_2-CO_2	0.45	444	193	10	110 (6,600)	0.74
			1,800	N_2-CO_2	0.14	−0.02	20	1	6 (360)	0.24
5	48	10 ml of broth	1,000 plus 1,000 CH[c]	H_2-CO_2	0.22	256	132	9	68 (4,080)	
				N_2-CO_2	0.13			4	6 (360)	

[a] The numbers are micrograms of acid produced.
[b] OD determined on a sample of withdrawn liquid culture.
[c] Casein hydrolysate.

TABLE 4. *Fermentation balances for H_2-CO_2 cultures*[a]

Expt	Substrates (μmol/ml)		H_2 and CO_2 accounted for in products				Substrate recovery (%)	
			Formate		Acetate			
	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2
1	319	135	17	17	224	112	76	95
2	270	137	4	4	228	114	86	86
3	362	129	10	10	400	200	113	163
4	444	173	9	9	416	208	96	125
5	256	132	5	5	248	124	99	98

[a] Values are the difference between the N_2 and H_2 cultures.

ports (personal communication through Rudolf Prins) that the subterminal ellipsoidal spores are quite different from the terminal spherical spores of *C. aceticum*. A culture of strain 22 has been sent to the Anaerobe Laboratory at Virginia Polytechnic Institute and also to the American Type Culture Collection.

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We are greatly indebted to Rudolf Prins for taking the culture to K. T. Wieringa at Wageningen, and to both for their efforts to resolve the question of identity with *C. aceticum*. The cultures counts of Mahmoud Saleh are also gratefully acknowledged.

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