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Effects of Cultivation Gas Phase on Hydrogenase of the Acetogen *Clostridium thermoaceticum*

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The effect of cultivation gas phase on the expression and activity of hydrogenase in heterotrophic cultures of *Clostridium thermoaceticum* was examined. Of the five gas phases tested, hydrogenase was maximal from cells cultivated under CO. Correlations were observed between the level of hydrogenase and the evolution of H_2 by growing cultures. Activity stains of polyacrylamide gels revealed a single hydrogenase band in CO_2 cells and multiple hydrogenase bands in CO cells.

Clostridium thermoaceticum is an acetogenic bacterium which has been the focal point of numerous studies to elucidate the path of homoacetate synthesis. Though originally isolated as a heterotroph (6), *C. thermoaceticum* is now known to possess considerable autotrophic properties (2, 5, 8, 11, 22, 27), and heterotrophic and autotrophic acetate synthesis merge at acetate III (Fig. 1). Carbon monoxide dehydrogenase and formate dehydrogenase are believed to play fundamental roles in acetogenic flow of both carbon and reductant (12, 18, 24, 28), although the physiological role of the recently discovered hydrogenase (4) remains less clear.

In a recent study, heterotrophically grown *C. thermoaceticum* was demonstrated to be competent in H_2 evolution when cultivation was under CO (18). Subsequently, we found that cell extracts from such cells had significantly higher levels of hydrogenase than did cells cultivated under CO_2. Considering the potential importance of hydrogenase in acetogenic energy metabolism, we initiated this study to ascertain the effects of cultivation gas phase on hydrogenase of *C. thermoaceticum*.

C. thermoaceticum was cultivated at 55°C in crimp-sealed 125-ml Wheaton serum bottles (total volume, 150 ml) containing 40 ml of glucose medium as previously described (18), except the phosphates were adjusted to 15 g of K_2HPO_4 and 15 g of KH_2PO_4 per liter. Sodium bicarbonate was not included in the medium to minimize the amount of bicarbonate-derived CO_2 in non-CO_2 cultures and to ensure a less variable initial pH. Medium (40 ml) was aseptically transferred to serum bottles, and after crimp sealing, the head space was replaced with the desired gas phase (filter sterilized), and the medium was prereduced with filter-sterilized sodium dithionite to a final concentration of 1 mM. The bottles were incubated at 55°C for 15 min before aseptic injection of 10% inoculum of log-phase cells which had been maintained in the phosphate-buffered medium under each of the gas phases tested. Cultures were not shaken or rolled during growth, and harvesting and preparation of cell extracts by lysozyme digestion in a Coy anaerobic chamber were as previously described (16). Cell extracts approximated 10 mg of protein per ml. Growth was monitored as the absorbancy at 660 nm, with a 1-cm path length. Hydrogenase assays, electrophoresis, and activity staining of polyacrylamide gels were performed by previously described methods (3, 4). Hydrogenase-specific activities were expressed as micromoles of H_2 oxidized per min per mg of protein. Protein was estimated by Coomassie brilliant blue staining (1), using bovine serum albumin as standard and lysozyme buffer (used to prepare cell extract) as the blank. Production of H_2 and consumption of CO were quantitated by thermal conductivity detection with a Hewlett Packard 5790A gas chromatograph and a Mole Sieve 13× 60-80 column (stainless steel; 2 m by 2 mm) with N_2 carrier gas (column and detector temperatures were 60 and 150°C, respectively). Consumption of CO_2 and H_2 were quantitated on Poropak Q 80-100; assay conditions were identical to those above. Gas solubilities at 55°C were calculated from standard tables, and the amount of gas produced or consumed was calculated by taking into account both gas and liquid phases (13).

Hydrogenase was maximal from cells cultivated in the presence of CO, the activity being five times that observed from cells cultivated under CO_2 (Table 1). Hydrogenase peaked in late-log phase and was reduced to near zero in stationary phase (Fig. 2). Considering the rather sharp activity profile of hydrogenase and its sensitivity to oxidation (4), it is not surprising that the enzyme initially went undetected in *C. thermoaceticum* (14).

Evolution of H_2 was concomitant with the increased level of hydrogenase of CO cultures; H_2 production from CO cells was eight- to ninefold greater than that of CO_2 cells (Table 1, culture A versus culture B). This CO-dependent evolution of H_2 is consistent with earlier findings (18) and suggests that H_2 production is hydrogenase mediated. The evolved reductant in CO cells may be glucose or CO derived; significantly, resting CO cells produced H_2 only if both glucose and CO were present (18).

When the initial cultivation gas phase was 100% H_2 (Table 1, culture D), no effect was observed on the level of hydrogenase. However, when an H_2-CO_2 (80:20) gas phase was utilized (culture E), hydrogenase was elevated and H_2 was consumed. Significantly, H_2 consumption was greatest in the presence of CO_2 (culture D versus culture E).

Cell extracts were subjected to polyacrylamide gel electrophoresis and stained for hydrogenase (Fig. 3). As in previous findings with bicarbonate cultures (4), cells cultivated under CO_2 yielded a single activity band (designated I). In contrast, a second hydrogenase band (designated II) was detected in CO cells. Regardless of cultivation gas phase, in situ CO dehydrogenase activity staining of polyacrylamide gels (3) revealed a single CO dehydrogenase band which did not coincide with the hydrogenase activity

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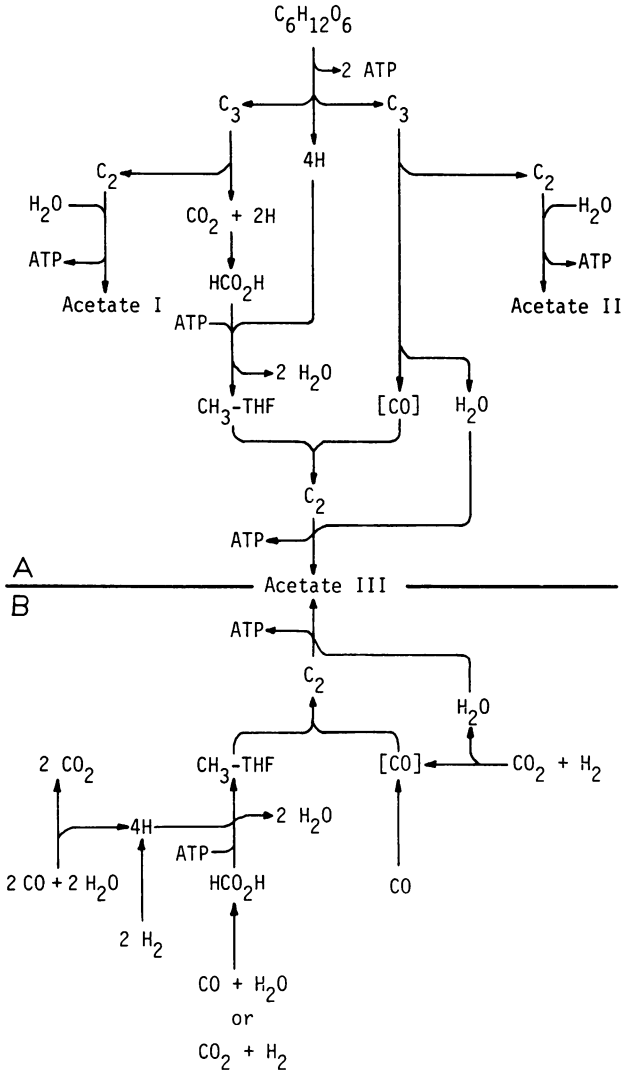

FIG. 1. Heterotrophic (A) and autotrophic (B) acetate synthesis by *C. thermoaceticum*. Abbreviations: THF, tetrahydrofolate; C_2, acetyl-coenzyme A, subsequently converted to acetate and ATP by the sequential actions of phosphotransacetylase (5) and acetate kinase (24); [CO], an enzyme-bound C_1 intermediate, the nature of which is currently unresolved.

bands (data not shown). Although the nature of the multiple hydrogenase bands observed from CO cells remains unknown, the multiple forms could be due to (i) activation or increased synthesis with subsequent aggregation of constitutive enzymes or (ii) synthesis of both constitutive and inducible enzyme forms. We are currently attempting to purify hydrogenase from *C. thermoaceticum* to address this matter.

Hydrogenase-mediated uptake of H_2 is prerequisite to autotrophic acetate synthesis from H_2 and CO_2 (Fig. 1B). Previous studies have demonstrated both H_2-dependent growth (11) and H_2-dependent acetate synthesis (22, 27) by *C. thermoaceticum*. In support of this role, H_2 consumption was also observed by growing heterotrophic cells in the present study (Table 1, cultures D and E). However, in heterotrophic cultures, the highest levels of hydrogenase occurred when cultivation was under CO and was concomitant with a decline in cell yield. This suggests that an increase in hydrogenase may occur in response to less than optimal heterotrophic environments.

TABLE 1. Hydrogenase levels from *C. thermoaceticum* cultivated under various gases

Culture	Initial gas phase	Hydrogenase (sp. act)[a]	Growth[b]	mmol of[c]:	
				H_2	CO_2
A	CO_2[d]	1.2	4.7	+0.4	+27.9
B	CO[d,e]	5.8[f]	3.3	+3.4	+35.4
C	N_2[d]	2.5	4.5	+1.9	+34.9
D	H_2[d]	1.0	5.1	−1.6	+4.4
E	H_2-CO_2[g]	2.3	6.0	−8.7	−7.3

[a] Maximum specific activity observed during growth.
[b] Maximum absorbancy at 660 nm observed.
[c] Millimoles of gas evolved (+) or consumed (−) per liter of liquid phase.
[d] 100%.
[e] 24.6 mmol of CO was consumed during growth.
[f] Specific activities as high as 25 have been observed from CO cells.
[g] 80:20.

In acetogenic synthesis of acetate, CO_2 serves as the principal terminal electron acceptor during homoacetate synthesis (Fig. 1). Environments rich in CO_2 may maintain this electron acceptor at levels approaching physiological saturation. Nevertheless, heterotrophic cultures produced CO_2 even when the initial cultivation gas phase was 100% CO_2 (Table 1, culture A). More (25 to 30%) CO_2 was produced when growth was under an environment not enriched with CO_2, i.e., with 100% N_2 (culture C). Furthermore, in N_2 cultures, production of H_2 was observed concomitant with the increased production of CO_2 (culture A versus culture C). When the initial gas phase was 100% CO, 25 mmol of CO per liter of culture was consumed during growth. Theoretically, CO is converted to acetate according

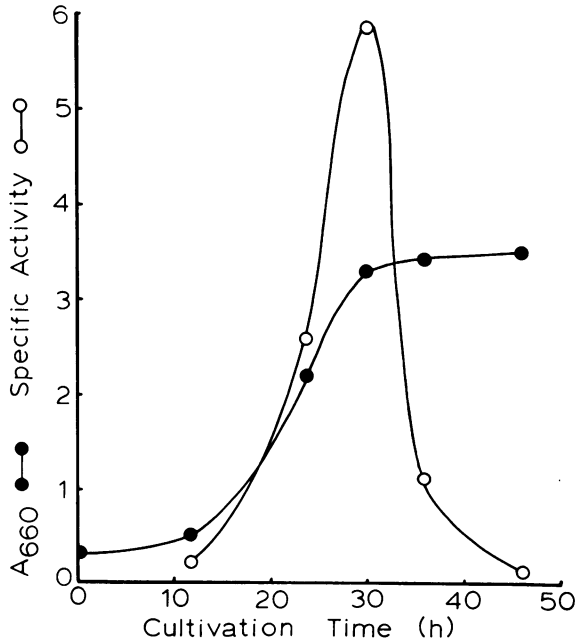

FIG. 2. Hydrogenase activity profile from CO cells. Culture was inoculated with late-log phase cells which had a specific activity of 5.5. A_{660}, Absorbancy at 660 nm.

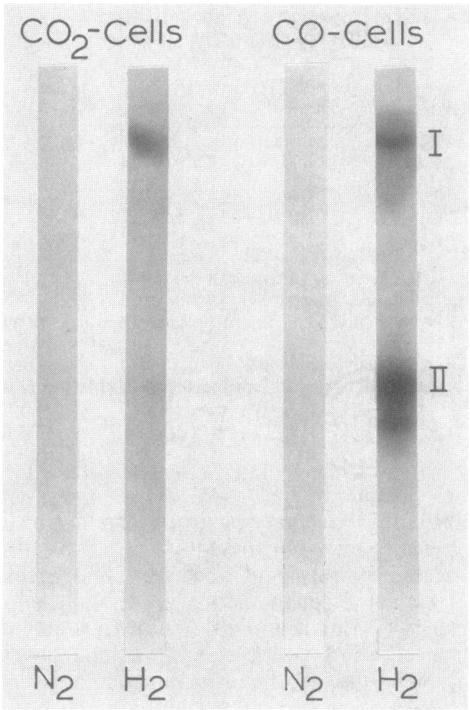

FIG. 3. Polyacrylamide gels of cell extracts from CO_2 or CO cells. Gels were stained for hydrogenase with methyl viologen and H_2; in control gels, H_2 was replaced with N_2 (4).

to the following stoichiometry: $4CO + 2H_2O \rightarrow CH_3CO_2H + 2CO_2$. Taking this into account, when the initial gas phase was 100% CO, the amount of CO_2 lost (evolved) from glucose fermentation was approximately the same as that observed during fermentation under 100% CO_2, the apparent increase being CO-derived (culture A versus culture B). Under such conditions, it is possible that CO dehydrogenase maintains a high intracellular level of CO_2 by CO oxidation. Conversely, in the presence of H_2 (cultures D and E), CO_2 was not evolved, indicating that flow (reduction) of CO_2 toward acetate is maximized with this exogenous reductant. Indeed, consumption rather than production of CO_2 was observed in H_2-CO_2 cultures, with H_2 consumption being stoichiometric to CO_2 consumption (culture E). In the present study, heterotrophic growth was maximized under such a gas phase.

We conclude from these and previous findings that hydrogenase of *C. thermoaceticum* functions in both the production and consumption of H_2 and that both heterotrophic and autotropic roles for the enzyme exist, subject to environmental conditions. Acetogenic hydrogenase might be involved in hydrogen cycling, interspecies hydrogen transfer, or oxidative phosphorylation (20, 26). That multiple hydrogenase bands are detected on polyacrylamide gels and environmental conditions influence their expression and activity add merit to such considerations. It is therefore of added interest that synthesis of active hydrogenase has been reported to be under redox control in a variety of microorganisms (7, 12, 17, 19, 21, 25) and that multiple hydrogenases have recently been demonstrated in the methanogens (9, 10).

Autotrophic synthesis of acetate would appear to yield no net ATP via substrate-level phosphorylation (Fig. 1B). This suggests that additional sites of ATP synthesis (e.g., electron transport phosphorylation) must exist en route to acetate, or consumption of ATP at the level of formyltetrahydrofolate synthetase is not mandatory in autotrophic growth, or both. A minimally defined medium has recently been developed for the cultivation of *C. thermoaceticum* (16), and studies utilizing minimally defined or autotrophic environments may yield additional insights on the physiological roles of hydrogenase in this acetogen.

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