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Carbonic Anhydrase in *Acetobacterium woodii* and Other Acetogenic Bacteria

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***Acetobacterium woodii*, *Acetohalobium arabaticum*, *Clostridium formicoaceticum*, and *Sporomusa silvacetica* were found to contain carbonic anhydrase (CA). Minimal to no CA activity was detected in *Moorella thermoautotrophica*, *Moorella thermoacetica* subsp. "*pratumsolum*," *Sporomusa termitida*, and *Thermoanaerobacter kivui*. Of the acetogens tested, *A. woodii* had the highest CA specific activity, approximately 14 U mg of protein^{-1}, in extracts of either glucose- or H_2-CO_2-cultivated cells. CA of *A. woodii* was cytoplasmic and was purified approximately 300-fold to a specific activity of 5,236 U mg of protein^{-1}. Intracellular acetate concentrations inhibited CA activity of *A. woodii* by 50 to 85%, indicating that intracellular acetate may affect in situ CA activity.**

Carbonic anhydrase (CA; carbonate hydrolyase, EC 4.2.1.1) is a zinc-containing enzyme found in animals, plants, bacteria, and archaea and catalyzes the following reaction: $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$ (8, 24, 33). Three classes (alpha, beta, and gamma) of CA have been resolved based on the amino acid sequence of the enzyme (1, 18). CA has multiple functions, including pH homeostasis, facilitated diffusion of CO_2, interconversion of CO_2 and HCO_3^-, and ion transport (8, 18, 37, 39, 42, 43). The crystal structures of human CA isoenzymes I and II, bovine isoenzymes II and III, murine CA isoenzyme V, and *Methanosarcina thermophila* CA have been determined (7, 20, 27, 49). Plant and animal CAs appear to be functionally equivalent. Most bacteria and archaea that contain CA can grow autotrophically, and highest CA levels are expressed under CO_2-limiting growth conditions (1, 24). The enzyme has been proposed to be involved in CO_2 or HCO_3^- uptake in bacteria that contain a periplasmic CA (1, 18). Aerobic bacteria that oxidize organic material to CO_2 generally do not contain CA (24). CA is induced in the methanogen *M. thermophila* during growth on acetate, and it has been proposed that CA of *M. thermophila* is required for acetate/H^+ symport, acetate/HCO_3^- antiport, or efficient removal of cytoplasmic CO_2 (1, 2, 24).

Acetogens are a heterogeneous group of obligately anaerobic bacteria that use the acetyl coenzyme A Wood/Ljungdahl pathway for the reductive synthesis of acetate from CO_2 (15, 47). CO_2 is the primary electron acceptor of acetogens and thus plays an important role in the energy conservation of this bacteriological group (14, 34). Because CA is known to play important roles in the metabolism of CO_2 in other microorganisms, the main objective of the present study was to determine if acetogens contain CA.

Cultivation of bacteria. All organisms were cultivated under anaerobic conditions in 1-liter infusion flasks containing 500 ml of medium; growth was measured as the optical density at 660 nm. *Thermoanaerobacter kivui* (DSM 2030), a thermophile belonging to cluster V of the *Clostridium* subphylum (11, 31, 46), was cultivated at 55°C in an undefined, carbonate-buffered medium (pH 6.5) containing 20 mM glucose (48). *Moorella thermoautotrophica* (DSM 1974) and *Moorella thermoacetica* subsp. "*pratumsolum*" (19) belong to cluster VI of the *Clostridium* subphylum (11, 19, 44, 46) and were cultivated at 55°C in an undefined, carbonate-buffered medium (pH 6.7) containing 10 mM fructose (12). *Clostridium formicoaceticum* (DSM 92) belongs to cluster XI of the *Clostridium* subphylum (3, 11, 46) and was cultivated at 37°C in an undefined, carbonate-buffered medium (pH 7.9) containing 10 mM fructose (36). *Sporomusa silvacetica* (DSM 10669) and *Sporomusa termitida* (DSM 4440) belong to cluster IX of the *Clostridium* subphylum (11, 29, 46). *S. silvacetica* was cultivated at 30°C in an undefined, carbonate-buffered medium (pH 6.8) containing 10 mM fructose (12, 29). *S. termitida* was cultivated at 30°C in an undefined, carbonate-buffered, dithiothreitol-reduced medium (pH 7.3) containing 20 mM mannitol (10). *Acetohalobium arabaticum* (DSM 5501) belongs to the family *Haloanaerobiaceae*, grows in 15 to 18% NaCl (50), and was cultivated at 37°C in an undefined medium (pH 7) containing 20 mM sodium lactate (26). *Acetobacterium woodii* (DSM 1030) belongs to cluster XV of the *Clostridium* subphylum (5, 11, 46) and was cultivated at 30°C in an undefined, carbonate-buffered medium (pH 6.7) containing 20 mM glucose (38). *A. woodii* was cultivated on a rotary shaker (100 rpm) to increase cell yields; specific activities of CA of *A. woodii* were not affected by shaking.

Cell extracts and CA assay. Cells were harvested at the end of exponential growth phase by centrifugation at 8,000 × *g*. Cell pellets were suspended in buffer A (50 mM sodium phosphate [pH 7.6] containing 1 μM zinc sulfate, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 μg of DNase I ml^{-1}) to a final concentration of 0.5 g (wet weight) of cells ml^{-1} and passed three times through a chilled French pressure cell at 3,000 kPa. Cell lysates were centrifuged at 20,000 × *g* for 15 min; the resulting supernatant fluids constituted the cell extracts. CA activity was measured electrometrically at room temperature (1, 45). The reaction mixture was at a volume of 1 ml and contained 20 mM Veronal-NaOH (pH 8.3), 1 μM zinc sulfate, and 10 to 100 μl of cell extract. For assays with highly active cell extracts (e.g., with extracts from *A. woodii*), 10 μl of cell extract was used per assay. The reaction was initiated by the addition of 0.5 ml of CO_2-saturated water; the

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TABLE 1. Specific activities of CA in cell extracts of acetogenic bacteria

Organism	Cultivation substrate	Sp act (U mg of protein^{-1})
A. woodii DSM 1030	Glucose	13.8 ± 1.2
	H_2-CO_2	15.2 ± 0.6
Acetohalobium arabaticum DSM 5501	Lactate	2.1 ± 0.4
C. formicoaceticum DSM 92	Fructose	1.4 ± 0.5
M. thermoacetica subsp. "*pratumsolum*"	Fructose	<0.02[a]
M. thermoautotrophica DSM 1974	Glucose	<0.02[a]
S. silvacetica DSM 10669	Fructose	0.8 ± 0.1
S. termitida DSM 4440	Mannitol	<0.02[a]
T. kivui DSM 2030	Glucose	0.12 ± 0.02[b]

[a] Below the detection limit of the CA assay.
[b] The specific activity increased slightly to 0.17 ± 0.04 U mg of protein^{-1} at 55°C.

time required for the pH to decrease from 8.0 to 7.0 (t) was recorded. Units of activity were calculated with the equation $(t_c - t)/t$, where t_c (control) was the time required for the pH to decrease from 8.0 to 7.0 without cell extract. Values are the means of three to five replicates. Protein was measured colorimetrically (9).

CA activities of various acetogens. The thermophiles *T. kivui*, *M. thermoautotrophica*, and *M. thermoacetica* subsp. "*pratumsolum*" and the termite gut acetogen *S. termitida* displayed minimal to no CA activity (Table 1). *S. silvacetica*, *C. formicoaceticum*, and *Acetohalobium arabaticum* displayed CA activities similar to those of other CA-containing bacteria (1, 24). *A. woodii* had an exceptionally high CA specific activity. Cultivation at the expense of glucose or H_2-CO_2 yielded similar CA activity levels in *A. woodii*. Throughout growth on glucose, the CA activity of *A. woodii* remained relatively constant (data not shown), indicating that CA activity did not change during different phases of growth. In addition, CA specific activities were not stimulated when *A. woodii* was cultivated on glucose under CO_2-limited conditions (i.e., in an undefined medium buffered with phosphate rather than carbonate salts and not containing supplemental CO_2). Acetazolamide (2 mM), a potent CA inhibitor (35, 41), had no apparent effect on glucose-dependent growth of *A. woodii*. However, growth on H_2-CO_2 by *A. woodii* was completely inhibited by 2 mM acetazolamide, suggesting that CA may be important for the optimal autotrophic growth of this acetogen. Because of the high CA activity in *A. woodii*, CA from this acetogen was chosen for further evaluation.

Characterization of the CA activity of *A. woodii*. Intact cells exhibited a much lower CA activity than did cell extracts (0.35 ± 0.1 [mean ± standard deviation] versus 13.8 ± 1.2 U mg of protein^{-1}, respectively). After centrifugation of cell extracts at 150,000 × g for 90 min, less than 0.5% of the CA activity was found in the particulate fraction; approximately 99% of the CA activity was recovered in the soluble fraction. Because acetazolamide (pK, 7.4) is only moderately diffusible through the cytoplasmic membrane (35, 41), inhibition of CA by acetazolamide should be progressive rather than immediate with intact cells if CA is cytoplasmic. Cell suspensions preincubated in buffer A containing acetazolamide showed a progressive decline in CA activity (Fig. 1). In contrast, cyanide (pK, 9.4), which in the protonated form freely permeates membranes and inhibits CA (24), instantaneously inhibited the CA activity of intact cells (Fig. 1). Collectively, these findings suggested that the CA of *A. woodii* is localized in the cytoplasm. CA activity in *A. woodii* cell extracts remained fully active after 6 months of storage at −20°C.

CA inhibitors (32) were found to have the following molar concentrations required for 50% inhibition of CA activity in cell extracts: sodium acetate, 1.1×10^{-1}; sodium formate, 3.0×10^{-2}; sodium azide, 8.8×10^{-5}; potassium iodide, 8.0×10^{-4}; potassium cyanide, 7.7×10^{-5}; sodium cyanate, 2.2×10^{-5}; acetazolamide, 4.4×10^{-6}; and sulfanilamide, 6.1×10^{-3}. Based on results with human erythrocyte CA isoenzyme II, acetate and formate bind by electrostatic attraction near the zinc atom in the hydrophobic part of the active site of CA (32). Acetate and formate were less effective inhibitors of *A. woodii* CA activity (50% inhibitory concentrations, 1.1×10^{-1} and 3.0×10^{-2} M, respectively) than the other anions tested. The intracellular acetate concentration of *A. woodii* during growth on glucose decreases from approximately 350 mM at the beginning of growth to approximately 145 mM at the end of growth (6). In CA assays with 350 and 150 mM acetate, the specific activities of CA in cell extracts were 14 and 45%, respectively, of that in control assays without acetate; these values imply that the intracellular levels of acetate might influence CA activities in situ. Intracellular acetate concentrations in *T. kivui* can be as high as 800 mM (28); although this acetogen had relatively low CA activity, it must also be noted that such a high intracellular concentration of acetate might reduce CA activity to negligible levels.

Purification of the CA of *A. woodii*. To verify the occurrence of CA in acetogens, CA activity from *A. woodii* was purified with fast protein liquid chromatography (FPLC) system LC500 (Pharmacia, Uppsala, Sweden). All purification steps were conducted under aerobic conditions at 4°C. Cell extract of glucose-cultivated cells was centrifuged at 150,000 × g for 1 h. Solid ammonium sulfate was added to the supernatant fluid to 1.5 M. The mixture was equilibrated for 30 min and then centrifuged at 18,000 × g for 15 min. The supernatant fluid was loaded on a 1- by 10-cm ethylamino-Sepharose column (22, 23) and batch eluted with 50 ml of buffer B (100 mM potassium phosphate [pH 7.6] containing 1 μM zinc sulfate, 0.1 mM PMSF, 1 mM dithiothreitol, and 1.5 mM ammonium sulfate). The eluate was centrifuged at 12,000 × g for 15 min, and the supernatant fluid was loaded at an injection rate of 0.5 ml

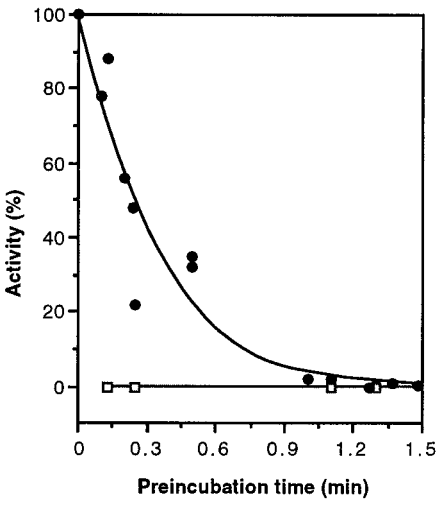

FIG. 1. Effects of acetazolamide (●) and cyanide (□) on the CA activity of *A. woodii* cell suspensions. Cells were preincubated with 2 mM acetazolamide or 2 mM cyanide for the times indicated and then tested for CA activity. The assay contained 7.8 mg of cell protein, and 100% activity equalled 2.7 U.

TABLE 2. Purification of CA from *A. woodii*

Purification step	Volume (ml)	Amt of protein (mg)	Sp act (U mg of protein^{-1})	Total activity (U)	Recovery (%)	Fold purification
Cell extract	2.3	81	18	1,450	100	1
Cytoplasmic fraction	2.0	57	21	1,206	83	1.2
Ammonium sulfate fraction	2.2	25	41	1,001	69	2.3
Ethylamino-Sepharose	50.0	17	59	996	68	3.3
Phenyl-Superose	4.0	0.36	1,808	651	45	101
Mono Q	2.0	0.11	5,236	576	40	293

min^{-1} onto a phenyl-Superose HR 10/10 FPLC column (Pharmacia) equilibrated with buffer B. The column was washed with 10 ml of buffer B. CA was subsequently eluted with a 47.5-ml decreasing linear gradient of 1.5 M $(NH_4)_2SO_4$ to buffer C (10 mM Tris-HCl [pH 7.6] containing 1 μM zinc sulfate, 0.1 mM PMSF, and 1 mM dithiothreitol) at 0.5 ml min^{-1}; the activity was eluted between 0.7 and 0.85 M $(NH_4)_2SO_4$. Active fractions were pooled, dialyzed against buffer C, and loaded onto a Mono Q HR 5/5 anion-exchange FPLC column (Pharmacia) equilibrated with buffer C. The column was washed with 10 ml of buffer C, and CA was subsequently eluted with a 47.5-ml increasing linear gradient of 0 to 1 M NaCl at 0.5 ml min^{-1}. The CA activity was eluted between 0.3 and 0.35 M NaCl.

CA activity was purified approximately 300-fold by this procedure, with an overall recovery of 40% (Table 2). Two prominent bands (approximately 20 and 30 kDa) were visible by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis and Coomassie brilliant blue R-250 staining (30). Further efforts to obtain a pure protein by gel filtration were not successful. CA from *M. thermophila* is 22.9 kDa (1); CA isoenzyme II from human erythrocytes is 30 kDa (4). The specific activity of the partially purified CA from *A. woodii* (5,236 U mg of protein^{-1}) approximated those of purified *M. thermophila* CA and purified human erythrocyte CA isoenzyme II (4,872 and 7,870 U mg of protein^{-1}, respectively) (1). CA of *A. woodii* remained fully active after several weeks of storage at 4°C.

Possible role of CA in acetogens. CO_2 is of particular importance to the acetyl coenzyme A pathway, and many acetogens cannot grow acetogenically under either heterotrophic or autotrophic conditions in the absence of exogenous CO_2 (3, 16, 40). Thus, one physiological function for CA in acetogens might be to increase intracellular CO_2 levels, as has been suggested for the cyanobacterium *Synechococcus* sp. strain PCC7942 (18). Such a CO_2-concentrating mechanism might be more essential when CO_2 must be used as both a carbon source and a terminal electron acceptor. Because acetogens produce large amounts of acetate during growth, CA might also be involved in regulating intracellular pH.

Certain bacteria that can metabolize acetate to CO_2 contain CA (24). These organisms take up acetate and excrete HCO_3^- or CO_2 and might contain an acetate/HCO_3^- antiporter (1, 24) that is similar to the Cl^-/HCO_3^- antiporters in erythrocytes and mucosa cells (13, 21). Acetogens must excrete acetate and take up HCO_3^- or CO_2. Thus, an acetate/HCO_3^- antiporter could also be envisioned for acetogenic bacteria, with cytoplasmic CA activity being indirectly coupled to acetate transport. Carrier-mediated acetate transport occurs in *A. woodii*, *Acetobacterium paludosum*, and *Acetohalobium arabaticum* (6, 26). Although the properties of the acetate carriers are unknown, energy conservation by acetate excretion has not been observed for acetogens (6, 17, 25). Acetate excretion by *T. kivui* appears to occur only by passive diffusion (26), and CA activity in *T. kivui* was minimal. CA activity was also negligible in the other thermophilic acetogens, *M. thermoautotrophica* and *M. thermoacetica* subsp. "*pratumsolum*." Because the equilibrium between CO_2 and HCO_3^- is achieved faster at higher temperatures, CA might not play a significant physiological role in thermophilic acetogens. The lack of detectable CA in *S. termitida* suggests that gut acetogens may contain minimal amounts of this enzyme. Environmental factors such as temperature, pH, and CO_2-HCO_3^- availability might influence the physiological role of CA in acetogens. A greater understanding of how such environmental factors impact acetogenesis and the in situ growth of acetogens might help explain why *A. woodii* has a comparatively high CA activity.

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