Tolerance and Metabolic Response of Acetogenic Bacteria toward Oxygen

Arno Karnholz, Kirsten Küsel, Anita Gößner, Andreas Schramm, and Harold L. Drake*

Department of Ecological Microbiology, BITOEK, University of Bayreuth, 95440 Bayreuth, Germany

Received 27 July 2001/Accepted 13 November 2001

The acetogens *Sporomusa silvacetica*, *Moorella thermoacetica*, *Clostridium magnum*, *Acetobacterium woodii*, and *Thermoanaerobacter kivui* (i) grew in both semisolid and liquid cultivation media containing O₂ and (ii) consumed small amounts of O₂. Low concentrations of O₂ caused a lag phase in growth but did not alter the ability of these acetogens to synthesize acetate via the acetyl coenzyme A pathway. Cell extracts of *S. silvacetica*, *M. thermoacetica*, and *C. magnum* contained peroxidase and NADH oxidase activities; catalase and superoxide dismutase activities were not detected.

Acetogens have been termed obligate or strict anaerobes. They do not grow aerobically, are isolated mostly from anoxic habitats, and utilize a pathway (the acetyl coenzyme A pathway) that contains enzymes that are extremely sensitive to O₂ (6, 7, 22). However, acetogens (i) can be readily isolated from leaf litter and the mineral soil of well-drained, oxic soils (8, 9), (ii) can tolerate periods of oxygenation in soils (21), (iii) are active in termite guts that have steep oxygen gradients (20), and (iv) occur in high numbers in transiently oxygenated rhizosphere sediments colonized by sea grass (12). These observations suggest that certain acetogens must cope with O₂ under in situ conditions. In preliminary studies, the classic acetogen *Moorella thermoacetica* was found to reduce resazurin in O₂-supplemented medium (A. Gößner and H. L. Drake, unpublished data), and the objective of the present study was to determine the tolerance and metabolic response of model acetogens toward O₂ (for a preliminary report of this study, see A. Karnholz, K. Küsel, and H. L. Drake, Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000, abstr. I-91, p. 401, 2000).

Organisms, media, and growth conditions. The acetogens used in this study were selected because each is a well-described model acetogen that has been isolated from a different habitat. The temperatures of incubation for *Sporomusa silvacetica* (DSM 10669; isolated from soil), *M. thermoacetica* (DSM 1974; isolated from horse manure), *Clostridium magnum* (DSM 2767; isolated from fresh water sediment), *Acetobacterium woodii* (DSM 1030; isolated from a marine estuary), and *Thermoanaerobacter kivui* (DSM 2030; isolated from lake sediment) were 30, 55, 30, 30, and 55°C, respectively. The acetogens were cultivated in a carbonate-buffered, undefined (U) medium containing yeast extract, vitamins, and trace metals but no reducing agents (4). Medium was dispensed under CO₂ into 27-ml crimp seal culture tubes (7 ml of medium per tube) or 1-liter infusion bottles (500 ml of medium per bottle; used for preparation of cell extracts), which were then sealed and autoclaved; the pH was approximately 6.7. Anoxic aqueous stock solutions of glucose or fructose (prepared under argon)

were filter sterilized and added to the medium by syringe injection by using O₂-free techniques. Culture tubes and bottles containing liquid medium were incubated in a horizontal, static position. Culture tubes were shaken vigorously before optical densities were measured. *Escherichia coli* K-12 (DSM 423) was cultivated aerobically in nutrient broth (8 g per liter; Difco Laboratories, Detroit, Mich.) at pH 6.8 and 37°C.

Measurement of O₂ in semisolid medium with microsen-
sors. Semisolid U medium was U medium supplemented with 5 g of Gelrite (Carl Roth GmbH, Karlsruhe, Germany) per liter. The 27-ml crimp seal culture tubes containing 10 ml of semisolid U medium were autoclaved, cooled to approximately 50°C, and then inoculated with 0.5 ml of freshly grown culture. As soon as the inoculated medium had solidified at room temperature, sterile O₂ was injected by syringe until a final concentration of 21% (vol/vol) was reached. The tubes were incubated vertically, and O₂ in the semisolid medium was measured with a microelectrode immediately after the tubes were opened. The microelectrode setup consisted of a micromanipulator-controlled O×10 sensor (5- to 10-μm tip diameter; Unisense, Aarhus, Denmark) and a PA 2000 picoammeter (Unisense) that was attached to a strip chart recorder. The microelectrode was calibrated with air-saturated (for 100% control) and N₂-saturated (for 0% control) water that had the same temperature as the culture tubes (i.e., either 30 or 55°C), and O₂ concentrations were calculated according to standard tables (<http://www.unisense.com/support/pdf/gas-tables.pdf>). The detection limit was 1 μM O₂. Since tubes were only assayed once, a series of replicate tubes were inoculated for each organism so that O₂ concentrations could be assessed periodically.

Enzyme assays and analytic methods. Cell extracts were prepared under anoxic conditions (10). Cell extracts used for enzyme assays were obtained from cells grown in U medium supplemented with 10 mM fructose (for *S. silvacetica*) or 10 mM glucose (for *M. thermoacetica* and *C. magnum*) and with 0.5% (vol/vol) O₂ in the gas phase. Catalase, peroxidase, NADH oxidase, and superoxide dismutase activities were assayed according to standard protocols (1, 2, 18, 19) at room temperature (22°C). The assays with extracts from *M. thermoacetica*, however, were conducted at 50°C. The catalase, peroxidase, NADH oxidase, and superoxide dismutase activi-

* Corresponding author. Mailing address: Department of Ecological Microbiology, BITOEK, University of Bayreuth, 95440 Bayreuth, Germany. Phone: 49-0-921-555-640. Fax: 49-0-921-555-793. E-mail: harold.drake@bitoe.uni-bayreuth.de.

FIG. 1. O₂ gradients in cultures of *S. silvacetica* (A), *M. thermoacetica* (B), and *C. magnum* (C) in semisolid U medium with headspaces containing 21% (vol/vol) O₂. Tubes were incubated vertically. Open and solid symbols are O₂ in uninoculated and inoculated culture tubes, respectively. Gradients were determined at the following times subsequent to incubation: 1 (circles), 6 (triangles), and 48 (squares) h (A); 1 (circles), 6 (triangles), and 72 (squares) h (B); 1 (circles), 6 (triangles), and 12 (squares) h (C). Growth had reached a maximum height at the last time interval.

ties are expressed, respectively, in the following units: micromoles of H₂O₂ consumed per minute, milligrams of pyrogallol oxidized per minute, micromoles of NADH oxidized per minute, and micromoles of nitrotriazolium blue chloride not reduced per minute. Growth was measured as the optical density at 660 nm (OD₆₆₀); the optical path width (i.e., the inner diameter of the culture tubes) was 1.6 cm. Uninoculated medium served as a reference. Protein in cell extracts was deter-

FIG. 2. Effect of O₂ on the growth of *S. silvacetica* (A), *M. thermoacetica* (B), and *C. magnum* (C) cultivated in nonreduced U medium. Culture tubes were incubated horizontally and shaken prior to each measurement. The initial concentrations (percentages by volume) of O₂ in the headspaces of culture tubes were as follows: 0 (●), 1.1 (○), 1.9 (▼), and 2.8 (▽) (A); 0 (●), 0.5 (○), 1.0 (▼), and 2.0 (▽) (B); 0 (●), 1.0 (○), 1.9 (▼), and 2.9 (▽) (C). Data points are the mean values of three replicate cultures.

mined colorimetrically (3). The amounts of substrates and products present in culture fluids and headspaces were determined by high-performance liquid chromatography and gas chromatography (4, 11, 17). The results are representative of replicate experiments.

FIG. 3. Growth and substrate-product profiles of *C. magnum* cultivated in nonreduced U medium with (open symbols) and without (solid symbols) O₂ in the headspace. (A) Circles, O₂; squares, optical density; triangles, H₂. (B) squares, glucose; circles, acetate. Data points are the average values of duplicate cultures.

Effect of O₂ on the growth of acetogens. When nonreduced, semisolid medium was inoculated with *S. silvacetica*, *M. thermoacetica*, *C. magnum*, *A. woodii*, or *T. kivui* and then incubated with a gas phase that contained 21% (vol/vol) O₂ (equivalent to the concentration of O₂ in air), the extent of the oxidized surface of the medium was less in inoculated tubes than it was in uninoculated controls (Fig. 1 and data not shown). Growth, determined visually as an increase in opacity or in the formation of colonies within the semisolid medium was apparent only in the portions of the tubes with negligible O₂. For example, as shown in Fig. 1C, the growth of *C. magnum* at 12 h was apparent at and below a depth of 13 mm, the portion of the tube where O₂ was not detectable, but growth was not apparent between depths of 0 and 12 mm, where O₂ was detectable. Culture tubes were under positive pressure during incubation, and the slight increase (i.e., peak) in the concentration of O₂ in the first few millimeters of the semisolid medium was due to the initial release of O₂ as the medium equilibrated to atmospheric pressure when the tubes were opened.

S. silvacetica, *M. thermoacetica*, *C. magnum*, *A. woodii*, and *T. kivui* also grew in nonreduced liquid culture medium containing small amounts of O₂ in the gas phase (Fig. 2 and data not shown). Increasing amounts of O₂ caused an increase in the lag phase of growth and a decrease in the final optical densities achieved. As illustrated by the large error bars in some of the growth curves in Fig. 2, growth became less re-

TABLE 1. Effect of O₂ on growth and product profiles of acetogens cultivated in U medium^a

Acetogen	O ₂ (% [vol/vol] in headspace)		Max ΔOD ₆₆₀ ^b	Substrate consumed (mM)	Acetate produced (mM) ^c	Acetate/substrate ratio ^d	Acetate/biomass ratio ^e
	Initial	Final					
<i>S. silvacetica</i>	0.0	0.0	0.47 (60)	4.5 ^f	11.4	2.5	24
	0.6	0.0	0.46 (64)	4.6 ^f	11.7	2.5	25
	1.0	0.2	0.43 (78)	4.5 ^f	11.9	2.6	28
<i>M. thermoacetica</i>	0.0	0.0	0.33 (86)	5.2 ^g	13.3	2.6	40
	0.2	0.0	0.29 (104)	5.0 ^g	12.5	2.5	43
	0.7	0.3	0.15 (138)	5.4 ^g	12.9	2.4	86
<i>C. magnum</i>	0.0	0.0	0.55 (12)	3.6 ^g	11.0	3.1	20
	0.5	0.1	0.46 (15)	3.5 ^g	11.1	3.2	24
	0.9	0.2	0.40 (26)	3.5 ^g	11.0	3.1	28

^a Values are the means of data for three replicate experiments.

^b Values in parentheses are the times (in hours) required to reach the maximum OD.

^c Values are corrected with data for control cultures lacking substrates.

^d The theoretical ratio of acetate produced to substrate consumed for homoacetogenesis is 3.0.

^e The concentration of acetate produced (millimolar) divided by the OD₆₆₀ of the culture.

^f The substrate was fructose.

^g The substrate was glucose.

TABLE 2. Oxidative stress enzymes in cell extracts

Organism	Enzyme level (mU/mg of protein) ^a			
	Catalase	Superoxide dismutase	Peroxidase	NADH oxidase
<i>S. silvacetica</i>	0	0	0.34 ± 0.01	8.0 ± 0.6
<i>M. thermoacetica</i>	0 ^b	0 ^b	1.13 ± 0.15	32.0 ± 1.2
<i>C. magnum</i>	0	0	0.06 ± 0.02	141 ± 6
<i>E. coli</i> K-12	22,527 ± 657	0.51 ± 0.16	66 ± 4	220 ± 28

^a Values are the means ± standard deviations of data from three replicate experiments.

^b No activity was observed at room temperature as well.

producible once the concentration of O₂ became at least partially inhibitory. Of the five acetogens tested, *A. woodii* and *T. kivui* were the most sensitive to O₂ and did not grow in non-reduced U medium when O₂ in the gas phase exceeded 0.3 and 0.5% (vol/vol), respectively.

Metabolic response of acetogens to O₂. *C. magnum* displayed the shortest doubling times of the acetogens examined (Fig. 2), so it was chosen for a detailed evaluation of the potential effects of O₂ on the formation of products during growth (Fig. 3). When the gas phase of cultures was supplemented with approximately 1% O₂ (vol/vol), O₂ was consumed throughout lag, log, and stationary phases of growth and trace levels of H₂ were produced (Fig. 3A). O₂ was not consumed in uninoculated controls. In contrast to the case with anoxic cultures, the initial presence of O₂ in the gas phase of cultures caused a delay in the conversion of glucose to acetate; however, the final ratio of acetate produced to glucose consumed for both anoxic controls and O₂-supplemented cultures approximated 3 (Fig. 3B), a value indicative of homoacetogenesis, C₆H₁₂O₆ → 3 CH₃COOH.

S. silvacetica, *M. thermoacetica*, *A. woodii*, and *T. kivui* also consumed low amounts of O₂ (Table 1 and data not shown). As with *C. magnum*, the presence of low amounts of O₂ did not alter the ratios of acetate produced to glucose consumed for *S. silvacetica* and *M. thermoacetica*. Acetate-to-biomass ratios can be used to evaluate the bioenergetics and growth efficiencies of acetogens (4). In general, the amount of acetate production that was required for growth increased when the headspaces of cultures was supplemented with O₂ (Table 1), indicating that maintenance energy requirements increased in response to oxidative stress.

Enzyme activities possibly associated with aerotolerance. Cell extracts of *S. silvacetica*, *M. thermoacetica*, and *C. magnum* contained various levels of peroxidase (which consumes H₂O₂) and NADH oxidase (which consumes O₂) activities; catalase (which consumes H₂O₂) and superoxide dismutase (which consumes O₂^{•−}) activities were not detected (Table 2).

Concluding remarks. The capacity of so-called strict anaerobes to tolerate and consume trace levels of O₂ was first demonstrated for sulfate-reducing bacteria (16). Since acetogens occur in, and can be easily isolated from, habitats that are subject to transient fluxes of O₂, it is not surprising that acetogens have the ability to consume low amounts of O₂ and to grow in medium supplemented with low concentrations of O₂. In addition to the acetogens evaluated in the present study, *Clostridium glycolicum* RD-1, an abundant culturable acetogen that inhabits the sea grass root, also displays tolerance to O₂

(13). *C. glycolicum* RD-1 can tolerate up to 6% (vol/vol) O₂ in the headspace of cultures and undergoes a metabolic shift towards classic ethanol and lactate fermentations when challenged with oxic conditions (13). It has recently been reported that methanogens and acetogens that inhabit the termite gut can likewise tolerate and consume traces of O₂ (14; H. Boga and A. Brune, Abstr. Annu. Meet. Verein. Allgem. Angewand. Mikrobiol., Biospectrum, Abstr. 15. P. 11. 33, p. 143, 2000).

The occurrence of NADH oxidase activity in cell extracts indicates that the acetogens tested can catalytically consume O₂. O₂ can yield H₂O₂ and O₂^{•−} within cells, and several enzymes are known to rid cells of these toxic products. Peroxidase activity was low but detectable. The absence of catalase and superoxide dismutase in acetogens was not entirely unexpected, since these enzymes produce O₂. Rubrerythrin (which consumes H₂O₂) and rubredoxin oxidoreductase (which consumes O₂^{•−}) are alternative oxidative-stress defense proteins in the sulfate reducer *Desulfovibrio vulgaris* and do not produce O₂ (15). Genes for similar proteins have been identified in *M. thermoacetica* (5). A full understanding of the biochemical processes that are responsible for the aerotolerance of acetogens will require additional investigations.

We thank Carola Matthies for helpful discussions and evaluation of the manuscript.

Support for this study was provided by the German Ministry of Education, Science, Research, and Technology (PT BEO 51-0339476C).

REFERENCES

- Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**:276–287.
- Beers, R. F., Jr., and I. W. Sizars. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195**:133.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Daniel, S. L., T. Hsu, S. I. Dean, and H. L. Drake. 1990. Characterization of the H₂- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoacetatum* and *Acetogenium kivui*. *J. Bacteriol.* **172**:4464–4471.
- Das, A., E. D. Coulter, D. M. Kurtz, Jr., and L. G. Ljungdahl. 2001. Five-gene cluster in *Clostridium thermoacetatum* consisting of two divergent operons encoding rubredoxin oxidoreductase-rubredoxin and rubrerythrin—type A flavoprotein—high-molecular-weight rubredoxin. *J. Bacteriol.* **183**:1560–1567.
- Drake, H. L. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA Wood/Ljungdahl pathway: past and current perspectives, p. 3–60. In H. L. Drake (ed.), *Acetogenesis*. Chapman and Hall, Inc., New York, N.Y.
- Drake, H. L., K. Küsel, and C. Matthies. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Leeuwenhoek, in press.
- Göfner, A. S., R. Devereux, N. Ohnemüller, G. Acker, E. Stackebrandt, and H. L. Drake. 1999. *Thermicanus aegyptius* gen. nov., sp. nov., isolated from oxic soil, a fermentative microaerophile that grows commensally with the thermophilic acetogen *Moorella thermoacetica*. *Appl. Environ. Microbiol.* **65**:5124–5133.
- Kühner, C. H., C. Frank, A. Griefhammer, M. Schmittroth, G. Acker, A. Göfner, and H. L. Drake. 1997. *Sporomusa silvacetica* sp. nov., an acetogenic bacterium isolated from aggregated forest soil. *Int. J. Syst. Bacteriol.* **47**:352–358.
- Kühner, C. H., C. Matthies, G. Acker, M. Schmittroth, A. S. Göfner, and H. L. Drake. 2000. *Clostridium akagii* sp. nov. and *Clostridium acidisoli* sp. nov.: acid-tolerant, N₂-fixing clostridia isolated from acidic forest soil and litter. *Int. J. Syst. Evol. Microbiol.* **50**:873–881.
- Küsel, K., and H. L. Drake. 1995. Effects of environmental parameters on the formation and turnover of acetate by forest soils. *Appl. Environ. Microbiol.* **61**:3667–3675.
- Küsel, K., H. C. Pinkart, H. L. Drake, and R. Devereux. 1999. Acetogenic and sulfate-reducing bacteria inhabiting the rhizosphere and deep cortex cells of the sea grass *Halodule wrightii*. *Appl. Environ. Microbiol.* **65**:5117–5123.
- Küsel, K., A. Karnholz, T. Trinkwalter, R. Devereux, G. Acker, and H. L.

- Drake.** 2001. Physiological ecology of *Clostridium glycolicum* RD-1, an aerotolerant acetogen isolated from sea grass roots. *Appl. Environ. Microbiol.* **67**:4734–4741.
14. **Leadbetter, J. R., and J. A. Breznak.** 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. *Appl. Environ. Microbiol.* **62**:3620–3631.
15. **Lumppio, H. L., N. V. Shenvi, A. O. Summers, G. Voordouw, and D. M. Kurtz, Jr.** 2001. Rubrerythrin and rubredoxin oxidoreductase in *Desulfovibrio vulgaris*: a novel oxidative stress protection system. *J. Bacteriol.* **183**:101–108.
16. **Marschall, C., P. Frenzel, and H. Cypionka.** 1993. Influence of oxygen on sulfate reduction and growth of sulfate-reducing bacteria. *Arch. Microbiol.* **159**:168–173.
17. **Matthies, C., A. Freiberger, and H. L. Drake.** 1993. Fumarate dissimilation and differential reductant flow by *Clostridium formicoaceticum* and *Clostridium aceticum*. *Arch. Microbiol.* **160**:273–278.
18. **Stanton, T. B., and N. S. Jensen.** 1993. Purification and characterization of NADH oxidase from *Serpulina* (*Treponema*) *hyodysenteriae*. *J. Bacteriol.* **175**:2980–2987.
19. **Stellmach, B., W. Gottschick, F. Battermann, and K. Zabel.** 1988. Bestimmungsmethoden enzyme, p. 222–223. Steinkopff Verlag, Darmstadt, Germany.
20. **Tholen, A., and A. Brune.** 1999. Localization and in situ activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). *Appl. Environ. Microbiol.* **65**:4497–4505.
21. **Wagner, C., A. Griefshammer, and H. L. Drake.** 1996. Acetogenic capacities and the anaerobic turnover of carbon in a Kansas prairie soil. *Appl. Environ. Microbiol.* **62**:494–500.
22. **Wood, H. G., and L. G. Ljungdahl.** 1991. Autotrophic character of acetogenic bacteria, p. 201–250. In J. M. Shively and L. L. Barton (ed.), *Variations in autotrophic life*. Academic Press, San Diego, Calif.