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Physiological Ecology of *Clostridium glycolicum* RD-1, an Aerotolerant Acetogen Isolated from Sea Grass Roots

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An anaerobic, H_2-utilizing bacterium, strain RD-1, was isolated from the highest growth-positive dilution series of a root homogenate prepared from the sea grass *Halodule wrightii*. Cells of RD-1 were gram-positive, spore-forming, motile rods that were linked by connecting filaments. Acetate was produced in stoichiometries indicative of an acetyl coenzyme A (acetyl-CoA) pathway-dependent metabolism when RD-1 utilized H_2-CO_2, formate, lactate, or pyruvate. Growth on sugars or ethylene glycol yielded acetate and ethanol as end products. RD-1 grew at the expense of glucose in the presence of low initial concentrations (up to 6% [vol/vol]) of O_2 in the headspace of static, horizontally incubated culture tubes; the concentration of O_2 decreased during growth in such cultures. Peroxidase, NADH oxidase, and superoxide dismutase activities were detected in the cytoplasmic fraction of cells grown in the presence of O_2. In comparison to cultures incubated under strictly anoxic conditions, acetate production decreased, higher amounts of ethanol were produced, and lactate and H_2 became significant end products when RD-1 was grown on glucose in the presence of O_2. Similarly, when RD-1 was grown on fructose in the presence of elevated salt concentrations, lower amounts of acetate and higher amounts of ethanol and H_2 were produced. When the concentration of O_2 in the headspace exceeded 1% (vol/vol), supplemental H_2 was not utilized. The 16S rRNA gene of RD-1 had a 99.7% sequence similarity to that of *Clostridium glycolicum* DSM 1288^T, an organism characterized as a fermentative anaerobe. Comparative experiments with *C. glycolicum* DSM 1288^T demonstrated that it had negligible H_2- and formate-utilizing capacities. However, carbon monoxide dehydrogenase was detected in both RD-1 and *C. glycolicum* DSM 1288^T. A 91.4% DNA-DNA hybridization between the genomic DNA of RD-1 and that of *C. glycolicum* DSM 1288^T confirmed that RD-1 was a strain of *C. glycolicum*. These results indicate that (i) RD-1 metabolizes certain substrates via the acetyl-CoA pathway, (ii) RD-1 can tolerate and consume limited amounts of O_2, (iii) oxic conditions favor the production of ethanol, lactate, and H_2 by RD-1, and (iv) the ability of RD-1 to cope with limited amounts of O_2 might contribute to its survival in a habitat subject to daily gradients of photosynthesis-derived O_2.

Sea grasses colonize shallow coastal marine environments (2, 26, 61) and thus are rooted in reduced, anoxic sediments where high rates of sulfate reduction can yield high concentrations of sulfide (5). Sediments colonized by the sea grass *Halodule wrightii* contain high numbers of readily culturable acetogens and acetate-utilizing sulfate reducers, and these numbers are significantly higher than in unvegetated sediments (38). Colonization of the sea grass rhizosphere by bacteria might give those organisms ready access to plant-derived substrates that could supply the energy needed for nitrogen fixation, thus yielding a beneficial plant-microbe interaction (11, 12). Although most rhizobacteria likely colonize root tips or outermost root cell layers (9), microautoradiographs of sea grass root thin sections hybridized with ^{33}P-labeled probes revealed the presence of acetogens, clostridia, and sulfate-reducing bacteria in both the rhizoplane and deep cortex cells of sea grass roots (38).

The O_2 produced by leaf photosynthesis is transported to the roots and generates transient O_2 gradients around the roots (1, 33). Thus, anaerobic endorhizobacteria likely experience periods of elevated O_2 tension, suggesting that such bacteria must cope with O_2. Indeed, exposure of sea grass roots to O_2 has minimal affect on root-surface-associated, sulfate-reducing activity (5). This observation is consistent with other findings demonstrating that some sulfate-reducing bacteria are aerotolerant (10, 19, 28).

Most acetogens have been isolated from habitats with stable anoxic conditions (e.g., sediments or sewage sludge) (21, 50). However, acetogens colonize the leaf litter and mineral soil of oxic forest soils (34, 37, 47), tolerate periods of oxygenation in soils (60), and are active in termite guts that have steep O_2 gradients (7, 56). The objectives of this study were to (i) isolate an acetogen from sea grass roots, (ii) determine its survivability under oxic conditions, and (iii) investigate its physiological response and protective mechanisms to elevated O_2 tensions.

MATERIALS AND METHODS

Collection of sea grass roots. Sea grasses (*H. wrightii*) were sampled in clear, plastic cores from a depth of about 1 m below the surface of the overlying brackish water near Big Sabine Point in Santa Rosa Sound, located in northwestern Florida. The cores were released into a sterile glass dish, and the roots were carefully separated from the sediment. The pH of the sediment was 6.7, and the water temperature was 33°C. Healthy roots, white and free of lesions, were

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excised with a sterile razor blade and washed twice in sterile phosphate-buffered saline (120 mM sodium phosphate and 0.85% NaCl, pH 7.2).

Medium composition and growth conditions. The anoxic, carbonate-buffered, undefined (U) medium contained yeast extract, vitamins, and trace metals but did not contain reducing agents (16). U_{salt} medium was U medium supplemented with NaCl (20 g liter^{-1}) and $MgCl_2$ (2 g liter^{-1}). The defined medium was U medium without yeast extract. Media were dispensed under CO_2 into 27-ml 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 approximated 6.7. Tryptic soy broth (TSB) medium (28 g of Bacto TSB without glucose [Difco Laboratories, Detroit, Mich.] per liter) was made anoxic by boiling and cooling under 100% argon. The pH was adjusted with H_3PO_4 or NaOH to the indicated pH. Anoxic stock solutions of substrates (prepared under argon) were filter sterilized and were added by syringe injection using O_2-free techniques. Unless otherwise indicated, culture tubes and bottles were incubated in a horizontal, static position, cultivation was in anoxic U medium, and the temperature of incubation was 30°C. *Escherichia coli* K12 (DSM 423) was cultivated in peptone-beef extract medium (5 g liter^{-1}) (Difco Laboratories) at pH 7.0.

Alternative electron acceptors, reduction of C_2H_2, and toxic effects of oxygen. The dissimilation of nitrate or sulfate was evaluated with TSB medium supplemented with 10 mM glucose and 5 mM $NaNO_3$ or 5 mM Na_2SO_4, respectively. The reduction of Fe(III) was determined by assessing the growth-dependent production of white Fe(II) precipitates in medium formulated for Fe(III)-reducing bacteria containing U growth factors (8). Nitrogenase activity was determined with a modification of the C_2H_2 reduction method (30, 41, 58). Sterile O_2 was added to the headspace of culture tubes to the concentrations (vol/vol) indicated.

Enrichment cultures. Washed roots (5 g) were brought into an O_2-free chamber (Mecaplex, Grenchen, Switzerland) (100% N_2 gas phase; room temperature) and were homogenized with a grinder in 45-ml basal salt solution (16). The root suspension was serially diluted and transferred to culture tubes containing U_{salt} medium and H_2-CO_2 (80:20 [vol/vol]) in the headspace. Stable acetogenic enrichment cultures were obtained from the highest growth-positive dilution series and were subsequently streaked onto solidified U_{salt} medium. Isolated colonies were picked with a sterile needle and were transferred to liquid U_{salt} medium; cultures were subsequently restreaked onto solidified U_{salt} medium, and isolated colonies were again transferred to liquid U_{salt} medium. This procedure was repeated two additional times, and isolates were examined microscopically for purity.

Electron microscopy. Cells were cultivated in U_{salt} medium or on solidified U_{salt} medium (1% Gelrite; Carl Roth GmbH, Karlsruhe, Germany); both media were supplemented with 10 mM glucose. For negative staining, cells were fixed for 30 min in liquid medium by adding glutaraldehyde to a final concentration of 2% (vol/vol). Cells were harvested by gentle centrifugation (1,000 × *g*; 15 min), adsorbed to carbon film (59), and stained with aqueous uranyl acetate solution (2% [wt/vol]). For preparation of thin sections, cells were fixed in glutaraldehyde-OsO_4 (39, 57).

Preparation of cell extracts and enzyme assays. Cell extracts were prepared under anoxic conditions (35, 42). Membranous and cytoplasmic fractions were prepared under oxic conditions (27). Oxidoreductase activities were assayed spectrophotometrically at 20°C in Tris-HCl (50 mM, pH 7.5) buffer containing 0.5 mM benzyl viologen (16). Assay tubes contained 20% H_2 (vol/vol, with N_2 in gas phase) for hydrogenase, 20% CO (vol/vol, with N_2 in gas phase) for carbon monoxide dehydrogenase or 4 mM sodium formate for formate dehydrogenase (100% N_2 in gas phase). These enzyme activities are expressed in micromoles of substrate oxidized minute^{-1}.

Catalase, peroxidase, NADH oxidase, and superoxide dismutase activities were assayed according to standard protocols (3, 4, 52, 53). Catalase, peroxidase, NADH oxidase, and superoxide dismutase activities are expressed in the following units, respectively: 1 μmol of H_2O_2 consumed min^{-1}, 1 mg of pyrogallol oxidized min^{-1}, 1 μmol of NADH oxidized min^{-1}, and 1 μmol of unreduced Nitro Blue Tetrazolium chloride min^{-1}. RD-1 was grown in U medium with 10 mM glucose and 5% (vol/vol) O_2 in the headspace when these enzymes were evaluated.

Redox difference spectra. Membranous and cytoplasmic fractions were reduced with sodium dithionite, and redox difference spectra were obtained with a Uvikon 930 (Kontron Instruments, Milan, Italy) double-beam recording spectrophotometer (34).

G+C content. Cells were treated with penicillin G (250 μg ml^{-1}) 3 h before harvesting. DNA extraction included treatments with lysozyme/proteinase K and RNase/proteinase K (43). The G+C content was determined by high-performance liquid chromatography using nonmethylized lambda DNA for calibration (45, 54).

Phylogenetic analysis. A total of 1,317 bases of the 16S rRNA gene of RD-1 were sequenced. DNA extraction, PCR-mediated amplification of the 16S rRNA gene, and purification of the PCR products were performed according to published protocols (20, 49). Cells were lysed by boiling at approximately 100°C. Purified PCR products were sequenced by using an ABI PRISM Ready Reaction Dye Terminator kit (Applied Biosystems, Foster City, Calif.). Sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A DNA sequencer. Alignment of the sequence data and sequence similarity calculations were performed using the tools of the ARB software package (http://www.mikro.biologie.tu-muenchen.de).

DNA-DNA hybridzation. DNA-DNA hybridization was determined by spectrophotometric reassociation kinetics according to published protocols (13, 17, 24, 31).

Additional analytical methods. Growth was measured as optical density at 660 nm; the optical-path width (inner diameter of culture tubes) was 1.6 cm. Uninoculated medium served as a reference. Protein in cell extracts was determined colorimetrically (6). Substrates and products were determined by high-performance liquid chromatography and gas chromatography (16, 36, 44). Nitrate was measured colorimetrically (14). Sulfate was analyzed by ion chromatography (36). Results are representative of replicate experiments.

Accession numbers. RD-1 has been deposited at the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under accession number DSM 13865. The 16S rRNA gene sequence of RD-1 has been deposited at the EMBL Nucleotide Sequence Database (Cambridge, United Kingdom) under accession number AJ291746.

RESULTS

Acetogenic enrichment cultures and isolates. When root homogenates were serially diluted in U_{salt} medium containing H_2-CO_2, stable enrichment cultures that produced 1 mol of acetate per 5 mol of H_2 consumed were obtained from the two highest growth-positive dilutions, 10^{-4} and 10^{-5}. Two gram-positive, rod-shaped isolates, RD-1 (obtained from the 10^{-5} growth-positive dilution) and RD-3 (obtained from the 10^{-4} growth-positive dilution), converted H_2-CO_2 to acetate in stoichiometries indicative of an acetyl coenzyme A (acetyl-CoA) pathway-dependent metabolism: $4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$ (21). Colonies of both isolates were shiny, convex, white to beige, and 2 to 3 mm in diameter. Initial screening of growth substrates indicated that RD-1 and RD-3 were the same organism, and RD-1 was selected for further characterization.

Morphology and ultrastructure of RD-1. Cells were 4 by 0.8 to 6 by 0.8 μm (Fig. 1A) and were motile in wet mounts. Thin sections revealed a multilayered cell wall but no outer membrane (Fig. 1A). Cells formed terminal spores (Fig. 1B); free spores were rarely observed in cultures under the light microscope. Up to seven cells of RD-1 were often tethered to one another by connecting filaments (Fig. 1C). The connecting filament consisted of a core and a surrounding sheath (Fig. 1D). A morphological continuity was apparent between the surface of the cell and the sheath (Fig. 1E). However, the sheath was not always present, indicating that it could be dissociated from the core. The negatively stained core usually revealed a darkened center (Fig. 1D), which might have been due to a high affinity of the inner portion of the core for uranyl acetate.

Growth properties of RD-1. Growth was observed after cultures were heated for 15 min at 80°C, confirming the occurrence of spores. Growth could not be maintained in defined medium, indicating that RD-1 required some undefined factors for growth. In TSB medium, growth occurred at pH 6.1 to

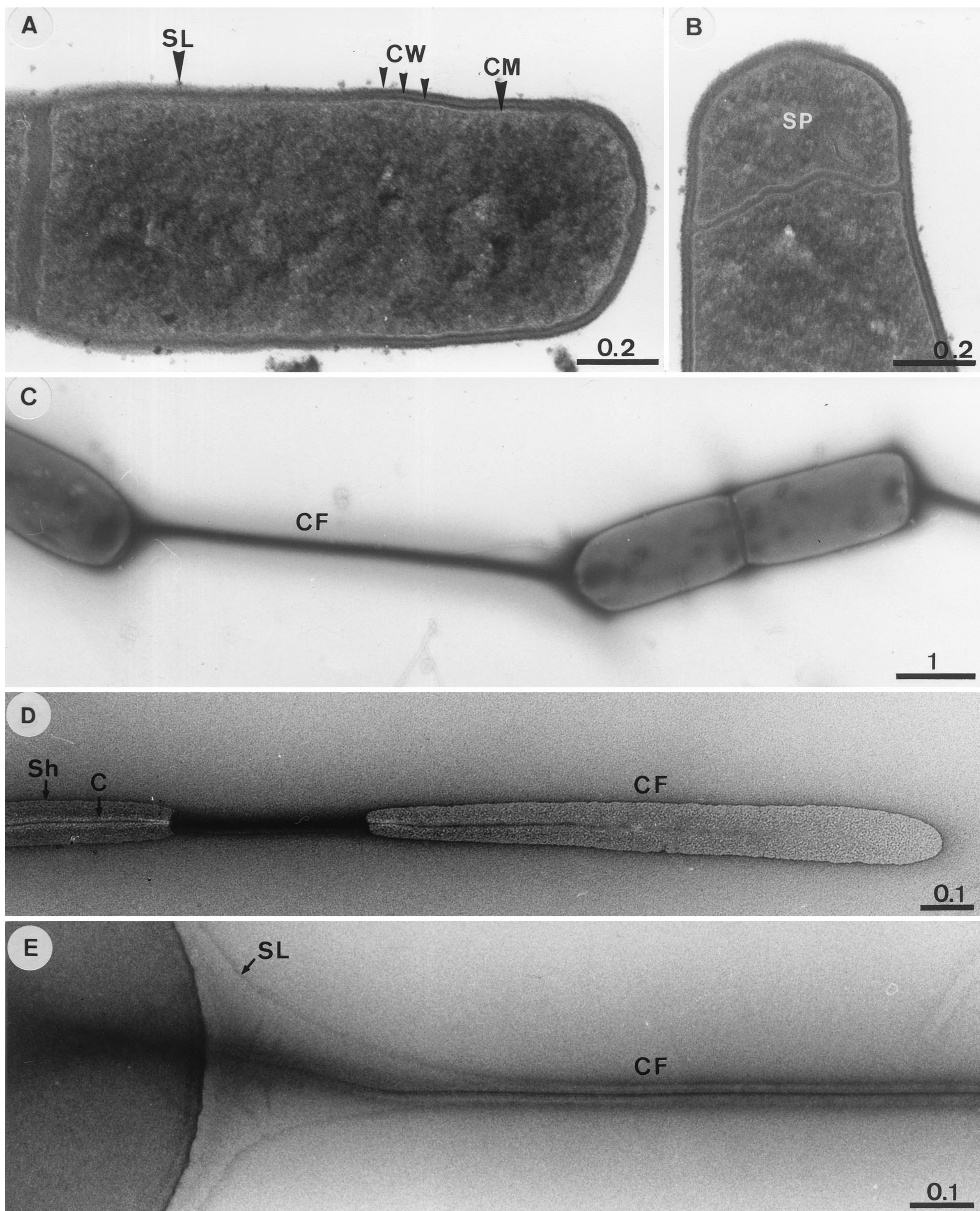

FIG. 1. Transmission electron micrographs of RD-1. (A) Thin section of a dividing cell. (B) Thin section showing formation of a terminal spore. (C to E) Negatively stained specimens revealing the ultrastructural features of connecting filaments. Abbreviations: SL, surface layer; CW, cell wall; CM, cytoplasmic membrane; SP, prespore; CF, connecting filament; Sh, outer sheath; C, core. Bars are in micrometers.

TABLE 1. Product profiles of RD-1 cultivated in U_{salt} medium[a]

Substrate	Optical density at 660 nm	Substrate consumed (mM)	Acetate produced (mM)	Ethanol produced (mM)	H_2 produced (mmol liter of culture fluid^{-1})	Acetate/substrate ratio[b]
H_2	0.03	42.5	9.8	ND[c]	0	0.23 (0.25)
Formate	0.01	15.4	4.7	0	0	0.31 (0.25)
Pyruvate	0.05	3.7	5.7	0	ND	1.54 (1.25)
Glucose	0.41	5.0	9.6	3.3	Trace levels	1.9 (3.0)
Xylose	0.22	5.4	8.7	2.3	Trace levels	1.6 (2.5)

[a] Lactate was not detected in any culture. Values were corrected with control values obtained from cultures lacking additional substrates and are means of three replicates. The standard deviations were less than 10%.

[b] Values in parentheses are the theoretical acetate-to-substrate ratios if substrates were converted to acetate via the acetyl-CoA pathway.

[c] ND, not determined.

8.2; growth did not occur at pH 5.6 and 8.5. The optimal pH was 7.0 to 7.5. RD-1 grew at 25 to 42°C in U_{salt} medium (pH 7.0) supplemented with fructose; no growth occurred at 20 and 45°C. At pH 7.0, growth rates and cell yields were optimal at 37 to 40°C; under these conditions, the doubling time was 2.9 h.

Substrate range and fermentation stoichiometries of RD-1. The following substrates were utilized in U_{salt} medium: fructose, glucose, maltose, xylose, pyruvate, lactate, formate, and H_2-CO_2. RD-1 produced acetate from H_2-CO_2, formate, pyruvate, and lactate in stoichiometries indicative of acetogenesis (22) (Table 1 and data not shown). Fructose, glucose, maltose, and xylose were fermented to acetate and ethanol and small amounts of H_2 (Table 1 and data not shown). The presence of elevated salt concentrations affected the ratio of acetate produced to fructose consumed (Table 2). Smaller amounts of acetate but greater amounts of ethanol and H_2 were produced when RD-1 was grown on fructose in U_{salt} medium than when RD-1 was grown on fructose in U medium. Growth on yeast extract (i.e., U medium without supplemental substrates) yielded acetate and isovalerate. Ethylene glycol was fermented to acetate and ethanol, and propylene glycol was fermented to propionate and propanol and small amounts of acetate. Neither growth nor utilization of substrate was observed with galactose, sucrose, melitose, cellobiose, mannose, lactose, vanillate, syringate, ferulate, methanol, ehanol, propanol, 2,3-butandiol, citrate, succinate, fumarate, butyrate, oxalate, acetate, or CO. RD-1 did not dissimilate nitrate, sulfate, or Fe(III) and did not produce methane. In medium lacking inorganic nitrogen compounds, RD-1 did not reduce C_2H_2, indicating that RD-1 did not produce nitrogenase.

Effect of O_2 on growth of RD-1. Glucose-dependent growth rates and cell yields were not significantly affected by up to 6% (vol/vol) O_2 in the headspace of static, horizontally incubated tubes that were periodically shaken prior to measuring optical densities (Fig. 2A). When culture tubes were continuously shaken (60 rpm), RD-1 grew in the presence of up to 4% (vol/vol) O_2 in the headspace (Fig. 2B).

Effect of O_2 on physiological capabilities of RD-1. When RD-1 was cultivated on glucose with 0.3, 2.1, 2.8, 3.8, 4.9, or 5.8% (vol/vol) O_2 in the headspace of static, horizontally incubated tubes, O_2 decreased to 0, 1.5, 2.0, 3.1, 3.9, or 4.7% (vol/vol), respectively, by the end of incubation (2 days). O_2 was not consumed in uninoculated controls. RD-1 consumed O_2 concomitantly with exponential growth and the consumption of glucose (Fig. 3).

Acetate production decreased, higher amounts of ethanol were produced, and H_2 (0.6 mmol l^{-1} culture fluid) and lactate (0.4 mM) became significant end products in the presence of O_2 (Table 3). The recovery of reductant was low in cultures containing O_2. Thus, unrecovered reductant derived from the oxidation of glucose appeared to be stored in unidentified products or biomass. The addition of up to 0.5% (vol/vol) O_2 to the headspace of cultures did not affect the capacity of RD-1 to consume H_2 (Fig. 4). H_2 was not consumed when O_2 in the headspace of cultures exceeded 1% (vol/vol).

Oxidative stress enzyme activities of RD-1. NADH oxidase, superoxide dismutase, peroxidase, and catalase activities in the cytoplasmic fraction of RD-1 approximated 4.3, 0.4, 0.1, and 0 U mg^{-1} of protein, respectively. These enzyme activities were not detected in the membrane fraction. NADH oxidase, superoxide dismutase, peroxidase, and catalase activities in the cytoplasmic fraction of *E. coli* K12 approximated 3.3, 0.5, 0.5, and 230 U mg^{-1} of protein, respectively.

TABLE 2. Effect of increasing salt concentrations on the fructose (5 mM)-dependent product profiles of RD-1[a]

Additional concn of NaCl and $MgCl_2$, respectively (g liter^{-1})	Products			Acetate:fructose ratio	Recovery of reducing equivalents (%)
	Acetate (mM)	Ethanol (mM)	H_2 (mmol liter of culture fluid^{-1})		
0, 0	11.7	2.3	0.09	2.34	101
5, 0.05	11.3	2.4	0.11	2.26	99
10, 0.1	11.1	2.9	0.15	2.22	103
15, 0.15	9.9	3.2	0.13	1.98	98
20, 0.2	9.6	3.0	0.18	1.92	93

[a] Fructose was totally consumed in all cultures. Lactate was not detected in any culture. Values were corrected with control values obtained from cultures lacking fructose and are means of three replicates. The standard deviations were less than 10%.

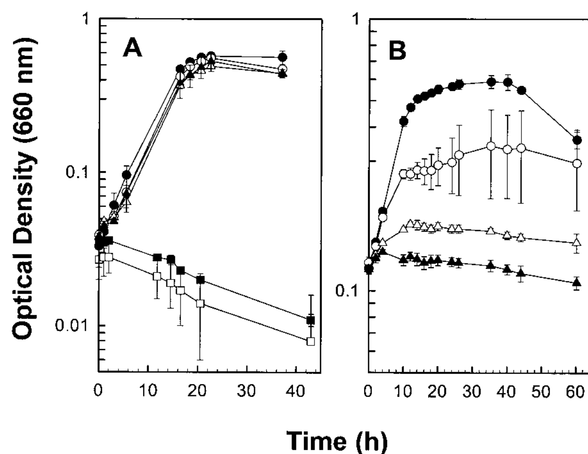

FIG. 2. Effect of O_2 on the glucose-dependent growth of RD-1. Culture tubes were either incubated horizontally and shaken prior to each measurement (A) or continuously shaken (B). Data are the means of three replicates (± standard deviations). The initial concentrations of O_2 (vol/vol) in the headspace were ●, 0%; ○, 4%; △, 5%; ▲, 6%; ■, 7%; and □, 9% (A) or ●, 0%; ○, 2%; ▲, 3%; and △, 6% (B).

Oxidoreductase activities and redox difference spectra of membranes of RD-1. Carbon monoxide dehydrogenase, hydrogenase, and formate dehydrogenase activites in cell extracts obtained from fructose-cultivated cells of RD-1 approximated 0.6, 0.9, and 0.2 U mg^{-1} of protein, respectively. No absorption maxima indicative of cytochromes were detected in the membranous or cytoplasmic fractions of RD-1 (data not shown).

Phylogenetic analysis, G+C content, DNA-DNA hybridization, and protein profiles. The 16S rRNA gene sequence of RD-1 was most closely related to that of bacteria that group in cluster XI of the genus *Clostridium* (15). The highest sequence similarity value (99.7%) was to *C. glycolicum* DSM 1288^T. The DNA base composition of RD-1 was 31.6 (±0.8) (n = 3) mol%. The G+C mol% of the DNA of *C. glycolicum* DSM 1288^T is 29 (32). DNA-DNA hybridization experiments revealed a 91.4% genome sequence similarity between RD-1 and *C. glycolicum* DSM 1288^T. Cells of RD-1 and of *C. glycolicum* DSM 1288^T that had been cultivated on fructose yielded nearly identical protein profiles when cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown).

Metabolic capacities of *C. glycolicum* DSM 1288^T. In U medium, *C. glycolicum* DSM 1288^T fermented fructose (10 mM) to acetate (21 mM) and ethanol (2.2 mM). In U_{salt} medium, fructose (10 mM) was fermented to acetate (17.8 mM), ethanol (2.5 mM), and trace levels of H_2. In contrast to RD-1, *C. glycolicum* DSM 1288^T did not catalyze the H_2- or formate-dependent formation of acetate. Nonetheless, carbon monoxide dehydrogenase and hydrogenase activities were detected in cell extracts of fructose-cultivated cells of *C. glycolicum* DSM 1288^T (activities approximated 1.1 and 0.6 U mg^{-1} of protein, respectively). The temperature optimum of *C. glycolicum* DSM 1288^T was 30°C.

DISCUSSION

RD-1 was isolated as an anaerobe with acetogenic capabilities yet was also able to grow in the presence of 4 and 6% (vol/vol) O_2 in the headspace of continuously and periodically shaken culture tubes, respectively. The capacity of RD-1 to consume small amounts of O_2 without an apparent lag phase suggests that this metabolic capacity was constitutive. Many so-called obligately anaerobic bacteria and archaea exhibit some degree of aerotolerance (48). NADH oxidase is used by the aerotolerant anaerobe *Brachyspira hyodysenteriae* for the consumption of O_2 (51). Facultative bacteria and certain obligate anaerobes contain superoxide dismutase or NADH oxidase (48, 51). Superoxide dismutase, peroxidase, and NADH oxidase activities were detected in the cytoplasmic fraction of RD-1, indicating that RD-1 can disproportionate superoxide and reduce hydrogen peroxide or O_2. The oxidative stress enzyme catalase, present in certain methanogens (40), was not

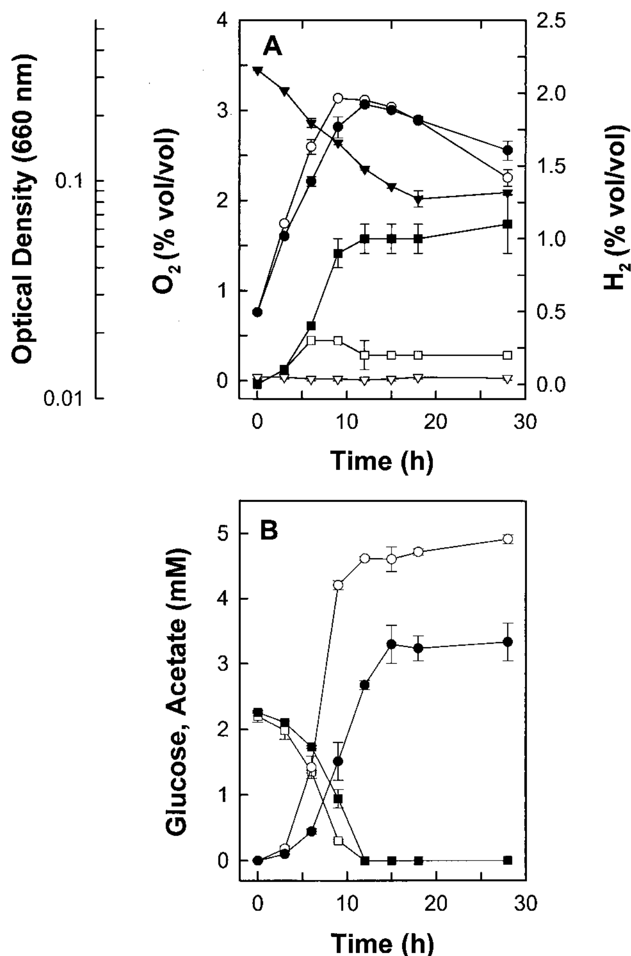

FIG. 3. Effect of O_2 on the glucose-derived product and growth profiles of RD-1. Data are the means of three replicates (± standard deviations). (A) ▼, O_2 in cultures containing supplemental O_2; ▽, O_2 in cultures without supplemental O_2; ●, optical density in cultures containing supplemental O_2; ○, optical density in cultures without supplemental O_2; ■, H_2 in cultures containing supplemental O_2; □, H_2 in cultures without supplemental O_2. (B) ■, glucose in cultures containing supplemental O_2; □, glucose in cultures without supplemental O_2; ●, acetate in cultures containing supplemental O_2; ○, acetate in cultures without supplemental O_2. Acetate values were corrected with control values from cultures lacking glucose. The small amounts of ethanol formed from 2 mM glucose could not be accurately quantitated.

TABLE 3. Effect of increasing O_2 concentrations on the glucose (5 mM)-dependent product profiles of RD-1[a]

Initial O_2 concn (%)	O_2 consumed (mmol liter of culture fluid^{-1})	Products (mM)				Acetate/glucose ratio	Recovery of reducing equivalents (%)[b]
		Acetate	Ethanol	Lactate	H_2		
Anoxic	NA[c]	12.1	0.8	0	0.02	2.42	89
0.3	0.29	11.6	0.8	0	0.02	2.32	85 (86)
2.0	2.11	7.5	2.6	0.3	0.54	1.50	81 (88)
2.8	2.22	6.4	2.9	0.3	0.52	1.28	75 (83)
3.7	2.17	6.8	1.5	0.4	0.53	1.36	66 (73)

[a] Glucose was totally consumed in all cultures. Values were corrected with control values obtained from cultures lacking glucose and are means of three replicates. The standard deviations were less than 10%.
[b] Values in parentheses take into account the amount of reductant that was theoretically required for the reduction of O_2.
[c] NA, not applicable.

detected in RD-1. Desulfoferrodoxin and rubrerythrin function together as an oxidative stress defense mechanism in the sulfate reducer *Desulfovibrio vulgaris* (42), and genes for similar proteins have been identified in the acetogen *Moorella thermoacetica* (A. X. Das and L. G. Ljungdahl, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. H-117, p. 374, 2000). In comparison to the ability of RD-1 to tolerate relatively high amounts of O_2, the acetogens *Acetobacterium woodii*, *M. thermoacetica*, *Clostridium magnum*, and *Sporomusa silvacetica* can only tolerate 0.5, 1, 2, and 2% (vol/vol) O_2, respectively, in the headspace of culture tubes when cultivated on glucose (A. Karnholz, K. Küsel, and H. L. Drake, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. I-91, p. 401, 2000; unpublished data). Methanogenic (40) and acetogenic (H. Boga and A. Brune, Abstr. Ann. Meet. Verein. Allgem. Angewand. Mikrobiol. BioSpectrum, abstr. 15.P.11.33, p. 143, 2000) termite gut isolates can grow and consume O_2 in agar-O_2-gradient tubes. Thus, certain acetogens, like other so-called obligate or strict anaerobes, have the capacity to cope with oxidative stress, and it should not be considered a paradox that acetogens occur in habitats subject to fluxes of O_2.

Under anoxic conditions, RD-1 utilized both ethanol fermentation and the acetyl-CoA pathway as terminal, electron-accepting processes when grown on glucose (Fig. 5). In contrast, growth on pyruvate did not yield ethanol as a product. Thus, the simultaneous engagement of acetogenesis and fermentation was substrate dependent. When RD-1 was grown on glucose in the presence of O_2, ethanol and, to a lesser extent, lactate and H_2 became the main reduced end products, indicating that reductant flow was diverted away from the acetyl-CoA pathway when redox conditions became more oxic (Fig. 5). Ethanol and lactate fermentation are also important energy-conserving processes for the acetogen *Ruminococcus productus* U1, and the simultaneous engagement of multiple catabolic processes might contribute to the in situ competitiveness of acetogens (22).

Many of the enzymes of the acetyl-CoA pathway are very sensitive to O_2 (21), and H_2-dependent acetogenesis by RD-1 was more sensitive to O_2 than was fermentation. O_2 is likewise inhibitory to the H_2-dependent acetogenic capabilities of certain termite gut isolates (Boga et al., Abstr. Ann. Meet. Verein.

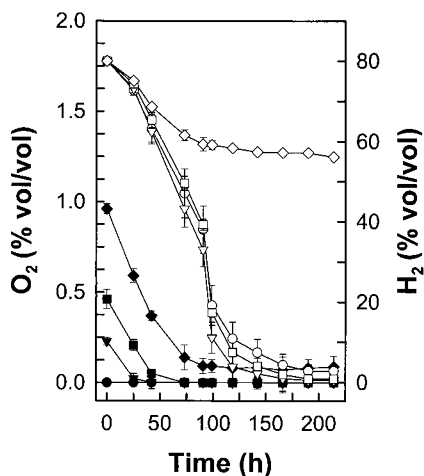

FIG. 4. Effect of O_2 on the consumption of H_2 by RD-1. Culture tubes contained an H_2-CO_2 gas phase (80:20%, vol/vol). Symbols: ◆, ■, and ▼, O_2 in cultures with supplemental O_2; ●, O_2 in cultures without supplemental O_2; ◇, □, and ▽, H_2 in cultures containing 1, 0.5, and 0.25% (vol/vol) O_2, respectively; and ○, H_2 in cultures without supplemental O_2.

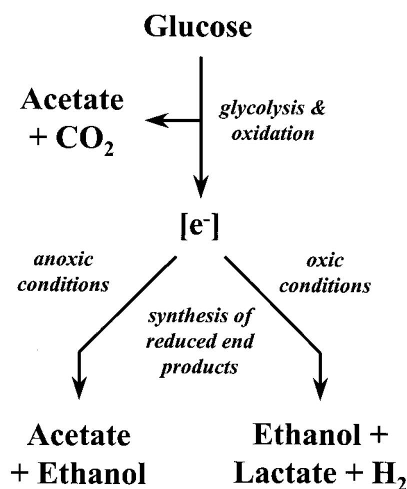

FIG. 5. Hypothetical scheme illustrating how oxic conditions cause a shift in the flow of reductant ([e$^-$]) by RD-1. The acetate formed under oxic conditions is derived from the oxidation of glucose (upper portion of scheme) rather than reductive synthesis from CO_2 via the acetyl-CoA pathway (depicted as the acetate that is produced as a reduced end product under anoxic conditions (lower left branch of figure).

Allgem. Angewand. Mikrobiol. BioSpectrum, abstr. 15.P.1133, p. 143, 2000). The ionic strength of the medium also caused a shift in the metabolism of RD-1 towards the synthesis of ethanol. However, lactate was only formed from sugars when RD-1 was subjected to oxidative stress.

Molecular characterization of RD-1 indicated that it was a strain of *C. glycolicum*. Strains of *C. glycolicum* have been isolated from soil, mud, bovine intestine, human feces, and human wounds (23, 25). *C. glycolicum* DSM 1288^T is described as a saccharolytic fermenter that produces acetate, ethanol, CO_2, and H_2 (29). In contrast to RD-1, *C. glycolicum* DSM 1288^T had negligible H_2- and formate-utilizing capacities. RD-1 and *C. glycolicum* DSM 1288^T also had dissimilar temperature optima. Although acetogenic capacities were not readily apparent with *C. glycolicum* DSM 1288^T, the organism nonetheless contained carbon monoxide dehydrogenase. Carbon monoxide dehydrogenase activity is also present in the nonacetogenic butyrate fermenter *Clostridium pasteurianum* (18).

A phylogenetically uncharacterized H_2-utilizing acetogen, *Clostridium* sp. strain 22 (ATCC 29797), might be a strain of *C. glycolicum* (46; information from the American Type Culture Collection [Manassas, Va.] Bacteriology Program). Strain 22 was isolated on H_2-CO_2 from sewage sludge, is a gram-positive rod that forms subterminal spores, and has a pH optimum of 8.0 (46). Since RD-1 formed terminal spores and had a pH optimum of 7.0 to 7.5, RD-1 and strain 22 appear to be dissimilar. *C. glycolicum* DSM 1288^T might have lost its ability to reductively synthesize acetate from H_2-CO_2. Most acetogens grow poorly on H_2-CO_2, in part because of the thermodynamic constraints of the carbonyl branch of the acetyl-CoA pathway (21), and certain acetogens lose their ability to efficiently utilize H_2 after prolonged cultivation in the laboratory on H_2-CO_2 (39).

Phylogenetically, acetogens are not tightly clustered but are widely dispersed throughout the low-G+C-content, gram-positive bacteria (55). To date, no general 16S rRNA gene probe is available to detect acetogens in natural samples. The 16S rRNA gene probes used to evaluate the occurrence of acetogens in sea grass roots (38) are specific for acetogenic species of *Acetobacterium* (probe AW), the acetogen *Eubacterium limosum* (probe AW), and *Clostridium* species that group in cluster I of their genus (probe Clost I), respectively (15). About 60% of the epidermal cells, 24% of the outermost cortex cell layers, and 2% of the deeper cortex cell layers of *H. wrightii* roots were colonized with acetogens that hybridized with probe AW (38). However, based on 16S rRNA gene sequence similarities, RD-1 grouped in cluster XI of the genus *Clostridium* (15) and would not be detected by the 16S rRNA gene probes AW and Clost I. Further studies will be required to elucidate the number and types of acetogens associated with sea grass rhizospheres and to understand how these microorganisms affect the viability of sea grasses.

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