Trophic interaction of the aerotolerant anaerobe *Clostridium intestinale* and the acetogen *Sporomusa rhizae* sp. nov. isolated from roots of the black needlerush *Juncus roemerianus*

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Acetogens were enumerated from root homogenates of the black needlerush *Juncus roemerianus* obtained from a nearly pristine salt marsh. An isolated colony, ST1, yielded acetogenic activity and was initially thought to be a pure culture; however, ST1 was subsequently found to be composed of an aerotolerant fermentative anaerobe (RC) and an acetogen (RS^T) (T indicates type strain). The two spore-forming mesophiles were separated by selective cultivation under conditions favouring the growth of either RC or RS^T. The 16S rRNA gene sequence of RC was 99 % similar to that of *Clostridium intestinale*, indicating that RC was a new isolate of this clostridial species. The rRNA gene sequence most similar to that of RS^T was only 96 % similar to that of RS^T and was from a species of the acetogenic genus *Sporomusa*, indicating that RS^T was a new sporomusal species; the name *Sporomusa rhizae* sp. nov. is proposed. RC grew at the expense of saccharides. H₂-forming butyrate fermentation was the primary catabolism utilized by RC under anoxic conditions, while homolactate fermentation was the primary catabolism under oxic conditions. RC consumed O₂ and tolerated 20 % O₂ in the headspace of shaken broth cultures. In contrast, RS^T was acetogenic, utilized H₂, lactate and formate, did not utilize saccharides, and could not tolerate high concentrations of O₂. RS^T grew by trophic interaction with RC on saccharides via the uptake of H₂, and, to a lesser extent, lactate and formate produced by RC. Co-cultures of the two organisms yielded high amounts of acetate. These results indicate that (i) previously uncharacterized species of *Sporomusa* are associated with *Juncus* roots and (ii) trophic links to O₂-consuming aerotolerant anaerobes might contribute to the *in situ* activities and survival strategies of acetogens in salt marsh rhizospheres, a habitat subject to gradients of plant-derived O₂.

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INTRODUCTION

Acetogens are bacteria that utilize the acetyl-CoA 'Wood–Ljungdahl' pathway for the reductive synthesis of acetate from CO₂ as an energy-conserving, terminal electron-accepting process (Wood & Ljungdahl, 1991; Drake, 1994). These organisms have been classically thought of as obligate anaerobes, and most acetogens have been isolated from habitats having relatively stable anoxic conditions (Drake

et al., 2004). However, acetogens also occur in (i) aerated litter and soils (Kuhner *et al.*, 1997; Küsel *et al.*, 1999a; Peters & Conrad, 1995; Wagner *et al.*, 1996), (ii) termite guts that have steep O₂ gradients (Brune *et al.*, 1995; Tholen & Brune, 1999), and (iii) the rhizosphere of the sea grass *Halodule wrightii* (Küsel *et al.*, 1999b, 2001), a habitat subject to transient periods of oxidation due in part to the transport of photosynthesis-derived O₂ to the root from which the O₂ can diffuse to the rhizoplane and adjacent sediments (Armstrong *et al.*, 1991; Kraemer & Alberte, 1995). The colonization of such habitats by acetogens indicates that these so-called obligate anaerobes can tolerate periods of oxygenation. Indeed, several acetogens have been recently shown to be capable of survival and growth under mildly oxic conditions (Küsel *et al.*, 2001; Karnholz *et al.*, 2002; Drake *et al.*, 2002; Boga & Brune, 2003).

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Abbreviation: MPN, most probable number.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of RC (1480 bp sequenced) and RS^T (1387 bp sequenced) are AM158323 and AM158322, respectively.

The occurrence of acetogens in the rhizoplane and root cortex cells of *H. wrightii* (Küsel *et al.*, 1999b) and the detection of formyltetrahydrofolate synthetase genes (indicative of acetogens: Lovell, 1994) from the roots of various salt marsh plants (Leaphart *et al.*, 2003) suggests that acetogens have heretofore unknown trophic links in the rhizospheres of marine and estuarine plants. The main objectives of the present study were to (i) determine if acetogens were associated with the roots of black needlerush *Juncus roemerianus*, a common subdominant plant in marshes along the Atlantic and northern Gulf of Mexico coasts of temperate north America, (ii) isolate and characterize an acetogen from *J. roemerianus* roots, and (iii) determine the physiological responses and protective mechanisms of this acetogen to oxidative stress.

METHODS

Sampling site. Samples were collected from the North Inlet estuary, near Georgetown, South Carolina, USA (79° 12' W, 33° 20' N). Detailed descriptions of this nearly pristine salt marsh system are available (Dame & Kenny, 1986; Piceno *et al.*, 1999). Low elevations of the North Inlet salt marsh estuary are dominated by *Spartina alterniflora*. Other wetlands plants occur in mixed and monotypic stands between the terrestrial biome and the short form *S. alterniflora* zone. These species include *Spartina patens*, *Salicornia virginica*, *Borrchia frutescens* and *J. roemerianus* (*Juncus* hereafter).

Medium composition and cultivation. Anoxic, undefined medium (UM) contained yeast extract, vitamins, trace metals and reducing agents (Daniel *et al.*, 1990). Media were dispensed under CO₂ into 27 ml culture tubes (7 ml medium per tube) or 1 litre infusion bottles (500 ml medium per bottle, used for preparation of cell extracts), which were then sealed and autoclaved; the pH approximated 6.7. Anoxic, filter-sterilized stock solutions of substrates (prepared under argon) were added by syringe injection using O₂-free techniques. Unless otherwise indicated, culture tubes and bottles were incubated in a horizontal, static position, cultivation was in UM, and the incubation temperature was 30 °C. Sterile O₂ was added to the headspace of culture tubes to the concentrations (v/v) indicated. Shaken cultures were maintained on a rotary shaker at 200 r.p.m.

Root homogenates. *Juncus* ramets with intact root zone sediments were collected from a monotypic stand at the Crab Haul Creek site of the North Inlet Microbial Observatory (<http://www.nimo-sc.org>). Roots were shaken and rinsed with sterile deionized water to remove sediment, then immediately frozen on dry ice. The frozen roots were shipped to the University of Bayreuth and stored at -80 °C. Frozen roots were transferred into a Mecaplex (Grenchen, Switzerland) O₂-free chamber (100% N₂ gas phase; room temperature). Root homogenates and serial dilutions of root homogenates were prepared as previously described (Küsel *et al.*, 2001). In brief, (i) 3 g (fresh weight) of roots were ground using a sterile glass grinder, (ii) the product was transferred to a bottle (containing 27 ml dilution buffer) that was sealed, (iii) the headspace of the bottle was replaced with sterile CO₂, and (iv) the contents of the bottle were mixed using an end-over-end shaker for 1 h. Such root homogenates were used for the preparation of dilution series and subsequent inoculation of media in enumeration studies.

Enumeration and acetogenic enrichment protocols. Most probable number (MPN) tubes were incubated in the dark and scored positive based on consumption of substrate or formation of products (Küsel *et al.*, 1999b). MPN values are the means of three

or four replicates and were calculated from standard tables (Alef, 1991). The protocol for the acetogenic enrichment from which strains RC and RS^T were obtained was as previously described (Küsel *et al.*, 1999b, 2001). Inoculum for the acetogenic enrichment was obtained from a highest growth-positive H₂/CO₂ MPN tube; the initial H₂/CO₂ enrichment contained 15 mM bromoethanesulfonate to inhibit the growth of methanogens (Sparling & Daniels, 1987). The H₂/CO₂ enrichment was streaked onto solidified medium, and colonies were picked and transferred to liquid medium for subsequent cultivation on the substrates indicated. Analysis of acetate confirmed the acetogenic conversion of substrates.

Preparation of cell-free extracts, enzyme assays and redox difference spectra. Cell-free extracts were prepared under anoxic conditions (Kühner *et al.*, 2000). Membranous and cytoplasmic fractions were prepared under oxic conditions (Frösl *et al.*, 1996). Redox-difference spectra were obtained with a Uvikon 930 (Kontron Instruments) double-beam recording spectrophotometer (Kühner *et al.*, 1997). Hydrogenase and formate dehydrogenase activities were assayed spectrophotometrically (Daniel *et al.*, 1990); units for these enzyme activities are µmol substrate oxidized min⁻¹. Catalase, peroxidase, NADH oxidase and superoxide dismutase activities were assayed according to standard protocols (Beauchamp & Fridovich, 1971; Beers & Sizors, 1952; Stanton & Jensen, 1993; Stellmach *et al.*, 1988), and units for these activities are, respectively: 1 µmol H₂O₂ consumed min⁻¹, 1 mg pyrogallol oxidized min⁻¹, 1 µmol NADH oxidized min⁻¹, and 1 µmol nitrotriazolium blue chloride not reduced min⁻¹. RC was grown in UM that lacked reducing agents and contained 10 mM glucose and 3% (v/v) O₂ in the headspace when these enzymes were evaluated. RS^T was grown in UM that lacked reducing agents and contained 10 mM *N*-acetylglucosamine and 0.3% (v/v) O₂ in the headspace when these enzymes were evaluated. Cell extracts of *Escherichia coli* K-12 (DSM 423) [cultivated in peptone-beef extract medium (5 g l⁻¹; Difco) at pH 7.0] were used as positive controls.

Phylogenetic analysis. Genomic DNA was purified from RC and RS^T using the Promega Wizard Genomic DNA Purification Kit. Near full-length 16S rRNA gene sequences were then PCR amplified using the *Bacteria* domain-specific oligonucleotide primers 27F (forward primer) and 1492R (reverse primer) (Lane, 1991). The PCR reaction system consisted of the following: 1 ng template DNA µl⁻¹, 0.025 U Expand High Fidelity DNA polymerase (Boehringer Mannheim) µl⁻¹, 1 × Expand HF buffer, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, and 0.25 pmol each primer µl⁻¹, in a final volume of 25 µl. The thermal cycling programme was described previously (Dang & Lovell, 2000). Cloning and amplified rRNA gene restriction analysis of cloned amplicons were as previously described (Dang & Lovell, 2000) with the following exceptions: (i) Taq DNA polymerase (Qiagen) was used instead of AmpliTaq Gold DNA polymerase (Perkin-Elmer) and (ii) amplicons were digested with *Hae*III and *Msp*I.

16S rRNA gene sequencing. Plasmid DNA was purified from each unique clone (Qiagen) and plasmid quality and quantity were determined by agarose gel electrophoresis and fluorometry, respectively. The DNA insert was sequenced in both directions using a SequiTherm EXCEL II Long-Read LC DNA sequencing kit (Epicentre Technologies) and a Li-Cor DNA4000LS sequencer. Reported 16S rRNA gene sequences most similar to those determined in this study were identified using the advanced BLAST search program (Altschul *et al.*, 1990) of the US National Center for Biotechnology Information GenBank database (Bilofsky & Burks, 1988). Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Distance matrices were constructed using Jukes-Cantor or Kimura models, with complete deletion of gaps and missing data, giving 1137 positions scored (MEGA version 2.1) (Kumar *et al.*, 2001). Neighbour-joining and maximum-parsimony (utilizing the mini-mini heuristic

search method) phylogenetic trees were built from the distance matrices. Reliability of tree topology was estimated using bootstrapping (1000 bootstrap replicates).

Additional methods. Electron micrographs were obtained with a Zeiss CEM 902A (Oberkochen, Germany) (Göbner *et al.*, 1999, Kuhner *et al.*, 2000); cells were negatively stained with uranyl acetate (Valentine *et al.*, 1968). Growth was measured as OD₆₆₀; the optical path width (inner diameter of culture tubes) was 1.6 cm. Uninoculated medium was used as reference. Protein was determined colorimetrically (Bradford, 1976). Substrates and products were determined by high-performance liquid chromatography and gas chromatography (Daniel *et al.*, 1990; Küsel & Drake, 1995; Matthies *et al.*, 1993). Unless otherwise indicated, data are the means of duplicate experiments.

Accession numbers. RC has been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, and the American Type Culture Collection (ATCC), Manassas, VA, USA, under accession numbers DSM 16614 and ATCC BAA-1027, respectively. RS^T has been deposited at the DSMZ and ATCC under accession numbers DSM 16652 and ATCC BAA-1028, respectively. The 16S rRNA gene sequences of RC (1480 bp sequenced) and RS^T (1387 bp sequenced) have been deposited at the EMBL Nucleotide Sequence Database (Cambridge, UK) under accession numbers AM158323 and AM158322, respectively.

RESULTS

Enumeration of acetogens

Root homogenates yielded 2×10^2 (range: 6×10^1 – 7×10^2) and 1×10^3 (range: 3×10^2 – 4×10^3) MPN g (fresh wt root)⁻¹ for H₂- and vanillate-utilizing acetogens, respectively. MPN values for acetate- and malate-utilizing sulfate-reducers approximated 2×10^3 (range: 3×10^2 – 8×10^3) and 2×10^3 (range: 4×10^2 – 9×10^3) MPN g (fresh wt root)⁻¹, respectively.

ST1, a mixed culture of two organisms, RC and RS^T

A colony that had been obtained from an acetogenic enrichment (see Methods) yielded an isolate (designated ST1) that displayed acetogenic activity on both glucose and H₂/CO₂. ST1 was thought to be a pure culture, as it was obtained after restreaking four times on solidified medium and was composed of weakly Gram-positive, rod-shaped cells that appeared to be uniform under a light microscope. Anaerobic growth of ST1 yielded acetate on H₂, and butyrate and acetate on glucose (both butyrate and acetate are expected products of certain acetogens: Drake *et al.*, 2004). However, two observations suggested that ST1 was composed of two organisms. (i) ST1 tolerated 21 % O₂ in the gas phase of shaken broth cultures and produced lactate from glucose under highly oxic conditions. Although certain acetogens can tolerate and reduce low concentrations of O₂ (Küsel *et al.*, 2001; Karnholz *et al.*, 2002; Drake *et al.*, 2002; Boga & Brune, 2003), the level of tolerance for O₂ by ST1 and production of lactate under oxic conditions suggested that it might contain an aerotolerant lactate-forming anaerobe, the lactate being subject to consumption by an acetogen under anoxic conditions, as has been documented for the

trophic interaction of the fermentative aerophile *Thermicanus aegyptius* and the acetogen *Moorella thermoacetica* (Göbner *et al.*, 1999). (ii) Two dissimilar partial 16S rRNA gene sequences were obtained from ST1 and related to species of *Clostridium* and *Sporomusa* (data not shown).

The two organisms were separated by culturing ST1 repeatedly on either 5 mM glucose with 12 % O₂ in the headspace (four sequential transfers, each during the stationary phase of growth) or 5 mM lactate under anoxic conditions (four sequential transfers, each during the exponential phase of growth), after which each culture was serially diluted in the same medium. The highest growth-positive dilution of each dilution series was then examined for purity and growth properties. The oxic glucose dilution series yielded an isolate (hereafter referred to as RC) that grew anaerobically on glucose but not H₂, and the anoxic lactate dilution series yielded an isolate [hereafter referred to as RS^T (^T indicates type strain)] that grew anaerobically on H₂ but not glucose.

Morphologies of RC and RS^T

Both RC and RS^T stained weakly Gram-positive and formed endospores; free refractile spores were occasionally seen in both cultures under a light microscope. Cells of RC were approximately 2.8 µm long and 0.6 µm wide (Fig. 1a). Cells of RS^T were slightly curved, flagellate, sometimes paired, and approximately 3.5 µm long and 0.8 µm wide (Fig. 1b, c).

Phylogenetics of RC and RS^T

The two closest phylogenetic relatives of RC were *Clostridium intestinale* and *Clostridium fallax* (99 % and 92 % 16S rRNA gene sequence similarity to that of RC, respectively) (Fig. 2), and it was concluded that RC was a new isolate of *C. intestinale*. The two organisms yielding sequences most similar to that of RS^T were *Sporomusa aerivorans* and *Sporomusa malonica* (96 % and 95 % 16S rRNA gene sequence similarity to that of RS^T, respectively) (Fig. 2). It was

Fig. 1. Transmission electron micrographs of RC (a) and RS^T (b, c). SP, spore. Bars, 1 µm.

Fig. 2. Phylogenetic tree showing the affiliations of near full-length 16S rRNA gene sequences from RC and RS^T to similar sequences identified using BLAST. The Jukes–Cantor model was used to construct the neighbour-joining tree, which was bootstrapped 1000 times. Bootstrap scores above the branches are those from neighbour joining and refer to the adjacent node. Scores below the branches are from a maximum-parsimony tree constructed utilizing the Jukes–Cantor model and the mini-mini heuristic search algorithm, and also bootstrapped 1000 times. Bootstrap values below 70 % are not shown. The *Treponema primitia* strain ZAS-1 16S rRNA gene sequence was used as the outgroup. Numbers in parentheses indicate sequence accession numbers. The scale bar indicates 2 % estimated sequence divergence.

concluded that RS^T constitutes a new species of *Sporomusa*; the name *Sporomusa rhizae* is proposed.

Optimum growth, substrate/product profiles and O₂ tolerance of RC

The optimum temperature and temperature range for the growth of RC were 35 °C and 10–50 °C, respectively. The optimum pH and pH range for growth were 8.5 and 5.5–10.5, respectively. The doubling time of RC under optimal

conditions with glucose as substrate was approximately 4 h; the final pH of such cultures approximated 4.8. Cells of RC lysed rapidly, as evidenced by a sharp decrease in optical density following the exponential phase of growth.

Cellobiose, glucose, fructose and *N*-acetylglucosamine supported the growth of RC; acetate, butyrate, lactate, formate and H₂ were fermentation products (Table 1, and data not shown). Ethanol was not detected in RC cultures. The formation of butyrate and H₂ as the main reduced end

Table 1. Product profiles of RC

Initial substrate concentrations approximated 5 mM. Due to the rapid lysis of RC, the observed maximum optical densities likely do not correspond to the maximum achieved during growth. CO₂ was not determined.

Substrate consumed (mM)	Maximum OD ₆₆₀	Products (mM)					Recovered reductant (%)
		Butyrate	Lactate	Acetate	Formate	H ₂	
Control*	0.03	0.1	0	0.1	0	0.4	NA
Cellobiose (4.9)	0.90	2.2	0.5	4.0	3.0	16.6	50
Glucose (5.6)	0.63	2.0	0.2	2.6	2.3	10.9	64
Fructose (5.4)	0.36	1.7	0.2	2.0	1.4	10.2	56

NA, not applicable.
*No substrate added.

products from saccharides under anoxic conditions was indicative of butyrate fermentation (Buckel, 2005; Dürre, 2005). However, the relatively low recovery of reductant in RC cultures suggested that RC might have produced an undetected end product or a storage compound. RC did not grow on stachyose, sucrose, raffinose, vanillate, syringate, citrate, xylose, butyrate, propionate, lactate, pyruvate, betaine, ethanol, acetate or H₂.

RC was aerotolerant. Growth on glucose in the presence of O₂ was concomitant with the consumption of O₂, a decrease in the amount of butyrate and H₂ produced, and an increase in the amount of lactate formed (Table 2). Although high amounts of O₂ curtailed the growth of RC, cells remained metabolically active under highly oxidic conditions, as shown by the production of lactate as nearly the sole reduced end product detected in both static and shaken cultures containing approximately 20 % O₂ in the headspace (Table 2).

Optimum growth, substrate/product profiles and O₂ tolerance of RS^T

The optimum temperature and temperature range for the growth of RS^T were 35 °C and 15–40 °C, respectively. The optimum pH and pH range for growth were 7.5 and 5.5–9.0, respectively. The doubling time of RS^T under optimal conditions with *N*-acetylglucosamine as substrate was approximately 8 h; the final pH of such cultures approximated 5.4. Lysis of RS^T cells was minimal.

Vanillate, syringate, citrate, *N*-acetylglucosamine, betaine, lactate, formate and H₂ supported the growth of RS^T (Table 3, and data not shown). Acetate was the main reduced end product formed by RS^T, a trait indicative of acetogens. Traces of butyrate, isobutyrate and propionate were also detected in cultures of RS^T. Although lactate was utilized, growth and acetate production with this substrate

was minimal. Certain acetogens (e.g. *M. thermoacetica*) can use CO, but some (e.g. *Thermoanaerobacter kivui*) cannot (Daniel *et al.*, 1990). CO was not utilized by RS^T, and growth in unsupplemented and vanillate-supplemented UM was inhibited by CO (Table 3). RS^T did not grow on raffinose, stachyose, sucrose, glucose, fructose, xylose, butyrate, propionate, ethanol, acetate, oxalate or glyoxylate.

RS^T grew in medium containing small amounts of O₂, and growth under such conditions was concomitant with the consumption of O₂ (Table 4). A metabolic shift did not occur in response to O₂. Under static conditions, *N*-acetylglucosamine-dependent growth of RS^T was less sensitive to O₂ than was H₂-dependent growth. Growth at the expense of *N*-acetylglucosamine or H₂ was equally sensitive to O₂ under shaken conditions. Acetate production ceased when the growth of RS^T was inhibited by elevated amounts of O₂. The spores of RS^T appeared to be highly resistant to O₂, as evidenced by the ability of cultures that had been incubated with 6 % O₂ in the headspace for 12 months (during which time no growth occurred and no O₂ was consumed) to be revived when transferred to anoxic conditions (data not shown).

Cytochromes and enzyme activities

Cytochromes were not detected in the membranes or cytoplasmic fraction of RC. Sodium dithionite-reduced minus air-oxidized spectra of membranes derived from RS^T revealed absorption maxima at 432, 482 and 561 nm, indicating that membranes of RS^T contained a *b*-type cytochrome. Membranous *b*-type cytochrome is indicative of the genus *Sporomusa* (Möller *et al.*, 1984; Breznak *et al.*, 1988; Kuhner *et al.*, 1997; Boga *et al.*, 2003). Cytochromes were not detected in the cytoplasmic fraction of RS^T. Cell-free extracts of RC and RS^T contained peroxidase activity (0.9 and 1.1 units per mg protein, respectively) and NADH

Table 2. Effect of O₂ on the glucose-dependent product profiles of RC

UM did not contain reducer and was supplemented with approximately 5 mM glucose. Due to the rapid lysis of RC, the maximum optical densities observed likely do not correspond to the maximum achieved during growth. CO₂ was not determined.

Glucose consumed (mM)	Maximum OD ₆₆₀	O ₂ (%)		Products (mM)				
		Initial	End	Butyrate	Lactate	Acetate	Formate	H ₂
Static								
5.1	0.52	0	0	2.3	0.1	2.8	0.8	9.6
5.2	0.22	10.7	7.2	0.7	4.2	3.2	0.5	2.3
4.6	0.05	21.2	18.5	0	7.6	0.9	0.2	0.2
Shaken								
4.7	0.47	0	0	2.2	0.1	2.4	0.6	17.1
4.7	0.35	3.3	0	0.4	4.7	2.3	0.1	1.5
4.7	0.15	5.9	2.3	0	5.8	2.4	0.1	0
2.0	0.05	12.1	10.9	0	2.6	0.5	0	0
1.4	0.00	20.3	20.3	0	2.1	0.2	0	0

Table 3. Product profiles of RS^T

Initial substrate concentrations approximated 5 mM; H₂ and CO were approximately 300 mbar overpressure in the headspace. CO₂ was not determined.

Substrate consumed (mM)	Maximum OD ₆₆₀	Acetate formed (mM)	Acetate ratio*	Recovered reductant (%)
Control†	0.08	5.3	NA	NA
Citrate (5.0)	0.26	16.0	10.7/11.2	95
Vanillate (3.2)‡	0.12	8.3	3.0/2.4	125
N-Acetylglucosamine (4.9)	0.56	19.6	14.3/19.6	73
Lactate (1.7)	0.09	6.1	0.8/2.6	31
Formate (6.0)	0.10	6.6	1.3/1.5	87
H ₂ (26.2)	0.12	10.6	5.3/6.6	81
CO (0)	0.02	1.3	–	–
Vanillate (0) + CO (0)	0.01	1.1	–	–

NA, Not applicable.

*Ratio represents the amount of acetate produced after correction for the amount of acetate formed in control culture lacking substrate divided by the amount of acetate expected via homoacetogenesis (as predicted from the amount of substrate utilized: Drake *et al.*, 2004).

†No substrate added.

‡Protocatachuate was the main aromatic product.

oxidase activity (5.6 and 32.2 units per mg protein, respectively). Catalase and superoxide dismutase were not detected in extracts of RC or RS^T. Hydrogenase activity (measured in the direction of uptake) was present in

cell-free extracts of RC and RS^T (87 and 2730 units per mg protein, respectively). Formate dehydrogenase activity was only detected in extracts of RS^T (7340 units per mg protein).

Table 4. Effect of O₂ on the growth of RS^T

UM did not contain reducer and was supplemented with N-acetylglucosamine (approx. 5 mM) or H₂ (approx. 300 mbar overpressure in the headspace). CO₂ was not determined.

Substrate consumed (mM)	Maximum OD ₆₆₀	O ₂ (%)		Acetate formed (mM)
		Initial	End	
Static				
N-Acetylglucosamine (5.1)	0.51	0	0	20.6
N-Acetylglucosamine (5.1)	0.49	0.6	0	20.3
N-Acetylglucosamine (5.3)	0.54	1.1	0	20.5
N-Acetylglucosamine (4.8)	0.53	2.5	0.9	17.5
N-Acetylglucosamine (0.1)	0.00	5.0	5.0	0.2
H ₂ (20.8)	0.10	0	0	9.5
H ₂ (24.0)	0.11	0.4	0	9.1
H ₂ (24.2)	0.10	0.8	0	9.0
H ₂ (0)	0.00	1.9	1.9	0.1
H ₂ (0)	0.00	3.9	3.8	0
Shaken				
N-Acetylglucosamine (5.3)	0.50	0	0	22.9
N-Acetylglucosamine (0.1)	0.00	0.5	0.4	0
N-Acetylglucosamine (0)	0.00	0.9	0.9	0
H ₂ (23.9)	0.12	0	0	10.9
H ₂ (1.2)	0.00	0.3	0.1	1.6
H ₂ (0.8)	0.00	0.8	0.8	0

Trophic interaction of RC and RS^T

RC utilized saccharides that RS^T did not, and RS^T grew at the expense of products formed by RC, i.e. H₂, formate and lactate. These properties suggested that of RC and RS^T would be metabolically linked and yield acetate and butyrate under co-culture conditions. Indeed, consumption of approximately 5 mM glucose by glucose-maintained co-cultures was concomitant with the production of approximately 6 mM acetate and 3 mM butyrate [these values are corrected for the amounts of acetate (4.5 mM) and butyrate (0.1 mM) produced by co-cultures in unsupplemented UM] under anoxic conditions. H₂ was a transient product in anoxic co-cultures, and lactate and formate were essentially not detected (Fig. 3a and data not shown). The maximum concentration of H₂ in anoxic co-cultures approximated 30 % that of anoxic RC cultures (Fig. 3a, Table 1). The high levels of hydrogenase and formate dehydrogenase

in RS^T were consistent with this strain's apparent use of RC-produced H₂ and formate in co-cultures. Glucose-maintained co-cultures consistently yielded approximately 50 % more butyrate than glucose-maintained RC cultures, and the recovery of glucose-derived reductant in co-cultures approximated 90 %, a value higher than that obtained in RC cultures (see above). These results suggested that the presence of H₂-consuming RS^T enhanced the production of butyrate by RC.

Lactate became a transient product in O₂-enriched (3 % in headspace) shaken co-cultures during the initial period when both glucose and O₂ were consumed (Fig. 3b). H₂ and butyrate were not apparent until the concentration of O₂ had decreased, and lactate and H₂ were subject to utilization when O₂ reached negligible levels. Acetate and butyrate were the two main reduced end products of O₂-enriched shaken co-cultures. These findings suggested that RC might aid

Fig. 3. Glucose-dependent product profiles of shaken co-cultures of RC and RS^T under anoxic (a) and O₂-enriched (b) conditions. Medium was UM without reducing agents. Error bars show the variation of triplicate cultures; symbols are placed at mean values (the error bar is smaller than the size of the symbol where no bar is apparent). ◆, OD₆₆₀; ◇, O₂; ○, glucose; ●, lactate; △, butyrate; ▲, acetate; □, H₂.

vegetative cells of RS^T in survival under highly oxic conditions, because pure cultures of RS^T did not grow under such conditions (Table 4).

DISCUSSION

The isolation of RC and RS^T as a commensal co-culture was fortuitous. However, this isolation illustrates the potential association of these and similar anaerobes in the rhizosphere of estuarine plants and is reminiscent of *T. aegyptius* and *M. thermoacetica*, which were also fortuitously isolated as a commensal co-culture (Gößner *et al.*, 1999). Glucose-dependent homoacetogenesis (i.e. the conversion of glucose to three molecules of acetate) is a hallmark of acetogens and is the basis by which the model acetogen *M. thermoacetica* was cultivated during the decades of research with it that resolved the acetyl-CoA pathway (Drake & Daniel, 2004). The inability of RS^T to utilize simple sugars demonstrates that it must be integrated to a food web via other trophic links under *in situ* conditions. The trophic interaction of RC and RS^T theoretically provides such a link (Fig. 4). H₂ appeared to be the primary trophic link between RC and RS^T under anoxic conditions. In contrast, formate appears to be the primary trophic link in co-cultures of the thermophiles *T. aegyptius* and *M. thermoacetica* (Gößner *et al.*, 1999).

RC is considered to be a strain of *C. intestinale* and utilized H₂-forming butyrate fermentation under anoxic conditions. RC's capacity to produce butyrate appeared to be enhanced by the presence of the H₂-utilizer RS^T. Clostridia have been classically referred to as spore-forming obligate anaerobes (Hippe *et al.*, 1992; Madigan & Martinko, 2005; Jones &

Keis, 2005). RC's tolerance to highly oxic conditions illustrates that clostridial species in the rhizosphere of estuarine plants can have extraordinary tolerance to O₂. Under highly oxic conditions, the catabolism of RC shifted to a nearly homolactate fermentation, a form of anaerobic catabolism utilized by a variety of aerotolerant fermenters (Jensen *et al.*, 2001; van Niel *et al.*, 2002; Matthies *et al.*, 2004; Madigan & Martinko, 2005). Butyrate fermentation necessitates the use of O₂-sensitive hydrogenase (Buckel, 2005), and RC's capacity to engage homolactate fermentation is likely the primary mechanism for its ability to cope with high amounts of O₂. The type strain of *C. intestinale* (ATCC 49213) was isolated from cattle faeces and, like RC, is aerotolerant (Lee *et al.*, 1989). However, the production of H₂, metabolic shifts in response to O₂, and quantitative product profiles have not been reported for *C. intestinale* (Lee *et al.*, 1989; Hippe *et al.*, 1992).

RS^T was identified as a new species of *Sporomusa*, and the name *Sporomusa rhizae* is proposed. All species of *Sporomusa* are acetogens. In contrast, clostridial acetogens do not form a monophyletic functional group (i.e. the closest phylogenetic neighbour of a clostridial acetogen might be a clostridial species that lacks the acetyl-CoA pathway) (Drake *et al.*, 2004; Drake & Küsel, 2005). Species of *Sporomusa* have been obtained from a variety of habitats, including silage (Möller *et al.*, 1984), the termite gut (Breznak *et al.*, 1988; Boga *et al.*, 2003) and soil (Kuhner *et al.*, 1997). The isolation of a species of *Sporomusa* from the roots of an estuarine plant extends the habitat range and illustrates the versatility of this spore-forming acetogenic genus.

Clostridium glycolicum RD1, a clostridial acetogen isolated from root homogenates of the seagrass *H. wrightii*, can tolerate up to 6 % O₂ in the gas phase and consume small amounts of O₂ (Küsel *et al.*, 2001; Drake & Küsel, 2005). RS^T likewise tolerated and reduced low concentrations of O₂. Other sporomusal species are also capable of reductively consuming O₂ (Drake *et al.*, 2002; Karnholz *et al.*, 2002; Boga *et al.*, 2003). Acetogenesis is dependent upon the acetyl-CoA pathway, and many of the enzymes involved in this reductive pathway are highly sensitive to oxidation (Wood & Ljungdahl, 1991; Ragsdale, 1991). Acetogens have several mechanisms for coping with oxidative stress. For example, *C. glycolicum* RD1 engages ethanol and lactate fermentations when challenged with O₂ (Küsel *et al.*, 2001). RS^T did not undergo such a metabolic shift and likewise had a higher sensitivity to O₂ than *C. glycolicum* RD1. Acetogens possess a variety of oxidoreductases that can reduce O₂ and its toxic by-products peroxide and superoxide. For example, the model acetogen *M. thermoacetica* contains peroxidase, NADH oxidase, rubredoxin oxidoreductase (a superoxide reductase) and rubrerythrin (a peroxidase), and also has the ability to dissimilate nitrate to ammonium (a more redox-positive half-cell reaction than that of CO₂/acetate) as an alternative terminal electron-accepting process (Das *et al.*, 2001, 2005; Karnholz *et al.*, 2002; Silaghi-Dumitrescu *et al.*, 2003; Drake & Daniel, 2004).

Fig. 4. Scheme illustrating the postulated trophic interaction of *C. intestinale* RC and *S. rhizae* RS^T.

Marine and estuarine plants grow in sediments that are constantly or frequently water saturated, and anoxia can occur at depths of only a few millimetres in flooded sediments. Plants growing in anoxic sediments ventilate their root systems to provide O₂ for root respiration (Blom, 1999; Jackson & Armstrong, 1999). Ventilation occurs via the aerenchyma, a tissue containing interconnected intercellular gas-filled spaces that can occupy as much as 70 % of the plant tissue volume. Diffusive and convective airflows through the aerenchyma of stems, rhizomes and roots have been demonstrated for several species (Christensen *et al.*, 1994; Hwang & Morris, 1991). In the case of plants having stomata, such as *Juncus* and other emergent grasses, ventilation is not dependent upon photosynthesis and thus occurs throughout the day–night cycle. Many of these ventilating plants appear to leak O₂ from root surfaces (Lovell, 2005 and references therein). Thus, it is likely that anaerobic bacteria growing on and in the roots of ventilating plants (i) must be innately aerotolerant (as is RC), (ii) grow in association with O₂-consuming organisms (as exemplified by RS^T's trophic growth with RC), or (iii) have other adaptations protecting them from O₂ toxicity (e.g. through development of protective microenvironments, a mechanism exploited by anaerobes in a number of O₂-rich environments: Jorgensen, 1977; Paerl & Pinckney, 1996).

In conclusion, this study provides an example of a symbiotic interaction between an aerotolerant fermentative *Clostridium* and an acetogenic *Sporomusa* that not only permits commensal utilization of saccharides with production of acetate, the key substrate for terminal carbon oxidation processes in marine and estuarine sediments (Gibson, 1990; Laanbroek & Pfennig, 1981; Sorensen *et al.*, 1981), but also confers protection to the more O₂-sensitive partner by the O₂-tolerant partner (Fig. 4). Such symbiotic, protective interactions are relatively unexplored, but may be both commonplace and essential to the activities of acetogens and other anaerobes in environments subject to transient fluxes of O₂.

Description of *Sporomusa rhizae* sp. nov.

Sporomusa rhizae [rhi'zæ. N.L. gen. fem. n. *rhizae* (from Gr. fem. n. *rhiza*, root), of the root; to indicate a spore-forming banana (per epithet of *Sporomusa*) from roots]. An acetogenic bacterium. Cells are slightly curved flagellate rods (3.5 × 0.8 µm), form endospores, and contain a membranous *b*-type cytochrome. Grows at 15–40 °C (optimum at 35 °C) and at pH 5.5–9.0 (optimum at pH 7.5). Grows anaerobically on vanillate, syringate, citrate, *N*-acetylglucosamine, betaine, lactate, formate and H₂. Acetate is primary reduced end product. Tolerates and consumes (i.e. reduces) small amounts of O₂. Peroxidase, NADH oxidase, hydrogenase and formate dehydrogenase positive. The type strain RS^T (=DSM 16652^T = ATCC BAA-1028^T) (i) was isolated from a root homogenate prepared from the black needle-rush *Juncus roemerianus*, and (ii) grows by trophic interaction with the aerotolerant fermentative *Clostridium intestinale* RC (fortuitously co-isolated with RS^T).

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