Clostridium scatologenes strain SL1 isolated as an acetogenic bacterium from acidic sediments

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A strictly anaerobic, H_2-utilizing bacterium, strain SL1, was isolated from the sediment of an acidic coal mine pond. Cells of strain SL1 were sporulating, motile, long rods with a multilayer cell wall. Growth was observed at 5–35 °C and pH 3·9–7·0. Acetate was the sole end product of H_2 utilization and was produced in stoichiometries indicative of an acetyl-CoA-pathway-dependent metabolism. Growth and substrate utilization also occurred with CO/CO_2, vanillate, syringate, ferulate, ethanol, propanol, 1-butanol, glycerine, cellobiose, glucose, fructose, mannose, xylose, formate, lactate, pyruvate and gluconate. With most substrates, acetate was the main or sole product formed. Growth in the presence of H_2/CO_2 or CO/CO_2 was difficult to maintain in laboratory cultures. Methoxyl, carboxyl and acrylate groups of various aromatic compounds were O-demethylated, decarboxylated and reduced, respectively. Small amounts of butyrate were produced during the fermentation of sugars. The acrylate group of ferulate was reduced. Nitrate, sulfate, thiosulfate, dimethylsulfoxide and Fe(III) were not utilized as electron acceptors. Analysis of the 16S rRNA gene sequence of strain SL1 demonstrated that it is closely related to *Clostridium scatologenes* (99·6% sequence similarity), an organism characterized as a fermentative anaerobe but not previously shown to be capable of acetogenic growth. Comparative experiments with *C. scatologenes* DSM 757^T demonstrated that it utilized H_2/CO_2 (negligible growth), CO/CO_2 (negligible growth), formate, ethanol and aromatic compounds according to stoichiometries indicative of the acetyl-CoA pathway. CO dehydrogenase, formate dehydrogenase and hydrogenase activities were present in both strain SL1 and *C. scatologenes* DSM 757^T. These results indicate that (i) sediments of acidic coal mine ponds harbour acetogens and (ii) *C. scatologenes* is an acetogen that tends to lose its capacity to grow acetogenically under H_2/CO_2 or CO/CO_2 after prolonged laboratory cultivation.

Keywords: *Clostridium scatologenes*, acetogenesis, carbon monoxide dehydrogenase, Gram-positive bacteria, acid mine ponds

INTRODUCTION

Acetogenic bacteria are obligate anaerobes that use the acetyl-CoA pathway and reduce CO_2 to acetate as their main product of respiration (Wood & Ljungdahl, 1991; Drake, 1994). Most acetogens have been isolated from strictly anaerobic habitats like marine or estuarine sediments, freshwater ponds or anoxic sewage sludge (Drake, 1994; Schink, 1994). Phylogenetically they do not form a distinct unit, but are intermixed within the large phylogenetic group of Gram-positive clostridia (Tanner & Woese, 1994). Based on comparative 16S rRNA gene sequence analyses and phenotypic data, the extremely heterogeneous genus *Clostridium* was taxonomically restructured and new nomenclature for many acetogens was proposed (Collins *et al.*, 1994; Willems & Collins, 1995, 1996). According to the taxonomic rearrangement, acetogens are present in eight different clusters (Willems &

The EMBL accession number for the 16S rRNA gene sequence of strain SL1 is Y18813.

Collins, 1996). Based on their phylogenetic proximity, closely related clostridial and acetogenic species might possess similar physiological capabilities.

In this study, an H_2-utilizing acetogen, isolate SL1, was isolated from an acidic sediment. Analysis of the 16S rRNA gene sequence of SL1 indicated that the isolate was closely related to *Clostridium scatologenes*. Comparative evaluation of *C. scatologenes* DSM 757^T revealed that it was capable of acetogenic growth and contained CO dehydrogenase, indicating that *C. scatologenes* should be classified as an acetogen.

METHODS

Field site and sampling. Sediments were obtained from an acidic coal mine pond located in the Lusatian mining area in east central Germany. The pH of the lake water and of the upper iron-rich sediment was approximately 3 and 3·2, respectively. The maximum summer temperature of the upper sediment was 12 °C. No oxygen was detected at the water/sediment interface. Replicate sediment cores were collected in February 1997 with a gravity corer in plexiglas tubes (i.d. 5·9 cm), transported to the laboratory and sectioned under an N_2 atmosphere within 24 h.

Enrichment cultures. Upper sediment from five replicate cores was pooled under anoxic conditions and 40 g (fresh wt) sediment was transferred to sterile 150 ml infusion bottles (Merck) inside a Mecaplex anaerobic chamber (100 % N_2 gas phase). Bottles were closed with rubber stoppers and screw-cap seals, flushed with sterile argon for 15 min and incubated in the dark at 12 °C with an initial overpressure of 20–25 kPa argon at room temperature for 90 d. Sterile H_2 [28 mmol (l culture)$^{-1}$] was added as electron donor. After the complete consumption of H_2, aliquots of the sediment were added to anaerobic FePPi medium [approx. 1 g sediment to 9 ml medium (see below)]. The enrichment cultures were supplemented with H_2 [28 mmol (l culture)$^{-1}$] and incubated at 15 °C. After two transfers, enrichment cultures were streaked onto solid U medium [solidified with 1·5 % agar (see below)]. Isolated colonies were transferred to and maintained in liquid U medium for assessment of substrate/product profiles. Cultures were considered to be pure based on uniform colony and cell morphologies.

Composition of media. Anaerobic media were prepared by the modified Hungate technique (Hungate, 1969). FePPi medium (Caccavo *et al.*, 1994) contained (l^{-1}): $NaHCO_3$, 2·5 g; NH_4Cl, 1·5 g; KH_2PO_4, 0·6 g; KCl, 0·1 g; yeast extract, 0·5 g; vitamins (Drake, 1994), 10 ml; trace metals (Drake, 1994), 10 ml; soluble ferricpyrophosphate [$Fe_4(P_2O_7)_3$], 3 g. The gas phase was N_2/CO_2 (80 : 20, v/v); the final pH of the medium was approximately 6·7. U medium is a non-reduced, undefined medium and contained (l^{-1}): $NaHCO_3$, 7·5 g; KH_2PO_4, 0·5 g; $MgCl_2.6H_2O$, 0·05 g; NaCl, 0·4 g; NH_4Cl, 0·4 g; $CaCl_2.2H_2O$, 0·01 g; yeast extract, 1·0 g; B vitamin solution (Drake, 1994), 5·0 ml; trace element solution (Drake, 1994), 5·0 ml. The gas phase was 100 % CO_2 and the pH after autoclaving was approximately 6·9. TSB medium contained tryptic soy broth (Difco), without dextrose, at a final concentration of 2·75 g l^{-1}; the initial pH of the medium was adjusted to different values, as indicated. The gas phase was 100 % N_2. Substrates were added as sterile stock or as sterile gas. For substrate utilization studies, most substrates were added at an initial concentration of 5–10 mM; sodium formate and ethanol were added at 25 and 15 mM, respectively. The concentrations of H_2 and CO were as indicated. Unless otherwise indicated, the incubation temperature was 30 °C.

Analytical techniques. Headspace gases were measured with Hewlett Packard 5980 series II gas chromatographs (Küsel & Drake, 1995). Gas values included the total amounts in both liquid and gas phases. In this study, no distinction was made between CO_2 and its carbonate forms. Concentrations were corrected for the changing liquid/gas phase volume ratio due to liquid samplings (0·5 ml). Aliphatic acids, aromatic compounds, alcohols and sugars were determined with Hewlett Packard 1090 series II high-performance liquid chromatographs (Küsel & Drake, 1995). Nitrate and sulfate were analysed by ion chromatography (Küsel & Drake, 1995). Reduction of Fe(III) was determined visually by a change of the yellow medium to colourless and the formation of a white Fe(II) carbonate precipitate, and also analytically by measuring the accumulation of Fe(II). Fe(II) was measured by the phenanthroline method (Tamura *et al.*, 1974). The pH was measured with an Ingold U457-S7/110 combination pH electrode. Growth was monitored as OD_{660} with a Spectronic 501 spectrophotometer (Bausch and Lomb). Protein content was determined by the method of Bradford (1976).

Electron microscopy. Strain SL1 was cultivated at 30 °C in U medium supplemented with fructose (10 mM). Cells were fixed by adding glutaraldehyde to a final concentration of 2 % (v/v) and harvested by centrifugation. For negative staining (Valentine *et al.*, 1968), an aqueous solution of uranyl acetate (2 %, w/v; pH 4·6) was used. For thin-section preparations, pelleted cells were embedded in agar (2 %, w/v) and fixed in glutaraldehyde/OsO_4 (Traub *et al.*, 1976). After staining for 7 min with 2 % uranyl acetate and, subsequently, for 5 min with lead citrate (Reynolds, 1963), specimens were examined in a model CEM 902A microscope (Zeiss).

Preparation of cell extract and enzyme assays. Cells were cultivated in U medium supplemented with either ethanol (10 mM) or vanillate (10 mM). Cultures were centrifuged and the cell pellet was washed and incubated in lysozyme buffer as described previously (Kuhner *et al.*, 1997). Hydrogenase and CO dehydrogenase activities were assayed at 30 °C in anaerobic Tris/HCl buffer (100 mM, pH 8·5) containing 1 mM benzyl viologen and 1 mM dithiothreitol; the gas phase was 100 % H_2 for hydrogenase assays and 100 % CO for CO dehydrogenase assays (Drake, 1982a).

Preparation and redox difference spectra of membranes. Cells were cultivated in U medium supplemented with fructose (10 mM). Membranes were prepared from cell extracts by ultracentrifugation under aerobic conditions (Fröstl *et al.*, 1996). Washed membranes were reduced with sodium dithionite and reduced-minus-oxidized spectra were obtained with a model Uvikon 930 double-beam recording spectrophotometer (Kontron Instruments) at room temperature.

SDS-PAGE. Cells were cultivated in U medium supplemented with either fructose (10 mM), vanillate (10 mM) or lactate (10 mM) and cell extracts were prepared by lysozyme digestion for electrophoretic analysis. Preparation of gels and one-dimensional electrophoresis were according to standard protocols (Laemmli, 1970).

Isolation and G+C content of DNA. DNA was isolated by standard methods (Cashion *et al.*, 1977). The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989).

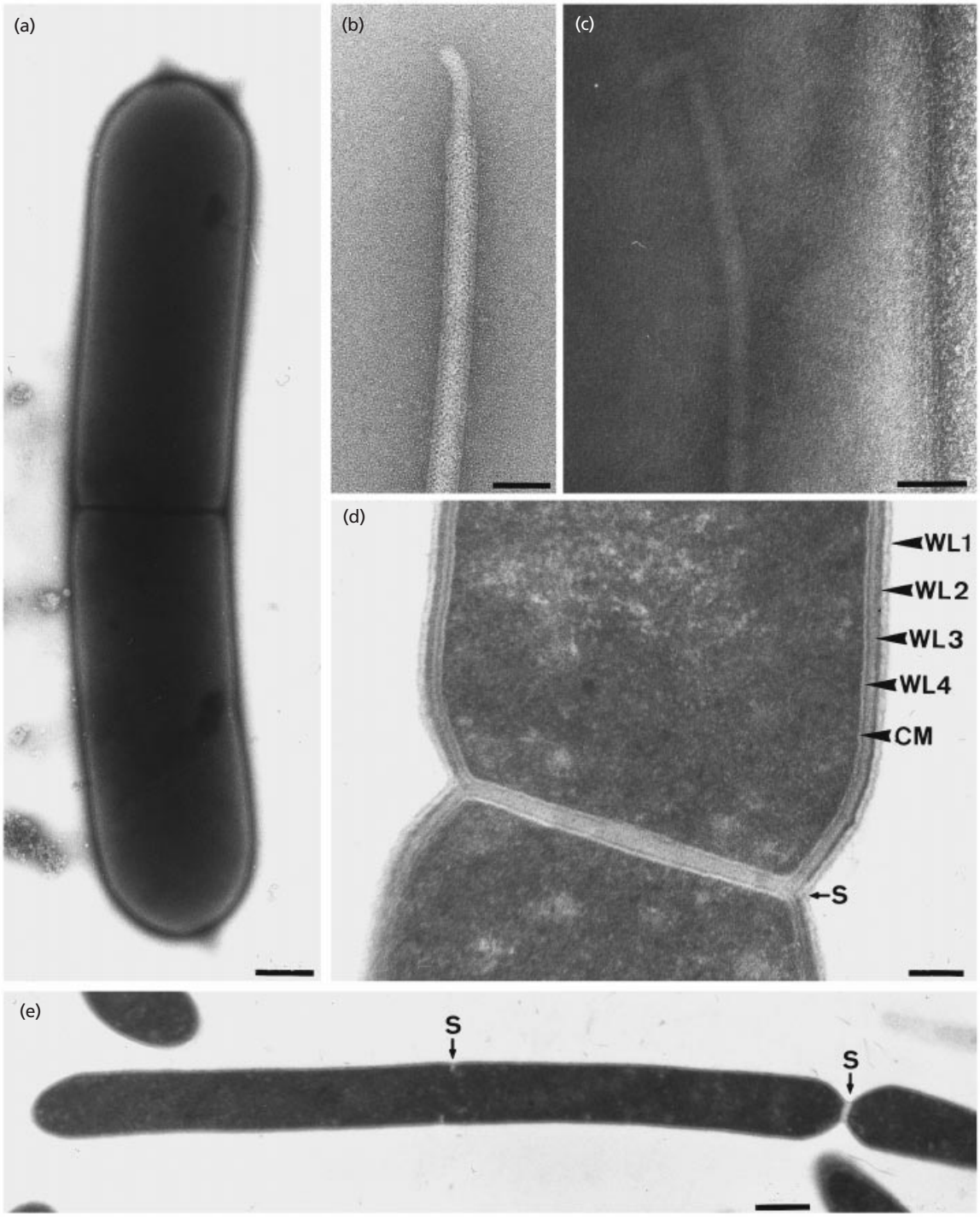

Fig. 1. Electron micrographs of strain SL1. (a–c) Negatively stained cells showing (a) cell division, (b) an isolated flagellum revealing the flagellar filament and hook, and (c) a laterally inserted flagellum. (d, e) Thin section micrographs of dividing cells. Bars: 0·5 (a, e), 0·1 (c, d) and 0·05 μm (b). WL, cell wall layer; CM, cytoplasmic membrane; S, septum.

16S rRNA gene sequencing and construction of the dendrogram. Genomic DNA was extracted, amplified by PCR and purified (Rainey *et al.*, 1996). Purified PCR products were sequenced by using an ABI PRISM Ready Reaction Dye Terminator kit (Applied Biosystems). Sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A DNA sequencer. Alignments of the sequence were done manually and determination of similarity values were done by the ae2 editor (Maidak *et al.*, 1996). Phylogenetic dendrograms were constructed according to the method of DeSoete (1983) and by the neighbour-joining method contained in the PHYLIP

package (Felsenstein, 1993; Saitou & Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of neighbour-joining data by performing 500 resamplings (Felsenstein, 1985). Accession numbers of reference organisms are included in Fig. 4.

RESULTS

Enrichment and isolation of strain SL1

Enrichment cultures in FePPi medium consumed supplemental H_2 within 7 d and produced acetate. Fe(III) was not reduced. After three subsequent transfers in liquid FePPi medium, the enrichment was streaked onto solidified U medium. A colony was picked and serially diluted in liquid U medium. The consumption of supplemental H_2 occurred within 3 d in all growth-positive dilutions and was coincident with the production of acetate. The mean H_2/acetate ratio was approximately 4·2:1, a value indicative of H_2-dependent acetogenesis (Wood & Ljungdahl, 1991; Drake, 1994). Isolate SL1 was obtained after consecutively restreaking (three times) an isolated colony obtained from the highest positive dilution. Colonies were shiny, convex, beige and had a maximum diameter of 3–4 mm.

Morphology of strain SL1

Isolate SL1 was a motile, long, thin rod (Fig. 1). Single cells were 3–4 μm in length; chains of cells were up to 10 μm long (Fig. 1e). Flagellar staining (Blenden & Goldberg, 1965) and electron microscopy revealed peritrichous flagella. Cells were motile in wet mounts. In old cultures, terminal and free spores were observed. Cells stained weakly Gram-negative (Clark, 1973) and thin sections revealed a multilayer cell wall (Fig. 1d).

General growth properties of strain SL1

Isolate SL1 grew at 5–35 °C; no growth was observed at 40 °C. Rates and yields of growth were optimal at 25–30 °C. Growth was observed after a sporulated culture was heated for 15 min at 80 °C. In U medium at pH 6·8 supplemented with fructose (10 mM), the doubling time at both 25 and 30 °C was approximately 5·3 h. Growth could not be maintained in U medium lacking yeast extract. In TSB medium strain SL1 grew at pH 3·9–7·0. No growth was observed at pH 3·4 and 7·8. Rates and yields of growth were optimal at pH 5·8 to 6·9. No growth or substrate consumption were observed in the presence of oxygen. In U and TSB medium, strain SL1 produced a dung-like odour similar to skatole. When grown on tryptophan in U medium, the skatole-like odour was more intense.

Substrate range and fermentation stoichiometries of strain SL1

In U medium, the following substrates were utilized: cellobiose, glucose, fructose, xylose, arabinose, mannose, vanillate, syringate, ferulate, ethanol, propanol, 1-butanol, glycerine, lactate, gluconate, pyruvate, for-

Table 1. Acetate/substrate stoichiometry of strain SL1 and *C. scatologenes* DSM 757^T cultivated in U medium

Organism	Substrate	Total incubation time (h)	ΔOD_{660}*	Substrate consumed (mM)†	Acetate produced (mM)‡	Acetate/substrate ratio§
Strain SL1	Fructose	50	0·90	8·5	20·3	2·39 (3)
	Arabinose	50	0·79	8·1	19·8	2·44 (2·5)
	Ethanol	120	0·15	16·8	23·8	1·42 (1·5)
	Formate	100	0·17	26·3	6·9	0·26 (0·25)
	Pyruvate	50	0·21	1·7	2·1	1·24 (1·25)
	Vanillate	100	0·18	9·2	7·2	0·78 (0·75)
	H_2	380	0·06	28·0	8·0	0·29 (0·25)
	CO	380	0·08	27·6	7·3	0·26 (0·25)
C. scatologenes	Fructose	50	0·79	8·4	15·4	1·83 (3)
	Arabinose	50	0·69	7·8	15·6	2·00 (2·5)
	Ethanol	120	ND	11·8	11·8	1·00 (1·5)
	Formate	100	ND	24·3	3·3	0·14 (0·25)
	Vanillate	100	0·12	8·4	5·8	0·69 (0·75)
	H_2	2000	Negligible‖	15·4	3·1	0·20 (0·25)
	CO	2900	Negligible‖	27·1	3·9	0·14 (0·25)

* Values are corrected for the ΔOD_{660} in controls lacking additional substrates. ND, Not determined.

† Values are the mean of two or three replicates.

‡ Values are corrected for the amount of acetate formed in controls lacking additional substrates.

§ Values in parentheses are the theoretical acetate/substrate ratios.

‖ During the first 2 d, growth was similar to controls lacking substrate and then levelled off.

Table 2. Effect of different media on the product profiles of strain SL1 and *C. scatologenes* DSM 757^T cultivated on sugars

The initial concentration of sugars was 10 mM and decreased to non-detectable levels after 3 d incubation.

Organism	Medium	Substrate	Acetate produced (mM)*	Butyrate produced (mM)*	H_2 produced (mM)*	Recovery of reducing equivalents (%)
Strain SL1	U	Fructose	21·4 ± 0·6	1·2 ± 0·0	0	81
		Arabinose	25·0 ± 0·5	0·5 ± 0·2	0	95
	TSB	Fructose	12·8 ± 0·4	3·2 ± 0·2	Trace	69
		Arabinose	13·7 ± 0·3	2·4 ± 0·1	Trace	72
C. scatologenes	U	Fructose	18·5 ± 0·8	1·6 ± 0·1	0	75
		Arabinose	22·0 ± 1·2	0·9 ± 0·0	0	88
	TSB	Fructose	8·4 ± 0·8	3·5 ± 0·1	2·4 ± 0·8	57
		Arabinose	10·1 ± 0·1	3·2 ± 0·2	0·4 ± 0·1	66

* Values are the mean (± SD) of three replicates and are corrected for the amount of product formed in controls lacking additional substrates. Trace, non-quantifiable levels detected.

mate, H_2/CO_2 and CO/CO_2. In all cases, optical densities were greater than those observed in the absence of supplemental substrates and acetate was the major end product (Table 1 and data not shown). Butyrate was also formed during the fermentation of sugars. In TSB medium, strain SL1 produced higher amounts of butyrate than in U medium (Table 2). Products formed from yeast extract or TSB were acetate, butyrate and iso-valerate. Neither growth nor substrate utilization were observed with melizitose, saccharose, lactose, maltose, galactose, trimethoxybenzoate, 4-hydroxybenzaldehyde, methanol, 2,3-butanediol, succinate, fumarate, citrate, oxalate, glyoxylate, acetate or butyrate.

Vanillate was O-demethylated and decarboxylated to catechol (Fig. 2a). When fructose and protocatechuate were provided simultaneously as substrates, protocatechuate was decarboxylated to catechol during fructose consumption (Fig. 2b). The aromatic ring recovery was approximately 95%, indicating that the aromatic ring was not subject to further transformation. The vanillate/acetate ratio was approximately 4:3·1 (Table 1), a value indicative of vanillate-dependent acetogenesis (Drake, 1994). Syringate was sequentially O-demethylated to 5-hydroxyvanillate and gallate (data not shown).

Maintenance of H_2 and CO cultures of strain SL1

The capacity of strain SL1 to utilize H_2 and CO decreased with prolonged cultivation. For example, strain SL1 consumed 28 mmol H_2 (l culture)$^{-1}$ within 5 d of when it was originally isolated and grown under H_2; this time increased to 4 weeks after six sequential transfers under H_2. In contrast, the capacity of strain SL1 to utilize aromatic compounds was stable in maintained cultures.

Reduction of alternative electron acceptors by strain SL1

Ferulate was reduced to hydroferulate and further O-demethylated to hydrocaffeate (Fig. 3). When isolate SL1 was grown in the presence of nitrate (5 mM), nitrate was not utilized and acetate production was not appreciably affected, indicating that nitrate is not used as an alternative electron acceptor. Similar results were obtained with sulfate, thiosulfate, dimethylsulfoxide and Fe(III), indicating that they too were not used as alternative electron acceptors.

Enzyme activities and redox difference spectra of membranes of strain SL1

The CO dehydrogenase activities in cell extracts obtained from fructose-, vanillate- and ethanol-cultivated cells was approximately 0·3, 3·8 and 6·3 μmol min^{-1} (mg protein)$^{-1}$, respectively (Table 3). Hydrogenase and formate dehydrogenase activities in extracts of ethanol-grown cells were greater than those of fructose- and vanillate-grown cells (Table 3). No absorption maxima indicative of a type *b* or type *c* cytochrome were detected in the membrane or cytoplasmic fraction of strain SL1 (data not shown).

16S rRNA gene sequence and G+C content of strain SL1

Phylogenetic analysis of the almost complete 16S rRNA gene sequence (93·3% of the *Escherichia coli* sequence) with the database of 16S rRNA gene sequences (Maidak *et al.*, 1996) indicated that strain SL1 is a member of the low G+C Gram-positive bacteria. The highest sequence similarity value (99·6%) was to *C. scatologenes* (Fig. 4); the similarities

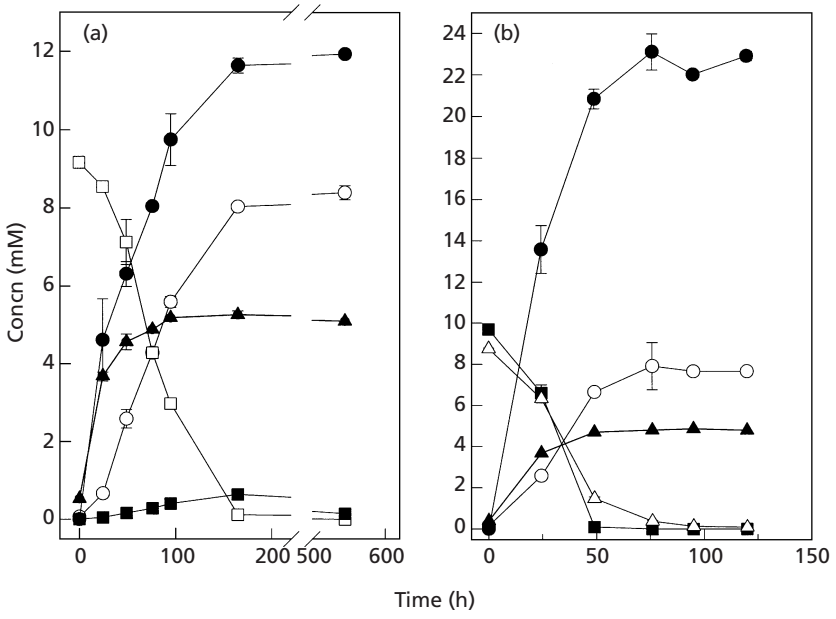

Fig. 2. Substrate/product profiles of strain SL1 cultivated in U medium supplemented with vanillate (a) and with fructose and protocatechuate (b). Data are the means (±SD) of three replicates. □, Vanillate; ■, protocatechuate; ○, catechol; ●, acetate; △, fructose, ▲ acetate in controls lacking additional substrate.

of strain SL1 to *Clostridium magnum* and *Clostridium pasteurianum* were 95·1 and 93·8 %, respectively. The DNA G+C content of strain SL1 was 30·3 mol %. The G+C content of *C. scatologenes* is 27 mol % (Johnson & Francis, 1975).

Comparative protein profiles of SL1 and *C. scatologenes* DSM 757^T

Cells of strain SL1 and of *C. scatologenes* DSM 757^T that had been cultivated on fructose, lactate or vanillate yielded nearly identical protein profiles when cell extracts were subjected to SDS-PAGE analysis (data not shown).

Comparative evaluation of the acetogenic capacities of *C. scatologenes* DSM 757^T

Based on the 16S rRNA gene sequence and protein profile similarities between strain SL1 and *C. scatologenes*, *C. scatologenes* DSM 757^T was analysed for its acetogenic capacities. In U medium, *C. scatologenes* DSM 757^T grew slower than strain SL1. *C. scatologenes* DSM 757^T grew more rapidly and produced higher cell yields in TSB medium compared to U medium. *C. scatologenes* DSM 757^T utilized the following substrates in U medium: cellobiose, glucose, fructose, arabinose, mannose, vanillate, ethanol, 1-butanol, lactate, pyruvate, formate, H_2/CO_2 and CO/CO_2. The acetate/substrate ratios obtained with *C. scatologenes* DSM 757^T were lower than those obtained with strain SL1 (Table 1). TSB medium lacked supplemental CO_2 and the recovery of reductant on TSB medium was less than that obtained with U medium for both *C. scatologenes* DSM 757^T and strain SL1 (Table 2). In addition, H_2 was produced when both strains were cultivated in TSB medium but not in U medium; the production of H_2 was greater with *C. scatologenes* DSM 757^T (Table 2). Vanillate was O-demethylated and decarboxylated to catechol, yielding acetate.

H_2 and CO were consumed very slowly by *C. scatologenes* DSM 757^T and growth on these substrates was negligible (see Table 1). Nonetheless, consumption of H_2 and CO was concomitant to the production of acetate. Despite the difficulty in demonstrating the growth of *C. scatologenes* DSM 757^T under H_2 and CO, and despite the decreased acetate/substrate ratios of *C. scatologenes* DSM 757^T, CO dehydrogenase, hydrogenase and formate dehydrogenase activities in cell extracts of *C. scatologenes* DSM 757^T were similar to those of strain SL1 (Table 3).

DISCUSSION

C. scatologenes has been isolated from soil and other habitats and is named for its production of skatole, an organic compound (3-methyl-1-H-indole) that has a dung-like odour (Weinberg & Ginsbourg, 1927; Smith, 1975). Strain SL1 produced a similar odour. *C. scatologenes* is described as an obligately anaerobic, Gram-positive, spore-forming, motile rod with a length of 3–21 μm (Holdeman *et al.*, 1977). As reported for other clostridial cells (Cato *et al.*, 1986), cells of strain SL1 stained weakly Gram-negative; however, thin sections did not reveal an outer membrane (Fig. 1d). The extended length of strain SL1 observed by light microscopy was likely due to predivisional elongation of the cells (Fig. 1e).

C. scatologenes has been characterized as both a saccharolytic and proteolytic fermenter that produces acetate and butyrate as main products and caproate, formate, propionate, iso-valerate and valerate as trace products (Holdeman *et al.*, 1977). However, the capacity of *C. scatologenes* to grow acetogenically on

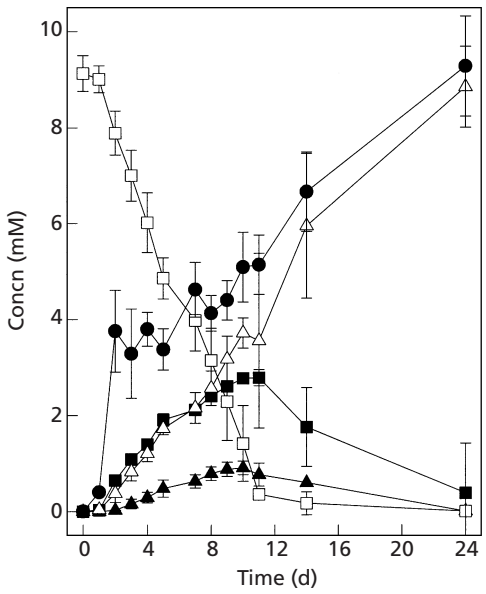

Fig. 3. Substrate/product profiles of strain SL1 cultivated in U medium supplemented with ferulate. Data are the means (±SD) of three replicates. □, Ferulate; ■, hydroferulate; △, hydrocaffeate; ▲, caffeate; ●, acetate. In controls lacking additional substrate, the concentration of acetate was approximately 2·2 mM.

C_1 compounds like H_2/CO_2, CO/CO_2, formate or O-methyl groups of aromatic compounds has not been reported in the past. The substrate utilization capacities of *C. scatologenes* DSM 757^T and strain SL1 were similar; however, the capacity of both strains to utilize H_2 and CO was minimal and difficult to sustain. Neither strain grew autotrophically (i.e. under H_2/CO_2 without yeast extract). Many other acetogens (e.g. *Ruminococcus productus* and *Ruminococcus hansenii*; Bernalier *et al.*, 1996) also display weak autotrophic capacities. In microcosms prepared from sediments of this cold, acidic coal mine pond, acetogens are capable of consuming H_2 at 12 °C (Küsel *et al.*, 1998). The isolation of strain SL1, an acetogen that is capable of growth over broad temperature and pH ranges, corroborates the possibility that acetogenesis is an ongoing process in this ecosystem. Relatively little information is available on the growth of acetogens under low pH and low temperature conditions (Phelps & Zeikus, 1984; Kotsyurbenko *et al.*, 1993; Wiegel, 1994).

Both strain SL1 and *C. scatologenes* DSM 757^T utilized various side chains of aromatic compounds. Many acetogens can utilize O-methyl groups of aromatic compounds (Frazer, 1994) or reduce the carbon–carbon double bond of acrylate side chains of aromatic compounds (Drake *et al.*, 1994). Decarboxylation of benzoate derivatives has also been reported for the thermophilic acetogens *Moorella thermoacetica* (Hsu *et al.*, 1990) and *Moorella thermoautotrophica* (Fröstl *et al.*, 1996). Small amounts of butyrate were formed by strain SL1. Butyrate, together with acetate, is also produced by the acetogen *Eubacterium limosum* (Sharak-Genthner *et al.*, 1981) and closely related '*Butyribacterium methylotrophicum*' (Zeikus *et al.*, 1980). *C. scatologenes* DSM 757^T also produced small amounts of H_2 in TSB medium supplemented with sugars; the production of H_2 by *C. scatologenes* grown on peptone/yeast extract/glucose (PYG) broth has been reported (Holdeman *et al.*, 1977). Many acetogens, including *M. thermoacetica* (Martin *et al.*, 1983; Daniel *et al.*, 1990), produce trace levels of H_2. The loss of reductant as H_2 by *C. scatologenes* DSM 757^T in TSB medium indicates that (i) the flow of reductant to the acetyl-CoA pathway can be suppressed by a rich organic medium or (ii) certain conditions induce a very efficient H_2-producing hydrogenase. Significant levels of H_2 were not produced by strain SL1, indicating that strain SL1 and *C. scatologenes* DSM 757^T are not physiologically identical.

That *C. scatologenes* is an acetogen is consistent with its phylogenetic branching adjacent to the acetogen

Table 3. Enzyme activities in cell extracts of strain SL1 and *C. scatologenes* DSM 757^T cultivated on various substrates

Cells were grown in U medium supplemented with 10 mM substrate (as indicated) and harvested in late exponential phase.

Organism	Growth substrate	Specific activity [U (mg protein)$^{-1}$]*		
		CODH	H_2ase	FDH
Strain SL1	Fructose	0·27±0·15	3·02±0·87	0·02±0·01
	Vanillate	3·76±1·42	8·70±0·76	0·09 ± 0·02
	Ethanol	6·26±2·11	23·13±1·86	0·42±0·17
C. scatologenes	Vanillate	2·48±0·87	9·45±0·26	0·12±0·02
	Ethanol	0·74±0·05	26·69±2·13	1·01±0·21

* Values are the mean (±SD) of three replicates. Control values were approximately 0·00±0·01 U (mg protein)$^{-1}$. CODH; CO dehydrogenase; H_2ase, hydrogenase; FDH, formate dehydrogenase.

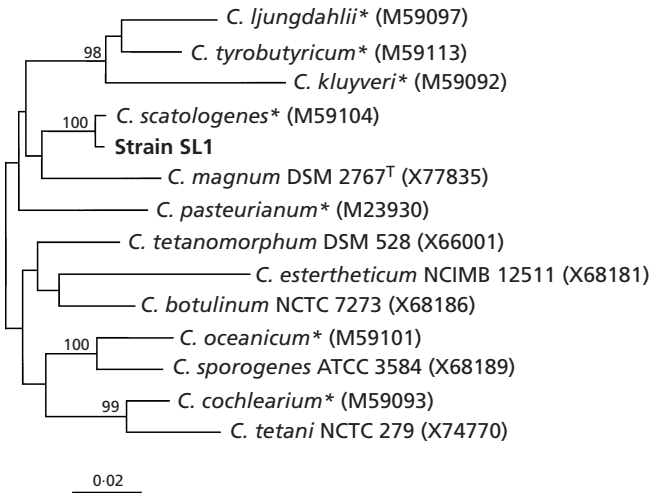

Fig. 4. Dendrogram showing the phylogenetic position of strain SL1 (based on analysis of the 16S rRNA gene). Scale bar, 12 nt substitutions per 100 nt. Numbers at the branching points refer to bootstrap values > than 95% (500 resamplings). *, Strain numbers not available. Accession numbers of 16S rRNA gene sequences are given in parentheses.

Clostridium magnum (Fig. 4). *C. magnum* was initially isolated as a heterotrophic acetogen (Schink, 1984) but was later shown to be capable of lithotrophic growth under H_2/CO_2 (Bomar *et al.*, 1991). The acetogen *M. thermoacetica* was also isolated as an obligate heterotroph (Fontaine *et al.*, 1942) and later shown to grow autotrophically under H_2/CO_2 and CO/CO_2 (Daniel *et al.*, 1990). Likewise, the acetogen *Clostridium formicoaceticum* was thought to be incapable of autotrophic growth (Andreesen *et al.*, 1970) until it was shown to grow autotrophically under CO/CO_2 (Lux & Drake, 1992). In general, most acetogens grow very poorly under H_2/CO_2, in part because of the thermodynamic constraints of reducing CO_2 to the CO level on the carbonyl branch of the acetyl-CoA pathway (Drake, 1994). In addition, despite the obligatory occurrence of CO dehydrogenase in acetogens, some acetogens cannot grow in the presence of CO [e.g. *Thermoanaerobacter* (*Acetogenium*) *kivui*; Daniel *et al.*, 1990]. Thus, it is not surprising that the capacity of *C. scatologenes* to grow acetogenically in the presence of H_2 or CO has thus far gone undetected and is difficult to maintain. That strain SL1 tends to lose its ability to efficiently utilize H_2 or CO indicates that *C. scatologenes* loses this lithotrophic capacity upon prolonged cultivation in the laboratory.

The acetogenic capacity of certain clostridial species may be the result of (i) divergent evolution from an ancestral species or (ii) the independent occurrence of the acetyl-CoA pathway among different clostridial groups (Tanner & Woese, 1994). The capacity to fix CO_2 via the acetyl-CoA pathway is common to other anaerobic prokaryotes like methanogens (Fuchs, 1994) and the similarity between the metal centres and the reactions catalysed by both acetogenic and methanogenic bacteria might be of evolutionary significance (Ragsdale, 1994). It is interesting to note that the butyrate fermenter *Clostridium pasteurianum* is not a close phylogenetic relative of *C. scatologenes* but nonetheless can consume (i.e. oxidize) CO during growth (Thauer *et al.*, 1974) and has a nickel-containing CO dehydrogenase (Diekert *et al.*, 1979; Drake 1982b). The nickel-containing CO dehydrogenase (i.e. acetyl-CoA synthase) of acetogens is of central importance to the acetyl-CoA pathway (Wood & Ljungdahl, 1991; Ragsdale, 1994). The results of this study indicate that clostridial species not previously known to be acetogens may nonetheless be capable of engaging the acetyl-CoA pathway under certain conditions. Given the wide distribution of acetogens in distantly related genera (Collins *et al.*, 1994; Willems & Collins, 1995, 1996), this possibility exists for non-clostridial anaerobes as well.

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REFERENCES

Andreesen, J. R., Gottschalk, G. & Schlegel, H. G. (1970). *Clostridium formicoaceticum* nov. spec. isolation, description and distinction from *C. aceticum* and *C. thermoaceticum*. *Arch Microbiol* **72**, 154–174.

Bernalier, A., Willems, A., Leclerc, M., Rochet, V. & Collins, M. D. (1996). *Ruminococcus hydrogenotrophicus* sp. nov., a new H_2/CO_2-utilizing acetogenic bacterium isolated from human feces. *Arch Microbiol* **166**, 176–183.

Blenden, D. C. & Goldberg, H. S. (1965). Silver impregnation stain for *Leptospira* and flagella. *J Bacteriol* **89**, 899–900.

Bomar, M., Hippe, H. & Schink, B. (1991). Lithotrophic growth and hydrogen metabolism by *Clostridium magnum*. *FEMS Microbiol Lett* **83**, 347–350.

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.

Caccavo, F., Jr, Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F. & McInerney, M. J. (1994). *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. *Appl Environ Microbiol* **60**, 3752–3759.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Cato, E. P., George, W. L. & Fiengold, S. M. (1986). Genus *Clostridium* Prazmowski 1880, 23^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Clark, G. (1973). *Staining Procedures used by the Biological Stain Commission*, 3rd edition. Baltimore: Williams & Wilkins.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J.,

Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.

Daniel, S. L., Hsu, T., Dean, S. I. & Drake, H. L. (1990). Characterization of the H_2- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. *J Bacteriol* **172**, 4464–4471.

DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.

Diekert, G. B., Graf, E. G. & Thauer, R. K. (1979). Nickel requirement for carbon monoxide dehydrogenase formation in *Clostridium pasteurianum*. *Arch Microbiol* **122**, 117–120.

Drake, H. L. (1982a). Demonstration of hydrogenase in extracts of the homoacetate-fermenting bacterium *Clostridium thermoaceticum*. *J Bacteriol* **150**, 702–709.

Drake, H. L. (1982b). Occurrence of nickel in carbon monoxide dehydrogenase from *Clostridium pasteurianum* and *Clostridium thermoaceticum*. *J Bacteriol* **149**, 561–566.

Drake, H. L. (1994). Acetogenesis, acetogenic bacteria, and the acetyl-CoA 'Wood/Ljungdahl' pathway: past and current perspectives. In *Acetogenesis*, pp. 1–15. Edited by H. L. Drake. New York: Chapman and Hall.

Drake, H. L., Daniel, S. L., Matthies, C. & Küsel, K. (1994). Acetogenesis: reality in the laboratory, uncertainty elsewhere. In *Acetogenesis*, pp. 273–302. Edited by H. L. Drake. New York: Chapman and Hall.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–789.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package), version 3.5.1. Seattle: Department of Genetics, University of Washington.

Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J. & Ritter, G. J. (1942). A new type of glucose fermentation by *Clostridium thermoaceticum* n. sp. *J Bacteriol* **43**, 701–715.

Frazer, A. C. (1994). O-demethylation and other transformations of aromatic compounds by acetogenic bacteria. In *Acetogenesis*, pp. 445–483. Edited by H. L. Drake. New York: Chapman and Hall.

Fröstl, J. M., Seifritz, C. & Drake, H. L. (1996). Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. *J Bacteriol* **178**, 4597–4603.

Fuchs, G. (1994). Variations of the acetyl-CoA pathway in diversely related microorganisms that are not acetogens. In *Acetogenesis*, pp. 507–520. Edited by H. L. Drake. New York: Chapman and Hall.

Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn, pp. 1–156. Blacksburg: Anaerobe Laboratory, Virginia Polytechnic Institute and State University.

Hsu, T., Daniel, S. L., Lux, M. F. & Drake, H. L. (1990). Biotransformations of carboxylated aromatic compounds by the acetogen *Clostridium thermoaceticum*: generation of growth-supportive CO_2 equivalents under CO_2-limited conditions. *J Bacteriol* **172**, 212–217.

Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.

Johnson, J. L. & Francis, B. S. (1975). Taxonomy of the clostridia: ribosomal nucleic acid homologies among the species. *J Gen Microbiol* **88**, 229–244.

Kotsyurbenko, O. R., Nozhevnikova, A. N. & Zavarzin, G. A. (1993). Methanogenic degradation of organic matter by anaerobic bacteria at low temperature. *Chemosphere* **27**, 1745–1761.

Kuhner, C. H., Frank, C., Grießhammer, A., Schmittroth, M., Acker, G., Gößner, A. & Drake, H. L. (1997). *Sporomusa silvacetica* sp. nov., an acetogenic bacterium isolated from aggregated forest soil. *Int J Syst Bacteriol* **47**, 352–358.

Küsel, K. & Drake, H. L. (1995). Effects of environmental parameters on the formation and turnover of acetate in forest soils. *Appl Environ Microbiol* **61**, 3667–3675.

Küsel, K., Dorsch, T. & Stackebrandt, E. (1998). Characterization of microbial communities and of two isolates from sediments of acidic coal mine ponds. In *Abstracts of the* 98*th Annual Meeting of the American Society for Microbiology* 1998, abstract N-89, p. 380. Washington, DC: American Society for Microbiology.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lux, M. F. & Drake, H. L. (1992). Re-examination of the metabolic potentials of the acetogens *Clostridium aceticum* and *Clostridium formicoaceticum*: chemolithoautotrophic and aromatic-dependent growth. *FEMS Microbiol Lett* **95**, 49–56.

Maidak, B. L., Olsen, G. L, Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1996). The Ribosomal Database Project (RDP). *Nucleic Acids Res* **24**, 82–85.

Martin, D. R., Lundie, L. L., Kellum, R. & Drake, H. L. (1983). Carbon monoxide-dependent evolution of hydrogen by the homoacetate-fermenting bacterium *Clostridium thermoaceticum*. *Curr Microbiol* **8**, 337–340.

Mesbah, M., Premachandran, U. & Whitman, B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Phelps, T. J. & Zeikus, J. G. (1984). Influence of pH on terminal carbon metabolism in anoxic sediments from a mildly acidic lake. *Appl Environ Microbiol* **48**, 1088–1095.

Ragsdale, S. W. (1994). CO dehydrogenase and the central role of this enzyme in the fixation of carbon dioxide by anaerobic bacteria. In *Acetogenesis*, pp. 88–126. Edited by H. L. Drake. New York: Chapman and Hall.

Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage; proposal for *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 1088–1092.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J Cell Biol* **7**, 208.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schink, B. (1984). *Clostridium magnum* sp. nov., a non-autotrophic homoacetogenic bacterium. *Arch Microbiol* **137**, 250–255.

Schink, B. (1994). Diversity, ecology, and isolation of acetogenic bacteria. In *Acetogenesis*, pp. 273–302. Edited by H. L. Drake. New York: Chapman and Hall.

Sharak-Genthner, B. R., Davis, C. L. & Bryant, M. P. (1981). Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol- and H_2-CO_2-utilizing species. *Appl Environ Microbiol* **42**, 12–19.

Smith, L. D. S. (1975). Common mesophilic anaerobes, including *Clostridium botulinum* and *Clostridium tetani*, in 21 soil specimens. *Appl Environ Microbiol* **29**, 590–594.

Tamura, H., Goto, K., Yotsuyanagi, T. & Nagayama, M. (1974). Spectrophotometric determination of iron(II) with 1,10-phenanthroline in the presence of large amounts of iron(III). *Talanta* **21**, 314–318.

Tanner, R. S. & Woese, C. R. (1994). A phylogenetic assessment of the acetogens. In *Acetogenesis*, pp. 254–272. Edited by H. L. Drake. New York: Chapman and Hall.

Thauer, R. K., Fuchs, G., Käufer, B. & Schnitker, U. (1974). Carbon-monoxide oxidation in cell-free extracts of *Clostridium pasteurianum*. *Eur J Biochem* **45**, 343–349.

Traub, W. H., Acker, G. & Kleber, I. (1976). Ultrastructural surface alterations of *Serratia marcescens* after exposure to polymyxin B and/or fresh human serum. *Chemotherapy* **22**, 104–113.

Valentine, R. C., Shapiro, B. M. & Stadtman, E. R. (1968). Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*. *Biogeochemistry* **7**, 2143–2152.

Weinberg, M. & Ginsbourg, B. (1927). *Données récéntes sur les microbes anaérobies et leur role en pathologie*, pp. 1–291. Paris: Masson et Cie.

Wiegel, J. (1994). Acetate and the potential of homoacetogenic bacteria for industrial applications. In *Acetogenesis*, pp. 484–504. Edited by H. L. Drake. New York: Chapman and Hall.

Willems, A. & Collins, M. D. (1995). Phylogenetic placement of *Dialister pneumosintes* (formerly *Bacteroides pneumosintes*) within the *Sporomusa* subbranch of the *Clostridium* subphylum of the gram-positive bacteria. *Int J Syst Bacteriol* **45**, 403–405.

Willems, A. & Collins, M. D. (1996). Phylogenetic relationships of the genera *Acetobacterium* and *Eubacterium* sensu stricto and reclassification of *Eubacterium alactolyticum* as *Pseudoramibacter alactolyticus* gen. nov., comb. nov. *Int J Syst Bacteriol* **46**, 1083–1097.

Wood, H. G. & Ljungdahl, L. G. (1991). Autotrophic character of acetogenic bacteria. In *Variations in Autotrophic Life*, pp. 201–255. Edited by J. M. Shively & L. L. Barton. San Diego: Academic Press.

Zeikus, J. G., Lynd, L. H., Thompson, T. E., Krzycki, J. A., Weimer, P. J. & Hegge, P. W. (1980). Isolation and characterization of a new, methylotrophic acidogenic anaerobe, the Marburg strain. *Curr Microbiol* **3**, 381–386.