Clostridium mayombei sp. nov., an H_2/CO_2 acetogenic bacterium from the gut of the African soil-feeding termite, *Cubitermes speciosus*

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Abstract. *Clostridium mayombei* sp. nov., a previously undescribed H_2-oxidizing CO_2-reducing acetogenic bacterium, was isolated from gut contents of the African soil-feeding termite, *Cubitermes speciosus*. Cells were anaerobic, Gram positive, catalase and oxidase negative, endospore-forming motile rods which measured $1 \times 2 - 6 \mu m$ and which had a DNA base composition of 25.6 mol% G + C (strain SFC-5). Optimum conditions for growth on $H_2 + CO_2$ were at 33°C and pH 7.3, and under these conditions cells produced acetate according to the equation: $4 H_2 + 2 CO_2 \rightarrow CH_3COOH + 2 H_2O$. Other substrates supporting good growth included carbohydrates (e.g. glucose, xylose, starch), sugar alcohols, and organic and amino acids, and with these substrates acetate was almost always the principle fermentation product. Comparative analysis of 16S rRNA nucleotide sequences confirmed that *C. mayombei* was closely related to various members of the genus *Clostridium*. However, morphological and physiological differences between *C. mayombei* and other homoacetogenic clostridia were deemed significant enough to warrant creation of a new taxon. Results are discussed in light of the diversity of H_2/CO_2 acetogens recently isolated from various termites, and in terms of the relative importance of H_2/CO_2 acetogenesis to termite nutrition.

Key words: *Clostridium mayombei* — Soil-feeding termites — *Cubitermes speciosus* — Gut microbe — Hydrogen — Acetogenic bacteria

The ability of H_2/CO_2 acetogenic bacteria to outcompete H_2/CO_2 methanogenic bacteria for H_2 is commonly observed for the hindgut fermentation of wood-feeding termites (Breznak and Kane 1990). This activity is important to termite nutrition, inasmuch as up to $1/3$ of the insects'

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respiratory requirement can be met by the oxidation of acetate derived from bacterial H_2/CO_2 acetogenesis (Breznak and Switzer 1986). By contrast, rates of bacterial H_2/CO_2 acetogenesis in the guts of soil-feeding and fungus-cultivating termites are considerably less, and rates of CH_4 emission are substantially higher, than those of their wood-feeding counterparts (Brauman et al., in prep.).

To learn more about termite gut acetogens in general, and to evaluate factors which might affect their competitiveness for H_2 in situ, attempts were made to isolate such bacteria from wood-feeding termites. As a result, two species of H_2/CO_2 acetogens were recently obtained and recognized as new taxa: *Sporomusa termitida* from *Nasutitermes nigriceps* termites (Breznak et al. 1988) and *Acetonema longum* from *Pterotermes occidentis* (Kane and Breznak 1991). Detailed studies with *S. termitida* suggested that its ability to grow mixotrophically (Breznak and Blum 1991), rather than a particularly high affinity or low threshold for H_2 (Breznak et al. 1988; Cord-Ruwisch et al. 1988), might also have a bearing on its ability to outprocess methanogens for H_2 *in situ*.

Notwithstanding the isolation of H_2/CO_2 acetogens from wood-feeding termites, it seemed prudent, for comparative purposes, to attempt to obtain analogous bacteria from termites in which H_2/CO_2 acetogenesis was not a dominant feature of the hindgut fermentation. In the present paper, we describe the isolation and characteristics of strain SFC-5, an H_2/CO_2 acetogen from guts of the African soil-feeding termite, *Cubitermes speciosus*. Unlike *S. termitida* and *A. longum*, strain SFC-5 was Gram positive and exhibited other properties typical of members of the genus *Clostridium*. However, strain SFC-5 did not correspond closely to any H_2/CO_2 acetogenic *Clostridium* previously described. Accordingly, it is proposed herein that strain SFC-5 constitute the type strain of a new species, *Clostridium mayombei*. [A preliminary report of these findings has been presented (M. D. Kane and J. A. Breznak, 1989, Abstr. Annu. Meet. Am. Soc. Microbiol. p. 234).]

Materials and methods

Termites

C. speciosus (Termitidae) was collected from the Mayombe tropical rain forest near Dimonika, People's Republic of Congo. Termites were degutted on site, and the guts were immediately immersed in an anoxic salts solution consisting of (g/l): KH_2PO_4 (0.2), NH_4Cl (0.3), KCl (0.5), NaCl (1.0), $MgCl_2 \cdot 6 H_2O$ (0.4), $NaHCO_3$ (2.5) and $CaCl_2 \cdot 2 H_2O$ (0.15). Dissected guts were transported to Michigan over a period of ten days, where they were used within 48 h of receipt.

Isolation of strain SFC-5

Strain SFC-5 was isolated by using media with (AC-K1) and without (AC-K2) the inclusion of 2-bromoethanesulfonate (BES), as described for the isolation of A. longum (Kane and Breznak 1991).

Growth and nutrition studies

CO_2/bicarbonate-buffered medium AC-K4 was used for growth studies. It was identical to AC-K1 medium (Kane and Breznak 1991), except that the amount of trypticase was decreased to 1.0 g/l and rumen fluid was omitted. Nutritional studies were performed by using AC-K5 medium which was identical to AC-K4, except that the amounts of trypticase and yeast extract were each increased to 2.0 and 1.0 g/l, respectively. Other procedures were as described previously (Kane and Breznak 1991).

Fermentation studies

The stoichiometry of H_2/CO_2 fermentation by growing cells was done by using AC-K5 medium and an H_2/CO_2 (80/20, vol/vol) atmosphere. Fermentations of glucose, xylose and sodium succinate were performed by using an initial gas phase of 100% N_2 and AC-K5 medium modified by omitting $NaHCO_3$, but including 3-(N-morpholino)propanesulfonic acid buffer (adjusted to pH 7.4; sterilized separately) at a final concentration of 10 mM. Material balance calculations included a correction for products formed from cells grown in basal medium containing no additional substrate. Other aspects were as previously described (Kane and Breznak 1991).

Other procedures

DNA base composition was determined by differential scanning calorimetry as described by Mackey et al. (1988), except that stainless steel sample pans were used with a Perkin-Elmer model DSC7 differential scanning calorimeter. Enterobacter agglomerans (from this laboratory's culture collection) was used as a control and was grown aerobically on plates of Brain-Heart Infusion agar (Difco). It displayed a T_{max} of 96.7 C which agreed well with that reported by Mackey et al. (1988).

Other experimental procedures, including comparative 16S rRNA sequence analyses, were performed as described previously (Kane and Breznak 1991).

Results

Isolation of bacteria

After two weeks incubation, primary enrichments for H_2/CO_2 acetogenic bacteria in AC-K1 medium (containing BES) exhibited turbidity, negative pressure in the headspace, and acetate production up to 30 mM (with periodic replenishment of H_2/CO_2). By contrast, similar enrichments in AC-K2 medium (without BES) exhibited production of CH_4 and contained F_{420}-fluorescent cells. From the former enrichments, seven strains of H_2/CO_2 acetogenic bacteria were isolated by using agar roll tubes. All strains exhibited similar morphology and Gram stain reaction, so one of these (strain SFC-5) was chosen for further study.

Morphology

Subsurface colonies of H_2/CO_2-grown cells were white to slightly yellow, oval-shaped with smooth edges, and about 2 mm in diameter. Individual cells were straight rods measuring $1 \times 2 - 6$ μm (Fig. 1 a, b). Cells were motile, and electron microscopy of negatively stained preparations revealed peritrichous flagella (not shown). In older cultures (≥ 7 d), cells were somewhat longer (10 – 15 μm) and slightly curved. Cells stained Gram positive, and electron micrographs of thin sections revealed a typical Gram positive cell wall morphology (Fig. 1 b). In addition, cells formed central to subterminal, oval endospores which only slightly swelled the sporangium (Fig. 1 a, b). Viable cells could be recovered from sporulated cultures held at 80°C for 10 min. Moreover, dipicolinic acid (DPA) extracted from sporulated cultures and prepared as the calcium chelate exhibited a UV spectrum identical to that of authentic calcium DPA.

Growth and nutritional studies

Strain SFC-5 was a strict anaerobe and grew only in O_2-free medium to which a reductant (e.g. dithiothreitol, 1 mM final concentration) had been added. Growth required the addition to media of trypticase or yeast extract (1.0 – 2.0 and 0.5 – 1.0 g/l, respectively), but best results were obtained when both constituents were added. Addition of rumen fluid (5%, v/v) to the growth medium had no effect on growth. Cells grew in AC-K4 medium within a temperature range of 15 to 45°C (optimum, 33°C) and pH (initial) range of 5.5 to 9.3 (optimum, 7.3).

When cells were grown at 30°C in AC-K5 medium with H_2/CO_2 as substrate, they: exhibited a doubling time of 5 h; achieved a final $O.D._{600 nm}$ of 0.47 – 0.50 (equiv. 160 – 180 μg cell mass/ml); and produced 18 to 23 mM acetate (without replenishment of H_2/CO_2) (Fig. 2). Cell yields and acetate production were considerably less when cells were grown in the same medium with N_2/CO_2 in the headspace.

Strain SFC-5 also grew on a variety of other substrates, including sugars, sugar alcohols, and organic and amino acids (Table 1). The major fermentation product from most substrates was acetate. A trace of isovalerate was also produced. However, cells formed propionate from succinate (see below) and isobutyrate from valine. Molar growth yields of strain SFC-5 on selected substrates are included in Table 2.
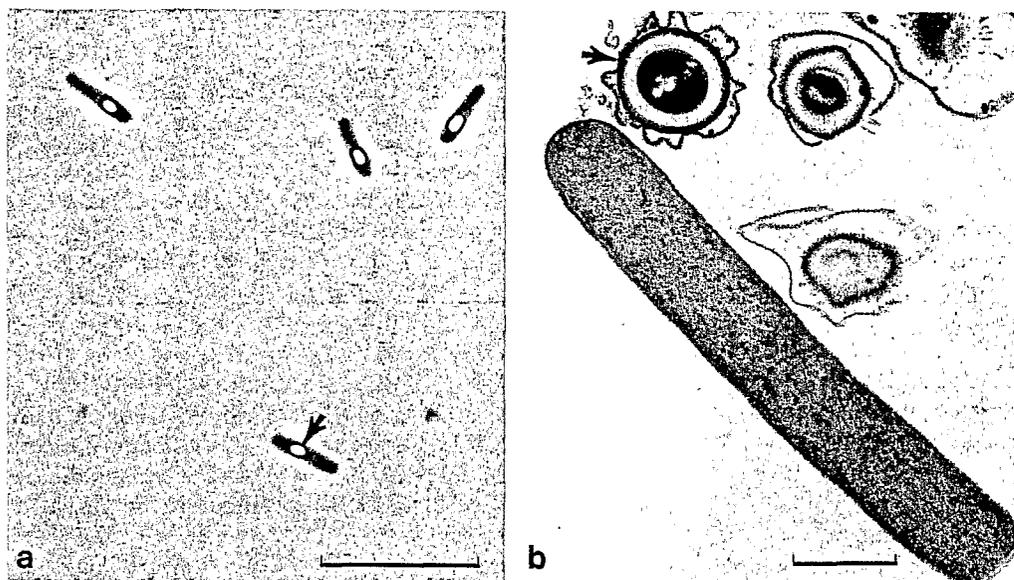
Fig. 1a, b. Morphology of strain SFC-5. a Phase contrast micrograph; bar = 10 μm. b Transmission electron micrograph of a thin section; bar = 1.0 μm. Note endospores (*arrows* a, b)
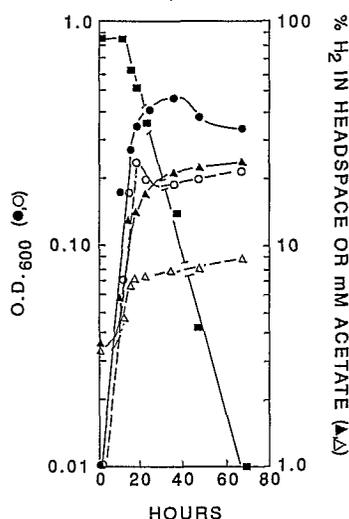

Fig. 2. Utilization of H_2 by strain SFC-5 for growth and acetogenesis. Cells were grown at 30°C in bottles containing 245 ml AC-K5 medium and 463 ml gas phase at 1 atm. The initial gas phase was either H_2/CO_2 (80/20, v/v; *closed symbols, solid lines*) or N_2/CO_2 (*open symbols, broken lines*)

Table 1. Substrates used as energy sources by strain SFC-5. Compounds were supplied at 5 to 10 mM, except for H_2/CO_2 (80/20, v/v)

Used for growth by strain SFC-5
H_2/CO_2, glucose, fructose, xylose, maltose, cellobiose[a], salicin, esculin, dextrin, starch, sorbitol, dulcitol, glycerol, formate, pyruvate, malate, succinate, syringate[a], alanine, glutamate, serine, and valine.

Tested, but not used
Mannose, rhamnose, ribose, melibiose, raffinose, arabinose, galactose, lactose, sucrose, trehalose, L-fucose, lactate, citrate, oxaloacetate, fumarate, D-gluconate, acetate, oxalate, gallate, caffeate, 3-hydroxybenzoate, benzoate, 3,4,5-trimethoxybenzoate, pyrogallol, methanol, ethanol, propanol, mannitol, ethylene glycol, adonitol, erythritol, butanol, isobutanol, pectin, xanthine, betaine and N-N-dimethylglycine

[a] Poor growth on this substrate

Fermentation stoichiometries

Cells of strain SFC-5 used H_2/CO_2 as an energy source for growth and acetogenesis according to the equation:
$4 H_2 + 2 CO_2 \rightarrow CH_3COOH + 2 H_2O$ (Table 2). The ability of strain SFC-5 to effect a complete synthesis of acetate from $H_2 + CO_2$ was confirmed by incubating H_2/CO_2 grown cells with $H_2 + {}^{14}CO_2$. Under these conditions, cells formed 1.70 μmol ^{14}C-acetate per h per mg protein. Degradation of the ^{14}C-acetate by the Schmidt reaction revealed that ^{14}C-label was incorporated into both carbon atoms of acetate in approximately equal amounts (data not shown).

Strain SFC-5 was also homoacetogenic, or nearly so, when fermenting organic compounds such as glucose or xylose (Table 2). By contrast, cells fermented succinate to propionate + CO_2 (Table 2). This mildly exergonic reaction, first demonstrated to support the growth of *Propionogenium modestum* (Schink and Pfennig 1982), has also been demonstrated for the H_2/CO_2 acetogens *Sporomusa termitida* (Breznak et al. 1988) and *S. malonica* (Dehning et al. 1989).

16S ribosomal RNA sequence analysis

The nucleotide sequence of the 16S rRNA from strain SFC-5 was determined and compared with those of *Sporomusa termitida* and *Acetonema longum* (Kane and Breznak 1991) and certain other bacteria. 1050 unambiguous nucleotide positions were used in the analysis. The 16S rRNA of strain SFC-5 was not closely related to that of *S. termitida* or *A. longum*, but did show a distinct and close relationship with one member of the genus *Clostridium*. The evolutionary distance between

Table 2. Molar growth yields and fermentation stoichiometries for strain SFC-5 grown on selected substrates

Substrate	Growth yield (g dry cell mass/ mol substrate fermented)	Product (mmol/100 mmol substrate fermented)				
		H_2	CO_2	Acetate	Propionate	% C recovery
H_2 (+ 50 mmol CO_2)[a]	1.9	—	—	22.9	0.0	91.6
Glucose	43.1	13.4	6.7	251.5	0.0	85.0
Xylose	27.5	0.0	0.0	206.4	0.0	82.6
Sodium succinate	0.9	0.0	58.2	0.0	122.8	106.7

[a] Assumed for calculation of material balance

strain SFC-5 and this close relative, *Clostridium lituseburense* (unpublished sequence obtained from C. R. Woese, rRNA Database Project, Univ. Illinois, Urbana USA; GenBank accession number M59107), was 5.1 evolutionary distance units (= 5.1 changes per 100 nucleotide positions). The evolutionary distance between strain SFC-5 and five other *Clostridium* species examined was ≥ 11.8, and that between strain SFC-5 and either *S. termitida* or *A. longum* was ≥ 23.7. For reference, evolutionary distance values of 5.1, 11.8, and 23.7 correspond to similarity values of 95.0%, 89.1% and 79.7%, respectively (the 16S rRNA-inferred phylogeny of *S. termitida, A. longum* and these five *Clostridium* species has been described in a separate communication; Kane and Breznak 1991). The 16S rRNA sequence for strain SFC-5 has been deposited with GenBank (accession number M62421); it can also be obtained by writing us directly.

Other characteristics

Strain SFC-5 was catalase and oxidase negative. Cytochromes were not detected in crude cell extracts, and neither sulfate nor nitrate was not used as a terminal electron acceptor. The DNA base composition of strain SFC-5 was 25.6 mol% G + C.

Discussion

Based on its morphology, physiology, and 16S rRNA nucleotide sequence, strain SFC-5 exhibits a close phylogenetic relationship with members of the genus *Clostridium*, especially *C. lituseburense*. However, along with a number of differences in substrate utilization between the two organisms, *C. lituseburense* growing in a peptone-yeast extract-glucose medium forms major amounts of butyrate, acetate, and isovalerate, with minor production of formate, propionate, and isobutyrate (Cato et al. 1986). By contrast, strain SFC-5 is essentially homoacetic when fermenting glucose (or xylose) in a similar complex medium (i.e. AC-K5 medium; Table 2). In fact, homoacetogenic carbohydrate fermentations such as those exhibited by strain SFC-5 are relatively uncommon among the more than 80 described species of *Clostridium* (Cato et al. 1986). To date, only five species and one unnamed isolated of this genus carry out such

fermentations, and strain SFC-5 differs from each in several respects. For example, *Clostridium thermoaceticum* (Fontaine et al. 1941) and *C. thermoautotrophicum* (Wiegel et al. 1981) are moderate thermophiles that grow optimally at 55−60°C, whereas strain SFC-5 does not grow above 45°C. Cells of the other four homoacetogenic clostridia are clearly distinguishable from those of strain SFC-5 on the basis of their morphology. *C. aceticum* (Adamse 1980; Braun et al. 1981), *C. formicoaceticum* (Andreesen et al. 1970) and *Clostridium* strain CV-AA1 (Adamse and Velzeboer 1982) all form round, terminal endospores which markedly swell the sporangium, whereas those of strain SFC-5 are oval, usually subterminal in location, and swell the cells only slightly or not at all. Although endospores of *C. magnum* (Schink 1984) are also oval and often subterminal, the cells themselves have a much greater width to length ratio than those of strain SFC-5, especially when the former produces endospores. Moreover, neither *C. magnum* nor *C. formicaceticum* use H_2/CO_2 as a substrate mixture for growth and acetogenesis. Therefore, on the basis of the morphological and physiological differences described above, it is proposed that strain SFC-5 be recognized as the type strain of the new species, *Clostridium mayombei*, a description of which is given below.

It is clear that H_2/CO_2 acetogenic bacteria in termite guts do not all belong to a single, neatly defined taxon. Indeed, recent efforts to isolate such organisms from termites have already yielded three distinct, and heretofore unrecognized, species: *Sporomusa termitida* (Breznak et al. 1988); *Acetonema longum* (Kane and Breznak 1991); and *Clostridium mayombei* (this study). Perhaps this is not surprising, since the termites from which they were isolated were: *Nasutitermes nigriceps*, an arboreal, wood-feeding, phylogenetically "higher" termite; *Pterotermes occidentis*, a phylogenetically lower "dry wood" termite; and *Cubitermes speciosus*, a soil-feeding higher termite. Given that there exist nearly 2000 species of termites on Earth whose biology, behavior, and nutritional ecology are quite diverse (Lee and Wood 1971), there may well be a high degree of coevolution between termites and their intestinal acetogens which is reflected, in part, by the phenotypic diversity of the latter. This certainly seems to be the case for the cellulolytic, intestinal protozoa of lower termites (Honigberg 1970). Moreover, we have recently discovered that rates of H_2/ CO_2 aceto- versus methanogenesis by the hindgut

microbiota vary significantly with the feeding guild into which various species of termites have evolved (Brauman et al., in prep.). Nevertheless, an interesting property shared by all H_2/CO_2 acetogens isolated from termites so far is the ability to form endospores. Inasmuch as periodic ecdysis (molting) of termites includes expulsion of the chitinous lining of the hindgut, as well as much, if not all, of the anoxic hindgut contents, the ability of the strictly anaerobic acetogens to form endospores may contribute to their survival when voided and may help ensure eventual reinoculation and recolonization of the gut.

We do not yet know the specific and quantitative contribution that *C. mayombei* makes to the nutrition and vitality of its soil-feeding host, nor do we know the number of vegetative cells versus endospores of *C. mayombei* in guts of *C. speciosus*. The relatively long period of time between collection and dissection of the termites, and the receipt by us of the gut samples, preclude any such definitive statements at this time. However, the importance to *C. speciosus* of intestinal H_2/CO_2 acetogenesis per se, whether catalyzed by *C. mayombei* in situ or not, is likely to be modest, because in *C. speciosus* and other soil-feeding termites methanogenesis, rather than acetogenesis, appears to be the major electron (H_2) sink reaction of the hindgut fermentation (Brauman et al., in prep.). If *C. mayombei* does contribute to its host's nutrition, it may be more relevant to the fermentative dissimilation of organic compounds in the gut. In any case, our steadily increasing knowledge about some of Earth's most abundant mesofauna, including those termites which are among the most ecologically important soil animals in the tropics, as well as the increased representation in culture of some of their seemingly important gut microorganisms such as acetogens, would appear to set the stage for some interesting future studies on the autecology of termite gut microbes and their significance intermite nutrition and global carbon cycling.

Description of Clostridium mayombei sp. nov.

Clostridium mayombei sp. nov. [may.omb'e.i. N.L. neut. adj. *mayombei*, pertaining to the Mayombe tropical rainforest, People's Republic of Congo, which is home to the termite (*Cubitermes speciosus*) from whose gut this bacterium was isolated].

Straight to slightly curved rods with rounded ends, and measuring $1 \times 2 - 6$ µm. Cells occurring singly or in pairs. Motile by peritrichous flagella. Gram positive. Heat-resistant endospores are formed that are 1 µm in diameter, oval, and subterminal to terminal in location. Colonies grown on $H_2 + CO_2$ are oval shaped with smooth edges, about 2 mm in diameter, and white to light yellow in color.

Strict anaerobe. Catalase and oxidase negative. Facultative chemolithotroph. Ferments $H_2 + CO_2$ to acetate. Also ferments glucose, fructose, xylose, maltose, cellobiose, dextrin, starch, sorbitol, dulcitol, glycerol, formate, pyruvate, malate, syringate, alanine, glutamate, serine, salicin, and esculin yielding acetate as the major acid product. Succinate is fermented to propionate $+ CO_2$, and valine is fermented to isobutyrate as major acid product.

Does not respire anaerobically with nitrate or sulfate. Cytochromes not detected. Temperature optimum, 33°C (range 15 to 45°C); pH optimum, 7.3 (range 5.5−9.3). Trypticase and yeast extract required in media for good growth. $G + C$ content of $DNA = 25.6$ mol% (strain SFC-5; differential scanning calorimetry method).

Source: Gut contents of the soil-feeding termite, *Cubitermes speciosus*, collected from the Mayombe tropical rainforest, People's Republic of Congo.

Type strain: Strain SFC-5, DSM 6539, deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig.

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