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NATURE AND NUTRITIONAL SIGNIFICANCE
OF ACIDOGENIC AND METHANOGENIC BACTERIA
IN GUTS OF TERMITES AND COCKROACHES

presented by

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NATURE AND NUTRITIONAL SIGNIFICANCE OF ACIDOGENIC AND METHANOGENIC BACTERIA IN GUTS OF TERMITES AND COCKROACHES

By

Matthew David Kane

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Department of Microbiology and Public Health
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ABSTRACT

NATURE AND NUTRITIONAL SIGNIFICANCE OF ACIDOGENIC AND METHANOGENIC BACTERIA IN GUTS OF TERMITES AND COCKROACHES

By

Matthew David Kane

Research described herein investigated the occurrence, activities and specific identities of members of two groups of strictly anaerobic, hydrogenotrophic bacteria which compete for H_2 in the guts of termites: H_2/CO_2 acetogenic bacteria, which use H_2 to reduce CO_2 to acetate (a major source of carbon and energy for termites); and H_2/CO_2 methanogenic bacteria, which use H_2 to reduce CO_2 to methane (a compound which cannot be utilized by termites).

H_2/CO_2 acetogenic bacteria outcompeted H_2/CO_2 methanogenic bacteria for H_2 in the hindguts of wood- and grass-feeding termites, and up to one-third of the respiratory requirement of some termite species could be met by oxidation of acetate derived from bacterial H_2/CO_2 acetogenesis. By contrast, fungus-cultivating, or soil-feeding termites emitted significantly more methane, and exhibited significantly lower rates of H_2/CO_2 acetogenesis than their wood-feeding counterparts.

Two novel H_2/CO_2 acetogenic bacteria were isolated in pure culture from termite guts, and the general morphological and physiological characteristics, nutrition, and molecular phylogeny of the isolates were studied in detail. Results have led to the description of *Acetonema elongata*, gen. nov., sp. nov., a Gram negative, endospore-forming rod isolated from gut contents of the phylogenetically lower, wood-feeding termite, *Pterotermes occidentis* (Kalotermitidae), and

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Clostridium mayombei, sp. nov., a Gram positive, endospore forming rod isolated from gut contents of the higher, soil-feeding termite, *Cubitermes speciosus* (Termitidae).

Additional studies with the omnivorous cockroach *Periplaneta americana* (Blattidae) demonstrated that numbers and activities of specific gut bacteria depended upon the insect's diet and life stage. For example, immature cockroaches emitted more methane than adult cockroaches, particularly when insects were fed diets high in plant fiber. By contrast, lactic and acetic acid production by lactic acid bacteria in the foregut was a significant process in cockroaches fed a diet low in plant fiber. Results of these studies demonstrated that, in guts of termites and cockroaches, microbial community structure and function is related to the developmental stage and diet of the host, and may make a significant contribution to the insect's nutrition.

With love -

For my parents and my grandmothers,

and in memory of my grandfathers.

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ACKNOWLEDGEMENTS

My deepest gratitude to my family and friends for your love, support and encouragement. And, of course, heartfelt thanks to my dogs for always being there when I needed them.

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I was very fortunate to do collaborative work with Dr. David Stahl of the University of Illinois, and Dr. David White of the University of Tenessee. Thank you for your help now, and in the future.

A special note of thanks to my friend, colleague and sidekick, Dr. Alain Brauman, and to my friend and colleague, Dr. Marc Labat (and both of your families) for everything in Marseille, Paris, Brazzaville and Dimonika. I hope there will be a sequel!

My associations with all of the scientists listed above were made possible only because I worked in the laboratory of a universally well liked and greatly admired microbiologist, Dr. John Breznak. He is living proof that a great scholar, researcher and teacher can also be a wonderful human being. For all that you have taught me about science and about life, and for accepting with grace and good humor all of those "shpilkes" I gave you, I thank you, John.

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INTRODUCTION

If biological success is measured either by numbers of individuals, or diversity of species, then insects are undoubtedly the most successful group of animals in the history of life on earth. There are more species of insects than all other animal species combined (48). This observation is especially remarkable in light of the fact that insects do not colonize marine habitats, a limitation which restricts them to approximately 30 percent of the planet's surface area.

The extreme phylogenetic radiation exhibited by insects has been accompanied by extensive diversification in dietary habit and nutritional physiology. Several intriguing behavioral and physiological adaptions have been employed by insects to assist them in this dietary and nutritional diversification, including the establishment of nutritionally based symbiotic relationships with microorganisms, some of which are described below.

Insects have supplemented the nutritive potential of their food by establishing mutualistic relationships with fungi (58,80), protozoa (36,44,98), and/or bacteria (14,17,29). Some of the insects for which nutritional relationships with microbes have been examined include: ants (58); beetles (5); cockroaches (29); cranefly larvae (49,83); crickets (49,87); millipedes (85); termites (14,15,17,96) and wood-boring wasps (52). Many other insect-microbe nutritional interactions probably remain to be discovered, and even those that have been identified need to be examined more closely to have a better understanding of the nature

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and diversity of this fascinating biological phenomenon. A complete synopsis of insect-microbe nutritional symbioses is beyond the scope of the present review, so the following passages deal only with those nutritional relationships that microorganisms have established with the insect groups used for this study, specifically, termites and cockroaches.

I. Termites and Cockroaches, and Their Nutritional Interactions with Microorganisms

Nutritionally based insect-microbe interactions involving termites (Isoptera), and cockroaches [Dictyoptera (ancestral relatives of termites)], with fungi, protozoa and bacteria, have been the subject of intensive study (summarized in references 14,15,17,29 and 58). The first section of this review will focus on the nutritional relationships of microorganisms with termites, and to a lesser extent, cockroaches, and the second section will discuss in detail certain competing bacterial processes in the guts of these two insect groups.

There are over 2200 living species of termites, one or more of which can be found in various habitats over two-thirds of the earth's land surface (96). They cause considerable damage to agriculture and architecture, but as decomposers of large amounts of biomass termites are also important to the recycling of nutrients, particularly in tropical regions (34,97). Their food consists of living, dead, decomposing, or highly decomposed plant matter, i.e., substances that can generally be described as lignocellulosic in composition (95). Termites are social insects, and in the nest of each species, morphologically differentiated castes perform different functions. Food

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resources are collected by termites belonging to a "worker" caste, which then feed themselves as well as other members of the termite nest (95,96). Although a few termites are polyphagous, and eat a variety of living or dead plant materials, termite biologists recognize at least three categories of specialization with regard to feeding behavior (95,96):

1) Termites that eat living, dead or decomposing vegetation (trees, grass and/or leaves). Wood-feeding species are the most well studied termites of this group due to their economic impact (34).

2) Termites that cultivate and consume aerobic, cellulolytic basidomycete fungi of the genus *Termitomyces*, which, in addition to plant materials, are consumed by the termite. This habit is limited to, but pervasive among, members of the subfamily, Macrotermitinae.

3) Termites that eat soil and presumably derive nutrition from soil organic matter (humus). Although their nutrition is understood the least, they constitute approximately 45 percent of all termite species (96) and are especially numerous and active in tropical habitats.

The currently accepted classification of termites recognizes five families of "lower", or more primitive, termites (Masto-, Kalo-, Hodo-, Rhino- and Serritermitidae) and one family of "higher", or more advanced, termites (Termitidae) (50,51). The lower termite families together constitute about 25 percent of all living species, the remainder belong to the Termitidae. There is some correlation between phylogenetic classification and diet, in that the lower termites are all wood-feeders, whereas the higher termites include plant- (wood-, grass- and/or leaf-) feeding, fungus-cultivating, and soil-feeding representatives (95).

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Only a small fraction of the more than 2200 termite species have been studied with respect to nutritional ecology and symbiotic interaction with microorganisms, so it is difficult to generalize about such relationships. However, one property apparently shared by all termites, regardless of feeding behavior or phylogenetic classification, is the presence of an anaerobic microbial community in the hindgut region of their alimentary tract, a community which can be extraordinarily diverse (6,14,20,58). Lower termites harbor both anaerobic bacteria and protozoa, including cellulolytic flagellates, in their hindguts. By contrast, the hindguts of higher termites lack permanent populations of protozoa, and contain only bacteria (14,23,96). One of the most conspicuous groups of bacteria observed in wet mounts of gut contents from both higher and lower termites are spirochetes (16,20,30). Unfortunately, no termite gut spirochetes have ever been obtained in pure culture, so their function(s) with respect to gut processes is still uncertain (16). The role of other termite gut microbes in host nutrition is discussed in detail below.

Cockroaches are believed to be termites' closest phylogenetic relatives (50,51). Although most cockroaches are generally considered to be omnivorous, members of one genus, *Cryptocercus*, are strictly wood-feeders (24). Like the phylogenetically lower termites, *Cryptocercus punctulatus* harbors a diverse population of anaerobic bacteria and cellulolytic protozoa in its hindgut (24,44). Others, such as the omnivorous American cockroach, *Periplaneta americana*, have only bacteria in their hindguts (29).

Our understanding of the overall nutritional relationships that termites and cockroaches have established with microorganisms is

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generally incomplete. However, the role that microorganisms play in certain key aspects of the digestive and metabolic activities of these insects has become increasingly better understood. The remainder of this section will summarize our current knowledge about the role of microbes in the nitrogen and carbohydrate metabolism of termites and cockroaches.

I. 1. *Role of microorganisms in the nitrogen metabolism of termites and cockroaches*

Wood-feeding termites digest a resource that contains up to 100 times less N (on a dry weight basis) than that exhibited by termite tissues (15). Consequently, termites have evolved ways of utilizing the activites of their gut microbiota to aquire and/or conserve combined N.

One method of termite N aquisition is through N_2-fixation activities of gut-associated bacteria (15,18,38,72,75,79). Results show that N_2-fixation rates for some wood-feeding species are significant enough to support up to 50% of the termite's N requirements (15,79). By contrast, most of the dietary nitrogen of fungus-cultivating termites is obtained directly from digestion of ingested fungal tissue (26). It is possible that termites might also aquire N by digestion of part of their gut microbiota, or through assmiliation of combined N (eg. amino acids) excreted by gut bacteria (59).

Mechanisms for nitrogen conservation involving gut microbes can also be important to termite N metabolism. In studies with the lower, wood-feeding termite, *Reticulitermes flavipes*, Potrikus and Breznak found that, although this termite has the ability to synthesize uric

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acid (a common nitrogenous excretory product of insects), the compound is not voided in the termite's feces, (73). Instead, uric acid transported via Malpighian tubules from its site of synthesis (fat body tissue) to the gut is immediately and completely fermented to acetate, CO_2, and NH_3 by anaerobic bacteria (74). Uric acid nitrogen liberated by hindgut bacteria is ultimately assimilated back into termite tissues, although the principle mechanism for this last recycling step remains to be established.

Unfortunately, so little is understood about the nutrition of soil-feeding termites, that any statement concerning the role of their gut microbes in nitrogen metabolism would be purely speculative.

A system analagous to that of wood-feeding termites, involving the conservation of uric acid nitrogen through the activities of bacterial symbionts, may exist for cockroaches as well. As with termites, uric acid does not appear to be a major excretory product of most cockroach species, although they synthesize the purine, and store it in fat body tissue (62-64). Intracellular bacteria (bacteroids) present in specialized cells in fat body tissue (mycetocytes) (33) have been implicated as agents of uric acid degradation and mobilization in cockroaches (25). However, studies of this system have been obstructed by the unavailability of authentic mycetocyte bacteria in pure culture. Until direct evidence for bacterial uricolysis is forthcoming, the nature of the symbiosis between cockroaches and their mycetocyte bacteria will remain uncertain.

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I. 2. *Role of microorganisms in the digestion of carbohydrates by termites and cockroaches*

Termites are faced with the formidable task of digesting relatively refractory lignocellulosic substances as their major carbon and energy source. For plant-feeding and fungus-cultivating termites cellulose is the main component which must be digested, and their symbiotic interactions with microorganisms, to various degrees, help them to do this.

Evidence suggests that lower, wood-feeding termites depend largely on anaerobic, cellulolytic protozoa to hydrolyze ingested cellulose (17,23). A landmark in the study of lower termites was the relatively recent isolation by M. A. Yamin of *Trichomitopsis termopsidis* and *Trichonympha sphaerica*, dominant anaerobic protozoa from the hindgut of the lower termite, *Zootermopsis angusticollis*, and the subsequent demonstration that these two protozoa converted crystalline cellulose to acetate, H_2 and CO_2 (69,99,100). Cellulase and other polymer-hydrolyzing enzyme activities (eg. amylase, xylanase and protease) have also been detected in crude extracts of *T. termopsidis* (68,102).

Our understanding of the overall utilization of cellulose by lower, wood-feeding termites is based largely on Yamin's work and studies with the gut microbiota of another lower termite, *R. flavipes*. The dissimilation of wood-polysaccharides in the hindgut of *R. flavipes* is essentially a homoacetic fermentation of cellulose, as depicted in Fig. 1. This diagram is based on a model proposed by Odelson and Breznak that has received good experimental support (67). Initially, wood polysaccharides (principally cellulose) are endocytosed by

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Figure 1. The Odelson-Breznak model for symbiotic degradation of wood polysaccharides (principally cellulose) by anaerobic protozoa and bacteria in the hindgut region of the alimentary tract of *R. flavipes*. Thickness of arrows signifies the relative contributions of microbial activities to the digestive process. See text for details. Modified from reference 67.

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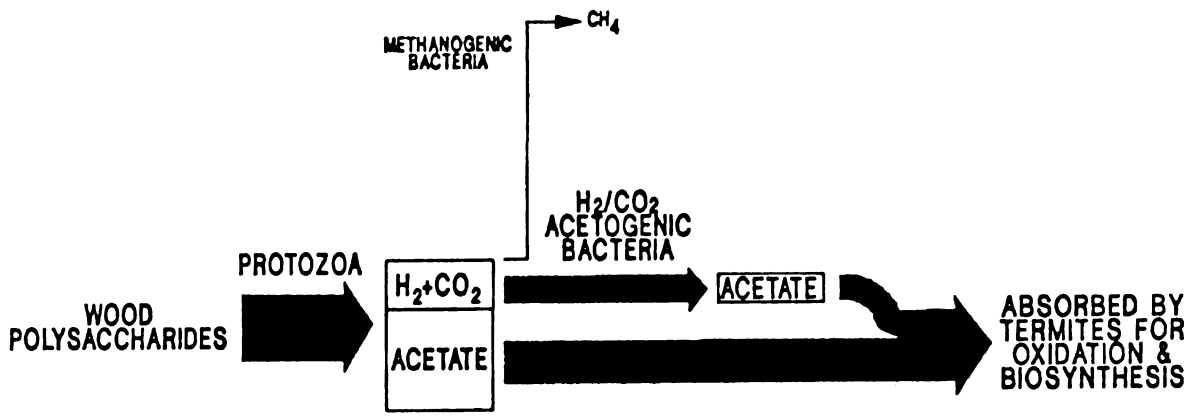
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protozoa, which then hydrolyze the cellulose and ferment the liberated glucosyl units to acetate, H_2 and CO_2. H_2 and CO_2 produced by protozoa are then consumed mainly by H_2-oxidizing, CO_2-reducing, acetogenic bacteria to form additional acetate (although some H_2/CO_2 is converted to methane by methanogenic bacteria) (21,67). Acetate is then used by the termite as an important source of carbon and energy (65). In fact, the entire respiratory requirement of *R. flavipes* termites can be met by the oxidation of microbially produced acetate. According to this scheme, roughly two-thirds of hindgut acetate is derived from activities of protozoa, and one-third is derived from bacterial H_2/CO_2 acetogenesis. The novel bacterial component of this model will be discussed in detail in the second section.

Although this simplified model may be conceptually appealing, it obscures the fact that the hindgut microbial community of lower termites is extremely diverse (14,20). The specific contribution of some of the other abundant organsisms in the gut (eg. spirochetes) to host nutrition, or to the functional stability and activity of the total hindgut microbial community, may also be of critical importance to the overall health and development of the insect.

Cellulose digestion in the higher wood-feeding termites must be different than that in the lower termites, because of the absence of hindgut protozoa. Moreover, the origin of enzymes that comprise the cellulase repertoire in wood-feeding Termitidae appears to include the termite itself. For example, *Nasutitermes* species that have been examined secrete cellulase enzymes in the midgut (43,60,66). Treatment of *Nasutitermes* with antibacterial drugs had little or no immediate deleterious effect on cellulase activity (43). Thus, in the Termitidae,

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cellulose hydrolysis may not depend exclusively on the presence of cellulolytic microorganisms. On the other hand, unpublished results from our laboratory suggest that cellulolytic gut bacteria may be widespread among wood-feeding Termitidae. Moreover, presence of a morphologically diverse bacterial community in the hindgut of wood-feeding representatives of the Termitidae (30) suggests that extensive bacterial fermentation of carbohydrates probably occurs in the hindgut of higher termites. Acetate and other typical bacterial fermentation products were detected in hindguts of one higher termite species examined (67). Consequently, details of the nutritional interaction between higher termites and their gut bacteria still remain to be clarified.

In the subfamily, Macrotermitinae, a major benefit derived from the cultivation of *Termitomyces* fungal gardens is the aquisition, by ingestion of fungal tissue, of some components of the cellulase enzyme complex not made by the termite itself (58). For example, *Macrotermes natalensis* and *Macrotermes subhyalinus* each make an endoglucanase, as do the fungi which they cultivate and consume, but both termites aquire from the fungi an exoglucanase which they do not make themselves (58). The reverse situation appears to be true for *Macrotermes mulleri*, which makes its own exoglucanse, but aquires an endoglucanase by ingesting its *Termitomyces* fungus (77,78).

As with the higher, wood-feeding termites, fungus-cultivating termites harbor a community of bacteria in their hindguts, as well as in the so-called mixed-segment region (a region of overlap between the posterior midgut and anterior hindgut that occurs in some termites) (79). However, almost no information exists concerning the role of

bacteria in the nutrition of Macrotermitinae. Not only is it possible that hindgut bacterial fermentation could be part of the termite's digestive strategy, but hindgut or mixed-segment-associated cellulolytic bacteria could, in theory, also contribute to the initial digestive step of cellulose hydrolysis.

Soil-feeding termites also have an anaerobic hindgut bacterial community and harbor bacterial populations in the mixed segment (6,7). The food of such termites has not been well defined in chemical terms, but probably consists of residues of lignins and tannins that comprise the organic component of soil referred to as humus. Some interesting circumstantial evidence suggests that anaerobic, methanogenic, bacterial consortia capable of degrading aromatic compounds from ingested soil could be important to carbohydrate metabolism in the guts of soil-feeding termites (11). However, fundamental aspects of the nutrition and digestive processes of these fascinating animals have not been investigated in detail. Therefore, it is difficult, if not impossible, to assess the role and quantitative significance of bacteria in their nutrition.

The relationship between microorganisms and wood-eating cockroaches belonging to the genus *Cryptocercus* is similar to that of the phylogenetically lower, wood-feeding termites (14,24). Knowlege of the nutritional relationship between microbes and cockroaches other than *Cryptocercus* is almost exclusively based on studies with two omnivorous species, *P. americana* and *Eublaberus posticus*. These two cockroaches have dense, anaerobic bacterial populations in their hindguts (29). Moreover, bacterial fermentation products (eg. volatile fatty acids) occur in hindguts, and can be transported across the hindgut epithelium

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of cockroaches (10,103).

Although many bacteria have been isolated from the hindguts of *P. americana* and *E. posticus* (29), the contribution of such bacteria to cockroach nutrition is unclear, in part because of the omnivorous and complex nature of the insects' diet (laboratory specimens are usually reared on a diet of dog chow and water). In one study, *P. americana* cockroaches were administered metronidazole, a drug which essentially eliminated all anaerobic bacteria from the alimentary tract. The health of adult cockroaches was unaffected, but the growth of immature *P. americana* was retarded. Moreover, the guts of metronidazole-fed immature specimens were degenerate (9). However, it was not known if gut bacteria were important to the carbohydrate metabolism of immature cockroaches. Another intriguing explanation for these results is that metronidazole eliminates cockroach gut bacteria which produce vitamins or growth factors that are important to the development of immature cockroaches. Obviously, more work is needed to better understand the contribution of cockroach gut bacteria to host nutrituion.

II. H_2/CO_2 Acetogenic and Methanogenic Bacteria, and Their Role as Terminal Consumers of H_2 in the Guts of Termites and Cockroaches

In the previous section, it was evident that the contribution of gut microorganisms to the carbohydrate metabolism of termites and cockroaches is best understood for lower, wood-feeding termites such as *R. flavipes*. A novel aspect of the model describing symbiotic degradation of cellulose by *R. flavipes* was the role accorded to H_2/CO_2 acetogenic bacteria as consumers of H_2 produced by protozoa (Fig. 1). To appreciate

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why bacterial H_2/CO_2 acetogenesis is such a novel aspect of this model, it is necessary to first discuss the role of H_2-consuming bacterial reactions in the terminal steps of anaerobic microbial decomposition processes.

Anaerobic decomposition of plant polymers occurs in a wide variety of aquatic and terrestrial habitats (45), and in the digestive tracts of many animals, including termites and cockroaches (15,104). During intermediate steps of this process, fermentative and fatty acid- and alcohol-oxidizing microorganisms may produce large amounts of H_2. However, H_2-consuming bacteria effectively remove H_2, keeping H_2 partial pressures in natural environments quite low (92). Removal of H_2 is a crucial component of anaerobic microbial decomposition processes, because the intermediate, H_2-producing reactions of anaerobic microbial food webs will proceed less efficiently, or not at all, if H_2 is allowed to accumulate (32,92).

Bacteria responsible for H_2-consumption in anaerobic environments include those that can reductively dissimilate NO_3^{-1}, SO_4^{-2}, or CO_2. Bacterial competition for H_2 in anoxic habitats is thought to be governed by: 1) the availability of NO_3^{-1} and SO_4^{-2} (CO_2 is not usually limiting); 2) the free energy yield associated with reduction each of these three electron acceptors [NO_3^{-1} reduction > SO_4^{-2} reduction > CO_2 reduction (28,86,105)]; 3) the affinity of NO_3^{-1}-, SO_4^{-2}- or CO_2-reducing organisms for H_2 and 4) the minimum H_2 partial pressure (or H_2 threshold) at which the reduction of each of these three electron acceptors will proceed (41). In environments that are low in NO_3^{-1} and SO_4^{-2} (such as most animal gastrointestinal tracts), CO_2 reduction is the *de facto* terminal, H_2-consuming (electron sink) process.

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processes are known to occur in anaerobic habitats: H_2/CO_2 methanogenesis and H_2/CO_2 acetogenesis, each of which is described below.

II. 1. *H_2/CO_2 methanogenesis and H_2/CO_2 methanogenic bacteria*

In most anaerobic environments low in NO_3^{-1} and SO_4^{-2}, CO_2 reduction to methane, rather than acetate, is the main, terminal, H_2-consuming reaction (Reaction 1). Reasons for this are discussed in section II. 3.

$$4H_2 + CO_2 \text{ ---> } CH_4 + 2H_2O \ (\hat{=} \ G^{o\prime} = -135.6 \text{ kj/reaction [86]}) \quad (1)$$

There are currently 43 species of bacteria described as of 1988 that can grow via Reaction 1, all of which belong to a physiologically and phylogenetically coherent group of strictly anaerobic archaebacteria whose growth is obligately methanogenic (8). Most methanogenic bacteria can grow on $H_2 + CO_2$, and some can also produce methane from acetate, formate, methanol, ethanol, CO and methylamines (8,88,89). During the past ten years, a great deal has been learned about the biochemistry, phylogeny, distribution and ecology of methanogenic bacteria, most of which is summarized in recent reviews (eg. 8,88).

Termites and cockroaches emit detectable amounts of methane, and H_2/CO_2 methanogenic bacteria have been observed in gut contents of representatives of both insect groups (29,67). The quantitaive significance of H_2/CO_2 methanogenesis in the guts of termites and cockroaches is also discussed in section II. 3.

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II. 2. *H_2/CO_2 acetogenesis and H_2/CO_2 acetogenic bacteria*

An alternative to CO_2 reduction to methane is CO_2 reduction to acetate by H_2/CO_2 acetogenic bacteria (Reaction 2). The biochemistry of H_2/CO_2

$$4H_2 + 2CO_2 \text{ ---> } CH_3COOH + 2H_2O \ (\hat{=} \ G^{o\prime} = -104.6 \ [86]) \qquad (2)$$

acetogenesis has been thoroughly investigated (see reviews by Wood's group [93,94] and Fuchs [39]). Although most biochemical studies have been done by using a few selected species (*Clostridium thermoaceticum* and *Acetobacterium woodii*), many new H_2/CO_2 acetogenic bacteria have been described recently (Table 1). Unlike H_2/CO_2 methanogenesis, H_2/CO_2 acetogenesis is not restricted to a phylogenetically coherent group, but is a property of several distantly related taxa of anaerobic bacteria which include both Gram positive and negative genera, several endospore-forming representatives, and mesophilic and thermophilic species.

As a group, H_2/CO_2 acetogenic bacteria can metabolize more than 60 different compounds including sugars, organic and amino acids and alcohols (56, and references contained therein). Most species (eg. *A. woodii*) convert carbohydrates to acetate as the principal fermentation product and, therefore, have been called "homoacetogens". However, a few others (eg. *E. limosum*) form both acetate and butyrate (82). It is also significant that when H_2/CO_2 acetogens are growing heterotrophically, they can be H_2-producers rather than H_2-consumers (32). For example, Lee and Zinder have demonstrated that one H_2/CO_2 acetogen can perform Reaction 2 in the reverse direction when grown in coculture with an H_2-consuming methanogen (54).

Table 1

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Table 1. List of H_2/CO_2 acetogenic bacteria described previous to this study, and some of their properties.

Isolate	Original Source	Cell Wall Type[a]	Endospore formation[b]	$T_{opt.}$ (°C)	Reference
Acetate-oxidizing rod (AOR)	Thermal digestor	?[c]	-	60	54
Acetitomaculum ruminus	Bovine rumen	+	-	38	40
Acetoanaerobium noterae	Oil drilling sediment	-	-	37	84
Acetobacterium carbinolicum	Freshwater seds./sludge	+	-	27	35
wieringae	Sewage digestor	+	-	30	12
woodii	Marine estuary	+	-	30	4
Acetogenium kivui	Lake sediment	-	-	66	55
Butyribacterium methylotrophicum	Sewage digestor	+	+[d]	37-40	101
Clostridium aceticum	Mud	-[e]	+	30	1,13
strain CV-AA1	Sludge	-[e]	+	30	2
thermaceticum	Horse manure	+	+	55-60	37
thermautrotrophicum	Mud/soil	+	+	56-60	90
Eubacterium limosum	Sheep rumen/sludge	+	-	39	82
Peptostreptococcus productus	Sewage digestor	+	-	37	57
Sporomusa acidovorans	Distillery effluent	-	+	35	70
malonica	Pond sediments	-	+	30	31
ovata	Sugar beet leaf silage	-	+	35-37	61
paucivorans	Lake sediments	-	-	n.d.[f]	42
sphaeroides	River sediments	-	+	34	61
termitida	Termite guts	-	+	30	22

[a] +, Gram positive; -, Gram negative.

[b] +, forms endospores; -, does not form endospores.

[c] Cell wall of the AOR is not characteristic of either Gram positive or Gram negative bacteria.

[d] Spores are atypical.

[e] Cells stain Gram negative, and electron micrographs are inconclusive. The true cell wall type has not been determined by other methods.

[f] n.d., not determined.

Clostridium formicaceticum (3) and *C. magnum* (81) are two homoacetogenic anaerobes that are unable to grow on $H_2 + CO_2$, but are undoubtedly closely related to some of the *Clostridium* species listed in Table 1. In addition, H_2/CO_2 methanogenic bacteria and some sulfate-reducing bacteria can form acetyl-CoA from $H_2 + CO_2$ for autotrophic synthesis of cell carbon (essentially a form of H_2/CO_2 acetogenesis), but such bacteria rely on methanogenesis or sulfate reduction to generate energy for growth (39).

Despite recent advances in our understanding of the microbiology and biochemistry of H_2/CO_2 acetogenic bacteria, almost no information exists concerning their ecology or their role as H_2-consumers in nature. They have been isolated from a wide variety of anaerobic habitats (Table 1), but it is not yet clear whether H_2/CO_2 acetogens that have been isolated using $H_2 + CO_2$ as the carbon and energy source, are, in fact H_2-consumers (or H_2-producers) *in situ*.

Theoretical considerations suggest that H_2/CO_2 methanogensis should always outcompete H_2/CO_2 acetogenesis for H_2 because H_2/CO_2 methanogenesis (Reaction 1) is thermodynamically more favorable than H_2/CO_2 acetogenesis (Reaction 2) (28). This is especially true under conditions which involve "typical" natural concentrations of substrates and products found in anoxic habitats (32,105). Moreover, methanogenic bacteria have a 10 to 100-fold lower threshold for H_2 than do their acetogenic counterparts (19,41), despite the fact that the H_2-V_{max}/K_m values of these two groups of hydrogenotrophic bacteria are in the same range (22). However, there are reports of a few habitats where rates of H_2/CO_2 acetogenesis compare favorably to, or even exceed rates of H_2/CO_2 methanogenesis. These include certain anaerobic freshwater lakes and

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sediments (27,46,71), and the gastrointestinal tracts of humans (53), rodents (76), baleen whales (91) and wood-feeding termites (21).

II. 3. *Competition for H_2 in the guts of termites and cockroaches*

The Odelson-Breznak model of symbiotic cellulose utilization by *R. flavipes* termites (Fig. 1) suggested that H_2/CO_2 acetogenesis outcompetes H_2/CO_2 methanogenesis for H_2 in termite hindguts. This suggestion was prompted by the observation that, relative to the overall rate of cellulose fermentation in the hindgut, *R. flavipes* termites evolved quantitatively insignificant amounts of methane and H_2 (62). Moreover, evaluation of the rates of acetate formation in guts and termite respiration rates, as well as the respiratory quotient exhibited by live insects, suggested that three acetate molecules were formed from each glucosyl unit of cellulose fermented (67). Thus, Odelson and Breznak postulated that H_2/CO_2 acetogenesis was the source of a significant amount (up to one-third) of hindgut acetate.

This speculation was subsequently confirmed by Breznak and Switzer (21), who also showed that rates of H_2-dependent reduction of $^{14}CO_2$ to ^{14}C-acetate by *R. flavipes* gut contents totally outcompeted rates of H_2-dependent reduction of $^{14}CO_2$ to $^{14}CH_4$. In addition, they demonstrated that H_2/CO_2 acetogenesis in *R. flavipes* guts was inhibited by O_2 or by feeding termites antibacterial drugs. By contrast, conditions which resulted in the loss of cellulolytic protozoa from guts had little effect on H_2/CO_2 acetogenesis. These results strongly implicated anaerobic bacteria as the agents of H_2/CO_2 acetogenesis. It was also found that H_2/CO_2 acetogenesis outcompeted H_2/CO_2 methanogensis

in the guts of three other lower, and two higher, wood-feeding termite species, and in the gut of the wood-feeding cockroach, *C. punctulatus*, but not in the gut of the omnivorous cockroach, *P. americana* (21).

Unfortunately, and for reasons that are not entirely understood, attempts to isolate H_2/CO_2 acetogenic bacteria from gut contents of *R. flavipes*, or other lower, wood-feeding termite species were unsuccessful. However, a new H_2/CO_2 acetogenic isolate (*Sporomusa termitida*) was recently obtained from gut contents of a higher, wood-feeding termite, *Nasutitermes nigriceps* (22).

III. Statement of Purpose

The present study was undertaken in an effort to better understand the microbiology, ecology and nutritional significance of bacterial H_2/CO_2 acetogenesis in guts of wood-feeding termites such as *R. flavipes*. It was hoped that such information might also help clarify the ability of H_2/CO_2 acetogenic bacteria to outcompete H_2/CO_2 methanogenic bacteria for H_2 in this habitat. Chapter 1 examines how widespread H_2/CO_2 acetogenesis and methanogensis are among a diverse sampling of termite species, and to what extent diet and feeding behavior are associated with H_2 consumption by termite gut bacteria. Experiments were performed with 23 different species, including fungus-cultivating and soil-feeding termites, in addition to higher and lower, wood- and grass-feeding representatives.

Access to a wide variety of termite species also afforded an opportunity to make further attempts to isolate H_2/CO_2 acetogenic bacteria from termite gut contents (a project that previously had met with only limited success). Chapters 2 and 3 describe two novel H_2/CO_2

acetogenic bacteria that were obtained in pure culture. Chapter 2 describes the first H_2/CO_2 acetogenic bacterium isolated from gut contents of a phylogenetically lower, wood-feeding termite; and Chapter 3 describes the first H_2/CO_2 acetogen isolated from gut contents of a higher, soil-feeding termite. Morphological, physiological and molecular phylogenetic characteristics of these two new isolates are compared to those of *Sporomusa termitida*, an H_2/CO_2 acetogenic bacterium previously isolated from gut contents of the higher, wood-feeding termite, *Nasutitermes nigriceps* (22).

Unfortunately, many termites are difficult or virtually impossible to rear in the laboratory, and those that can be maintained outside of their natural habitat usually have somewhat inflexible dietary needs. Therefore, to study the effects of dietary changes on insect gut microbial ecology, and to learn more about the contribution of gut microorganisms to cockroach carbohydrate metabolism, experiments were performed with the omnivorous cockroach species, *P. americana*. The effect of high fiber diets on the activities and numbers of acidogenic and methanogenic bacteria in different regions of the alimentary tract of adult and immature *P. americana* are described in Chapter 4.

In summary, this study represented an integrated approach to examine factors (such as diet and developmental stage) that influence compatible and competitive interactions between acidogenic and methanogenic bacteria in the guts of termites and cockroaches.

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CHAPTER 1

RELATIONSHIP BETWEEN TERMITE DIET AND ACETOGENESIS AND METHANOGENESIS BY GUT BACTERIA

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ABSTRACT

Evolution of different dietary habits in termites is paralleled by fundamental differences in the activity of their hindgut microbiota. In hindguts of wood- and grass-feeding termites, CO_2-reducing acetogenic bacteria generally outcompeted methanogenic bacteria for available reducing equivalents (presumably H_2). By contrast, H_2/CO_2 acetogenesis was of little significance in hindguts of fungus-cultivating and soil-feeding termites, which evolved more methane than their wood-and grass-feeding counterparts. Given the large biomass of termites on Earth, these findings will help refine global estimates of CO_2 reduction in anaerobic habitats and the contribution of termites to atmospheric methane.

Termites are unevenly distributed over two-thirds of the earth's land surface, whereupon they are important decomposers of plant biomass, but may also incidentally cause extensive damage to dwellings and crops (*1,2*). Although perhaps best recognized for their ability to feed on sound wood, certain termite species obtain most of their nutrition by feeding on soil (humus) (*2,3*). Others cultivate and consume cellulolytic fungi which, when ingested with plant matter, provide important digestive enzymes for the insect (*3,4*). Nevertheless, a property shared by all termites examined thus far is the presence of a dense and diverse community of microbes in their hindgut (*3,5*). Indeed, the digestion of lignocellulosic food resources by termites appears to involve their gut microbiota, which includes bacteria and anaerobic, cellulolytic protozoa in phylogenetically "lower" termites, and bacteria alone in phylogenetically "higher" termites (*3,5*).

The contribution of gut microbes to termite digestion has recently been investigated in detail for the North American wood-feeding lower termite, *Reticulitermes flavipes*. Odelson and Breznak (*6*) demonstrated that acetate was the major product of microbial fermentation of wood polysaccharides (principally cellulose) in the hindgut. Subsequent oxidation of microbially produced acetate by termite tissues (Eq. 1) could support the entire respiratory requirement of *R. flavipes*. A model was proposed to describe the major metabolic

$$CH_3COOH + 2\ O_2 \text{ ---> } 2\ CO_2 + 2\ H_2O \qquad (1)$$

interactions among gut microbes of lower termites resulting in acetate production. This model suggested that cellulolytic protozoa ferment

each glucosyl unit of cellulose to acetate, H_2 and CO_2, [Eq. 2; demonstrated by using mixed cell suspensions as well as axenic cultures of termite hindgut protozoa (*7*)]. The H_2 and CO_2 were then believed to

$$C_6H_{12}O_6 \text{ ---> } 2CH_3COOH + 4H_2 + 2CO_2 \qquad (2)$$

(Cellulolytic protozoa)

be consumed by H_2/CO_2 acetogenic bacteria to form one additional acetate (Eq. 3; *6*). H_2 and CH_4 [the later of which is formed via CO_2 reduction by methanogenic bacteria (Eq. 4; *6,8,9*)] are also emitted by lower termites, but represent only a minor fate of reducing equivalents (i.e. $H^+ + e^-$) generated during wood polysaccharide fermentation (*6*). This model was confirmed by Breznak and Switzer (*10*) who showed that

$$4H_2 + 2CO_2 \text{ ---> } CH_3COOH + 2H_2O \qquad (3)$$

(H_2/CO_2 acetogenic bacteria)

$$4H_2 + CO_2 \text{ ---> } CH_4 + 2H_2O \qquad (4)$$

(Methanogenic bacteria)

H_2/CO_2 acetogenesis (Eq. 3) was also the main H_2-consuming (i.e. "electron sink") reaction in hindguts of three lower and two higher, wood-feeding termites. Moreover, pure cultures of H_2/CO_2 acetogenic bacteria have recently been isolated from termites and studied in detail (*20*, Chapters 2 and 3).

To assess the validity of this model for termites in general, we

recently examined a variety of tropical species that represented different patterns of food resource preference. Opportunities to sample freshly collected specimens of many of these species (especially those from remote regions) were rare, and therefore our efforts concentrated on examining a wide variety of species rather than different samples of one or a few species. Termites used in this study included wood-feeding members of three lower termite families (Hodo-, Kalo- and Rhinotermitidae) and wood-feeding, grass-feeding, fungus-cultivating and soil-feeding representatives of the higher termite family, Termitidae (*3,13*). Herein we show that the ability of H_2/CO_2 acetogenic bacteria to outcompete methanogenic bacteria for H_2 in termite hindguts is a widespread phenomenon among wood- and grass-feeding termites, but not among soil-feeding or fungus-cultivating termites which emit considerably more methane than their wood-feeding counterparts (*11*).

Methane production by the gut microbiota was usually estimated by measuring methane emission from live termites themselves, a technique which is noninvasive and minimizes disruption of the insects (*6*). However, this technique does not distinguish between H_2-dependent methane production, and methane produced from other substrates (eg. acetate), so it may overestimate *in situ* rates of methanogenesis from H_2 + CO_2. By contrast, because termite tissues readily oxidize acetate (*6*), H_2/CO_2 acetogenesis rates were determined by measuring the reduction of $^{14}CO_2$ to ^{14}C-acetate by anoxic homogenates of termite gut contents. This was done under two conditions: with exogenously supplied H_2; and with reductant (presumably H_2) produced endogenously by microbes present in the gut homogenates (*12*). It is important to note that the latter condition may seriously underestimate *in situ* rates of H_2/CO_2

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acetogenesis, because homogenization and dilution of gut contents probably disrupt important physical interactions between H_2-producing microbes and H_2-utilizing acetogenic bacteria (*15*).

H_2/CO_2 acetogenesis generally, and sometimes completely, outcompeted methanogenesis as the main H_2-consuming process in guts of 12 (of 13) wood-feeding termites and one grass-feeding termite, especially when measured in the presence of exogenously supplied H_2 (Table 1). For wood- and grass-feeding termites, the grand mean rate of ^{14}C-acetate formation from $^{14}CO_2$ (using endogenously produced H_2) by gut contents was 3-fold greater than the grand mean rate of CH_4 emission. When supplied with exogenous H_2, the grand mean rate of ^{14}C-acetate formation from $^{14}CO_2$ by gut contents of wood- and grass-feeding termites increased to 10-fold greater than the grand mean rate of CH_4 emission. By contrast, methane emission rates were greater than rates of H_2/CO_2 acetogenesis for 3 fungus-cultivating, and 5 (of 6) soil-feeding termites (Table 1). Grand mean rates of methane emission were 2 to 7 times greater than grand mean rates of ^{14}C-acetate formation for fungus-cultivating termites and 5 to 12 times greater for soil-feeding termites. As with wood-feeding termites, individual and grand mean rates of $^{14}CO_2$ reduction to ^{14}C-acetate by gut contents of fungus-cultivating or soil-feeding species were usually increased by the presence of exogenously supplied H_2.

Comparison of these hydrogenotrophic bacterial processes between termite dietary groups indicated that grand mean rates of $^{14}CO_2$ reduction to ^{14}C-acetate by gut contents from wood- and grass-feeding termites (with, or without exogenously supplied H_2) were significantly greater than those of fungus-cultivating or soil-feeding termites (Table

Table 1. Reduction of $^{14}CO_2$ to ^{14}C-acetate by termite gut contents, and CH_4 emission by live termites of different dietary guilds.

	μmol product x g termite^{-1} x h^{-1}		
	^{14}C-Acetate[a]		
Termite and Diet	From exogenously supplied H_2	From endogenously produced H_2	CH_4 Emitted[b]
WOOD-FEEDING TERMITES[c]:			
Coptotermes formosanus	1.66	0.10	0.01[d]
Prorhinotermes simplex	1.18	0.57	n.d.
Pterotermes occidentis	2.07	0.48	0.00
Reticulitermes flavipes	0.93 ± 0.43	0.09 ± 0.06	0.10
Zootermopsis angusticollis	0.33 ± 0.25	0.07 ± 0.02	1.30
Amitermes sp.	5.16	1.03	0.13
Gnathamitermes perplexus	1.83	0.13	0.21
Microcerotermes parvus	4.96 ± 1.34	1.16 ± 0.98	0.14
Nasutitermes arborum	2.29	3.00	0.13
Nasutitermes costalis	5.96	0.99	n.d.
Nasutitermes lujae	1.91	0.13	0.15
Nasutitermes nigriceps	3.68	0.89	0.24
Tenuirostritermes tenuirostris	0.98	0.05	0.11
GRASS-FEEDING TERMITE:			
Trinervitermes rhodesiensis	2.70	2.38	0.18
GRAND MEAN[e]:	**2.54 ± 0.47**	**0.79 ± 0.24**	**0.23 ± 0.10**
FUNGUS-CULTIVATING TERMITES:			
Macrotermes mulleri	0.05	0.01	0.25
Pseudacanthotermes militaris	0.23	0.16	0.67
Pseudacanthotermes spiniger	0.17	0.01	0.36
GRAND MEAN:	**0.17 ± 0.03**	**0.06 ± 0.05**	**0.43 ± 0.13**
SOIL-FEEDING TERMITES:			
Crenetermes albotarsalis	0.05	0.02	0.93
Cubitermes fungifaber	0.56	0.21	0.48
Cubitermes speciosus	0.02 ± 0.01	0.01 ± 0.01	0.85
Noditermes sp.	0.03	0.05	0.64
Procubitermes sp.	0.05	0.03	0.39
Thoracotermes macrothorax	0.07	0.01	1.09
GRAND MEAN:	**0.13 ± 0.10**	**0.06 ± 0.03**	**0.73 ± 0.11**

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Table 1 (continued).

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[a]Results are mean values of duplicate analyses for n = 1 except for the following species, which are mean values of duplicate analyses for n as indicated: *R. flavipes*, n = 20; *Z. angusticolis*, n = 3; *M. parvus*, n = 3; *N. lujae*, n = 2; *C. albotarsalis*, n = 2; *C. speciosus*, n = 3. For results where n $\geq$ 3, results are mean values $\pm$ standard deviation (14).

[b] Determined essentially as described previously (*6*), using mean values of duplicate analyses for n = 3 to 5.

[c]The first five species listed are classified as "lower" termites, and have a hindgut microbiota consisting of bacteria and cellulolytic, flagellate protozoa. The remaining species are classified as "higher" termites, and have a hindgut microbiota consisting only of bacteria (*3*).

[d]n.d., not determined.

[e]Grand means are $\pm$ standard error (14) and were calculated by using all of the individual analyses for each dietary group.

1; *14*). By contrast, grand mean rates of methane emission by soil-feeding, and, to a lesser extent, fungus-cultivating termites, were significantly greater than those of wood-feeding termites. It was also noteworthy that gut contents from all wood- and grass-feeding termites tested displayed readily detectable levels of H_2/CO_2 acetogenic activity, but one fungus-cultivating species, and 5 soil-feeding species exhibited almost no H_2/CO_2 acetogenesis, even when supplied with exogenous H_2. Conversely, all fungus-cultivating, and soil-feeding species examined evolved relatively high amounts of methane, but two wood-feeding species (*P. occidentis*, and *C. formosanus*) evolved little or none.

It might be argued that the relatively low rate of methane emission from wood-feeding termites is due to aerobic oxidation of this gas before it emanates from the insect. However, kinetic analysis of O_2 consumption by intact termites suggests that this is not the case (*6*). Moreover, we have measured rates of $^{14}CO_2$ reduction to $^{14}CH_4$ by anoxic gut contents from the following wood-feeding species: *R. flavipes*, *Z. angusticollis*, *M. parvus*, *N. lujae* and *N. nigriceps*; and from the soil-feeding termite, *C. speciosus* (*10,16*). In all cases, rates of $^{14}CO_2$ reduction to $^{14}CH_4$ by termite gut contents were less than those of methane emission by live termites, even when supplied with exogenous H_2. In addition, previously published rates of $^{14}CO_2$ reduction to $^{14}CH_4$ by gut contents from the wood-feeding *P. simplex* and *N. costalis* were considerably less than rates of ^{14}C-acetate formation from $^{14}CO_2$ (10). Unfortunately, due to logistical limitations we were unable to determine rates of $^{14}CO_2$ reduction to $^{14}CH_4$ by gut contents from fungus-cultivating species or other soil-feeding species.

It is not surprising that animals with anaerobic, fermentative microbial communities in their alimentary tract evolve methane. A classic example of this is the bovine rumen microbiota which evolves up to 200 liters CH_4 per animal per day (*22*). However, the ability of H_2/CO_2 acetogens to outcompete methanogens for H_2 in the guts of wood-feeding termites [and in certain other habitats including the colon of some humans (*19*)], is enigmatic. Thermodynamic and kinetic considerations suggest that in environments low in sulfate and nitrate, CO_2 reduction to methane (not acetate) should be the dominant, terminal H_2-consuming process (*8,18*). Methanogenesis from $H_2 + CO_2$ yields more energy (-135.6 kJ/reaction) than does acetogenesis (-104.6 kJ/reaction) (*8*), and the threshold of H_2/CO_2 acetogens for H_2 is more than 10-fold greater than that of methanogens (*18*). Moreover, the apparent K_m for H_2 uptake by H_2/CO_2 acetogenic bacteria is in the same range as (not unusually lower than) that of many methanogens (*20*). Clearly, factors other than thermodynamics and affinity for H_2 must be important to the success of H_2/CO_2 acetogens in ecosystems such as the guts of wood-feeding termites.

In an effort to understand why H_2/CO_2 acetogenesis outcompetes methanogensis in the guts of wood-feeding termites, we have isolated in pure culture three strains of H_2/CO_2 acetogenic bacteria - one each from the guts of a higher and lower wood-feeding termite, and one from the gut of a soil-feeding termite (*20*, Chapters 2 and 3). Results indicate that these isolates each represent new and different bacterial species and we are currently studying various aspects of their nutrition and physiology which may bear on their ability to compete for H_2 in termite guts (20).

Other investigators have suggested that CH_4 emission from termites is an important source of atmospheric methane. Estimates of termite contributions have ranged from less than 5 percent to greater than 40 percent of total annual global methane production (21). We feel that such estimates must be viewed with caution, because of uncertainties associated with global estimates of termite numbers and activities. Moreover, previous estimates were made without information on rates of acetogenesis and methanogenesis for termites of different dietary groups. From the present study it appears that, due to the hydrogenotrophic activity of acetogenic bacteria in hindguts, wood- and grass-feeding termites typically evolve less than 10 percent of the amount of methane that they might otherwise produce. By contrast, fungus-cultivating and soil-feeding termites evolve significantly more CH_4 per termite than do termites of the former dietary groups. As global estimates of populations of specific termite dietary groups become more reliable, these data will help to refine our understanding of the contribution of termites and their gut microbes to global methane production and CO_2 reduction in anaerobic habitats.

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11. Results for a few individual termite species were previously published as portions of a separate study ([*10*], and are included in two reviews in press: J.A. Breznak and M.D. Kane, *FEMS Microbiol. Rev.* [1990]; A. Brauman *et al.*, in *Microbiology and Biochemistry of Strict Anaerobes Involved in Interspecies Hydorgen Transfer*, [Plenum Publishing Corp., 1990].

12. All experiments were performed in East Lansing, Michigan on freshly collected or laboratory-maintained specimens (*R. flavipes*), or within 48 h of receipt of specimens provided as indicated (*13*), except for termites from Peoples' Republic of Congo, all of which were assayed recently in Brazzaville from freshly collected nests. The H_2/CO_2 acetogenesis assay has been described in detail previously (*10*) and is only summarized here. Guts from 20 to 60 termites were removed by using an anaerobic chamber and pooled in an anoxic buffered salts solution prior to homogenization. Reaction vials (8-ml) had a final liquid volume of 0.5ml, and contained

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1.2 μmol $NaH^{14}CO_3$, pH 7.2 (approx. specific activity, 6.5 x 10^5 dpm/μmol) and the contents of 2 to 4 homogenized termite guts. The atmosphere in reaction vials consisted of 100% N_2 (for determination of rates of ^{14}C-acetate formation from $^{14}CO_2$ by endogenously produced H_2) or 100% H_2 (for rates of ^{14}C-acetate formation from $^{14}CO_2$ catalyzed by exogenously supplied H_2). After termination of the reaction, the supernatant fluid was analyzed for ^{14}C-labelled products by high performance liquid chromatography.

13. Termites were collected from the follwing locations : *C. formosanus*, Lake Charles, Louisianna, USA (provided by L. Williams, USDA Gulfport, MS); *P. simplex*, Coral Gables, Florida, USA (provided by G. Prestwich, State Univ. New York, Stonybrook); *P. occidentis*, *Amitermes* sp., *G. perplexus* and *T. tenuirostris*, Santa Rita Range area, southwestern Arizona, USA (collected with the help of W. Nutting, U. Arizona); *R. flavipes*, Dansville, Michigan, USA; *Z. angusticolis*, San Francisco Bay Park, California, USA (provided by J. Traniello, Boston U.); *M. parvus* and *N. lujae*, forest near Brazzaville, Peoples' Republic of Congo; *N. arborum*, *M. mulleri*, *C. albotarsalis*, *C. speciosus*, *Noditermes* sp., *Procubitermes* sp. and *T. macrothorax*, Mayombe rainforest, Peoples' Republic of Congo; *N. costalis*, forest near Frijoles, Panama; *N. nigriceps*, forest in Lesser Antilles; *T. rhodesiensis* and *C. fungifaber*, savannah near Niari, Peoples' Republic of Congo; *P. militaris*, savannah near Brazzaville, Peoples' Republic of Congo; *P. spiniger*, sugar cane fields near Nkayi, Peoples' Republic of Congo.

14. Significant differences between means were verified by using tests of comparison for two sample means (R.G.D. Steel and J.H. Torrie *Principles and Practices of Statistics: A Biometrical Approach*, 2nd Edition, [McGraw-Hill, Inc., New York, 1980], pp.86-121). For all means identified as unequal, $\alpha \leq 0.05$, except for the grand mean rates of CH_4 emission from wood-feeding termites and soil-feeding termites, where $\alpha \leq 0.40$. Standard deviation and standard error were used as defined in *ibid*, pp. 8-38.

15. R. Conrad *et al.*, *Appl. Environ. Microbiol.* 50:595-601.

16. M.D. Kane, A. Brauman and J.A. Breznak, unpublished.

17. This mean value is for 20 separate determinations, and is slightly less than that previously reported (*10*) which was based on 6 determinations.

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20. J.A. Breznak *et al.*, *Arch. Microbiol.* **150**, 282 (1988); M.D. Kane and J.A. Breznak, *Abstr. Ann. Meet. Amer. Soc. Microbiol.* p.234 (1989); J.A. Breznak and J.M. Switzer, *Abstr. Ann. Meet. Amer. Soc.* Microbiol. *p.160 (1989).*

21. P.R. Zimmerman *et al.*, *Science* **218**, 563 (1982); R.A. Rasmussen and M.A. Kahill, *Nature* **301**, 700 (1983); P.R.Zimmerman and J.P. Greenberg, *Nature* **302**, 354 (1983); M.A. Kahill and R.A. Rasmussen, *Nature* **302**, 355 (1983); N.M. Collins and T.G. Wood, *Science* **224**, 84 (1984); P.R. Zimmerman *et al.*, *Science* **224**, 86 (1984); W. Seiler, in *Current Perspectives in Microbial Ecology*, M.J. Klug and C.A. Reddy, Eds., (American Society for Microbiology, Washington, D.C., 1984) pp.468-477; P.J. Fraser *et al.*, *J. Atmos. Chem.* 4, 295 (1986).

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CHAPTER 2

H_2/CO_2 ACETOGENIC BACTERIA FROM TERMITE GUTS: I. *Acetonema elongata*, GEN. NOV., SP. NOV., A GRAM NEGATIVE, ENDOSPORE-FORMING BACTERIUM FROM *Pterotermes occidentis*

Abstract

Strain APO-1, a previously undescribed H_2/CO_2 acetogenic bacterium, was isolated from gut contents of the wood-feeding termite *Pterotermes occidentis*. Cells of strain APO-1 were anaerobic, Gram negative, endospore-forming, motile rods which measured 0.30-0.40 x 6-60 μm. Cells were catalase positive, oxidase negative, and had 51.5 mol percent G + C in their DNA. Optimum conditions for growth on H_2 + CO_2 were at 33^{o} C and pH 7.8, and under these conditions cells produced acetate according to the following stoichiometry: $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$. Other substrates supporting good growth of strain APO-1 included glucose, ribose, and various organic acids. The major fermentation products were usually acetate and butyrate, but with certain substrates other products (eg. propionate, succinate, 1,2-propanediol) were also formed. Based on comparative analysis of 16S rRNA nucleotide sequences, strain APO-1 was somewthat related to members of the genus *Sporomusa* (another genus of Gram negative, endospore-forming acetogens). However, the morphological and physiological differences between strain APO-1 and the six known species of *Sporomusa* were significant. Consequently, it is proposed that a new genus, *Acetonema*, be established, with strain APO-1 as the type strain of the new species, *Acetonema elongata*. This newly described bacterium could potentially contribute to the nutrition of *P. occidentis* termites by forming volatile fatty acids (eg. acetate, propionate and butyrate) which are important carbon and energy sources for the insect.

Introduction

Microbial fermentation of wood polysaccharides in the hindgut of phylogenetically "lower" termites such as *Reticulitermes flavipes* (which harbor both bacteria and anaerobic, cellulolytic protozoa) has been described as essentially a homoacetic fermentation of cellulose; i.e., n $C_6H_{12}O_6$ ---> n $3CH_3COOH$ (Odelson and Breznak, 1983). The cellulolytic protozoa ferment each glucosyl unit of cellulose to 2 acetate + $4H_2$ + $2CO_2$. An additional acetate is formed from each 4 H_2 + 2 CO_2 by bacterial H_2/CO_2 acetogenesis. The latter process outcompetes methanogenesis as the major "electron sink" reaction in the hindguts of almost all "lower" termites examined to date, as well as in the hindgut fermentation of all wood-feeding "higher" termites (which harbor only bacteria in their hindguts) (Breznak and Switzer, 1986; Chapter 1). In an effort to understand why this is so, attempts have been made to isolate H_2/CO_2 acetogenic bacteria from termite guts for further study. Recently, the H_2/CO_2 acetogen *Sporomusa termitida* was isolated from the "higher" wood-feeding termite *Nasutitermes nigriceps*, (Breznak et al. 1988).

The present report describes the isolation and characterization of strain APO-1, an H_2/CO_2 acetogenic bacterium isolated from the guts of the "lower" wood-feeding *Pterotermes occidentis*. Although strain APO-1 resembled members of the genus *Sporomusa* in being a Gram negative, endospore-forming, H_2/CO_2 acetogen, its morphology was distinctly different from known species of *Sporomusa*, which are more or less uniform in cell size and shape. Moreover, physiological and molecular phylogenetic analyses prompted us to propose strain APO-1 as the type strain of a new genus and species, *Acetonema elongata*.

[A preliminary report of these findings has been presented (M. D. Kane and J. A. Breznak, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1989, I100, p.234)].

Materials and Methods

Termites

P. occidentis (Walker) (Kalotermitidae) was collected in the vicinity of the Santa Rita Range area near Tuscon, Arizona, USA. They were used within 72 hours of collection. Methane emission from live *P. occidentis* worker termites was determined as described previously (Odelson and Breznak, 1983).

Bacteria

Sporomusa termitida strain JSN-2 was described previously (Breznak, et al., 1988). *Clostridium mayombeii* strain SFC-5 is described in a companion paper (Chapter 4).

Isolation of strain APO-1

An anoxic homogenate of guts of *P. occidentis* worker termites was prepared with a buffered salts solution (BSS) as described previously (Breznak and Switzer, 1986). Serial ten-fold dilutions of gut homogenate were made in BSS and then inoculated into 18-mm serum-stoppered tubes containing 10.0 ml of anoxic AC-K1 or AC-K2 medium. AC-K1 medium was a $CO_2/NaHCO_3$-buffered medium containing inorganic salts, vitamins, yeast extract (0.5 g/l) and resazurin. It was identical to AC-19 medium (Breznak et al., 1988) except for the addition of

trypticase (BBL, Baltimore, MD; 2.0 g/l, and clarified bovine rumen fluid (50 ml/l). AC-K2 medium was identical to AC-K1 except that the methanogenesis inhibitor 2-bromoethane-sulfonate (BES; filter sterilized separately) was included at a final concentration of 50 mM. Tubes were incubated horizontally on a reciprocal shaker (100 rpm) at 30° C with 1.0 atm of H_2/CO_2 (80/20, vol/vol) in the headspace. From the highest dilution tube of AC-K2 medium showing depletion of H_2/CO_2 and production of acetate, roll tubes were prepared using AC-K2 medium solidified with 2% agar (Hungate, 1969). Isolated colonies were picked from roll tubes that had consumed H_2/CO_2 and were reinoculated into AC-K2 broth to test for H_2/CO_2 consumption and acetate production. Broth tubes showing the greatest depletion of gas and production of acetate were chosen for repeated passage in agar roll tubes. Cultures were considered to be pure after three successive passages in roll tubes, at which time phase contrast microscopy revealed a single morphological type. They were then transferred to AC-K1 medium (lacking BES) for routine culture at 30° C in 18-mm serum-stoppered tubes containing 10 ml medium and a headspace of H_2/CO_2 (80/20, vol/vol).

Growth and nutrition studies

AC-K3 basal medium was used for growth and nutrition studies. It was identical to AC-K1 medium, except that yeast extract and clarified rumen fluid were omitted. The medium was prepared under an O_2-free gas phase containing 20% CO_2 and had a final pH of 7.2, except where the pH was adjusted by varying the $CO_2/NaHCO_3$ ratio (Costilow, 1981) for determination of optimum pH for growth. For studies of substrate utilization, AC-K3 basal medium was dispensed into sterile,

18-mm screw cap tubes containing a predetermined amount of the substrate to be tested. Tubes were filled completely with AC-K3 basal medium so as to leave essentially no gas in the headspace. Molar growth yields of cells were estimated as described previously (Breznak et al., 1988).

Growth of cells was measured by determining the absorbance of cultures at 600 nm with a Bausch & Lomb Spectronic 20 colorimeter, or a Beckman DU spectrophotometer. Cell dry mass determinations were performed as previously described (Breznak et al., 1988).

Fermentation studies

The fermentation stoichiometry of cells growing with H_2/CO_2 was done by using bottles containing 245 ml AC-K3 basal medium and a 465 ml gas phase of H_2/CO_2 (80/20, vol/vol). Fermentations of glucose, rhamnose and potassium fumarate were done by growing cells under 100% N_2 in basal AC-K3 medium modified by omitting $NaHCO_3$ and including 3-(N-morpholino) propanesulfonic acid buffer (adjusted to pH 7.4 and filter sterilized separately) at a final concentration of 10 mM. Material balance calculations were corrected for the small amount of products formed by cells growning in modified AC-K3 medium containing no additional substrate.

Sequencing of 16S ribosomal RNA

Total nucleic acids were extracted from ca. 200 mg of cells by using hot phenol and were precipitated from the aqueous phase with ethanol. Nucleotide sequences were determined by the dideoxynucleotide - reverse transcriptase method of Lane et al. (1985). Nearly complete sequences were obtained by using 16S rRNA as the template and starting

reactions with one of seven oligonucleotide primers complimentary to universally conserved regions of the 16S rRNA (Montgomery et al., 1988). The sequences for *Sporomusa termitida* strain JSN-2, *Acetonema elongata* strain APO-1 and *Clostridium mayombeii* strain SFC-5 have been deposited with GenBank. They can also be obtained by writing us directly.

Sequence similarities and evolutionary distances were determined in collaboration with D. A. Stahl (Univ. Illinois, Urbana, USA) by the methods of Olsen et al., (1986) with the modifications of Montgomery et al., (1988). Unpublished sequences for the 16S rRNA of *Megasphaera elsdenii* and *Sporomusa paucivorans* were kindly made available to us by C. R. Woese, Univ. Illinois.

Chemical assays

H_2 and CH_4 were quantified by gas chromatography (Odelson and Breznak, 1983). In enrichment cultures, nutritional studies and H_2/CO_2 growth experiments, acetate production was determined by gas chromatography of culture supernatant fluids (Breznak and Switzer, 1986). For fermentation material balances, acetate, propionate and butyrate were determined by high performance liquid chromatography (HPLC) (Breznak and Switzer, 1986). 1,2-propanediol was also quantified by using the same HPLC system, wherein it displayed a retention time of 16.75 min. Protein was determined by the Folin reaction (Hanson and Phillips, 1981). Dipicolinic acid (DPA) was extracted from sporulated cultures and assayed spectrophotometrically as the calcium chelate (Lewis, 1967).

Lipopolysaccharide was determined by D. C. White (U. Tennessee,

Knoxville, USA) using gas chromatography and mass spectrometry (Parker et al., 1982)

Other procedures

Measurement of H_2-dependent reduction of $^{14}CO_2$ to ^{14}C-acetate by cell suspensions, and determination of the distribution of ^{14}C in acetate by Schmidt degradation, were performed as described previously (Breznak et al., 1988, Breznak and Switzer, 1986).

Polymyxin B sensitivity was assayed by the method of Wiegel and Quandt (1982).

For determination of cytochromes, crude cell extract preparations and $Na_2S_2O_4$-reduced minus oxidized spectra were performed as described previously (Breznak et al., 1988) except that a Gilford Response™ spectrophotometer was used.

DNA base composition was determined by B. Mannarelli (USDA-ARS, Peoria, Illinois, USA) using the buoyant density method (Mannarelli, 1988). Previously described methods were used for electron microscopy (Breznak and Pankratz, 1977), fluorescence microscopy (Doddema and Vogels, 1978), catalase and oxidase tests (Potrikus and Breznak, 1977), nitrate reduction (Smibert and Krieg, 1981) and sulfide production (Cline, 1969).

Chemicals

All chemicals were reagent grade and were purchased commercially.

Results

Enrichment and isolation of bacteria

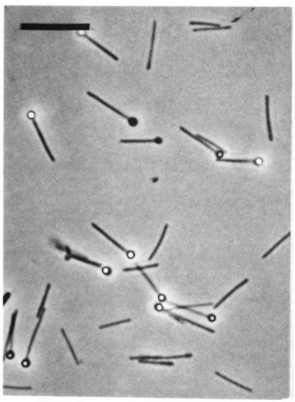
Live worker termites of *P. occidentis* emitted no detectable CH_4. Therefore, it was not too surprising that successful enrichments for H_2/CO_2 acetogenic bacteria were obtained from gut homogenates inoculated into both AC-K1 medium (lacking BES) and AC-K2 medium. After four weeks, enrichments exhibited turbidity, negative pressure and acetate production up to 24 mM (with periodic replenishment of H_2/CO_2) out to the fourth dilution tube suggesting an initial population of at least 10^6 H_2/CO_2 acetogens per ml gut fluid. No methane was produced, and no F_{420}-fluorescent cells were observed by microscopy in either medium. From enrichments in AC-K2 medium, three strains of H_2/CO_2 acetogenic bacteria were obtained. All three strains were similar in morphology and Gram stain reaction, so one of these (strain APO-1) was chosen for further study.

Colony and cell morphology

Colonies of H_2/CO_2 grown cells were 1-2 mm in diameter, circular with uneven edges, and opaque with a slight brown color. Cells of strain APO-1 were straight, thin rods measuring 0.30-0.40 x 6.0-60.0 μM (Fig. 1a & b). Cells stained Gram negative, and electron micrographs of thin sections revealed the presence of distinct inner (cytoplasmic) and outer membranes characteristic of Gram negative bacteria (Fig. 1c). The cell wall of strain APO-1 cells also produced "bleb"-like protrusions when cells were incubated in the presence of polymyxin B (1580 units/ml), a reaction that Wiegel and Quandt (1982) demonstrated to be

Figure 1a-c. Morphology of strain APO-1. a. Phase contrast micrograph; bar = 10.0 μm. b,c. Transmission electron micrographs of thin sections; bars =1.0 μm (b) and 0.1 μm (c). Note phase-bright, terminal, spherical endospores in a and b, and the outer membrane of the cell wall (c, *arrow*).

A



B

C

specific for Gram negative bacteria. Moreover, there was an appreciable amount of lipopolysaccharide associated with cell lipid (D. C. White, personal communication). Cells also formed spherical, terminal endospores that markedly swelled the sporangium (Fig 1a & b). Viable cells could be recovered from sporulated cultures held at 80° C for 10 min, and DPA extracted from sporulated cultures displayed UV absorption maxima and minima identical to those of authentic DPA.

The average length of strain APO-1 cells during early log phase growth on H_2/CO_2 was more than twice the average length of cells at the end of log phase, at which time up to 75% of cells had formed endospores (Fig. 2). Endospores were present only on cells $\leq$ 12 μm and, in general, such cells were more phase dark than cells of greater length (Fig. 1a). Cells grown on organic compounds such as glucose (below) were generally longer (up to 50 μm), less phase dark and formed fewer endospores than H_2/CO_2 grown cells.

In wet mount preparations cells exhibited little or no motility. However, when allowed to settle on the surface of an agar-covered slide, cells displayed rapid motility, presumably due to the presence of peritrichous flagella which were observed in some electron micrographs.

Growth and nutrition studies

Strain APO-1 was a strict anaerobe. The addition of trypticase (0.2%) or, to a lesser extent, yeast extract (0.2%) stimulated growth, but the addition of rumen fluid (5%) had little effect. With glucose as substrate (below), cells grew within a temperature range of 19 to 40° C and a pH range of 6.4 to 8.6. Optimum growth in AC-K3 medium, with H_2/CO_2 as substrate, occurred at 33° C and pH 7.8. At 30° C with H_2/CO_2

Figure 2. Cell length and endospore formation during growth of strain APO-1 in AC-K3 medium with H_2 + CO_2 as substrate. During growth (measured by determining $O.D._{600nm}$), samples of culture fluid were periodically removed for phase microscopy to measure cell length and to determine the percentage of cells with endospores.

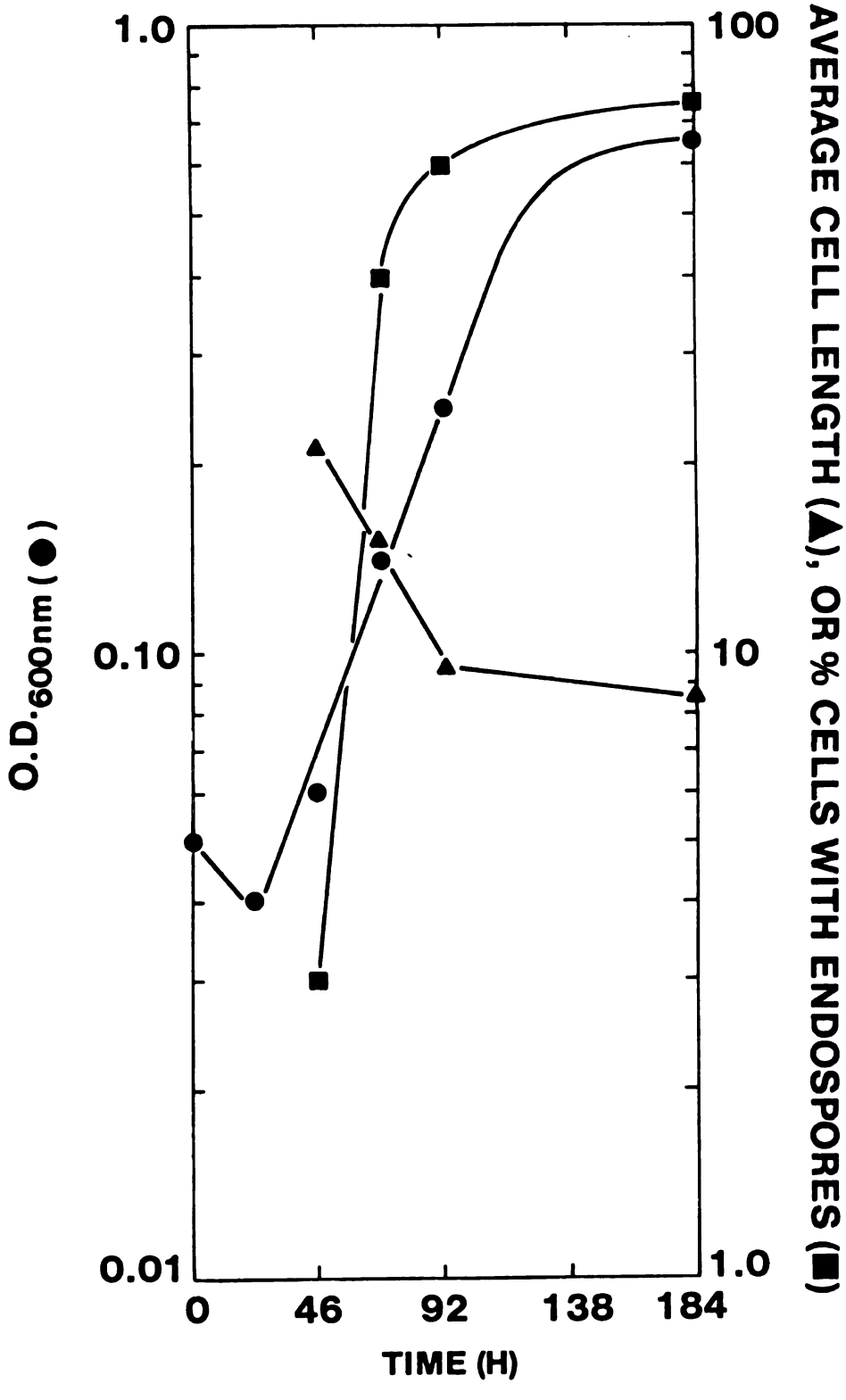

1.0
0.10
0.01
100
10
1.0
0
46
92
138
184
TIME (H)
O.D.600nm (●)
AVERAGE CELL LENGTH (▲), OR % CELLS WITH ENDOSPORES (■)

Figure 2.

Figure 3. Utilization of H_2 by strain APO-1 for growth and acetogenesis. Cells were grown on a shaker at 30^{o} C in bottles containing 245 ml AC-K3 medium and 463 ml gas phase at 1 atm. The gas phase was either H_2/CO_2 (80/20, vol./vol.; (*closed symbols, solid lines*) or N_2/CO_2 (*open symbols, broken lines*).

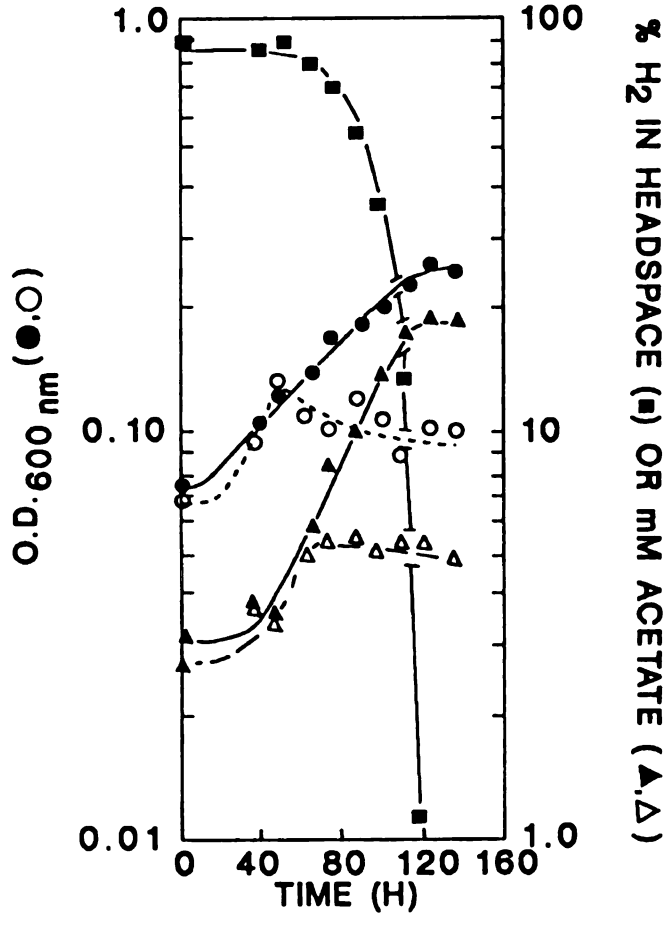

1.0
0.10
0.01
100
10
1.0
0
40
80
120
160
TIME (H)
O.D.600 nm (●,○)
% H2 IN HEADSPACE (■) OR mM ACETATE (▲,△)

Figure 3.

as substrate cells grew with a doubling time of 36h, achieved a final $O.D._{600nm}$ of 0.25 to 0.30 (approx. 100 to 125 μg dry mass/ml), and produced 18 to 23 mM acetate (without replenishment of H_2/CO_2) (Fig. 3). During growth the pH of the culture dropped from an initial value of 7.8 to a final value of 6.7. However, growth on H_2/CO_2 could not be initiated at 37° C, and growth was slightly suppressed when mid-log phase cells grown on H_2/CO_2 at 30° C were shifted to 37° C (Fig. 4a). By contrast, growth of cells on glucose was slightly stimulated by a similar temperature shift (Fig. 4b).

In addition to growth on H_2/CO_2, strain APO-1 grew heterotrophically on a range of other compounds (Table 1). Butyrate and acetate were the principle acids produced during fermentation of mannose, glucose, fructose, rhamnose, ribose, mannitol, pyruvate and oxaloacetate. However, acetate and propionate were the major acids produced by fermentation of citrate, fumarate and propanol. 1,2-propanediol was also a significant product from growth on rhamnose. Acetate was the sole product detected from growth on ethylene glycol and trimethoxybenzoate. Detailed balances for fermentations of some of these substrates are presented below. Molar growth yields of strain APO-1, determined for selected substrates, were as follows (g dry mass/mol substrate used): H_2 (+ CO_2), 1.0; glucose, 15.1, rhamnose, 4.2 and fumarate, 1.4. Doubling times of cells grown at 30° C with these substrates were (h): H_2/CO_2, 36; glucose, 8; rhamnose, 15.5 and fumarate, 40. Neither sulfate or nitrate were used as electron acceptors.

Figure 4. Effect of temperature on H_2/CO_2-grown (A) and glucose grown (B) cells of strain APO-1. Cells were grown in AC-K3 medium on a rotary shaker at 30° C (*closed symbols, solid lines*), and then shifted in mid-log phase (arrows) to 37° C (*open symbols, broken lines*).

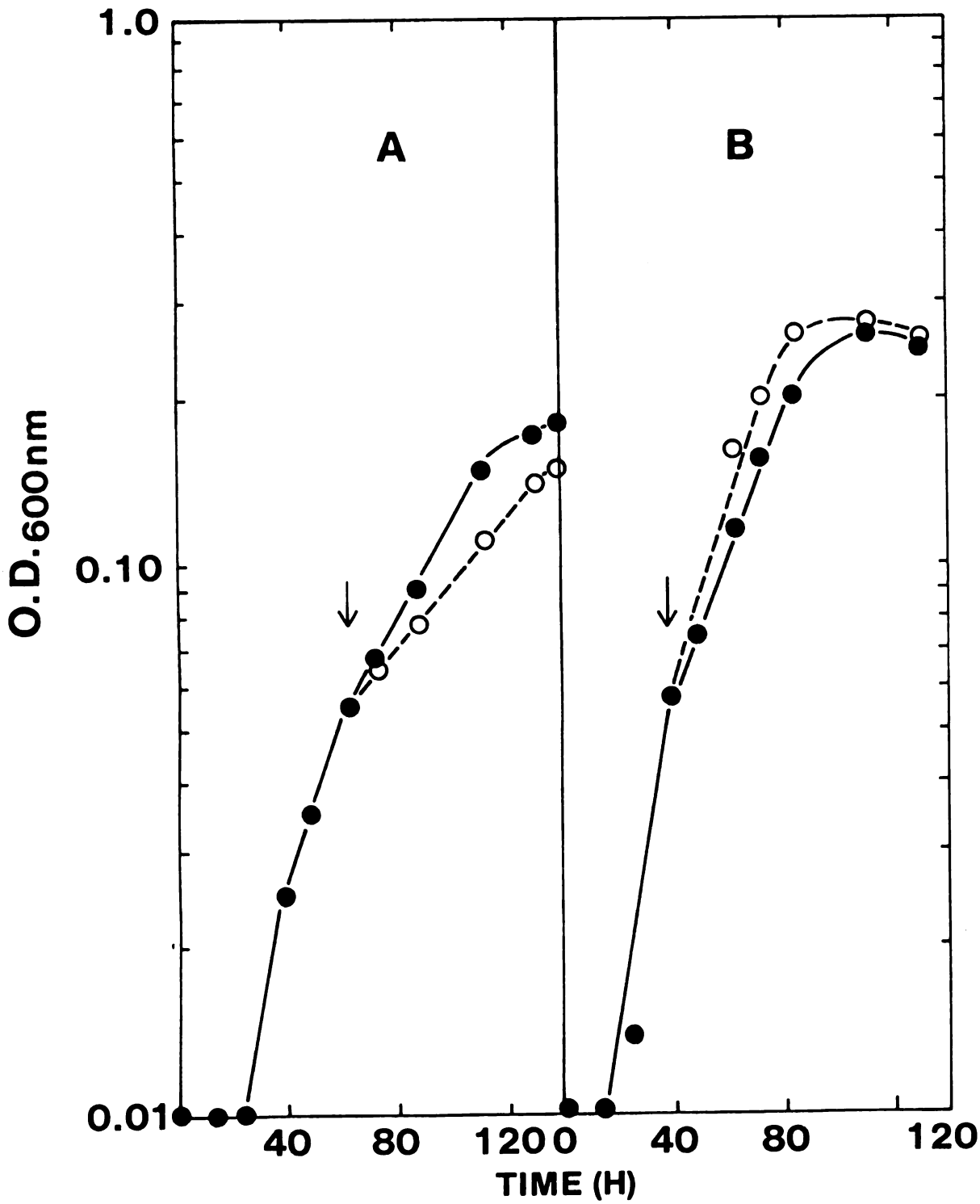


Figure 4.

Table 1. Substrates used for growth by strain APO-1[a].

Used by strain APO-1:

H_2/CO_2, glucose, fructose, mannose, rhamnose, ribose, citrate[b] pyruvate, oxaloacetate, fumarate, propanol[b], mannitol, ethylene glycol[b] and 3,4,5-trimethoxybenzoate[b].

Tested, but not used:

Melibiose, raffinose, maltose, cellobiose, arabinose, galactose, lactose, xylose, sucrose, trehalose, starch, L-fucose, formate, lactate, malate, D-gluconate, acetate, oxalate, succinate, gallate, syringate, caffeate, 3-hydroxybenzoate, benzoate, pyrogallol, methanol, ethanol, glycerol, adonitol, sorbitol, erythritol, butanol, isobutanol, dulcitol, pectin, xanthine, dextrin, betaine, salicin, esculin, N,N dimethylglycine and Casamino acids.

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[a]Compounds were supplied at a final concentration 5 to 10 mM [except for Casaminoacids (5 g/l final concentration) and H_2/CO_2 (80/20)].

[b]Poor growth on this substrate.

Fermentation balances

The stoichiometry of H_2/CO_2 utilization by strain APO-1 was consistent with that of other H_2/CO_2 acetogenic bacteria: 4 H_2 + 2 CO_2 ---> CH_3COOH + 2 H_2O (Table 2). Moreover, when H_2/CO_2 grown cell suspensions were incubated under H_2 with $^{14}CO_2$ as substrate, cells formed 85.6 nmol ^{14}C-acetate per h per mg cell protein and the ^{14}C-acetate was labeled in both carbons (54.3% CH_3-group and 61.2% COOH-group). These results confirmed that strain APO-1 could effect a total synthesis of acetate from H_2 + CO_2. However, strain APO-1 usually formed other major products in addition to acetate during heterotrophic growth on organic substrates. For example, fermentation of glucose yielded primarily butyrate, CO_2 and H_2 (Table 2) and was somewhat similar to the butyric fermentation carried out by *Eubacterium limosum* and certain clostridia (Kluyver and Schnellen, 1937; Gottschalk, 1986). The major end products of rhamnose fermentaion were 1,2-propanediol, acetate, butyrate and succinate. The reason for the relatively poor carbon recovery during analysis of the rhamnose fermentation (75.9%) is not yet known, however a similar anomoly sometimes accompanies rhamnose fermentation by clostridia (Ghazvinizadeh et al., 1972). On the other hand, fumarate fermentation by strain APO-1 was similar to that of *Sporomusa malonica* (Dehning et al., 1989) and was consistent with the theoretical equation 3 fumarate ---> 2 propionate + acetate + $4CO_2$.

Strain APO-1 was apparently also able to oxidize propanol to propionate and use the reducing equivalents liberated to reduce CO_2 to acetate. This reaction was first demonstrated with *Acetobacterium carbinolicum* (Eichler and Schink, 1984). However, growth of strain APO-1 was poor on propanol, so the fermentation was not investigated in detail.

Table 2. Fermentation products formed during growth of strain APO-1 with various substrates.

Substrate	Product (mmol/100mmol substrate fermented)							
	H_2	CO_2	Acetate	Propionate	Butyrate	Succinate	1,2-propanediol	%C recovery
H_2 (+ 50 mmol CO_2)[a]	-	n.d.[b]	22.7	1.3	0.4	0.0	0.0	101.2
Glucose	88.9	137.0	17.3	10.2	98.4	0.0	0.0	99.3
Rhamnose	16.3	27.5	31.4	0.0	18.4	10.4	83.2	75.9
Fumarate	0.0	131.5	17.0	69.8	0.0	0.0	0.0	93.7

[a]Assumed for calculation of material balance.

[b]n.d., not determined.

Analysis of 16S ribosomal RNA sequences

The nearly complete sequences of 16S rRNAs from strain APO-1, *Sporomusa termitida* strain JSN-2 (Breznak et al., 1988), and *Clostridium mayombeii* strain SFC-5 (Chapter 4) were compared with those of *Sporomusa paucivorans* (Olivier et al., 1985), *Megasphaera elsdenii* (Rogosa, 1971) (latter two sequences provided by C. R. Woese, U. Illinois) and the published sequence of *Bacillus subtilis* (Green et al., 1985) (Table 3). Of those organisms examined, the closest relatives to strain APO-1 were *S. paucivorans* and *S. termitida*. However, the evolutionary distance

between the two species of *Sporomusa* (0.051) was significantly less than the evolutionary distance between strain APO-1 and *S. paucivorans* (0.114) or *S. termitida* (0.133).

Table 3. Evolutionary distance between strain APO-1 and other selected eubacteria.

Organism	Evolutionary distance[a] from:				
	S. paucivorans	*S. termitida*	*M. elsdenii*	*B. subtilis*	*C. mayombeii*
Strain APO-1	0.114	0.133	0.152	0.166	0.214
S. paucivorans		0.051	0.143	0.162	0.178
S. termitida			0.145	0.185	0.209
M. elsdenii				0.192	0.216
B. subtilis					0.178

[a]0.1 evolutionary distance unit is equivalent to 0.1-nucleotide changes per position.

The phylogenetic tree resulting from these comparisons is presented in Appendix I. The analysis used to construct the tree in Appendix I included several other unpublished sequences kindly provided

by C.R. Woese. Strain APO-1 is related to a broadly defined, but phylogenetically coherent group that includes *S. termitida*, *S. paucivorans* and *Megashpaera elsdenii*. Analysis of 16S rRNA oligonucleotide catalogues has shown that bacteria in this group (which also includes *Sporomusa ovata*, *Sporomusa sphaeroides* and *Selenomonas ruminantium*) are not related to any Gram negative eubacteria, but show a distinct (although remote) relationship to bacteria in the "*Clostridium*" subdivison of Gram positive eubacteria (Stackebrandt et al., 1985).

Other characteristics

Cells were catalase positive, but oxidase negative. Cytochromes were not detected in crude cell extracts. The DNA base composition of strain APO-1 was 51.5% G + C.

Discussion

Taxonomy of strain APO-1

The ability to obtain energy for growth by formation of acetate from H_2 + CO_2 is found among several bacterial genera including: *Acetitomaculum*, *Acetoanaerobium*, *Acetobacterium*, *Clostridium*, *Desulfotomaculum*, *Eubacterium*, *Peptostreptococcus* and *Sporomusa* (Ljungdahl, 1986). Therefore, this distinctive physiological property is not highly determinative from a taxonomic standpoint. By contrast, possession of a truly Gram negative cell wall coinciding with the ability to form endospores is rare in a single bacterial taxon. Aside from the thermophilic halophile *Sporohalobacter* (Oren et al., 1987), the only bacteria currently known to exhibit both of these properties are

H_2/CO_2 acetogens of the genus *Sporomusa*. However, morphological and physiological differences between strain APO-1 and members of the genus *Sporomusa* are significant (Table 4).

Cells of *Sporomusa* are all curved rods of moderate diameter and no longer than 8μm. All, except *S. paucivorans*, form endospores which can be either subterminal or terminal in each species. By contrast, cells of strain APO-1 are thin, straight rods which are somewhat flexible when very long (20-60 μm). Endospores of strain APO-1 cells are always terminally located. Wet mounts of *Sporomusa* cells exhibit obvious tumbling motility due to the presence of lateral flagella. However, cells of strain APO-1 were only noticably motile when placed on the surface of agar covered slides.

Formate and methanol are metabolized by all six *Sporomusa* species, the latter compound supporting especially good growth, however neither of these substrates was utilized by strain APO-1 (Table 4). In addition, all sporomusas tested do not use sugars other than fructose (except *S. acidovorans*, which also uses ribose), but strain APO-1 used hexoses (fructose, glucose, mannose), ribose, and the methyl pentose rhamnose. Moreover, when utilizing sugars and certain organic acids, strain APO-1 formed butyrate as a major fermentation product, whereas the fermentations of sporomusas are almost always homoacetogenic and never result in the formation of significant amounts of butyrate.

The G + C content in the DNA of strain APO-1 was about 3 mol percent higher than that of the highest *Sporomusa*. Furthermore, the evolutionary distance between strain APO-1 and either *S. paucivorans* or *S. termitida* (as determined by comparative rRNA sequencing) was more

Table 4. Characteristics useful for distinguishing strain APO-1 from members of the genus *Sporomusa*.

Property	Strain APO-1	All *Sporomusa* species[a]
Cell shape:	thin, straight rods	curved rods
Length (μm):	6-60	2-8
Width (μm):	0.3-0.4	0.4-0.9
Cell wall composition:	Gram negative	Gram negative
Endospore location:	terminal	subterminal-terminal[b]
Growth on:		
$H_2 + CO_2$	+	+
Glucose	+	-
Mannose	+	-
Formate	-	+
Methanol	-	+
Butanol	-	+[c]
Betaine	-	+[c]
Mol % G + C in DNA:	51.5	41.3-48.6

[a]Inclusive *Sporomusa* species described to date: *S. sphaeroides* and *S. ovata* (Moller et al., 1984); *S. acidovorans* (Ollivier et al., 1985); *S. paucivorans* (Hermann et al., 1987); *S. termitida* (Breznak et al., 1988) and *S. malonica* (Dehning et al., 1989). [b]*S. paucivorans* does not form endospores. [c]Not determined for *S. acidovorans*.

than twice the evolutionary distance between *S. paucivorans* and *S. termitida* (Table 3). Although strain APO-1 is, in fact, somewhat related to these two *Sporomusa* species as determined by 16S rRNA sequence comparison, there are as yet no specific rules by which evolutionary distance measurements can be used to delimit different genera or species. Therefore, in light of the physiological and distinctive morphological differences between strain APO-1 and members of the genus *Sporomusa*, and taking into account the evolutionary distances inferred by comparative 16S rRNA sequencing, it is herewith proposed that a new genus, *Acetonema*, be established, with strain APO-1 as the type strain of the new species *Acetonema elongata*.

Ecological considerations

Although H_2/CO_2 acetogenesis outcompetes methanogenesis for H_2 produced during the hindgut microbial fermentations of wood-feeding termites, most termites emit some CH_4 (Chapter 1). Furthermore, enrichments for H_2/CO_2-utilizing bacteria from the guts of wood-feeding termites often result in methanogenic bacteria overgrowing acetogens, unless BES is added to the medium (Kane and Breznak, unpublished results). However, an interesting feature of *P. occidentis*, the termite used for this study, was that H_2/CO_2 acetogenesis occurred in the gut of this termite species to the virtual exclusion of methanogenesis (Chapter 1). Moreover, no F_{420}-fluorescent cells were observed in *P. occidentis* gut contents or in H_2/CO_2 enrichments from gut contents, and no CH_4 was produced in such enrichments (even in medium without BES). However, cells similar in morphology to *A. elongata* were

observed in gut contents of *P. occidentis* and appeared to dominate the H_2/CO_2-utilizing enrichments.

It seems likely that *A. elongata* contributes to the nutrition of *P. occidentis* termites by forming volatile fatty acids (eg. acetate, propionate and butyrate) which are important carbon and energy sources for the insect (Breznak and Odelson, 1983; Obrien and Breznak, 1984). In *R. flavipes* termites, acetate derived from H_2/CO_2 acetogenesis by gut bacteria could support up to 1/3 of the energy requirements of the insect (Breznak and Switzer, 1986). Rates of H_2/CO_2 acetogenesis by *P. occidentis* gut homogenates (with or without exogenously supplied H_2) were, in fact, more than twice those of *R. flavipes* gut homogenates (Chapter 1). However, culture methods may be ineffective in enumerating H_2/CO_2 acetogenic bacteria such as *S. termitida* or *A. elongata* in termite gut contents (Breznak et al., 1988). Consequently, we are investigating the possibility of using 16S rRNA-directed hybridization probes (Stahl et al., 1988), or analysis of membrane phospholipid fatty acid "signitures" (Guckert and White, 1986) as means of assessing populations of Gram negative endospore-forming H_2/CO_2 acetogens *in vivo*.

Description of Acetonema elongata, *gen. nov. sp. nov.*

Acetonema gen. nov. (A.ce.to.ne'ma. L.n. *acetum* vinegar; M.L.n. *nema* thread; M.L.neut.n. *Acetonema* vinegar-forming thread)

Thin, straight rods with rounded ends. Motile, multi-flagellate, but specific location of flagella insertion remains to be confirmed. Gram negative by staining and by cell wall ultrastructure. Lipopolysaccharide is present. Heat resistant endospores formed. Catalase positive, oxidase negative.

Strict anaerobes. Chemoorganotrophs. Ferment H_2 +CO_2 to acetate. Sugars and organic acids are preferred substrates, from which butyrate is usually also a major product.

Do not respire anaerobically with nitrate or sulfate. Cytochromes not detected. Mesophiles. DNA base composition: 51.5 mol% G + C (for the type species) Type species: *Acetonema elongata*. *Acetonema elongata* sp. nov. (e.lon'ga.ta. L.fem.part.adj. *elongata*

elongated, stretched out). Straight rods with round ends, 0.3-0.4 x 6-60 μm. Cells single or in

short chains of 3 or 4. Long cells can be highly flexible. Translational motility only observed for cells placed on agar-covered slides, not in wet mounts; more than one flagellum present. Mature endospores are 1 μm in diameter, spherical and terminal in location. Endospores resist heating to 80^{o} C for 10 min. Colonies of cells grown on H_2 + CO_2 are 1-2 mm in diameter, circular with uneven edges and opaque with a slight brown color.

Strict anaerobe. Oxidase negative, catalase positive. Can obtain energy for growth by acetogenesis from H_2 + CO_2. Also ferments mannose, glucose, fructose, rhamnose, ribose, mannitol, pyruvate and oxaloacetate to butyrate and acetate, but ferments citrate, fumarate and propanol to acetate and propionate. 1,2-propanediol is also a quantitatively significant product from fermentation of rhamnose. Acetate was the sole product detected from ethylene glycol and trimethoxybenzoate. H_2 and CO_2 may be produced during growth on organic compounds.

pH optimum, 7.8 (range 6.4 to 8.6); temperature optimum, 33° C (range 19 to 40° C). Trypticase or yeast extract required for good growth. DNA base composition: 51.5 mol% G + C (strain APO-1; buoyant density).

Source: Gut contents of the termite *Pterotermes occidentis*.

Type strain: Strain APO-1.

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CHAPTER 3

H_2/CO_2 ACETOGENIC BACTERIA FROM TERMITE GUTS: II. *Clostridium mayombei*, SP. NOV., ISOLATED FROM GUTS OF THE AFRICAN SOIL-FEEDING TERMITE, *Cubitermes speciousus*

Abstract

Strain SFC-5, a previously undescribed H_2/CO_2 acetogenic bacterium was isolated from gut contents of the African, soil-feeding termite *Cubitermes speciosus*. Cells of this strain were anaerobic, Gram positive, catalase and oxidase negative, endospore-forming, motile rods which measured 1 x 2-6 μm. 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 $4H_2 + 2CO_2 \text{ ---> } CH_3COOH + 2H_2O$. Growth also occurred between 15° and 45° C and pH 5.5 to 9.3, and other substrates supporting good growth included certain carbohydrates (eg. glucose, xylose, starch), organic and amino acids and alcohols. The major fermentation product was almost always acetate alone. Based on comparative analysis of 16S rRNA nucleotide sequences, strain SFC-5 was closely related to various members of the genus *Clostridium*. However, the morphological and physiological differences between strain SFC-5 and other homoacetogenic clostridia were significant. Consequently, it is proposed that strain SFC-5 constitute the type strain of a new species, *Clostridium mayombei*. The properties of *C. mayombei* are compared to those of *Sporomusa termitida* and *Acetonema elongata* (H_2/CO_2 acetogens previously isolated from the guts of wood-feeding termites) as part of a continuing study to determine why acetogenic bacteria compete effectively for H_2 in the guts of wood-feeding, but not soil-feeding, termites.

Introduction

The ability of H_2/CO_2 acetogenic bacteria outcompete H_2/CO_2 methanogenic bacteria for H_2 is a widespread phenomenon in the hindgut fermentations of wood- and grass-feeding termites (Chapter 1). This process is important to termite nutrition, inasmuch as up to 1/3 of the insects' respiratory requirement can be met by the oxidation of acetate derived from bacterial H_2/CO_2 acetogenesis in the hindgut (Breznak and Switzer, 1986). By contrast, this phenomenon does not appear to be as important to termites which feed on the humic component of soil. Rates of microbial H_2/CO_2 acetogenesis in the guts of soil-feeding termites were considerably less, and rates of CH_4 emission were substantially higher, than those of their wood- and grass-feeding counterparts (Chapter 1).

To learn more about termite gut acetogens in general, and to begin to evaluate factors which might affect their competetiveness for H_2 *in situ*, H_2/CO_2 acetogenic bacteria were sought from the guts of wood-feeding termites. Two Gram negative, endospore-forming isolates, *Sporomusa termitida* (from *Nasutitermes nigriceps*) and *Acetonema elongata* (from *Pterotermes occidentis*), were obtained and studied in detail (Breznak et al, 1988; Chapter 2). Although the competetiveness of *S. termitida* for H_2 could not be attributed to an unusually high affinity or low threshold for H_2 (Breznak *et al.*, 1988; Cord-Ruwisch *et al.*, 1988), certain other aspects of their physiology which may bear on their success in the termite gut (eg. the ability to grow mixotrophically), are the subject of a continuing investigation (Breznak and Switzer, 1989). However, it also seemed useful to obtain, for comparative purposes, H_2/CO_2 acetogens from the guts of termites in which this

activity was not a dominant electron sink reaction. Accordingly, we attempted to isolate such bacteria from the hindguts of soil-feeding termites.

In the present study, 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. elongata*, strain SFC-5 was Gram positive, and exhibited other properties characteristic of members of the genus *Clostridium*. However, strain SFC-5 did not correspond closely to any H_2/CO_2 acetogenic *Clostridium* that has been described previously, so it is propsed that strain SFC-5 constitute a new species, *Clostridium mayombei*.

[A preliminary report of these findings has been presented (M. D. Kane and J. A. Breznak, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, I100, p.234)].

Materials and Methods

Termites

C. speciosus (Termitidae) specimens were 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 anaerobic salts solution consisting of (g/l) KH_2PO_4 (0.2), NH_4Cl (0.3), KCl (0.5), NaCl (1.0), $MgCl_2.6H_2O$ (0.4), $NaHCO_3$ (2.5) and $CaCl_2.2H_2O$ (0.15). Dissected guts were transported to Michigan over a period of ten days, where they were used within 48h of receipt.

Isolation of strain SFC-5

Isolation of strain SFC-5 was done by using media with (AC-K1) and without (AC-K2) the addition of 2-bromoethane sulfonate (BES) as described for the isolation of *A. elongata* (Chapter 2).

Growth studies

CO_2/bicarbonate-buffered medium (AC-K4) was used for growth studies. It was identical to AC-K1 medium (Chapter 2), except that the amount of trypticase was reduced to 1.0 g/l and rumen fluid was omitted. Other aspects of growth studies have been described previously (Chapter 2)

Nutrition studies

Nutrition studies were performed by using AC-K5 medium, which was identical to AC-K4 medium, except that amounts of trypticase and yeast extract were each increased to 2.0 and 1.0 g/l, respectively. Nutrition studies were performed as described in Chapter 2.

Fermentation studies

A fermentation balance of H_2/CO_2 grown cells was done by using AC-K5 medium and an H_2/CO_2 (80/20, vol/vol) atmosphere. The basal growth medium for material balances of glucose, xylose and sodium succinate fermentations was AC-K5 modified by omitting $NaHCO_3$, and including 3-(N-morpholino) propanesulfonic acid buffer (sterilized separately, adjusted to pH 7.4) at a final concentration of 10 mM. Incubation was under 100% N_2, and material balance calculations were corrected for the amount of

products formed from cells grown in basal medium containing no additional substrate.

Measurements of H_2-dependent reduction of $^{14}CO_2$ to ^{14}C-acetate by cell suspensions, and determination of the distribution of ^{14}C in acetate by Schmidt degradation, were performed as described previously (Breznak and Switzer, 1986 Breznak et al., 1988).

Chemical assays and other procedures

All chemical assays and other analyses (including comparative 16S rRNA sequence analysis) were performed as described in Chapter 2.

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, H_2/CO_2 enrichments in AC-K2 medium (without BES) exhibited production of CH_4 and contained F_{420}-fluorescent cells. From the former enrichments H_2/CO_2 acetogenic bacteria were isolated by using agar roll tubes. Seven strains of H_2/CO_2 acetogenic bacteria (strains SFC-1 through SFC-7) were isolated, all of which exhibited similar morphology and Gram stain reaction. Strain SFC-5, which grew slightly faster than the other strains, was chosen for further characterization.

Morphology

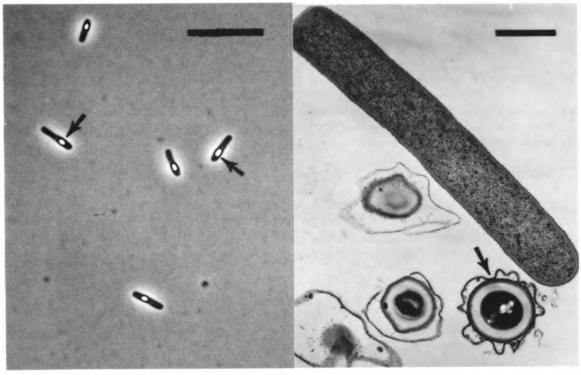
Subsurface colonies of H_2/CO_2 grown cells were white to slightly yellow, oval-shaped with smooth edges and about 2 mm in diameter. Cells of strain SFC-5 were straight, motile rods measuring 1.0 x 2-6 μm (Figs. 1a & 1b). In old cultures ($\geq$ 7d), longer (10-15 μm), slightly curved cells were also observed. Cells stained Gram positive, and electron micrographs of thin sections revealed a typical Gram positive cell wall morphology (Fig. 1b). In addition, cells formed central to subterminal, oval endospores which sometimes casused cells to swell slightly (Fig. 1a). Viable cells could be recovered from sporulated cultures held at 80^{o} C for 10 min. Moreover, dipicolinic acid (DPA) could be extracted from sporulated cultures and exhibited a UV spectrum similar to that of authentic DPA.

Growth and nutritional studies

Cells of strain SFC-5 were capable of growth only in O_2-free medium to which a reductant [eg.dithiothreitol (1.0 mM, final concentration)] had been added. Growth required the addition 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 to the medium at ratio of 2:1 (trypticase:yeast extract). Addition of rumen fluid (5%, vol./vol.) to the growth medium had no effect on growth. Optimum growth in AC-K4 medium occurred at 33^{o} C and pH 7.3. Cells also grew within a temperature range of 15 to 45^{o} C and a pH range of 5.5 to 9.3. No growth occured at 15 or 45^{o} C, or at a pH of 5.2 or 9.6.

Figure 1a, b. Morphology of strain SFC-5. a. Phase contrast micrograph; bar = 10.0 μm. b. Transmission electron micrograph of a thin section; bar = 1.0 μm. Note oval endospores (*arrows* a, b).

A B



When cells were grown at 30° C in AC-K5 medium with H_2/CO_2 as substrate, they exhibited a doubling time of 5h; achieved a final $O.D._{600\ n.m.}$ of 0.47-0.50 (approx. 0.160-180 μg dry mass/ml); and produced 18 to 23 mM acetate (without replentishment 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 cells also grew on a variety of other substrates, including sugars, organic and amino acids, and alcohols (Table 1). The major product formed from most substrates was acetate, although a trace amount of isovalerate was also formed. The only exceptions were that cells converted succinate to propionate + CO_2, and valine to isobutyrate. Molar growth yields for selected substrates are given in Table 2. Neither sulfate or nitrate were reduced by cells grown with glucose as substrate.

Fermentation balances

Cells of strain SFC-5 used H_2/CO_2 as a substrate for growth and acetogenesis according to the equation: $4H_2 + 2CO_2 \text{ ---> } CH_3COOH + 2H_2O$ (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 $H_2 + {}^{14}CO_2$ as substrates. Under these conditions, cells formed 1.70 μm ^{14}C-acetate per h per mg protein. Degradation of ^{14}C-acetate by the Schmidt reaction demonstrated that 40% of the ^{14}C was associated with the COOH-group and 43% with the CH_3-group.

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

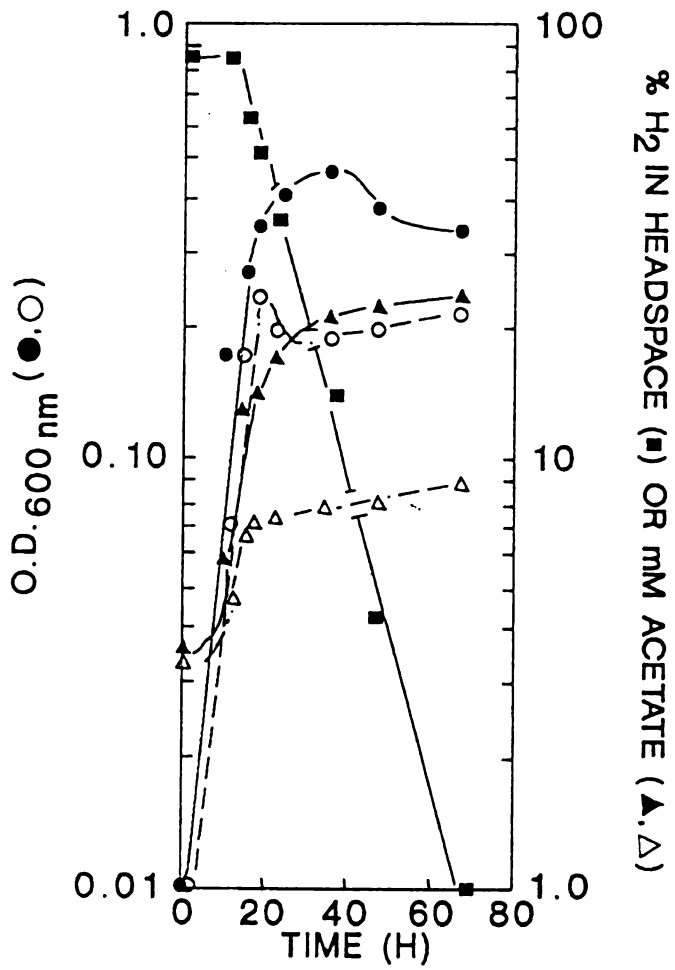

1.0
0.10
0.01
100
10
1.0
0
20
40
60
80
TIME (H)
O.D.600 nm (●,○)
% H2 IN HEADSPACE (■) OR mM ACETATE (▲,△)

Figure 2.

Table 1. Sustrates used for growth by strain SFC-5. Compounds were supplied at a final concentration of 5 to 10 mM, except for H_2/CO_2 (80/20, vol./vol.).

Used for growth by strain SFC-5:

H_2/CO_2, fructose, glucose, maltose, xylose, starch, cellobiose[a], sorbitol, dulcitol, glycerol, formate, pyruvate, malate, succinate, syringate[a], alanine, glutamate, serine, valine, salicin, dextrin and esculin.

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.

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[a]Poor growth on this substrate.

Table 2. Molar growth yields and fermentation balances for strain SFC-5 grown with various substrates.

Substrate	Growth yield (g dry cell matter /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	-	n.d.[b]	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.

[b]n.d., not determined.

Similar to most H_2/CO_2 acetogenic clostridia, strain SFC-5 produced acetate as the sole major product when fermenting organic compounds such as glucose or xylose (Table 2). In addition, cells fermented succinate according to the equation: $HOOCCH_2CH_2COOH \rightarrow CH_3CH_2COOH + CO_2$. This mildly exergonic reaction, first demonstrated for the bacterium *Propionogenium modestum* (Schink and Pfennig, 1982), has also been demonstrated for two H_2/CO_2 acetogens belonging to the genus *Sporomusa* (Breznak et al., 1988; Dehning et al., 1989).

16S ribosomal RNA sequence analysis

Comparison of the nearly complete sequence of the 16s rRNA from strain SFC-5 with those of *S. termitida*, *A. elongata* and certain other bacteria has been described in a companion paper (Chapter 2). The phylogenetic tree resulting from such comparisons is given in Appendix I. The analysis used to construct this tree included the unpublished 16s rRNA sequences from a variety of other clostridia, which were kindly provided by C. R. Woese, U. Illinois. Strain SFC-5 showed a distinct and close relationship with members of the genus *Clostridium*. The relationship between strain SFC-5 and its closest relative (*Clostridium lituseburense*) was 0.053 evolutionary distance units (= 0.05 changes per nucleotide position). The evolutionary distance between strain SFC-5 and six other clostridia was $\geq$ 0.117, and the evolutionary distance between strain SFC-5 and eubacteria from genera other than *Clostridium* was $\geq$ 0.193.

Other characteristics

Strain SFC-5 was catalase and oxidase negative. Cytochromes were not detected in crude cell extracts.

Discussion

Taxonomy

Inasmuch as cells of strain SFC-5 were strictly anaerobic, Gram positive, endospore-forming rods that did not carry out dissimilatory sulfate reduction, it seemed clear that this new isolate should be classified in the genus *Clostridium*. This conclusion was supported by

analysis of the 16S rRNA sequence of strain SFC-5, which revealed a close evolutionary relationship with various clostridia, especially *C. lituseburense* (Appendix I). However, the latter species is not homoacetogenic (with glucose as substrate), and uses mannose, but not xylose as a substrate for growth (Cato *et al.*, 1986). 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 isolate of this genus carry out homoacetogenic fermentations of carbohydrates, and strain SFC-5 differs from these bacteria in several respects. For example, *Clostridium thermaceticum* (Fontaine et al., 1941) and *C. thermautotrophicum* (Wiegel et al., 1981) grow optimally at 55-60^{o} C, whereas strain SFC-5 did not grow above 45^{o} C. Moreover, unlike strain SFC-5, these two thermophiles use lactate and galactose, but not maltose as growth substrates. Cells of the other four homoacetogenic clostridia are clearly distinguishable from those of strain SFC-5 with respect to 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) form round, terminal endospores which markedly swell the sporangium, whereas those of strain SFC-5 are oval, usually subterminal, and swell the cells only slightly or not at all. Although the endospores of *C. magnum* (Schink, 1984) are oval and often subterminal, the cells themselves have a greater width to length ratio than those of strain SFC-5, especially when the former produces endospores. Moreover, neither *C. magnum* or *C. formicaceticum* use H_2/CO_2 as a substrate for growth and acetogenesis. Other differences between strain SFC-5 and the four previously described

mesophilic clostridia are listed in Table 3. Due to the morphological and physiological differences described above, it is proposed that strain SFC-5 constitute a new species of *Clostridium*, named *Clostridium mayombei*, a concise description of which is given below.

Ecological considerations

C. mayombei and the two other H_2/CO_2 acetogenic bacteria isolated from termite guts clearly do not constitute a single taxon. Although *S. termitida*, *A. elongata* and *C. mayombei* are all motile and form endospores, examination of other properties exhibited by these isolates indicates that in fact, termite gut acetogens appear to be quite diverse (Table 4). From an ecological standpoint, the increased availability of H_2/CO_2 acetogenic bacteria from the guts of various termites provides an opportunity to begin to systematically examine factors which bear on the ability of such diverse bacteria to compete effectively for H_2 in the guts of wood-feeding, but not soil-feeding termites. Experiments with *S. termitida* indicated that this isolate does not have a higher affinity or lower threshold for H_2 than that of various H_2-utilizing methanogens (Breznak *et al.*, 1988). Nevertheless, other factors (such as the ability to grow mixotrophically) may be important to the success of H_2-utilizing acetogens in the guts of wood-feeding termites. The observation that *S. termitida* and *A. elongata* (both Gram negative bacteria isolated from the guts of wood-feeding termites) are relatively closely related to one another, but not to *C. mayombei* (Chapter 2), suggests that H_2-utilizing, Gram negative acetogens in the guts of wood-feeding termites may have some properties (not possessed by Gram positive acetogens such as *C. mayombei*) that

Table 3. Substrates useful for distinguishing strain SFC-5 from other mesophilic, homoacetogenic clostridia.[a,b]

Growth on:	Isolate				
	Strain SFC-5	*C. aceticum*	*C. formicoaceticum*	*C. magnum*	Strain CV-AA1
H_2 +CO_2	+	+	-	-	+
Ethanol	-	+	n.d.	-	n.d.
Glycerol	+	n.d.	n.d.	-	n.d.
Methanol	-	-	n.d.	-	+
Formate	+	+	+	-	n.d.
Fumarate	-	+	n.d.	-	n.d.
Succinate	+	-	n.d.	-	n.d.
Glucose	+	-	-	+	+
Maltose	+	-	n.d.	-	n.d.
Ribose	-	+	n.d.	-	n.d.
Sucrose	-	-	-	+	n.d.
Xylose	+	-	n.d.	+	n.d.

[a] +, growth; -, no growth; n.d., not determined.

[b] Data compiled from Andreesen et al., 1970; Adamse 1980; Braun et al., 1981; Adamse and Velzeboer, 1982 and this study.

Table 4. Characteristics useful for distinguishing H_2/CO_2 acetogenic bacteria isolated from termite guts.

Property	Isolate		
	S. termitida	*A. elongata*	*C. mayombei*
Cell dimensions (μm)	0.5-0.8 x 2-8	0.3 x 6-60	1 x 2-6
Cell wall type	Gram -	Gram -	Gram +
Endospore location	terminal/subterminal	terminal	subterminal
pH optimum/range	7.2/6.2-8.1	7.8/6.4-8.	7.3/5.5-9.3
Temp. optimum/range (^{o}C)	30/19-37	33/19-40	33/15-45
Catalase/oxidase	+/-	+/-	-/-
Growth on:			
$H_2 + CO_2$	+	+	+
Formate	+	-	+
Methanol	+	-	-
Lactate	+	-	-
Ethanol	+	-	-
Mannose	-	+	-
Ribose	-	+	-
Propanol	-	+	-
Maltose	-	-	+
Xylose	-	-	+
Starch	-	-	+
Glycerol	-	-	+

confer on them a competitive advantage over H_2/CO_2 methanogens *in vivo*. H_2 competition experiments between *C. mayombei*, *S. termitida* and *A. elongata* may also help clarify these issues.

It is intriguing that a close relative of *C. mayombei* is *C. lituseburense* (Appendix 1), a non-homoacetogenic bacterium which was isolated from West African soil (Cato, *et al.*, 1986). Possibly, some bacteria that occur in the guts of soil-feeding termites could more accurately be described as soil bacteria whose residence in the gut is only temporary. This may well be the case for *C. mayombei*, which is obviously not an effective competitor for H_2 in the guts of *C. speciosus* termites. On the other hand, H_2-independent fermentative activities of *C. mayombei* may contribute to the nutrition of *C.speciosus* termites, even if the bacterium's presence in the gut is of a transient nature. In any case, the isolation and characterization of *C. mayombei* increases our understanding of the diversity of H_2/CO_2 acetogenic bacteria as well as the intestinal microecology of one of the Earth's most abundant groups of termites. It also provides an important new organism for further studies on the ecological significance of H_2 consumption by acetogens and their competitiveness in natural habitats.

Description of Clostridium mayombei *sp. nov.*

Clostridium mayombei sp. nov. may.omb'e.i. M.L. adj. *mayombei*, pertaining to the Mayombe tropical rainforest (People's Republic of Congo), from which this bacterium was isolated by using the gut contents of *Cubitermes speciosus* termites.

Straight to slightly curved rods 1 x 2-6 μm, with rounded ends.

Cells single or in pairs. Motile, but location and number of flagella are uncertain. Gram positive. Heat resistant endospores 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 slightly yellow in color.

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

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 for good growth.

Source: Gut contents of the termite *Cubitermes speciosus* collected from the Mayombe tropical rainforest, Peoples' Republic of Congo.

Type strain: Strain SFC-5.

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CHAPTER 4

EFFECT OF HOST DIET ON THE PRODUCTION OF ORGANIC ACIDS AND METHANE BY COCKROACH GUT BACTERIA

ABSTRACT

The effect of high fiber diets on microbial processes in cockroach guts was investigated by feeding *Periplaneta americana* cockroaches milled cereal leaves (MCL), milled corn cob (MCC), or commercial bran-type breakfast cereal (KAB) in place of the commonly used laboratory diet of dog chow (PDC). The activities and numbers of specific gut bacteria varied significantly with the insect's diet and life stage. Acetate and lactate were the dominant organic acids present in gut fluid of adult cockroaches and occurred at concentrations up to 17 mM and 8 mM, respectively. These acids were most abundant in the gut fluid of PDC-fed insects and were generally highest in the foregut and midgut regions. The foreguts of PDC-fed cockroaches contained an abundant population of lactic acid bacteria which formed acetate and lactate from endogenous hexoses. When adult cockroaches were fed PDC amended with antibacterial drugs: i) the concentrations of acetate, lactate and total hexoses in gut fluid decreased significantly; ii) the numbers of lactic acid bacteria in the foregut also decreased significantly; and iii) the production of acetate and lactate by foregut homogenates was suppressed. It was estimated that acetate and lactate production by bacteria in the foregut of PDC-fed adult *P. americana* could support up to 14% of the insect's respiratory requirement. When insects were fed high fiber diets of KAB, MCL or MCC, bacterial production of acetate and lactate in the foregut diminished. The main electron sink processes accompanying the gut fermentation of *P. americana* were microbial reduction of CO_2 to CH_4, formate and acetate.

Methanogenesis occurred exclusively in the hindgut region and was favored when insects were fed high fiber diets. Moreover, larvae generally emitted more CH_4 per gram fresh weight than adult cockroaches, particularly when insects were fed the high fiber diets. These results indicate that host diet has a significant effect on the intestinal microecology of *P. americana* .

INTRODUCTION

Presence of the American cockroach, *Periplaneta americana*, in human dwellings causes damage and distress world wide. Nevertheless, because of its ubiquity, relatively large size and nonfastidious nature, *P. americana* has served as an important model for studies of insect physiology, biochemistry and behavior (23). This seemingly omnivorous and opportunistic insect is commonly reared in the laboratory on a diet of dog chow and water, yet the nutritional ecology of *P. americana* is poorly understood (a situation that prevails for most cockroach species).

Previous studies of *P. americana* have demonstrated the presence of bacteria in the foregut and midgut regions (3). However, the most dense and diverse bacterial flora occurs in the hindgut - a region wherein extensive attachment of bacteria to the gut wall is observed (13). Facultative and obligate anaerobic bacteria (including methanogens) have been described from the midgut and hindgut (4,6,13). Although several studies have suggested that the hindgut flora may contribute to the nutrition and development of *P. americana* (2,3,6,7,12), the biochemistry of such interactions has not been examined in detail (13).

Results from our laboratory indicated that microbial reduction of CO_2 to acetate (rather than to CH_4) represented the main electron sink reaction in the hindgut fermentation of wood-feeding termites (Chapter 1). In fact, in *Reticulitermes flavipes* termites acetate derived from CO_2 reduction could subsequently support up to 33% of the termite's respiratory requirement (8,20). By contrast, CO_2 reduction to methane was found to be the main eletron sink reaction in the hindgut

fermentation of *P. americana* (8,13). These and other observations (13) indicated that gut microbial fermentation patterns in *P. americana* were qualitatively and quantitatively different from those in termites. Consequently, this study was initiated in order to examine further the metabolic and nutritional relationships between *P. americana* and their gut bacteria, as well as to increase our limited understanding of the intestinal microecology of insects in general.

This report describes the production of organic acids and CH_4 by *P. americana* gut bacteria; the importance of the foregut as a site of bacterial metabolism; and the effects of high and low fiber diets on gut-associated bacterial processes.

MATERIALS AND METHODS

Animals. A colony of *P. americana* L. (Blattidae) was maintained in an insect rearing cage (no. 1450CS; Bio Quip Products, Santa Monica, CA) at 25° C. Insects were fed dog chow (Ralston Purina, St. Louis, MO) and water *ad libitum*.

Feeding experiments. To evaluate the effect of diet on gut microbe activity, individual cockroaches were held for up to two weeks before assay in wide-mouth 30-ml glass serum vials (no. 223553; Wheaton Scientific, Millville, NJ) containing a wetted cotton swab and an appropriate food pellet. Cotton swabs were remoistened daily. The vials were stoppered with non-absorbant cotton. Individuals were fed ca. 200-mg pellets of dog chow (PDC), ball-milled cereal leaves (MCL; no. C-7141; Sigma Chemical, St. Louis, MO), ball-milled corn cobs (MCC; a gift from R. Hespell, USDA, Peoria, IL) or 200-mg of All-Bran cereal

flakes (KAB; Kellogg, Battle Creek, MI) (Table 1). For treatment with antibacterial drugs, individuals were fed dog chow pellets wetted with 0.5 ml of a solution containing chloramphenicol, penicillin G and tetracycline (400 μg/ml each). Food pellets and cotton swabs were replaced periodically to discourage mold growth.

Table 1. Protein and fiber content of *P. americana* diets[a].

Diet	% Crude protein (wt./wt.)	% Crude fiber (wt./wt.)
PDC	21.0	4.5
MCL	2.5	15.0
KAB	14.1	31.7
MCC	2.7	33.4

[a] Data were supplied by the manufacturer/supplier, except for MCC (22).

$^{14}CO_2$ fixation assay and analysis of $^{14}CO_2$ fixation products. H_2-dependent fixation of $^{14}CO_2$ by cockroach gut microbiota was assayed as described previously (8) except that cockroaches were dissected in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI). Entire adult cockroach gut tracts were used for the assay. Soluble products of $^{14}CO_2$ fixation were separated by using high-performance liquid chromatography (8).

Gas emission measurements. Emission of CH_4 and H_2 by live cockroaches was measured by incubating individuals in stoppered 30-ml vials at 30° C. Periodically, 0.2 ml samples of headspace gas were removed for analysis of H_2 and CH_4 by gas chromatography (20). The headspace gas thus removed was replaced with an equal volume of air.

In other experiments, guts from adult cockroaches were removed by dissection in an anaerobic glove box and sectioned into foregut, midgut and hindgut regions. Individual gut sections were pooled from five animals and incubated at 30° C in stoppered 5-ml glass serum vials containing 1 ml of a buffered salts solution (BSS) (8) and an initial atmosphere of $95\%N_2/5\%H_2$ (v/v). CH_4 emission by gut segments was measured by sampling the headspace gas at the end of the experiment.

H_2S emission was measured by incubating live adults in stoppered 30-ml vials each containing a 1.0-ml stemmed glass well (no. K-882330-0000; Kontes, Vineland, NJ) suspended about 3 cm above the insect. Wells contained 0.75 ml of a 2% (wt/vol) solution of zinc acetate and a fluted piece of filter paper. Following 5 h incubation, the entire contents of each well was transferred to a vial containing 10 ml H_2O. The sulfide content of the solution was then determined by colorimetric assay (11). The detection limit for H_2S was determined to be 40 nmol x g (fresh weight insect)$^{-1}$ x h^{-1}.

Sampling of cockroach gut fluid. Adult *P. americana* were held at 2° C until they became immobilized (about 5 minutes). The legs were then removed and the alimentary canal was exposed by making a ventral incision extending from the terminal sternum to the prothorax, with care being taken not to tear any portion of the gut wall. Various regions of

the alimentary tract were then sampled by puncturing the gut wall with a 25 gauge syringe needle and immediately aspirating the gut contents into a 20 μl capillary pipet. Samples were then ejected into a small polypropylene centrifuge tube and centrifuged at 11,310 x g for 20 min at 4° C. 5.0 μl portions of supernatant fluid (hereafter referred to as extracellular gut fluid) were used for subsequent analyses (below).

Organic acid production by foregut homogenates. Intact foreguts were dissected out of five adult *P. americana* and pooled in a small glass tissue homogenizer containing 4.5 ml of Ringers solution, pH 7.8 (18). Foreguts were homogenized for 1 min, and 1 ml portions of the homogenate were added to 5-ml glass serum vials. The vials were sealed with butyl rubber stoppers and incubated at 30° C. One vial in each experiment was a heat-inactivated (100° C, 10 min) control. Reactions were terminated at appropriate times by placing the vials on ice. Homogenates were centrifuged at 11,310 x g for 20 min at 4° C, and the supernatant fluids (hereafter referred to as foregut homogenate fluid) were collected for analyses (below).

Quantification and characterization of bacteria. Homogenized foreguts from adult *P. americana* were serially diluted in sterile Ringers solution, pH 7.8, and 0.1-0.5 ml of each dilution were spread on plates of two different media. One medium was selective for lactobacilli (22). The other medium contained (g/100ml): tryptone (Difco Laboratories, Detroit, MI), 1.0, yeast extract (Difco), 0.5, beef extract (Difco), 0.1, sucrose, 10.0, agar, 2.0, and NaN_3 (sterilized

separately as a 1% solution), 0.005. Dilution and plating were done by using an anaerobic glove box.

Spread plates were placed in a Gas Pak jar (Becton-Dickinson) which contained an H_2 + CO_2 generator envelope. The jar was incubated at room temperature (22-25^{o} C) in the anaerobic glove box until colonies developed (3-4 days). After this time, colonies were enumerated and representative ones selected for further characterization were streaked for purification onto fresh plates of the same medium from which they had been picked. Pure culture isolates were inoculated into homologous broth medum for growth studies and for determination of organic acid production (see below). In order to test for gas production, test tubes (18 x 150 mm) containing inoculated liquid media were overlayed sequentially with 1.0 ml 2% agar, 1.0 ml vaspar and 1.0 ml 2% agar. Other characteristics of the bacteria were evaluated by routine microbiological methods(17).

Analysis of organic acids and hexoses. For analysis of volatile fatty acids (VFA's), 5 μl samples of extracellular gut fluid were mixed in small centrifuge tubes each containing 35 μl H_2O, 5 μl of 85% H_3PO_4, and 5 μl of a 10.0 mM isobutyrate solution (internal standard). For analysis of foregut homogenates, sample preparation was the same except that 40.0 μl of sample was used and no H_2O was added. Mixtures were then centrifuged at 11,310 x g at 4^{o} C for 10 min, and the supernatant fluid from each was used for quantification of VFA's by gas-liquid chromatography (8,20).

Lactic acid was assayed colorimetrically by the method of Barker (1) which included a sample clarification step, but which was scaled

down to accommodate small sample sizes. For this procedure, 3 to 5 μl gut fluid or 30 μl foregut homogenate were made up to 150μl with water prior to the clarification step. Lactic acid present in samples of gut fluid was also assayed enzymatically by using L-lactic dehydrogenase (EC 1.1.1.27) as described by Everse (14).

Lactic acid production by bacterial isolates grown in broth cultures was assayed by gas-liquid chromatography (19).

Total hexoses present in foregut homogenates were measured by the anthrone method (25) with glucose as a standard.

Electron Microscopy. Excised cockroach foreguts were ligated at each end with cotton thread, then immersed in a 2.5% (vol/vol) solution of glutaraldehyde for 2.5 h. Samples were subsequently dehydrated by immersion in a graded ethanol series (70%, 80%, 90%, 95%, 99% and 100%). The foreguts were then critical point dried under CO_2 and sliced midway between the two ligated ends using a razor blade. Foregut halves were mounted on studs (with the cut end facing up) and sputter coated with gold for 3 min. Coated samples were viewed by using a JEOL model JSM-35C scanning electron microscope.

RESULTS

Gas metabolism by cockroach gut microbiota. Cockroach whole-gut homogenates were capable of H_2-dependent $^{14}CO_2$ fixation, and formate and acetate accounted for virtually all of the $^{14}CO_2$ fixed (Table 2). The data in Table 2 represent strictly H_2-dependent fixation of $^{14}CO_2$

whereby tabulated values were corrected for $^{14}CO_2$ fixation which occurred (in controls) under an incubation atmosphere of 100% N_2 and which was supported by endogenously-produced H_2 or some other reductant. Consequently, since the endogenous rate of $^{14}CO_2$ fixation was often as much as 40% of that occurring under an atmosphere of 100% H_2, the values in Table 2 are probably minimum rates. PDC-fed cockroaches exhibited the highest rates of H_2-dependent formicogenesis and acetogenesis, but MCC-fed cockroaches had the highest acetate/formate ratio.

Table 2. Effect of diet on H_2-dependent fixation of $^{14}CO_2$ into organic acids by adult *P. americana* whole-gut homogenates.[a]

Diet	nmol $^{14}CO_2$ fixed into product x g (fresh weight insect)$^{-1}$ x h^{-1}			
	Total	Formate	Acetate	Formate + Acetate (% of Total)
PDC	177.0	156.5	11.5	94.9
MCL	63.6	59.1	5.5	101.6
KAB	57.7	51.7	4.3	97.1
MCC	46.9	35.1	9.9	96.6

[a]Tabulated values are corrected for $^{14}CO_2$ fixation under an incubation atmosphere of N_2.

P. americana reared on a diet of dog chow and water emitted 29.6 ± 24.2 nmol CH_4 x g (fresh weight insect)$^{-1}$ x h^{-1} (grand mean; n = 130). Although CH_4 emission rates varied considerably between individuals of similar weight, and from day to day for each individual, immature cockroaches (larvae) almost always produced more CH_4 per gram than did large (adult) cockroaches (Fig. 1). By contrast, cockroaches fed high fiber diets emitted CH_4 at rates 4 to 6 times greater than that of dog chow fed cockroaches and exhibited greater differences in CH_4 emission rates between immature and adult cockroaches. This was particularly true for those cockroaches fed MCC and KAB (Fig. 1).

Excised hindguts from dog chow fed adult cockroaches emitted 31.3 nmol CH_4 x g (fresh weight insect)$^{-1}$ x h^{-1}, a rate which fully accounted for the CH_4 emissions displayed by intact adult insects. Significant amounts of CH_4 could not be detected from excised foregut or midgut regions incubated under similar conditions, indicating that the hindgut microbiota was the origin of gut methanogenic activity.

Adult *P. americana* fed PDC did not evolve significant amounts of H_2 or H_2S.

Organic acids in cockroach gut fluid. Acetate and lactate were the predominant organic acids found in extracellular fluid from all regions of the gut of *P. americana*. Trace amounts of propionate, butyrate and iso-valerate were also detected. Concentrations of acetate and lactate were highest in the guts of dog chow reared cockroaches, and generally highest in the foregut and midgut regions (Table 3). However, concentrations of these acids were markedly reduced in the foregut and midgut, and to a lesser extent in the hindgut, when cockroaches were fed

Figure 1. Effect of diet on methane emission from *P. americana*. For two weeks before sampling, cockroaches were fed either: MCC, *circles*; KAB, *asteriscs*; MCL, *triangles*; or PDC (control), *boxes*.

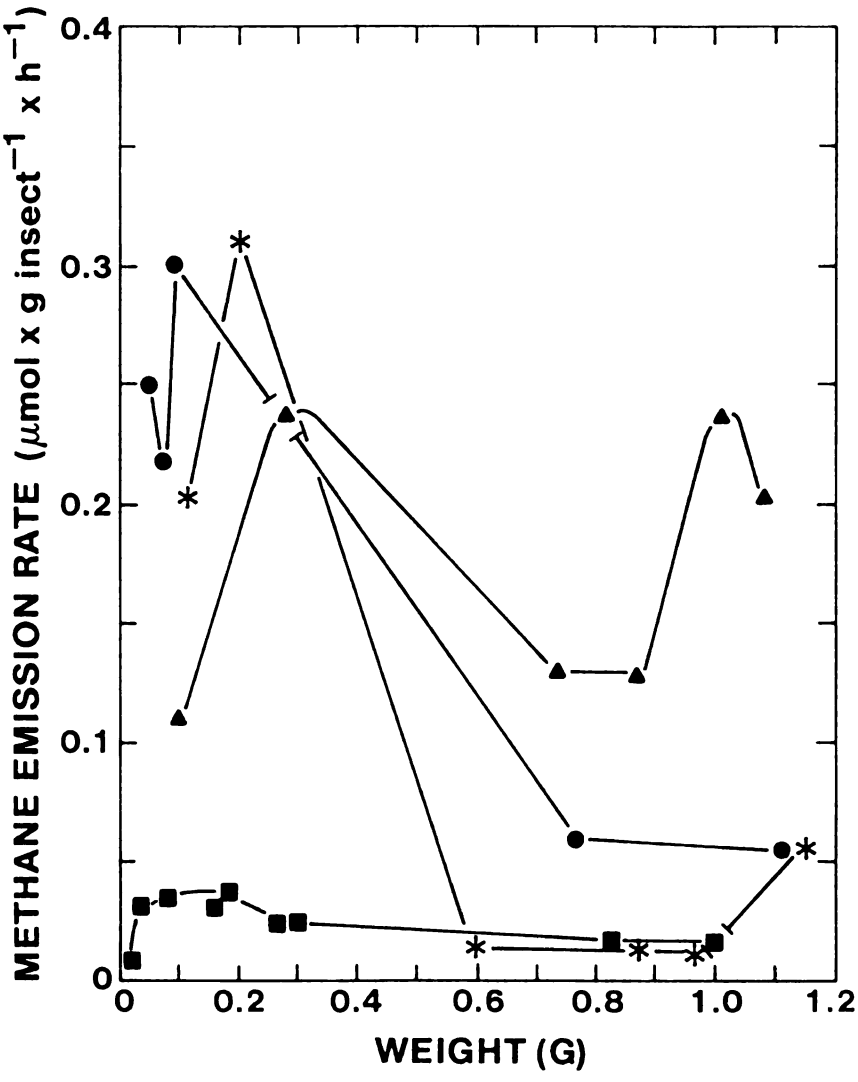

METHANE EMISSION RATE (μmol x g insect^{-1} x h^{-1})
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1.0
1.2
WEIGHT (G)

Figure 1.

Table 3. Acetate and lactate content of gut fluid of adult *P. americana*.[a]

Diet	Acetate (mM)		
	Foregut	Midgut	Hindgut
PDC	17.37 ± 2.13	14.09 ± 1.47	7.22 ± 2.27
PDC + Antibiotics[b]	4.76 ± 6.75	6.90 ± 1.79	5.06 ± 5.02
MCL	1.67 ± 0.11	11.68 ± 5.08	7.15 ± 2.24
KAB	7.48 ± 7.30	6.51 ± 5.04	4.95 ± 1.51
MCC	4.74 ± 4.93	8.93 ± 2.50	4.21 ± 1.94
Starved	12.28 ± 5.03	13.57 ± 5.20	0.94 ± 1.69
	Lactate (mM)		
	Foregut	Midgut	Hindgut
PDC	6.07 ± 3.11	8.21 ± 3.00	4.71 ± 2.52
PDC + Antibiotics[b]	< 3.00	< 3.00	< 3.00
MCL	5.07 ± 3.64	3.51 ± 2.80	< 3.00
KAB	< 3.00	< 3.00	< 3.00
MCC	6.18 ± 5.17	3.72 ± 1.82	< 3.00
Starved	< 3.00	< 3.00	< 3.00

[a]Values are the mean ± standard deviation of 3 or more determinations.

[b]Penicillin G, chloramphenicol and tetracycline (@ 400 μg/g dog chow).

dog chow containing antibacterial drugs (Table 3). This observation suggested that bacteria were primarily responsible for the presence of acetate and lactate in *P. americana* guts.

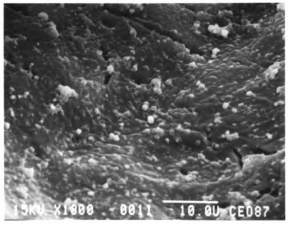
Although acetate and lactate concentrations were generally lower in cockroaches fed diets other than dog chow, such differences were not always dramatic. For example, concentrations of lactate in the foregut, and acetate in the midgut and hindgut, of animals fed cereal leaves were not significantly different than those of dog chow fed animals (Table 3). Interestingly, animals starved for two weeks still maintained relatively high levels of acetate in the foregut and midgut, but not in the hindgut.

SEM of cockroach foreguts. SEM of foreguts from PDC-reared *P. americana* revealed widespread, but generally sparse populations of bacteria attached directly to the foregut wall (Fig. 2A). In contrast, food particles in the foregut were extensively colonized by bacteria (Fig. 2B). Bacteria found on such particles were mainly coccoid in shape and formed a dense lattice of cells on the surface of the particles (Fig. 2C,D).

Production of acetate and lactate by cockroach foregut homogenates. Inasmuch as bacteria were suspected to be responsible for the high concentrations of lactate and acetate in the foregut of PDC-reared cockroaches, it was important to measure the production of these acids by resident foregut microbiota. When foregut homogenates from PDC-reared adult *P. americana* were incubated at 30^{o} C, acetate and lactate were produced and total hexoses (measured as glucose

Figure 2. Scanning electron micrographs of the luminal surface of the foregut of *P. americana* (A) and of bacteria attached to the surface of food particles in the foregut (B,C); D is higher magnification of the enclosed area in B. (Bar in A, B and C = 10 μm; bar in D = 1 μm.)

A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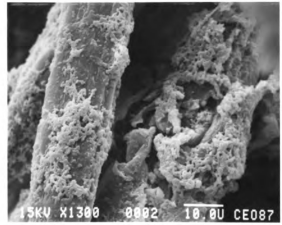
B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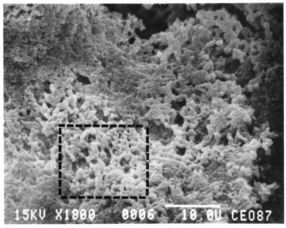
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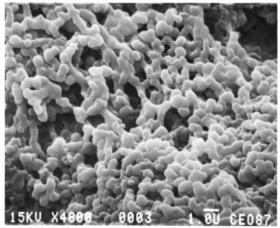
C


15KV X4000 0003 10.0U CE087

D

equivalents) decreased (Fig. 3A). Production of acetate and lactate alone in foregut homogenates from PDC fed animals could, in theory, account for about 40% of the total hexose used. By contrast, foregut homogenates from cockroaches fed PDC containing antibacterial drugs did not produce significant amounts of acetate or lactate and contained lower amounts of total hexoses (Fig. 3B). These results suggested that gut bacteria were responsible for the presence of a nutritionally significant hexose fraction in cockroach foreguts from which they produced acetate and lactate *in situ*.

Foregut homogenates from cockroaches fed MCL also produced acetate and a small amount of lactate, accompanied by a decrease in total hexoses (Fig. 3C). Acetate and lactate production in foregut homogenates from cockroaches fed MCL could theoretically account for about 60% of the total hexose used. Foregut homogenates from cockroaches that were starved, or fed KAB or MCC produced little or no acetate or lactate and contained lower amounts of total hexoses than foregut homogenates from PDC fed insects (Fig. 3, D-F). Zero time values for acetate and lactate in the time course assay were surprisingly similar between cockroaches fed different diets (Fig. 3, A-F). This observation seemed inconsistent with the considerable differences observed for steady state concentrations of these two acids in foregut fluid (Table 3). However, this discrepancy was more apparent than real. Owing to dilution in preparation of the foregut homogenate (see Materials and Methods), the maximum concentrations of acetate and lactate at the beginning of the time course experiment depicted by Fig.

Figure 3. Effect of diet on acetate and lactate production by foregut homogenates of adult *P. americana*. For two weeks prior to assay, cockroaches were fed either: A, PDC (control); B, PDC + antibiotics; C, MCL; D, KAB; E, MCC; or F, no food. Acetate (*circles*), lactate (*boxes*) and total hexose (measured in glucose equivalents) (*triangles*) are given in μmol per gram fresh weight insect.

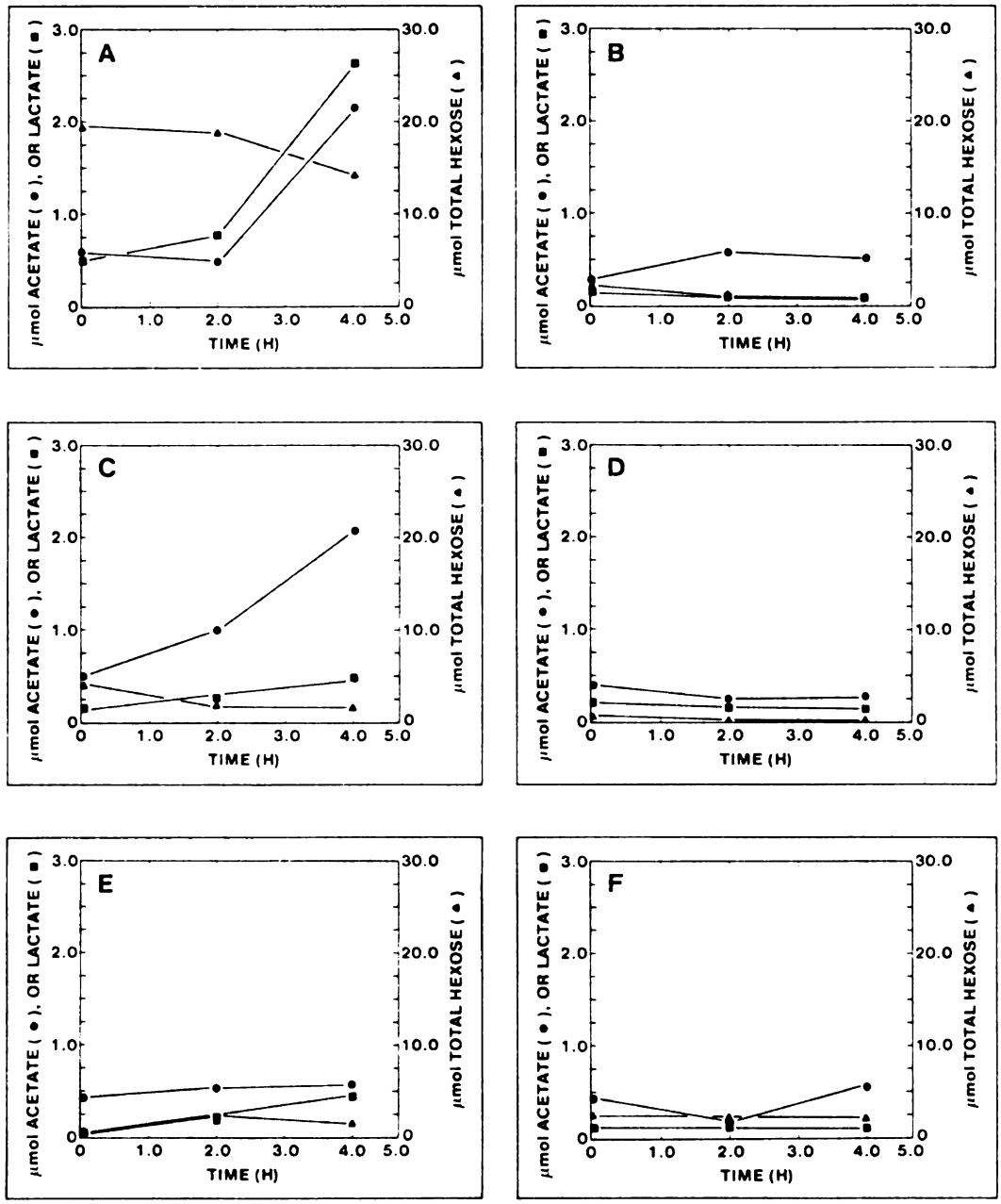

A
B
C
D
E
F
μmol ACETATE (●), OR LACTATE (■)
μmol TOTAL HEXOSE (▲)
TIME (H)
0
1.0
2.0
3.0
4.0
5.0
10.0
20.0
30.0

Figure 3.

Table 4. Effect of diet on populations of lactic acid bacteria in adult *P. americana* foreguts.[a]

Diet	Viable cells x 10^4 per g (fresh weight insect)$^{-1}$	
	Streptococci	Lactobacilli
PDC	23,200.0	72,800.0
PDC + Antibiotics	1.6	0.9
MCL	0.8	1,010.0
KAB	32.0	4.3
MCC	230.0	53.0
Starved	800.0	1,300.0

[a]Results are the values of individual analyses, except for insects fed PDC, where results are the mean for n = 3.

3 were close to the detection limits of the assays used, somewhat masking the significant differences observed when steady state concentrations of acetate and lactate in gut fluid were determined.

Lactic acid bacteria in cockroach foreguts. The presence and production of lactic and acetic acids in *P. americana* foreguts suggested that the resident bacteria observed by SEM (above) were saccharolytic lactic acid bacteria. This was verified by quantitative culture techniques employing selective, as well as rich non-selective, media. Foreguts of PDC-reared cockroaches contained nearly 10^9 bacteria per

gram fresh weight insect. All isolates examined were Gram positive, catalase negative, nonmotile rods and cocci that did not form endospores. All produced lactic acid from growth on glucose, and none required strict anaerobic conditions for growth. Four rod isolates and four coccus isolates were chosen for further characterization, and tenatively identified (by testing growth at various temperatures, and for acetate and/or gas production from glucose) as members of the genera *Lactobacillus* (both homo- and heterofermentative species), *Streptococcus* and *Enterococcus*. Lactic acid bacteria were significantly less abundant in the foreguts of cockroaches fed diets other than dog chow (Table 4). Suprisingly, the number of lactic acid bacteria in the foreguts of starved cockroaches was as higher than that in foreguts of cockroaches fed diets other than dog chow, but only about 2% of that of PDC reared cockroaches.

A water extract of PDC pellets from the cockroach rearing cage contained about 10^7 viable bacteria per gram PDC. Since adult *P. americana* consume about 18 mg PDC per day (4), they could ingest a quantity of organisms equivalent to about 0.01% (10^5 bacteria) of their resident population of lactic acid bacteria per day. This observation indicates that the lactic acid bacteria observed in the foregut are proliferating in that habitat and not merely transient organisms.

DISCUSSION

Results of this study showed that production of organic acids and CH_4 by gut bacteria of *P. americana* cockroaches were significantly affected by the insect's developmental stage and diet. In addition, the significance of the foregut as a site for lactate and acetate production by lactic acid bacteria was also demonstrated. The general trend accompanying a shift from PDC to a low protein-high fiber diet was: i) a decrease in populations of lactic acid bacteria in the foregut; ii) a decrease in total soluble hexose and a suppression of bacterial production of lactate and acetate in the foregut; and iii) an increase in bacterial methanogenic activity, and a modest decrease in H_2-dependent formico- and acetogenesis in the hindgut. The only exception to this trend was that a diet of MCL did not substantially supress acetate production by cockroach foregut bacteria.

The decrease in total hexose observed in foreguts of insects fed MCL, KAB and MCC probably reflects the lower amounts of soluble, anthrone-reactive material (soluble hexose monomers and polymers) present in the diets themselves. However, foreguts of cockroaches fed PDC + antibiotics also had lower amounts of soluble hexose compared to PDC-fed controls. This observation suggests that foregut bacteria may be capable of liberating anthrone-reactive material from certain ingested foodstuff such as PDC.

At present we cannot explain why starved *P. americana* maintained relatively high concentrations of acetate and lactate in the foregut and midgut regions (Table 3) as well as a significant population of lactic acid bacteria in the foregut. This is particularly confusing inasmuch

as lactic acid bacteria appear to colonize mainly food particles in the foregut (Fig. 2). Although food particles can have a residence time in the foregut of over 100 h (24), this does not explain the enigma of lactic acid bacteria remaining in the foregut following 2 weeks of food starvation.

A previous study demonstrated the presence of bacteria in the foregut of *P. americana* (3), however subsequent research emphasized the dense and diverse microflora present in the hindgut region (4,13) and the relationship of foregut bacteria to the host was not examined. An important finding in this study is that the foregut of adult cockroaches fed PDC contained significant populations of lactic acid bacteria which appear to heavily colonize certain food particles, and whose activities were responsible for high concentrations of acetate and lactate in this region. It seems likely that bacterial production of organic acids contributes to the lower pH of the foregut (5.4 - 6.8) compared to the rest of the alimentary canal (6.5 -7.6), as occurs in the crop of chickens (15). However, preliminary experiments by Greenberg et al. (17) found that cockroaches fed antibiotic-treated banana for 5 d also retained a low pH in the foregut region. Therefore, non-bacterial (i.e. host) factors apparently also influence the pH of the foregut.

P. americana possess the enzymes necessary for complete oxidation of acetate and lactate (26,27). [Although only low levels of lactic dehydrogenase (LDH) are present in most cockroach tissue, such is not the case for the midgut, which exhibits high LDH activity (9,10), and is the primary sight of metabolite absorption in most insects (5)]. Based on acetate and lactate production rates (0.52 and 0.40 umol x g [fresh weight insect]$^{-1}$ x h^{-1}, respectively; calculated from Fig. 3A) it

can be estimated that the subsequent uptake and oxidation of these acids by *P. americana* could support as much as 14% of the animal's respiratory requirement.

An additional interesting aspect of the present study was the discovery that immature (larval) cockroaches produced significantly more CH_4 on a per gram basis than did adult cockroaches, particularly when cockroches were fed diets other than PDC. Bracke *et al* (6) observed that loss of the cockroach hindgut anaerobic community by feeding insects metronidozole adversely affected the development of larvae, but did not seem to affect adult cockroaches. It seems likely that the nutritional requirements of immature *P. americana* differ from adult animals, and this is reflected by changes in activities or populations of hindgut anaerobic bacteria, including methanogens.

Undoubtedly, considerable research remains before the complex nutritional interactions between cockroaches and their intestinal microflora are fully appreciated. Nevertheless, the present study underscores the importance of using a carefully defined diet, and considering the potential contributions of gut bacteria, in studies of cockroach nutrition and physiology.

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APPENDIX

ANALYSIS OF 16S rRNA SEQUNECES OF H_2/CO_2 ACETOGENIC BACTERIA ISOLATED FROM TERMITE GUTS

Table 1. Nearly complete sequences of 16S rRNA from H_2/CO_2 acetogenic bacteria isolated from termite guts. SFC-5, *Clostridium mayombei* strain SFC-5 (Chapter 3); APO-1, *Acetonema elongata* strain APO-1 (Chapter 2); JSN-2, *Sporomusa termitida* strain JSN-2 (Breznak *et al.*, 1988, Chapter 2). The sequence of *Bacillus subtilus* (B.subt; provided by C. R. Woese, U. Illinois) is given as a reference.

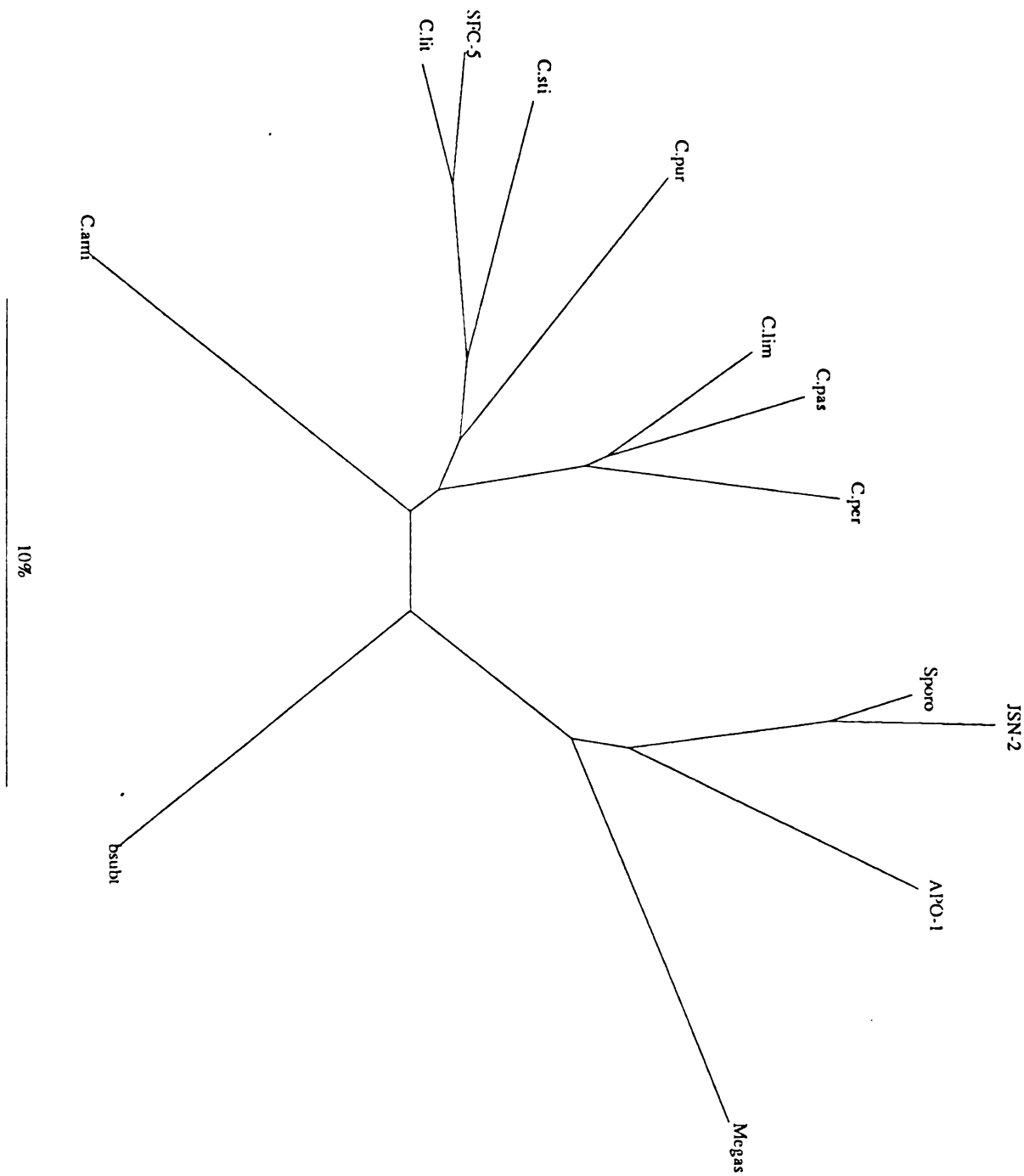
SFC-5	--------UGAG	AGUUUGAUCC	UGGCUCAGGA	UnnNCGCUGG	CGGCG-UGC-CU	44
APO-1	-------UgGAG	AGUUUGAuCC	UGGCUCAGGA	CGAACGCUGG	CGGCG-UGC-UU	45
JSN-2	----UAUUGGCG	CGUUUnanCC	UGGCUCAGGA	CGAACnCUGG	CGGCG-UGC-CU	48
B.subt	--UUUAUCGGAG	AGUUUGAUCC	UGGCUCAGGA	CGAACGCUGG	CGGCG-UGC-CU	50
SFC-5	NACNCAUGCN	AGUCGAGCGA	-------UUCU	C-UUCG-GAGaA	------GAGCG	83
APO-1	nACNCNUGCN	UGUCGAACGG	-----GGAGUU	A-GCAA-UANCU	CC----AAGUG	88
JSN-2	AACACAUGCA	AGUCGAACGG	------AGUNU	A-GCAA-UAACU	N---CUUAGUG	91
B.subt	AAUACAUGCA	AGUCGAGCGG	ACAGG-UGGGA	G-CUUG-CUCCC	G-AUGUUAGCG	100
SFC-5	GCGGACGGGU	GAGUnACGCG	UGGGUAACCU	GCCUCAUACA	CAUGGAUAAC	133
APO-1	GCGAAcGGGU	GAGUAACGCG	UAGACNAUCU	GCCCUUGAGA	ngGGGACAAC	138
JSN-2	GCGAACGGGU	GAGUAACGCG	UAGACAACCU	GCCUCUUAGC	UGGGGACAAC	141
B.subt	GCGGACGGGU	GAGUAACACG	UGGGUAACCU	GCCUGUAAGA	CUGGGAUAAC	150
SFC-5	AUACC-GAAA-G	GUNUGCUAAU	ACAGGAUGAU	-AUAAGAGA-UUC	-ACAU-MGA-UUU	184
APO-1	GUUCC-GAAA-G	GAGCGCUaAU	ACCGAAUgUU	-GUGGAGAG-AKU	-GCAU-NGU-UUC	189
JSN-2	ACCGC-GAAA-G	UGGUGCUAAU	ACCGAAUGUG	-GUAACUCG-GUU	-GCAU-AAC-GGA	192
B.subt	UCCGG-GAAA-C	CGGGGCUAAU	ACCGGAUGGU	-UGUUUGA--ACC	-GCAU-GGU-UCA	200
SFC-5	AUUA-UCAAA-G	C---------UCCG------	---GCGGUAU	GAGAUGGACC	CGCGUCUGAU	226
APO-1	UUUA-CUAAA-G	GUGGCCUCU-AUNU-NUNAG	CUACCACUCA	AGGAGGGGUC	UGCGUCUGAU	247
JSN-2	GUUA-AGAAA-G	AUGGCCAC--UCUa-AAGAA	CUAUCGCUAA	GAGAUGGGUC	UGCGUCUGAU	249
B.subt	AACA-UAAAA-G	GUGGC-----UUCG-----G	CUACCACUUA	CAGAUGGACC	CGCGGCGCAU	250
SFC-5	UAGCUAGUUG	GUA-AGGUAAU	GGCUUACCAA	GGCGACGAUC	AGUAGCNGAC	276
APO-1	UAGCUAGUUG	GUG-AGGUAAC	GGCUCACNAA	GGCNACGAUC	AGUAGCCGGU	297
JSN-2	UAGCUGGUUG	GUG-AGGUAAC	GGAUCACCAA	GGCGACGAUC	AGUAGCCGGU	299
B.subt	UAGCUAGUUG	GUG-AGGUAAC	GGCUCACCAA	GGCAACGAUG	CGUAGCCGAC	300
SFC-5	CU-GAGA-GGG-U	GAUcGGCCAC	AUUGGAACUG	AGACACGGUC	CNaACUCCUA	326
APO-1	CU-GAGA-GGA-U	GAACGGCCAC	ACUGGGACUG	AGACACGGCC	NNGACUCCUA	347
JSN-2	CU-GAGA-GGA-U	GAACGGCCAC	ACUGGGACUG	AGACACGGCC	NNGACUCCUA	349
B.subt	CU-GAGA-GGG-U	GAUCGGCCAC	ACUGGGACUG	AGACACGGCC	CAGACUCCUA	350
SFC-5	ggGGAGGCAG	CAGUGGGGAA	UAUUGCACAA	UGGGC-GAAA-G	CCUNAUGCAG	376
APO-1	CGGGAGGCAG	CAGUGGGGAA	UCUUCCGCAA	UGGGC-GAAA-G	CCUGACGGAG	397
JSN-2	CGGGAGGCAG	CAGUGGGGAA	UCUUCCGCAA	UGGAC-GAAA-G	UCUGACGGAG	399
B.subt	CGGGAGGCAG	CAGUAGGGAA	UCUUCCGCAA	UGGAC-GAAA-G	UCUGACGGAG	400
SFC-5	CAACGCCGCG	UGAGUGAUGA	AGGCC-UUCG-G	GUCGUAAAAC	UCUGUCCUCA	426
APO-1	CAACGCCGCG	UGAGUGAAGA	AGGUu-uUCG-G	AUUGUAAAGC	UCUGUCUUUC	447
JSN-2	CAACGCCGCG	UGAGUGAAGA	AGGCC-UUCG-G	GUCGUAAAGC	UCUGUCGUUU	449
B.subt	CAACGCCGCG	UGAGUGAUGA	AGGUU-UUCG-G	AUCGUAAAGC	UCUGUUGUUA	450

SFC-5	AGGAAG----	----------	--AUAA-------	-------UGAC	GGUACUUGA-G	450
APO-1	GGGACGAAUG	UGCGGGUGGU	-AAAUA--AUCAC	UC-GUAAUGAC	GGUACCGAA-G	497
JSN-2	GGGACGAACG	UNGYCUAUGU	-GAAUA--AURUA	GA-CUAAUGAC	GGUACCAAA-G	499
B.subt	GGGAAGAACA	AGUACCGUUC	-GAAUA--GGGCG	GU-ACCUUGAC	GGUACCUAA-C	500
SFC-5	GAGGAAGCCC	CGGCUAA-CUA	CGUGCCAGCA	GCCGCGGUAA	UACGUAGGGG	500
APO-1	GAGGC-GCCA	CGGCUAA-CUA	CGUGCCAGCA	GCCGCGGUAA	UACGUAGGUG	546
JSN-2	GAGGAAGCCA	CGGCUAA-CUA	CGUGCCAgCA	GCCGCGGUAA	UACGUAGGUG	549
B.subt	CAGAAAGCCA	CGGCUAA-CUA	CGUGCCAGCA	GCCGCGGUAA	UACGUAGGUG	550
SFC-5	GNNNGCGUUA	UCCGGAUUUA	CUGGGCGUAA	AGGGUGCGUA	GGUGGUUUCU	550
APO-1	GCNAGCGUUG	UCCGGAAUgA	UUGGGCGUNA	AGCGCGCGCA	GGCGGGUUUA	596
JSN-2	GCAAgcGUUG	UCCGGAAUUA	UUGGGCGUAA	AGGGCGUGUC	GGUGGCCUGG	599
B.subt	GCAAGCGUUG	UCCGGAAUUA	UUGGGCGUAA	AGGGCUCGCA	GGCGGUUUCU	600
SFC-5	UAAGUC-AGGA	GUGAAAGGCU	ACGG-CUNAA-C	CGUAGUA--AG	CUCUUGAAAC	599
APO-1	UAAGUC-UGUU	GUGAAAAUGC	GGGG-CUnaA-C	YCCGUAGG-GU	CAAUGGAAAC	645
JSN-2	UAAGUC-GUGU	GUCUAAGUGC	GAAG-CUcAA-C	UUCGUAUG-GG	CGCAGGAAAC	649
B.subt	UAAGUC-UGAU	GUGAAAGCCC	CCGG-CUCAA-C	CGGGGAGG-GU	CAUUGGAAAC	650
SFC-5	UGGGAGAC-UU	GAGUGCCGGA	GAGGNNAGUG	GAAUUCCUAG	UGUAGCG-GUG	649
APO-1	UGUKAAUC-UU	GAGUGCAGGA	GAGGAAAGCG	GAAUUCCCAG	UGUAGCG-GUG	695
JSN-2	UGUCCAgC-UU	GAGUGCAGGA	GAGGAAAGUG	GAAUUCCCAG	UGUAGCG-GUG	699
B.subt	UGGGGAAC-UU	GAGUGCAGAA	GAGGAGAGUG	GAAUUCCACG	UGUAGCG-GUG	700
SFC-5	AAA-UGCGUAG	CACUUAGGAG	GAACaCCAG-U	AGCGAAGGCg	GCUUUCUGGA	699
APO-1	AAA-UGCGUNG	AUAUUGGGAG	GAACACCAG-U	GGCGAAGGCG	GCNUUCUNGA	745
JSN-2	AAA-UGCGUAG	AUAUUGGGAG	GAACACCAG-U	GGCGAAGGCG	ACUUUCUgGA	749
B.subt	AAA-UGCGUAG	AGAUGUGGAG	GAACACCAG-U	GGCGAAGGCG	ACUCUCUGGU	750
SFC-5	CUGUAACUGA	CACUGAGGCA	CGAAAGCGUG	GGGA-CGAAC	AGGAUUAGAU	748
APO-1	CUGUUACUNA	CGCUGAGGCG	CAAAAGCCAG	GGUAGUGAAC	GGGAUUAGAU	795
JSN-2	CUGUGUCUGA	CACUGAGGCG	CGAAAGCCAG	GGGAGCGAAC	GGGAUUAGAU	799
B.subt	CUGUAACUGA	CGCUGAGGAG	CGAAAGCGUG	GGGAGCGAAC	AGGAUUAGAU	800
SFC-5	ACCCUnGUAG	UCCACGCCGU	AAACGAUGAG	UACUAGGUGU	NNGG----UUN	795
APO-1	ACCCCGGUAA	UCCUAGCNGU	NAACGAUGGG	UACUAGGUGU	AGGAGG-UAUC	845
JSN-2	ACCCCGGUAG	UCCUGGCCGU	AAACGAUGGG	UACUAGGUGU	AGAGGG-UAUC	849
B.subt	ACCCUGGUAG	UCCACGCCGU	AAACGAUGAG	UGCUAAGUGU	UAGGGGG-UUU	850
SFC-5	CCCC-CCaUCG	GUGCCGCAgc	UAACGCAUUA	AGUACUCCGC	CUGGGGAGUA	845
APO-1	GACC-CnUCCU	GUGCNGGAGU	UAACGCAAUA	AGUAccnAGC	CUGGGGAGUA	895
JSN-2	GACC-CcUUCU	GUGCCGGAGU	UAACGCAAUA	AGUaCCCCGC	CUGGGGAGUA	899
B.subt	CCG-CCCCUUA	GUGCUGCAGC	UAACGCAUUA	AGCACUCCGC	CUGGGGAGUA	900

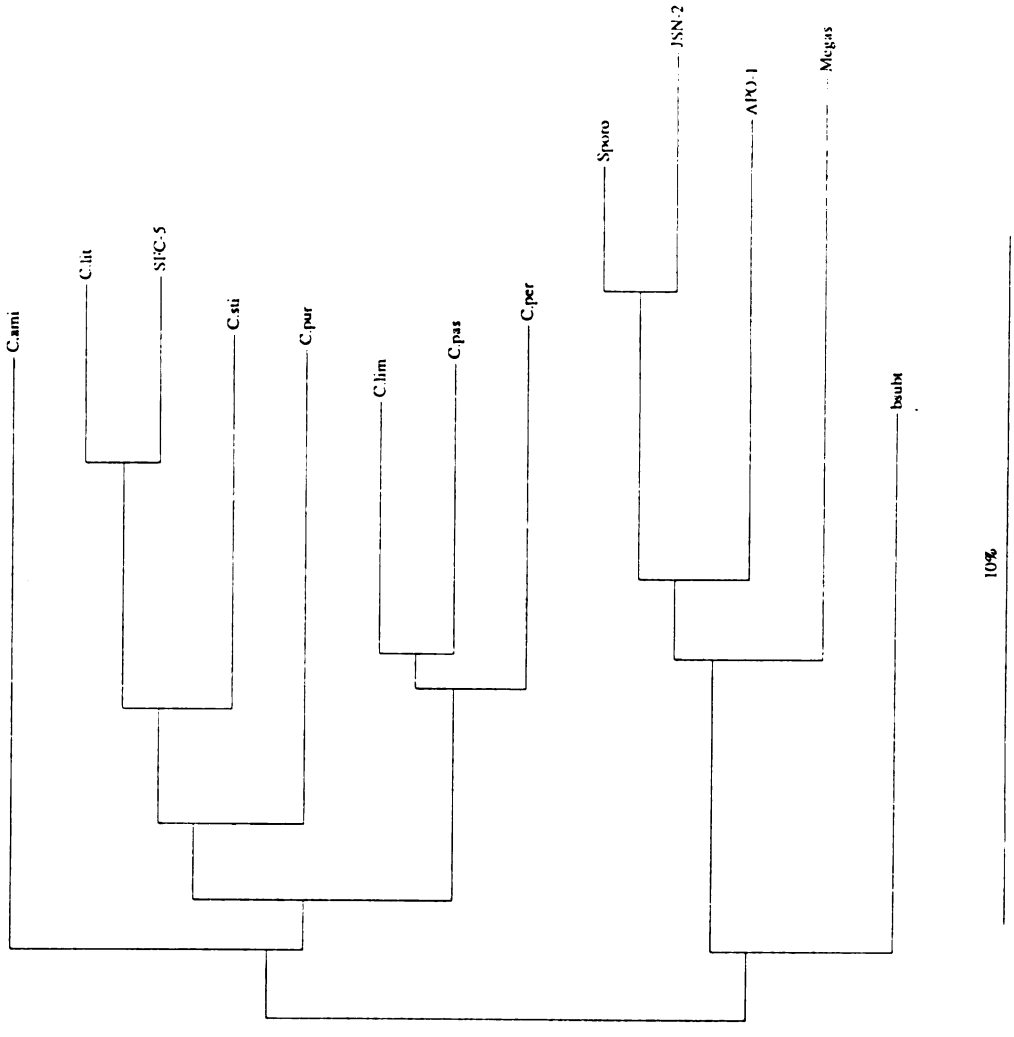
SFC-5	CGCUC-GCAA-G	AGUGAAACUC	AAA-GGAAUUG	ACGGGGACCC	G-CACAAGUAG	895
APO-1	CGGCC-GCAA-G	GUUGAAACnn	AAA-GGAAUUG	ACGGGGNcCC	G-CACAAGCGG	945
JSN-2	CGGCC-GCAA-G	GUUGAAACUC	AAA-GGAAUUG	ACGGGGnCCC	G-CNcnAGcGG	949
B.subt	CGGUC-GCAA-G	ACUGAAACUC	AAA-GGAAUUG	ACGGGGGCCC	G-CACAAGCGG	950
SFC-5	CGGAGCAUGU	GGUUUAAUUC	GAAGCAACGC	GAAGAACCUU	A-CCUAAGCUU	945
APO-1	UGGAGUaUGU	NGUUUNAUUC	GANNNAACGC	GAAGAACCUU	A-CCAGGGCUU	995
JSN-2	UGGCGUCUGU	NGUUUAAUUA	GAnGCAACGC	GAAGAACCUU	A-CCAGGGCUU	999
B.subt	UGGAGCAUGU	GGUUUAAUUC	GAAGCAACGC	GAAGAACCUU	A-CCAGGUCUU	1000
SFC-5	GACAUC-CUUU	U-GACCGCUGC-	-CUNAUC-GCA---	U-Y----UUC-UUCG	-GGG-ACAGAAGU	994
APO-1	RACAUC-GCCW	R-AA-GAUCCUA	--GAGA--UAGGGA	G------YYY-UUCG	-GGR-A-CAGGUa	1043
JSN-2	GACAUU-GAGC	G-AA-AGGUUCA	--GAGA--UGaAUC	C-C-U--AUC-UuCG	-GAU-A-CGCGAA	1049
B.subt	GACAUC-CUCU	G-AC-AAUCCUA	--GAGA--UAGGAC	G-U-C--CCC-UUCG	-GGG-G-CAGAGU	1050
SFC-5	GACAGGUGGU	GCAUGGUUGU	CGUCAGCUCG	UGUC-GUGA-GA	UGUUGGGUUA	1044
APO-1	GACAGGUGGU	GCAUGGCWRU	NGNNAGCUNG	UGUN-GUNA-GA	UNUUGGGnnn	1093
JSN-2	AACAGGUGGU	GCAUGGCUGU	CGUCAGAUAG	uGUC-GUGA-GA	UGUUGGGUUa	1099
B.subt	GACAGGUGGU	GCAUGGUUGU	CGUCAGCUCG	UGUC-GUGA-GA	UGUUGGGUUA	1100
SFC-5	AGUCCCGCAA	CGAGCGCAAC	CCUUGCCUUU	AGUUGCCAGC	A--UUAAGU-UGG	1094
APO-1	nnnnnnnnnn	nnnnnnnnnn	nnnnnnnnnn	nnnnnnnnnn	nn-nnnnnnnnn	1145
JSN-2	AGUCCCGCAA	CGAGCGCAaC	CCUUAUCCUU	UGUUGCCAGC	GC-GUAAAG-GCG	1150
B.subt	AGUCCCGCAA	CGAGCGCAAC	CCUUGAUCUU	AGUUGCCAGC	A--UUCAGU-UGG	1150
SFC-5	GCACUCUAGA	GGGACUGCcA	GG--GAUAA-CCU	G-GAGGAAGGU	GGGGAUGACG	1144
APO-1	nnnnnnnnnn	nnnnnnnnnn	nn--nnnnn-nnn	n-nnnnnnnnn	nnnnnnnnnn	1195
JSN-2	GGAaCUCNAG	GGAGCUGCgG	CA-nGAGAA-UGC	G-GUGGaaGGc	GGGGAUGACG	1201
B.subt	GCACUCUAAG	GUGACUGCCG	GU--GACAA-ACC	G-GAGGAAGGU	GGGGAUGACG	1200
SFC-5	UNaAAUCAUC	AUGCCCCUUA	UGCUUAGGGC	UACACACGUG	CUACAAUGGG	1194
APO-1	nnnAGUCAUC	AUGCCCcUUa	UGUCCUGGGC	UNCNNNCGUu	CUNCNAUGGG	1245
JSN-2	UCAAGUCAUC	AUGCCCCUaA	UGUCCUGGGC	UACACACGUN	CUACaAUGGG	1251
B.subt	UCAAAUCAUC	AUGCCCCUUA	UGACCUGGGC	UACACACGUG	CUACAAUGGA	1250
SFC-5	UGGUA-CAGAG	GGCAGCCAAG	UC-GUGA-GGCC	GAGCUAAUCC	C-UUAAGCCA	1243
APO-1	CUCUuANNGAG	GGAAGCAAAG	CC-GCGA-GGCC	AAGCCaAUCC	CAAAAACGAG	1296
JSN-2	CUUANACAAAG	CGAAGCAAGC	CU-GCGA-AGGU	AAGCAAAACG	CAGAAAUAaG	1302
B.subt	CAGAA-CAAAG	GGCAGCGAAA	CC-GCGA-GGUU	AAGCCAAUCC	CACAAAUCUG	1300
SFC-5	UUCUCAGUUC	GGAUUGUAGG	CUGAAACUCG	CCUKCAUGAA	GCUNGAGUUN	1293
APO-1	AUCUNNGUUC	GGAUCGGAGG	CUgCNNCUcG	CCUuNgUGAA	GUCGGAAUCg	1346
JSN-2	CUCUCRGUUC	GGANGGcAGG	CUGCAaCUCG	CCUcCGUGAA	GUCGGaAUCG	1352
B.subt	UUCUCAGUUC	GGAUCGCAGU	CUGCAACUCG	ACUGCGUGAA	GCUGGAAUCG	1350

SFC-5	CUAGUAAUCG	CAGAUCaG-AA	UGCUGCGGUN	AAUGCGUUCC	C	1334
APO-1	CUaGUaAUCG	CAGAUCaG-CA	UGCUNCGGU			1375
JSN-2	CUAGUAAUCG	CAGG				1366
B.subt	CUAGUAAUCG	CGGAUCAG-CA	UGCCGCGGUG	AAUACGUUCC	CGGGCCUUGU	1400
SFC-5						1334
APO-1						1375
JSN-2						1366
B.subt	ACACACCGCC	CGUCACACCA	CGAGAGUUUG	UAACACCCGA	AG-UCGGUGAG	1450
SFC-5						1334
APO-1						1375
JSN-2						1366
B.subt	-GUAACCU---UUU	----AGGAGCCAGC	CGC-CGAAGGU	GGGACAGAUG	AUUGGGGUGA	1500
SFC-5						1334
APO-1						1375
JSN-2						1366
B.subt	AGUCGUAACA	AGGUAGCCGU	AUCGGAAGGU	GCGGCUGGAU	CACCUCCUUU	1550
SFC-5						1334
APO-1						1375
JSN-2						1366
B.subt	CU					1552

Figure 1a, b. 16S rRNA phylogenetic tree of H_2/CO_2 acetogenic bacteria from termite guts with various other eubacteria presented in bush (a) and bar(b) formats. The scale bars are in percent nucleotide substitutions per sequence position. SFC-5, *Clostridium mayombei* strain SFC-5 (Chapter 3); APO-1, *Acetonema elongata* strain APO-1 (Chapter 2); JSN-2, *Sporomusa termitida* strain JSN-2 (Breznak *et al.*, 1988, Chapter 2). The following sequences were kindly provided by C. R. Woese, U. Illinois: Sporo, *Sporomusa paucivorans*; Megas, *Megasphaera elsdenii*; bsubt, *Bacillus subtilis*; C.ami, *Clostridium aminovalericum*; C.lit, *Clostridium lituseburense*; C.sti, *Clostridium sticklandii*; C.pur, *Clostridium purinilyticum*; C.lim, *Clostridium limosum*; C.pas, *pasteurianum*; C.per, *Clostridium perfringens*. The tree was constructed using the least squares method [Fitch, W. M. and E. Margoliash (1967), Construction of phylogenetic trees: a method based on mutational distances as estimated from cytochrome c sequences is of general applicatability. *Science* **155**:279-284].


C.lit
SFC-5
C.sti
C.pur
C.amf
C.lim
C.pas
C.per
Sporo
JSN-2
APO-1
Megas
bsubt
10%

A


C.ami
C.lit
SHC-5
C.sti
C.pur
C.lim
C.pas
C.per
Sporo
JSN-2
AIO-1
Megas
baubi
10%

B