11-1-2007

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Applied and Environmental Microbiology, Nov. 2007, p. 6819–6828 Vol. 73, No. 21
0099-2240/07/$08.00+0 doi:10.1128/AEM.00786-07

Stenoxybacter acetivorans gen. nov., sp. nov., an Acetate-Oxidizing Obligate Microaerophile among Diverse O_2-Consuming Bacteria from Termite Guts[▽]

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Received 9 April 2007/Accepted 25 August 2007

In termite hindguts, fermentative production of acetate—a major carbon and energy source for the insect—depends on efficient removal of inwardly diffusing oxygen by microbes residing on and near the hindgut wall. However, little is known about the identity of these organisms or about the substrate(s) used to support their respiratory activity. A cultivation-based approach was used to isolate O_2-consuming organisms from hindguts of *Reticulitermes flavipes*. A consistently greater (albeit not statistically significant) number of colonies developed under hypoxia (2% [vol/vol] O_2) than under air, and the increase coincided with the appearance of morphologically distinct colonies of a novel, rod-shaped, obligately microaerophilic β-proteobacterium that was <95% similar (based on the 16S rRNA gene sequence) to its closest known relative (*Eikenella corrodens*). Nearly identical organisms (and/or their 16S rRNA genes) were obtained from geographically separated and genetically distinct populations of *Reticulitermes*. PCR-based procedures implied that the novel isolates were autochthonous to the hindgut of *R. flavipes* and comprised ca. 2 to 7% of the hindgut prokaryote community. Representative strain TAM-DN1 utilized acetate and a limited range of other organic and amino acids as energy sources and possessed catalase and superoxide dismutase. On solid medium, the optimal O_2 concentration for growth was about 2%, and no growth occurred with O_2 concentrations above 4% or under anoxia. However, cells in liquid medium could grow with higher O_2 concentrations (up to 16%), but only after proportionately extended lag phases. The genetic and physiological distinctiveness of TAM-DN1 and related strains supports their recognition as a new genus and species, for which the name *Stenoxybacter acetivorans* gen. nov., sp. nov. is proposed.

The vitality of wood-feeding termites depends upon a dense and phylogenetically diverse community of hindgut microbes, which contribute to the insects' nitrogen economy and assist in the degradation of wood polysaccharides (cellulose and hemicelluloses) to short-chain fatty acids used by their hosts for energy (9, 11). In most termites, microbially produced acetate dominates the hindgut fatty acid pool, comprising up to 98 mol% of all volatile fatty acids and occurring at concentrations as high as 80 mM (33). Acetate is absorbed through the hindgut epithelium, serves as the major oxidizable energy source for the insect, and is capable of supporting up to 100% of the termite's daily respiratory requirements (33, 50). However, incomplete oxidation of polysaccharides to the level of acetate is favored by maintenance of anoxia in the hindgut lumen (33, 50).

Early measurements of the redox potential of hindguts, together with the presence of strictly anaerobic microbes, led to the hypothesis that termite guts were devoid of oxygen (7, 53). By contrast, other data suggested that some portion(s) of the hindgut must contain O_2. For example, aerotolerant, facultative, and even O_2-requiring microorganisms were frequently represented among isolates (13, 43, 51, 53), and degradation of lignin model compounds in vivo was found to be O_2 dependent (14). It was not until direct measurements were made with microelectrodes that fine-scale profiles of O_2 gradients within intact hindguts were obtained (13, 17). The results demonstrated the presence of a peripheral hypoxic zone, with O_2 occurring at partial pressures of about 20×10^2 to 30×10^2 Pa (ca. 20 to 30% air saturation) at the gut wall, which decreased steeply to anoxia a distance of 150 to 200 μm inward. This indicated that a majority (approximately 60%) of the hindgut contained significant levels of O_2, undoubtedly a result of its small size and relatively large surface-to-volume ratio (12). Such results also implied that some members of the microbiota on and/or near the hindgut wall constituted an important "oxygen sink" (12, 13, 17). Consistent with this interpretation were earlier experiments showing that hindguts rapidly became completely oxic (implied by the color of redox dyes) when termites were fed antibacterial drugs (53); and exposure of termites to hyperbaric levels of O_2 resulted in the death of hindgut spirochetes and protozoans (both groups have been reported to be anaerobes) but led to a 6- to 10-fold increase in overall hindgut bacterial colony counts on culture plates incubated in air (53). However, such treatments decreased the ability of termites to survive on a diet of wood or cellulose.

It now seems clear that O_2-consuming members of the peripheral hindgut microbiota are critical in creating and maintaining anoxic conditions in the luminal region for an essentially homoacetic conversion of wood polysaccharides by carbohydrate fermenters and CO_2-reducing acetogens (27, 50). However, our understanding of this O_2-consuming microbiota is meager, as is the nature of the substrate(s) that fuels their respiratory activity, although acetate itself is a likely candidate

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▽ Published ahead of print on 7 September 2007.

given its abundance and high rate of oxidation to CO_2 in termite hindguts (50), as well as the abundance of (not-yet-characterized) acetate oxidizers inferred from most-probable-number enumerations (51). Accordingly, we sought to explore the nature of O_2 consumers in more detail by using acetate as a substrate in isolation media, as well as in incubation atmospheres that included hypoxia (2% O_2) to accommodate the possibility that some members of the O_2-consuming microbiota might be so highly adapted to life in hypoxia that they do not grow in air (21% O_2). The results of this effort led to the isolation of several previously uncultivated bacteria from termite guts, including a microaerophilic, acetate-oxidizing member of the β-*Proteobacteria*, *Stenoxybacter acetivorans* gen. nov., sp. nov., which is a significant member of the O_2-consuming bacterial microbiota and whose description is the subject of this paper. A companion paper (55) reports on the physiological ecology of *S. acetivorans*, including the expression of relevant genes in situ.

MATERIALS AND METHODS

Termites. *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) was collected in Dansville and Spring Arbor, MI; Raleigh, NC; Woods Hole, MA; and Janesville, WI. *Reticulitermes santonensis* (Feytaud), which appears to be synonymous with *R. flavipes* (3), was collected near Forêt de la Coubre, France; *Coptotermes formosanus* Shiraki (Rhinotermitidae) was collected near Ft. Lauderdale, FL; and *Zootermopsis angusticollis* (Hagen) (Termopsidae) was obtained from a laboratory culture (29). *R. flavipes* and *C. formosanus* were either degutted within hours of collection or maintained in laboratory nests (10, 33). *R. santonensis* was maintained in the laboratory (56) for 1 year prior to use.

Isolation and cultivation of bacteria. Guts from 50 worker larvae were extracted with sterile forceps under an hypoxic, CO_2-enriched atmosphere (2% O_2, 5% CO_2, 93% N_2) within a vinyl chamber (Coy Laboratory Products, Grass Lake, MI). Extracted guts were transferred to and homogenized in a sterile glass tissue homogenizer containing 2 ml of a buffered salts solution (BSS) composed of (per liter) 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.5 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 1.0 g NaCl, 0.62 g $MgCl_2 \cdot 6H_2O$, 2.84 g Na_2SO_4, and 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS). The pH of the BSS was adjusted to pH 7.0, and the solution was preincubated under the CO_2-enriched hypoxic atmosphere for at least 12 h before use, during which time the pH decreased to about 6.5, which was close to the pH of the hindgut fluid (pH 6.4) (13). Serial 10-fold dilutions of gut homogenate were prepared in BSS, and 0.1-ml aliquots of each dilution were spread onto six plates of ACY medium, which consisted of BSS amended with 10 mM sodium acetate, 0.05% (wt/vol) Bacto Casamino Acids, 0.05% (wt/vol) Bacto yeast extract, 0.01% (vol/vol) each of a trace element solution and a vitamin solution (49), and 1.5% (vol/vol) Bacto agar. The pH of ACY medium was adjusted to 7.0. Three plates from each dilution were retained within the hypoxic chamber, and the remaining plates were placed in glass desiccator jars containing CO_2-enriched air (5% [vol/vol] CO_2, 95% air). Every 2 days, the atmosphere in the jars was replenished. All incubations were at 22 to 23°C.

After ca. 20 days, when new colony formation was subsiding, plates containing well-separated colonies were selected for colony enumeration, isolation, and identification. The number of colonies having a similar morphology on each plate was noted, and an effort was made to have at least one pure culture representative of each colony type. For each colony, duplicate plates of homologous medium were streaked; one plate was incubated under hypoxia, and the companion plate was incubated in CO_2-enriched air. In addition, specific groups of organisms were targeted for isolation by using a "plate wash PCR" (49) with the following 16S rRNA gene forward primers: Acd31f (5′-GAT CCT GGC TCA GAA TC-3′) for *Acidobacteria* (4), End197f (5′-GCA GCA ATG CGT TTT GAG-3′) for *Endomicrobia* (this study), Pla40f (5′-CGG RTG GAT TAG GCA TG-3′) for *Planctomycetes* (K. Huizinga, personal communication), and Ver53f (5′-TGG CGG CGT GGW TAA GA-3′) for *Verrucomicrobia* (49). The reverse primer in all cases was the general bacterial reverse primer 1492r (see below). The purity of isolates was based on uniform colony and cell morphology; the latter was determined by phase-contrast microscopy. Preliminary screening of isolates for acetate utilization was done by streaking onto plates of ACY medium with and without acetate. Conspicuously better growth on acetate-containing ACY medium was a presumptive indicator of acetate utilization. Isolated strains of the novel acetate-oxidizing, microaerophilic member of the β-*Proteobacteria* described here (*S. acetivorans* gen. nov., sp. nov.) were assigned designations with the prefix "TAM."

DNA extraction and PCR-related procedures. For rapid phylogenetic identification of isolates, a portion of colony material was removed with a sterile inoculating loop, suspended in 500 μl of sterile BSS, and centrifuged for 10 min at 4,000 × *g*. The supernatant liquid was removed, and total DNA in the cell pellet was extracted and purified by using a Bactozol DNA extraction kit as recommended by the manufacturer (Molecular Research Center, Cincinnati, OH). The precipitated DNA was dissolved in 250 to 500 μl of sterile water (pH 7.5), occasionally aided by incubation at 60°C for 1 to 12 h.

For each isolate, the 16S rRNA gene was amplified by PCR using the general primers 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′), which target regions of the gene common to most *Bacteria* (54). Each 25-μl reaction mixture contained 25 to 100 ng DNA template, 1× reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 0.2 μM of each primer, and 0.625 U *Taq* polymerase (Invitrogen). The PCR mixtures were subject to (i) 3 min at 95°C, (ii) 30 cycles of 45 s at 95°C, 45 s at 56°C, and 45 s at 72°C, and (iii) 5 min at 72°C. A 5-μl sample of each PCR mixture was analyzed by electrophoresis on 1.0% agarose gels prepared in 0.5× Tris-borate-EDTA with 10 μg/ml ethidium bromide (39). PCR products were visualized by UV transillumination, and images were captured by using a Kodak 290 electrophoresis documentation and analysis system (Eastman Kodak).

The total number of *S. acetivorans*, *Acidobacteria*, or *Verrucomicrobia* cells in *R. flavipes* and their primary anatomical location were estimated by a dilution-to-extinction PCR approach. DNA was extracted from 50 to 100 freshly collected intact termites, termite hindguts (from which the midgut had been detached at the midgut-hindgut junction with a sterile scalpel), and degutted termite bodies by using a MoBio Ultraclean soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) after homogenization in a Mini-BeadBeater-8 (BioSpec Products, Inc., Bartlesville, OK) operating at full speed for 45 s according to the DNA isolation protocol. Purified DNA was normalized on a per-termite-equivalent basis and was serially diluted in buffer (10 mM Tris-HCl [pH 8.0] containing 50 ng/μl calf thymus DNA as a carrier). As a control, 100 ng purified DNA from *S. acetivorans* TAM-DN1 or *Verrucomicrobia* strain TAV-1 (49) was also serially diluted in buffer. Each dilution was used as the template in a PCR with forward primer TAM203f (5′-GCT TCG CAA GGA CCT CAC-3′; targeting the 16S rRNA gene of *S. acetivorans* strains [see below]), Ver53f, or Acd31f combined with reverse primer 1492r (see above). The PCR mixture was identical to that described above, and a total of 30 PCR cycles was used. Five-microliter samples of each PCR mixture were analyzed by electrophoresis on a 1.0% agarose–0.5× Tris-borate-EDTA gel stained with 1× Gelstar nucleic acid stain (Cambrex, East Rutherford, NJ). Fluorescent bands of PCR products were visualized, captured, and analyzed as described above. An estimate of the in situ abundance of the organisms was then made by comparing the extinction point (i.e., the smallest amount of template DNA yielding a visible amplimer) of purified TAM-DN1 or TAV-1 DNA to that of homologous target DNA in serially diluted termite samples, as well as the determined genome sizes and 16S rRNA gene copy numbers of *S. acetivorans* (3.2 Mb and four copies, respectively [see below]) and *Verrucomicrobia* strain TAV-1 (4.0 Mb and one copy, respectively [see below]) and a presumed 3.0-Mb genome and one 16S rRNA gene copy for *Acidobacteria* (18).

To evaluate the autochthony (i.e., indigenousness) of *S. acetivorans* for *R. flavipes*, termites were freshly collected from a forested area in Dansville, MI, along with termite "nest soil" (i.e., soil at the interface of a fallen log on which termites were feeding and through which they were actively tunneling) and separate "nonnest soil" that showed no evidence of termite activity. Soil samples were collected with sterile spatulas and placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) kept on ice, and DNA was extracted from termite guts (100 guts) and soil samples (1 g) within hours of collection by using the MoBio Ultraclean soil DNA extraction kit as described above. The purified DNA was used in PCRs performed as described above with the *S. acetivorans*-targeting 16S rRNA gene forward primer TAM203f and general reverse primer 1492r. PCR-amplified DNA was cloned into *Escherichia coli* TOP10 using the plasmid vector pCR2.1 (TA clone kit; Invitrogen). The partial sequences of randomly selected clones were determined using the TAM203f primer. Only sequences longer than 500 bp were used for subsequent analyses. Sequences were imported into the ARB software package (30) and aligned, and phylogenetic trees were constructed (see below) using 503 shared nucleotide positions. The statistical program LIBSHUFF was used to determine relatedness of the clones, and a nonparametric estimate of library similarity was obtained using the method of Yue and Clayton as implemented by SONS (40, 41).

Sequencing and phylogenetic analysis. Before amplimers were sequenced, unreacted deoxynucleoside triphosphates and PCR primers were digested and dephosphorylated using ExoSap-IT by following the supplier's instructions (USB, Cleveland, OH).

Partial 16S rRNA gene sequences for each isolate were determined with Applied Biosystems cycle sequencing technology (Applied Biosystems, Foster City, CA), using the general bacterial primer 8f. Sequence chromatograms were checked for quality, and the initial identity of each isolate was determined by using the BLAST search tool in GenBank (2) or the Ribosomal Database Project (http://cme.rdp.msu.edu) (16). A total of 29 strains of *S. acetivorans* were obtained from *R. flavipes* collected in Dansville and Spring Arbor, MI, Raleigh, NC, Woods Hole, MA, and Janesville, WI, as well as from *R. santonensis*. Of these 29 strains, a subset of 15 strains representing the apparent phylogenetic breadth of the group was chosen for nearly full-length sequencing of the 16S rRNA gene. Fourfold coverage of each nucleotide position was obtained by using the method described previously (49) but omitting primers F2, R4*, Acd31f, and Ver53f and adding primer 8f. Individual 16S rRNA gene sequence reads for each isolate were manually edited and assembled by using the Contig Assembly Program contained within BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

For phylogenetic analyses, the partial or nearly full-length 16S rRNA gene sequence of each isolate or clone was aligned in ARB (http://www.arb-home.de/) (30). Ambiguities in sequence alignments were corrected manually, where possible, and only unambiguously aligned positions were used for phylogenetic analyses. Maximum likelihood phylogenetic trees were constructed and estimates of internal branch support were obtained in ARB using the TREE-PUZZLE routine (30, 42).

Termite microsatellite DNA analysis. Eight degutted *Reticulitermes* worker larvae from each of the six collection sites were placed individually in 550 μl Bead solution (MoBio Ultraclean soil DNA kit; MoBio Labs) in separate Bead tubes and were homogenized in a Mini-BeadBeater-8 operating at full speed for 1 min. Purified DNA was obtained using the MoBio Ultraclean soil DNA kit protocol. The resulting DNA was used in individual, nonmultiplexed PCRs with 5′-Hex-labeled PCR primers 5-10, 6-1, 11-1, 11-2, and 21-1 (52). Each 50-μl reaction mixture contained 10 ng of termite DNA, 1× reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 0.2 μM of each primer, and 0.625 U *Taq* polymerase (Invitrogen). The PCR was performed as follows: (i) 3 min at 94°C; (ii) 35 cycles of 30 s at 94°C, 50 s at 60°C, and 1.0 min at 72°C; and (iii) 5 min at 72.0°C. PCR products were precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of 100% ethanol. The DNA pellet was washed in 75% ethanol, air dried, and dissolved in 50 μl sterile water (pH 7.0). Two hundred to 300 ng of the purified PCR product(s) was separated by using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Peak sizes were determined with the Genotyper software (Applied Biosystems), and alleles at each locus were binned according to fragment size (±1.5 bp to accommodate variability during electrophoretic separation). The number of alleles at each locus was categorized using a Microsoft Excel spreadsheet. For all possible alleles, "1" was used when an allele was present in a given termite and "0" was used when an allele was absent. Based on this table, the Jaccard coefficient (Jc) was calculated for all pairwise comparisons by using EstimateS (15). A distance matrix was created by using Microsoft Excel, where the Jaccard distance was calculated as follows: 1 − Jc. This matrix was uploaded into MEGA (26) for cluster analysis.

Genomic properties. The genome sizes of *S. acetivorans* TAM-DN1 and *Verrucomicrobia* strain TAV-1 were estimated by pulsed-field gel electrophoresis of restriction endonuclease-digested total DNA (genomic DNA kit; QIAGEN Inc., Chatsworth, CA) (8, 22). The 16S rRNA gene copy number was determined by methods described previously (25), using the restriction enzymes Sma2 and Hae2 (New England Biolabs, Ipswich, MA). The G+C content was determined by high-performance liquid chromatography (HPLC) analysis (31), as modified by Graber et al. (22).

Cultivation, nutrition, and physiological studies. Routine cultivation of TAM strains was in BYA medium consisting of BSS (see above), 0.05% (wt/vol) Bacto yeast extract, and 10 mM sodium acetate. Isolates were typically grown in 50-ml sterile Erlenmeyer flasks containing 15 ml BYA medium and were incubated at 22 to 23°C with shaking (250 rpm) in the CO_2-enriched hypoxic atmosphere of the vinyl glove box (see above). The pH of uninoculated BYA medium kept in the CO_2-enriched hypoxic atmosphere was 6.5.

Substrate utilization by TAM-DN1 was tested by using 18-mm anaerobe tubes (catalog no. 2048-00150; Bellco) with butyl rubber stoppers containing 5 ml of BYA medium in which acetate was replaced by the test substrate at a concentration of 10 mM. The tube headspace (ca. 22 ml) contained 2% O_2, 5% CO_2, and 93% N_2. Test cultures were inoculated (1%, vol/vol) with an exponential-phase culture growing in BYA medium and then were incubated at 22 to 23°C with the tubes held horizontally in a reciprocal shaker operating at 150 rpm. Cell yields were determined by measuring the optical density of cultures at 600 nm with a Milton Roy Spectronic 20 colorimeter and converting the value to cell density by means of an experimentally determined standard curve. An increase of at least 50% in the cell yield above the "no-substrate" control yield after two successive transfers was considered evidence of substrate utilization.

The ability of TAM-DN1 to utilize acetate and succinate simultaneously was tested by using 500-ml screw-cap bottles (catalog no. 5636-00533; Bellco) to which an 18-mm anaerobe tube (see above) was fused. The bottles contained 100 ml of BYA medium supplemented with 10 mM succinate and were inoculated (1%, vol/vol) with an exponential-phase culture growing in BYA medium. Incubation was at 22 to 23°C in the CO_2-enriched hypoxic chamber (see above) with shaking at 250 rpm. Concurrent with growth measurements (see above), 1-ml samples of culture fluid were removed and centrifuged at 12,000 × *g* for 10 min, and the supernatant was filtered through a 0.2-μm-pore-size filter and stored at −20°C until analysis. Organic acids were analyzed by HPLC (Waters, Milford, MA) on an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad, Hercules, CA) at 23°C, with 4 mM H_2SO_4 as the eluent (0.6 ml/min). Organic acids were detected and quantified with a Waters 2487 UV detector operating at 210 nm and calibrated with homologous standards.

The oxygen tolerance of TAM-DN1 was determined from the ability of cells to grow in liquid medium or on plates under defined headspace concentrations of O_2. For growth in liquid, 5 ml of anoxic BYA medium was dispensed into 18-mm anaerobe tubes with butyl rubber stoppers containing a headspace consisting of air (21% [vol/vol] O_2) or 100% N_2. Into the tubes containing 100% N_2, air or pure oxygen was injected to obtain a final headspace concentration of O_2 of 0.5, 1, 2, 4, or 8% (after release of overpressure). The tubes were sterilized by autoclaving, inoculated (5%, vol/vol) with exponential-phase TAM-DN1 cells growing in BYA medium, and then incubated horizontally at 22 to 23°C with shaking at 150 rpm. Growth was monitored spectrophotometrically (see above). The duration of the lag phase was estimated as described by Lenski et al. (28). For growth on plates, 1.5% agar was incorporated into liquid BYA medium, which was then dispensed into Wolfe anaerobic agar bottle plates (4 ml/bottle) fitted with screw caps (catalog no. 2535-S0020; Bellco). The bottle plates were autoclaved and then placed on their sides to allow the medium to solidify. The bottle plates were then transferred into an anoxic chamber (10% H_2, 5% CO_2, 85% N_2; PlasLabs, Lansing, MI), uncapped, and streaked with an exponential-phase culture of TAM-DN1. Each bottle plate was then closed with a butyl rubber stopper held in place by a screw cap having a hole as an injection port. Pure, sterile O_2 was then injected into the bottle plates (average headspace volume, 60 ml) to obtain final concentrations of O_2 of 0.5, 1, 2, 4, 6, 8, 12, 16, and 21% in the headspace (after release of overpressure), after which the bottle plates were incubated in an upright position at 22 to 23°C.

The ability of strain TAM-DN1 to grow under anoxic conditions by fermentation or anaerobic respiration was tested by using 5 ml BYA medium that had been deoxygenated under a vacuum and added to 18-mm anaerobe tubes with butyl rubber stoppers under 100% N_2. After autoclaving, the BYA medium was supplemented with 10 mM (final concentration) glucose or one of the following potential electron acceptors from a sterile stock solution: potassium nitrate, potassium nitrite, sodium sulfate, or sodium fumarate. For some of the tubes containing fumarate, 30% hydrogen was included in the headspace. The positive controls were tubes of BYA medium supplemented with 2% O_2 by injection of air. The tubes were inoculated (2%, vol/vol) with an exponential-phase culture growing in BYA medium under hypoxia and then incubated at 22 to 23°C horizontally with shaking at 150 rpm.

Enzyme assays. *S. acetivorans* TAM-DN1 was grown in 100 ml BYA medium in 500-ml sidearm bottles (see above) under an hypoxic atmosphere with shaking at 250 rpm. At mid-log phase (at which growth was known to be acetate dependent, with ca. 5×10^8 cells/ml), cells from an entire culture were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, washed with 20 ml sonication buffer (10 mM EDTA, 50 mM Tris-HCl; pH 7.0), recentrifuged, and then resuspended in 10 ml of the same buffer. Cells were disrupted in an ice water bath by sonication (three 30-s treatments) with a Branson model 450 Sonifier (power setting, 5; 50% duty cycle) equipped with a 0.5-in. threaded-body step horn with a flat tip. The sonicate was centrifuged at 12,000 × *g* for 60 min at 4°C, and the resulting supernatant liquid, considered to be the crude cell extract, was transferred into a Slide-A-Lyzer dialysis cassette (capacity, 3 to 12 ml; 3,500-molecular-weight cutoff; Pierce, Rockford, IL) and dialyzed for 12 h in 2 liters of dialysis buffer (50 mM Tris-HCl, pH 7.0). The dialyzed crude extract was removed from the cassette and used immediately for assays of enzyme activities.

Catalase activity was assayed as described by Beers and Sizer (6). Superoxide dismutase activity was measured by the xanthine/xanthine oxidase-cytochrome *c* reduction method (20). NAD(P)H oxidase and peroxidase activities were assayed

TABLE 1. Total cultivable bacteria in gut homogenates of *Reticulitermes* species collected from different geographical locations

Termite	Origin	Total CFU(10^5)[a]	
		CO_2-enriched air[b]	Hypoxia[c]
R. flavipes	Dansville, MI	9.2 ± 1.0	12.0 ± 2.4
	Spring Arbor, MI	6.2 ± 2.7	6.4 ± 1.0
	Janesville, WI	10.0 ± 0.8	14.0 ± 1.7
	Raleigh, NC	12.0 ± 1.5	13.0 ± 2.6
R. santonensis	Forêt de la Coubre, France	15.0 ± 5.4	17.0 ± 3.2

[a] CFU per gut equivalent on plates of ACY medium. The values are means ± standard deviations ($n = 3$).
[b] 5% CO_2 and 95% air.
[c] 2% O_2, 5% CO_2, and 93% N_2.

as described previously (48). The qualitative, colorimetric test used for cytochrome *c* oxidase has been described previously (46).

Microscopy. Phase-contrast micrographs were prepared from wet mounts on agar-coated slides (36). Images were captured with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a SPOT charge-coupled device digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

For scanning electron microscopy, late-exponential-phase cells of *S. acetivorans* TAM-DN1 were harvested and then resuspended in 0.1 M sodium phosphate buffer (pH 7.4) and mixed with an equal amount of 4% glutaraldehyde. After fixation for 30 min, 1 drop of the fixed cells was placed on a poly-L-lysine-coated coverslip. The suspension was allowed to settle for 10 min, and the coverslip was washed with distilled water and placed in graded ethanol series (25, 50, 75, and 95% ethanol and then three changes of 100% ethanol) for not less than 5 min for each stage. Samples were then critical point dried in a Balzers critical point dryer (BAL-TEC AG, Liechtenstein) using liquid carbon dioxide as the transitional fluid. The glass coverslips were mounted on aluminum stubs, and samples were coated with gold (thickness, approximately 20 nm) by using an Emscope model SC 500 sputter coater (Quorum Technologies, East Sussex, United Kingdom). Electron micrographs were obtained with a JEOL 6400V scanning electron microscope with a LaB6 emitter (JOEL-USA, Inc., Peabody, MA).

For transmission electron microscopy, exponential-phase cells were harvested and resuspended in 0.1 M cacodylate buffer (pH 7.4) to which an equal amount of 4% glutaraldehyde was added. Following a 30-min fixation, cells were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h and dehydrated using a graded acetone series (30, 50, 70, 85, and 95% acetone for 15 min each and three 15-min 100% acetone steps). The cells were then embedded in Poly/Bed 812 (Polysciences, Warrington, PA), and 70-nm sections were cut. Sections were stained with uranyl acetate, followed by lead citrate (each at a concentration of 2% in 50% ethanol). Electron micrographs were obtained with a JEOL 2200FS 200-kV field emission transmission electron microscope.

Other procedures. Protein was quantified by the bicinchoninic acid method (47) with bovine serum albumin as a standard, and the final cell yields, expressed as dry weight, were based on the assumption that protein constitutes 55% of cell dry weight (32).

Accession numbers. The partial 16S rRNA gene sequences of all isolates and clones obtained in this study have been deposited in the EMBL, GenBank, and DDJB nucleotide sequence databases under accession numbers EF212897 through EF213020. Specific accession numbers are given below. *S. acetivorans* type strain TAM-DN1 has been deposited in the ATCC and DSMZ culture collections under accession numbers BAA-1483 and DSM 19021, respectively.

RESULTS AND DISCUSSION

Enumeration and isolation of putative O_2-consuming organisms. For *R. flavipes* collected from Dansville, MI, the total number of CFU on ACY isolation medium was about 30% higher for plates incubated under CO_2-enriched hypoxia (5% CO_2, 2% O_2, 93% N_2) than for plates incubated under 5% CO_2-enriched air (Table 1). Although not statistically significant with the number of experiments performed ($n = 3$), the consistent increase in CFU seen with hypoxic incubation coincided with the appearance of a colony type whose morphology was distinct enough to be easily differentiated from the other types (Fig. 1). Phase-contrast microscopy of cells comprising such colonies revealed that they were thin, nonmotile rods that were 0.5 by 5 μm (Fig. 2A). Scanning and transmission electron microscopy of representative strain TAM-DN1 revealed a gram-negative-type cell wall that included an outer membrane (Fig. 2B and C). Cells also possessed intracellular inclusions that resembled inclusions of the carbon and energy reserve polymer poly-β-hydroxybutrate (Fig. 2C). Such isolates could be subcultured only on plates incubated in hypoxia, and they displayed robust colony growth only if acetate was included in ACY medium.

Preliminary phylogenetic identification of 19 randomly selected strains (strains with designations beginning with "TAM") purified from such colonies revealed that their 16S rRNA genes were 99.7% similar to each other and grouped within the family *Neisseriaceae* in the phylum β-*Proteobacteria*. However, they were only 94.1% similar to their closest known relative, *Eikenella corrodens* (Fig. 3). The mean ± standard deviation in situ abundance of TAM strains in guts of Dansville-collected *R. flavipes*, based on plate counts of TAM-type colonies, was $1.0 \times 10^5 \pm 0.2 \times 10^5$ CFU · gut^{-1} ($n = 3$). This was in close agreement with the $2.2 \times 10^5 \pm 0.5 \times 10^5$ cells · gut^{-1} ($n = 2$) estimated by dilution-to-extinction PCR, based on the determined 3.3-Mb genome size and four *rrs* (16S rRNA-encoding gene) copies present in strain TAM-DN1 (data not shown). Therefore, TAM strains were estimated to comprise between 2 and 7% of the total prokaryotic population in *R. flavipes* guts, based on previously determined direct

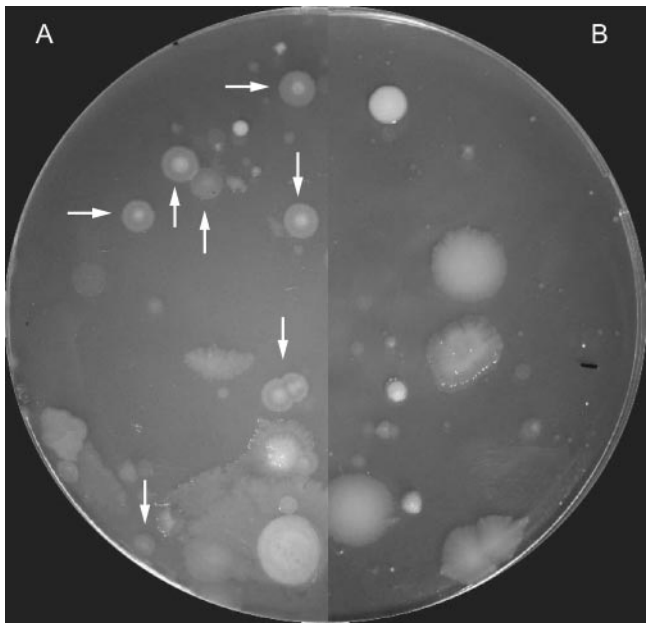

FIG. 1. Composite photograph of colonies on spread plates of ACY isolation medium incubated under CO_2-enriched hypoxia (A) or CO_2-enriched air (B). The arrows indicate a distinctive colony type seen only on plates incubated under hypoxia. Plates were inoculated with a dilution of gut homogenate of *R. flavipes* collected in Dansville, MI.

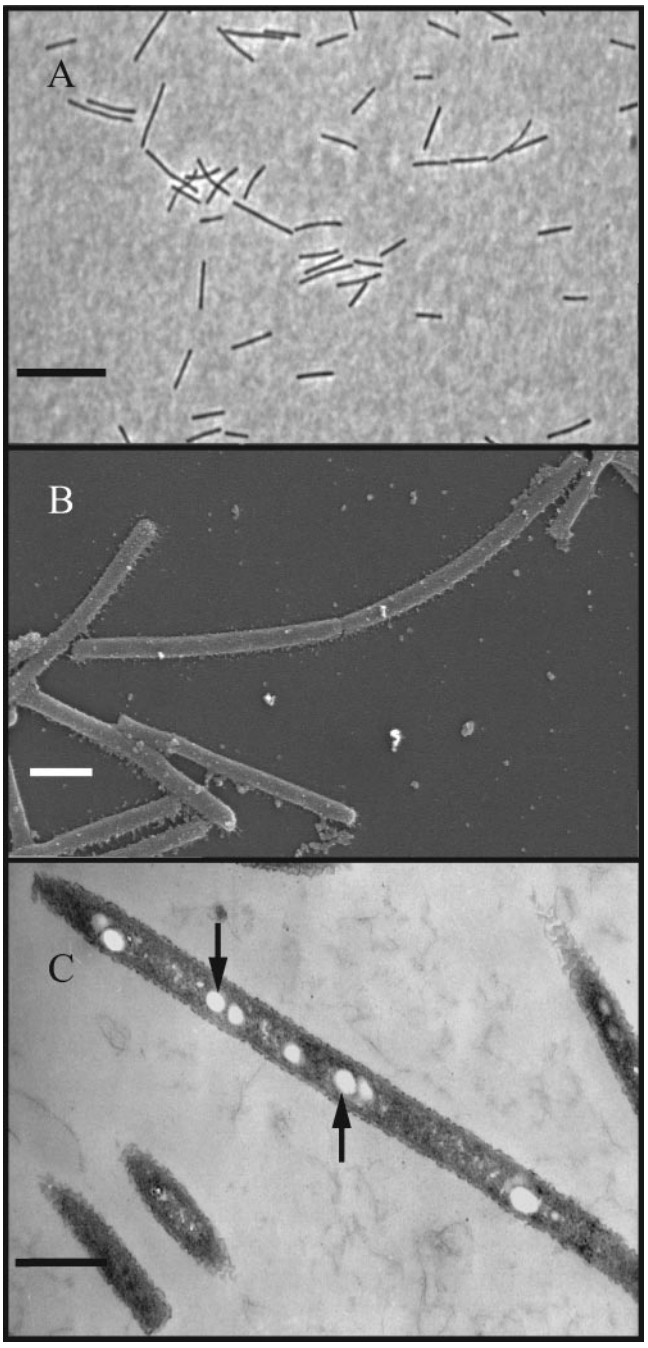

FIG. 2. Phase-contrast (A), scanning electron (B), and transmission electron (C) micrographs of strain TAM-DN1. The arrows in panel C indicate intracellular granules that resemble poly-β-hydroxybutyrate. Scale bars = 10 μm (A) and 1 μm (B and C).

microscopic counts (43, 51). Dilution-to-extinction PCR experiments also indicated that TAM strains were associated only with hindguts, not with degutted bodies or portions of midgut that are usually attached to extracted hindguts. Owing to their relative abundance, their confirmed association with hindguts, their phylogenetic novelty, and their apparent ability to oxidize acetate only under hypoxic conditions, suggesting that they might be true microaerophiles adapted to life within the hypoxic peripheral region of acetate-rich *R. flavipes* hindguts, strains of these bacteria were chosen for closer examination, and representative strain TAM-DN1 was subjected to the most detailed characterization.

The bulk of the other colonies on isolation plates were members of the *Streptococcaceae*, *Enterococcaceae*, and *Enterobacteriaceae* (Fig. 3); however, these colonies occurred with roughly equal frequency on plates incubated under CO_2-enriched air or hypoxia. Not surprisingly, only colonies of *Enterobacteriaceae* displayed more robust growth on acetate-containing ACY medium. Members of the *Streptococcaceae* and *Enterococcaceae* are capable of growth in the presence of oxygen and can use O_2 as an electron acceptor (51), but they are not known to oxidize acetate as an energy source. Their appearance on isolation plates was almost certainly supported by energy-yielding nutrients (e.g., sugars and amino acids) present in the yeast extract and Casamino Acids components of ACY medium.

With the exception of the TAM strains, the isolates reported above were typical of organisms isolated from termite guts previously or whose 16S rRNA genes were represented in clone libraries prepared from various termite species, including *R. flavipes* (1, 5, 19, 23, 24, 37, 38, 43, 44, 51, 56). A 16S rRNA gene clone closely related to that of the TAM isolates was obtained from a gut wall fraction prepared from *R. santonensis* (56). Otherwise, these organisms have not previously been isolated from termite guts, probably because most previous isolation efforts employed incubations in air or under anoxia.

Recent molecular phylogenetic surveys of termite gut microbes have also revealed the presence of organisms grouping within phyla whose members are not well represented in culture, including the phyla *Verrucomicrobia*, *Acidobacteria*, and *Planctomyces* and the candidate phylum *Endomicrobia* (24, 34). As such organisms might also be important for O_2 consumption in situ, we screened for their presence in earlier isolation attempts with gut homogenates of *R. flavipes* (from Dansville, MI). By using "plate wash PCR," members of the *Verrucomicrobia* (strain prefix TAV) and the *Acidobacteria* (strain prefix TAA) were isolated and described (49) and are included in Fig. 3. Results of dilution-to-extinction PCR analyses (data not shown) indicated that members of the *Verrucomicrobia* were associated entirely with, and presumably reside in, the gut and were not associated with degutted bodies. By contrast, members of the *Acidobacteria* were associated with guts and degutted bodies at roughly equal levels, suggesting that they may be transients acquired from soil. In any case, the gut-associated members of the *Verrucomicrobia* and *Acidobacteria* in *R. flavipes* appeared to be minor members of the prokaryotic community, representing only about 0.1 and 0.02% of the direct microscopic counts, respectively. *Endomicrobia* were not detected on isolation plates.

Autochthony of the TAM strain community. To determine whether TAM strains were autochthonous members of the *R. flavipes* community, 16S rRNA gene clone libraries were prepared from termite guts, termite nest soil, and non-termite-associated forest soil by using PCR with TAM strain-targeting primer sets, and the libraries were analyzed by the LIBSHUFF and SONS programs (40, 41). Results revealed that the forward primer TAM203f was not entirely specific for TAM

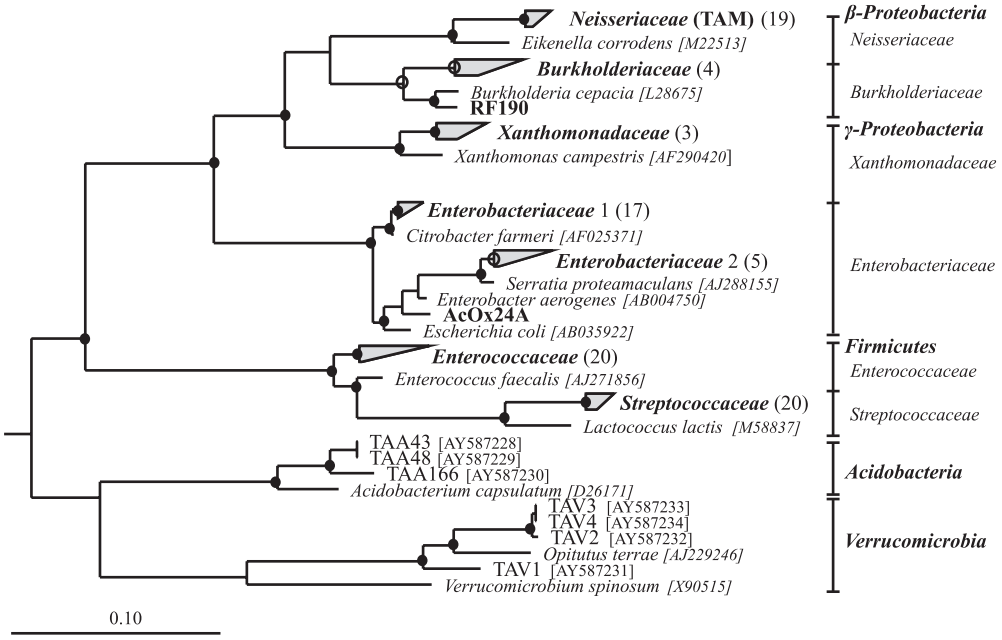

FIG. 3. Maximum likelihood-based 16S rRNA gene phylogeny of isolates obtained from *R. flavipes* collected from Dansville, MI (based on 405 shared nucleotide positions). Isolates obtained in this study are indicated by boldface type; TAA and TAV strains were from a previous study in our laboratory (49). Closely related isolates are condensed and indicated by gray blades showing the breadth and depth of phylogenetic diversity, with the number of isolates indicated in parentheses. Branch points with >75% support are indicated by a filled circle; branch points with 50 to 74% support are indicated by an open circle. GenBank accession numbers for reference species (in italics) and previously reported isolates from guts of *R. flavipes* (not italicized) are shown in brackets. Other GenBank accession numbers for isolates are as follows: TAM strains, EF212897 to EF212915; *Burkholderiaceae*, EF212928 to EF212932; *Xanthomonadaceae*, EF212933 to EF212935; *Enterobacteriaceae*, EF212936 to EF212958; *Enterococcaceae*, EF212959 to EF212978; and *Streptococcaceae*, EF212979 to EF212998. *Aquifex pyrophilus* (GenBank accession no. M83548) was used as an outgroup (not shown). Scale bar = 0.1 change per nucleotide.

strains; it also supported amplification of 16S rRNA genes from hitherto unknown β-*Proteobacteria* present in nest soil and forest soil (Fig. 4). Nevertheless, analyses clearly indicated the presence of a closely related (average 16S rRNA gene identity, 98.4%) TAM strain community in guts that was phylogenetically distinct from 16S rRNA genes amplified from termite nest soil or non-termite-associated forest soil. A Yue-Clayton nonparametric maximum likelihood similarity estimate revealed that clones from the forest and nest soils shared 23% ± 9.7% similarity, clones from forest soil and termite guts shared 7.5% ± 4.5% similarity, and all nest soil and termite gut clones shared 75.8% ± 10.7% similarity (means ± standard errors). Although clones from the termite gut and surrounding nest soil had a relatively high level of similarity, LIBSHUFF

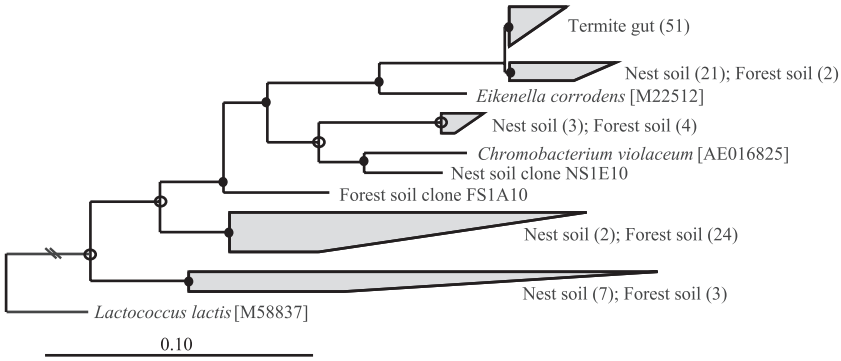

FIG. 4. Maximum likelihood-based phylogeny of PCR-amplified 16S rRNA gene clones obtained with a TAM strain-targeting primer set. Clones were obtained from *R. flavipes* guts (51 clones), nest soil (34 clones), and adjacent but non-termite-associated forest soil (34 clones) from the Dansville, MI, site. The phylogeny is based on 503 aligned nucleotides. Groups of closely related clones are condensed and indicated by gray blades, with the number of clones indicated in parentheses. Branch points with >75% support are indicated by filled circles. Branch points with 50 to 74% support are indicated by open circles. Sequences representative of the phylogenetic distribution of the clones have been deposited in the GenBank database under accession numbers EF212999 to EF213008 (forest soil), EF213009 to EF213016 (nest soil), and EF213017 to EF213020 (*R. flavipes* guts). Accession numbers of reference species are shown in brackets. Scale bar = 0.1 change per nucleotide.

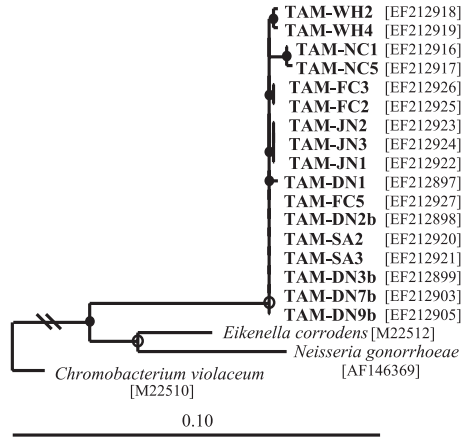

FIG. 5. Maximum likelihood-based 16S rRNA gene phylogeny of termite gut "TAM" strains having various origins. The phylogeny is based on 845 shared nucleotide positions. The isolates were from *R. flavipes* collected in Dansville, MI (strain designations containing DN), Raleigh, NC (NC), Spring Arbor, MI (SA), and Janesville, WI (JN), and from *R. santonensis* collected in Forêt de la Coubre, France (FC); partial 16S rRNA gene clones were obtained from gut homogenates of *R. flavipes* collected in Woods Hole, MA (WH). *Lactococcus lactis* (GenBank accession no. M58837) was used as an outgroup (not shown). Branch points with >75% support are indicated by filled circles. Branch points with 50 to 74% support are indicated by open circles. GenBank accession numbers are shown in brackets. Scale bar = 0.1 change per nucleotide.

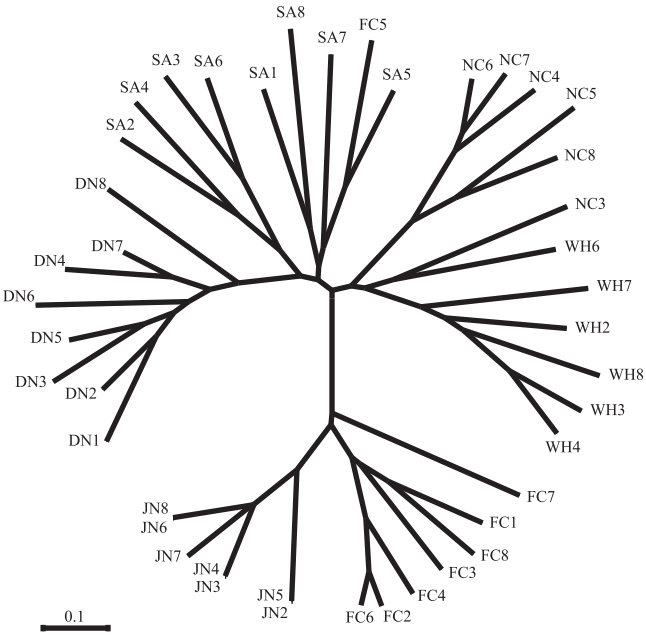

FIG. 6. Neighbor-joining analysis of five microsatellite loci (44 alleles) for 43 individual worker termites collected from different geographical locations. The two-letter source designations for *R. flavipes* and *R. santonensis* are the same as those described in the legend to Fig. 5.

revealed they were statistically different ($P = 0.001$). These results are consistent with the concept that TAM strains in hindguts of *R. flavipes* (from Dansville, MI) are autochthonous.

Geographical distribution. The apparent autochthony of TAM strains in *R. flavipes* from Dansville (see above) prompted a search for similar bacteria in geographically and/or genetically distinct populations of *R. flavipes* and other termite species. Like plating experiments with Dansville-collected specimens, plating experiments with *R. flavipes* collected from different sites in the United States, as well as with *R. santonensis* collected in France, yielded a consistently greater number of CFU when ACY medium isolation plates were incubated under hypoxia (Table 1). Again, the increase coincided with the appearance of TAM-like strains recognizable by their distinctive colony and cell morphology (Fig. 1 and 2A), which facilitated the isolation of pure-culture representatives. Based on 845 shared 16S rRNA nucleotide positions, these new TAM isolates, as well as TAM-like rRNA gene sequences amplified from gut homogenates of termites collected in Woods Hole, MA, were closely related (99.8% 16S rRNA gene identity) (Fig. 5).

To assess the genetic relatedness of the source termites themselves, five separate microsatellite DNA loci present in six to eight individual worker larvae from each of the six different sites were amplified by PCR and analyzed. In all, 44 distinct alleles were recognized for the five different loci. Clustering analysis revealed that almost all alleles grouped according to geographical origin and hence were from genetically distinct populations, presumably as a result of divergent evolution over long periods of time (Fig. 6). Interestingly, alleles from *R. flavipes* collected in Janesville, WI, grouped closely with alleles of *R. santonensis* collected in France, supporting the recent conclusion that *R. flavipes* and *R. santonensis* are synonymous (3). Within a collection site, however, there was also a relatively high degree of variation. This may have been due to the fact that the termites used for analysis were worker larvae collected while they were foraging, and termites from several genetically different colonies may have been foraging together on the same food source. No TAM-like isolates (or TAM-like 16S rRNA gene sequences PCR amplified from gut homogenates) were obtained from the Formosan termite, *C. formosanus* Shiraki, despite the fact that it is also a subterranean wood-feeding termite in the same family (Rhinotermitidae) as *Reticulitermes*, or from the western dampwood termite, *Z. angusticollis* (Hagen) (Termopsidae). These results further support the concept that TAM strains are autochthonous in *Reticulitermes* and suggest that these symbionts occupy an important ecological niche within hindguts.

Relationship to oxygen. One of the most striking and readily apparent properties of TAM strains was their robust growth under hypoxia but their inability to grow anaerobically (by fermentation or anaerobic respiration [see below]) or in air (or CO_2-enriched air). Accordingly, the O_2 sensitivity of TAM-DN1 was determined by measuring growth on or in acetate-containing solid or liquid BYA medium under various concentrations of oxygen. On solid medium, where cells are in direct contact with the headspace gas phase, TAM-DN1 was unable to form colonies under anoxia or with O_2 concentrations higher than 4% (Fig. 7A). At O_2 concentrations of 0.5% (not shown) and 4%, there were fewer colonies and the colonies did not extend as far along the streak line as the colonies grown with headspace O_2 concentrations of 1.0% (not shown) and 2.0%. Therefore, based on this qualitative analysis, the optimum O_2 concentration for growth on solid medium appeared

FIG. 7. Tolerance of *S. acetivorans* strain TAM-DN1 to oxygen. (A) Growth on solid BYA medium in Wolfe bottle plates with a headspace containing no oxygen (plate 1), 2% (vol/vol) oxygen (plate 2), 4% (vol/vol) oxygen (plate 3), and 6% (vol/vol) oxygen (plate 4). The same inoculum was used for each plate, and streaking was from the bottom of the bottle plate toward the mouth. (B) Growth in liquid BYA medium under an atmosphere containing no oxygen (tube 1), 2% (vol/vol) oxygen (tube 2), 4% (vol/vol) oxygen (tube 3), 8% (vol/vol) oxygen (tube 4), and 16% (vol/vol) oxygen (tube 5).

to be around 1 to 2% (vol/vol). By contrast, in broth cultures, where oxygen diffusion to individual cells is limited by a relatively large volume of liquid, a 1% (vol/vol) inoculum of 2% O_2-grown TAM-DN1 was able to initiate growth at O_2 concentrations as high as at least 16% (Fig. 7B) but not under air (21% O_2) (not shown) or under anoxia. However, when cultures were grown with oxygen limitation (≤8% [vol/vol] O_2), the duration of the lag phase increased linearly with the initial O_2 concentration, as did the final cell yield (Fig. 8). This observation indicates that within limits, cells can adapt to deal with elevated concentrations of O_2 (or reactive oxygen species generated by metabolism in the presence of elevated concentrations of O_2), and the time required for the adaptation is proportional to the O_2 concentration to which the cells are initially exposed. Once adapted, however, they apparently are able to consume all of the O_2 for energy generation, which is translated into a proportional increase in cell biomass. The biomass yields of TAM-DN1 for initial oxygen concentrations of 2, 4, and 8% were 19.2 ± 1.5, 22.3 ± 4.5, and 20.2 ± 5.2 g per mol oxygen consumed, respectively (means ± standard deviations; $n = 3$; corrected for the small amount of growth in medium without acetate). These biomass yields are similar to

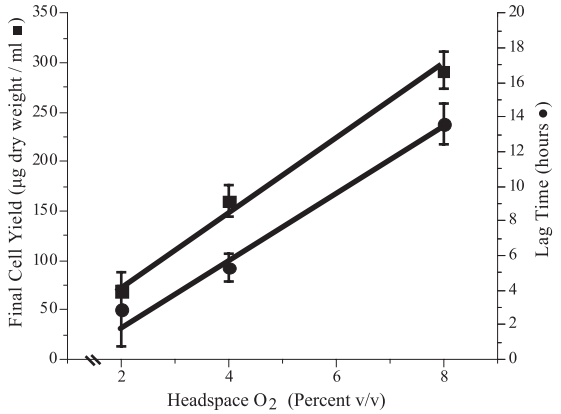

FIG. 8. Final cell yield and lag time of *S. acetivorans* strain TAM-DN1 grown under oxygen limitation (≤8% [vol/vol] O_2) in liquid medium. The error bars indicate standard deviations ($n = 3$).

the value reported for other bacteria grown on acetate (21.4 g per mol oxygen consumed) (35).

The microaerophilic nature of TAM-DN1 prompted assays for enzymes that protect against reactive oxygen species. When grown under 2% O_2, TAM-DN1 expressed catalase (99 U · mg protein^{-1}) and superoxide dismutase (32 U · mg protein^{-1}). No NAD(P)H oxidase or peroxidase activity was detected. Qualitative tests indicated that the cells were also oxidase positive, implying that they possessed a *c*-type cytochrome.

Overall, these observations indicate that TAM-DN1 and related strains are obligate microaerophiles and probably reside in the hypoxic region of hindguts, on or near the gut wall. Expression of oxyprotective enzymes, such as catalase and superoxide dismutase, as well as the ability to tolerate and adapt to headspace O_2 concentrations up to 16% in liquid media, may be important during trophallactic (termite-to-termite) transfer and colonization of guts of newly hatched larvae and recently molted colony mates, whose microbiota is essentially absent and drastically reduced, respectively, and whose O_2 content is almost certainly substantially higher than the O_2 content when the termites are fully colonized.

Substrate utilization by, and growth characteristics of TAM-DN1. The substrates utilized as energy sources by strain TAM-DN1 for growth included acetate, acetyl-acetate, succinate, butyrate, glutamate, glutamine, fumarate, and one or more components present in Casamino Acids (data not shown). Of these, the compounds known to be present in termite hindgut fluid include acetate (in situ concentration, 60 to 80 mM) and butyrate (2 mM) (33), as well as a number of free amino acids, including glutamate (1.7 mM) and glutamine (1.0 mM) (21, 45).

HPLC analysis of substrate utilization by TAM-DN1 revealed that cells were able to utilize acetate and succinate simultaneously when equimolar amounts (10 mM) of these two compounds were present in the culture medium (data not shown), and the final cell yields were twice as high (2.3×10^9 cells/ml) when the strain was grown on both substrates than when it was grown on acetate alone (1.2×10^9 cells/ml). The rate of acetate utilization by TAM-DN1 was 0.09 μmol · min^{-1} · mg protein^{-1} when succinate was present in the medium and 0.12 μmol · min^{-1} · mg protein^{-1}

without added succinate. These results suggest that in situ, cells do not need to be restricted to the substrate typically present at the highest concentration (acetate) but may simultaneously be able to use other oxidizable substrates available at lower concentrations. This ability may also be important when bacteria are colonizing newly hatched or recently molted colony mates.

The substrates not utilized for growth included cellobiose, maltose, glucose, xylose, arabinose, lactate, pyruvate, citrate, formate, propionate, 2-oxoglutarate, malate, maleic acid, benzoate, threonine, and glycine. Moreover, TAM-DN1 was not able to grow anaerobically on acetate using fumarate (with or without H_2 in the headspace), nitrate, nitrite, or sulfate as electron acceptors, nor was TAM-DN1 able to grow fermentatively on glucose.

In BYA liquid medium under 2% O_2, TAM-DN1 grew at 22, 30, and 37°C, but the shortest generation time (3.4 h) was observed at 30°C. No growth occurred at 42°C.

CO_2 was not required for growth, but the onset of growth in BYA medium was generally more rapid under a CO_2-enriched hypoxic atmosphere (i.e., 2% O_2, 5% CO_2, 93% N_2), such as that provided within the flexible vinyl glove box. However, the effect of CO_2 on the growth rate and final cell yield has not been tested yet. Stimulation by CO_2 may reflect a biosynthetic need for CO_2 fixation, which would be more obvious at low cell densities before the CO_2-producing activity per ml of culture is sufficient to satisfy the demand.

Proposal for a new taxon, *Stenoxybacter acetivorans* gen. nov., sp. nov. The molecular and physiological properties of TAM-DN1 and related strains are sufficiently distinct from those of any other known bacterium to warrant their classification as a new genus and species within the phylum β-*Proteobacteria*. The relatively distant (16S rRNA gene identity, 94.1%) evolutionary relationship to their closest named relative, *E. corrodens*, coupled with their obligately microaerophilic phenotype, relatively narrow substrate utilization spectrum, and apparent autochthony in guts of *Reticulitermes* species are fully consistent with this notion. Accordingly, we propose the name *Stenoxybacter acetivorans* for these bacteria (see below). In a companion paper (55), we report on experiments to test the hypotheses that *S. acetivorans* cells reside in the peripheral, hypoxic region of hindguts of *R. flavipes* and use acetate to fuel their O_2-consuming respiratory activity in situ.

Description of *Stenoxybacter* gen. nov. *Stenoxybacter* gen. nov. (Sten.o.xy.bac′ter. Gr. adj. *stenos*, narrow; Gr. adj. *oxys*, acid or sour and in combined words indicating oxygen [N.L. *oxygenium*]; N.L. masc. n. *bacter*, rod or bacterium; N.L. masc. n. *Stenoxybacter*, rod with a narrow oxygen range). The genus description is, at present, the same as that for the type species, *Stenoxybacter acetivorans*.

Description of *Stenoxybacter acetivorans* sp. nov. *Stenoxybacter acetivorans* sp. nov. (a.ce.ti.vo′rans. L. neut. n. *acetum*, vinegar or acetic acid; L. pres. part. *vorans*, devouring; N.L. pres. part. *acetivorans*, acetate consuming). Cells are rods (0.5 by 5 μm) with a gram-negative type of cell wall morphology that includes an outer membrane. Nonmotile. Strict aerobe; obligate microaerophile. Growth occurs on solid media in atmospheres containing 0.5 to 4.0% (vol/vol) oxygen (optimum oxygen concentration, 1 to 2%). No growth under ≥6% oxygen or under anoxia, either by fermentation or by anaerobic respiration with fumarate, nitrate, nitrite, or sulfate as the electron acceptor. On solid BYA medium, colonies are 4 to 5 mm diameter, have a crenate, low, convex morphology, and have a "ringed" appearance, with a cream-colored center surrounded by a ring of light orange, followed by a light blue edge. In liquid BYA medium, cells can adapt to headspace concentrations of oxygen up to 16%, but there are prolonged lag phases whose durations are proportional to the initial oxygen concentration. Cells possess cytochrome *c* oxidase (qualitative test), catalase, and superoxide dismutase enzyme activities. Growth occurs in liquid media at temperatures between 22 and 37°C (optimum temperature, 30°C); no growth occurs at 42°C. Growth onset is stimulated by inclusion of 5% CO_2 in the incubation atmosphere. Substrates utilized as energy sources include acetate, acetyl-acetate, succinate, butyrate, glutamate, glutamine, fumarate, and one or more components present in Casamino Acids. Cellobiose, maltose, glucose, xylose, arabinose, lactate, pyruvate, citrate, formate, propionate, 2-oxoglutarate, malate, maleic acid, benzoate, threonine, and glycine are not utilized. The size of the genome of strain TAM-DN1 is 3.3 Mb; the genome has a G+C content of 53.7 ± 0.3 mol% and possesses four 16S rRNA gene copies. The nucleotide sequence of the 16S rRNA gene of strain TAM-DN1 has been deposited in the GenBank database under accession number EF212897.

The type strain, isolated from guts of *R. flavipes* collected in Dansville, MI, is TAM-DN1; it has been deposited in the American Type Culture Collection, Manassas, VA, as strain BAA-1483 and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as strain DSM 19021. Similar strains have also been isolated from *R. flavipes* collected in Spring Arbor, MI, Janesville, WI, and Raleigh, NC, as well as from *R. santonensis* collected in Forêt de la Coubre, France.

ACKNOWLEDGMENTS

We thank E. Vargo for providing *R. flavipes* from North Carolina, A. Brune for providing *R. santonensis*, and N.-Y. Su for providing *C. formosanus*. We are also grateful to J. McKinlay for help with HPLC analyses of organic acids, K. Kim for determination of the genome sizes of TAM-DN1 and TAV-1, T. M. Schmidt and K. H. Friderici for advice on PCR primer design and microsatellite DNA analyses, C. Flegler and A. Pastor-Lecha for scanning electron and transmission electron microscopy, and H. G. Trüper for advice on nomenclature, etymology, and construction of Latinized names.

This research was supported by grants from the National Science Foundation (grant IBN-0114505) and Michigan State University Agricultural Experiment Station to J.A.B. and by an MSU College of Natural Science Recruiting Fellowship to J.T.W.

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