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# Nitrogen-Fixing *Enterobacter agglomerans* Isolated from Guts of Wood-Eating Termites[1]

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Two strains of facultatively anaerobic, $N_2$-fixing bacteria were isolated from guts of *Coptotermes formosanus* and identified as *Enterobacter agglomerans*. The deoxyribonucleic acid base composition of isolates was 52.6 and 53.1 mol% guanine plus cytosine. Both isolates and a known strain of *E. agglomerans* carried out a mixed acid type of glucose fermentation. $N_2$ fixation by *E. agglomerans* was inhibited by $O_2$; consequently, $N_2$ served as an N source only for cells growing anaerobically in media lacking a major source of combined N. However, peptone, $NH_4Cl$, or $KNO_3$ served as an N source under either aerobic or anaerobic conditions. It was estimated that $2 \times 10^2$ cells of *E. agglomerans* were present per termite gut. This value was 100-fold lower than expected, based on $N_2$ fixation rates of *E. agglomerans* in vitro and that of the intact termites. However, low recoveries of *E. agglomerans* may be related to the marked decrease in $N_2$ fixation rates observed when intact termites or their extracted guts were manipulated for the isolation of bacteria. It was concluded that the $N_2$-fixing activity of *E. agglomerans* may be important to the N economy of *C. formosanus*.

In 1973 Breznak et al. (7) demonstrated $N_2$ fixation in termites by using the acetylene ($C_2H_2$) reduction assay. These workers showed that the activity was associated with the termite gut, could be modulated by the amount of combined N in the diet of the termites and could be abolished by feeding the insects antibacterial drugs, indicating that termite gut bacteria mediated $N_2$ fixation. It was suggested that $N_2$-fixing bacteria or their metabolic products might be important as an N source for some termites since the food (wood) of the insects is relatively low in combined N. Benemann (4) also observed $N_2$ fixation in termites and reported variations in $C_2H_2$-reducing activity between different groups of *Kalotermes minor*. Recently, Breznak (6) found that $C_2H_2$-reducing activity of *Coptotermes formosanus* can vary over 200-fold, with high rates being exhibited by young, growing larvae. In fact, it was estimated that the amount of $N_2$ fixed by young larvae could, over the period of a year, allow the termites to double their N content if the fixation rate remained constant.

These findings and the suggestion that bacterial $N_2$ fixation might be important to some termites during their development (4, 6, 7) prompted a search for the organisms involved. The results of such an endeavor constitute the substance of the present paper.

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## MATERIALS AND METHODS

**Termites.** Formosan subterranean termites, *C. formosanus* Shiraki, were collected in the vicinity of Lake Charles, La., and maintained in the laboratory in the form of termite-infested wood. Externally undifferentiated larvae beyond the second instar (i.e., worker termites; 23) were used. The average fresh weight of workers was 2 mg. The same group of termites was used for all experiments.

**Isolation of *Enterobacter agglomerans*.** A successful enrichment medium consisted of equal portions of the following two solutions, which were sterilized separately. Solution 1 contained (milligrams per 100 ml of distilled water): glucose, ribose, glycerol, and mannitol, 800 each; $MgSO_4 \cdot 7H_2O$, 100; NaCl, 2; $FeSO_4 \cdot 7H_2O$, 3; $Na_2MoO_4 \cdot 2H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 13; sodium thioglycolate, 100; and resazurin, 0.2. Solution 2 contained 1% (vol/vol) of a vitamin solution (34) in 0.1 M potassium phosphate buffer, pH 7.0. The medium was prepared under $N_2$-$CO_2$ (95:5), using strict anaerobic techniques (22), and was contained in anaerobic culture tubes (Hungate type; Bellco Glass, Inc., Vineland, N.J.) at a volume of 5 ml/tube. The final pH of the medium was 6.9. Solid medium was prepared by incorporating 1.5% Ionagar no. 2 (Colab Laboratories, Inc.,

[1] Journal article no. 7760 from the Michigan Agricultural Experiment Station.

Chicago Hts., Ill.) and omitting thioglycolate.

Workers of *C. formosanus* were held in a sterile petri dish and irradiated for 15 s with a germicidal lamp (30 W; General Electric Co., Schenectady, N.Y.) positioned 45 cm from the insects. Termites were then transferred to an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.; 2), and their guts were removed by using sterile forceps. Ten guts were placed in a small tissue homogenizer containing 2.0 ml of sterile enrichment medium and homogenized for 1 to 2 min. A 0.1-ml amount of the homogenate (i.e., 0.5 gut equivalents) was inoculated into 4.9 ml of enrichment medium, and this constituted the $10^{-1}$ dilution. Thereafter, serial 10-fold dilutions were made up to $10^{-6}$. Syringe techniques (26) were used for all dilutions. Tubes were then secured horizontally in a gyratory water bath shaker (model G76; New Brunswick Scientific Co., New Brunswick, N.J.) operating at 176 rpm. The incubation temperature was 30°C.

The highest dilutions developing visible turbidity were tested for $C_2H_2$-reducing activity (see below). Positive cultures were again serially diluted to $10^{-8}$ and reincubated. Isolates were obtained from the second $10^{-8}$ dilution tubes by streaking roll tubes (22) and were considered to be pure cultures after four successive passages in roll tubes.

**Other bacterial strains.** Known strains of *E. agglomerans*, used for comparative purposes, were CDC 811-74 and CDC 156-74. The former strain belonged to biogroup G2, whereas the latter belonged to biogroup 2 (14). Both strains were obtained from W. H. Ewing, Center for Disease Control, Atlanta, Ga.

**Growth studies.** Basal medium GSV was used in all growth studies. Its composition was similar to that of the enrichment medium, except ribose, glycerol, mannitol, thioglycolate, and resazurin were omitted, and the glucose concentration was increased to 1%.

For anaerobic cultivation, GSV medium was prepared by boiling individual solutions for 10 min under a stream of $O_2$-free Ar or $N_2$, dispensing them into anaerobically maintained vessels, and combining appropriate solutions after heat sterilization. Usually, tubes containing 5 ml of medium were used. However, some experiments also made use of 1-liter Erlenmeyer flasks containing 300 ml of medium and equipped with serum stoppered sampling ports and gas ($N_2$) inlet and outlet tubes. For aerobic cultivation, media were prepared without anaerobic precautions and dispensed in 50-ml amounts into 300-ml-capacity Nephelo culture flasks equipped with a side arm (12 by 130 mm; Bellco Glass, Inc., Vineland, N.J.). Most cultures were incubated with shaking as described above. Anaerobic cultures in 1-liter Erlenmeyer flasks were vigorously stirred with a magnetic stirrer driving a stirring bar which was included in the culture vessel. The final pH of all media used in growth studies was 7.0 ± 0.1.

Media contained in tubes or Nephelo flasks were inoculated with 0.05% (vol/vol) of a broth culture containing between $1 \times 10^8$ and $4 \times 10^8$ cells/ml. A 1% inoculum was used for media in 1-liter Erlenmeyer flasks. Growth was measured turbidimetrically and by direct cell counts and in some cases also by viable cell counts and protein determinations. Turbidimetric measurements were made by using a Bausch & Lomb Spectronic 20 colorimeter operating at 660 nm. A Petroff-Hausser counting chamber was used for making direct cell counts. Viable cell counts were determined by diluting samples of culture fluid in nutrient broth (Difco Laboratories, Detroit, Mich.) and spreading 0.1 ml of appropriate dilutions (in triplicate) on plates of nutrient agar (Difco). Plates were then incubated aerobically at 30°C, and colonies were enumerated after 24 h. Protein was assayed with the Folin phenol reagent after treatment of samples with NaOH (19). Crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as a protein standard.

**Analyses of fermentation products.** Cells were grown under Ar in GSV medium in which 0.1% $NH_4Cl$ (sterilized separately as a 10% solution) was incorporated. Gaseous products were determined by use of a fermentation "train" (27). At the end of the fermentation, the medium was clarified (27) and the clarified fermentation liquor (CFL) was assayed directly for glucose (31), acetoin and diacetyl (33), and glycerol (28).

Ethanol was qualitatively identified and quantified in neutral volatile distillates (27) of CFL by gas chromatography. A Varian model 2440 gas chromatograph, equipped with an $H_2$ flame ionization detector, was used. The column was stainless steel (0.125 inch by 5 feet [ca. 0.32 by 152.4 cm]) containing Porapak Q (80/100 mesh; Waters Associates Inc., Milford, Mass.). $N_2$ was the carrier gas (30 ml/min), and temperatures were as follows: column, 170°C; injector and detector, 205°C each.

Neutral ether extracts (27) of CFL were assayed for 2,3-butanediol by gas chromatography as described above, except that temperatures were: column, 200°C; injector and detector, 250°C each. The limit of detection using this system was 0.5 μmol of butanediol per ml of neutral ether extract.

Organic acids were extracted from acidified CFL with ether (27) and were qualitatively identified and quantified by gas chromatography. A Varian model 1420 gas chromatograph, equipped with a thermal conductivity detector, was used. The column was stainless steel (0.125 inch by 6 feet [ca. 0.32 by 182.9 cm]) and contained 15% SP 1220-1% $H_3PO_4$ on Chromosorb W AW (100/120 mesh; Supelco, Inc., Belefonte, Pa.). Helium was the carrier gas (25 ml/min), and temperatures were: column, 135°C; injector and detector, 165°C each. Volatile fatty acids were applied to the column as ether solutions. Nonvolatile acids were first methylated and extracted into $CHCl_3$ (20) prior to application. Colorimetric assays were also used for the estimation of pyruvate and oxaloacetate (18) and lactate (3).

**Biochemical tests.** Most biochemical tests were performed by the methods of Edwards and Ewing (13). Sugar fermentation reactions were evaluated by using phenol red broth base (Difco) in which 1% of the test sugar was incorporated. Sugars were filter sterilized separately as 10% solutions, except esculin, which was sterilized with the broth base.

KCN sensitivity was determined as described by

Holding and Collee (21). Nitrate reduction was tested as described by Lennette et al. (25), using nitrate reduction broth.

**Determination of G+C content in DNA.** The mole percent guanine plus cytosine (G+C) in deoxyribonucleic acid (DNA) was determined by M. Mandel, using the buoyant density method (30).

**$C_2H_2$ reduction assays.** The ability of samples to reduce $C_2H_2$ to $C_2H_4$ was taken as presumptive evidence for $N_2$ fixation (29). $C_2H_4$ was measured by using flame ionization gas chromatography (29) and stainless-steel column (0.125 inch by 5 feet) containing Porapak N (80/100 mesh; Waters Associates, Inc.). $N_2$ was the carrier gas (30 ml/min), and the column temperature was 62°C.

$C_2H_2$-reducing activity in enrichment cultures was measured by injecting 0.3 $cm^3$ of $C_2H_2$ into tubes through the rubber septa and then reincubating cultures for 24 h before assaying for the presence of $C_2H_4$ in the gas phase. For growth studies, assays were performed by injecting culture samples into serum stoppered, Ar-filled vials at a volume of 2.5 to 5% of the vial capacity. $C_2H_2$ was then introduced in an amount yielding 0.05 atm of $C_2H_2$ in the gas phase, and samples were incubated for 1 h at 30°C on a reciprocating shaker operating at 88 oscillations/min. The reaction was terminated with 0.4 ml of 50% trichloroacetic acid/ml of sample, and the head space gas was immediately assayed for $C_2H_4$. $O_2$ inhibition studies were performed in a similar manner, except that $C_2H_2$ and varying amounts of $O_2$ were injected into Ar-filled vials before the introduction of cells. $O_2$ used in these studies was found to be free from detectable $H_2$ and CO by gas chromatographic analysis (24).

$C_2H_2$ reduction assays of intact termites or their extracted guts were performed in a manner similar to that described previously (7).

**Other experimental procedures.** The Gram stain was performed with Kopeloff reagents (20). Motility of cells was determined by direct observation of wet mounts of broth cultures, using phase-contrast microscopy.

For electron microscopy, cells were negatively stained (5) and examined with a Philips EM 300 electron microscope.

The pH of termite gut macerates was measured by placing 10 guts in 0.05 ml of deionized, glass-distilled water on a sheet of dental wax. Guts were then minced with a scalpel, and the pH of the resulting macerate was measured with a combination pH electrode (model 6020; Ingold Electrodes, Inc., Lexington, Mass.) adapted to a Radiometer pH meter (model 26; The London Co., Copenhagen, Denmark).

## RESULTS

**Isolation of bacteria.** In three independent experiments the highest dilutions of gut homogenate yielding growth in primary enrichments were the $10^{-3}$ dilutions (two experiments) and the $10^{-2}$ dilution (one experiment). These also showed $C_2H_2$-reducing activity and were tentatively judged to contain $N_2$-fixing cells. From $10^{-3}$ dilution tubes two isolates of putative $N_2$-fixing bacteria were obtained, and these (strains C-1 and C-2) were used for further study. For approximating the numbers of $N_2$-fixing bacteria per termite gut, the three series of diluted gut homogenates were considered as one series (three tubes per dilution) from a single termite population. Consultation of a three-tube most-probable-number table (10) indicated that approximately $2 \times 10^2$ $N_2$-fixing bacteria were present per termite gut.

Numerous additional attempts were made to obtain growth and $C_2H_2$ reduction activity in primary dilution tubes greater than $10^{-3}$. Aerobic enrichments were unsuccessful in this regard, as were the following modifications of the medium: (i) omission of thioglycolate or substitution of this compound with dithiothreitol (0.025%) or glutathione (0.05%); (ii) supplementation of the enrichment medium with 0.05% cholesterol plus 0.3% sodium succinate plus 0.03% yeast extract, 0.3% sodium succinate plus 0.005% casein hydrolysate, 0.1% ethanol plus 0.4% sodium fumarate plus 0.1% $Na_2SO_4$, or 0.001% yeast extract alone; (iii) substitution of sugars in the enrichment medium with 0.5% sodium pyruvate plus 0.5% sodium formate plus 0.03% glutamine, or 1% sodium lactate plus 0.03% glutamine plus 0.005% serine. Although some of these modified media (i.e., those containing amino acids or yeast extract) yielded visible growth at $10^{-6}$ dilution, none showed $C_2H_2$-reducing activity. Subcultures from such tubes, in media containing lesser amounts of combined nitrogen, either yielded no growth or sparse growth, but never $C_2H_2$ reduction.

**General characteristics of isolates.** Isolates were gram-negative, nonsporeforming, facultatively anaerobic rods, 0.5 by 1.0 $\mu$m in size. Cells were motile, and electron microscopy revealed the presence of peritrichous flagella. Cells grown aerobically on nutrient agar formed colonies that were 2 to 4 mm in diameter, round, and white to cream color.

Biochemical reactions of isolates are shown in Table 1. In addition, sugar fermentation tests indicated that both strains fermented arabinose, dulcitol, esculin, glucose, inositol, maltose, mannitol, rhamnose, salicin, sucrose, trehalose, and xylose. Only strain C-1 fermented lactose. Neither strain fermented adonitol, erythritol, raffinose, or sorbitol. These data indicated that isolates were strains of *E. agglomerans* (14). In accord with the biogroup designations of Ewing and Fife (14), strain C-1 was assigned to aerogenic biogroup G2 (indole negative, Voges-Proskauer negative), whereas

TABLE 1. *Biochemical reactions of strains C-1 and C-2*

| Test or substrate | Reaction[a] of strain: | |
|---|---|---|
| | C-1 | C-2 |
| TSI[b] | | |
| Slant | Acid | Acid |
| Base | Acid | Acid |
| Gas | + | − |
| $H_2S$ | − | − |
| Urease | − | − |
| Indole | − | − |
| Methyl red (37°C) | ± | − |
| Voges-Proskauer (37°C) | − | − |
| Citrate (Simmons) | + | + |
| KCN | − | − |
| Lysine decarboxylase | − | − |
| Ornithine decarboxylase | − | − |
| Arginine dihydrolase | − | − |
| Jordan's tartrate | − | − |
| Nitrate to nitrite | + | + |
| Oxidation-fermentation | F | F |
| Oxidase | − | − |

[a] +, Positive reaction; ±, weak positive reaction; −, negative reaction; F, fermentation.
[b] TSI, triple sugar iron agar (Difco).

strain C-2 was assigned to anaerogenic biogroup 2 (nitrate positive, indole negative, Voges-Proskauer negative).

**Fermentation products.** To buttress the contention that termite isolates were strains of *E. agglomerans* and to learn more about the physiology of the isolates, the products of glucose fermentation by growing cells were compared with those of a known strain of *E. agglomerans* (Table 2). Major fermentation products of all strains were $CO_2$, $H_2$, lactate, acetate, ethanol, and succinate. Small amounts of formate, pyruvate, oxaloacetate, acetoin, and diacetyl were also formed. Whereas strains C-1 and CDC 811-74 formed significant amounts of glycerol, strain C-2 formed only trace amounts of this compound. None of the strains formed 2,3-butanediol. Carbon recoveries, based on fermentation products alone, were roughly 90%. Oxidation-reduction indexes were close to 1.0, a value that would be expected from a fermentation of glucose.

**G+C content of DNA.** The moles percent G+C values in the DNAs of termite isolates were 53.1 (strain C-1) and 52.6 (strain C-2). Values for known strains of *E. agglomerans* were 56.1 (strain CDC 811-74) and 57.1 (strain CDC 156-74). Although the values for termite isolates were slightly lower than those of the two known strains tested, more comprehensive analyses of the DNA base composition of *E. agglomerans* (i.e., the Herbicola-Lathyri bacteria) have revealed values ranging from 52.6 to 57.7 mol% G+C (32). Consequently, we will refer to termite isolates as *E. agglomerans* strains C-1 and C-2 for the remainder of the paper.

**Growth studies and $C_2H_2$ reduction tests.** Growth studies were used to verify the $N_2$-fixing ability of isolates and to characterize this activity. GSV basal medium, containing glucose, N-free salts, and vitamins, was used. Peptone, $NH_4Cl$, or $KNO_3$, when added to GSV, served as an N source for cells growing either aerobically, or anaerobically under Ar (Table 3). Cell yields under these conditions ranged from $4 \times 10^8$ to $1 \times 10^9$ cells/ml, with higher yields being obtained aerobically. No growth occurred in unsupplemented GSV medium aerobically or under Ar (Table 3). However, when

TABLE 2. *Fermentation products of termite isolates and E. agglomerans CDC 811-74*

| Product | mmol/100 mmol of glucose fermented | | |
|---|---|---|---|
| | Strain C-1 | Strain C-2 | *E. agglomerans* CDC 811-74 |
| $CO_2$ | 103.2 | 107.3 | 101.4 |
| $H_2$ | 103.0 | 103.5 | 101.4 |
| Lactate | 26.7 | 51.7 | 32.7 |
| Acetate | 47.0 | 32.7 | 27.3 |
| Ethanol | 67.9 | 71.2 | 65.9 |
| Succinate | 14.0 | 10.4 | 15.0 |
| Formate | 25.0 | 7.0 | 5.7 |
| Glycerol | 15.5 | 0.3 | 15.1 |
| Pyruvate | 4.7 | 6.0 | 3.1 |
| Oxaloacetate | 0.6 | 3.9 | 2.1 |
| Acetoin | 0.3 | 0.2 | 0.5 |
| Diacetyl | 0.5 | 0.4 | 0.4 |
| 2,3-Butanediol | 0.0 | 0.0 | 0.0 |
| Carbon recovered (%) | 93.4 | 92.6 | 86.4 |
| O-R index[a] | 1.0 | 1.0 | 0.9 |

[a] O-R, Oxidation-reduction.

TABLE 3. *Specific growth rates[a] of E. agglomerans strains isolated from termites*

| Addition[b] to GSV basal medium | Doublings × $h^{-1}$ at initial gas phase | | | | | |
|---|---|---|---|---|---|---|
| | Strain C-1 | | | Strain C-2 | | |
| | Air | 100% $N_2$ | 100% Ar | Air | 100% $N_2$ | 100% Ar |
| Peptone | 1.56 | 0.47 | 0.61 | 1.64 | 0.59 | 0.59 |
| $NH_4Cl$ | 1.17 | 0.30 | 0.40 | 1.14 | 0.38 | 0.47 |
| $KNO_3$ | 0.68 | 0.34 | 0.44 | 0.47 | 0.35 | 0.43 |
| None | 0.00 | 0.16 | 0.00 | 0.00 | 0.13 | 0.00 |

[a] During exponential growth.
[b] Sterilized separately and incorporated at a final concentration of 0.2%.

100% $N_2$ was used as the initial gas phase, cells exhibited specific growth rates of 0.13 to 0.16 (Table 3) and reached densities of $4 \times 10^8$ cells/ml. Provision of combined N sources to cells growing under $N_2$ increased their specific growth rates 1.8- to 4.5-fold (Table 3) and almost doubled the cell yields. Although not shown in Table 3, only cells growing in unsupplemented GSV under $N_2$ exhibited $C_2H_2$-reducing activity.

When strain C-2 was grown under $N_2$ in GSV basal medium, an exponential increase in the optical density of the culture coincided with exponential increases in protein, viable cell number, and $C_2H_2$-reducing activity (Fig. 1). The latter reached a maximum of 250 nmol of $C_2H_4$ formed per (h × ml) at the late exponential phase of growth and declined thereafter. It can be calculated from the data in Fig. 1 that the greatest $C_2H_2$-reducing activity (normalized to viable cell number) occurred at 33 h and was 104 nmol of $C_2H_4$ formed per ($h \times 10^8$ cells). Formation of $C_2H_4$ was dependent on the presence of $C_2H_2$. Almost identical results were obtained with strain C-1.

These data indicated that termite isolates fix $N_2$ only under anaerobic conditions, in media lacking a major source of combined N, and that $C_2H_2$ reduction reflected this activity.

**$O_2$ inhibition of $C_2H_2$ reduction.** Because strains C-1 and C-2 could not grow aerobically in unsupplemented GSV medium (Table 3), it was suspected that $O_2$ inhibited $N_2$ fixation. To test this, cells growing anaerobically under $N_2$-fixing conditions (i.e., in unsupplemented GSV under $N_2$) were placed in vials containing Ar, $C_2H_2$, and various amounts of $O_2$. As shown in Fig. 2, as little as 0.01 atm of $O_2$ almost completely inhibited $C_2H_2$ reduction by strain C-1. Virtually identical results were obtained with strain C-2. These data indicated that *E. agglomerans* behaves in a manner similar to that of many other $N_2$-fixing, facultative anaerobes.

**Effect of anaerobiosis on $C_2H_2$ reduction by intact termites and their extracted guts.** Intact termites and extracted termite guts were made anaerobic, and their $C_2H_2$-reducing activity was tested. Some of the anaerobic conditions simulated those encountered in the procedure for the isolation of *E. agglomerans* (Table 4).

Introduction of termites into the anaerobic glove box rendered them unconscious and resulted in a 100-fold decrease of their normal $C_2H_2$-reducing activity. The activity was not reacquired by reexposure to air, even though the animals regained consciousness (Table 4,

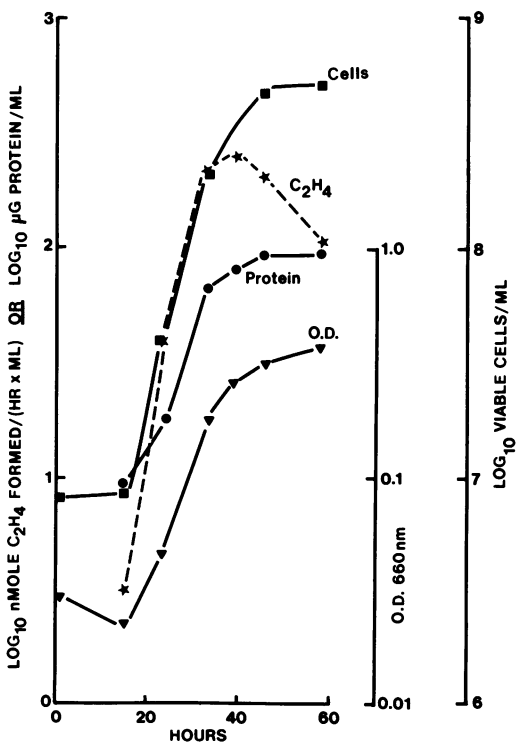


FIG. 1. *Growth and $C_2H_2$ reduction exhibited by E. agglomerans strain C-2. OD, Optical density.*

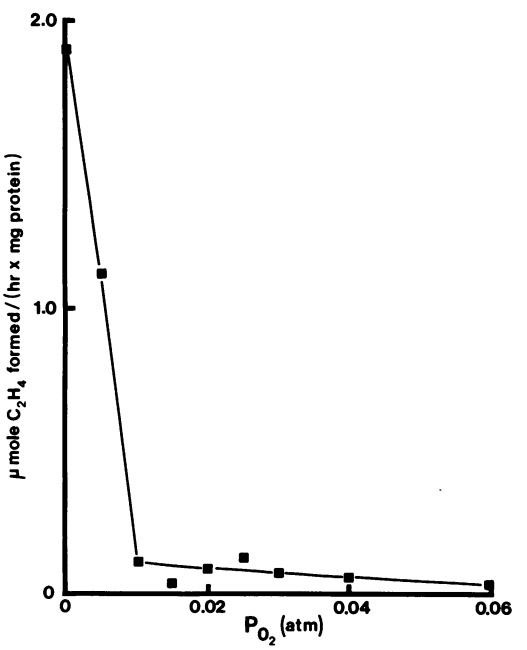


FIG. 2. *$O_2$ inhibition of $C_2H_2$ reduction by cells of E. agglomerans strain C-1. Each point on the curve represents the average value of three separate determinations.*

TABLE 4. *Effect of anaerobiosis on $C_2H_2$ reduction by C. formosanus and their extracted guts*

| Expt no. | Specimen[a] | Treatment | Atmosphere[b] in assay vial | $C_2H_2$-reducing activity[c] |
|---|---|---|---|---|
| 1 | IT | Untreated (control) | Air | 0.611[d] |
| 2 | IT | Admitted into glove box[e] and immediately assayed | Ar-$H_2$ (90:10) | 0.006 |
| 3 | IT | As for expt 2[e], but reexposed to air prior to assay[f] | Air | 0.000 |
| 4 | IT | Gassed with Ar for 1 min prior to assay[e] | Ar | 0.252 |
| 5 | IT | Gassed with $N_2$ for 1 min prior to assay[e] | $N_2$ | 0.118 |
| 6 | TG | Guts removed in glove box and immediately assayed | Ar-$H_2$ (90:10) | 0.003 |
| 7 | TG | As for expt 6, but reexposed to air prior to assay | Air | 0.000 |
| 8 | TG | Guts removed and assayed aerobically | Air | 0.000 |

[a] Groups of 25 specimens were used in each experiment: IT, intact termites; TG, termite guts.
[b] Atmospheres also contained $C_2H_2$ which was injected at zero time (see text).
[c] Nanomoles of $C_2H_4$ formed per (hour × 25 specimens).
[d] Equivalent to 12.21 nmol of $C_2H_4$ formed per (h × g [fresh weight]).
[e] Termites were rendered unconscious by this treatment.
[f] Termites regained consciousness during the 1-h assay period.

experiments 1, 2, and 3). Although $H_2$ (a known inhibitor of nitrogenase [9]) constituted 10% of the glove box atmosphere, its presence was not alone responsible for the loss of $C_2H_2$-reducing activity, since even a brief exposure of intact termites to pure Ar or $N_2$ also had a dramatic inhibitory effect (Table 4, experiments 4 and 5). Extracted termite guts showed a similar response, even if extracted and assayed aerobically (Table 4, experiments 6, 7, and 8).

The loss of $C_2H_2$-reducing activity by intact termites did not appear to result from a drastic change in the pH of their gut contents during anaerobiosis. Gut macerates of untreated termites had pH's of 6.5 and 7.0 (two separate determinations), whereas those from termites kept under Ar for 1 h had a pH of 6.9.

## DISCUSSION

Although *E. agglomerans* has been isolated from a variety of intestinal and extra-intestinal habitats (15, 16), to our knowledge this is the first demonstration of *E. agglomerans* in termite guts and the first quantitative analysis of the fermentation products of this species. Unlike most *Klebsielleae*, the tribe to which the genus *Enterobacter* belongs (11), our strains of *E. agglomerans* did not produce 2,3-butanediol as a major fermentation product. Rather, the products formed (Table 2) were typical of a mixed acid fermentation (12), with a significant production of glycerol by two of the three strains assayed.

It is noteworthy that Aho et al. (1) also documented $N_2$ fixation by *E. agglomerans*, using isolates obtained from decaying white fir trees. These workers further substantiated the $N_2$-fixing ability of isolates by demonstration of $^{15}N_2$ incorporation into growing cells. French, on the other hand, recently reported the isolation of $N_2$-fixing bacteria from Australian termites, but did not state the identity of the isolates, their numbers in guts, or their magnitudes of $N_2$ fixation (17).

Although *E. agglomerans* was the only $N_2$-fixing bacterium we isolated from termite guts, we are reluctant to conclude that it is the major $N_2$ fixer in this habitat, simply because it was isolated from relatively low dilutions of gut homogenate. Calculations based on maximum rates of $C_2H_2$ reduction by *E. agglomerans* in vitro [104 nmol of $C_2H_4$ formed per (h × $10^8$ cells)] indicated that a population of 2.3 × $10^4$ cells/gut would be necessary to account for the activity observed with intact termites [0.611 nmol of $C_2H_4$ formed per (h × 25 termites); Table 4]. Based on most-probable-number estimates, our isolation attempts yielded 100-fold fewer cells per gut than the expected value, even when aerobic isolation procedures were used or when the enrichment medium was extensively modified. It is significant, however, that removal of termite guts, even under anaerobic conditions, resulted in a 99 to 100% decrease in the $C_2H_2$-reducing activity of the preparation (Table 4). A 60 to 100% loss in activity occurred even when intact termites were made anaerobic prior to assay (Table 4). Benemann (4) also observed a 90% loss of $C_2H_2$-reducing activity when *K. minor* was incubated anaerobically. The inhibitory effect of anaerobiosis on $C_2H_2$-reducing activity of termites may have a bearing on our inability to retrieve *E. agglom-*

*erans* in greater numbers. Interestingly, recovery of *E. agglomerans* in numbers 100-fold lower than expected paralleled the 100-fold decrease in $C_2H_2$-reducing activity observed when the insects were made anaerobic.

Low recoveries of $N_2$-fixing *E. agglomerans* may also result from incomplete dispersion of bacterial aggregates during preparation of gut homogenates. Electron microscopy of guts of *C. formosanus* (8) has revealed the presence of dense bacterial aggregates that adhere strongly to the epithelium. If *E. agglomerans* forms such aggregates, much more vigorous preparatory procedures may be necessary for their dispersal. However, preliminary experiments employing 0.04% Trition X-100 or 0.01% Tween 80 in the homogenizing solution or blending gut homogenates in a microblender assembly were unsuccessful in this regard. Finally, the presence of a true microaerophilic $N_2$-fixing bacterium in guts, in numbers greater than those of *E. agglomerans*, has not been discounted and is a possibility currently being investigated in our laboratory.

In view of these considerations, we prefer to be conservative at this time and conclude that *E. agglomerans* may be important to the N economy of *C. formosanus*.

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## ADDENDUM IN PROOF

French et al. (J. Gen. Microbiol. 95:202-206, 1976) recently implicated *Citrobacter freundii* as a nitrogen-fixing agent in guts of Australian termites. However the number of *C. freundii* cells per termite gut was not determined.

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