sample size bias of Pamilo's method. It also gives greater weight to loci with multiple alleles and to loci with evenly distributed allele frequencies. Each colony was weighted equally.
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17. Some species are believed to have cycles of within-colony relatedness. There may be many queens in newly founded colonies, but attrition reduces the number considerably before any new queens are produced [M. J. West-Eberhard, *Proceedings of the Seventh International Congress* (International Union for the Study of Social Insects, London, 1973), p. 396; see also Forsythe (*8*)].
18. Dissections of two colonies of *Polybia sericea* showed 12 and 146 fertilized queens, the latter figure extrapolated from a partially dissected colony (*9*). *Polybia occidentalis* also may have queens numbering in the hundreds, though usually fewer (*8*, tables 1.3, 1.8, and 3.5).
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20. We thank D. Kirsch for field assistance and C. R. Solís for help with data management. T. Blohm graciously provided living quarters and access to his ranch. We also thank K. Jaffé, A. Fernández, and J. González for facilitating our research in Venezuela. Supported by NSF grant BSR86-05026.

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Nitrogen Fixation by Anaerobic Cellulolytic Bacteria

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Four strains of anaerobic nitrogen-fixing, cellulose-fermenting bacteria were isolated in pure culture from freshwater mud and soil. Nitrogenase activity was demonstrated in these strains and also in several previously described anaerobic cellulolytic bacteria isolated from various natural environments. These are the first anaerobic bacteria known to use cellulose as an energy source for nitrogen fixation. Because cellulose is a plant polysaccharide that abounds in nature, these results raise the possibility that nitrogen-fixing, cellulose-fermenting bacteria may be widespread and thus play a major role in carbon and nitrogen cycling.

THE REDUCTION OF ATMOSPHERIC dinitrogen (N_2) to ammonia (NH_3) by bacteria (biological nitrogen fixation) is a key transformation in the cyclic turnover of this element in natural environments. It is estimated that nitrogen fixation by symbiotic and free-living (nonsymbiotic) bacteria accounts for approximately 60% of the 2×10^8 metric tons of nitrogen fixed annually on our planet by biological and nonbiological processes (*1*).

Free-living, nitrogen-fixing bacteria are widely distributed in nature and add substantial amounts of combined nitrogen to the environments they inhabit (*2*). For example, Bormann and co-workers (*3*) reported that more than half of the nitrogen added to a northern hardwood forest ecosystem each year may derive from the activity of free-living, nitrogen-fixing bacteria in the soil, with the remaining nitrogen added mainly through precipitation.

Nitrogen fixation by free-living heterotrophic bacteria in natural ecosystems may be limited by the availability of oxidizable growth substrates (*4*) that serve as energy sources for the reduction of N_2 to NH_3 and for other growth processes. Surprisingly, even though cellulose occurs abundantly in natural environments where nonsymbiotic nitrogen fixation has been observed, it has not been determined whether this plant polysaccharide is widely used as an energy source by nitrogen-fixing bacteria. Photosynthesis yields annually up to 1.5×10^{11} tons of dry plant material worldwide, almost half of which consists of cellulose (*5*). The degradation of this vast amount of cellulose is carried out almost exclusively by microorganisms. Recently, Waterbury and co-workers (*6*) showed that cellulose serves as a growth substrate for a nitrogen-fixing aerobic bacterium that exists in a symbiotic relation with shipworms. Their findings demonstrate that these two complex physiological processes, nitrogen fixation and cellulose degradation, can be performed by a single bacterium. The objective of the present study was to determine whether free-living anaerobic cellulolytic bacteria that are widespread in terrestrial environments fix nitrogen when they utilize cellulose as the fermentable substrate for growth.

We isolated four strains of anaerobic cellulolytic bacteria from forest soil and freshwater mud, using a procedure that selected for nitrogen-fixing strains. We prepared enrichment cultures by serially diluting soil or mud samples into anaerobic culture tubes containing a liquid growth medium, designated MW-C (*7*), which lacked a source of combined nitrogen, included cellulose as the fermentable substrate, and was maintained in an N_2 atmosphere. After 7 to 14 days of incubation at 30°C, enrichment cultures showed significant disappearance of cellulose. Spent medium and remaining cellulose fibers from enrichment cultures were serially diluted into melted (40° to 45°C) cellulose soft agar medium in tubes. The contents of these tubes were poured onto plates of agar medium within an anaerobic chamber (*7*). After 2 to 4 weeks of incubation at 30°C, colonies surrounded by zones of clearing appeared in the otherwise opaque medium. These colonies were transferred by streaking

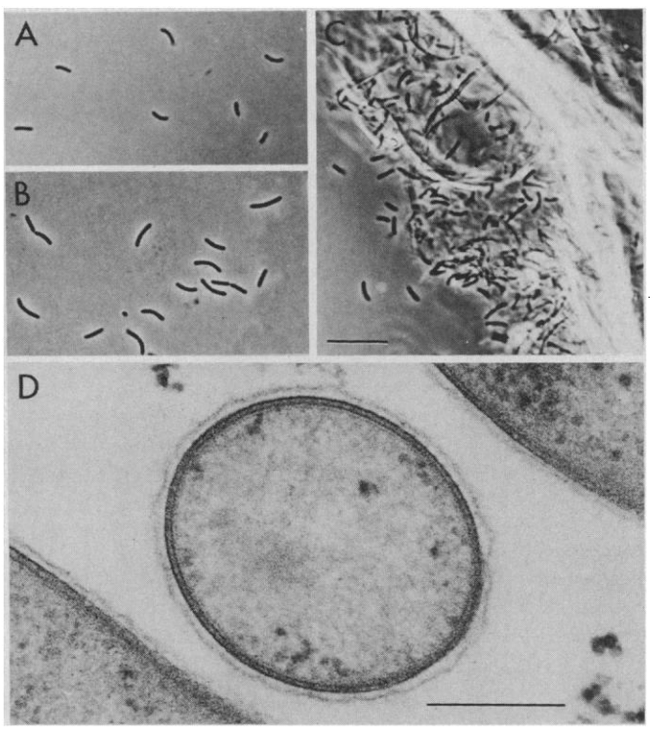

Fig. 1. (**A** through **C**) Phase-contrast photomicrographs of nitrogen-fixing cellulolytic isolates (wet-mount preparations), strains (A) B1B, (B) B3B, and (C) B1C. All phase-contrast micrographs are at the same magnification. Scale bar, 10 μm. (**D**) Transmission electron micrograph of a thin section of strain B1A cells stained with uranyl acetate. Scale bar, 0.2 μm. All cells were cultured to late-exponential phase in cellobiose-containing medium MJOU-CB (*15*). Strain B1C cells (C) are entangled in cellulose fibers introduced into the culture along with the inoculum [a 7-day culture in cellulose medium MJOU-C (*15*)].

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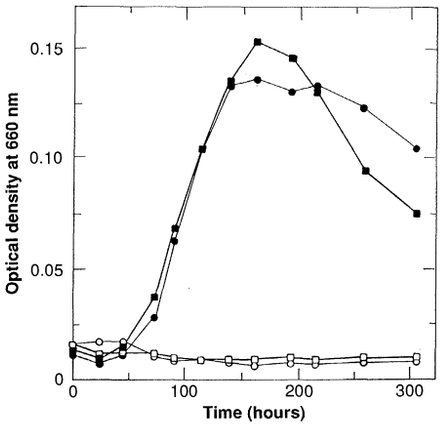

Fig. 2. N_2-dependent growth of nitrogen-fixing strains. Strains C7 (circles) and B3B (squares) were cultured in cellobiose-containing medium MJOUC-CB (*15*) in an argon (open symbols) or N_2 (solid symbols) atmosphere. A medium containing the soluble sugar cellobiose rather than cellulose was used to simplify growth measurements. Final growth yields of cultures grown in an N_2 atmosphere were 1.2×10^8 to 1.6×10^8 cells per milliliter. Control cultures, which contained 0.2% NH_4Cl, grew in both argon and N_2 atmospheres. Ammonium-repressible nitrogenase activity was present in cells grown in an N_2 atmosphere with cellobiose as the energy source.

onto plates of cellobiose agar medium (7) and restreaked at least three times to obtain pure cultures. Colonies were then transferred into liquid cellulose medium (MW-C) to confirm whether the isolate was cellulolytic. Stock cultures of the isolates were maintained in this medium. Three isolates (B1A, B1B, and B1C) were obtained from mud from the bottom of a shallow pond (Beaver's Pond, Shutesbury, Massachusetts) and one (strain B3B) from forest soil near Beaver's Pond.

The isolates resembled one another morphologically (Fig. 1, A through C). All were motile curved rods measuring 0.6 to 0.8 μm by 3 to 6 μm. Under the growth conditions used, spores were never observed either within cells or free in culture supernatant fluids. Cells of all four strains stained Gram-negative. Electron microscopy of thin sections of strain B1A cells (Fig. 1D) showed that the cytoplasmic membrane was surrounded by a multilayered cell wall that included inner, densely stained layers and an outer, less densely stained surface layer. Thus, the cell envelope ultrastructure of this isolate differed from typical tripartite (cytoplasmic membrane–peptidoglycan–outer membrane) cell walls of most Gram-negative anaerobic bacteria [for example, most members of the family Bacteroidaceae (*8*)] but resembled that of other Gram-negative mesophilic cellulolytic bacteria (*9, 10*) and certain Gram-negative and Gram-variable clostridia (*11*).

All isolates were obligately anaerobic and fermented polysaccharides, hexoses, and pentoses that are commonly present in plant materials (cellulose, xylan, cellobiose, D-glucose, D-galactose, D-mannose, D-fructose, D-xylose, L-arabinose, and D-ribose) (*12*). None of the strains utilized maltose, glycerol, or amino acids as fermentable substrates. Further phenotypic and genotypic characterization of the isolates is required to determine their taxonomic position.

We demonstrated the presence of nitrogenase in the isolates by means of the acetylene reduction test (*13*). Cells reduced acetylene to ethylene when growing in medium MW-C but not when growing in the same medium supplemented with 0.2% ammonium chloride (NH_4Cl). For example, a 10-day-old culture of strain B3B in medium MW-C reduced 1310 nmol of acetylene per hour per milligram of cell protein. The occurrence of NH_4^+-repressible acetylene reduction indicated that nitrogenase was present in the isolates. Inasmuch as the isolates grew in a medium lacking combined nitrogen and containing cellulose as the fermentable substrate, we concluded that they utilized this polysaccharide as the energy source for N_2 fixation.

Several previously described obligately anaerobic cellulolytic species [*Clostridium papyrosolvens*, from estuarine sediments of the River Don in Aberdeenshire, Scotland (*14*); *Clostridium* strain C7, from mud of a freshwater swamp in Woods Hole, Massachusetts (*9*); and strain JW-2, from wetwood of an American elm in Amherst, Massachusetts (*10*)], were cultured in a defined, cellulose-containing medium designated MJOU-C (*15*). These species did not grow in medium MW-C used to isolate nitrogen-fixing cellulolytic bacteria, possibly because a reducing agent was not added to medium MW-C. The previously described cellulolytic species, as well as the new isolates, reduced acetylene to ethylene when grown in medium MJOU-C but not when grown in the same medium supplemented with NH_4Cl, a result that indicated the presence of nitrogenase in these bacteria (Table 1). Moreover, growth in a medium lacking combined nitrogen was dependent on N_2 (Fig. 2). The results of this experiment demonstrated that N_2 served as the nitrogen source for growth. Furthermore, the experiment provided additional evidence, independent of the acetylene reduction assay, for the presence of nitrogenase in these bacteria. Thus, cellulolytic bacteria from a variety of environments synthesized active nitrogenase and apparently incorporated N_2 into cell material during growth.

In the present study we did not attempt to enumerate nitrogen-fixing cellulolytic bacteria in specific terrestrial environments. The results of such experiments, which would involve viable counts of cellulolytic bacteria, probably would not be meaningful because these bacteria often adhere to or are entangled in cellulose fibers (Fig. 1C). Furthermore, the growth requirements of diverse nitrogen-fixing cellulolytic bacteria are not known. However, our finding of nitrogenase activity in cellulose-fermenting bacteria isolated from a variety of environments is consistent with the view that nitrogen-fixing cellulolytic bacteria are widespread in nature. Low levels of acetylene reduction have been reported in two additional cellulolytic bacteria, *Clostridium cellobioparum* and *C. thermocellum*, when they are cultured in a cellobiose-containing medium (*16*). This activity is not repressed by NH_4^+ in *C. thermocellum*, a result that casts doubt on whether the observed activity was due to a

Table 1. Nitrogenase activity in cellulose-fermenting bacteria. Cells were cultured in an N_2 atmosphere at 30°C for 6 days (strain B3B) or 11 days (strain C7) in 5 ml of growth medium in 16-ml screw-cap tubes sealed with rubber septa. Then tubes were flushed with N_2 and vented to atmospheric pressure. Subsequently, 1.1 ml of gas were removed from each of the sealed tubes and replaced with 1.1 ml of acetylene (produced by hydrating calcium carbide crystals). The cultures were incubated at 30°C, and gas samples removed at time intervals were assayed for the ethylene content with a gas chromatograph (Varian 3700). Gases were separated on a Porapak N column (Supelco) at 50°C (N_2 carrier gas flow rate, 25 ml/min) and detected with a flame-ionization detector.

Strain	Medium (*15*)	Experiment	Cell density ($\times 10^8$ cells per milliliter)	Acetylene reduced (nmol/hour)	Specific activity (nmol/hour per milligram of cell protein)
B3B	MJOU-C	1	1.5	67	520
		2	2.0	65	600
B3B	MJOU-C	1	5.6	0*	0
	+0.2% NH_4Cl	2	4.8	0*	0
C7	MJOU-C	1	1.1	48	700
		2	1.1	37	540
C7	MJOU-C	1	2.3	0*	0
	+0.2% NH_4Cl	2	2.9	0*	0

*No activity detected.

nitrogenase. Nitrogen fixation by *C. cellobioparum* and *C. thermocellum* during growth on cellulose was not reported.

Inasmuch as environments rich in cellulose are frequently deficient in nitrogen (for example, peat soils, agricultural and municipal wastes, and composts), cellulose-fermenting bacteria that satisfy their nitrogen requirements through the fixation of N_2 would be expected to have a strong selective advantage over those that require a source of combined nitrogen. Because vast amounts of cellulose are available in a wide variety of environments, it is possible that cellulolytic, nitrogen-fixing bacteria are widespread in nature and that they play a significant role in nitrogen and carbon cycling. The potential use of these bacteria in improving soil fertility and in the conversion of lignocellulosic wastes into useful products remains to be explored.

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An in Vitro System for Accurate Methylation of Internal Adenosine Residues in Messenger RNA

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Some internal adenosine residues in messenger RNA are methylated posttranscriptionally in the nucleus. Most of the methylated adenosine residues in prolactin mRNA are in the 3′ untranslated region. The site of methylation in the 3′ end of prolactin mRNA was determined. This methylation reaction is highly specific; of the three adenosine residues in consensus sequences located in the 3′ end, only one is methylated. An in vitro methylation system was developed in which bovine prolactin mRNA, synthesized in vitro with T7 RNA polymerase, was accurately methylated in a HeLa cell nuclear extract. The adenosine residue that was methylated in vitro was the same as the one methylated in vivo. This cell-free system, which accurately methylates the N^6-position of adenosine residues in mRNA, will allow further study of the mechanism of adenosine methylation.

N^6-METHYLADENOSINE (m^6A) IS A posttranscriptional modification of adenosine that occurs in most eukaryotic cellular and viral mRNAs with one to three m^6A residues per 1000 nucleotides (*1–3*). The methylated adenosine is usually found within an AAC or GAC consensus sequence (*4–6*). Localization studies performed on a few specific mRNAs indicate that the distribution of m^6A is nonrandom. For example, in Rous sarcoma virus genomic RNA there are 10 to 15 m^6A residues clustered in the 3′ end of the RNA (*3*). Similarly, in bovine prolactin mRNA, most of the m^6A residues are located in the 3′ untranslated sequence of 138 nucleotides (*7*). This region of prolactin mRNA contains only 3 consensus sequences of a total of 27 sequences present throughout the mRNA. Therefore, the site of methylation appears to be specific and perhaps dictated by more than just the 3-nucleotide consensus sequence. However, the mechanism and function underlying this modification of mRNA remains obscure.

In an attempt to study the mechanism of m^6A formation in mRNA molecules we established an in vitro methylation system with synthetic mRNA substrates and HeLa cell nuclear extracts. Bovine prolactin mRNA was synthesized in vitro with T7 RNA polymerase (Fig. 1A) and incubated with a HeLa cell nuclear extract in the presence of the methyl donor S-[^{3}H]adenosylmethionine (SAM). Under the conditions described in the legend to Fig. 1B, 70 to 80% of the radioactive methyl groups were incorporated into internal adenosine residues (Fig. 1B) and 5% of the input RNA was methylated. High-performance liquid chromatography (HPLC) showed that the predominant methylated component is m^6A with a low level (7% to 8%) of the methylated nucleotides as 2′-O-methyl-

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