microorganisms to lignocelluosic substrates are unknown, it is clear that alkaline hydrogen peroxide treatment removes a significant barrier to attachment, allowing more rapid cell wall colonization and digestion.

The finding that alkaline hydrogen peroxide renders plant fibers more digestible by ruminants suggests that many alternative feed sources, including crop residues and other cellulosic plant biomass, may be used in animal production. Utilization of cellulose in this way makes available a nearly inexhaustible feed supply. Furthermore, the ability to feed highly digestible cellulosic materials to ruminants would eliminate competition between present animal production practices and the demands of an expanding world population for cereal grains.

References and Notes

1. G. Chambat, J. D. Joseleau, F. Barnoud, *Phytochemistry* **20**, 241 (1981); E. B. Cowling, *Biotechnol. Bioeng. Symp.* **5**, 163 (1975); A. H. Gordon, A. J. Hay, D. Dinsdale, J. S. D. Bacon, *Carbohydr. Res.* **57**, 235 (1977); H. G. Jung and G. C. Fahey, Jr., *J. Anim. Sci.* **57**, 206 (1983); S. P. Rowland, *Biotechnol. Bioeng. Symp.* **5**, 183 (1975).
2. J. M. Gould and S. N. Freer, *Biotechnol. Bioeng.* **26**, 628 (1984).
3. P. Nakatsubo, I. D. Reid, T. K. Kirk, *Biochem. Biophys. Res. Commun.* **102**, 484 (1981); L. J. Forney, C. A. Reddy, M. Tien, S. D. Aust, *J. Biol. Chem.* **257**, 11455 (1982); H. Kutsuki and M. H. Gold, *Biochem. Biophys. Res. Commun.* **109**, 320 (1982); M. Tien and T. K. Kirk, *Science* **221**, 661 (1983); J. K. Glenn, M. A. Morgan, M. B. Mayfield, M. Kuwahara, M. H. Gold, *Biochem. Biophys. Res. Commun.* **114**, 1077 (1983).
4. W. G. Pond, R. A. Merkel, L. D. McGilliard, V. J. Rhodes, in *Animal Agriculture: Research to Meet Human Needs in the 21st Century* (Westview, Boulder, Colo., 1980), p. 165.
5. Treatment with alkaline hydrogen peroxide consisted of suspending material in distilled water containing 1 percent (weight to volume) hydrogen peroxide. Sodium hydroxide was added to bring the suspension to *p*H 11.5, and the mixture was stirred gently at room temperature. After 16 hours of incubation the insoluble residue was collected, washed repeatedly until the filtrate was neutral, and oven-dried (80°C).
6. A. Z. Mehrez and E. R. Orskov, *J. Agric. Sci. Cambridge* **88**, 645 (1977). Substrate (3 g) is placed in 7 by 15 cm nylon bags (20 to 90 μm pore size) and suspended in the rumen of a fistulated steer. Rate of digestion is calculated by regressing the natural log of percentage potentially digestible material remaining over time. Extent of digestion is calculated by measuring disappearance of substrate after 48 hours incubation in the rumen.
7. In experiment 1, low-straw diets were composed of wheat straw (36 percent, treated or untreated), ground corn (46.2 percent), soybean meal (11.7 percent), liquid cane molasses (5.5 percent), calcium carbonate (0.7 percent), and vitamin supplement (0.2 percent); high-straw diets were composed of wheat straw (72 percent, treated or untreated), ground corn (3.5 percent), soybean meal (18.2 percent), liquid cane molasses (5.5 percent), calcium carbonate (0.2 percent), dicalcium phosphate (0.4 percent), and vitamin supplement (0.2 percent). Diets fed in experiments 2 and 3 were essentially the same as those in experiment 1, except that urea was added (0.5 percent at the expense of soybean meal) to ensure optimal ruminal ammonia levels.
8. National Research Council, *Nutrient Requirements of Domestic Animals, Nutrient Requirements of Sheep* (National Academy of Sciences, Washington, D.C., ed. 5, 1975).
9. Gross energy data were obtained with a Parr 1241 adiabatic bomb calorimeter.
10. H. K. Goering and P. J. Van Soest, *U.S. Dep. Agric. Handb. 379* (1970).
11. J. B. Robertson and P. J. Van Soest, *J. Anim. Sci.* **45** (suppl. 1), 254 (1977).
12. K. J. Cheng, D. Dinsdale, C. S. Stewart, *Appl. Environ. Microbiol.* **38**, 723 (1979).
13. Samples (~25 ml) were withdrawn directly from the rumen through a fistula, and the suspended straw particles were collected by centrifugation, washed in 0.1*M* phosphate buffer (*p*H 7.0), and fixed in 4 percent glutaraldehyde for 24 hours. The samples were postfixed in 2 percent OsO_4 for 3 hours, dehydrated by sequential ethanol washes (25 percent concentration steps), and critical-point-dried (CO_2). After being coated with gold and palladium, the straw particles were examined with an ISI model SS130 scanning electron microscope.
14. M. J. Latham *et al.*, *Appl. Environ. Microbiol.* **35**, 1166 (1978); *ibid.*, p. 156; D. E. Akin and H. E. Ramos, *ibid.* **29**, 692 (1975).

15. The expert technical assistance of L. Dexter is gratefully acknowledged. Valuable assistance in the production of treated wheat straw was also provided by R. Montgomery and W. Bury. Supported by the U.S. Department of Agriculture under agreement 58-519B-3-1237, administered by the Northern Regional Research Center, Peoria, Ill., and by the University of Illinois, Urbana.

10 June 1985; accepted 5 September 1985

Synthesis of Fibrils in Vitro by a Solubilized Cellulose Synthase from *Acetobacter xylinum*

Abstract. *A digitonin-solubilized cellulose synthase was prepared from* Acetobacter xylinum. *When this enzyme was incubated under conditions known to lead to active synthesis of 1,4-β-D-glucan polymer (cellulose), electron microscopy revealed that clusters of fibrils were assembled within minutes. Individual fibrils are 17 ± 2 angstroms in diameter. Evidence that the fibrils were freshly synthesized and cellulosic in nature was their incorporation of the tritium from UDP-[^3H]glucose (UDP, uridine 5′-diphosphate), their binding of gold-labeled cellobiohydrolase, and an electron diffraction pattern with 004, 200, and 012 reflections (characteristic of cellulose synthesized in vivo) but missing 110 and $\bar{1}10$ reflections. The small size of the fibrils is atypical of native* A. xylinum *cellulose microfibrils. The fibrils synthesized in vitro resemble, in morphology and size, the fibrillar cellulose produced when* A. xylinum *is cultured in the presence of agents that interfere with the normal process of crystallization of the microfibrils. The solubilized enzyme unit may therefore be producing a basic fibrillar structure that, in vivo, interacts laterally with other fibrils to produce native cellulose microfibrils.*

FONG CHYR LIN
R. MALCOLM BROWN, JR.*
Department of Botany,
University of Texas,
Austin 78713-7640
JAMES B. COOPER
DEBORAH P. DELMER*
ARCO Plant Cell Research Institute,
6560 Trinity Court,
Dublin, California 94568

*To whom correspondence should be addressed.

Cellulose, a polymer of 1,4-β-D-glucan, is the most abundant biopolymer on Earth. It is the primary component of such products as wood, cotton, and paper. Cellulose exists in nature as microfibrils, which form the structural framework of the cell walls of plants, some algae, and certain fungi (*1*). Among the few bacteria that synthesize cellulose, *Acetobacter xylinum* has been studied intensely because it produces abundant amounts in growth medium. The cellulose is extruded from a row of pores aligned along the longitudinal axis of the cell (*2*) and forms an extracellular pellicle, which consists of ribbons of cellulose microfibrils. Freeze etching of *A. xylinum* cells has revealed in the outer membrane a row of particles, complementary to the pores, which may take part in the synthesis of cellulose (*3*).

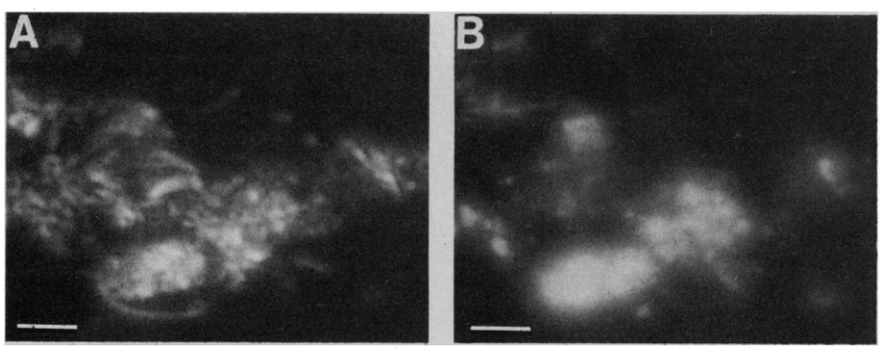

Fig. 1. (A) Dark-field microscopy of in vitro reaction product. Scale bar, 1.0 μm. (B) Fluorescence microscopy image of the product in the presence of 0.5 percent Tinopal LPW (Ciba-Geigy). Scale bar, 1.0 μm.

Many attempts have been made to synthesize cellulose in vitro (*4*), but only recently, by means of membrane fractions from *A. xylinum* (*5*), has a convincing high-rate synthesis of a 1,4-β-D-glucan polymer been achieved. These studies have demonstrated a complex regulation of cellulose synthase, mediated by GTP (guanosine 5′-triphosphate), in which this enzyme is solubilized in a form that still possesses its regulatory properties (*6*). Further studies (*7*) have clarified the role of GTP. It is converted to an unusual guanyl oligonucleotide by an activator-forming enzyme distinct from cellulose synthase, and this derivative, tentatively characterized as a cyclic oligonucleotide composed of GMP (guanosine 5′-monophosphate) residues, is the true activator of cellulose synthase (*8*). The in vitro product is clearly an alkali-insoluble 1,4-β-D-glucan polymer (*5, 6*); however, no information has become available on the crystalline structure or microfibrillar nature of the product.

We present evidence that fibrils are indeed assembled in vitro by the activated, solubilized enzyme unit from *A. xylinum*. For these studies, the enzyme unit was prepared and incubated with substrate and activator under conditions leading to the synthesis of 1,4-β-D-glucan polymer (*9*), and the synthesis of cellulose was followed by light and electron microscopy.

Dark-field microscopy shows that, within 30 minutes, a product is made that scatters light (Fig. 1A). With fluorescence microscopy, the synthesized materials flouresce intensely with Tinopal LPW, one of a class of fluorescent dyes that bind to β-glucans (Fig. 1B) (*10*). We also found that reaction mixtures containing enzymes but lacking substrate have a weak fluorescence.

Electron microscopy of negatively stained material reveals an accumulation of fibrils (Fig. 2A) that are not observed when UDP-glucose (uridine 5′-triphosphate glucose) or activator is omitted from the reaction. Fibrils synthesized in vitro appear in clusters. The basic fibrils measure only 17 ± 2 angstroms (Å) in diameter, and they tend to associate with each other laterally to produce small aggregates (Fig. 2B). The accumulated fibrils are too small to be called typical microfibrils, but the appearance of the material shown in Fig. 2A is quite similar to that of the in vitro product from digitonin-solubilized chitin synthase (*11*).

Evidence that the fibrils are cellulosic in nature comes from studies with gold-labeled, highly purified cellobiohydrolase I (CBH-Au) (*12*). Clusters of fibrils synthesized in vitro are specifically labeled with CBH-Au (Fig. 3A). The specificity of this binding supports the conclusion that the fibrils are indeed cellulose (*12*). The presence of cellulose is further supported by the observation that the in vitro material is susceptible to degradation by the cellobiohydrolase if incubation is prolonged beyond even a few minutes (Fig. 3B). In addition, if UDP-glucose is omitted from the incubation medium, no structures are specifically labeled with CBH-Au.

The addition of tritium-labeled UDP-glucose (UDP-[^{3}H]glucose) to the incubation mixture, followed by CBH-Au labeling, unidirectional shadowing, and autoradiography, shows that the colloidal gold and silver grains both coincide with the shadow-cast in vitro product (Fig. 4) (*13*). The dual label provides evidence that the product is a glucan derived from supplied UDP-glucose; it does not represent any preexistent fibrils in the preparation.

Additional evidence for the cellulosic nature of the in vitro product comes from our electron diffraction studies (*14*). A diffraction pattern from bacterial cellulose is shown in Fig. 5A. According to the indexing convention of Woodcock and Sarko (*15*), the following reflections are observed: 004 (2.60 Å), 200 (3.90 Å), 012 (4.41 Å), 110 (5.20 Å), and $\bar{1}10$ (6.16Å). A diffraction pattern from the in vitro product is shown in Fig. 5B. A characteristic 004 meridional reflection

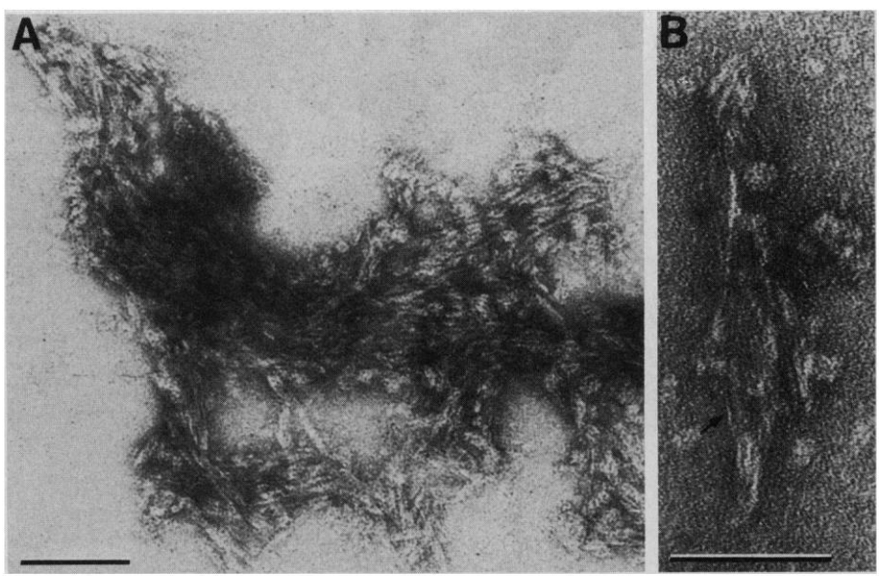

Fig. 2. (A) Electron micrograph of negatively stained in vitro product showing the morphology of fibrils, which tend to cluster in clumps. Scale bar, 0.1 μm. (B) Micrograph similar to (A) except at higher magnification. Scale bar, 0.1 μm. Arrow denotes 17-Å fibril.

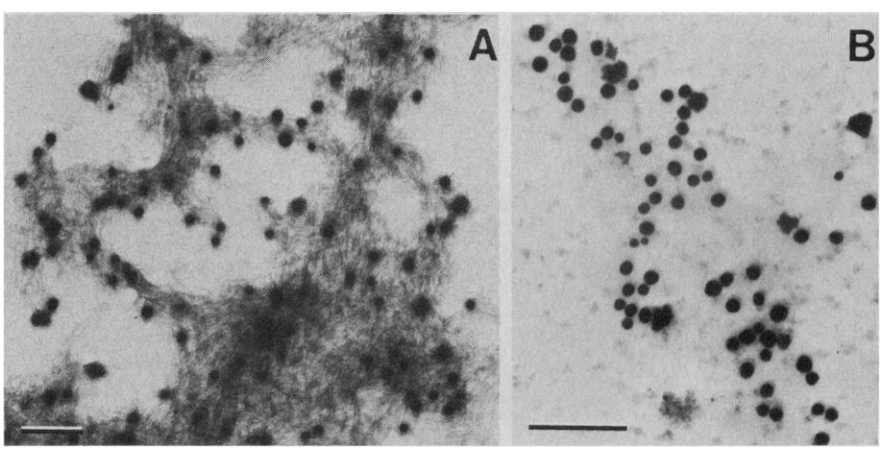

Fig. 3. (A) Electron micrograph of negatively stained in vitro product after incubating 1.5 minutes in CBH-Au solution (*12*) showing CBH-Au complexes associated with clustered fibrils. Scale bar, 0.1 μm. (B) CBH-Au degrades the product after 20 minutes. Compare the product seen here with that seen in (A). Scale bar, 0.1 μm.

(2.57 Å) is present as well as the 200 (3.83 Å) and the 012 (4.25 Å) reflections. Conspicuously absent are the two 110 and $\bar{1}10$ equatorial reflections. These data support the evidence that the in vitro product is, indeed, cellulose; however, the lack of the 110 and $\bar{1}10$ reflections agrees with the ultrastructural evidence for the very small fibrillar size. The *d* spacing for the 200 reflection of the in vitro product suggests it may be cellulose I, since cellulose II has a characteristic *d* spacing of 4.03 Å for this reflection. The presence of a 200 reflection from the in vitro product is evidence for the glucan chains being organized into sheets.

The diameter of the in vitro fibrils is similar to that of the smallest fibril produced in vivo by *A. xylinum* when crystallization is disrupted by fluorescent brighteners (*16*). Since it is thought that these brighteners interfere with crystallization only after extrusion of the product from the pores, this suggests that each solubilized enzyme unit is producing a basic fibril of low crystallinity that contains only 12 to 25 glucan chains (*17*). The enzyme unit may be equivalent to a single particle in the row in the outer membrane of the intact cell. Many particles are observed within the fibrillar network, but because of the impurity of the solubilized enzyme preparation we used, it would be premature to attempt to distinguish which of these particles may be the synthase enzyme.

In conclusion, we report the visualization of the formation of cellulose fibrils in vitro. The enzyme mixture used in their formation is quite labile, and the reaction rates are linear with respect to time for only 8 to 15 minutes (*6, 7*); this may account for the very short length of the fibrils observed. A detailed study of the time course of the synthesis as well as an investigation of the stability of the reaction is needed. It will also be interesting to see if typical native microfibrils can be formed in vitro when the enzyme complex is further purified and associated with other such components as membranes or proteoliposomes. Structural studies of purified cellulose synthase will also add greatly to our understanding of the mechanism of the directed chain polymerization of cellulose.

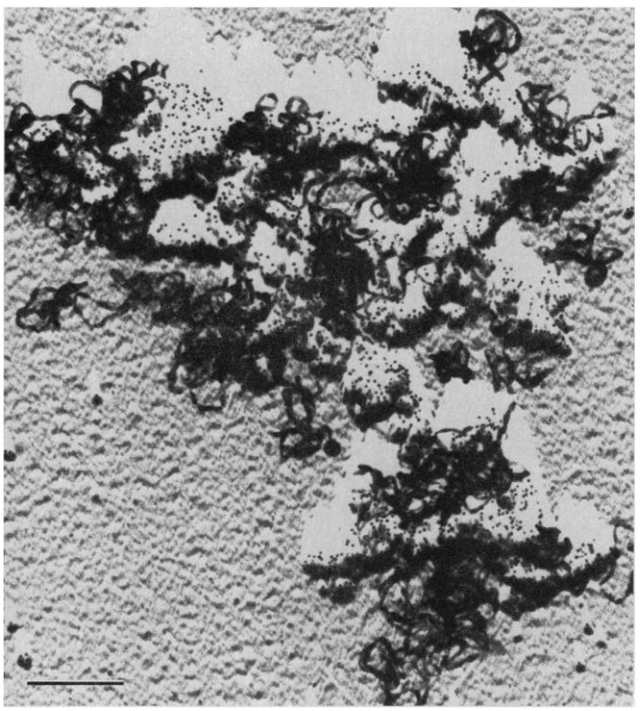

Fig. 4. Autoradiograph of cellulose fibrils synthesized in vitro after a combination of gold labeling and unidirectional shadow-casting. The incorporation of the radioactive substrate UDP-[^{3}H]glucose and the binding of CBH-Au to the product can be seen. Scale bar, 0.5 μm.

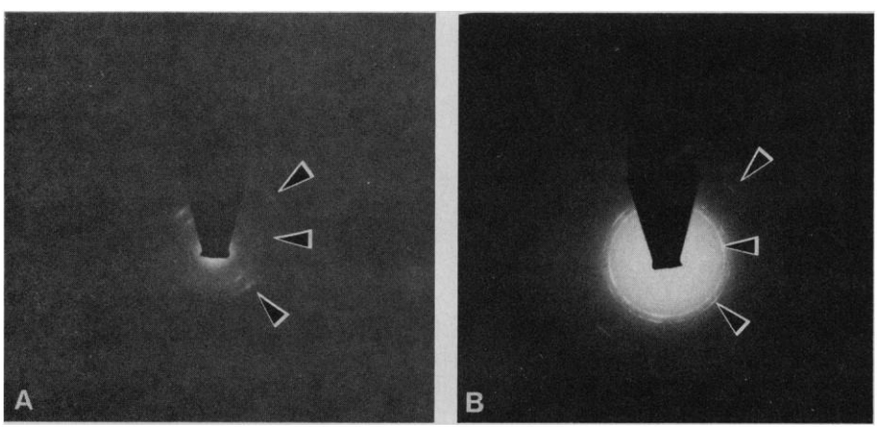

Fig. 5. (A) Electron diffraction pattern of cellulose synthesized in vivo by *A. xylinum*. The upper arrow points to the 004 meridional reflection, the middle arrow to the 012 reflection, and the lower arrow to the three equatorial reflections, 200, 110, and $\bar{1}10$. (B) Electron diffraction pattern of the in vitro product from *A. xylinum*. The upper arrow points to the 004 meridional reflection; the middle arrow to the 012 reflection, and the lower arrow to the 200 reflection.

References and Notes

1. R. D. Preston, *The Physical Biology of Plant Cell Walls* (Chapman & Hall, London, 1974); A. Frey-Wyssling, *The Plant Cell Wall* (Borntraeger, Berlin, 1976).
2. K. Zaar, *J. Cell Biol.* **80**, 773 (1979).
3. R. M. Brown, Jr., J. H. M. Willison, C. L. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4565 (1976).
4. D. P. Delmer, *Adv. Carbohydr. Chem. Biochem.* **41**, 105 (1983); G. W. Gooday, *Prog. Ind. Microbiol.* **18**, 85 (1983).
5. Y. Aloni, D. P. Delmer, M. Benziman, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6448 (1982).
6. Y. Aloni, R. Cohen, M. Benziman, D. P. Delmer, *J. Biol. Chem.* **258**, 4419 (1983).
7. P. Ross *et al.*, *FEBS Lett.* **186**, 191 (1985).
8. In unpublished work done in collaboration with C. Weinhouse of the Hebrew University, M. Thelen, J. B. Cooper, and D. P. Delmer recently showed that the *A. xylinum* cellulose synthase can be activated directly by guanyl oligonucleotide after electrophoretic separation and assay of the enzyme in native acrylamide gels by the procedure of Delmer *et al.* (*J. Cell Sci.*, in press).
9. The strain of *A. xylinum* used for the in vitro studies was supplied by M. Benziman, Hebrew University, Jerusalem, Israel, and it was the same strain used by Benziman's group for their studies on cellulose synthase (*5, 6, 7*). Growth of the bacterium and preparation of digitonin-solubilized enzyme was performed as described (*6*) except that the cells were disrupted in a French pressure cell at 20,000 pounds per square inch. Guanyl oligonucleotide was prepared as described (*7*). Incubations, containing 75 m*M* tris (*p*H 8.6), 15 m*M* $MgCl_2$, 10 m*M* UDP-glucose, 1 m*M* $CaCl_2$, 1 m*M* EDTA, 0.5 percent digitonin, and about 20 μg/ml of guanyl oligonucleotide, were carried out in Eppendorf pipette tips at room temperature for 20 to 30 minutes. A small drop of the reaction product was then placed on a carbon-coated Formvar copper grid, drained, and stained with 1 percent aqueous uranyl acetate containing 0.1 mg/ml bacitracin. We have succeeded in preparing a solubilized enzyme preparation and activator from two other strains of *Acetobacter* that produced fibrils of similar size and morphology.
10. I. D. Rattee and M. M. Breur, *The Physical Chemistry of Dye Absorption* (Academic Press, New York, 1974), pp. 181–182.
11. E. Cabib, A. Duran, B. Bowers, in *Fungal Walls and Hyphal Growth*, J. H. Burnett and A. P. Trinci, Eds. (Cambridge Univ. Press, Cambridge, 1979), pp. 189–201.
12. The CBH-Au complex was prepared by the method of M. Gretz (personal communication), which is similar to that described [H. Chanzy, B. Henrissat, R. Vuong, *FEBS Lett.* **172**, 193 (1984)]. The reaction product was mounted on a carbon-coated Formvar grid, washed by several drops of double-distilled H_2O (DD H_2O), incubated in a solution of CBH-Au complex at *p*H

7.0 for 1.5 to 2.5 minutes, and then washed directly with DD H_2O several times. The grid subsequently was stained with 1 percent aqueous uranyl acetate for electron microscopy. Purified CBH I was supplied by M. Schulein, Novo Industrias, Bagsvaerd, Denmark. It was highly purified and was shown to be very specific for cellulose and a few other 1,4-β-polymers. The presence of other 1,4-β-polymers in this in vitro system is excluded because only UDP-glucose is available as a substrate in the incubation mixture. The glucose was shown to be incorporated into the product by autoradiography (*13*).

13. For autoradiography, the grid was prepared as described (*12*), but with radioactive substrate. It was shadow-cast unidirectionally with platinum-carbon at a 10° angle. Then the grid was autoradiographed as reported [B. M. Kopriwa, *Histochemie* **37**, 1 (1973)]. Although individual fibrils were not distinguished after shadowing, this procedure was found necessary to preserve the integrity of the in vitro product during the autoradiographic developing and fixing procedures.
14. For electron diffraction studies, a MoO_3 standard was used to calibrate the *d* spacings. For controls, cellulose produced by *A. xylinum* was cleaned with 1*N* HCl and then a mixture of methanol and chloroform (1:1 by volume), washed with DD H_2O, frozen in liquid nitrogen, ground to a fine powder, and dried onto carbon-coated grids. The in vitro material was placed directly onto grids and air-dried. For electron diffraction analysis, a transmission electron microscope (Philips EM 420) was operated at 120 kV with a condenser aperture of 30 μm, the condenser-1 lens current set at position "5" in microprobe position, and a beam current of less than 5 μA. For exposure, the low-dose technique of beam shift was employed; the diffraction pattern was recorded on electron microscope film (Mitsubishi MEM, from Japan) and developed (Mitsubishi Gekkol developer) for 4 minutes at 20°C.
15. C. Woodcock and A. Sarko, *Macromolecules* **13**, 1183 (1980).
16. C. H. Haigler, R. M. Brown, Jr., M. Benziman, *Science* **210**, 903 (1980).
17. H. Chanzy, K. Imada, R. Vuong, *Protoplasma* **94**, 299 (1978); H. Chanzy, K. Imada, A. Mollard, R. Vuong, F. Barnoud, *ibid.* **100**, 303 (1979).
18. We thank M. Gretz, K. Kudlicka, C. Haigler, M. Benziman, C. Weinhouse, S. Kuga, and P. Goff for their assistance in perfoming the experiments and preparing this report. We also thank T. Itoh for supplying the Mitsubishi MEM film and A. Sarko for assisting in the interpretation of the electron diffraction patterns. Supported in part by grants to R.M.B. from Johnson and Johnson.

12 August 1985; accepted 7 October 1985

Cell-Assisted Growth of a Fastidious Spiroplasma

Abstract. *The Colorado potato beetle spiroplasma, which is not cultivable in conventional cell-free media, grew in tissue culture media in the presence of several coleopteran and lepidopteran insect cell lines. The cultured organisms attained titers of 1.2 × 10^9 spiroplasmas per milliliter of culture at the 100th passage and retained infectivity and a high capacity for translational motility at the 15th passage. Cell culture systems may facilitate the isolation of other presently uncultivable microorganisms and may be useful in the study of the role of microbial physiology and behavior in pathogenicity.*

KEVIN J. HACKETT
DWIGHT E. LYNN
Insect Pathology Laboratory, U.S. Department of Agriculture, Beltsville, Maryland 20705

Although wall-less prokaryotes *(Prokaryotae:Mollicutes)* have recently been accorded the status of a division *(Tenericutes)* (*1*), they have heretofore been thought to be a numerically minor taxon. Recent observations suggest that this may be far from the case. Two mollicute families—spiroplasmas *(Spiroplasmataceae)* and acholeplasmas *(Acholeplasmataceae)*—have been shown to be widespread in insects (*2*) and to have diverse host relationships (*3*). Although some mollicutes are pathogenic to their insect hosts, others are ovarially transmitted mutualists (organisms that have mutually beneficial association with each other) and many appear to be commensals (supply food or protection to other organisms without damage or benefit to themselves) (*4*). The pathogenicity of certain mollicutes for arthropods suggests that these organisms might be useful in the biological control of insect pests (*5*). However, since as many as half of the spiroplasmas observed microscopically in insects are currently uncultivable (*2*), it is difficult to study their pathogenicity. Those spiroplasmas that are cultivable can be grown in cell-free media, but they usually require very complex, and often specific, nutrients. We report here the successful use of insect tissue culture for the isolation of a previously uncultivable, highly fastidious (having complex nutritional requirements) mollicute, the Colorado potato beetle spiroplasma (CPBS).

Search for a microbial control agent for the Colorado potato beetle, *Leptinotarsa decemlineata*, a devastating pest of potatoes (*6*), led to the discovery of CPBS in 1982 (*3*). CPBS is (i) host-specific, (ii) able to achieve titers of approximately 10^{10} spiroplasmas per milliliter of gut fluid, (iii) transmissible to both larval and adult beetles via regurgitated gut fluid or feces, and (iv) able to persist on leaves and in hibernating adults. Its pathogenicity and biological control potential have yet to be assessed.

The morphology of CPBS has attracted considerable attention. In contrast to other known spiroplasmas, which are helical, CPBS has a characteristic spiral shape that is associated with an unusually high capacity for translational motility. Another unusual characteristic of CPBS is its ability to move through the meshlike peritrophic membrane lining the beetle midgut to attach to microvilli (*2*). Although these observations suggest that CPBS might differ substantially from known spiroplasmas, its membership in the *Spiroplasmataceae* was confirmed by dark-field and electron microscopic determination of its size (about 130 by 5000 nm), by the presence of a single membrane, and by the lack of a cell wall or periplasmic fibrils in antibiotic-free media (*2*). As tested by the spiroplasma deformation test (*7*), CPBS was serologically distinct from spiroplasmas in groups I to XI and all other available putative serogroups (represented by strains DU-1, CC-1, EC-1, I-92, CB-1, DF-1, TN-1, PUP-1, and I-25) (*4*, *8*).

Although we were not able to cultivate CPBS in any of the many (*9*) currently available mollicute media, we were able to improve CPBS maintenance by amending the media with gut fluids from the Colorado potato beetle. Addition to the media of filtered extracts from potato leaves (the natural diet of the beetles) did not improve CPBS growth. We therefore suspected that critical growth factors were being supplied by the insect gut cells and hypothesized that factors supplied by cultured insect cells might permit sustained growth of CPBS.

We obtained inocula of CPBS by dissecting and rupturing Colorado potato beetle guts in cell culture media and filtering the extracts through membrane filters (pore size, 450 nm). The insect cell culture systems are described in Table 1. Cell cultures were split once per week and were maintained at 23°C until used. We transferred 0.4-ml aliquots of inoculum into 4.0 ml of 0- or 4-day-old cell cultures and incubated them at 26°C; cultures were passaged every 4 days. For daily quantification of spiroplasma growth, a 3-μl drop of culture was placed on a microscope slide and covered with an 18-mm^2 cover glass (Corning No. 1½). The number of spiroplasmas in 50 randomly selected microscopic fields (X1250, Zeiss dark-field microscopy) was counted, and titers were calculated (*10*).

Growth of the CPBS isolate (LD-1) in culture media overlying the *Diabrotica* cells was at first moderate, reaching titers of 8 × 10^7 spiroplasmas per milliliter of culture at the 10th passage, but reached titers of 1.2 × 10^9 spiroplasmas per milliliter at the 100th passage. Large numbers of aggregated spiroplasmas that formed medusa-like spheres were present in later passages. LD-1 from the 98th passage, inoculated into cell culture at an initial titer of 4.4 × 10^5 spiroplasmas per milliliter, doubled every 28.7