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.
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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.*

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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

Table 1. Insect cell culture systems.

Insect cell line	Designation	Reference	Tissue derivation	Medium†	Peak titer‡ (spiroplasmas per ml)
Coleoptera					
*Diabrotica undecimpunctata**	IPLB-DU182A	(*15*)	Embryos	1	8×10^7
*D. undecimpunctata**	IPLB-DU182E	(*15*)	Embryos	1	8×10^7
Heteronychus arator	DSIR-HA1179	(*16*)	Embryos	2	2×10^7
Lepidoptera					
Trichoplusia ni	IAL-TND1	(*17*)	Imaginal discs	2	4×10^7
T. ni	IPLB-TN-R[2]	(*18*)	Embryos	3	2×10^8
Plodia interpunctella	IAL-PID2	(*19*)	Imaginal discs	2	8×10^7
Manduca sexta	MRRL-CH1	(*20*)	Embryos	3	2×10^8

*Used for primary isolation. †Medium 2 contains 3:1 Goodwin's IPL-52B:IPL-76 medium (*21*) plus 9 percent fetal bovine serum, glycerol (2 g/liter), DL (−) sodium glycerophosphate · $6H_2O$ (1 g/liter), polyvinylpyrrolidone K90 (0.1 g/liter), *N*-acetyl-D-glucosamine (1 g/liter), glutamine (2 g/liter), folic acid (4 mg/liter), and NaCl (366 mg/liter) to adjust osmolality. Medium 1 has the same composition as medium 2, but the osmolality was adjusted with mannitol (20 g/liter) instead of NaCl. Medium 3 is Grace's medium (*22*) supplemented with lactalbumin hydrolysate (3 g/liter), yeast autolysate (3 g/liter), and 9 percent fetal bovine serum. ‡Strain LD-1 was passaged nine times (1:10) in the IPLB-DU182E *Diabrotica* cell culture systems. Values are for LD-1 growth during subsequent passage into each of the cell culture systems. The different cell lines were seeded at different cell densities and had different growth rates; the growth obtained in these studies was therefore not necessarily optimal for any given cell line.

hours during lag phase, with a minimum exponential-phase doubling time of 7.5 hours (4 days after inoculation). Although LD-1 spiroplasma retained its translational motility in cell cultures, its spiral morphology changed to a helical morphology after ten passages. Nevertheless, cell-cultured LD-1 maintained infectivity, as evidenced by establishment of spiroplasmas from the 15th passage in ten of ten beetles inoculated by mouth; ten of ten control beetles, inoculated with cell culture alone, remained uninfected by spiroplasmas. The multiplying forms in the inoculated beetle guts regained their spiral morphology and exhibited conspicuous translational motility.

Although only *Diabrotica* cell lines were used for primary isolation, another coleopteran and four lepidopteran cell lines (Table 1) also supported excellent LD-1 growth. We were not able to isolate LD-1 in any of the cell-free media in the absence of insect cells. The media used for the various cell lines differed considerably in the types and amounts of amino acids, vitamins, salts, organic acids, sugars, peptones, and other components. LD-1 growth at the 10th passage varied tenfold (2×10^7 to 2×10^8 spiroplasmas per milliliter) among the different cell culture systems, with growth in systems containing modified Grace's medium initially appearing best. However, after ten additional passages of the organism in each of these cell culture systems, LD-1 grew equally well in all systems, attaining titers of about 2×10^8 organisms per milliliter.

According to our measurements, beetle gut-fluid osmolality (320 mOsm) and acidity (*p*H 5.6) correspond fairly well to that of the tissue culture media used (350 to 400 mOsm and *p*H 6.5, decreasing to *p*H 5.1 within 10 days after inoculation with LD-1). Compared to standard spiroplasma media, such as M1A and SP-4 (*9*) which are relatively low in defined amino acids (1,000 to 5,000 mg/liter) and high in peptones and extracts (13,000 to 17,000 mg/liter), media used in our cell culture systems are high in defined amino acids (9,000 to 10,000 mg/liter) and low in peptones and extracts (6,000 to 7,000 mg/liter). DelGiudice *et al.* (*11*) found that the apparent cell dependence of *Mycoplasma hyorhinis* strain 1050 was actually a manifestation of the inhibitory effect of peptones and extracts commonly used in bacterial media; reduction in concentrations of these inhibitors and substitution with tissue cell extracts permitted growth. Perhaps the low concentrations of these components in our media are responsible, in part, for the success of our insect cell culture systems.

Cell-free media that had been conditioned for 2, 24, or 48 hours by the growth of insect cells failed to support LD-1 growth; perhaps at least one essential cell-supplied factor is labile. Presumably, LD-1 gains access to growth factors from the insect cells via cell secretion or lysis. Although Clark showed (via transmission electron microscopy) that LD-1 adheres to and penetrates the membrane of the gut microvilli of the Colorado potato beetle (*2*), we have observed (via dark-field and fluorescent microscopy after staining cells with Hoechst 33258) only infrequent attachment to, and no penetration of, cultured cells.

Several workers (*12, 13*), using spiroplasmas that could be readily cultivated in cell-free media, have carried out studies of the cytopathic effects of those microorganisms for cell or organ cultures. This work began in 1982 when Steiner *et al.* (*12*) studied the interactions of corn stunt spiroplasma with cultured *Drosophila* cells. McGarrity and Kotani (*14*) recently demonstrated that *Spiroplasma mirum*, a relatively fastidious organism that is usually isolated in the rich SP-4 cell-free medium, could be isolated in nutrient-poor media that had been conditioned by the growth of insect cells.

Our study, which documents the successful cultivation of a highly fastidious spiroplasma, demonstrates that tissue cultures can be used to isolate mollicutes that cannot be cultivated in any other way. Cell culture systems, such as the ones described here for LD-1 culture, appear to have great potential for promoting isolation of other presently uncultivable mollicutes. Cell culture systems are similar to natural systems in many ways and may provide excellent in vitro models for studying the role of microbial physiology and behavior in pathogenicity.

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The Insulin Receptor Contains a Calmodulin-Binding Domain

Abstract. *Substantial evidence suggests that calcium has a pivotal role in regulating the initial events through which insulin alters plasma membrane metabolism. Because binding of insulin to its receptor represents the initial site of insulin action in the plasma membrane, studies were undertaken to determine whether the insulin receptor is a calmodulin-binding protein. Preparations enriched in the insulin receptor and calmodulin-binding proteins were isolated from detergent-solubilized rat adipocyte membranes by chromatography with wheat germ agglutinin agarose and calmodulin-conjugated Sepharose, respectively. Substantial purification of a manganese-dependent, insulin-sensitive phosphoprotein of 95K identified as the β subunit of the insulin receptor was accomplished. Binding and photocovalent cross-linking of iodine-125–labeled calmodulin to these affinity-purified preparations and to isolated plasma membranes, followed by immunoadsorption with insulin receptor antibodies bound to protein A Sepharose, resulted in significant purification of a binding complex of 110K to 140K. These results indicate that the adipocyte insulin receptor or a polypeptide closely associated with the receptor is a calmodulin-binding protein.*

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An association between Ca^{2+} and the action of insulin in the adipocyte plasmalemma is supported by observations that (i) insulin increases plasma membrane Ca^{2+} binding (*1*, *2*), (ii) the hormone decreases the activity (*3–6*) and phosphorylation (*7*) of a calmodulin-sensitive, high-affinity (Ca^{2+} and Mg^{2+}) adenosinetriphosphatase (ATPase), and (iii) extracellular Ca^{2+} is required for maximal insulin-stimulated glucose transport (*8*) and binding of insulin to (*9*) and phosphorylation of (*10*) the insulin receptor. The involvement of calmodulin is supported by the recent demonstration that insulin induces concentration-dependent increases (up to 75 percent) in binding of [^{125}I]calmodulin after addition of the hormone to adipocyte plasma membranes (*11*).

The insulin receptor is a heterologous molecule composed of two α (130K) and two β (95K) subunit glycoproteins linked by disulfide bonds (*12*). Binding studies with [^{125}I] insulin used in conjunction with cross-linking reagents or photoaffinity probes have suggested that the α subunit is the major insulin-binding domain (*13–16*). The β subunit is a tyrosine-specific protein kinase, and binding of insulin to its receptor stimulates this activity as well as phosphorylation of the β subunit (*17–20*). More importantly, the ability of insulin to stimulate this phosphorylation in detergent-solubilized membranes and purified receptor preparations provides a simple and convenient means to identify and monitor purification of the receptor.

Using insulin-stimulated phosphorylation as a marker, we determined that the β subunit of the insulin receptor was enriched in detergent-solubilized adipocyte membrane proteins isolated by affinity chromatography with calmodulin-conjugated Sepharose. As shown in lanes 3 and 4 in Fig. 1, an insulin-sensitive phosphoprotein of 95K was substantially purified by this approach. The specificity of this purification process is supported by the fact that the material applied to calmodulin Sepharose was characterized by several phosphoproteins (lanes 1 and 2 in Fig. 1). Moreover, similar results were observed after chromatography of solubilized membrane proteins with wheat germ agglutinin (WGA) agarose (lanes 5 and 6 in Fig. 1). The latter approach has been extensively used for enriching the insulin receptor (*17, 18, 21, 22*). Definitive identification of the insulin-sensitive 95K phosphoprotein illustrated in Fig. 1 as the β subunit was provided by its immunochemical isolation with antibody to the insulin receptor both before and after affinity chromatography. Thus, these results indicate that the insulin receptor binds to calmodulin in detergent-solubilized rat adipocyte membranes.

As evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, the profiles of proteins purified on calmodulin Sepharose and WGA agarose were markedly different. WGA-purified proteins were characterized by the presence of four major bands, of which the α and β subunits of the insulin receptor represented at least 50 percent of the stained protein. On the other hand, silver-stained gels of proteins purified on calmodulin Sepharose were characterized by a diverse array of proteins (12 major bands), of which the α and β subunits represented not more than 10 percent of the stained material. Thus, because the amounts of protein in the phosphorylation reactions depicted in lanes 3 and 4 [calmodulin-binding proteins (calmodulin BP's)] and lanes 5 and 6 (WGA BP's) in Fig. 1 were identical (0.5 μg per assay), the insulin receptor and hence the phosphorylated β subunit were present in greater amounts in the WGA BP preparation.

Using insulin-stimulated phosphorylation of the β subunit as a marker for the insulin receptor, we monitored the recovery of the receptor before adsorption to, and after elution from, WGA agarose and calmodulin Sepharose. Approximately 80 to 90 percent of the receptors present in detergent-solubilized plasma membranes were recovered from WGA agarose. When the WGA-purified receptor preparation was passed over calmodulin Sepharose, 25 to 50 percent of the receptors were recovered. These results suggest that only portions of the insulin receptor molecules bind to calmodulin Sepharose. Although it is tempting to speculate that the lack of quantitative binding reflects heterogeneity in the structure and calmodulin-binding properties of the receptor (for example, dissociation of α and β subunits), the reason for this apparent incongruity is unclear.

Photoaffinity labeling studies indicated that calmodulin binds to a protein in both calmodulin Sepharose– and WGA agarose–purified material which undergoes substantial purification after immunoadsorption with antibodies that recognize the insulin receptor. Calmodulin Sepha-