Mycoplasma lactucae sp. nov., a Sterol-Requiring Mollicute from a Plant Surface

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Strain 831-C4T (T = type strain), isolated from the surface of lettuce plants (Lactuca sativa) obtained from a retail food market, was shown to be a sterol-requiring mollicute. Morphological examination of this organism by electron and dark-field microscopic techniques showed that it consists of small, nonhelical, nonmotile, pleomorphic coccoid cells, with individual cells surrounded by a single cytoplasmic membrane. No evidence of a cell wall was observed. The organism grew rapidly in all conventional culture medium formulations for mollicutes in either aerobic or anaerobic environments. The optimum temperature for growth was 30°C, but multiplication occurred at 18 to 37°C. Strain 831-C4T catabolized glucose, but hydrolysis of arginine or urea could not be demonstrated. The genome size of strain 831-C4T was determined to be about 569 megadaltons, while the base composition (guanine-plus-cytosine content) of the DNA was 30.0 mol%. Recent studies in which we compared the 16S rRNA sequences of strain 831-C4T with those of more than 40 other mollicutes indicated that this organism is phylogenetically related to the Spiroplasma-Mycoplasma mycoides clade. Strain 831-C4T was serologically unrelated to the type strains of previously described Mycoplasma species and to 18 other unclassified sterol-requiring isolates cultivated from various animal, plant, or insect sources. Strain 831-C4T (= ATCC 49193) is the type strain of Mycoplasma lactucae sp. nov.

The presence of nonhelical, wall-less procaryotes (class Mollicutes) on plant surfaces was first well documented in 1979 (8, 15) when organisms with the general features of both Acholeplasma and Mycoplasma species were isolated from the surfaces of several tropical floral plants. Some of the strains described in these studies were serologically related to certain previously established Acholeplasma species that had at that time been found only in vertebrate hosts (e.g., Acholeplasma axanthum, Acholeplasma oculi, and Acholeplasma laidlawii). Other isolates discovered on plant surfaces eventually were found to represent distinct new Acholeplasma species having no obvious association with vertebrates (e.g., Acholeplasma florum) (14, 28). Shortly thereafter, other acholeplasmas, representing both vertebrate-associated and new putative plant-associated species, were reported to be present on the surfaces of vegetables (17; also see the review in reference 21).

The natural occurrence of acholeplasmas in the guts and hemolymphs of insects was established by Clark and colleagues in work carried out in the early 1980s (5, 25). These observations indicated that a wide variety of unclassified acholeplasmas can be found in insect gut fluids and on plant (especially flower) surfaces, leading to speculation that an exchange of these mollicutes occurs between insect and plant hosts during feeding excursions. We recently characterized one of these new acholeplasmas, Acholeplasma entomophilum, and identified it as an important part of the gut flora of a variety of insects and a frequent resident on plant surfaces (22; J. G. Tully, R. F. Whitcomb, D. L. Rose, K. J. Hackett, E. Clark, R. B. Henegar, P. Carle, and J. M. Bové, Zentrabl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A, in press).

The question of whether sterol-requiring Mycoplasma species participate in a similar insect-plant interaction was partially resolved recently with documentation of the presence of sterol-requiring mollicutes in insect guts or in hemolymph (25). One of the isolates (strain ELCN-1), which was found in the hemolymph of the firefly beetle Elychnia corrusa, has been characterized and has been named Mycoplasma elychnieae (23). In an accompanying paper, three other sterol-requiring mollicutes isolated from firefly beetles are characterized and named as new Mycoplasma species (29). Although the occurrence of these four Mycoplasma species on a plant host has yet to be established, several sterol-requiring mollicutes have been cultivated from plant or flower surfaces. A representative strain from a cluster of four related isolates cultivated from flowers of several tropical plants in Florida is characterized and assigned to the genus Mycoplasma in an accompanying paper (24).

In this report, we describe the features of a second sterol-requiring mollicute, which was isolated from the surface of lettuce collected at a retail food market, and propose that it be considered a new Mycoplasma species.

MATERIALS AND METHODS

Mycoplasma strains. Strain 831-C4T (T = type strain) was isolated from lettuce (Lactuca sativa) collected from a neighborhood vegetable stand in Columbus, Ohio. The geographic origin of the lettuce is unknown. The isolate was purified by conventional filtration-cloning techniques (18). The type strains of previously described Mycoplasma spe-
cies and a collection of currently unclassified, nonhelical, sterol-requiring mollicutes of diverse animal, plant, or insect origin were also used in various parts of this study (for a list of these species see reference 23).

**Culture medium and cultivation techniques.** Lettuce samples were tested on the day that they were purchased or were stored at 4°C for 2 to 4 days. The technique used for isolation of mollicutes from vegetables in this study was modified slightly from that described previously (17). Briefly, a single leaf of iceberg head lettuce was placed in a 1-liter flask containing 25 ml of SP-4 medium (27). The SP-4 broth also contained 500 μg of thallous acetate per ml and 1.0 mg of sodium ampicillin (Wyeth Laboratories, Philadelphia, Pa.) per ml. The lettuce-broth mixture was allowed to incubate for 4 h at 35°C, after which the contents were poured through a funnel containing several folds of cheesecloth covered with glass wool, and the filtrates were collected by vacuum pressure. In order to reduce possible electrotactic adherence of mollicutes, the sterile glass wool and cheesecloth had been rinsed with distilled water and fresh SP-4 broth prior to separation of plant material from washings. The filtrate was then sequentially passed through polycarbonate filters (diameter, 47 mm; Nuclepore Corp., Pleasanton, Calif.) with average pore sizes of 800, 600, and 400 nm. A 0.4-ml volume of each of the final 400-nm filtrates was inoculated into 12 ml of fresh SP-4 medium in a 4-dram (14.8-ml) screw-cap vial.

The vials were incubated at 37°C and examined daily for changes in turbidity or a color shift in the phenol red pH indicator. One vial exhibited a color change in the indicator to the acid side within 2 days, and broth from this vial was plated onto SP-4 agar medium. The agar plate was incubated at 35°C (in an atmosphere containing 95% nitrogen and 5% carbon dioxide), and colonies with the typical fried-egg morphology were observed after about 2 days. Broth cultures of this isolate (strain 831-C4T) were selected for purification, and early passages of the triply cloned line were preserved by lyophilization. For characterization studies, a lyophilized culture of strain 831-C4T was revived and passed twice in SP-4 broth at 30°C. The other culture media employed included the Edward formulation of conventional 20% horse serum mycoplasma broth (9), serum fraction broth containing 1% bovine serum fraction (20), and serum-free media supplemented with fatty acid mixtures (14, 22). A solid medium of each of these formulations was made by adding 0.8% Noble agar (Difco Laboratories, Detroit, Mich.). Agar cultures were incubated at 30°C, either aerobically (with 5% carbon dioxide in a GasPak system [BBL Microbiology Systems, Cockeysville, Md.]) or under anaerobic conditions (hydrogen GasPak system).

To measure the temperature requirements for growth of strain 831-C4T, a series of 10-fold dilutions of the organism in SP-4 broth were prepared. One series of the diluted mycoplasma was incubated at each of six selected temperatures (10, 18, 25, 30, 32, 35, and 37°C). The relative number of organisms, measured by noting color changes (red to yellow) and turbidity in the highest dilution of SP-4 broth (color-changing units [CCU] per milliliter), was recorded after purification, and early passages of the triply cloned line were subcultured, the organism was plated onto conventional blood agar and incubated aerobically at 37°C. After 2 to 10 days, the plates were examined for bacterial colonies and evidence of reversion.

**Morphological studies.** Strain 831-C4T was grown overnight at 30°C in SP-4 broth, and the resulting logarithmic-phase cultures were examined at a magnification of ×1,250 by dark-field microscopy. For electron microscopic examination, the organism was grown in approximately 10 ml of horse serum broth, and the cells were pelleted by centrifugation. The cell pellet was then fixed for 2 h in 3% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, embedded in Epon-araldite, sectioned, and stained with 1% aqueous uranyl acetate and Reynolds lead citrate.

**Sterol requirement.** Growth requirements for sterol were assayed by using a standard broth culture method (19) at 30°C. The protein contents of mycoplasma cell pellets were assayed with a protein kit (Bio-Rad Laboratories, Richmond, Calif.). A growth inhibition test with 1.5% digitonin, as an indirect measure for sterol requirement, was also performed (19).

**Tests for biological and biochemical properties.** The procedures used to demonstrate carbohydrate fermentation and arginine and urea hydrolysis have been described previously (1). The procedures used to assess filtration characteristics (18), for the hemadsorption assay (11), and for the film and spot reaction (10) also have been described previously.

**Serological tests.** Antisera to strain 831-C4T was raised in rabbits by using recommended techniques for mollicutes (16). Hyperimmune antisera to all 80 previously described *Mycoplasma* species and to 18 other unclassified mycoplasmas were available from the reference collection maintained at the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. (see list in reference 23). Antisera in this collection were utilized in standard disk growth inhibition tests (6) with strain 831-C4T, in which we used a mycoplasma agar medium containing the 1% bovine serum fraction supplement and aerobic incubation at 30°C. Direct agar plate immunofluorescent tests (12) were performed on strain 831-C4T colonies on horse serum agar medium by using fluorescein-conjugated antisera to the mycoplasmas referred to above (23). Finally, disk growth inhibition tests with strain 831-C4T were also performed with antisera to 11 recognized *Acholeplasma* species (22).

**Genomic analysis.** Techniques for extraction and purification of chromosomal DNA from mollicutes have been described previously (3). Procedures for determining genome size by renaturation kinetics of denatured DNA have also been described previously (2). The guanine-plus-cytosine content of purified DNA of strain 831-C4T was determined by using buoyant density techniques (4). Purified DNA from *Spiroplasma citri* (genome size, approximately 1,000 megadaltons; base composition, 26 mol% guanine plus cytosine) was used as a reference standard.

**RESULTS AND DISCUSSION**

**Cultural and morphological properties.** Strain 831-C4T grew rapidly and extremely well in all types of mycoplasma broth media, including those containing fetal bovine serum (e.g., SP-4 medium), horse serum, or bovine serum fraction supplements. Broth cultures usually reached peak logarithmic-phase growth levels in 8 to 18 h at 30°C. Satisfactory growth was also observed on solid media prepared from all of these formulations. However, growth could not be sustained beyond three consecutive passages in serum-free formulations or in serum-free media containing supplements of the Tween 80 fatty acid mixture. Growth occurred over a temperature range of 18 to 37°C, with optimum growth observed at 30°C. Colonies of strain 831-C4T on horse serum agar exhibited typical fried-egg morphology (Fig. 1). Growth
on solid medium was observed on agar plates incubated aerobically at 30°C or when the plates were placed in a GasPak system under an anaerobic atmosphere.

Logarithmic-phase cultures of strain 831-C4T in SP-4 medium examined by dark-field microscopy showed predominantly coccoid forms. A few nonmotile, filamentous forms were seen, but no evidence of helical structures was apparent. Cells of the organism sedimented from broth cultures and examined by electron microscopic techniques again showed predominately coccoidal elements. No evidence of a cell wall was observed in these preparations; representative round cells varied from 300 to 500 nm in diameter and were surrounded by a single cytoplasmic membrane (Fig. 2).

**Sterol requirement.** The responses of strain 831-C4T to cholesterol supplements in serum-free SP-4 medium are shown in Table 1. No growth was apparent in the base broth alone, while enhanced growth was observed when 1 to 5 µg of cholesterol per ml was present. Cholesterol concentrations greater than 5 µg/ml seemed to be slightly inhibitory. A 6-mm zone of growth inhibition was observed with strain 831-C4T in the digitonin test, again confirming the need for sterol as a growth supplement.

**Biochemical and biological properties.** Strain 831-C4T rapidly fermented glucose with the production of acid and a consequent reduction in the pH of the culture medium. No evidence of arginine or urea hydrolysis was observed. Strain 831-C4T did not produce the film and spot reaction, but colonies of the organism on agar medium hemadsorbed guinea pig erythrocytes. Passage of a strain 831-C4T broth culture containing 10⁹ CCU/ml through either a 450- or 300-nm pore size membrane filter reduced the viable cell titer only 10-fold (10⁸ CCU/ml). Filtrates recovered after passage of the strain through a 220-nm pore size membrane filter contained 10⁷ CCU/ml, and a few cells of strain 831-C4T were also able to pass through a 100-nm pore size filter (titer, 10⁷).

**FIG. 1.** Colonies of strain 831-C4T on SP-4 agar medium after 3 days of incubation at 37°C in an aerobic environment. Bar = 100 µm.

**FIG. 2.** Electron micrograph of a sectioned and stained cell pellet of strain 831-C4T. Sections were stained with 2% aqueous uranyl acetate and Reynold lead citrate. The arrow indicates the unit membrane. Bar = 100 nm.

A timed growth curve study performed in SP-4 broth with an initial inoculum of 1.6 x 10⁴ CCU/ml showed that a peak titer of 1.4 x 10⁹ CCU/ml was reached within 30 h at 35°C.

**Serological tests.** Growth inhibition and plate immunofluorescent tests were carried out with antisera or conjugates to mycoplasmas and acholeplasmas listed recently (23). These tests indicated that strain 831-C4T is not related serologically to 80 previously established species in the two genera or to a group of 18 unclassified, nonhelical, sterol-requiring mollicutes that represent putative species in the genus *Mycoplasma.*

**Genome size and DNA base composition.** Genome size measurements on the DNA of strain 831-C4T, in which renaturation kinetics were used, indicated an average genome value of about 569 megadaltons (individual values ranged from 487 to 673 megadaltons). The base composition (guanine-plus-cytosine content) of the DNA of strain 831-C4T was determined to be 30 ± 1 mol%, as measured by buoyant density.

**Habitat.** The single mollicute strain characterized in this study is suspected to have been present on the leaf surface of lettuce. However, until further isolations representative of this organism are made from plant surfaces, insects, arthropods, or other environments or locations, the true habitat and the possibility that this organism is involved in a plant surface-insect cycle will remain unknown.

**TABLE 1.** Growth response of strain 831-C4T to cholesterol

<table>
<thead>
<tr>
<th>Supplement(s) added to serum-free base medium</th>
<th>Cholesterol concn (µg/ml)</th>
<th>Amt of protein (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse serum (20%)</td>
<td>Control</td>
<td>1.99</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>IG*</td>
</tr>
<tr>
<td>Albumin (1%), Tween 80 (0.01%), and palmitic acid (10 µg/ml)</td>
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<td>0.853</td>
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<tr>
<td></td>
<td>5</td>
<td>2.381</td>
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<td></td>
<td>10</td>
<td>2.228</td>
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<td></td>
<td>20</td>
<td>2.228</td>
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* All cultures were incubated at 30°C.

* IG, Insufficient growth.
Mycoplasma lactucae sp. nov. Mycoplasma lactucae (lac’tu·ce·ae; L. fem. n. lactuca, lettuce; L. gen. n. lactucae, of lettuce, referring to the plant from which the organism was first isolated). Cells consist primarily of coccoid forms, varying in size from 300 to 500 nm in diameter, with only occasional short, nonhelical, pleomorphic filaments. Cells lack true cell walls. Nonmotile. Colonies on solid medium was first isolated. Cells consist primarily of coccoid forms, a representative plant isolate in a number of useful metabolic studies on mollicutes (7; J. D. Pollack, K. D. Beaman, V. V. Tryon, and J. Robertson, Proc. 4th Congr. Int. Org. Mycoplasmol.; Yale J. Biol. Med. 57:891, 1984).

A recent extensive phylogenetic analysis of conserved sequence subunits of the 16S rRNAs of more than 40 mollicutes (26) has shown that strain 831-C4T is a member of the Spiroplasma-Mycoplasma mycoides clade of mollicutes. This organism is evolutionarily especially close to the aforementioned floral isolate strain MIT (24) and to a cluster of other mollicutes that have important insect-plant associations. The information available in this broad phylogenetic study provides strong support for the assignment of strain 831-C4T to the genus Mycoplasma.

The properties described here for strain 831-C4T fulfill the essential criteria (13) for species of the class Mollicutes, including absence of cell wall, filterability, lack of reversion to walled bacteria when the organism is grown in antibiotic-free media, penicillin resistance, and production of typical colonial forms on agar. The growth requirement for sterol or serum and the lack of helicity place this organism in the family Mycoplasmataceae. The inability of strain 831-C4T to hydrolyze urea, in conjunction with the properties outlined above, mandates assignment of the organism to the genus Mycoplasma. Finally, a serological comparison of strain 831-C4T with a collection of all of the previously described Mycoplasma species and with other unclassified strains that probably represent putative species in the genus Mycoplasma demonstrated that the new isolate is unrelated to other organisms in the genus. Therefore, we propose the name Mycoplasma lactucae for this organism. The taxonomic description below summarizes the properties of the organism.

Mycoplasma lactucae sp. nov. Mycoplasma lactucae (lac’tu·ce·ae; L. fem. n. lactuca, lettuce; L. gen. n. lactucae, of lettuce, referring to the plant from which the organism was first isolated). Cells consist primarily of coccoid forms, varying in size from 300 to 500 nm in diameter, with only occasional short, nonhelical, pleomorphic filaments. Cells lack true cell walls. Nonmotile. Colonies on solid medium containing 0.8% Noble agar usually have the appearance of fried eggs. Chemoorganotroph. Acid is produced from glucose. Does not hydrolyze arginine or urea. Film and spot reaction negative.

Agar colonies hemadsorb guinea pig erythrocytes. Cholesterol or serum is required for growth. The temperature range for growth is 18 to 37°C; optimum growth occurs at 30°C. Serologically distinct from previously described Mycoplasma species. Isolated from lettuce (L. sativa) collected from a retail produce market; natural habitat unknown. Pathogenicity for plants or insects has not been determined.

The average genome size is 569 megadaltons. The guanine-plus-cytosine content of the DNA is 30 ± 1 mol%, as determined by the buoyant density method.

The type strain is strain 831-C4 (= ATCC 49193).

ACKNOWLEDGMENT

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LITERATURE CITED


