Acholeplasma entomophilum sp. nov. from Gut Contents of a Wide Range of Host Insects

JOSEPH G. TULLY,1* DAVID L. ROSE,2 PATRICIA CARLE,2 JOSEPH M. BOVÉ,2 KEVIN J. HACKETT,3 AND ROBERT F. WHITCOMB3

Mycoplasm Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Frederick Cancer Research Facility, Frederick, Maryland 21701; Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de Recherche Agronomique, Pont-de-la-Maye, France; Insect Pathology Laboratory, U.S. Department of Agriculture, Beltsville, Maryland 20705

Eleven sterol-nonrequiring mollicute strains isolated from gut contents of representatives of nine insect genera and two strains isolated from flower surfaces (Bidens sp.) were found to be serologically similar to an insect-derived acholeplasma (strain TACT [T = type strain]) described briefly in an earlier report. Strain TACT, isolated from gut fluids of a tabanid fly (Tabanus catenatus) and shown in earlier studies to belong to the class Mollicutes, lacked a sterol requirement for growth and catabolized glucose, but did not hydrolyze arbutin, arginine, or urea. Most strains of the strain TACT cluster grew on serum-free medium alone or on serum-free medium supplemented with a 0.04% Tween 80 fatty acid mixture. The strains grew over a temperature range of 23 to 32°C (optimum, 30°C) but did not grow at 37°C. The guanine-plus-cytosine content of the deoxyribonucleic acid was 30 mol%. Strain TACT was serologically unrelated to the ten previously established species in the genus Acholeplasma and to six other unclassified sterol-nonrequiring strains isolated from plant or insect hosts. The data suggest that this group of new acholeplasmas is widespread in insects and may be disseminated among various plant surfaces during feeding excursions. Strain TAC (= ATCC 43706) is proposed as the type strain of Acholeplasma entomophilum sp. nov.

Acholeplasmas (class Mollicutes, order Acholeplasmatales) have become important models in basic studies on wall-less procaryotes, particularly in relation to membrane structure and function, microbial physiology, virus carriage, and procaryote evolution (6, 19, 20, 27, 29, 35). In contrast to most other mollicutes, acholeplasmas require no cholesterol or serum supplements for growth (12, 22, 23). In the early history of this group, the ability of acholeplasmas to grow in simple media was frequently invoked in describing them as "saprophytes." This suspected mode of existence was also used to explain their recovery from soil and sewage (see review in reference 26). Later, however, it was established that a variety of serologically and genetically distinct acholeplasmas occurred in association with numerous animal hosts, and it was postulated that the presence of acholeplasmas in soil and sewage came from animal contact (12). Reports of the occurrence of acholeplasmas on, and perhaps in, plants (reviewed in reference 27) began to appear soon after other wall-less procaryotes were found in plant tissues (8). However, since most of these mollicutes had the features of Acholeplasma laidlawii, a species originally found in both animal tissues and saprophytic environments, it was not certain what role plants played in the ecology of acholeplasmas (27). In 1979, the recovery of Acholeplasma axanthum, A. oculi, and A. laidlawii from decaying crown tissues of coconut palms afflicted with "lethal yellowing disease" (10) provided substantial evidence for plant involvement in acholeplasma maintenance in nature. These and other Acholeplasma species also were subsequently recovered from the surfaces of flowers (18, 28, 33) and fresh vegetables (25). However, three of the strains derived from flower surfaces were discovered to be serologically related but distinct from other classified acholeplasmas. These organisms were later characterized and named Acholeplasma florum (17).

The occurrence of acholeplasmas on plant surfaces, particularly in locations with little access to animal, sewage, or soil contamination (e.g., crown tissues of palms) (10), strongly implicated insect dissemination of acholeplasmas. This supposition was recently confirmed when three new acholeplasma isolates (TACT [T = type strain], YJS, and PS-1) and two strains of A. florum were cultivated from insect gut fluids (4). These findings, which completed the missing ecologic link in the maintenance and dispersion in nature of these mollicutes, were confirmed in another recent report. In these studies (30), 23 acholeplasmas which had been isolated from gut fluids of various insect hosts by T. B. Clark and his associates were partially characterized; 11 isolates from 10 different insect species were related to strain TACT, 2 isolates were related to strain YJS, 2 isolates were identified as A. florum, and 8 insect acholeplasmas were unrelated to all currently known Acholeplasma species or unclassified strains.

In this report we describe the taxonomic properties of strain TACT, which was selected as representative of a group of 14 isolates, and propose that it be given status as a new species in the genus Acholeplasma.

MATERIALS AND METHODS

Acholeplasma strains. Techniques for primary isolation of acholeplasmas and other mollicutes from insect tissues have been reported previously (16). Details of the isolation of strain TACT from a tabanid fly (Tabanus catenatus) and some preliminary characteristics of purified (triply cloned) strain TACT have been presented previously (4). The isolation from specific insect hosts of 11 additional acholeplasma strains and two strains from flowers, all serologically related to strain TACT, was outlined earlier in a brief report (30). The type strains of established Acholeplasma species employed in this study included A. axanthum S-743, A. equife-

* Corresponding author.
tale C112, A. florum L1, A. granulatum BTS39, A. hippocon C1, A. laidlawii PG8, A. modicum PG49, A. morum 72-043, A. oculi 19L, and A. parvum H23M. Several unclassified acholeplasmas strains of plant or insect origin were also included in serological comparisons of new isolates; these were palm isolate J-233 (10), flower isolate F-7 (J. C. Vignault, C. Saillard, and J. M. Bové, unpublished data), vegetable strain 0502 (25), insect-derived acholeplasma strains YJS and PS-1 (4), and strain PUPA-2, which is representative of three new serologically distinct acholeplasmas isolated from gut fluids of fireflies (Photuris pennsylvania) by T. B. Clark and K. J. Hackett (30).

Media and cultivation procedures. Most insect-derived acholeplasmas were grown in primary culture on either SM-1 or MD medium (31) at 26 to 30°C. Following several early passages, a majority of strains could be transferred to conventional acholeplasma media containing 1% bovine serum fraction (29) or to a serum-free medium consisting of mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.) supplemented with palmitic acid (10 µg/ml), 0.5% (vol/vol) bovine serum albumin (fatty acid poor; Sigma Chemical Co., St. Louis, Mo.), and 0.01 to 0.04% (vol/vol) Tween 80 fatty acid mixture (Atlas Chemical Co., Wilmington, Del.) (4, 17, 28, 29). A solid medium was prepared by adding 0.8% Noble agar (Difco Laboratories, Detroit, Mich.) to the serum fraction or serum-free broth base prior to sterilization. Agar cultures of the acholeplasmas usually were incubated at 30°C under anaerobic environments. Serial 10-fold dilutions of stock cultures of strain TACT and several other insect-derived isolates adapted to growth in serum-free broth supplemented with 0.04% Tween 80, glucose, and phenol red indicator were incubated at 23, 30, 32, or 37°C. The relative number of organisms, measured by recording color change (red to yellow) and turbidity in the highest 10-fold broth dilution (color-changing units per milliliter), was recorded after an incubation period of 5 days.

Morphological studies. Broth cultures of strain TACT and other related acholeplasmas were examined by dark-field microscopy.

Sterol requirement. Sterol requirements for growth were determined by a broth culture method (23).

Tests for biological and biochemical properties. The procedures used to determine carbohydrate fermentation and arginine and urea hydrolysis have been described previously (1). Production of β-D-glucosidase involved in hydrolysis of arbutin was assayed by a fluorogenic test (2, 24). Carotenoid production was tested by a method described previously (21). The procedures used for the hemadsorption assay (15) and film and spot reaction (14) have been described previously.

Serological tests. Antiserum to strain TACT was raised in rabbits. Hyperimmune antisera to established Acholeplasma species and to six other unclassified acholeplasmas came from the reference collection maintained at the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. Disk growth inhibition tests, in which we used these antisera and strain TACT as an antigen, were carried out on serum fraction agar plates by using techniques described previously (5). In addition, direct plate immunofluorescence tests (7) were performed on strain TACT grown on agar medium by utilizing fluorescein-conjugated antisera to the acholeplasmas listed above. Finally, disk growth inhibition tests were also performed with antisera to about 75 species in the genus Mycoplasma (see reference 13 for a list of the species and unclassified sterol-requiring mycoplasmas employed in these tests).

DNA base composition. The guanine-plus-cytosine content of the purified deoxyribonucleic acid (DNA) of strain TACT was determined by buoyant density measured by equilibrium centrifugation in a CsCl gradient (3). Purified DNA from Spiroplasma citri (26 mol% guanine plus cytosine) was used as a reference in the procedure.

RESULTS AND DISCUSSION

Cultural and morphological properties. Broth cultures of strain TACT grew rapidly, producing visibly turbid cultures within 24 to 48 h in serum-containing medium at 30°C. Less turbidity, but satisfactory growth, was apparent in medium containing a bovine serum fraction supplement or in serum-free broth supplemented with 0.04% Tween 80. Little or no growth occurred in serum-free medium alone, as was reported in some previous studies on strain TACT (4). The other 13 similar isolates also grew well in Tween-supplemented serum-free base media. Strain TACT did not require cholesterol for growth, since Tween 80 supplements to serum-free medium produced growth almost equal to that obtained in cholesterol-containing medium (Table 1). Growth occurred over a temperature range of 23 to 32°C, with the greatest number of organisms (108 color-changing units per ml) obtained in broth cultures incubated at 30°C. The amount of growth observed in broth cultures maintained at 37°C was small (<106 color-changing units per ml). Colonies of strain TACT on agar media exhibited typical "fried egg" morphology (Fig. 1). Dark-field microscopy of broth cultures at 24 to 48 h revealed a mixture of small to medium pleomorphic coccoid forms. The cellular morphology of strain TACT was examined previously by electron microscopy (4). These studies confirmed a coccoid form as the predominant shape for the strain; cells of the organism were surrounded by a single cytoplasmic membrane and were devoid of cell wall material.

Biochemical and biological properties. Strain TACT and most other insect- or flower-derived isolates of the group showed strong fermentative activity on media containing glucose. However, mannose was not fermented by strain TACT, and no arginine or urea hydrolysis was apparent. Tests for β-D-glucosidase were negative, indicating that arbutin was not hydrolyzed. Carotenoid and film and spot reaction tests were negative. However, colonies of strain TACT on agar medium showed positive hemadsorption of guinea pig erythrocytes. In studies re-

<table>
<thead>
<tr>
<th>Cholesterol concn in medium (µg/ml)</th>
<th>Amt of cell protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>1.56</td>
</tr>
<tr>
<td>5.0</td>
<td>1.81</td>
</tr>
<tr>
<td>10.0</td>
<td>2.18</td>
</tr>
<tr>
<td>20.0</td>
<td>2.18</td>
</tr>
<tr>
<td>Control*</td>
<td>3.12</td>
</tr>
</tbody>
</table>

* Amount of protein in the cell pellet obtained from 100 ml of growth medium.

** Serum-free medium.

* Serum-free medium supplemented with 0.5% albumin and 10 µg of palmitic acid per ml.

* Serum-free medium supplemented with 0.5% albumin, 10 µg of palmitic acid per ml, and 0.04% Tween 80.

* Medium containing 1% bovine serum fraction.
strain TAC\textsuperscript{T} and related acholeplasmas from insect guts have been shown to be members of the class Mollicutes (11). The ability of these organisms to grow in the absence of cholesterol or serum supplements indicates that they belong to the order Acholeplasmatales, family Acholeplasmataceae, and genus Acholeplasma (29). We propose, therefore, that these organisms be designated Acholeplasma ento-mophillum sp. nov. The taxonomic description given below summarizes the properties of the organism.

Acholeplasma entomophillum sp. nov. (en.to.mo’phil.um. Gr. n. entom. insect; Gr. v. phylein love; Lat. fem. adj. entomophillum insect loving). Cells are pleomorphic but primarily coccoid, varying from 300 to 800 nm in diameter. Cells lack true cell walls. Nonmotile. Colonies on solid medium containing 0.8% Noble agar usually have the appearance of fried eggs. Chemoorganotroph. Acid produced from glucose, but not mannose. Does not hydrolyze arginine, urea, or arbutin. Carotenoids are not produced when cells from 1-liter volumes of liquid broth are examined. Film and spot reaction negative.

Agar colonies hemadsorb guinea pig erythrocytes. Cholesterol not required, although some strains require 0.4% Tween 80 fatty acid supplements for growth in serum-free media.

Temperature range for growth is 23 to 32°C, with optimum growth at about 30°C.

Serologically distinct from other Acholeplasma species. Isolated from gut contents of tabanid flies, beetles, butterflies, honey bees, and moths and from flowers. Apparently nonpathogenic for the insect hosts. Pathogenicity for other insects has not been determined.

The guanine-plus-cytosine content of the DNA is 30 ± mol%, as determined by buoyant density measurements. The type strain is strain TAC (= ATCC 43706).

FIG. 1. Colonies of strain TAC\textsuperscript{T} on 1% bovine serum fraction agar medium after 3 days of incubation at 30°C.

ported previously (4), strain TAC\textsuperscript{T} was filterable through 220-nm porosity membrane filters, was resistant to 500 U of penicillin per ml and did not revert to bacterial or cell wall-containing forms when it was grown in antibiotic-free culture media.

**Serological testing.** Disk growth inhibition tests, in which we compared strain TAC\textsuperscript{T} against antisera to 10 established Acholeplasma species and against antiserum of six unclassified acholeplasmas, did not show evidence of serological cross-reactions. These results were also confirmed in epi-immunofluorescence tests when agar colonies of strain TAC\textsuperscript{T} were treated with conjugated antisera to the 16 acholeplasmas listed above. Strain TAC\textsuperscript{T} was also employed in disk growth inhibition tests in which antiserum to type strains of approximately 75 Mycoplasma species were utilized. These tests also indicated that strain TAC\textsuperscript{T} was unrelated to any established sterol-requiring species in the genus Mycoplasma.

**DNA characteristics.** The guanine-plus-cytosine content of the DNA of strain TAC\textsuperscript{T}, determined by averaging the results obtained from three separate buoyant density measurements, was found to be 30 ± 1 mol%.

**Habitat.** As reported elsewhere (30), acholeplasma strains serologically related to strain TAC\textsuperscript{T} have been isolated from a variety of insect hosts, including representatives of at least nine different genera. These observations provide substantial evidence that the organism is prevalent in the insect-plant environment. Isolations of acholeplasmas serologically identical to strain TAC\textsuperscript{T} appear to far outnumber reported isolations of A. florum (4, 17, 30). Although equal numbers of isolations were attempted from insect hemolymph and gut fluids, all of the isolated organisms were derived from guts.

The widespread dissemination of the organism probably occurs during feeding excursions on infected plant surfaces. Strain TAC\textsuperscript{T} organisms are not known to be pathogenic for their insect hosts. Whether they might be pathogenic for other insects remains to be determined, either through field studies or by experimental insect inoculations (16). However, it has been clearly established that some acholeplasmas can persist and multiply in insects (9, 32, 34).

From the properties described here and elsewhere (4, 30),


