Acholeplasma brassicae sp. nov. and *Acholeplasma palmae* sp. nov., Two Non-Sterol-Requiring Mollicutes from Plant Surfaces

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Two mollicutes (strains 0502^T [T = type strain] and J233^T), which were isolated from the surfaces of broccoli (*Brassica oleracea* var. *italica*) plants or the crown tissues of the coconut palm (*Cocos nucifera*), were capable of sustained growth in serum-free (or cholesterol-free) mycoplasma broth media. Examination by electron and dark-field microscopic techniques revealed that the cells of each strain were small, nonhelical, nonmotile, pleomorphic, and coccoid and that each cell was surrounded by a single cytoplasmic membrane. No evidence of a cell wall was found. The organisms were filterable and grew rapidly in most conventional mycoplasma culture medium formulations containing horse or fetal bovine sera under either aerobic or anaerobic conditions. The optimum temperature for growth of both organisms was 30°C, but multiplication occurred over a temperature range from 18 to 37°C. Both strains catabolized glucose, but did not hydrolyze arbutin, arginine, or urea. The genome size of strain 0502^T was 1,215 kbp, and the DNA base composition (guanine-plus-cytosine content) was 35.5 mol%. The genome size of strain J233^T was 1,610 kbp, and the DNA base composition was 30.0 mol%. The two isolates were not serologically related to each other or to the type strains of 11 previously described *Acholeplasma* species. Strain 0502 (= ATCC 49388) is the type strain of *Acholeplasma brassicae* sp. nov., and strain J233 (= ATCC 49389) is the type strain of *Acholeplasma palmae* sp. nov.

The presence of nonhelical, wall-less procaryotes (class *Mollicutes*) on plant surfaces was first documented in 1979 (10, 15) when what at the time were thought to be both sterol-requiring and non-sterol-requiring mollicutes were isolated from several tropical and subtropical plants. According to prevailing taxonomic concepts, all of the non-sterol-requiring organisms were regarded as *Acholeplasma* species. Indeed, some of the non-sterol-requiring isolates obtained from plant surfaces were later shown to be related to *Acholeplasma* species previously isolated from vertebrate hosts (e.g., *Acholeplasma axanthum*, *Acholeplasma oculi*, and *Acholeplasma laidlawii*) (10, 23). Other strains that grew in the absence of cholesterol or serum appeared to be members of putative new *Acholeplasma* species (5, 23, 25, 29, 31, 33).

The mollicutes from plants that appeared to be sterol-requiring organisms were at first referred to the genus *Mycoplasma*. Subsequent research, however, showed that the members of this group belonged to two distinct clusters, neither of which consisted of true *Mycoplasma* species. The strains in one cluster were mollicutes that, although they were not able to grow in a serum-free base broth medium, grew rapidly and continuously if small amounts (0.04%) of polyoxyethylene sorbitan monooleate (Tween 80) were added to the base medium (21, 25, 31, 33). Later, a large number of strains that had similar growth requirements were isolated from other plant and insect hosts from temperate regions. At first, these strains were mistaken for acholeplasmas, because Tween 80

could apparently replace sterol in the growth media used. However, this cluster of organisms was eventually shown to be phylogenetically distinct from the acholeplasmas (34). At the present time, these strains have been placed in 11 species, all of which have been assigned to the recently proposed new genus *Mesoplasma* in the class *Mollicutes* (30, 32).

Extensive studies of the second group of plant isolates showed that these mollicutes had more strict growth requirements for cholesterol or serum; no growth stimulation was observed after addition of Tween 80 to serum-free media. Later, additional mollicutes with similar nutritional needs were isolated from plants and insects in temperate regions (36). Five new species were recognized within this cluster of mollicutes, and recently these organisms were assigned to a second new genus (*Entomoplasma*) in the class *Mollicutes* (30).

Two of the plant surface strains obtained in the 1979 (10) and 1982 (23) studies have been examined further to confirm the initial identification of these organisms as true *Acholeplasma* species. In this report, we describe the properties of these strains and propose that these organisms should be designated new *Acholeplasma* species.

MATERIALS AND METHODS

Mycoplasma strains. Strain 0502^T (T = type strain) was isolated from broccoli plants in Columbus, Ohio, in 1982 (23). Strain J233^T was derived from an isolate (originally designated E847) obtained from crown tissues of a coconut palm infected with lethal yellowing disease in Jamaica in 1978 (10). A triply cloned (26) culture of strain J233^T was prepared in England and subsequently was deposited in the American Type Culture Collection as strain ATCC 33684. Later, this purified strain was used in a number of pathogenicity studies (8, 9). The original strain J233^T culture examined in the United States was

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triply cloned and later was also deposited in the American Type Culture Collection as strain ATCC 49389^T. Although strains J233^T and E847 are serologically identical and represent the same species, the characterization study described in this paper was carried out with cloned strain J233^T (= ATCC 49389^T). Strain 0502^T was also purified by conventional filtration-cloning techniques. The following type strains of previously described *Acholeplasma* species were used in various parts of this study and were obtained from the collection of the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md.: *Acholeplasma axanthum* S-743^T, *Acholeplasma cavigenitalium* GP3^T, *Acholeplasma equifetale* C112^T, *Acholeplasma granularum* BTS39^T, *Acholeplasma hippikon* C1^T, *Acholeplasma laidlawii* PG8^T, *Acholeplasma modicum* PG49^T, *Acholeplasma morum* 72-043^T, *Acholeplasma multilocale* PN525^T, *Acholeplasma oculi* 19L^T, and *Acholeplasma parvum* H23M^T.

Culture medium and cultivation techniques. The general techniques used for isolating mollicutes from plant sources have been described previously (7). Strain 0502^T was cultivated by washing plant tissue with SP-4 broth (35), filtering the wash fluid through a sterile 450-nm-pore-size membrane filter, and incubating the preparation at 37°C. Strain J233^T was grown initially on conventional mycoplasma media supplemented with 10% horse serum (11), but growth also occurred in bovine serum fraction media (28) that were supplemented with or lacked 0.01% Tween 80. Characterization studies were performed with either serum fraction or SP-4 broth media, containing 500 U of penicillin per ml. Solid media were prepared with 0.8% Noble agar (Difco Laboratories, Detroit, Mich.) and were incubated under aerobic and anaerobic conditions (GasPak system; BBL Microbiology Systems, Cockeysville, Md.).

An incubation temperature of 30°C was used throughout this study. The techniques used for determining the optimum growth temperatures and the temperature ranges for growth (from 10 to 37°C) for the two strains (in SP-4 broth) have been described previously (36). Early passages of strains 0502^T and J233^T were also maintained for at least five consecutive passages on horse serum broth that lacked penicillin and other antibiotics. At each subculturing, the organisms were 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. Strains 0502^T and J233^T were grown overnight at 30°C in SP-4 broth, and the resulting logarithmic-phase cultures were examined by dark-field microscopy, using a magnification of $\times 1,250$. For electron microscopic examination, each 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 for 1 h in 1% osmium tetroxide, dehydrated in acetone, embedded in Epon-araldite, sectioned, and stained with 1% aqueous uranyl acetate and Reynold's lead citrate (36).

Sterol requirement. Growth requirements for sterol were determined by a standard broth culture method (19, 27), using serum-free broth medium supplemented with either 0.01% Tween 80 fatty acid mixture or cholesterol; various concentrations of cholesterol were tested. All of the broth cultures used in these tests were incubated at 30°C. The protein contents of mycoplasma cell pellets were determined with a protein kit (Bio-Rad Laboratories, Richmond, Calif.).

Tests for biological and biochemical properties. The procedures used to study carbohydrate fermentation (1) and hydrolysis of arbutin (20), arginine, and urea (1) have been described

FIG. 1. Colonies of two acholeplasmas on 20% horse serum agar incubated for 3 days at 30°C under anaerobic conditions. (A) Strain J233^T. (B) Strain 0502^T. Bar = 100 μ m.

previously. The techniques used to assess filtration characteristics (26) and hemadsorption (12) also have been described previously.

Serological tests. Antiserum to strains 0502^T and J233^T was raised in rabbits by using standard techniques (22). Hyperimmune antisera to all 11 previously described *Acholeplasma* species (see above) were obtained from the reference collection maintained at the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. The antisera were used in standard disc growth inhibition tests (6) performed with strains 0502^T and J233^T by using an agar medium containing 1% bovine serum fraction and anaerobic incubation at 30°C. Direct agar plate immunofluorescence tests (13) were performed with strain 0502^T or J233^T agar colonies and fluorescein-conjugated antisera to other acholeplasmas (see above).

Genomic analysis. The techniques used to extract and purify chromosomal DNAs from mollicutes and to determine genome sizes by pulsed-field gel electrophoresis have been described previously (3, 18). The guanine-plus-cytosine contents of the purified DNAs of strains 0502^T and J233^T were determined by buoyant density, melting temperature, and high-pressure liquid chromatography (HPLC) methods (4). Purified DNA from *Spiroplasma citri* (genome size, approximately 1,780 kbp; base composition, 26 mol% guanine plus cytosine) was used as a reference standard.

RESULTS AND DISCUSSION

Cultural and morphological properties. Strains 0502^T and J233^T grew rapidly on mycoplasma medium formulations containing serum (fetal bovine serum or horse serum) or bovine serum fraction. Sustained growth also occurred in serum-free mycoplasma broth (21). Growth occurred over a temperature range of 18 to 37°C, and optimum growth was observed at 30°C. The colonies obtained on horse serum or SP-4 agar formulations exhibited typical fried-egg morphology (Fig. 1).

Logarithmic-phase cultures of strains 0502^T and J233^T in SP-4 medium examined by dark-field microscopy contained predominantly coccoid forms. The cells sedimented from broth cultures and examined by electron microscopy also were predominantly coccoid. No evidence of a cell wall was observed in these preparations; the diameters of representative

FIG. 2. Electron micrographs of sectioned and stained cell pellets of two acholeplasmas. The sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The arrowheads indicate the unit membrane. (A) Strain J233^T. (B) Strain 0502^T. Bar = 100 nm.

round cells varied from 300 to 500 nm, and each cell was surrounded by a single cytoplasmic membrane (Fig. 2).

Sterol requirement. The growth responses of strains 0502^T and J233^T in serum-free base media containing fatty acid or cholesterol supplements are shown in Table 1. Both strains exhibited some growth in the serum-free base broth alone and produced greater cell yields when this medium contained palmitic acid and albumin. The growth yields did not significantly increase when cholesterol was added. The lack of a sterol requirement for both strains was also observed in another study (21), in which these organisms were evaluated for sustained growth through 23 serial dilutions in media without serum.

Biochemical and biological properties. Both strain 0502^T

and strain J233^T rapidly fermented glucose, with consequent decreases in the pH values of the culture media. No evidence of mannose fermentation or hydrolysis of arbutin, arginine, or urea was observed. Strain 0502^T and J233^T colonies on agar medium did not hemadsorb guinea pig erythrocytes. Filtration studies were performed with each strain. For strain 0502^T the unfiltered control broth and the broth media after filtration through membranes having pore sizes of 450, 300, 220, and 100 nm contained 1×10^9 , 1×10^9 , 1×10^8 , 1×10^3 , and 0 color-changing units/ml, respectively; the corresponding values for strain J233^T preparations were 1×10^9 , 1×10^9 , 1×10^9 , 1×10^6 , and 0 color-changing units/ml, respectively. No evidence of reversion to bacterial forms was obtained with either strain.

Serological tests. Extensive growth inhibition and plate immunofluorescence tests, which were performed with antisera or conjugates to the previously described acholeplasmas, revealed that strains 0502^T and J233^T were not related serologically to each other or to 11 previously described *Acholeplasma* species. Antisera prepared to 0502^T and J233^T produced homologous growth inhibition zones that were 3 to 4 mm wide. In several previous studies of strain J233^T (9, 10), we observed some low-level serologic cross-reactions in both growth inhibition and immunofluorescence tests between this organism and antisera or conjugates to strain 19L^T of *A. oculi*. However, these results were not consistently reproducible, and other phenotypic markers (such as no arbutin hydrolysis) suggested that strain J233^T was not closely related to *A. oculi* strains.

Genomic analysis. The following genome sizes were determined by pulsed-field gel electrophoresis: strain 0502^T, 1,215 kbp; and strain J233^T, 1,610 kbp. The DNA base compositions determined by the three techniques were 35.5 mol% for strain 0502^T and 30.0 mol% for strain J233^T. The genome sizes of the two organisms are consistent with the data in other reports

TABLE 1. Growth responses of strains J233^T and 0502^T to cholesterol^a

Supplement(s) added to serum-free base medium	Cholesterol concn (μg/ml)	Amt of protein (mg/100 ml)	
		Strain 0502 ^T	Strain J233 ^T
Bovine serum fraction (1%)	(Control)	5.57	2.00
None	0	1.28	0.94
Albumin (1%) and palmitic acid (10 μg/ml)	0	4.60	2.20
Albumin, palmitic acid, and Tween 80 (0.01%)	0	6.13	2.57
Albumin, palmitic acid, and cholesterol	1	5.97	1.63
	5	6.38	1.38
	10	5.97	1.40
	20	6.38	1.40

^a All cultures were incubated at 30°C.

which have indicated that the genome sizes of acholeplasmas range from 1,200 to 1,500 kbp (2, 16, 17).

Recently, a phylogenetic study of strain J233^T, in which a sequence analysis of a conserved protein gene (*rps3*) (24) was performed, indicated that this organism is closely related to *A. laidlawii*, thus providing additional support for classification of J233^T as a species in the genus *Acholeplasma*. This study was also of considerable interest since it showed that the plant-pathogenic members of the class *Mollicutes*, which are called mycoplasma-like organisms or phytoplasmas, are more closely related to strain J233^T (and *A. laidlawii*) than to other members of the *Mollicutes*.

Habitat. At the present time, the two mollicutes characterized in this study are known only as inhabitants of plant surfaces. However, on the basis of previous experience, it is quite likely that one or more insect hosts will eventually be found to be colonized with each organism. The occurrence of mollicutes on plant surfaces probably results primarily from insect contamination of plant tissue during insect feeding excursions, and characterization and availability of specific reagents to identify such mollicutes frequently provide new information on the ecology of mollicutes (5).

In some previous studies, it was shown that strain J233^T infections in leafhoppers (*Euscelidius variegatus*) could be maintained for 2 to 3 weeks following experimental inoculation, although no evidence was obtained that the organism was pathogenic for the insect host (8, 9).

The following properties of strains 0502^T and J233^T described in this paper fulfill the essential criteria (14) for placing species in the class *Mollicutes*: absence of a cell wall, filterability, lack of reversion to walled bacteria when the organisms are grown in antibiotic-free media, penicillin resistance, and production of typical colonial forms on agar. The lack of a growth requirement for sterol or serum and the lack of helicity place these organisms in the family *Acholeplasmataceae* and the genus *Acholeplasma* (28). Finally, a serologic comparison of the two strains with a collection of antisera to all previously described *Acholeplasma* species demonstrated that strains 0502^T and J233^T are not related to other organisms in the genus.

We therefore propose that these two mollicutes should be designated new *Acholeplasma* species. The taxonomic descriptions below summarize the properties of each organism.

Description of *Acholeplasma brassicae* sp. nov. *Acholeplasma brassicae* (bras'si-cae. L. fem. gen. n. *brassicae*, of cabbage, referring to the plant origin of the organism). Cells are primarily coccoid, ranging in diameter from 300 to 500 nm. 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 arbutin, arginine, or urea. Agar colonies do not hemadsorb guinea pig erythrocytes.

Cholesterol or serum is not required for growth.

The temperature range for growth is 18 to 37°C; optimum growth occurs at 30°C.

Serologically distinct from other *Acholeplasma* species.

Isolated from broccoli (*Brassica oleracea* var. *italica*) obtained at a commercial vegetable market.

Pathogenicity for plants and insects has not been determined.

The average genome size is 1,215 kbp. The guanine-plus-cytosine content of the DNA is 35.5 mol%, as determined by buoyant density, melting temperature, and HPLC methods.

The type strain is 0502 (= ATCC 49388).

Description of *Acholeplasma palmae* sp. nov. *Acholeplasma palmae* (pal'mae. L. fem. gen. n. *palmae*, of a palm tree, referring to the plant from which the organism was isolated).

Cells are primarily coccoid, ranging in diameter from 300 to 500 nm. 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 arbutin, arginine, or urea. Agar colonies do not hemadsorb guinea pig erythrocytes.

Cholesterol or serum is not required for growth.

The temperature range for growth is 18 to 37°C; optimum growth occurs at 30°C.

Serologically distinct from other *Acholeplasma* species.

Isolated from the crown tissues of a palm (*Cocos nucifera*) tree with lethal yellowing disease in Jamaica.

Apparently not pathogenic for some leafhoppers; plant pathogenicity has not been determined.

The average genome size is 1,610 kbp. The guanine-plus-cytosine content of the DNA is 30.0 mol%, as determined by buoyant density, melting temperature, and HPLC methods.

The type strain is J233 (= ATCC 49389).

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