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Isolation of Acholeplasmas and a Mycoplasma from Vegetables

NORMAN L. SOMERSON,[1]* JOHN P. KOCKA,[1] DAVID ROSE,[2] AND RICHARD A. DEL GIUDICE[3]

Department of Medical Microbiology and Immunology, The Ohio State University College of Medicine, Columbus, Ohio 43210[1]; Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205[2]; and Biological Carcinogenesis Program, Frederick Cancer Research Center, Frederick, Maryland 21701[3]

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The isolation of *Mollicutes* from food has not been reported. To isolate *Mollicutes* in the presence of high levels of unwanted bacteria, we first incubated fresh vegetables in liquid culture media containing lysozyme, ampicillin, and thallous acetate. Culture fluids were then separated from the vegetable samples, subjected to one freeze-thaw cycle, and passed through a filter of 0.4-μm porosity. Filtered samples were cultured in SP4 medium and in a conventional medium containing horse serum. With this procedure 21 acholeplasma isolations representing three species were obtained from endive, broccoli, and kale. Of 35 food samples tested, 11 were positive for acholeplasmas; acholeplasmas isolated from 6 of these samples were recovered only in SP4 medium. In seven single vegetable samples, two or more *Acholeplasma* spp. were isolated.

A. laidlawii was isolated from all three vegetables and *A. axanthum* was found in broccoli and kale. Four isolates were serologically identified as *A. oculi*. *Mycoplasma verecundum* was the only *Mycoplasma* species recovered. Several isolates could not be typed serologically, as they reacted with antisera to both *A. morum* and *A. hippikon*. These isolates may include new *Acholeplasma* spp.

In the last 10 to 15 years, investigators have reported the recovery of a large variety of cell wall-deficient organisms from plants (12, 15). Little is known about the extent to which the edible parts of plants contain such microbes. High levels of contaminating bacteria in ground level crops have undoubtedly complicated or prevented demonstration of mycoplasmas and acholeplasmas in vegetables.

The order *Mycoplasmatales* includes organisms in the genera *Mycoplasma* and *Acholeplasma*. Mycoplasmas require sterol for growth; acholeplasmas will grow in the absence of sterol. Both groups of cell wall-deficient organisms are widely distributed in nature.

We report the development of a method to isolate acholeplasmas and mycoplasmas from fresh vegetables that are heavily contaminated with bacteria. This is the first description of a technique in which lysozyme is used to inhibit bacterial growth in mycoplasma (or acholeplasma) culture media. Using this procedure, we have recovered a *Mycoplasma* and several *Acholeplasma* spp. from fresh vegetables.

MATERIALS AND METHODS

Food samples. Vegetables were purchased from local retail markets and from farm stands. Samples were tested on the day purchased or stored in a refrigerator for 2 to 4 days. We examined kale, endive, radishes, spinach, broccoli, cabbage, and cauliflower. Lettuce was used only in the experiments in which bacterial contamination was investigated.

Organisms. *Acholeplasma laidlawii* PG8 and PG9, *Mycoplasma arginini* G230, *M. bovirhinis* PG43, and *M. bovigenitalium* PG11 were the prototype strains from our laboratory stocks. They were used for determining the efficiency of our isolation procedure and in lysozyme susceptibility tests. *Staphylococcus epidermidis*, *Escherichia coli*, and *Saccharomyces cerevisiae* were obtained from the culture collections of the Department of Medical Microbiology and Immunology and the Department of Microbiology at Ohio State University, Columbus, Ohio. *Streptococcus* ADA L, a stable L-form, was obtained from the National Institutes of Health, Bethesda, Md.

Culture media and reagents. SP4 was the primary medium in preliminary experiments concerned with reducing bacterial contamination and in recovering mycoplasmas and acholeplasmas. SP4 medium has been previously described for the isolation of a spiroplasma (17) and for culturing *M. pneumoniae* from throat specimens (16). A solid SP4 medium was prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 1.0%.

The Hayflick medium formulation was also used to isolate *Mollicutes* from vegetables. This medium has been used for isolating *M. pneumoniae* and other mycoplasmas (2). This broth medium was modified to contain 10% horse serum, 1.25% yeast extract, 1.0% glucose, and 0.002% phenol red. L-Arginine hydro-

chloride was added at a 0.1% (wt/vol) final concentration for the culture of *M. arginini*. For growing the *Streptococcus* ADA L-form, 4% (wt/vol) sodium chloride was added to the SP4 or modified Hayflick medium.

All ingredients were pretested for their ability to support the growth of type cultures. Whenever possible, a single commercial lot of each ingredient was used throughout the study.

S. epidermidis was grown on a soybean-casein digest (Trypticase soy agar, BBL Microbiology Systems, Cockeysville, Md.) containing 7.5% (wt/vol) sodium chloride, *S. cerevisiae* was isolated on Littman Oxgall agar (Difco), and *E. coli* was grown on MacConkey agar.

Antimicrobial agents. Unless stated, mycoplasma media contained thallous acetate (Fisher Scientific Co., Cincinnati, Ohio) at 500 μg/ml and sodium ampicillin injectable (Wyeth Laboratories, Philadelphia, Pa.) at a 1.0-mg/ml final concentration.

In some preliminary experiments, media contained disodium carbenicillin (Beecham-Massengill, Bristol, Tenn.) and sodium methicillin (Bristol Laboratories, Syracuse, N.Y.), each at a 1-mg/ml final concentration.

Lysozyme (chicken egg white) was purchased from Schwarz/Mann (Orangeburg, N.Y.) and was reported to have an activity of 11,000 U/mg. This enzyme was used at a final concentration of 2 mg/ml. In a preliminary experiment, *M. arginini*, *M. bovirhinis*, *M. bovigenitalium*, and *A. laidlawii* were unaffected by this level of enzyme. The activity of the lysozyme in SP4 medium after a 4-h incubation period at 35°C was found to be 85 to 91% of the initial value (4).

Quantitation of viable mycoplasmas. An estimate of the number of viable mycoplasmas inoculated into broth medium or recovered from wash fluids was obtained by making serial 10-fold dilutions of the samples in SP4 or Hayflick broth. Determinations were performed in 1-dram (15-mm-diameter) screw-capped vials, and the number of acid or alkaline color-changing units was determined by observing the shift in the color of the phenol red indicator (9, 13). For confirmation that the pH shift produced in the culture fluids did not result from bacterial contamination, 0.1-ml samples were inoculated onto agar media of the Hayflick formulation described earlier. Agar plates were incubated at 35°C and examined after 7 days for typical "fried-egg" colonies.

Glass wool filtration. Glass wool and cheesecloth filters were used to retain soil and other large particulate matter released into the culture media from the food samples. The filtration apparatus was modified from that used by Larkin et al. (8) to consist of a 300-ml column chromatography addition funnel containing a 1-in. (ca. 2.54-cm) layer of glass wool supported by cheesecloth. The apparatus was moistened with distilled water and autoclaved. In an attempt to reduce adsorption of *Mollicutes*, the apparatus was wetted with approximately 50 ml of sterile broth medium just before the filtrations.

Biochemical and serological identification of isolates. Presumptively identified *Mollicutes* isolates were classified as *Acholeplasma* or *Mycoplasma* spp. based on their response to 1.5% digitonin (6). Species were determined in growth inhibition tests (3) and in epifluorescence techniques (5).

RESULTS

Reduction of microbial contamination of foods. In initial isolation attempts from chopped, homogenized, or whole iceberg lettuce, we found large amounts of bacteria and fungi. Gram stains revealed gram-positive cocci in clusters, large numbers of gram-negative rods, and forms morphologically resembling yeasts. The initial use of glass wool and cheesecloth filtration did not reduce bacterial contamination, though this procedure did clarify the culture medium by removing soil and other large particulate matter.

In efforts to reduce the microbial populations, we used antimicrobial compounds followed by a freeze-thaw treatment and monitored the effectiveness of these decontamination procedures. Suspensions of *S. epidermidis*, *E. coli*, and *S. cerevisiae* were placed into 25 ml of SP4 broth. Lysozyme, ampicillin, and thallium were added to the culture medium, and the mixtures were incubated at 35°C for 4 h. The mixtures were filtered through glass wool; the filtrates were then frozen in liquid nitrogen and thawed in a 37°C water bath. The enumeration of each microbial species was determined (i) before incubation, (ii) before the freeze-thaw step, and (iii) after the freeze-thaw step (Table 1). *E. coli* was most sensitive to the prolonged exposure to antibacterial compounds; *S. epidermidis* and *S. cerevisiae* were less affected, but after the final freeze-thaw step in the sequence, they were reduced by approximately 2 logs as compared with initial counts.

We retested the effectiveness of the sequence of antibacterial and freeze-thaw steps, using samples of lettuce. The usefulness of lysozyme was also evaluated under the cultural conditions just described. The initial bacterial population was reduced by <0.5 log when lysozyme was omitted or replaced with methicillin. A 99% reduction in the microbial counts was observed when treatment with the three antimicrobial agents was followed by a freeze-thaw step. In a

TABLE 1. Reduction in numbers of microbial prototype species in SP4 medium after sequential antimicrobial and freeze-thaw treatments

Organism	Bacterial count (log_{10} CFU/ml) during sampling sequence[a]:		
	Before incubation in antimicrobial agents	After 4-h incubation	After freeze-thaw
E. coli	6	<2	<2
S. epidermidis	6.7	5.3	4.9
S. cerevisiae	5.3	4.7	3.0

[a] As determined on selective media described in the text. Differences of >1 log were considered significant.

similar experiment, a broth culture with $10^{7.4}$ colony-forming units (CFU) of bacteria per ml obtained from lettuce leaves contained only $10^{3.7}$ CFU/ml after the 4-h incubation and the freeze-thaw steps.

Recovery of acholeplasmas added to lettuce. Lettuce leaves were added to 25 ml of SP4 broth without antibiotics. After 0.5 h, lysozyme, ampicillin, thallous acetate, and 1.0 ml of a broth culture of *A. laidlawii* B were added. After the 4-h incubation period, the culture was passed through glass wool; then the filtrate was subjected to a freeze-thaw cycle and filtered through polycarbonate membranes of 0.4-μm porosity.

After the freeze-thaw cycle (Table 2), populations of bacteria and acholeplasmas had each been reduced by approximately 2 logs. The filtration step that followed the freeze-thaw cycle was extremely effective in eliminating bacteria. After the complete procedure, the bacterial population was reduced by more than 5 logs, and the population of *A. laidlawii* was reduced by 2 to 3 logs.

Elimination of a stable L-form by the isolation procedure. Lysozyme and antibiotics that affect cell wall synthesis can generate cell wall-deficient forms from the contaminating bacteria and complicate the isolation of *Mollicutes*. *Streptococcus* ADA L, an L-form stable in 4% NaCl culture media, was used as an indicator of L-form sensitivity to the isolation procedure.

Streptococcus ADA L was grown for 18 h in Hayflick medium containing 4% NaCl (wt/vol). Samples of this culture were inoculated into fresh Hayflick medium and into SP4 broth, each without added NaCl. These salt-free cultures were subjected to the entire isolation process (Table 3). At each procedural step, samples were examined in media containing 4% NaCl to determine the number of surviving L-forms.

The isolation process was as effective in removing *Streptococcus* ADA L as it had been in eliminating bacteria. A 4-h exposure to culture fluids without added NaCl produced a 2- to 3-log reduction, as determined by enumeration on media containing salt; freeze-thaw produced the loss of another log, and the last filtration step further reduced the number to less than 10^2 CFU/ml. The final filtrate was mixed with an equal volume of SP4 or Hayflick broth and incubated at 35°C. No growth of L-forms was detected after 7 days of incubation in either medium. Thus, it is unlikely that L-forms generated from bacteria would survive the isolation procedure.

Complete isolation procedure. Each food sample was divided into two equal portions. One food portion was placed in a 1-liter Erlenmeyer flask containing 25 ml of SP4 broth medium, and the other was placed in 25 ml of Hayflick culture medium (Fig. 1). The two medium formulations contained ampicillin and thallous acetate, with lysozyme used only in the initial step.

Each flask was incubated for 4 h, and the contents were poured through a column chromatography addition funnel containing glass wool over cheesecloth. Liquid nitrogen was used for freezing the filtrates; frozen filtrates were thawed in a 37°C water bath. The broth from each flask was then divided into four 6-ml aliquots and passed through a prefilter of 12-μm porosity and a polycarbonate filter of 0.4-μm porosity. Each filtrate was collected in a 4-dram (21-mm-diameter) vial. Sterile culture medium (6 ml) was added to each of the screw-capped vials, and the cultures were incubated at 35°C. If there was a color shift in the phenol red pH indicator, the fluids were inoculated into fresh broth and also onto mycoplasma agar medium. Since there were two flasks in each sample, a total of eight

TABLE 2. Recovery of bacteria and acholeplasmas during steps in the procedure for the isolation of *Mollicutes*

Organisms	Counts[a] during sampling sequence[b]		
	Before incubation in antimicrobial agents	After freeze-thaw	After 0.4-μm filtration
Bacteria	7.3	5.5	<2
Acholeplasmas	5.1	3.5	2.5

[a] For bacteria, $\log_{10}$ CFU per milliliter, as determined on Trypticase soy agar. For acholeplasmas, $\log_{10}$ color-changing units per milliliter. The number of acid or alkaline color-changing units was determined by a shift in the color of the phenol red indicator.

[b] Refers to Fig. 1, isolation procedure diagram.

TABLE 3. Elimination of *Streptococcus* ADA L-form during steps of the isolation procedure[a]

Sampling sequence[b]	*Streptococcus* ADA L-form counts ($\log_{10}$ CCU/ml)[c]	
	Modified Hayflick broth	SP4 broth
Before incubation	5.6	5.6
After 4-h incubation	2.5	3.5
After glass wool filtration ..	2.5	3.8
After freeze-thaw	1.5	2.5
After 0.4-μm filtration	ND	1.8
After incubation for 7 days in medium without NaCl .	ND	ND

[a] *Streptococcus* ADA L-form is a stable laboratory-adapted L-form strain.

[b] Refers to Fig. 1, isolation procedure diagram.

[c] As determined in media containing 4% NaCl. CCU, Color changing units; ND, none detected.

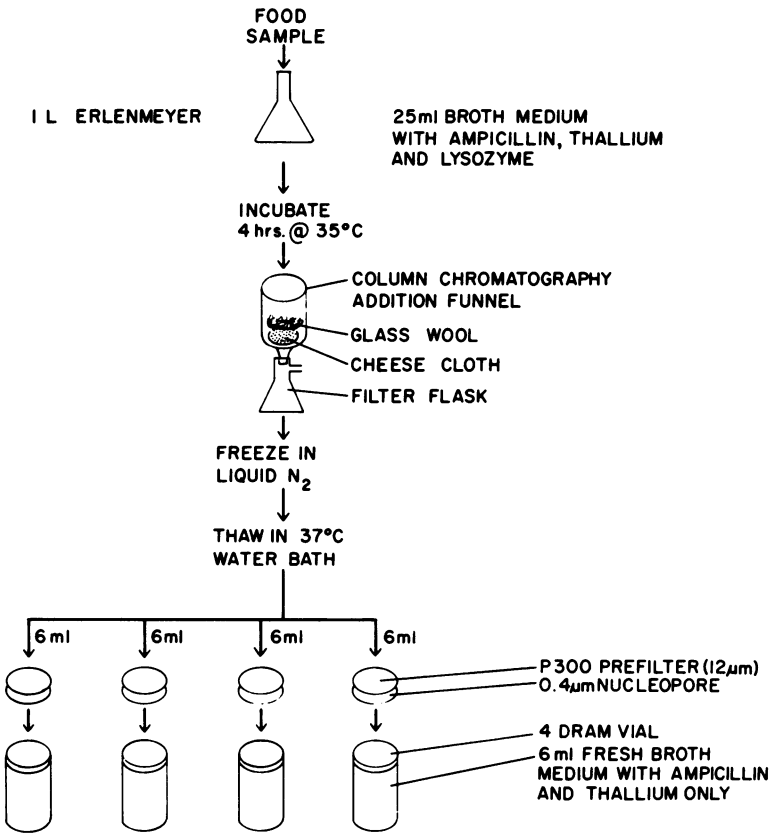

FIG. 1. Procedure for the isolation of *Mollicutes* from vegetables.

vials served as cultures for each isolation attempt.

Isolation of acholeplasmas and a *Mycoplasma* sp. from farm crops. Vegetables were examined for the presence of mycoplasmas. Of 35 food samples tested, 11 were positive for acholeplasmas (Table 4); 7 of 8 kale, 3 of 6 broccoli, and 1 of 8 endive samples contained acholeplasmas. Of 11 positive samples, 4 were isolated on both SP4 and Hayflick medium. In one case, an acholeplasma specimen was obtained by using the Hayflick formula alone. Had we not included the SP4 formula, 6 of the 11 isolations cultured only on SP4 medium would have been missed.

A. laidlawii, *A. oculi*, and *A. axanthum* were the commonly known acholeplasmas that we isolated. *A. laidlawii* was the most frequently isolated organism. We were able to reisolate these organisms from the original food samples, from culture fluids frozen after the final filtration, and from separately purchased vegetables obtained from the same source.

Four acholeplasma isolates could not be identified; in immunofluorescence tests, three of these showed a weak cross-reaction to *A. morum* (11) and *A. hippikon*. In a growth inhibition test (3), there was a small clear zone around the disk containing antiserum to *A. morum*. Antiserum against *A. hippikon* was not available for a growth inhibition test.

One isolate from kale did not show a relationship to any of the *Acholeplasma* spp. The only *Mycoplasma* sp. that we found was repeatedly isolated from a kale sample and was identified as *M. verecundum*.

DISCUSSION

Thallous acetate in combination with a β-lactam antibiotic was ineffective in reducing bacterial growth in fresh vegetable samples. In attempts to isolate *Mollicutes* from fresh vegetables (data not shown), there was no combination of antibacterial compounds (used as a sole treatment) that prevented bacterial overgrowth on agar or in broth media.

Freezing and thawing in the presence of lysozyme was used previously in extracting polysomes from *E. coli* (10). We used these treatments in a new method to significantly reduce the numbers of bacteria from vegetables. We added lysozyme to thallous acetate and ampicillin in culture medium. The use of lysozyme to

TABLE 4. Isolate identifications

Isolate source	Isolate identification
Endive 1	*A. laidlawii*
Broccoli 1	*A. axanthum*
	Acholeplasma sp.[a]
Broccoli 2	*A. laidlawii*
	A. axanthum
	A. oculi
	Acholeplasma sp.[a]
Broccoli 3	*A. axanthum*
	A. laidlawii
Kale 1	*A. laidlawii*
	A. oculi
	A. axanthum
Kale 2	*A. axanthum*
	A. oculi
Kale 3	*A. laidlawii*
	Acholeplasma sp.[a]
	M. verecundum
	Acholeplasma sp.[b]
Kale 4	Undetermined
Kale 5	*A. laidlawii*
	A. oculi
	A. axanthum
Kale 6	*A. laidlawii*
Kale 7	Undetermined

[a] Reacts with both *A. morum* and *A. hippikon* in epifluorescence.

[b] Unknown *Acholeplasma* sp.

reduce the numbers of bacteria in cultures for the recovery of *Mollicutes* has not been previously reported. The incubation period of 4 h was considered necessary because it apparently allowed for multiplication of acholeplasmas or mycoplasmas and for antibacterial action. Surviving bacteria probably were more susceptible to the physical stress of freeze-thawing because of prolonged exposure to antimicrobial agents. The osmolarity of the culture medium appears to be prohibitive to any L-forms generated. A final filtration through 0.4-μm polycarbonate filters drastically reduced the number of surviving bacteria. Our early experiments indicated that acholeplasmas and mycoplasmas survive this combination of treatments; in fact, in some experiments not reported here, the numbers of acholeplasmas actually increased with these treatments, possibly as a result of the breaking up of aggregates by freeze-thaw action.

Vegetables were contaminated with more than one genus of *Mollicutes*. Some of our vegetable samples contained two or more *Acholeplasma* spp.; one specimen contained four species. The isolation procedure may favor rapidly growing organisms, and if acholeplasmas are present, their overgrowth may prevent the detection of the generally slower-growing *Mycoplasma* spp.

A. laidlawii is recognized as having a wide distribution in nature, and its isolation was not surprising (14). However, the isolation of *M. verecundum*, first reported from cattle (7), is the first account of this organism being recovered from a nonanimal source. There were three isolates that showed weak serological reactions to both *A. morum* and *A. hippikon*, species originally isolated from cell culture and from horses, respectively. We cannot explain this result, since these two species do not cross-react with each other in either growth inhibition or fluorescence tests and are distinct in DNA-DNA homology studies (1). *A. axanthum* and *A. oculi* have been isolated from coconut palms (15); these findings, combined with our results, suggest that acholeplasmas may have a much wider ecological distribution. One other isolate (from kale) appeared to be unrelated to any of the *Acholeplasma* spp. for which we possessed antisera; it may be a new species.

Our report describes a technique that permitted the first isolation of acholeplasmas and a mycoplasma from vegetables consumed by humans.

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