

Characterization of thermophilic actinomycetes isolated from residential heating and humidification systems

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Seven thermophilic actinomycete isolates from heating-humidification systems of individuals with definite or suspected "environment-associated" hypersensitivity chest disease were characterized. Five of the isolates were Thermoactinomyces sp., 4 exhibiting characteristics identical to those of Thermoactinomyces vulgaris. One isolate was a Micromonospora sp. on the basis of biochemical cell wall composition, and another morphologically resembled Promicromonospora. Two additional thermophilic actinomycete isolates, Micropolyspora faeni, strain A-94, known to be an important source of "farmer's lung" antigen, and a newly described species, Thermoactinomyces sacchari, the most important source of "moldy bagasse" antigen, were also characterized. These 2 isolates were completely different from the home heater-humidifier isolates in gross and microscopic characteristics. Results of "metabolic" and "cellular" antigenic analyses, using specific hyperimmune rabbit antisera, revealed shared "cellular" and "metabolic" antigens among all home humidifier isolates, but not between these isolates and M. faeni. Thus, the testing of human sera against any single heating-humidifier Thermoactinomyces sp. would likely detect the presence of precipitins against many closely related strains, but not against M. faeni.

In recent months an increasing number of hypersensitivity pneumonitides have been reported in patients exposed to "actinomycete-contaminated" home heating and humidification systems.¹⁻⁴ Actinomycetes are ubiquitous in distribution, and members of the genera *Micromonospora* and *Streptomyces* capable of growing below 37° C. have been isolated from the air of houses and outdoor environments.⁵

However, thermophilic actinomycetes have been cultured with regularity from "contaminated" home heating-humidification systems⁶ of patients with hypersensitivity pneumonitis, and have been shown to be associated with the patients' illnesses based on clinical and immunologic evidence plus positive provocative inhalation challenge tests.¹⁻⁴

In the current report we characterize 7 thermophilic actinomycete isolates from heating-humidification systems of home and work areas of individuals with definite or suggestive evidence of "environment-associated" hypersensitivity chest disease. Two additional thermophilic actinomycete isolates, *Micropolyspora*

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TABLE I. Sources of isolates studied

Isolate number	Isolate source
M-1	Water from heating and air conditioning unit of office in which several executives developed hypersensitivity pneumonitis and/or chronic bronchitis
M-2	Water from furnace humidifier of home in which patient with "home-associated" intermittent bronchitis resided
M-3	Water from office air conditioner of physician with severe respiratory distress similar to asthma, associated only with office environment
M-4	Furnace humidifier water from home in which patient with intermittent recurrent bronchitis, related to home environment, resided
M-5	Residue from furnace humidifier of patient with severe asthma that occurred only in home environment
M-6	Residue from window air conditioning unit of patient with "home-associated" bronchospastic pulmonary disease
N-1	Residual water from New Jersey home humidification unit of patient with severe recurrent hypersensitivity pneumonitis
L-1	<i>T. sacchari</i> repeatedly isolated from bagasse, Lockport, La.; known rich source of bagasse antigen in a form of hypersensitivity pneumonitis (bagassosis)
E-1	<i>M. faeni</i> , A-94 hay isolate, Corbaz, Gregory, and Lacey type strain; known rich source of "moldy hay" antigen in a form of hypersensitivity pneumonitis (farmer's lung)

faeni, strain A-94, known to be an important source of "farmer's lung" antigen, and a newly described species, *Thermoactinomyces sacchari*, the most important source of "moldy bagasse" antigen, were also studied for comparative purposes.

MATERIALS AND METHODS

Isolation procedures

Isolates M-1 to M-6 were obtained by culturing samples from furnace duct dust, air return filter dust, humidifier water, and humidifier residue from homes or work environment areas of affected individuals residing in the Milwaukee area. Isolate N-1 was kindly supplied by Drs. Tourville, Leudemann, and Weiss. Isolate L-1 (*T. sacchari*) was grown from a Louisiana bagasse sample, and isolate E-1 (*M. faeni*) was a gift from Dr. J. Lacey. Sources of all isolates are listed in Table I. Isolates M-1 to M-6 were obtained by incubating aliquots of each dust or water sample in trypticase soy, V-8, half-strength nutrient, casamino acid and glucose yeast extract agar, and broth at 55° C. Some thermophilic bacterial isolates were obtained from most dust and water samples, but these were not studied. Thermophilic actinomycete isolates from samples listed in Table I were obtained as either single colonies or as multiple colonies identical in gross and microscopic morphology.

Gross and microscopic slide culture morphology

Isolates were subcultured on the above media at 37°, 45°, 49°, and 55° C. After recording gross colony morphology on all media, morphologic characteristics for identification were determined by examination of unstained living slide cultures incubated at 45° and 55° C. Measurements of spores, sporophores, and hyphal width were made on these cultures. The presence and characteristics of aerial, substrate, and submerged mycelium were determined by use of a × 45 objective and × 10 ocular. For studies to determine sporulation on aerial or substrate mycelium only, slide cultures were flooded in inverted position with 2 per cent celloidin, fixed and hardened in 95 per cent alcohol plus 5 per cent chloroform. Celloidin sheets were transferred to a clean slide, flooded with lactic acid fuchsin solution, coverslip

placed, and sealed with clear nail polish. This procedure was especially valuable for examining aerial sporulation and for determination of the presence and type of sporophore. Morphologic classification was based on criteria for identification of genera of the Actinomycetales as listed by Williams, Davies, and Cross.⁷

Presumptive physiologic decomposition tests and cell wall hydrolysates

Current classification of thermophilic actinomycetes is dependent on many variables,⁸⁻¹⁰ and ability to grow at various temperatures may be a function of nutritional requirements rather than a basic characteristic of the microorganism. Because of this we also attempted to identify our isolates according to carbohydrate assimilation tests and cell wall amino acid, amino sugar, and monosaccharide composition.

Growth resulting from incubation of all isolates was washed 5 times with distilled water and agitated with glass beads (0.25 to 0.30 mm. diameter) in a model MSK Braun cell homogenizer (Bronwell Scientific Co., Rochester, N. Y.) at 4,000 cycles per minute for 4 minutes. Microscopic observations revealed complete cellular disruption by this procedure. Cell walls were separated from cytoplasmic debris by centrifugation (6,000 g × 1 hour at 4° C.), and the supernate was immediately lyophilized and stored in a desiccator. Cell walls were washed 6 times with 0.2M NaCl, once with 1M NaCl, and 10 times with distilled water followed by lyophilization. Portions of lyophilized cell wall preparations were treated with trypsin (1 mg. trypsin per 10 mg. cell walls) (Worthington Biochemical Corp. Freehold, N. J.) at 37° C. for 2 hours, followed by extensive washing with distilled water and re-lyophilization.

Additional cell preparation aliquots were extracted with chloroform:methanol (2:1) to remove readily extracted lipids, washed extensively with methanol and water, and re-lyophilized.

Monosaccharides were determined qualitatively and quantitatively by thin-layer and gas-liquid chromatographic techniques. Quantitative thin-layer chromatography was performed using the procedure of Kraeger and Hamilton,¹¹ whereby 2N aqueous HCl hydrolysates were chromatographed on phosphate-buffered glass-filter paper, ITLC type SG (Gelman Instrument Co., Ann Arbor, Mich.) in chloroform-methanol-pyridine-water (130:40:1:5). Portions of each sample were also treated according to the procedure of Becker, Lechavalier, and Lechavalier¹² with 2N H₂SO₄ and chromatographed as described for quantitative analysis. For quantitation, trimethylsilyl ether derivatives were prepared from the products of either a 1N methanolic HCl hydrolysis (80° for 40 hours) or a 2N methanolic HCl hydrolysis (100° C. for 4 hours) using dulcitol as the internal standard.^{13, 14} The derivatives were extracted into chloroform before chromatography. A Barber-Colman gas chromatograph equipped with a flame ionization detector was employed with the following conditions: column packing, 3 per cent OV-1 on 80/100 Chromosorb W HP (Superleo, Inc., Bellefonte, Pa.); column temperature, 175° C.; injector temperature, 255° C.; detector temperature, 235° C. Results were expressed as per cent of cell wall.

Amino acids and amino sugars were determined quantitatively in a modified Technicon amino acid analyzer¹⁵ following hydrolysis of 5 mg. samples in 4N HCl for 4 hours at 110° C. The time and concentration of acid for hydrolysis were determined by preliminary experiments.

Glucosamine was not always detected in the cell wall hydrolysates by the amino acid analyzer, and the following procedure was used to demonstrate its presence; 5 mg. samples were hydrolyzed as mentioned and evaporated in a desiccator over sodium hydroxide. The evaporated hydrolysate was taken up in water and chromatographed on glass-fiber paper,¹¹ together with authentic muramic acid and glucosamine standards in chloroform-methanol-ammonia-water (130:63:7:4). Guide strips, cut from the paper and developed with ninhydrin spray reagent (Gelman Instrument Co., Ann Arbor, Mich.), were used to locate the areas of paper containing glucosamine and muramic acid in the hydrolysates. These areas were eluted with water, lyophilized, and rechromatographed on the phosphate-buffered glass-fiber paper¹¹ in isopropanol-water (80:20) or on silica gel thin-layer plates (Brinkman Instrument Co., Westbury, N. Y.) in chloroform:methanol:17 per cent ammonia (40:40:20).

TABLE II. Gross and microscopic colonial morphology of thermophilic actinomycete isolates on primary medium (trypticase soy agar) at 45° and 55° C.

Isolate number	Colonial morphology	Microscopic morphology
M-1	Abundant aerial mycelium and sporulation; profuse sporulation on agar surface and submerged mycelium; surface growth bone white; reverse ocher; no diffusible pigment	Submerged mycelium delicate (1.0 to 1.5 μ), bearing scanty monospores, aerial mycelium heavily intertwined with hooking of hyphae; predominantly sessile monospores, some on short sporophores; occasional chains of 4 to 5 spores
M-2	Surface growth granular, clear to white; some autolysis of central aerial and substrate mycelium at 48 hr.; abundant growth at 45 and 55° C.	Aerial hyphae scanty and bearing monospores after 48 hr.; substrate hyphae 2 to 3 μ diameter bearing round to oval monospores with occasional chains of bispores, both sessile and on short sporophores; submerged (vegetative) mycelium delicate; freely branching and bearing monospores; ovate intercalary spores present
M-3	Abundant growth at 24 and 48 hr.; surface growth bone white; reverse ocher; no diffusible pigment; abundant aerial sporulation	Primarily monospores on aerial and submerged mycelium; some doublets and rare triplets in chains on aerial mycelium; monospores primarily sessile but short sporophores common; spores round to globose, approximately 0.8 μ diameter
M-4	Abundant growth at 24 and 48 hr.; surface growth bone white; reverse ocher; no diffusible pigment; abundant aerial sporulation	Primarily monospores on aerial and submerged mycelium; some doublets and rare triplets in chains on aerial mycelium; monospores primarily sessile but short sporophores common; spores round to globose, approximately 0.8 μ diameter
M-5	Surface growth granular, clear to white; only scanty growth with closely opposed colonies after 48 hr. incubation at 55° C.; growth rapid, however, at 45° C.	Substrate sporulation very heavy; round to oval monospores approximately 1.0 μ diameter; heavy substrate sporulation interfering with study of submerged mycelium; ovate intercalary spores present; some submerged hyphae fragmenting into 1 to 2 μ segments within hyphal membrane; aerial hyphae short and bearing single spores, sessile or on short sporophores
M-6	Very scanty aerial mycelium at 24 and 48 hr.; surface growth bone white; reverse ocher; no diffusible pigment	Submerged mycelium; primarily sessile round to oval monospores 1.0 to 1.5 μ in diameter; mycelium abundant with crowded hyphae angling deep into agar; very heavy substrate sporulation; aerial mycelium very scanty, bearing occasional monospores either sessile or on sporophores of 2 to 4 μ length
N-1	Abundant growth at 24 and 48 hr.; surface growth bone white; reverse ocher; no diffusible pigment; abundant aerial sporulation	Primarily monospores on aerial and submerged mycelium; some doublets and rare triplets in chains on aerial mycelium; monospores primarily sessile but short sporophores common; spores round to globose approximately 0.8 μ diameter
L-1*	Buff-tan slimy, translucent, wrinkled surface; growth moderate; autolysis of scanty aerial mycelium; reverse translucent buff-tan	Aerial mycelium short; substrate mycelium abundant; aerial and substrate mycelium contains single globose spores 0.5 to 1.0 μ usually on short sporophores; hyphae approximately 1 μ in diameter
E-1	Pale yellow raised colonies, no diffusible pigment, short white aerial mycelium, good growth at 48 hr.	Substrate mycelium dense, 0.8 μ in diameter, short, straight chains of globose to oval spores approximately 0.8 to 1.0 μ diameter; aerial mycelium abundant with short spore chains extending laterally and terminally

*Characterized on half strength nutrient and yeast extract.

Glucosamine, muramic acid, and alanine have similar Rf values in the solvent system of Kraeger and Hamilton,¹¹ but it serves to separate them from other amino acids for subsequent chromatography. The technique employed for amino acid quantitation did not distinguish between the LL and meso isomers of diaminopimelic acid. This was determined by descending paper chromatography on Whatman No. 1 filter paper, as described by Hoare and Work¹⁶ using LL and meso standards. Results of amino acid and amino sugar quantitation were obtained as μ moles per milligram and expressed as per cent total μ moles amino acids present.

Rabbit antiactinomycete sera

Antisera were prepared by immunizing 2 to 3 Kg. white female New Zealand rabbits with homogenized, lyophilized, pure culture growths of the respective thermophilic actinomycete isolates in Freund's complete adjuvant (3 to 5 animals per isolate). Rabbits received 0.6 ml. of emulsion containing 5.0 mg. actinomycete antigen intramuscularly every 2 weeks for 5 months. Sera were harvested and frozen at -4° C. Pooled antisera against each actinomycete isolate were tested by agar gel double diffusion as described previously using 0.75 per cent Oxoid ionagar No. 2.¹⁷

Actinomycete antigen

Growths were harvested from broth cultures after 5 days, centrifuged, washed 3 times with distilled water, and agitated with glass beads (0.25 to 0.30 mm.) in a Braun cell homogenizer at 4,000 cycles per minute for 4 minutes. Lyophilized material was resuspended in 0.9 per cent saline solution and emulsified in Freund's complete adjuvant.

"Cellular" antigen for gel double diffusion studies consisted of lyophilized pure culture actinomycete growth, homogenized to the point of complete cell wall disruption, suspended in veronal buffer pH 8.3, 20 mg. per milliliter, incubated at room temperature for one hour, and centrifuged to remove excess particulate matter.

"Metabolic" antigen consisted of either trypticase soy or yeast extract broth in which the organisms had grown for 8 days. Broth was centrifuged at 2,500 r.p.m. for one hour to remove all growth, followed by dialysis, Seitz filtration, lyophilization, and reconstitution in veronal buffer at pH 8.3, 20 mg. per milliliter, for double-diffusion testing. In all cases rabbit hyperimmune sera were also tested against fresh trypticase soy and yeast extract broths prepared in the same manner but not used for growth of microorganisms.

RESULTS

Gross colonial and microscopic slide culture morphology of all isolates is listed in Table II. Representative streak plate and slide cultures plus electron photomicrographic spore preparations of several isolates are illustrated in Figs. 1 to 3. The L-1 *T. sacchari* bagasse isolate and *M. faeni* (A-94) hay isolate were completely different from all home heater-humidifier isolates in gross and microscopic characteristics in agreement with previously described reports.^{6, 18} The cell wall pattern of isolate L-1 (*T. sacchari*) was of a Type III variety, whereas a Type IV pattern was noted for isolate E-1 (*M. faeni*).^{12, 19}

All heating unit isolates grew well at 45° , 49° , and 55° C., with scanty slow growth on some plates at 37° C. Growth was most abundant on the primary isolation medium, trypticase soy, but was also good on glucose yeast extract and nutrient agar. Casamino acid and V-8 agar yielded slow, scanty growth initially, but growth became abundant after 7 days.

Home heater isolates M-3, M-4, and N-1 were virtually identical in colonial and microscopic morphology, and growth was rapid and abundant at 24 hours on the primary isolation medium, trypticase soy agar, at 55° and 45° C. Cell wall analyses shown in Table III were of a Type III variety^{12, 19} of Lechevalier, Lechevalier, and Becker, containing major amounts of glucosamine, muramic

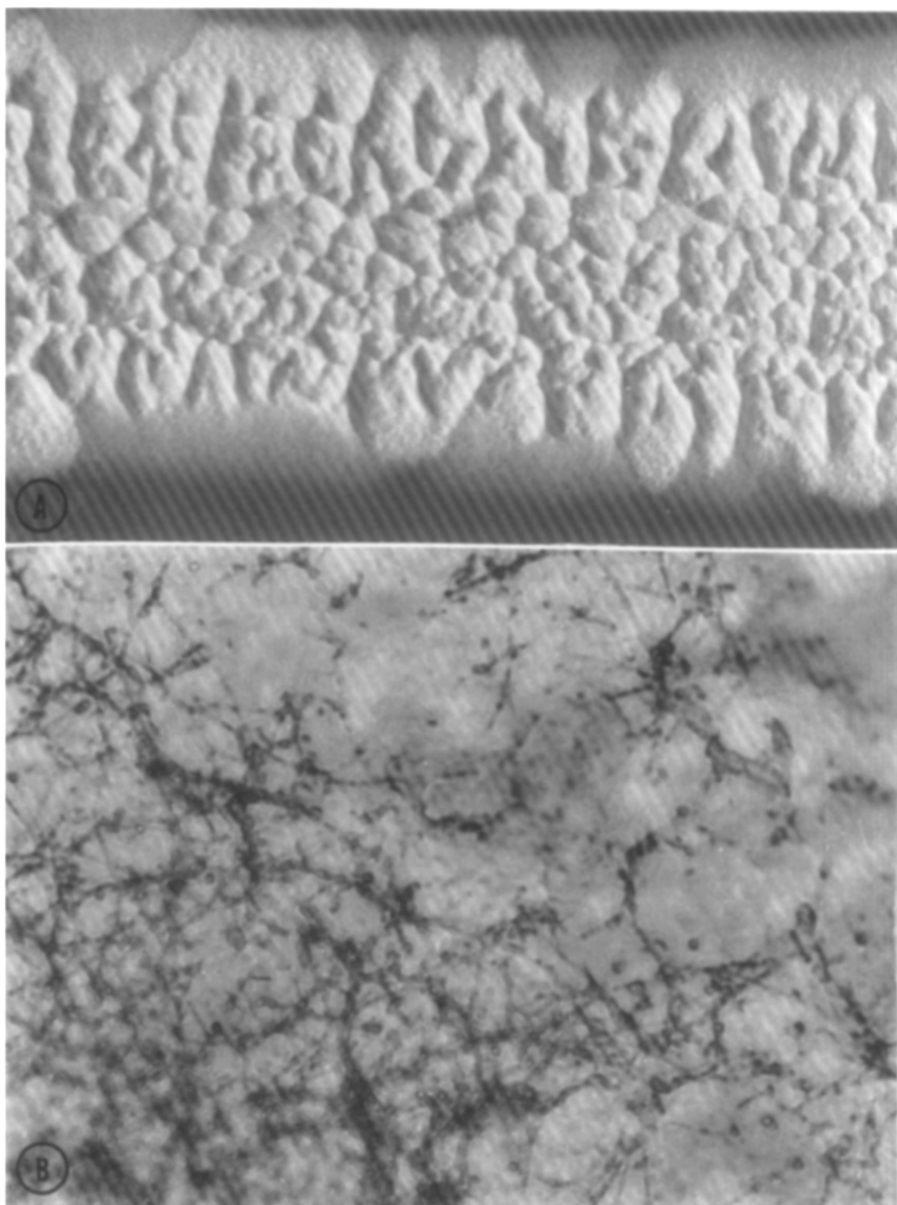


FIG. 1. **A**, Streak plate culture of isolate M-1 on trypticase soy agar incubated for 24 hours at 55° C. Surface was bone white and reverse ochre without diffusible pigment. Gross colonial and microscopic morphology of this isolate was similar to that of isolates M-3, M-4, and N-1. **B**, Slide culture microscopic morphology; isolate M-1; vegetative (submerged) mycelium. Trypticase soy agar incubated for 24 hours at 55° C. Note abundant sporulation with predominance of monospores and some sporulation by means of fragmentation within hypha into round or oval spores.

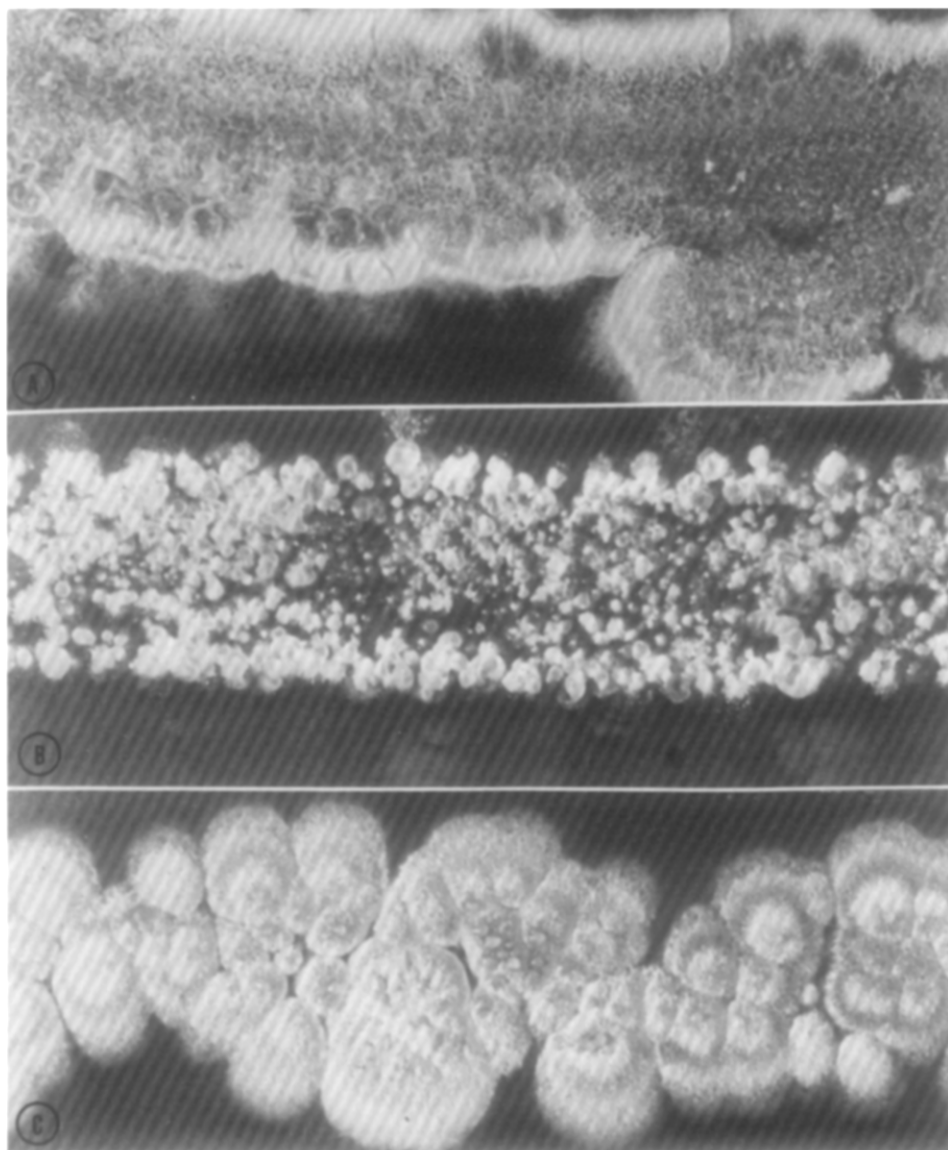


FIG. 2. Streak plate cultures of, **A**, isolate M-5, **B**, isolate M-2, and, **C**, isolate M-6 on trypticase soy agar after 24 hours of incubation at 55° C. Note quite different colonial characteristics, M-5 demonstrating a coarsely granular-clear to white surface growth, M-2 a fine granular surface growth with central autolysis, and M-6 a bone white surface growth similar to M-1, M-3, M-4, and N-1.

acid, alanine, and glutamic acid plus the meso isomer of 2, 6-diaminopimelic acid. Two of these isolates contained small amounts of galactose and trace amounts of glucose. The combination of characteristics indicates that all of these isolates were *T. vulgaris*.

Isolate M-1 closely resembled M-3, M-4, and N-1 in colonial morphology, but

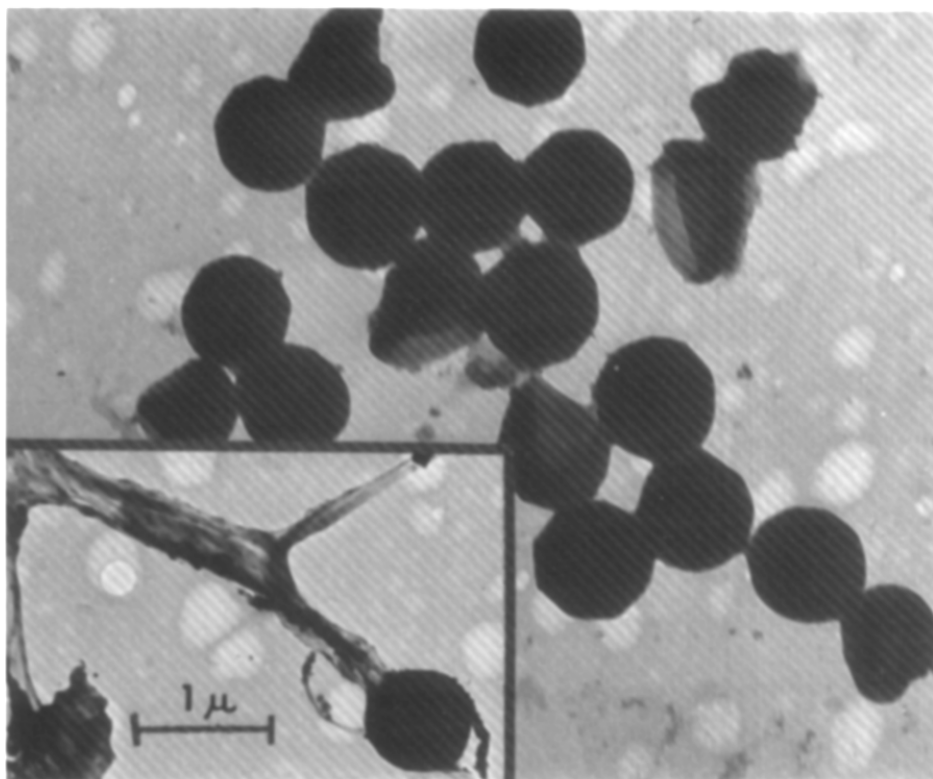


FIG. 3. Electron photomicrograph, short aerial mycelium spore chains of isolate M-1. Note round to globose spores approximately 0.8μ in diameter.

some differences were noted in microscopic morphology as listed in Table II. After numerous subcultures during a period of more than one year, M-1 was restudied and appeared to be stable. It also possessed a Type III cell wall (Table III). We believe the isolate should be classified as *T. vulgaris*.

Isolates M-2 and M-5 exhibited a granular-clear to white surface growth on trypticase soy and nutrient agar, and both differed from the bone white colonies of M-1, M-3, M-4, M-6, and N-1 home humidifier isolates (Table II). At 48 hours, however, autolysis of the central aerial and substrate mycelium of isolate M-2 was evident (Fig. 2). This was not true for isolate M-5 (Fig. 2). Morphologically, isolate M-2 appeared to be closely related to *T. sacchari* but not identical.

Isolate M-5 morphologically resembled *Promicromonospora*.²⁰ Its cell wall composition did not conform to any known type but was compatible with the *Actinomyces* variety (Type V of Lechevalier¹⁹ or Type I of Yamaguchi²¹) in possessing only a faint trace of diaminopimelic acid, major amounts of lysine, alanine, and glutamic acid, and galactose, as has been reported for *Promicromonospora*.²²

Isolate M-6 belonged to the genus *Thermoactinomyces* on the basis of morphology, since occasional single spores were noted on aerial mycelium in addition to heavy substrate sporulation.²² Cell wall analysis was, however, not compatible

TABLE III. Amino acid, amino sugar, and monosaccharide actinomycete cell wall content

	Isolate number and content								
	M-1	M-2	M-3	M-4	M-5	M-6	N-1	L-1	E-1 (A-94)
<i>Amino acids and amino sugars (% total μmoles/mg.)</i>									
Hydroxyproline	—	—	—	—	—	—	—	—	—
Aspartic acid	0.6	1.3	2.9	0.9	2.3	8.1	3.9	9.1	1.3
Threonine	0.1	0.4	0.5	0.2	0.9	2.3	1.7	3.5	0.2
Serine	0.8	1.3	1.3	1.0	1.4	6.1	3.4	2.9	0.5
Muramic acid	5.7	5.3	3.9	6.8	0.3	4.0	4.2	2.9	9.3
Glutamic acid	21.0	17.1	11.2	25.9	10.1	10.8	16.0	18.2	20.9
Proline	tr.	—	tr.	tr.	tr.	—	tr.	tr.	0.4
Glycine	3.1	2.8	4.5	4.3	18.3	13.9	—	0.9	1.1
Alanine	18.6	19.4	21.8	17.2	9.7	14.8	11.6	15.9	26.5
Glucosamine	7.6	11.0	9.0	10.5	tr.	3.3	16.3*	3.8	12.3
Valine	2.2	11.2*	0.6	6.8	2.8	0.2	1.1	1.8	tr.
Cysteine	2.2	0.6	0.1	0.3	0.9	0.6	0.6	0.1	tr.
Methionine	tr.	—	tr.	tr.	0.5	0.2	—	0.2	tr.
α , ϵ diaminopimelic acid†	26.2	20.4	23.0	16.2	tr.	16.3	7.3	14.9	21.6
Isoleucine	0.8	0.1	0.2	0.3	2.3	1.3	—	0.3	0.7
Leucine	0.2	1.4	2.8	1.1	18.3	4.8	4.5	8.0	1.1
Tyrosine	0.6	0.6	0.6	0.6	3.2	3.9	3.9	1.6	0.2
Phenylalanine	2.6	1.6	1.9	3.3	2.3	5.2	3.4	2.4	0.5
Ornithine	tr.	0.1	tr.	0.5	8.7	0.1	tr.	—	—
Lysine	1.3	1.3	2.7	1.8	11.5	5.7	5.6	4.2	0.3
Histidine	0.7	0.4	0.8	0.7	5.0	2.1	1.7	1.9	0.3
Arginine	0.6	0.4	1.6	0.9	2.3	4.1	6.2	5.3	0.3
Unknowns	2.7	4.8	9.6	0.6	tr.	0.7	5.6	2.4	—
<i>Monosaccharides (% cell wall)</i>									
Arabinose	—	—	—	—	—	—	—	—	9.4
Galactose	2.9	1.7	2.2	2.9	1.0	—	—	tr.	6.2
Glucose	0.3	tr.	0.7	2.1	—	—	—	—	—
Unknowns	—	—	—	—	—	1.0‡	—	—	—
Cell wall type	III	III	III	III	V?	II	III	III	IV

tr. = trace; — = not detected.

*Likely includes some unknown.

†Meso isomer in all isolates.

‡Likely mannose.

with this taxon, revealing a Type II (Micromonospora) pattern. If one accepts some aerial sporulation within the genus *Micromonospora*, isolate M-6 is a member of this genus.

In attempting to further characterize our heating unit isolates on carbon utilization medium of Shirling and Gottlieb,¹⁰ none grew in a describable manner with any of the added carbon sources. After inoculation and incubation at 45° C. and 55° C. for 5 days, with daily inspection no difference could be seen between the negative controls and any of the added carbon sources.

Analysis of "cellular" and "metabolic" antigens of our isolates by Ouchterlony double diffusion revealed the following: Both "cellular" and "metabolic" antigens of home humidifier isolates M-1 through M-6 and N-1 were shared with each other (Fig. 4) and with isolate L-1 (*T. sacchari*). "Cellular" and "metabolic" antigens of isolate E-1 (*M. faeni*), a known rich source of "moldy hay" antigen, reacted against homologous antisera but not against any of the home heating-humidifier isolates (Fig. 4) or L-1 (*T. sacchari*) antisera. Lyophilized, dialyzed trypticase soy and yeast extract broths, which had not been used for

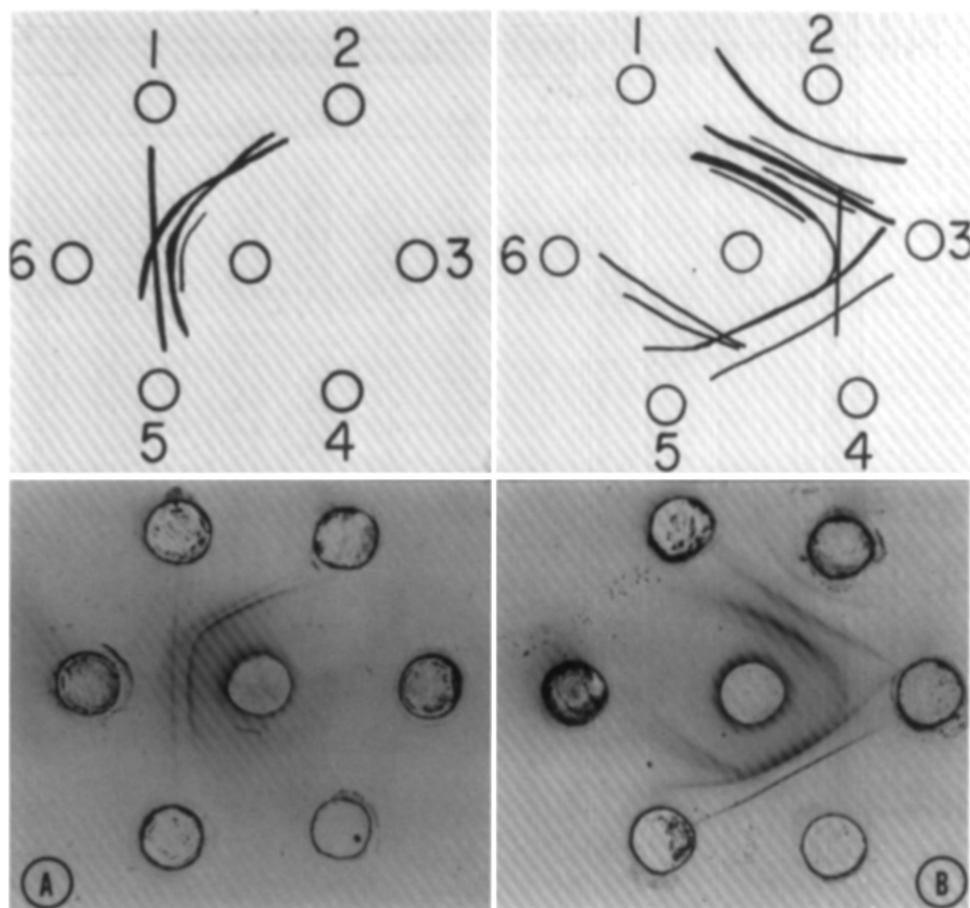


FIG. 4. Differences between "cellular" and "metabolic" antigens of several home heater-humidifier thermophilic actinomycetes and *M. faeni*. Peripheral wells in **A** and **B** contain the following antigens: (1) *M. faeni* "cellular," (2) M-5 "metabolic," (3) M-5 "cellular," (4) M-1 "metabolic," (5) M-1 "cellular," and (6) *M. faeni* "metabolic." Center well in **A** contains rabbit anti-*M. faeni* serum and in **B**, anti-M-4 serum. Note lack of shared antigens between *M. faeni* and home humidifier-heater isolates (**A**). Anti-M-4 serum (**B**) reacts with "cellular" and "metabolic" antigens of M-5 and M-1 isolates but not with *M. faeni*.

actinomycete growth, revealed no precipitin arcs against any of our rabbit anti-actinomycete sera, indicating that precipitin arcs produced using "metabolic" antigens were not the result of immunization with trace broth impurities in the washed actinomycete antigen preparations.

DISCUSSION

Our data concerning biochemical cell wall composition, gross and microscopic morphologic characteristics, and temperature requirements indicate that home heater-humidifier isolates M-1, M-2, M-3, M-4, and N-1 were *Thermoactinomyces* sp. Isolate M-6 was a *Micromonospora* based on biochemical cell wall criteria. Isolate M-5 remains unclassified, but its morphologic and biochemical cell wall

criteria are compatible with *Promicromonospora*.²⁰ As stated by Luedemann²³ with regard to the genus *Micromonospora*, "there is no easy solution to what a species is or should be until the investigator has had a sufficient number of geographically diverse, but diagnostically similar strains to compare." Our descriptions of M-5 and M-6 are based only on single isolates, and we feel that accurate identification should be attempted only after other similar isolates are obtained for study. Bagasse isolate L-1 was identical to the newly described *T. sacchari*. Our cell wall analysis provides the first evidence that this species possesses a Type III cell wall. Prior to the description of *T. sacchari*, we had identified our most antigenically potent bagasse actinomycete isolates as *Micromonospora (Thermoactinomyces) vulgaris*.²⁴ This was due in part to equating our results of whole cell amino acid, aminosugar, and monosaccharide analysis with published data on cell wall hydrolysates. Our current results indicate that these isolates are *T. sacchari* and not *M. vulgaris*. For several years prior to the description of *T. sacchari* by Lacey, it was accepted by some that *T. vulgaris* and *M. vulgaris* were synonymous and that *T. vulgaris* was the only species in the genus *Thermoactinomyces*. The slight variations in cultural and physiologic behavior between *T. vulgaris* and *M. vulgaris*, such as sugar fermentation, growth characteristics on various media, amylase activity, and gelatin liquefaction, were considered by Küster and Locci²⁵ not to exceed the normal variation within a species. If this fact is to be accepted, most of our home heater-humidifier isolates (M-1, M-3, M-4, and N-1) are *T. vulgaris* based on morphologic and biochemical cell wall characteristics identical to those of this species.

Results of "cellular" and "metabolic" antigenic analysis of our home humidifier isolates are of interest in indicating considerable cross reactivity. Walbaum, Biguet, and Tran Van Ky,²⁶ in an extensive immunoelectrophoretic analysis of *Thermopolyspora polyspora* (sic *M. faeni*) with reciprocal absorption studies, showed some shared "cellular" antigens between this organism and a *T. vulgaris* isolate. Barboriak, Fink, and Scribner,²⁷ on the other hand, using Ouchterlony double-diffusion and sonified unextracted actinomycete growth in saline solution as antigen, noted no shared antigens between one unidentified humidifier isolate and *T. vulgaris* or *M. faeni*. We also employed only simple Ouchterlony double diffusion in our antigenic analysis and failed to note any shared "cellular" or "metabolic" antigens between *M. faeni* and any of our *Thermoactinomyces*, home heating-humidifier isolates. However, isolates L-1 (*T. sacchari*) and N-1 (*T. vulgaris*) both shared "metabolic" and "cellular" antigens with all M humidifier isolates. Differences in strains, growth media employed, duration of incubation, antigen preparation, and method of analysis could have accounted for these discrepancies. However, our *M. faeni* "metabolic" antigen reacted only with rabbit hyperimmune serum prepared against this organism. This latter finding is in agreement with the results of Walbaum, Biguet, and Tran Van Ky²⁶ and indicates that a positive precipitin reaction against "metabolic" antigen of *M. faeni* and *T. vulgaris* is evidence of a prior immunization against both species. Based on our results given previously, the testing of human sera against any single heating-humidifier unit *Thermoactinomyces* sp., using "cellular" and "metabolic" antigens, would likely detect the presence of precipitins against

many closely related strains or isolates but not against *M. faeni*. Since *M. faeni* has not been isolated from home and office environments, this point would seem to be of no major diagnostic consequence.

Preliminary studies in our laboratory, however, suggest that serum precipitins can be detected against a variety of organic dust, fungal, and actinomycete antigens in some of the normal population, using more sensitive gel diffusion techniques.²⁸ The use of such varying gel diffusion techniques and antigen preparations, plus the known sources of false positive gel diffusion reactions, points to the need for standardization of antigen preparations, reference antisera, and techniques for use in the presumptive diagnosis of human hypersensitivity pneumonitides.

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