A Scheme for the Identification of Thermophilic Actinomycetes Associated with Hypersensitivity Pneumonitis

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A scheme has been developed for the identification of thermophilic actinomycetes associated with hypersensitivity pneumonitis. Eighty strains, 10 *Micropolyspora faeni*, 6 *Saccharomonospora viridis*, 52 *Thermoactinomyces candidus*, 7 *T. vulgaris*, 4 *T. sacchari*, and 1 *T. dichotomica*, either isolated from patients' environment or received as authentic strains, were studied. In addition to the cultural and microscopic morphology in various media, each strain was subjected to an array of biochemical tests. These tests included decomposition of tyrosine, xanthine, hypoxanthine, gelatin, casein, esculin, and arbutin. Using a rapid thin-layer chromatography method, the isomer of diaminopimelic acid and sugar in the whole cell hydrolysate were studied. The thermophilic actinomycetes can be identified in a reasonable period of time using a combination of all these tests.

Thermophilic actinomycetes from self-heated hay and compost have been known to microbiologists for a long time. Not much attention, however, was given to their ecology, physiology, and biochemical characteristics until it was found that farmer's lung was caused by the inhalation of dust from moldy hay containing spores of thermophilic actinomycetes, particularly *Micropolyspora faeni* and *Thermoactinomyces vulgaris* (19, 20). It has also been reported that *T. vulgaris* and *T. candidus* (13a), growing in the heating and air conditioning systems of buildings or homes, can cause hypersensitivity pneumonitis in susceptible individuals (1, 2, 8). Other thermophilic actinomycetes species, namely *T. sacchari* and *Saccharomonospora viridis*, have also been implicated in hypersensitivity pneumonitis of man (16, 18, 22).

Previously, most descriptions of the species of thermophilic actinomycetes were based on their morphological features alone. Küster and Locci (15) studied 104 strains of *Thermoactinomyces* species belonging to *T. vulgaris*, *T. thalophilus*, *T. thermophilus*, and *T. monosporus* and found that all these different species are synonyms of *T. vulgaris*. Cross et al. (7) described the thermophilic actinomycete responsible for farmer's lung hay antigen and classified it as *M. faeni*. Although in recent years considerable progress has been made in the taxonomy

of actinomycetes, no comparable attempt was made in the identification of thermophilic actinomycetes.

The current investigation is carried out to study in detail the morphological, physiological, and biochemical characteristics of thermophilic actinomycetes associated with hypersensitivity pneumonitis in an attempt to devise a scheme for differentiating the various species.

MATERIALS AND METHODS

Strains. Eighty strains of thermophilic actinomycetes belonging to three genera and six species were included in the study. The identity and source of the strains are given in Table 1. Duplicate sets of all the strains were maintained at room temperature and at 4 C, and subcultures were made every 3 months. All the strains except *T. sacchari* and *S. viridis* were grown in Trypticase soy agar (TSA) at 55 C. *T. sacchari* strains were grown in half-strength nutrient agar at 50 C, while *S. viridis* strains were grown in TSA at 50 C. Fresh subcultures were used for all the tests.

Morphology. Colony morphology of the organisms were studied by growing them on TSA, TSA with 0.2% yeast extract, Trypticase soy broth (TSB), nutrient agar, half-strength nutrient agar, and blood agar (BA). Cultures were incubated at 45, 50, and 55 C for up to 2 weeks and studied for rate of growth, aerial mycelial production, sporulation, pigment production, hemolysis in BA, and pellicle formation in broth. Colony morphology was also studied by examining the undisturbed culture under a

TABLE 1. Source of strains included in the study and their final identification

Strain no.	Source and identification when received ^a	Identification
T-103, T-104, T-105, T-110, T-111, T-112, T-113, T-115, T-116, T-119, T-120, T-121, T-122, T-123, T-141, T-142, T-149, T-162, T-189	Air samples collected by Anderson sampler from homes and house dust	<i>Thermoactinomyces candidus</i>
T-102, T-118	Home humidifier water	<i>T. candidus</i>
T-106 (ATCC-7868), T-129, T-130, T-131, T-133, T-134, T-135, T-136, T-137, T-138, T-139, T-140, T-160, T-163, T-164, T-165, T-168, T-192	Dust samples from heating units and air conditioners	<i>T. candidus</i>
T-143, T-144, T-158, T-159, T-161, T-166, T-190	Wood shavings	<i>T. candidus</i>
T-125, T-132	Unknown	<i>T. candidus</i>
T-108	Moldy cattle feed	<i>T. candidus</i>
T-124	Mushroom compost	<i>T. candidus</i>
T-170	HAL - <i>T. vulgaris</i> P54W	<i>T. candidus</i>
T-191	MC - <i>T. vulgaris</i> (Greer)	<i>T. candidus</i>
T-101	MC - <i>T. vulgaris</i>	<i>T. vulgaris</i>
T-151	MC - <i>T. vulgaris</i> H/S strain	<i>T. vulgaris</i>
T-126	House dust	<i>T. vulgaris</i>
T-147 (ATCC-15733)	<i>T. vulgaris</i>	<i>T. vulgaris</i>
T-155	JL - <i>T. vulgaris</i> A1270	<i>T. vulgaris</i>
T-156	JL - <i>T. vulgaris</i> A64	<i>T. vulgaris</i>
T-167	Air conditioner	<i>T. vulgaris</i>
T-145 (ATCC-27349)	<i>T. sacchari</i>	<i>T. sacchari</i>
T-171	HAL - <i>T. sacchari</i> PriM	<i>T. sacchari</i>
T-198 (ATCC-27376)	<i>T. sacchari</i>	<i>T. sacchari</i>
T-199 (ATCC-27375)	<i>T. sacchari</i>	<i>T. sacchari</i>
T-172	HAL - <i>T. dichotomica</i> N1595	<i>T. dichotomica</i>
T-150 (ATCC-15347)	<i>Thermopolyspora polyspora</i>	<i>Micropolyspora faeni</i>
T-152, T-186	MC - <i>M. faeni</i>	<i>M. faeni</i>
T-153, T-154	JL - <i>M. faeni</i>	<i>M. faeni</i>
T-178, T-179, T-180, T-181	HAL - <i>M. faeni</i> , A-91, A-92, A-94, V-4066	<i>M. faeni</i>
T-193	Isolated from contaminated antigen	<i>M. faeni</i>
T-127	MC - <i>Thermomonospora viridis</i>	<i>Saccharomonospora viridis</i>
T-128, T-148	House dust	<i>S. viridis</i>
T-146 (ATCC-15386)	<i>Thermomonospora viridis</i>	<i>S. viridis</i>
T-157	JL - <i>S. viridis</i> A-66	<i>S. viridis</i>
T-182	HAL - <i>S. viridis</i> 4047	<i>S. viridis</i>

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microscope with a $\times 45$ objective. Gram-stained preparations from cultures and slide cultures were also examined whenever necessary.

Incubation. Unless otherwise stated, all the tests were done by incubating the inoculated plates and tubes at 50 C for 7 days. During incubation all the plates were store in plastic bags to avoid drying.

Decomposition of casein. This was carried out according to the method of Gordon and Smith (12).

Decomposition of tyrosine, xanthine, hypoxanthine, and adenine. The media and methods followed were the same as those of Gordon et al. (13) and Kurup and Schmitt (14), except that TSA was used as the basal medium. Cultures were inoculated

at the center of the plates and after incubation the clearance of tyrosine, xanthine, hypoxanthine, and adenine crystals around and beneath the colony was examined.

Decomposition of gelatin. TSA, supplemented with 0.4% (wt/vol) gelatin, was inoculated and incubated. The plates were then flooded with a reagent having the following composition (11): mercuric chloride, 15 g; concentrated HCL, 20 ml; and distilled water, 100 ml. A clear zone around the colony indicated gelatin decomposition.

Hydrolysis of starch. Ten grams of potato starch, suspended in 100 ml of cold distilled water, was added to 900 ml of TSA, autoclaved, and made into

plates. After inoculation and incubation the plates were flooded with Grams iodine solution (5). A clear zone around the colony indicated starch hydrolysis.

Hydrolysis of esculin. Esculin (0.1% wt/vol) and ferric citrate (0.05% wt/vol) were added to TSA. Plates were inoculated and incubated for 2 weeks. Hydrolysis of esculin was evidenced by blackening of the medium around the colony (5).

Splitting of arbutin. The medium used was the same as for esculin hydrolysis except that arbutin was substituted for esculin. The results were interpreted as in the hydrolysis of esculin.

Hydrolysis of hippurate. The medium and method used was the same as described by Gordon and Horan (10) except that the inoculated broth was incubated at 50 C for 2 weeks.

Resistance to lysozyme. TSB containing 0.005% (wt/vol) of lysozyme was inoculated with each strain and incubated at 50 C for 2 weeks. A plain TSB tube inoculated with each strain served as a control. The lysozyme solution was prepared according to the method of Gordon (9).

Production of deoxyribonuclease. Deoxyribonuclease test agar (BBL) was prepared according to the manufacture's instructions. The medium was inoculated and incubated. The plates were then treated with 5 to 8 ml of normal HCL. A clear zone around the colony indicated the production of deoxyribonuclease (5).

Urease production. Urease production was tested by inoculating urease test medium (6) and incubating for up to 1 week. An uninoculated tube was also incubated as a control. Development of a pink color indicated the production of urease.

Resistance to novobiocin. Novobiocin (Albany-cin, Upjohn Co.), incorporated in TSB to a final concentration of 100 µg/ml, was inoculated with the test strains. A control without the antibiotic was inoculated and incubated in the same manner. Resistance to novobiocin was evidenced by an apparently similar growth, both in test and control tubes.

Sensitivity to antibiotics. TSA plates were uniformly swabbed with a light suspension of spores. Disks impregnated with ampicillin, streptomycin, chloramphenicol, and gentamicin were placed over the inoculated plates and incubated at 45 C for 18 to 24 h. The zone of inhibition was measured and the results were recorded as sensitive or resistant (3).

Resistance to heating for varying periods of time. Spores of the thermophilic actinomycetes suspended in distilled water were heated in a boiling water bath. Cultures were transferred from the heated suspension onto TSA plates after 10, 30, 60, and 120 min of heating. The plates were incubated and examined for 1 week for evidence of growth. Cultures from an unheated suspension served as a control.

Hydrolysis of tributyrin. This was tested according to the method described by Kurup and Schmitt (14) with the exception that TSA was used as the basal medium.

Hydrolysis of chitin and cellulose. These were tested in TSA with 0.1% (wt/vol) chitin or cellulose. After 1 week of incubation, plates were observed for

clearance of chitin or cellulose around and beneath the colony.

Acid production from carbohydrates. The method followed was essentially the same as the one described by Gordon and Smith (12). Representative strains of each species were inoculated and incubated for 2 weeks. The following carbohydrates were tested: adonitol, arabinose, fructose, galactose, glucose, glycerol, lactose, maltose, mannitol, sucrose, and xylose.

Sugar utilization. Sugar utilization was studied by incorporating 1% (wt/vol) each of glucose, arabinose, sucrose, lactose, xylose, and maltose into TSA. Growth in TSA was recorded as "+" and any sugar supplemented media showing heavier growth than on TSA was recorded as "++".

Whole cell analysis for diaminopimelic acid and sugars. Thermophilic actinomycetes were grown in TBS (100-ml quantities in 500-ml Erlenmeyer flasks) at 55 C for 3 to 5 days. The growth was harvested by centrifugation and washed in sterile distilled water to remove all the medium. Approximately 50 mg (wet weight) and 200 mg (wet weight) of the growth was used for the diaminopimelic acid (DAP) and sugar analysis, respectively. Hydrolysate for DAP analysis was prepared according to the method of Becker et al. (4) and for sugar analysis according to the method of Lechevalier (17). Ascending rapid thin-layer chromatography was followed for both DAP and sugar analysis. Gelman (ITLC-SA, 5 by 20 cm) sheets were spotted with 5 µl of the hydrolysate and developed for 2 to 2.5 h in *n*-butanol, acetic acid, and water (4:1:1) for DAP. The sheets were then air-dried, sprayed with ninhydrin reagent (0.2% ninhydrin in acetone), and heated at 100 C for 3 min. Five microliters of a 0.01 M standard DAP (Nutritional Biochemicals Co.) was also spotted along with the hydrolysate. Five microliters of the hydrolysate for sugar analysis was spotted, as in the case of DAP, and developed in a solvent system of chloroform, methanol, and water (30:38:2) for 2 to 2.5 h. The sheets were air-dried and sprayed with acid aniline phthalate (3.25 g of phthalic acid in 100 ml of water saturated with *n*-butanol and 2 ml of aniline). The sheets were then heated at 100 C for 5 min. Five microliters of a 0.1% solution of ribose, xylose, arabinose, galactose, glucose, and rhamnose were also developed along with the hydrolysate.

RESULTS

All strains of *T. candidus* and *T. vulgaris* grew fast in all media tested, and developed a colony of about 5 cm in diameter in 3 to 5 days (Fig. 1 and 2). Strains of *M. faeni* grew moderately fast and attained a size of 2 to 3 cm in diameter (Fig. 3), whereas the growth of *T. sacchari*, *S. viridis* (Fig. 4), and *T. dichotomica* was very slow and attained a size of 1 to 2 cm in diameter in 1 week on most media tested. A yellowish growth with fluffy mycelium distinguishes *T. dichotomica* from all other strains studied. *S. viridis* strains grew slowly and only

FIG. 1. One-week-old culture of *T. candidus* on TSA.

FIG. 2. One-week-old culture of *T. vulgaris* on TSA.

four of six strains consistently produced blue-green pigment in TSA. A majority of the strains of *S. viridis* failed to grow or grew poorly at 55 C and above in all media tested. Regardless of the media used, *T. sacchari* grew slowly and produced only minimal aerial mycelia. *T. vulgaris* and *T. candidus* strains produced elaborate primary and secondary mycelia. Frequently the primary mycelium broke into unicellular arthrospores. Unicellular spores, produced on both the substrate and aerial mycelia, were either attached directly on the filaments or on short stalks. *M. faeni* showed moderate growth in all media tested. The primary growth was yellowish and usually raised. Occasionally, spots of white aerial myce-

lium developed on prolonged incubation. Chains of spores, usually 5 to 15, were produced in abundance from both aerial and substrate mycelium (Fig. 5). *T. candidus* and *T. vulgaris* grew well on BA and produced large zones of hemolysis, whereas *M. faeni* grew well but failed to produce any hemolysis. *T. sacchari*, *T. dichotomica* and *S. viridis* grew poorly on BA and failed to hemolyze it. All species produced surface growth and some sediment in broth cultures.

Results of the physiological and biochemical tests are given in Table 2. All tested strains failed to grow or showed scanty growth on media used to test for acid production from carbohydrates. No recognizable difference in growth was noted in TSA and TSA supplemented with

FIG. 3. Ten-day-old culture of *M. faeni* on TSA.

FIG. 4. Ten-day-old culture of *S. viridis* on TSA with 0.2% yeast extract.

FIG. 5. Chains of spores produced by *M. faeni*. $\times 1,200$

TABLE 2. Physiological characteristics of thermophilic actinomycetes

Property	No. of strains positive					
	<i>T. candidus</i> (52 strains)	<i>T. vulgaris</i> (7 strains)	<i>T. sacchari</i> (4 strains)	<i>T. dichotomica</i> (1 strain)	<i>M. faeni</i> (10 strains)	<i>S. viridis</i> (6 strains)
Decomposition of casein	52	7	4	1	0	6
Tyrosine	0	7	0	0	3	2
Xanthine	0	0	0	0	7	0
Hypoxanthine	0	7	0	0	10	0
Adenine	0	0	0	0	0	0
Tributylin	0	0	0	0	0	0
Cellulose	0	0	0	0	0	0
Chitin	0	0	0	0	0	0
Hydrolysis of starch	0	7	4	1	0	0
Gelatin	52	7	4	1	10	6
Decomposition of esculin	52	0	1	0	9	0
Splitting of arbutin	52	0	0	0	8	0
Production of DNase	26	5	0	0	6	0
Urease	11	3	0	0	2	3
Reduction of nitrate	0	0	0	0	10	1
Resistance to heating at 100 C						
10 min	52	7	4	0	0	0
30 min	51	5	1	0	0	0
60 min	49	3	0	0	0	0
120 min	33	3	0	0	0	0
Resistance to novobiocin 30 μ g/ml	52	7	4	1	0	0
100 μ /ml	52	7	4	1	0	0
Sensitivity to ampicillin	50	7	4	1	8	6
Streptomycin	52	7	4	1	9	6
Chloramphenicol	39	7	4	1	9	6
Gentamicin	52	7	4	1	10	6
Resistance to lysozyme	52	7	4	1	8	0
Cell wall type	III	III	III	III	IV	IV

TABLE 3. *Morphological and physiological characters for the presumptive identification of thermophilic actinomycetes*

Property	Organism					
	<i>T. candidus</i>	<i>T. vulgaris</i>	<i>T. sacchari</i>	<i>T. dichotomica</i>	<i>M. faeni</i>	<i>S. viridis</i>
Colony-color	White	White	Colorless to white	Yellow	Yellow	Blue-green
Spores	Single	Single	Single	Single	Chain	Single
Aerial hypha	+	+	—	+	—	+
Hydrolysis of casein	+	+	+	+	—	+
Hypoxanthine	—	+	—	—	+	—
Starch	—	+	+	+	—	—
Esculin	+	—	—	—	+	—

sugars. *Meso* DAP was detected in all strains tested. *M. faeni* and *S. viridis* showed galactose and arabinose in the whole cell hydrolysate, whereas none of the other strains showed these sugars.

DISCUSSION

All six species belonging to the 3 thermophilic genera can be identified with accuracy using the morphological, physiological, and biochemical characteristics described. *M. faeni* and *S. viridis* are sensitive to heating at 100 C and to the action of novobiocin. Both species have a type 4 cell wall. The differentiation between the two species is not difficult when *S. viridis* produces the characteristic blue-green pigment. *S. viridis* strains invariably hydrolyze casein as against *M. faeni* strains which do not. Microscopically the single spores, produced on the aerial hyphae by *S. viridis*, can be differentiated from *M. faeni* which produce chains of spores on both the substrate and aerial mycelium. Decomposition of hypoxanthine and xanthine and resistance to lysozyme are some of the additional features which separate *M. faeni* from *S. viridis*. All species belonging to the genus *Thermoactinomyces* are resistant to prolonged boiling and grow in medium containing over 100 µg of novobiocin per ml. All species of *Thermoactinomyces* hydrolyzed casein. *T. dichotomica* can be easily differentiated by the yellow color of the mycelium and dichotomously branching sporophores. *T. candidus* can be distinguished from other species by its ability to decompose esculin and arbutin and its inability to hydrolyze starch. Tyrosine and hypoxanthine are decomposed by *T. vulgaris* but not by the other species. In addition, *T. sacchari* can be identified by its morphology, particularly slow growth of the colony, lack of any visible aerial mycelium, and early lysis of aerial mycelium. Carbohydrate utilization, reported by Lacey (16) to differentiate *T. sacchari* from *T. vulgaris*, was found to be of questiona-

ble value in the present study due to inconsistent results. Our results in this respect are comparable to the one reported by Seabury et al. (21).

Some *M. faeni* strains grew at 37 C, although the rate of growth was slow. Unless microscopic examination and physiological tests are performed, differentiation of *Nocardia* strains from *M. faeni*, on the basis of colonial morphology alone, is extremely difficult. Reports on the isolation of *M. faeni* from sputum and lung biopsy specimens are noteworthy in this context (22). Some strains of *Thermomonospora* may be confused with *Thermoactinomyces* in their colonial morphology. Both of these genera possess a type 3 cell wall. Resistance to novobiocin and tolerance to high temperatures for prolonged periods are very useful criteria for differentiating *Thermoactinomyces* from *Thermomonospora*.

In conclusion, morphological and physiological tests, combined with thin-layer chromatography analysis for DAP and sugars, make it possible to identify thermophilic actinomycetes within 1 to 2 weeks. Table 3 lists the important features useful in the presumptive identification of thermophilic actinomycetes.

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