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Motile Nocardoid *Actinomycetales*

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The properties of 42 strains of nocardoid (nocardioform) bacteria were compared. The results indicate that the organism previously called *Nocardia turbata* does not belong to the genus *Nocardia* nor does it fit into any of the previously described genera.

Many bacteria have a tendency to branch. Even *Escherichia coli* kept at low temperature may do so (23). Other bacteria form true branching filaments as their normal growth pattern. These include: (i) the *Hyphomicrobiales*, with narrow (0.2 μm) branching filaments producing wider bacterial cells by budding, (ii) the *Mycoplasmatales*, with defective or no cell walls, and (iii) the *Actinomycetales*, whose murein-containing cells elongate into branching filaments.

As has been pointed out previously (14), one can separate actinomycetes into six groups on the basis of the major constituents found in their cell walls. On the basis of morphology and chemical constitution of cell wall preparations, one can recognize 18 genera of aerobic actinomycetes, as analyzed by H. A. Lechevalier and M. P. Lechevalier [H. Prauser (ed.), *The Actinomycetales*, in press]. The organisms grouped in these 18 genera are morphologically the most evolved of the known actinomycetes and do not present major taxonomic problems at the generic level.

Cell wall composition and morphology do not enable one to differentiate easily between the genera *Nocardia* and *Mycobacterium* and certain corynebacteria and their relatives. These organisms, which present a spectrum of morphology ranging from hyphal to bacteroid, represent a major taxonomic riddle.

The present study is a comparison of some properties of 42 strains of such bacteria. In particular, the problem has been to establish the identity of nocardoid bacteria forming motile elements, especially (i) the so-called "motile nocardia" of Ørskov, which was called *Nocardia turbata* by Erikson (5) and which was considered to be a *Cellulomonas* by Jones and Bradley (12), and (ii) the C_4 group of organisms on which we previously published preliminary data (9). By nocardoid or Prauser's term, "nocardioform" (*The Actinomycetales*, in press), we refer to the tendency of the hyphae of such organisms to fragment into small units, a characteristic of certain of the members of the genus *Nocardia*. No members of the true nocardiae were included in this study. We have reported previously (13) on these organisms which include, in addition to the asteroides-farcinica group, *N. brasiliensis* and *N. caviae*. The true nocardiae have, in general, hyphae which are abundantly branched, and there is usually formation of aerial mycelium; they have a type IV cell wall and a type A sugar pattern (13). Our results show that the "C_4 group" of organisms belongs to the genus *Mycoplana* and that the "turbatae" belong to neither the genus *Nocardia* nor *Cellulomonas*. The genus *Oerskovia* is proposed to accommodate these organisms (21).

MATERIALS AND METHODS

Organisms. The organisms included in this study are listed in Table 1. The media indicated were used either as broths or solidified by the addition of 15 g of agar per liter of medium. All organisms were grown aerobically at 28 or 37 C except for the two strains of *Actinomyces* which were grown anaerobically at 37 C in Brewer jars using the "Gaspak" (BBL). Minimal and maximal temperatures for growth were determined by incubating the various organisms on maintenance medium at 10, 24, 37, 42, and 55 C.

Morphology. Each organism was examined microscopically after various lengths of incubation on as many of the media listed in Table 1 as would support its growth. Light photomicrographs are of undisturbed cells growing on solid media. For electron microscopic observation, cellular suspensions were fixed by contact for 1 hr with 1% formaldehyde which had been

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TABLE 1. *Some properties of nocardoid bacteria*

Organism[a]	Maintenance medium[b]	Motile	Gram stain	Temp for growth (C)		Viability in water or in buffer after		Anaerobic growth
				Min	Max	8 hr, 50 C	4 hr, 60 C	
Mycobacterium tuberculosis H37 Ra	YD	−	+	37	42	+	+	−
M. smegmatis ATCC 607	N	−	+	10	42	+	+	−
M. rhodochrous IMRU 502	YD	−	+	10	42	+	+	−
Corynebacterium pseudotuberculosis IMRU 1076	Ben	−	+	10	42	+	+	±
C. pointsettiae ATCC 9682	BH	−	+	10	37	+	+	−
C. flaccumfaciens-aurantiaca ATCC 12813	BH	+	±	10	42	+	+	±
C. lacticum IMRU 630	YD	−	+	10	37	+	+	−
C. michiganense ATCC 4450	YD	−	+	10	28	−	−	−
Actinomyces israelii ATCC 12102; ATCC 10048	AB	−	+	24	42	+	+	+
Arthrobacter globiformis ATCC 8010	N	−	±	10	28	−	−	−
Cellulomonas gelida ATCC 488; *C. flavigena* ATCC 482; *C. biazotea* ATCC 486	BH	+	±	24	42	−	−	+
C. fimi IMRU 22	N	+	±	24	37	−	−	−
Erysipelothrix insidiosa ATCC 11917	BH	−	+	24	42	+	−	−
Intrasporangium calvum K-7 KIP	Ben	−	+	10	42	+	+	−
Intrasporangium species LL 12-17	SE	−	+	24	42	+	−	−
Rothia dentocariosa D10B; XPGA	Ben	−	+	24	42	+	−	±
Oerskovia turbata SSIC 891, IMRU 689; IMRU 761; IMRU 762; LL-11-49; LL-Y13-3; LL-Y13-4; LL-17-11; IMRU 763; IMET 7130; IMET 7006; IMET 7133; IMET 7135	SE	+	+	10	37	+	−	−
Mycoplana bullata ATCC 4278; NCIB 9440; *M. dimorpha* ATCC 4279; NCIB 9439; LL-C3; LL-C4; LL-4-9; LL-5-20; LL-8-32	Ben	+	−	24	42	+	−	−

[a] ATCC, American Type Culture Collection; IMRU, Culture Collection, Institute of Microbiology, Rutgers, University, The State University of New Jersey; LL, Lechevaliers' collection; NCIB, National Collection of Industrial Bacteria; K, L. V. Kalakoutskii, Moscow; IMET, Culture Collection, Institut für Mikrobiologie und Experimentelle Therapie, Jena; SSIC, State Serum Institute of Copenhagen.

[b] Abbreviations: AB, actinomyces broth (BBL); N, nutrient medium (Difco); BH, brain heart infusion medium (Fisher Scientific); YD, yeast extract-glucose medium (24); Ben, Bennett's medium (9); Pab, pablum medium (15); SE, soil extract medium (6).

neutralized with calcium carbonate. After fixation, the cells were centrifuged and washed with distilled water. Drops were placed on Formvar-coated grids, and, after about 15 min, the grids were washed by flotation on water. Shadowing was carried out with germanium. Grids were examined with an Akashi 50E-1 electron microscope.

Staining. The Gram stain procedure was as modified by Hucker (3). The acid-fast stain used was a modified Ziehl-Neelsen method (6).

Thermotolerance. Thermotolerance was determined by maintaining cellular suspensions at 50 and 60 C for 4 and 8 hr before transferring to maintenance medium and incubating for up to 3 weeks.

Production of catalase. Production of catalase was detected after flooding 5-day-old slants of the organisms, grown on the appropriate maintenance medium, with 3 ml of 3% hydrogen peroxide and observing the evolution of gas.

Physiological tests. The physiological test procedures used have been previously described (13, 22).

Chemical tests. Cell wall analyses were carried out by the method of Becker et al. (2). Lysine, glycine, aspartic acid, ornithine, and diaminobutyric acid were separated by using either a modification of the system of Hoare and Work (10) substituting 11.6 N HCl for 10 N and developing at 4 C for 30 hr (system 1), or by first developing in butanol-pyridine-water-acetic acid (60:40:30:3) (2) for 48 hr (system 2), followed by system 1 for 24 hr. Amino acids were revealed using 0.4% ninhydrin in water-saturated butanol. The identification of sugars present in whole cell hydrolysates was as described by Lechevalier (13), who used an *n*-butanol-pyridine-water-toluene (5:3:3:4) paper chromatographic system (system 3). Ribitol and arabinose were resolved with the system of Partridge (19), which is the upper phase of *n*-butanol-ethyl alcohol-water (4:1:5), or with system 2. Glycerol and rhamnose were seprated by using the isopropanol-boric acid (7:1) system of Ikawa et al. (11) or with system 3. Reducing sugars and polyols were detected with a periodate-benzidine spray containing 0.1% periodate (7), or the Bean-Porter modification of this reagent (1).

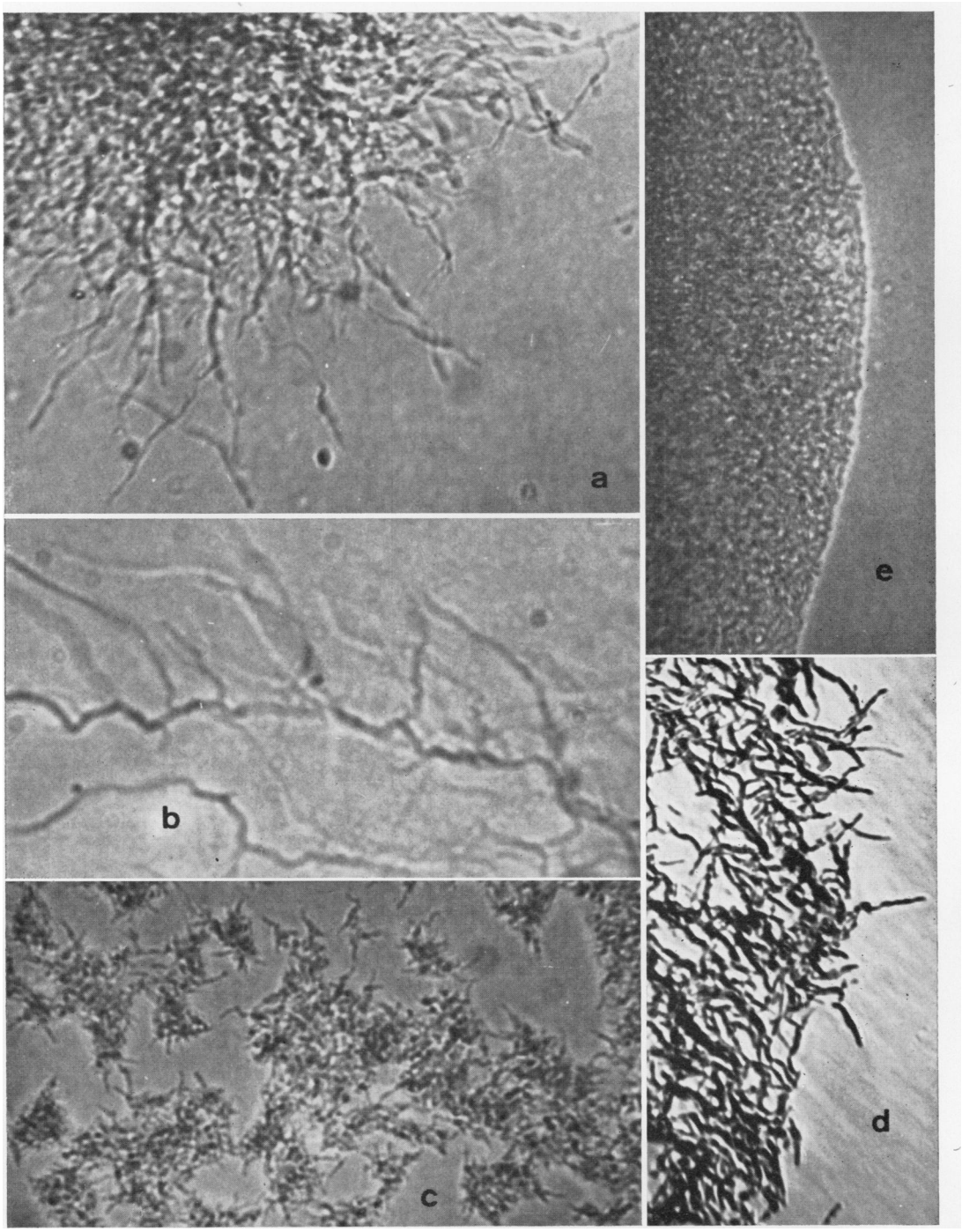

FIG. 1. *Photomicrographs of undisturbed 24-hr plate cultures on solid media. Bright field microscopy. Magnification, 900×, except b, which is 1,800×. (a) Oerskovia turbata SSI 891; Czapek agar (24) plus 0.2% yeast extract (Difco); (b) same, IMET 7130, half-strength nutrient agar (0.5 N); (c) Cellulomonas gelida ATCC 488, 0.5 N agar; (d) Mycobacterium smegmatis 607, 0.5 N agar; (e) Arthrobacter globiformis ATCC 8010, yeast extract-glucose agar.*

Determination of guanine and cytosine content. Since most of the strains (*Corynebacterium*, *Cellulomonas*, *Nocardia*, *Mycobacterium*, and *Mycoplana*) contain lipidic substances in their cells, they were first treated with acetone, chloroform, and ether, followed by drying. The dry cells were ground with a mortar and pestle and suspended in the saline ethylenediaminetetraacetic acid (EDTA) of Marmur (17) to an optical density (OD) of 10 (20 to 50 ml). These suspensions were incubated with lysozyme (0.05 mg/ml) overnight at 28 C and further lysed with Pronase (1 mg/ml) and sodium dodecyl sulfate (SDS; 1%). After addition of Pronase and SDS, the suspensions were heated for 10 min at 60 C and then incubated for 7 to 12 hr at 37 C. If complete lysis occurred, the mucous lysate was gently shaken with an equal amount of 80% phenol [80% phenol in 10 × SSC buffer (standard saline citrate, 0.15 M NaCl + 0.015 M sodium citrate) adjusted to *p*H 8]; otherwise, unlysed cell debris was removed by centrifugation prior to phenolization. The samples were centrifuged, the upper clear layer transferred to a beaker, and twice its volume of cold ethyl alcohol was added. By gently stirring with a glass rod, the crude deoxyribonucleic acid (DNA) was collected and then transferred to 10 to 20 ml of 0.1 × SSC, depending on how much DNA was present. After the DNA had dissolved (usually 12 hr later), 0.1 volume 10 × SSC was added to make the salt concentration 1 × SSC. Ribonuclease solution (1 mg/ml of saline) was added to give a final concentration of 10 μg/ml and the whole solution was incubated for 30 min at 37 C. To remove ribonuclease and remaining protein, the DNA was gently shaken with chloroform-isoamyl alcohol (24:1, v/v), followed by centrifugation at 12,000 × *g* for 20 min. Chloroform (bottom layer) was then removed with a Pasteur pipette, and the DNA solution was poured into a breaker and again treated with ethyl alcohol. The precipitate was collected and dissolved as indicated above. The DNA preparation was stored in the refrigerator with a drop of chloroform until used for melting determinations. Before melting, the DNA solution was diluted with SSC to an OD of about 0.3 to 260 nm (50% absorbance). Melting was carried out in a Beckman DU spectrophotometer with constant temperature equipment.

RESULTS AND DISCUSSION

No effort was made to obtain quantitative data about the branching of the organisms. The observers simply noted that the turbatae and the intrasporangia were the most frequently branched organisms, followed by the mycoplanae. These three groups of organisms were the only ones exhibiting secondary branching as far as could be seen by observation of whole mounts with the electron microscope. Branching was less in all other organisms and nil in *Mycobacterium tuberculosis* H37Ra and *Erysipelothrix insidiosa*. Photomicrographs grouped in Fig. 1 give an idea of the microscopic appearance of the growth of some of the organisms studied, ranging from the highly filamentous and branched turbatae to the very smooth *Arthrobacter globiformis*. *Cellulomonas gelida* and *Mycobacterium smegmatis* represent intermediates in the development of branching.

Motility was observed in the four strains of *Cellulomonas*, the nine strains of *Mycoplana*, and the seven strains of turbatae. In addition, motility was observed in *Corynebacterium flaccumfaciens-aurantiaca*.

Flagellation was as follows. *Cellulomonas* species. Motile elements (1.5 by 0.5 μm) usually monotrichous, but up to three randomly distributed flagella observed. *Corynebacterium flaccumfaciens-aurantiaca*. Most often the motile elements (1.8 by 0.5 μm) were tapered rods bearing at the wide end (0.5 μm) a single subpolar flagellum or, more rarely, a tuft of up to four flagella inserted in the same general area. The narrow end of these motile cells was extended into an amorphous tail, leaving one under the impression that the cells, propelled by their flagellum, had left behind a streak of extracellular slime. *Mycoplana* species. Rods (1.8 × 0.5 μm) with a subpolar tuft of flagella. Turbatae. Small motile elements (1.1 by 0.4 μm) mainly monotrichous, longer motile elements, usually peritrichous.

Our observations on flagellation are in agreement with previously published data (*Bergey's Manual of Determinative Bacteriology*, 7th ed.), except that our most recent data on the flagellation of turbatae are in better agreement with the data of Leifson (16) than we previously reported (9).

The Gram stain reactions were as reported in the literature: all the organisms studied were gram-positive except the cellulomonads and *C. flaccumfaciens-aurantiaca*, which were gram-variable (gram-negative becoming gram-positive),

TABLE 2. *Major constituents of cell walls of motile nocardoid actinomycetes and related genera*[a]

Cell wall type	DAB[b]	Lysine	Ornithine	Aspartic acid	Glycine	*Meso*-DAP[b]	L-DAP	Arabinose	Galactose
I					+		+		
IV						+		+	+
V		+	+		—[c]				
VI		+		+	—				
VII	+	+		+	—				
VIII				+	—				

[a] All preparations contained major amounts of alanine, glutamic acid, glucosamine, and muramic acid.

[b] DAB, 2,4-diaminobutyric acid.

[c] Glycine is variably present in these groups.

[d] DAP, 2,6-diaminopimelic acid.

TABLE 3. *Major components of cell wall and whole cell preparations from nocardoid bacteria*

Organism	Cell wall type	Whole cell sugars and polyols									GC content
		Galactose	Glucose	Mannose	Arabinose	Fucose	Ribose	Rhamnose	Glycerol	Ribitol	
											%
Intrasporangium calvum 7 KIP	I	+[a]	+	−	−	−	+	−	+	−	ND[b]
Intrasporangium species 12-17		−	−	−	−	−	−	−	+	+	70.7
Mycobacterium rhodochrous 502	IV	+	+	+	+	−	+	−	+	−	68.7
M. smegmatis 607		+	+	+	+	−	+	−	±	−	68.8
M. tuberculosis H37 Ra		+	+	+	+	−	−	−	+	−	66.8
Corynebacterium pseudotuberculosis 1076		+	+	+	+	−	−	−	±	−	67.5
Actinomyces israelii 10048	V	+	+	−	−	−	+	−	+	−	ND
Corynebacterium poinsettiae 9682		+	+	+	±	−	+	+	+	−	72.8
C. flaccumfaciens-aurantiaca 12813		−	−	−	−	−	−	−	+	−	74.5
Cellulomonas fimi 22		±	+	−	±	−	+	−	+	−	76.1
C. gelida 488		−	+	+	±	−	+	−	+	−	74.2
Oerskovia turbata 891 (Ørskov's 27)	VI[c]	+	+	−	−	−	−	−	+	−	70.5
7130		+	+	−	−	−	−	−	+	−	70.5
689		+	+	−	−	−	−	−	+	−	71.7
761		±	−	−	−	−	±	−	+	−	75.1
762		+	±	−	±	−	+	−	+	−	71.2
11-49		+	+	+	±	−	+	−	+	−	72.4
Y-13-3		+	+	±	−	−	+	−	+	−	ND
Y-13-4		+	+	+	−	−	+	−	+	−	ND
17-11		+	+	+	−	−	−	−	+	−	ND
763		+	−	−	−	−	−	−	+	−	ND
7006		ND									71.5
7133		ND									72.0
7135		ND									70.5
Rothia dentocariosa D 10B		+	−	+	−	−	−	−	+	−	ND
R. dentocariosa XPGA		+	+	+	±	−	+	−	+	−	ND
Arthrobacter globiformis 8010		+	+	+	±	−	+	−	+	−	ND
Corynebacterium lacticum 630		+	+	+	±	−	+	+	+	−	70.3
Erysipelothrix insidiosa 11917	VII	−	−	−	−	−	−	−	−	−	ND
Corynebacterium michiganense 4450		+	−	−	±	+	+	+	+	−	67.3
Cellulomonas flavigena 482	VIII	±	−	±	−	−	+	−	+	−	74.6
C. biazotea 486		−	+	+	±	−	+	+	+	−	75.6
Actinomyces israelii 12102	—[d]	+	+	+	−	−	±	−	+	−	ND
Mycoplana dimorpha 9439	Gram-negative, *meso*-DAP	−	+	±	±	−	+	−	+	−	63.9
M. bullata 9440S		−	+	±	±	−	+	−	+	−	65.4
M. bullata 9440R		−	+	±	±	−	+	−	+	−	66.1
M. bullata 4278		−	−	−	−	−	+	−	+	−	68.6
M. dimorpha C3, C4, 4-9, 5-20, 8-32		+/−	+	+	−	−	+	−	+	−	ND

[a] Symbols: +, major amounts; ±, small amounts; ±, traces; −, absent; +/−, some strains +, some strains −.

[b] Not determined.

[c] Cell walls of all turbatae contained major amounts of galactose.

[d] Lysine + L-DAP, no ornithine.

and the mycoplanae, which were gram-negative.

Maximal and minimal temperatures for growth of the turbatae and the corynebacteria were mostly 37 and 10 C, whereas, for the cellulomonads and the mycoplanae, these figures were, in general, 42 and 24 C.

The most temperature-sensitive organisms were the cellulomonads. When suspended in water or buffer at 50 C, they were killed in 8 hr. The same

was true of *Corynebacterium michiganense*. Mycoplanae, turbatae, and rothiae were all killed by a similar exposure for 4 hr at 60 C. The mycobacteria and the remaining corynebacteria were still viable after the treatment at 60 C.

Most of the organisms were strictly aerobic. The two strains of *Actinomyces* and three of the four cellulomonads were capable of good growth under anaerobic conditions. *Cellulomonas fimi* was the exception, not being able to grow anaerobically. It is also the only one of the cellulomonads unable to grow at 42 C.

All organisms were catalase-positive except the two *Actinomyces* and the *Erysipelothrix*. Only the mycobacteria were acid-fast. Whole cell sugar hydrolysates showed no useful pattern. Glycerol was present in most cases, but ribitol was not detected except in one strain (Table 3).

As can be seen in Table 3, from the organisms studied here, which are intermediate between the actinomycetes and the true bacteria, cell wall preparations are often obtained that do not fall into one of the six previously described groups characteristic of actinomycetes (13). We propose two new types, VII and VIII, for certain of these (Table 2). Cell wall type VIII is unusual, containing no diamino acid. One should note, however, that ornithine was detected by one of us (Prauser) in cell wall preparations of *Cellulomonas biazotea* 486.

Intrasporangium calvum and a related strain (12–17) yielded cell wall preparations of type I. Mycobacteria and *Corynebacterium pseudotuberculosis* have a cell wall of type IV, *Actinomyces israelii* 10048 and certain corynebacteria and cellulomonads have a type V, and the rothiae and all of the turbatae, but no cellulomonads, have a type VI. The mycoplanae all contained *meso*-2,6-diaminopimelic acid (DAP) in addition to an array of amino acids typical of gram-negative bacteria. The balance of the strains were of type VI, VII, or VIII (Table 3). The cell wall analysis of *Actinomyces israelii* 12102 was at variance with that reported by others (4). Our strain, obtained from the American Type Culture Collection in November 1967, contained L-DAP in addition to lysine, but no ornithine.

Our strains C_3, C_4, 4–9, 5–20, and 8–32, all isolated from soil, belong to the genus *Mycoplana* and do not differ appreciably from the strains of *Mycoplana dimorpha* (Table 3) obtained from culture collections and were as described by Gray and Thornton (8).

Our results indicate little relationship between the turbatae and species of *Cellulomonas*. Morphologically, the turbatae are more filamentous and branch more extensively than the cellulomonads. Also significant are differences in: (i) the Gram stain reaction, (ii) flagellation, (iii) temperature requirements and temperature tolerances, (iv) cell wall composition, and (v) growth habits. Unlike Jones and Bradley (12), we would not place the turbatae in the genus *Cellulomonas*, but like Prauser (*The Actinomycetales, in press*), we find it quite difficult to suggest a proper generic assignment for them among presently described genera. Morphologically, because of the abundance of branching, we consider the turbatae and the mycoplanae as members of the *Actinomycetales*.

Turbatae are clearly not members of the genus *Nocardia*, which currently contains nonmotile organisms forming some aerial mycelium and having a type IV cell wall, as shown by Lechevalier and Prauser (13, 20; *The Actinomycetales, in press*). Rather, they are closer to the nonmotile nocardoid members of the genera *Promicromonospora* and *Intrasporangium*. As a consequence, Prauser et al. (21) proposed the new genus, *Oerskovia*, in honor of the first man to describe these organisms (18). The type strain of *Oerskovia turbata* is Ørskov's strain 27 which bears number 891 in the collection of the State Serum Institute of Copenhagen. Strains IMRU 689 and IMET 7130 appear to be replicates of strain 891.

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