Motile Nocardoid Actinomycetales

R. S. SUKAPURE, MARY P. LECHEVALIER, H. REBER, M. L. HIGGINS, H. A. LECHEVALIER, AND H. PRAUSER

Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903, and Institut für Mikrobiologie und Experimentelle Therapie, Deutsche Akademie der Wissenschaften zu Berlin, Jena, East Germany

Received for publication 17 June 1969

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 Örskov, which was called Nocardia turbata by Erikson (5) and which was considered to be a Cellulomonas by Jones and Bradley (12), and (ii) the C4 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 actinomycetes-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 “C4 group” of organisms belongs to the genus Mycoplana and that the “turbatae” belong to neither the genus Nocardia nor Cellulomonas. The genus Örskovia 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

---

1 Present address: Research Laboratories, Hindustan Antibiotics Ltd., Pimpri, Poona-18, India.
2 Present address: Institut für Bodenbiologie, 3301 Braunschweig-FAL, West Germany.
3 Present address: School of Medicine, Temple University, Philadelphia, Pa. 19140.
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 (5:3:1:4) 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 separated 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 ×); (c) Cellulomonas gelida ATCC 488, 0.5 × agar; (d) Mycobacterium smegmatis 607, 0.5 × 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 \times \text{SSC buffer} (\text{standard saline}, 0.15 M \text{NaCl} + 0.015 M \text{sodium citrate}) adjusted to pH 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 \times \text{SSC}, depending on how much DNA was present. After the DNA had dissolved (usually 12 hr later), 0.1 volume \(10 \times \text{SSC}\) was added to make the salt concentration \(1 \times \text{SSC}\). Ribonuclease solution (1 mg/ml of saline) was added to give a final concentration of 10 \( \mu \text{g/ml} \) 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-isooamyl alcohol (24:1, v/v), followed by centrifugation at 12,000 \( \times g \) for 20 min. Chloroform (bottom layer) was then removed with a Pasteur pipette, and the DNA solution was poured into a beaker 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 \( \mu \text{m} \)) usually monotrichous, but up to three randomly distributed flagella observed. Corynebacterium flaccumfaciens-aurantiaca. Most often the motile elements (1.8 by 0.5 \( \mu \text{m} \)) were tapered rods bearing at the wide end (0.5 \( \mu \text{m} \)) 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 \( \times 0.5 \mu \text{m} \)) with a subpolar tuft of flagella. Turbatae. Small motile elements (1.1 by 0.4 \( \mu \text{m} \)) mainly monotrichous, longer motile elements, usually peritrichous.

Our observations on flagellation are in agreement with previously published data (Berger'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**

| Cell wall type | DAB<sup>a</sup> | Lys- | Ornithinine | Aspartic acid | Glucose | Mur-DAP<sup>b</sup> | L-DAP | Ara-Bi- | Galac- |
|---------------|-----------------|-----|-------------|--------------|--------|-------------------|------|-------|-------|
| I             |                 |     |             |              | +      | +                 |      | +      | +      |
| IV            |                 |     |             |              | +      | +                 |      | +      | +      |
| V             | +               | +   |              |              | +      | +                 |      | +      | +      |
| VI            | +               | +   |              |              | +      | +                 |      | +      | +      |
| VII           | +               | +   |              |              | -<sup>c</sup> | +                             |      | -      | -      |
| VIII          | +               |     |              |              | -<sup>c</sup> | -                             |      | -      | -      |

<sup>a</sup> All preparations contained major amounts of alanine, glutamic acid, glucosamine, and muramic acid.

<sup>b</sup> DAB, 2,4-diaminobutyric acid.

<sup>c</sup> Glycine is variably present in these groups.

<sup>d</sup> DAP, 2,6-diaminopimelic acid.
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*. Myco-
planae, turbatae, and rothiae were all killed by a
similar exposure for 4 hr at 60 C. The myco-
bacteria 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 fimii*
was the exception, not being able to grow anaer-
obically. It is also the only one of the cellulo-
monads 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, contain-
ing no diamino acid. One should note, how-
ever, 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 pseudotuber-
culosus* 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 C9, C11, 4-9, 5-20, and 8-32, all
isolated from soil, belong to the genus *Mycoplasma*
and do not differ appreciably from the strains of
*Mycoplasma 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*. Mor-
phologically, the turbatae are more filamentous
and branch more extensively than the cellulomo-
nads. Also significant are differences in: (i) the
Gram stain reaction, (ii) flagellation, (iii) temper-
ate 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 Actinomyceetas, in press*),
we find it quite difficult to suggest a proper generic
assignment for them among presently described
genera. Morphologically, because of the abun-
dance of branching, we consider the turbatae and
the mycoplanae as members of the *Actinomy-
ceetas*.

Turbatae are clearly not members of the genus
*Nozardia*, which currently contains nonmotile
organisms forming some aerial mycelium and
having a type IV cell wall, as shown by Lecheva-
lier and Prauser (13, 20; *The Actinomyceetas, in
press*). Rather, they are closer to the nonmotile
nocardoid members of the genera *Promicro-
monospora* and *Intrasporangium*. As a con-
sequence, 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 Orskov'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.

ACKNOWLEDGMENTS

This investigation was supported by grant GB 5810 and GB
7675 of the National Science Foundation.

When most of this work was done, R. S. Sukapure was a
Pfizer Postdoctoral Fellow and H. Reber a Waksman-Merck
Postdoctoral Fellow at the Institute of Microbiology, Rutgers
University, The State University of New Jersey, New Bruns-
wick, N. J.

LITERATURE CITED

1. Bean, R. C., and G. G. Porter. 1959. Detection and dif-
ferratiation of sugars and polyols on single paper chromato-
grams. Anal. Chem. 31:1929-1930.

2. Becker, B., M. P. Lechevalier, and H. A. Lechevalier. 1965.
Chemical composition of cell-wall preparations from
strains of various form-genera of aerobic actinomycetes.
Appl. Microbiol. 13:236-243.

3. Conn, H. J., M. A. Darrow, and V. M. Emmel. 1960. Staining
procedures used by the biological stain commission. The
Williams & Wilkins Co., Baltimore.

4. Cummins, C. S. 1965. Ornithine in mucocpeptide of gram-
positive cell walls. Nature (London) 206:1272.

5. Erikson, D. 1954. Factors promoting cell division in a “soft”
mycelial type of *Nozardia: Nozardia turbata*. J. Gen.
Microbiol. 11:198-208.

6. Gordon, R. E., and M. M. Smith. 1955. Proposed group of
characters for the separation of Streptomycetes and Nozardia.
J. Bacteriol. 69:147-150.

7. Grado, C., and C. E. Ballou. 1961. Myco-inositol phosphates
obtained by alkaline hydrolysis of beef brain phosphoinosi-
tide. J. Biol. Chem. 236:54-60.

8. Gray, P. H. H., and H. G. Thornton. 1928. Soil bacteria that
decompose certain aromatic compounds. Zentralbl. Bak-
teriol. Parasitenk. Abt. II 73:82-84.

9. Higgins, M. L., M. P. Lechevalier, and H. A. Lechevalier.
1967. Flagellated actinomycetes. J. Bacteriol. 93:1446-1451.
10. Hoare, D. S., and E. Work. 1957. The stereoisomers of α,ε-
diaminopimelic acid. 2. Their distribution in the bacterial order Actinomycetales and in certain Eubacteriales. Biochem. J. 65:441–447.

11. Ikawa, M., J. W. Morrow, and S. J. Harney. 1966. Paper chromatographic system for the identification of glycerol in bacterial cell walls. J. Bacteriol. 92:812–814.

12. Jones, L. A., and S. G. Bradley. 1964. Phenetic classification of actinomycetes. Develop. Ind. Microbiol. 5:267–272.

13. Lechevalier, M. P. 1968. Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71:934–944.

14. Lechevalier, H. A., and M. P. Lechevalier. 1967. Biology of actinomycetes. Annu. Rev. Microbiol. 21:71–100.

15. Lechevalier, M. P., and H. Lechevalier. 1957. A new genus of the Actinomycetales: Waksmania. J. Gen. Microbiol. 17:104–111.

16. Leifson, E. 1960. Atlas of bacterial flagellation. Academic Press Inc., New York.

17. Marmur, J. 1961. A procedure for the isolation of deoxy-
ribonucleic acid from micro-organisms. J. Mol. Biol. 3:208–218.

18. Ørskov, J. 1938. Untersuchungen über Strahlenpilze, rein-
gezüchtet aus dänischen Erdproben. Zentralbl. Bakteriol. Parasitenk. Abt. II. 98:344–357.

19. Partridge, S. M. 1946. Application of the paper partition chromatogram to the qualitative analysis of reducing sugars. Nature (London) 158:270–271.

20. Prauser, H. 1967. DAP-freie, gelbe Actinomyzeten mit Tendenz zur Beweglichkeit. Z. Allg. Mikrobiol. 7:81–83.

21. Prauser, H., M. P. Lechevalier, and H. Lechevalier. 1970. Description of Oerskovia gen. n. to harbor Ørskov’s motile Nocardia. Appl. Microbiol. 19:534.

22. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill, Inc., New York.

23. Shaw, M. K. 1968. Formation of filaments and synthesis of macromolecules at temperature below the minimum for growth of Escherichia coli. J. Bacteriol. 95:221–230.

24. Waksman, S. A. 1950. The actinomycetes. Chron. Bot., Waltham, Mass.