Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

4. The Partially Acid-fast Proactinomycetes

By DAGNY ERIKSON

Rothamsted Experimental Station, Harpenden, Herts.

SUMMARY: About three hundred strains of proactinomycetes were isolated from Rothamsted soils and examined together with certain strains from the National Collection of Type Cultures and other sources. Although little more than 9% were partially acid-fast on immediate isolation, subcultivation on rich media such as milk or nutrient glucose broth increased the percentage to 31%. The strains showed a range of features, from the soft mycobacterial type of growth with transient vegetative mycelium and very sparse aerial mycelium, if any, to the harder, more actinomycete-like variety. Of the acid-fast species *Proactinomyces opacus*, *Proactinomyces salmonicolor*, and *Proactinomyces paraffinae* predominated. In all, ninety-two strains were observed continuously for nearly two years on a variety of complex and simple media, and were stained at frequent intervals. Acid-fast cell elements occurred more often in complex protein- and fat-containing media, and in chemically defined media containing paraffin or large quantities of glycerol. They varied in shape and size according to the species and the age and quality of the growth. It is thought that differences of permeability of the cytoplasmic membrane in different environments may account for these variations. No evidence was obtained of any 'resting spores' or 'chlamydospores' in the vegetative mycelium. Since there are no true spores in the aerial mycelium when it is present, the proactinomycetes in general may be regarded as asporogenous.

The proactinomycetes (Jensen, 1931, 1932), also known as *Actinomyces* Groups II*a* and II*b* (Ørskov, 1923) and *Nocardia* (Waksman & Henrici, 1943; Bergey, 1948), are distinguished morphologically from the true actinomycetes (*Streptomyces* Waksman & Henrici) by (1) the transient nature of the vegetative mycelium, which may break up into rods and cocci after 1–3 days, and so produce a soft growth resembling that of mycobacteria; and (2) the infrequent development of an aerial mycelium, which may fragment, but does not divide evenly into spores, and which is often only of microscopical dimensions.

Most workers agree that the sparse, often rudimentary, aerial mycelium cannot be regarded as a sporogenous phase. Yet genuine sporing actinomycetes can at times produce asporogenous variants, which develop at the most only a few undivided aerial hyphae exactly similar to those of proactinomycetes (Appleby, 1947; Erikson, 1948). It is not impossible that there should be conditions under which proactinomycetes are stimulated to greater aerial growth, and that the cell contents of some of the aerial filaments should divide and behave as happens in the corresponding hyphae of actinomycetes. It is, however, in the very variable shape and size of the cell elements of the substratum or vegetative growth on different media that the great plasticity of this group is expressed. In the older medical literature and in the more recent work of Krassilnikov (1934) on soil organisms allied to mycobacteria, there is

frequent mention of peculiar swollen spore forms such as 'chlamydospores', 'resting spores', 'cystites', 'arthrospores', which can be distinguished by their size and staining properties.

The property of acid-fastness has been reported for many members of this group. Together with angular division, it is one of the characteristics which allies them with the mycobacteria. Jensen (1931), when proposing the species *Proactinomyces paraffinae*, described certain branches of the vegetative mycelium as dividing up into acid-fast, spore-like bodies. On the other hand, it has long been known that the conidiospores of the aerial mycelium of certain species of actinomycetes are acid-fast (Lieske, 1921; Erikson, 1935; Basu, 1937). Bacterial endospores are also acid-fast. In this study a search was made in a large range of partially acid-fast proactinomycetes to determine: (*a*) which cell elements under a variety of cultural conditions are resistant to acid decolorization, using the method of Umbreit (1939); (*b*) whether they occur consistently and are capable of being interpreted as a sporogenous phase; (*c*) what factors favour the development of aerial mycelium.

Source of the strains studied

Conn & Dimmick (1947) state that 'partially acid-fast organisms, apparently related to mycobacteria, do occur in soil; but...they do not seem to make up part of the predominant soil flora'. Jensen (1931), who used a complex casein agar medium for isolation, also considered that they were rare, and that it was necessary to make a special search for them or to use selective methods such as adding paraffin to the soil. Recent work in this department, following that of Gray & Thornton (1928), suggests that they are fairly widely distributed. Thus, of eighteen *Proactinomyces* colonies picked at random from soil-extract agar plates poured by a colleague in the routine plating of Rothamsted soils, twelve were found to be partly acid-fast when grown on nutrient glucose broth. That they can be easily overlooked is shown by the following experiment. Three soil plots were sampled, and all presumed proactinomycete strains were immediately tested for acid-fastness in the first subculture on semisolid soil-extract agar. The yields were: Plot A, 26 strains, 5 of them acid-fast; Plot B, 37 strains, 2 acid-fast; Plot C, 28 strains, 1 acid-fast. But when these same ninety-one strains were subcultured three to four times on nutrient glucose broth or in milk and re-tested, the numbers acid-fast were: Plot A, 26 strains, 14 acid-fast; Plot B, 37 strains, 11 acid-fast; Plot C, 28 strains, 12 acid-fast. These findings were confirmed at intervals, with one or two exceptions, and eventually 33 of the 91 strains proved to be partially acid-fast on some medium. This apparent enhancement of acid-fastness after the first isolation from soil is contrary to the usual decrease in such staining properties in pathogenic organisms of this group obtained from animal material. In this last case, however, they are generally grown on rich media from the beginning.

Other experiments yielded similar results, and in all, during a period of nearly two years, about 300 proactinomycete strains were examined. In addition, a few strains were received through the kindness of Dr Turfitt (from soils) and of Dr Sharp (from human pathological material). Some National

Collection Type Cultures were included for comparison. From this total 82 strains were selected as showing acid-fast elements on some media. The majority could be assigned to known species listed in Bergey (1948) under the generic name *Nocardia*, and are as follows (the older nomenclature being retained for the present).

Proactinomyces minimus Jensen, 4 strains; *Proactinomyces opacus* den Dooren de Jong, 12 strains; *Proactinomyces polychromogenes* Vallee, 2 strains; *Proactinomyces paraffinae* Jensen, 14 strains; *Proactinomyces salmonicolor* den Dooren de Jong, 18 strains; *Proactinomyces coeliacus* Gray & Thornton, 4 strains; *Proactinomyces ruber* Krassilnikov, 3 strains; 18 unidentified.

National Collection Type Cultures: No. 576, *Actinomyces luteus* Christopherson & Archibald; No. 659, *Actinomyces caprae* Silberschmidt; No. 6115, *Actinomyces rhodnii* Erikson; No. 3488, *Proactinomyces paraffinae* Jensen; No. 3486, *Proactinomyces polychromogenes* Vallee; No. 2568, *Mycobacterium convolutum* Gray & Thornton; No. 2569, *Mycobacterium erythropolis* Gray & Thornton; No. 2566, *Mycobacterium crystallophagum* Gray & Thornton; No. 2563, *Mycobacterium agreste* Gray & Thornton; No. 2571, *Mycobacterium actinomorphum* Gray & Thornton; No. 525, *Mycobacterium phlei* Lehmann & Neumann.

Unidentified mycobacterium 'Hewison' (human source).

Since *Proactinomyces opacus*, *Proactinomyces salmonicolor*, and *Proactinomyces paraffinae* predominated in the soil strains, were clearly marked types, and together covered almost the complete range of the features characteristic of the genus, they were studied in detail.

Proactinomyces opacus

(*a*) *Cultural characters.* A soft cream to pink growth on nutrient agars. Most strains were of the *crystallophagum* type, moister and softer, as described by Jensen, and not so filamentous as his *opacus* strains. There is little doubt that they should be classed together. On chemically defined agar media such as starch-nitrate, ammonium lactate, and Oxford's (1946) ammonium acetate medium, growth was colourless and thin, and produced an initial mycelium the filaments of which were more or less quickly divided into short rods. The addition of 0·01 % $MnSO_4$ to Czapek's sucrose-nitrate agar usually stimulated the production of the pale pink pigment in the very moist, almost mucoid type of growth which was characteristic of these strains.

(*b*) *Incidence of acid-fast elements.* Each strain was tested daily for a week, and thereafter every week for three months, with the following results.

Nutrient glucose broth: small mycelia and branching filaments, variably acid-fast, up to the fourth day; from the fourth to the fourteenth day filaments scarce, generally not acid-fast, and short beaded rods, partly acid-fast, predominant; after the second week more acid-fast rods in the cream surface scum than in the copious bottom deposit; at three months very short rods, sometimes coccoid, almost all non-acid-fast in both surface and bottom growth; broth occasionally turbid in the first week, afterwards clear.

Milk: short rods, beaded, mostly not acid-fast, some branching filaments up to the third day; from the fourth to the fourteenth day gradual increase in length of filaments, branching mycelia, and general acid-fastness; at six weeks

surface growth shows mostly short rods and cocci which are somewhat more acid-fast than the comparable elements in the bottom growth; mostly non-acid-fast at three months.

Czapek salts (nitrate) + liquid paraffin: the branching filaments are more often not acid-fast and the short rods positive and variable.

Ammonium phosphate, Czapek salts + solid paraffin: mostly short rods, positive.

Czapek salts (nitrate) + increasing amounts of glycerol: short rods negative or variable up to 2·5 % glycerol; from 5 to 12·5 % coccoid rods, often in chains, constantly acid-fast.

Czapek salts (nitrate) + 1 % various carbon sources (sucrose, glucose, galactose, maltose, lactose, xylose, sorbitol, dulcitol): few branching filaments, mostly short rods, negative or occasionally variable, whether growth is good as in galactose, sorbitol, glucose, sucrose, maltose, or poor as in lactose, xylose, dulcitol; no acid produced.

Czapek salts (sucrose) + 0·1 % various nitrogen sources (sodium nitrate, ammonium phosphate, ammonium lactate, alanine, glycine, urea): similar, occasional branching filaments, mainly short rods, non-acid-fast, whether growth is poor (urea) or fair to good (all others).

Thus, acid-fast cell elements predominated at the period of maximum growth in a free air supply in complex media; in a chemically defined medium they could be found only when substances such as paraffin or large quantities of glycerol were added; in all instances the elements which retained the stain were normal vegetative cells—branching filaments and the short rods into which they divide as the result of population pressure; cocci or large swollen cells were very rarely seen.

(*c*) *Development of aerial mycelium.* The short simple aerial filaments, which on media like glucose, asparagine and starch-tryptone agar appear on the first to the third days then quickly wither away again, have been admirably depicted by Jensen (1931). I agree with him in finding no division into spores, and no difference in staining properties. Most of these strains produced a very thin dry growth on Oxford's ammonium acetate agar, which tended to inhibit vegetative division so that the mycelia remained intact for 1–3 weeks and gave rise to aerial filaments that were relatively long and sometimes branched but not divided. When a cover-slip was pressed over these growths, it was impossible to distinguish between substratum and aerial mycelium; there was no difference in refractility, density of protoplasm, width of filament, or thickness of cell wall.

Proactinomyces salmonicolor

(*a*) *Cultural characters.* A rich salmon pink to yellow-pigmented soft growth on nutrient agars, and a more pasty and sometimes crumbly consistency compared with *P. opacus*. Growth fair to good and usually coloured on the simpler media such as starch-tryptone and ammonium lactate agars; particularly good and characteristic on Czapek's sucrose nitrate agar + 0·01 % $MnSO_4$, becoming somewhat dry and considerably convoluted. One strain immediately after isolation produced a darkening of the medium, but this property was lost

on subsequent cultivation. The cells on this medium are remarkably broad, highly refractile, and intensely Gram-positive. The development of large swollen, spherical, pear-shaped and other irregular elements can be seen in hanging-drop broth cultures. They are to be found towards the centre of the drop after the initial division of the minute mycelium. At the margins of the drop where the film of liquid has spread thinly on the surface of the slip, the filaments tend to be longer and to spread in parallel bundles. The production of 'cystites' is used as a diagnostic criterion in Bergey's (1948) classification.

(*b*) *Incidence of acid-fast elements.* Nutrient glucose broth: minute mycelia and branching filaments up to 24 hr., mostly negative; few long filaments on the second day, generally acid-fast; from the third to the fourteenth day rods, often showing bipolar beading, and cocci, variable and negative; thereafter gradual loss of acid-fastness in beaded rods and chains of cocci; broth generally clear; no significant difference in staining reactions of top and bottom growth.

Milk: up to the third day generally short branching filaments and rods, mostly negative; from fourth to fourteenth day increase in length of filaments which are usually strongly beaded and acid-fast; no difference in bottom growth; later, rods and cocci, mainly negative.

Czapek solutions + liquid or solid paraffin: bipolar rods, long filaments, and chains of cocci, all usually acid-fast.

Czapek salts + increasing amounts of glycerol: filaments and rods, mainly negative up to 2·5 %; variable at 5 %; mostly rods, constantly acid-fast, from 7·5 to 12·5 %.

Chemically-defined solutions with various carbon and nitrogen sources: as for *P. opacus*, with the exception that cocci are generally produced; usually non-acid-fast; but the addition of a small quantity of $MnSO_4$ (0·01 %) to the sucrose nitrate medium resulted in numbers of beaded filaments, rods and cocci showing partial acid-fastness; four strains grew poorly in *m*-cresol as carbon source and were all acid-fast.

Here, although the developmental picture is of greater complexity than in the preceding species, acid-fastness is not restricted to any special shape of cell. The large, spherical, or irregularly swollen 'cystites' are relatively few, appear in the early stages of growth, and are seldom acid-fast. In the strongly beaded rods and filaments which are typical of this species in all cultures, it is generally the cell walls that retain the basic fuchsin for the longest periods, while the condensed beads of cytoplasm take the counterstain. The enhancing effect of such additions as paraffin and glycerol is not permanent; subculture from these media to starch-tryptone agar, on which the growth is usually non-acid-fast, yields cells that do not resist acid decolorization.

(*c*) *Development of aerial mycelium.* A few short, undivided aerial filaments appear occasionally on starch-tryptone, and more often and more persistently on ammonium acetate agar. On one-month-old Czapek sucrose nitrate plates the rather dry, convoluted pink growth of some strains developed a thin white frosting of aerial mycelium. Certain of the individual threads later produced septa at irregular intervals, but no differences in permeability or resistance to dyes could be established for these aerial cells.

Proactinomyces paraffinae

(*a*) *Cultural characters.* A hard, firm, yellowish growth with a visible amount of white aerial mycelium on most nutrient agars. On starch nitrate and glucose ammonium phosphate agars growth is thinner, and frequent bulbous swellings at the ends of the long initial filaments as described by Jensen can be seen. Angular branching and division into chains of cocci both occur within 2–7 days. On poorer media such as ammonium lactate and ammonium acetate agars, the original minute mycelium divides almost completely by the characteristic 'slipping' method into an assemblage of short rods, and only at the margins of the colony are longer filaments present, giving a rhizoid appearance to the growth. The production of chains of acid-fast cocci in the vegetative mycelium is claimed as a specific character.

(*b*) *Incidence of acid-fast elements.* Nutrient glucose broth: the general picture is similar to that of *P. salmonicolor*, except that acid-fastness is more infrequent; in the early stages minute mycelia, branching filaments and rods may all show acid-fastness; gradually cocci become predominant, but like the former cell elements they are variably acid-fast.

Milk: as in the other two species, a gradual increase in positive staining reactions and filament length, followed by loss of acid-fastness and division into rods and coccoid chains.

Chemically defined solutions + paraffin: this species grows exceptionally well, as its name implies, but the enhancement of acid-fast staining is not so marked. All the cell elements, branching filaments, rods, and cocci, have been noted as variably acid-fast.

Chemically defined solutions + increasing amounts of glycerol: acid-fastness is general at the higher levels, 7·5–12·5 %, and the predominant picture is of short rods.

Chemically defined solutions + various sources of carbon and nitrogen: most strains are negative throughout, but the addition of 0·01 % $MnSO_4$ to Czapek's sucrose nitrate causes certain strains to show an appreciable number of acid-fast elements, mainly short rods. This species grows fairly well in *p*- and *o*-cresol, and particularly so in *m*-cresol; in the last very short rods sometimes show acid-fast staining.

(*c*) *Development of aerial mycelium. P. paraffinae*, in the relative ease with which it forms aerial mycelium on a variety of media, approximates to the true actinomycetes (*Streptomyces*). Observation of microcultures on starvation media such as water agar, showed at 12 hr., simple vegetative mycelium; 20 hr., greater branching of mycelium, one aerial hypha; 42 hr., 5–10 aerial hyphae, all simple and undivided; 66 hr., aerial hyphae with irregular sub-division of contents, no distinction in staining properties from vegetative filaments. From month-old, dried-up plates of $MnSO_4$ sucrose nitrate agar entirely covered with a veil of aerial mycelium, certain individual filaments were removed by means of a micromanipulator. They were all exceedingly fragile, and with the gentlest handling broke up into irregular cylindrical

elements which, when cultured in droplets of nutrient glucose broth, elongated to form a new mycelium indistinguishable from that produced by cells of the normal substratum growth.

DISCUSSION

Representatives of the other species listed at the beginning were also tested under similar conditions, and the picture was much the same. Acid-fast elements could be found in most strains on the same favourable media, and they could be intact mycelia, mycelial fragments, isolated branching filaments, short rods, or cocci, according to the nature of the species, the age and quality of its growth on the various substrates. No consistent type of acid-fast cell could be found for any species in the whole range, and no evidence for any peculiar forms of 'resting spores'. All the proactinomycetes were less resistant to acid ethanol decolorization than the true mycobacteria (*cf.* Umbreit, 1939). The gradual waxing and waning of the property of acid-fastness during prolonged cultivation in such media as milk suggests the validity of the theory of Yegian & Vanderlinde (1947) that this property is dependent upon the permeability of the cytoplasmic membrane. Micro-organisms which may grow out of the nutritive medium into the air, as do all the actinomycetes, frequently show at different stages of growth a patchy staining with vital dyes such as dilute methylene blue. Finally, the observations of previous workers that the aerial mycelium, when present, is not spore-bearing have been confirmed.

This work was done by the author as a member of the scientific staff of the Agricultural Research Council.

I wish to thank Mr A. V. Garcia for the large collection of isolates from soil which formed the basic material of this study. I also wish to thank Miss Enid Wilsher for technical assistance.

REFERENCES

Appleby, J. C. (1947). An asporogenous variant of *Streptomyces griseus*. *J. gen. Microbiol.* **2**, 80.

Basu, C. C. (1937). Notes on a new strain of *Actinomyces* obtained by blood culture. *Ind. J. med. Res.* **25**, 325.

Bergey, D. H. (1948). (Edited by Breed, R. S., Murray, E. G. D. & Hitchens, A. P.) *Manual of Determinative Bacteriology*, 6th ed. London: Baillière, Tindall and Cox.

Conn, H. J. & Dimmick, I. (1947). Soil bacteria similar in morphology to *Mycobacterium* and *Corynebacterium*. *J. Bact.* **54**, 291.

Erikson, D. (1935). The pathogenic aerobic organisms of the *Actinomyces* group. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 203.

Erikson, D. (1948). Differentiation of the vegetative and sporogenous phases of the Actinomycetes. 3. Variation in the *Actinomyces coelicolor* species-group. *J. gen. Microbiol.* **2**, 252.

Gray, P. H. H. & Thornton, H. G. (1928). Soil bacteria that decompose certain aromatic compounds. *Zbl. Bakt.* Abt. 2, **73**, 74.

Jensen, H. L. (1931). Contributions to our knowledge of the Actinomycetales. II. The definition and subdivision of the genus *Actinomyces*, with a preliminary account of Australian soil actinomycetes. *Proc. Linn. Soc. N.S.W.* **56**, 345.

Jensen, H. L. (1932). Contributions to our knowledge of the Actinomycetales. IV. The identity of certain species of *Mycobacterium* and *Proactinomyces*. *Proc. Linn. Soc. N.S.W.* **57**, 364.

KRASSILNIKOV, N. A. (1934). Die Entwicklungsgeschichte der Bodenmykobakterien. *Zbl. Bakt.* Abt. 2, **90**, 428.

LIESKE, R. (1921). *Morphologie und Biologie der Strahlenpilze.* Leipzig: Borntraeger.

ØRSKOV, J. (1923). *Investigations into the Morphology of the Ray Fungi.* Copenhagen: Levin and Munksgaard.

OXFORD, A. E. (1946). Note on the production of soluble blue pigment in simple media by *Actinomyces coelicolor*. *J. Bact.* **51**, 267.

UMBREIT, W. W. (1939). Studies on the *Proactinomyces*. *J. Bact.* **38**, 73.

WAKSMAN, S. A. & HENRICI, A. T. (1943). The nomenclature and classification of the actinomycetes. *J. Bact.* **46**, 337.

YEGIAN, D. & VANDERLINDE, R. J. (1947). The nature of acid-fastness. *J. Bact.* **54**, 777.

(*Received* 15 *November* 1948)