STUDIES ON THE PROACTINOMYCES

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Received for publication January 26, 1939

The taxonomy of the actinomycetes has been subject to unending controversy, and further confusion has been introduced by the formation of a group intermediate between Actinomyces and Corynebacterium and Mycobacterium which was termed Proactinomyces. It is to the validity and composition of this group of organisms that these studies were directed. Two virtually identical systems of separation, differing only in nomenclature and using morphological characters for their main subdivisions, are those proposed by Orskov (1923, 1938) and Jensen (1931a and b, 1932) illustrated in table 1. Since the nomenclature of Jensen seems based on sounder taxonomic principles it has been used in this paper. The earlier physiological studies of Waksman (1919) and the morphological studies of Drechsler (1919) were confined essentially to the “Actinomyces” group of Jensen and may serve, therefore, as further physiological subdivisions of this group, with the exception of one culture, “Act. asteroides,” which is a Proactinomyces.

The members of the genus Proactinomyces are distinguished from the members of the genus Actinomyces by the former’s inability to form spores in the aerial mycelium. Spores are defined as “bodies identical to one another in form having a special mode of formation” and are to be distinguished from the so-called fragmentation “spores.” Eighteen strains of acid-fast and 11 strains of non acid-fast Proactinomyces which sometimes

1 Journal Series Paper, N. J. Agricultural Experiment Station, Department of Soil Chemistry and Microbiology.
2 Now with the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin.
formed aerial mycelia, together with 10 species selected at random from among the true *Actinomyces*, were subjected to a wide variety of cultural and environmental conditions to determine whether some condition might not be found in which spore formation could be induced. These were cultivated, at 28°C., in tubes, plates and slide cultures, upon Czapek’s, starch, egg albumin, nutrient, glycerol and water agars, as described by Waksman.

### TABLE 1

**Nomenclature of actinomycetes**

<table>
<thead>
<tr>
<th>CHARACTER</th>
<th>ORSKOV (1923, 1938)</th>
<th>JENSEN (1931a and b, 1932)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Spore formation in aerial mycelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Single spores born at end of branch</td>
<td>Micromonospora</td>
<td>Micromonospora</td>
</tr>
<tr>
<td>B. Spores formed in chains at end of mycelial hyphae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Vegetative mycelium undivided</td>
<td>Cohnistreptothrix</td>
<td>Actinomyces</td>
</tr>
<tr>
<td>b. Vegetative mycelium divided</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. No spores formed in aerial mycelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. “Stable” mycelium (β form)</td>
<td>Actinomyces</td>
<td>Proactinomyces</td>
</tr>
<tr>
<td>1. Long hyphae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Colony growth in liquid media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Actinomyces-like colony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. “Unstable” mycelium (α form)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Short mycelium if formed at all</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Bacterial (diffuse) growth in liquid media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Bacteria-like colony</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1919) as well as on potato slants and on Frazier’s gelatin (Fred and Waksman, 1928). They were observed for a period of three months at approximately weekly intervals for spore formation, both by direct microscopic observation and by use of the Orskov contact cover-slip technic (1923). In no case was there any indication of spore formation among the *Proactinomyces* strains. Under similar conditions, strains of true *Actinomyces* produced...
STUDIES ON THE PROACTINOMYCES

a macroscopic (in a few cases only microscopic) aerial mycelium which showed abundant spore formation within a week. In a few cases, especially on rich media, spore formation was slightly delayed, but was generally evident by the second week. Therefore, pending valid evidence to the contrary, the line of distinction between the Proactinomyces and the Actinomyces proper is probably a sufficiently constant and practical distinction to be of value in their separation.

The genus Proactinomyces is separated from Corynebacterium and Mycobacterium upon the basis of the absence of mycelial formation in the latter. While, admittedly, such a distinction appears eminently suitable, it has in practice resulted in considerable confusion, which arises, in part, at least, from the lack of recognition that many bacteria, when placed under conditions which somewhat restrict free movement, may (especially in early stages of growth), form structures which may be easily mistaken for mycelium. The observations of McCarter and Hastings (1935), of Krassilnikow (1934), and even of Orskov (1938) on his group “IIb” leave little doubt that strains which would generally be considered bacteria, may form, under given conditions of growth and given techniques of observation, structures which have been called “mycelium.” The lack of an adequate definition of what constitutes a mycelium is a real cause of confusion. A “chain” or alignment of cells extending no more than 5–10 cell lengths and rapidly disintegrating so that the structures are not detectable after 18 to 26 hours is often considered equivalent to a mycelium 200 to 300 μ long which remains without marked fragmentation for several weeks. Jensen attempts to circumvent this difficulty by defining as Proactinomyces those forms which show a “constant” formation of mycelia while placing the forms showing only occasional mycelia among the bacteria. A moment’s reflection will indicate that such a character is too dependent upon given cultural conditions to be of much aid in separation.

It has become increasingly apparent that the Proactinomyces are composed of two rather distinct types of forms, one of which is difficult to distinguish from the Corynebacterium or Mycobacterium, and which is usually termed the “unstable mycelium group.”
(here called "α" Proactinomyces). The other group can be distinguished from Actinomyces by lack of spore formation, but its properties do not greatly resemble those of the first type. This latter group is usually termed "stable mycelium group" (here called "β" Proactinomyces). A list of the properties of these two groups is given in table 1.

Since it is recognized that simple mycelium formation is not an adequate criterion for separation from the Corynebacterium and Mycobacterium, and since the α and β groups of Proactinomyces are distinguished by the rather vague distinctions of "stable" and "unstable" mycelium or "slow" and "rapid" disintegration, a search for better criteria seemed desirable. Of these, three appeared suitable: (1) aerial mycelium, (2) diffuse growth in liquid media, (3) structure of colony. Aerial mycelium was present quite consistently (though sometimes microscopic) among the "stable mycelium" group, while it was never found among the "unstable mycelium" types. This character, if sufficiently constant, could be a desirable characteristic for separation. While such is Orskov's contention (1923, 1938), strains are found which show typical β structure (and virtually no signs of disintegration even when cultured for as long as 3 to 4 months) yet never produce even traces of microscopically observable aerial mycelium. Other strains show aerial mycelium only under particular circumstances, so that production of aerial mycelium cannot actually be used as a criterion for separation, since it is dependent, to a large extent, on the cultural conditions.

The lack of diffuse growth in fluid media seems a more suitable index of mycelium formation and it is surprising that it has not been previously used as a method of separation. It has been frequently noted that while bacteria always produce some degree of turbidity, actinomycetes do not. These show rather flaky or colony-like growths, but no turbidity. In this study it was found that all β type strains produced no turbidity while all α types did. Such a separation, based upon turbidity is hardly

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* The difference between α and β types in this respect is actually more fundamental than is at first apparent. An Actinomyces in liquid media forms "colonies" composed in the most part of a single individual (perhaps even a single
adequate, however, since many mycobacteria produce no turbidity, since R and S cultures often differ in turbidity, and since the O/R potential of the media often determines whether a "non-turbid" pellicle or diffuse growth is observed. Yet those familiar with the growths of an actinomycete in liquid media will recognize that the complete absence of turbidity plus the formation of colony-like structures are a constant character of Actinomyces and a similar type of growth among the \( \beta \) forms of Proactinomyces might serve as one of the criteria of separation. That this criterion is not entirely a plus or minus character is apparent since variations have been reported (Jensen, 1931a; Topping, 1937; Wright, 1937) to occur which resulted in turbid-forming variants.

The variants of Proactinomyces polychromogenus (Jensen, 1931a) obtained from the Lister Institute Collection ("filamentous," "normal" and "bacterial" forms) do not show turbidity in fluid media, but are rather of the \( \beta \) type (stable mycelium). The cultures of Topping (1937) (no. 114R and S) belong to the \( \alpha \) group, while the remaining report (Wright, 1937) emphasizes that the turbidity-forming variants seem distinctly unusual and are very possibly entirely comparable to the variations which one occasionally finds in bacterial cultures. Such conversions from one species to another are not too uncommon and while they serve to indicate relationships, they do not invalidate entirely the aid in separation obtained by observations on liquid media.

Finally, the difference in the consistency of the colony is quite distinct between the \( \alpha \) and \( \beta \) groups, that of the \( \alpha \) type being generally soft, translucent and gummy, whereas the \( \beta \) type is hard, waxy and cannot be normally separated into fragments with the inoculating needle, yet this distinction seems somewhat too vague for adequate separation.
After studying and comparing various reputed *Proactinomyces* types sent to the laboratory through the courtesy of many workers, the following diagram of their relationships may be suggested. This is very similar to that of Jensen and to that of Orskov, the fundamental points being entirely in agreement.

![Diagram of relationships between Corynebacterium, Proactinomyces, and Mycobacterium](image)

Attention is centered in this report on the β types, particularly those producing an orange to orange-red pigment, whose function in nature (aside from the pathogenic "Act. asteroides") can only be indicated as a probable attack on resistant compounds (Gray and Thornton, 1928; Jensen, 1932).

**Type I. Partially Acid-fast β Proactinomyces**

Jensen (1931b and 1932) found that certain forms among the *Proactinomyces* exhibited both a relatively and a partially acid-fast character; this was also encountered among certain forms in this study. It can be interpreted to mean that not only does the acid-fast character depend to a considerable extent upon the medium (in milk they are likely to be acid-fast, while on certain
STUDIES ON THE PROACTINOMYCES

synthetic media they may be non acid-fast), but also that a part of the mycelium may be acid-fast, whereas closely adjacent parts may be non acid-fast. It has been particularly noted that the aerial mycelium seems less readily decolorized than the submerged mycelium. It is decolorized in all cases, however, more rapidly by acid alcohol than is the case with the tubercle organism. A standard method of decolorizing, which has proved satisfactory for distinguishing between partially acid-fast and non acid-fast forms, consists in placing the stained slide (Carbol-Fuchsin, 2 per cent, steam for 5 minutes) in a stain jar containing 3 per cent HCl in alcohol for exactly 15 seconds. Wash in water immediately, and place in stain jar containing 0.1 per cent aqueous solution of Methylene blue for three minutes. By this method heavy clumps may not be entirely decolorized, but they offer no difficulty if the areas free from them are examined.

The organisms included in this group, in addition to being partially acid-fast, have several other related characters. They do not hydrolyze starch, which indeed seems correlated with the partial acid-fast character (Gordon and Hagan, 1936). They do not hydrolyze gelatin, and without exception possess the ability to utilize paraffin as the sole carbon source. This type was represented in this study by seventeen strains as follows:

3308A. *A. asteroides*. Waksman collection.
3013. Unidentified pathogen obtained from Dr. Broadhurst.
3306. *A. asteroides* Bering.
401. *A. farcinica* (Fitch 1932).
558. *A. gypsoides* (Henrici 1934).
559. *A. asteroides* (Henrici 1934).
568, 568a, 568b. Unidentified forms, isolated from potato scab in 1932 by Dr. Taylor at Cornell University.

Paraffin utilization was determined by inoculation into a liquid medium composed of the salts employed in Czapek's media with a drop of melted commercial paraffin per tube. While this paraffin probably was not entirely pure it served to distinguish the acid-fast cultures from the non-acid-fast cultures, since the latter showed no growth whatsoever.

Cultures 401 through 650 were obtained through the courtesy of Dr. W. A. Hagan, Cornell University.
The consistency of the growth of these organisms is crumbly and wax-like, as compared to the smooth, shiny, hard growths of the non acid-fast types. On Czapek's agar (sucrose-NaNO₃ medium), growth is scanty in the case of all strains, but sooner or later (usually within 5 days) a white aerial mycelium begins to form. Some strains produce more aerial mycelium than others but in no case was spore formation observed. The pigment of the cultures was orange to orange-red but no black color was produced on any of the media employed. There was considerable fragmentation of the mycelium after one week, at 28°C., into rod-shaped branching forms of varied lengths, but long strands were visible for as long as two months. Previous descriptions of A. asteroides leave little to add (Henrici, 1930; Henrici and Gardner, 1921; Waksman, 1919).

A few of the properties of the 17 strains falling into this group are summarized in table 2. Among these strains, several were described as distinct species and several were unidentified forms. Data are presented only of the growth on Czapek's synthetic agar (on which the organisms grow slowly) and on beef peptone agar (on which they grow rapidly). Growth on water-agar, paraffin agar and egg-albumin agar is almost identical to that on Czapek's; starch-ammonium sulfate and glycerol-nitrate agars are somewhat better nutrients and occupy an intermediate position, while Frazier gelatin agar gives growth very similar to that on beef-peptone. Data are given for the characters of the organisms after 5 and 20 days incubation. It will be noted that differences between the several strains are relatively minor and in reality reflect slightly different rates of development, rather than any differences in the kind of growth; there is probably no justification at the present time, for their further separation into separate species upon the basis of growth characters.

It is entirely possible that these strains differed from the type strain Proactinomyces asteroides (Eppinger) Gasperini when they were first isolated, but that cultivation on artificial media has caused the loss of these differentiating characters, which indeed has been shown to be the case with "Act. gypsoides" (Henrici, 1930). At present, however, they may be considered as but
# TABLE 2

Cultural characters of partially acid-fast β proactinomyces

<table>
<thead>
<tr>
<th>NUMBER</th>
<th>DESCRIPTION</th>
<th>SYNTHETIC AGAR§</th>
<th>NUTRIENT AGAR§</th>
<th>STARCH</th>
<th>GELATIN</th>
<th>MILK†</th>
<th>PARAPFVIN GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth</td>
<td>Color*</td>
<td>Aerial mycelium</td>
<td>Acid-fast</td>
<td>Fragmentation of mycelium</td>
<td>Amount of growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>20</td>
<td>5</td>
<td>20</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>3308A</td>
<td>A. asteroides</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>O-R</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>3013</td>
<td>No. 3 Henrici</td>
<td>±</td>
<td>2</td>
<td>O-Y</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>3306</td>
<td>A. asteroides Bering</td>
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<td>2</td>
<td>-O</td>
<td>O-R</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>401</td>
<td>A. forcinicus</td>
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<td>2</td>
<td>O-R</td>
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<td>±</td>
<td>+</td>
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<tr>
<td>558</td>
<td>A. gypsodes</td>
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<td>2</td>
<td>O</td>
<td>++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>559</td>
<td>A. asteroides</td>
<td>1</td>
<td>2</td>
<td>O</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>568</td>
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<td>2</td>
<td>O-R</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>568A</td>
<td>Unidentified</td>
<td>1</td>
<td>2</td>
<td>O-R</td>
<td>±</td>
<td>±</td>
<td>+</td>
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<tr>
<td>568B</td>
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<td>1</td>
<td>2</td>
<td>O-W</td>
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<td>+</td>
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<tr>
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<td>O</td>
<td>±</td>
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<td>+</td>
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<tr>
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<td>2</td>
<td>O</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>O</td>
<td>±</td>
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<td>+</td>
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<tr>
<td>649</td>
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<td>1</td>
<td>2</td>
<td>O</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>650</td>
<td>Unidentified</td>
<td>1</td>
<td>2</td>
<td>O</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3322B</td>
<td>Non-acid fast YPC</td>
<td>3</td>
<td>4</td>
<td>O</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* O, orange; W, white; R, red; Y, yellow.
† Slight production of a soluble yellow pigment diffusing into media.
‡ Observations at 30 days. Slow clearing after 4 weeks. Milk remains fluid.
§ Waksman (1919).
minor variants of the well-known type *Proactinomyces asteroides*, and without doubt, should be so designated.

Jensen (1932) has proposed the delineation of four other species within this group of organisms which are defined as follows:

Initial mycelium well-developed:

1. Vegetative mycelium, soft, little aerial mycelium:
   a. Red colored (yellow or white variants), *P. polychromogenus*.
   b. White to pale pink colored *P. opacus*, *P. erythropolis*.

2. Vegetative mycelium, hard:
   a. Yellow colored *P. paraffinae*.

The descriptions given by Jensen would lead one to consider *P. polychromogenus*, *P. opacus*, *P. erythropolis* as members of the α type. Examination of "*A. erythropolis*” (American Type Culture Collection no. 4277) confirms this suspicion. However, the cultures of *P. polychromogenus* from the Lister Institute and another kindly supplied by Dr. Jensen were found to be of the β mycelium type, and closely resembled the types studied here. All of which illustrates the confusion inherent in descriptions which employ "stable" or "unstable" mycelium or "hard" and "soft" mycelium as terms upon which separation is to depend.

The forms examined closely resemble *P. polychromogenus* (filamentous phase) and might be readily classed there. However, in view of the apparent instability of *P. polychromogenus* and the relative stability of the *P. asteroides* types here studied and in view of the more or less rare variations reported on the same types by Wright (1937), it is proposed to consider them as separate entities. *P. asteroides* is pathogenic while the pathogenicity of *P. polychromogenus* is unknown.

A readily applicable modification of the Jensen system would appear to be as follows:

Partially acid-fast types, non-proteolytic, non-diastatic, constantly utilize paraffin:

1. α mycelium types: *P. opacus*, *P. erythropolis*.

2. β mycelium types:
   a. Red colored *P. polychromogenus*, *P. asteroides*;
   b. Yellow colored *P. paraffinae*. 
STUDIES ON THE PROACTINOMYCES

TYPE II. NON ACID-FAST β PROACTINOMYCES

The non acid-fast forms, possessing diastatic and proteolytic properties and unable to use paraffin as the sole source of carbon were represented in this study by 10 strains. The origin of the strains is as follows:

3072. Unidentified (N. J. Collection).
3377. *A. salmonicolor* Millard and Burr.
3308B. "*A. asteroides,*" obtained from Dr. Broadhurst in 1936.
3322B. "*A. fradii,*" obtained from Bucherer, Holland, in 1935.
3382, 3383, 3393, 3395, 3396. (*P. flavescens.*)

The organisms here studied do not constitute an entirely homogeneous group and on casual examination would be considered as distinct and separate species. However, if the course of development is watched closely, it will be noted that the differences between them are more a matter of rate of development than of any qualitative differences between the strains. A case in point concerns the strains 3072, 3376, and 3382. On starch agar all three were identical and remained throughout their growth deep flesh-colored orange. On Czapek's synthetic medium they were identical in appearance for about five days, at which time 3376 began to show traces of black pigment until at 20 to 25 days the growth was almost entirely black except for a narrow margin of the original orange. Throughout its growth, 3072 remained orange, while 3382 showed an irregular black pigmentation (appearing among some of the replicates but not among others), which began at about the 7th to 8th day and was not complete for 30 to 40 days. Even such differences as those noted here were not constant since on continued repetition of the experiment it was noted that the black pigmentation was not a character of the strain, but seemed to be related to some other cause. For example, subcultures from the black form on the same media showed no trace of the black pigment for at least 40 days but retained its original characteristic orange; this was true of 3382, which indeed very rarely showed black pigmentation.

*Cultures 3382 to 3396 were isolated from Zuidersee soils and sent to Dr. Waksman for identification by Dr. Westerdijk.*
These variations in pigment production are not explainable at present, but they serve to confuse any system of classification unless they are specifically noted. Further, the actual shade of the orange pigment may vary with the different strains, but the general type of pigment was easily recognized.

In table 3, the characters of the strains examined are summarized; the same remarks apply as indicated for table 2. Jensen divided the group as follows:

Non-acid-fast organisms, constantly diastatic:
1. Non-proteolytic, no aerial mycelium: *P. mesentericus*;
2. Proteolytic:
   a. Rapid growers, broth turbid *P. actinomorphus*;
   b. Slower growers, broth clear *P. flaveescens*.

The same remarks apply to this separation as to the similar one for acid-fast forms and a suitable modification appears to be the following:

Non-acid-fast forms, constantly diastatic:
1. α mycelium types;
   a. Non-proteolytic *P. mesentericus*,
   b. Proteolytic *P. actinomorphus*,
2. β mycelium types:
   a. Yellow colored *P. flaveescens*,
   b. Red to orange (tendency to blacken) *P. maculatus*, n. sp.

The strains here studied are included under the term *Proactinomyces maculatus* since the original *Actinomyces maculatus* strain of Millard and Burr (1926) is typical of the group. These strains are more closely related to *Actinomyces salmonicolor* M & B, but the name *Proactinomyces salmonicolor* has been preempted. *Actinomyces maculatus* M & B is described as facultatively anaerobic, though the original strain of that organism does not now possess that character, and, in fact, is indistinguishable from *Actinomyces salmonicolor*. The description of the new representative of the species is as follows:

Filamentous organisms possessing a tough shiny colony which is cartilaginous, rarely producing aerial mycelium though in certain
### TABLE 3
Cultural characters of non-acid-fast β proactinomyces

<table>
<thead>
<tr>
<th>NUMBER</th>
<th>DESCRIPTION</th>
<th>SYNTHETIC AGAR†</th>
<th>NUTRIENT AGAR</th>
<th>STARCH</th>
<th>GELATIN</th>
<th>MILK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth</td>
<td>Color</td>
<td>Aerial mycelium</td>
<td>Acid-fast</td>
<td>Fragmentation of mycelium</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3072</td>
<td>Unidentified</td>
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<td>4</td>
<td>O-B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3376</td>
<td>A. maculatus M and B</td>
<td>2</td>
<td>4</td>
<td>O-B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3377</td>
<td>A. salmonicolor M and B</td>
<td>3</td>
<td>4</td>
<td>O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3383B</td>
<td>A. aerogenes</td>
<td>±</td>
<td>±</td>
<td>O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3322B</td>
<td>A. francis</td>
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<td>4</td>
<td>O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>4</td>
<td>O</td>
<td>-</td>
<td>-</td>
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<tr>
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<tr>
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<td>3</td>
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<td>O-B</td>
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<tr>
<td>3396</td>
<td>P. afosencus</td>
<td>2</td>
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<td>Y</td>
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<tr>
<td>3306A</td>
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<td>1</td>
<td>2</td>
<td>O-R</td>
<td>±</td>
<td>+</td>
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</table>

* O, orange; R, red; B, black; W, white; Y, yellow.
† Waksman (1910).
strains it may occur frequently. In the young colony an orange (varying from orange-yellow to orange-red) intercellular pigment is produced on all media, which may or may not change to black as the culture ages. The organisms hydrolyze starch and gelatin, do not utilize paraffin, do not digest milk, are non-acid-fast, and retain their mycelium form for long periods.

This species is designated as *Proactinomyces maculatus* (Millard and Burr) Nov. Comb. Umbreit.

Because of the variations in rates of growth and in pigment production, which has been noted earlier, further studies with representatives of the group were undertaken in an attempt to establish the factors responsible. While the attempts were unsuccessful, some of the information secured is of interest. Traces of certain metals, namely Cu, Fe, Mn, B, Mo and Va, were added to the basic medium (Czapek's sucrose-nitrate medium), in amounts equivalent to 1 mgm. of the metal per liter. The general technique in this and other growth experiments was to inoculate (usually in triplicate) a liquid medium with a suspension of the organism. After incubation (usually 2 to 3 weeks), the growth was weighed by filtering through weighed filter paper, drying at 80°C. for 24 to 48 hours and noting the increase in weight. In the case of the metals noted above the growth was never greater than that of the untreated control. Various types of extracts from manure, plants, fresh *Rhizopus* mycelium, cells of actinomycetes, germinated wheat, etc., sterilized either by heat or by filtration gave no increases in yield of cells and no indications of increasing the growth rate. The C/N ratio of the medium was varied without influencing the growth until limiting amounts of either nitrate or sucrose were reached. Since it was noted that growth in general seemed better on agar media than on the corresponding liquid media and since additions of small amounts of agar had no effect, it was thought possible that the surface available might be a factor in growth. Several experiments were conducted using glass beads, washed sand, asbestos and glass slides to provide surface. While occasionally an increase in growth was obtained by these measures, the results were inconsistent and seemed to point to surface as operating
indirectly through perhaps the O/R potential. Additions of reducing agents inhibited growth (cysteine and reduced iron were used) but in liquid media additions of potassium permanganate (final concentration M/100, M/1000) increased the rate of growth slightly. Aeration of the media with air inhibited growth, so that it was considered that free oxygen, high O/R potential, and surface (at least in an undisturbed medium) were necessary. Shake cultures using glucose or starch as an energy source with additions of oxidizing agents (KMnO₄, M/100, M/1000, M/10,000 final concentration; K₃Fe(CN)₆ 1/100, 1/1000; Fe citrate 1/10, 1/100, 1/1000) yielded growth only at the surface, and the actual amount of growth did not differ significantly from the controls. These results emphasize the necessity of free oxygen.

Various nitrogen sources were also studied. The compounds listed below were added in amounts to give 0.3 gram N/liter. NaNO₃ was the best source of nitrogen, followed by glutamic acid, egg albumin, zein, urea, leucine, dried blood, aspartic acid, alanine, ammonium sulfate, ammonium lactate, tyrosine, phenylalanine, casein, in the order given. No growth was obtained (strain 3382) on acetamide, caffeine, l-cystine, hippuric acid, or keratin and only traces on glycine. In all the experiments reported here the organism maintained its original orange pigment so that no information was gained as to the cause of blackening. The general course of development of the strains in this group may be briefly described as follows:

On Czapek's agar the strains show pigmentation, in from 2-5 days, on other media usually within 3 days; the pigment is usually flesh color with no trace of black. The mycelium remains without appreciable fragmentation for 20 to 30 days. Aerial mycelium is seldom present, and if present is likely to appear within the first ten days. For some unknown reason, occasionally a sub-culture will grow very slowly while another will grow rapidly. This could not be related to any factor except the amount of inoculum, which is difficult to control.

For groups of organisms where such inconsistent differences appear, it is necessary to study the entire course of development over a period of time, to include several replicates, and to repeat
the studies periodically. Culture 3396 seems to resemble *Pro-
actinomyces flavescens* though physiologically it is indistinguish-
able from the others.

**SUMMARY AND CONCLUSIONS**

An examination of several well-defined species and many un-
identified organisms belonging to *Proactinomyces* of Jensen has
shown that:

1. The criteria for separation of the group from *Actinomyces*
   proper (the absence of spore formation) is a valid one.

2. Because of the difficulty of determining and defining mycel-
   ium formation, the lower limits of the group are not adequately
distinguishable from the closely related *Corynebacterium* or
   *Mycobacterium*.

3. Within the *Proactinomyces* two groups are distinguishable.
   These have been separated as follows: (a) *α Proactinomyces*,
   characterized by short unstable mycelium, "soft" bacteria-like
   colony, diffused bacterial growth in liquid media. (b) *β Pro-
   actinomyces*, characterized by long stable mycelium, "hard"
   *Actinomyces*-like colony, typical *Actinomyces* (colony) growth
   in liquid media. The validity of the diagnostic features is
discussed in detail in the text.

4. The "stable" mycelium type (β) was studied more closely
   and it is shown that a further separation on the basis of partial
   acid-fastness is possible. Slight modifications of the present
   separations appear desirable and a further description and emen-
   dation of the species *Actinomyces maculatus* Millard & Burr is
given. A transfer of this species to the genus *Proactinomyces*
is proposed; e.g. *Proactinomyces maculatus* (Millard & Burr)
Nov. Comb. Umbreit.

The author takes this opportunity of expressing his indebted-
ness to the several workers who aided materially in the work
through contributions of cultures or criticisms of the manuscript,
and especially to Professor S. A. Waksman without whose con-
tinual guidance little could have been accomplished.
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