MICROBIAL TRANSFORMATIONS OF THE TOBACCO ALKALOIDS

I. Cultural and Morphological Characteristics of a Nicotinophile

PETER L. SGUROS

Biochemical Division of the Research Department, R. J. Reynolds Tobacco Company, Winston-Salem, North Carolina

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Only a few articles in the past ten years have attempted to define the relationships between autochthonous microflora and the alkaloid products of the tobacco plant. Almost from the moment of the germination of the tobacco seed the synthesis of nicotine begins in the root in significant quantities (Dawson, 1948). At maturity, the extent of total alkaloid production normally reaches 2 to 4 per cent of the dry weight of the plant tissue. Most of this percentage is represented by nicotine or structurally related compounds (figure 1). Although there are certain published conceptions, it is instinctive to rebel at the assumption that such materials are present without adequate reason. Furthermore, the number of known alkaloids in nature indicates that they represent another great category of natural materials which, for certain purposes of classification, may be aligned with such groups as amino acids, carbohydrates, and fatty acids. The significance of these materials in the life of the tobacco plant is unknown. While possibly fortuitous, it is interesting to note that such highly important compounds as pyridoxin and nicotinic acid bear close resemblance to many of the so-called "pyridine" alkaloids. To date, however, the preponderance of questions concerning the metabolic pathways of the tobacco alkaloids in plant and animal tissue remains essentially unanswered. To such relatively new and potentially important fields of knowledge the bacteria may provide the key.

This report describes an initial step in the study of the microorganism-alkaloid relationship: the isolation and characterization of the nicotinophilic bacterium, *Arthrobacter oxydans* (Sguros, 1954).

Conn and Dimmick (1947) described in this journal a new genus of the family *Corynebacteriaceae* which they named *Arthrobacter* (Fischer, 1895). The type species, *A. globiforme*, had been initially described as *Bacterium globiformis* Conn (Conn, 1925, 1928) and further characterized by Conn and Darrow (1930, 1935), Taylor and Lochhead (1937), Lochhead and Taylor (1938), Taylor (1938), Lochhead (1940, 1948), and Lochhead and Chase (1943). In addition to *A. globiforme*, Conn and Dimmick have transferred two species of soil corynebacteria (Jensen, 1934) to the genus *Arthrobacter* as *A. helvolum* and *A. tumescens*. Lochhead and Burton (1953) have added *A. pascens* and *A. terregens* to the genus, and the senior author is presently preparing the genus *Arthrobacter* for inclusion in the 7th edition of *Bergey's Manual* (Breed, 1954, *personal communication*). Detailed nutritional studies on *A. pascens* and *A. terregens* have been reported by Lochhead and Burton (1953) and Burton and Lochhead (1953). Sacks (1954) has recently isolated and characterized *A. citreus*.

Nicotine decomposing bacteria have been described by Weber (1935), Wenusch (1942, 1943), Bucherer (1942, 1943), Frankenburg (1948), Abdel-Ghaffar *et al.* (1952), and Wada and Yamasaki (1953, 1954). Only in the case of Bucherer was a thorough cultural study attempted, but little is presented concerning metabolic implications. The Japanese workers present the rudiments of a possible degradation pathway but do not actually characterize the responsible culture.

EXPERIMENTAL METHODS

Seven strains of *A. oxydans* were isolated from various places in the southeastern section of the United States and studied in parallel (table 1). The isolation medium consisted of nicotine 0.4, monobasic potassium phosphate 0.2, potassium chloride 0.5, and yeast extract (Difco) 0.01 per cent in tap water at pH 6.8. Magnesium and ferrous sulfates (0.0025 per cent) were added routinely. The addition of 1.5 per cent agar did

not detract from the isolating capacity. The medium was regularly sterilized in the autoclave for 20 minutes at 121 C.

Cultural studies were controlled through the parallel use of *A. globiforme*, ATCC 8010, and *A. tumescens*, ATCC 6947. Other comparisons were taken from the literature or personal communication.

Taxonomic characteristics were studied for the most part according to the *Manual of Methods for the Pure Culture Study of Bacteria* (1953). Morphological studies were performed by means of the Leitz Ortholux microscope (1,125 × and 1,425 × for both dark field and stained preparations) and the Leitz phase microscope. In the latter case, a glass cell 2.5 by 5.5 by 0.3 mm was fabricated to afford the indispensable air and moisture to the organisms in a drop of semisolid nutrient agar suspended from a coverslip. This was the only feasible method found for following the growth of a given cell through its life cycle at high magnification. The ordinary light microscope was used to examine the organisms for gram reaction, metachromatic granulation, flagella, spores, fat, acid-fastness, and capsulation. Motility and cell formations were investigated by dark field illumination.

Life cycle observations were made by almost continuous observation while gram staining reactions were studied at hourly intervals for the first 24 hours and every 24 hours following for more than one week. Other staining reactions were observed at 24 hour intervals. Dark field observations were made on cultures sampled as for gram staining above.

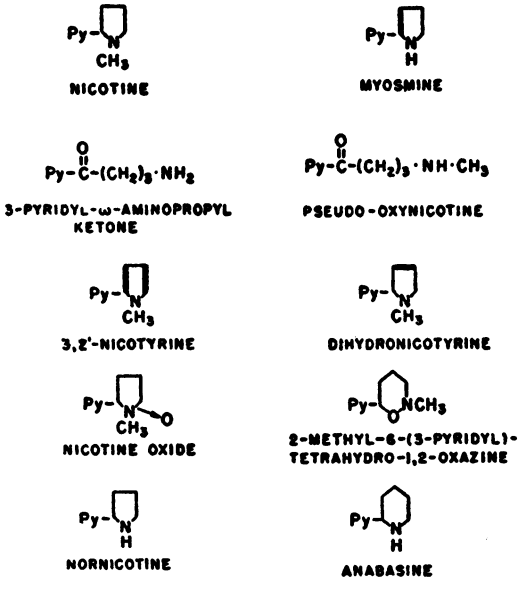

Figure 1. Certain of the known tobacco alkaloids (Py symbolizes the pyridine ring).

TABLE 1

Culture sources

Strain No.	Source	Source Type	Location
1	Air		North Carolina
4	Air		North Carolina
22	Air		North Carolina
117	Tobacco leaf	Flue-cured	North Carolina
122	Tobacco leaf	Flue-cured	South Carolina
127	Tobacco leaf	Flue-cured	North Carolina
131	Tobacco leaf	Burley	Kentucky

Semisolid, nutrient agar (0.7 per cent) was employed for the continuous study of the growing organism. Nutrient and nicotine-salts broths and agars were employed for the growth of organisms destined for staining procedures. Broth smears were flame-fixed while Bouin fixation was employed on plated agar cut-outs (Welshimer and Robinow, 1949).

Growth curve-morphology correlations were made during the growth of *A. oxydans* in shake culture. A washed suspension of resting cystites was used to inoculate 20 ml quantities of liquid media contained in 25 by 250 mm tubes. The tubes were then mounted in special racks on the platform of a rotary shaker and agitated at 270 rpm and 25 C. Both the nicotine isolating medium and nutrient broth were employed in these studies. The tubes were removed from the shaker at intervals and the turbidities determined on a Lumetron photoelectric colorimeter. The nicotine medium required the use of a 420 filter while a 620 filter was employed for the nutrient broth culture. Immediately following each reading, smears were made from the media and gram stained for microscopic observation.

RESULTS

While all strains of *A. oxydans* were very similar in cultural behavior pattern, it seems feasible to divide them into biotypes. Two of the seven strains are chrome yellow on all media tested while the rest are pearl gray or white. The former produce a viscid growth on agar while

the latter retain a butyrous consistency. It is also of interest to note that the yellow cultures grow very scantily upon potato glucose agar for almost a week and then seem to abruptly burst into luxuriant growth. The white cultures do not show this lag. Accordingly, the biotypes are called *A. oxydans* biotype *xanthum* and *A. oxydans* biotype *album*.

Morphology. Old broth and agar cultures, especially in synthetic media, are invariably coccus and coccoid in appearance, the cells varying in diameter from 1 to 3 μ (figure 2). Arrangements vary from single cells and pairs to chains and masses. Within 3 hours, following inoculation into fresh nutrient broth or semisolid agar, germination begins (figure 3). The surface of a given arthrospore or "cystite" (Jensen, 1934) is interrupted by one or two—infrequently three—protrusions which rapidly assume the shape of tapered, tubular appendages 1 to 4 μ in length and 0.5 to 1 μ in width. In many cases, the rod-like structure of the nascent tube is sharply differentiated from the spheroid structure of the cystite. Frequently such differentiations are more subtle and the cystite-tube combination appears simply as a clavate or tapered, uneven-sided rod. The germ tube may be straight or curved although graceful curvature is a more general character. It seems important to note that in this species the germ tubes are *not* commonly in diametric opposition with regard to their points of issuance from the cystite. Rather, these projections commonly appear at an obtuse angle. When the germ tubes are more mature, they appear swollen to the extent that the terminal cystite is no longer a distinct structure. This results in the germ tube-cystite-germ tube combination assuming the shape of a boot or boomerang, a form commonly observed in microscopic preparations. Another distinguishing feature of this species is the strong tendency for the cystites to remain attached in pairs and short chains regardless of the number of germ tubes emerging from them. This results in bizarre arrangements of myceloid nature (figure 4). Branching *per se* is actually not a prominent characteristic. Within 8 to 12 hours, a process of fragmentation of the myceloid appears to begin (figure 5). Initially, this may be detected by the separation of a rod form from the tip of the mature, elongated germ tube. Within 15 hours the myceloids appear to disintegrate into uneven, lesser masses of short rods, coccoids and cocci. Within one to three weeks the culture assumes a homogeneous appearance of cocci in single, paired, chain, or grape-like clusters. The whole cycle is schematically represented in figure 6. It will be noted that fragmentation of the myceloid may proceed to completion without a separation of the components by significant distances; hence, the appearance of chains and clusters. As has been mentioned above, the tenacious nature of this post-fragmentation association assumes determinative importance. The picture is essentially the same on 1.5 per cent nutrient agar although the myceloids are less exaggerated. Nicotine agar and broth media appear to reduce the complexity of the myceloids causing a more subtle transition through the cycle. An important characteristic of this species (Conn and Dimmick, 1947) is the gram reaction observed during the life cycle period. Cystites are gram negative although they usually contain a gram positive granule. The granules appear violet or reddish purple with alkaline methylene blue stain. During germination these granules may increase enormously in size almost filling their cystites. The emerging germ tubes are very weakly gram negative at first but rapidly increase in counterstain affinity. Soon hereafter the very tips of the germ tubes develop their own, smaller, gram positive granule. The number of granules of the elongating germ tube appear to increase until fragmentation into cystites occurs leaving each with its own granule. The cycle is completed. In most cases, not disallowing procedure-induced artifact, the granule appears at the surface region of the mature, *resting* cystite as a dot or an oval, deeply stained body. During intermediate stages, rod forms appear to have the gram positive material at the surface region although it may be greatly stretched longitudinally. Seldom are cells observed which are totally gram positive, and even in such cases the intensity is very delicate. It appears doubtful that more than a single granule is normally present in each, nondividing cell (Clark and Mitchell, 1942) or that the large parental granule of the arthrospore is functionally different from the minute granule of the germ tube end. It is logical, due to the greater age of the distal portion of the germ tube, that this region should be the first to synthesize the new granule. The cystite granule is undoubtedly that described by Conn and Dimmick (1947) as occurring at the

"nodes" of the "branches". When a cystite germinates in two or three directions, the original granule remains at the apices. Finally, the granule may, within the *germinating* cystite, assume the *almost* exact shape and size of the cell; or, filling half of it, appear ovoid or fusiform; or remain as a small globule; or be seen as an elliptical form. In this species, the cystite is usually not filled entirely. It is distinctive that the granule favors only one edge of the cell, ordinarily being coincident with it. This follows, of course, from the above described granulation of the *resting* cystite. It is obvious that cystite germination and the size of the granule are related in a manner probably more than superficial. No attempt will be made at this time to discuss the nature of this relationship or the detailed sequence of the development of the granule. Clark and Mitchell (1942) have determined this granule in *A. globiforme* to be volutin.

No "tails" were observed as have been reported by Clark and Mitchell (1942) as well as by Taylor and Lochhead (1937). In this connection it is interesting to note that the "tailing sequence", as described in this early work, may be taken as a corroboration of the writer's observations if the "sequence" is merely reversed. It is assumed, of course, that the "tail" was actually a germ-tube. In this event the sequence could be developed as follows: (a) coccal cystite, (b) emergence of a faintly staining, young germ-tube, (c) mature germ-tube, swelling and with strong affinity for stain, (d) uneven-sided "rod form" with arthrospore structure no longer distinguished from the enlarged germ-tube (figures 4 and 6). Neither true motility nor flagella were ever observed. While this is contrary to the recent observations of Sacks (1953, 1954), it conforms well with descriptions of all other valid species known to this author. Negative results were also obtained in tests for capsules, spores, and acid-fastness. Fat stains gave positive results. Metachromatic granules were observed.

Cultural characteristics. Nutrient agar plate colonies may be punctiform or circular. They are smooth, entire or slightly undulate, convex, glistening, viscid, and opaque. One variety typically exhibits a bright yellow, insoluble pigment while the other is pearl gray or white.

Gelatin plate colonies are punctiform, convex, smooth, entire, opaque, and slowly liquefying.

Agar stroke growth is luxuriant, filiform, glistening, viscid, and yellow. Achromogenic strains are butyrous and pearl gray. A glassy, metallic sheen is acquired with age.

Nutrient broth growth typically exhibits a surface ring, strong turbidity, and viscid sediment. Achromogenic strains are slower in producing viscidity.

Acid production in *fermentation tests* is consistent among these strains only in fructose and sucrose. Acid in glucose is weak and transient. No gas is observed in any case. All of the conventionally employed phenol red broths containing mono-, di-, and polysaccharides as well as sugar alcohols and glucosides permit good growth and turn basic.

Gelatin stabs exhibit crateriform liquefaction which begins in 2 days and slowly reaches 50 per cent to 75 per cent completion without progressing significantly further.

Growth on nicotine-salts-yeast extract plates and slants is somewhat more abundant than described above but otherwise quite identical. In these cases, however, a deep blue, diffusible pigmentation characteristically augments the yellow or pearl white, nondiffusible colors. In time, the blue pigment turns reddish or yellow-brown. This blue material is formed during the oxidation of nicotine and is practically insoluble in all of the common immiscible solvents with the exception of benzyl alcohol. It is both pH and redox potential sensitive. In shaken broth cultures the color change to brown is quite gradual, but if these cultures are removed from the shaker in the "blue stage", the organisms reduce their own product to a yellow-brown within a few hours. Shaking again restores the blue color.

No growth can occur in the absence of oxygen on Brewer's medium in a hydrogen atmosphere. If this deprivation is allowed to continue for a few days, the organisms appear irreparably damaged. None but surface growth has ever been observed in stab cultures of various media.

Litmus milk turns basic with reduction of the litmus. This is usually followed by slow clearing.

Nitrates are strongly reduced.

Hydrogen sulfide is not produced.

Indole is not produced. *Methyl red* and *Voges-Proskauer* tests are negative, but *citrate* supports growth.

Catalase reaction is strong.

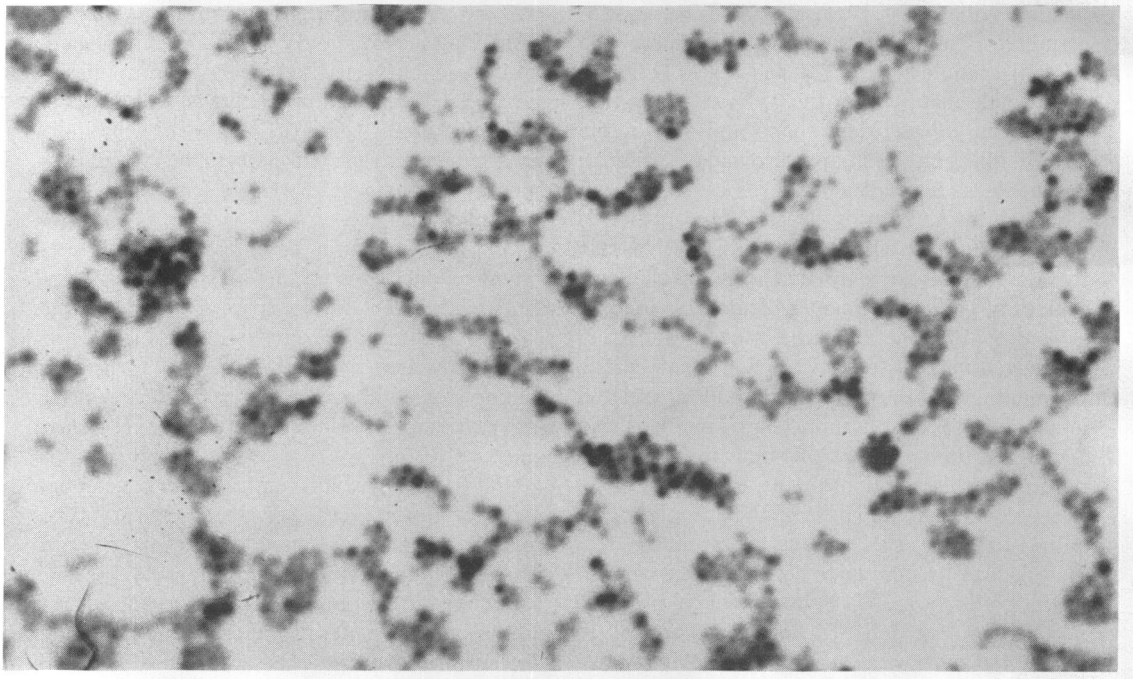

Figure 2. Arthrobacter oxydans in dormant, cystite phase. Old nicotine agar culture gram stained. Ca 1,500 ×.

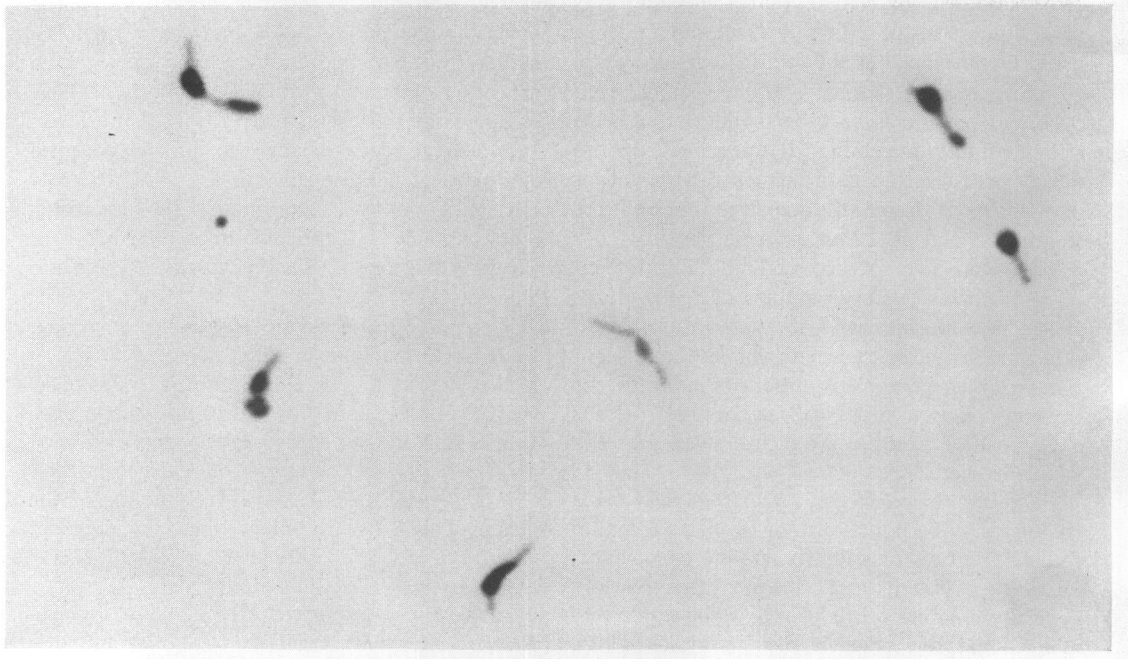

Figure 3. Arthrobacter oxydans germinating from cystites. Six hour nutrient agar culture gram stained. Ca 3,200 ×.

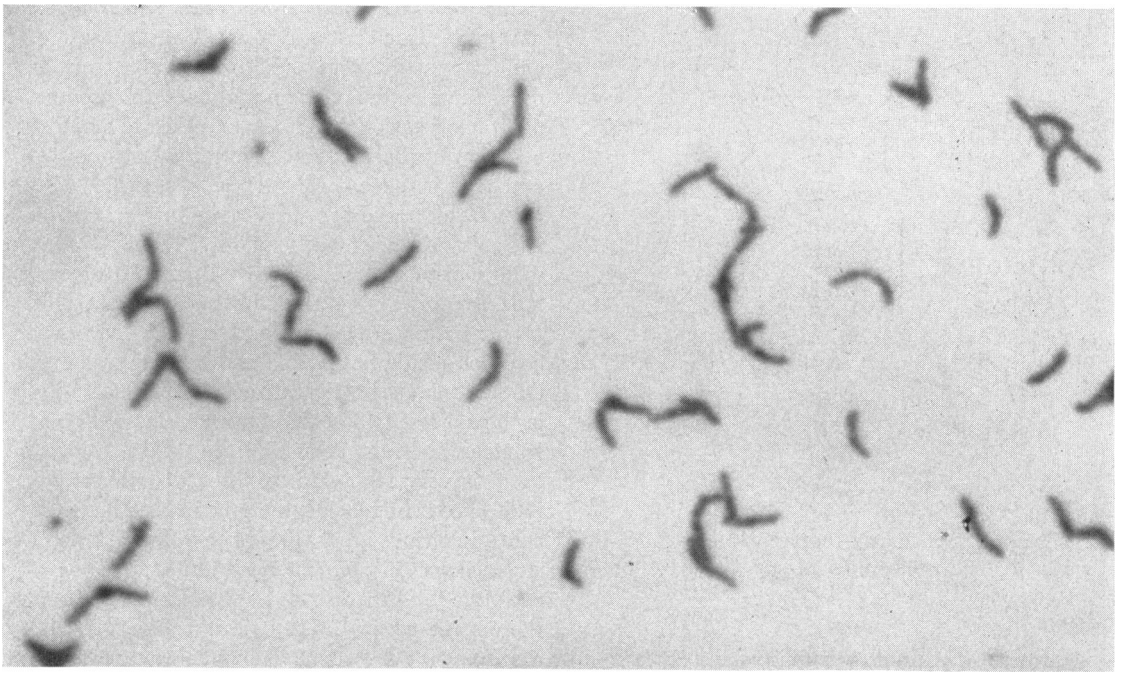

Figure 4. Arthrobacter oxydans in myceloid stage. Ten hour nutrient agar culture gram stained. Ca 1,500 ×.

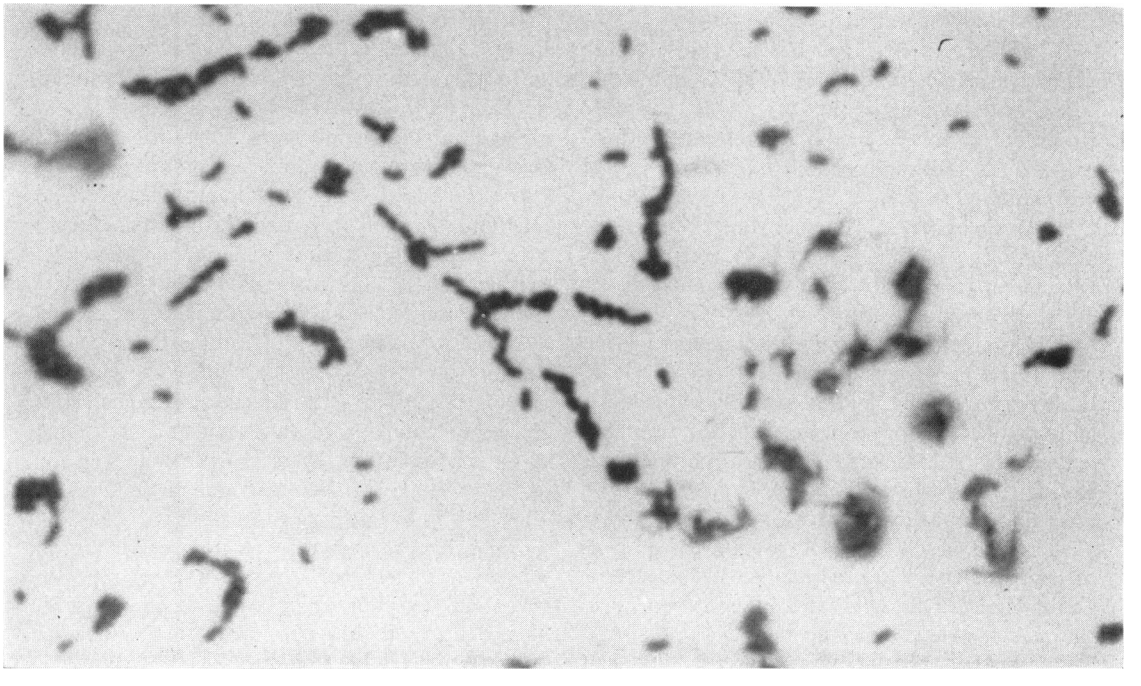

Figure 5. Arthrobacter oxydans in early fragmenting stage. Nineteen hour nutrient agar culture gram stained. Ca 1,500 ×.

Figure 6. Schematic representation of the life cycle as observed in a single field by phase microscopy. Organism in semisolid agar.

Growth does not occur in the presence of phenol or cresol but is fair in the presence of naphthalene and good on ethanol agar. Ammonium salts and amino acids can serve as nitrogen sources.

Starch is hydrolyzed.

Potato glucose agar. Growth is luxurious, but the yellow variety exhibits a lag of about one week.

In nutrient media, growth is favored by pH 7 while in nicotine-salts media pH 6 gives an optimal response. In either case, 25 C is the optimal growth temperature.

The sources (table 1) are air and tobacco leaf although the habitat is probably soil. The organisms appear hardy, and their characteristics are stable in stock culture storage.

It is interesting to note that upon one occasion after plating a suspension from a "high alkaloid" burley sample on nicotine-salts agar media, a large population appeared that was almost a pure culture of *A. oxydans* in both chromogenic varieties. The white variety predominated by a large margin in this instance.

The control cultures, *A. globiforme* and *A. tumescens*, behaved in a manner which left no doubt that *A. oxydans* is a different species of the genus. While morphologies and gram stain characteristics are similar, there is sufficient difference to warrant a separate speciation. This is most apparent in *A. tumescens* which is of smaller cell size in most respects, rendering its life cycle transformations much more subtle. Culturally, this organism grows very scantily upon the nicotine medium and does not produce any pigmentation. While its growth rate on most common media is much slower than that of *A. oxydans*, it nevertheless liquefies gelatin with far greater rapidity. Growth in broth is weak without surface phenomena. *A. globiforme*, while morphologically more similar to *A. oxydans*, fails to grow upon the nicotine medium. The type species grows moderately well on most common media but with a cream chromogenesis. Of the three species, its rate of gelatin liquefaction is the slowest. No acid is observed in sucrose or fructose. Nitrate reduction, in this strain at least, is very restricted or absent. Starch is not hydrolyzed. In general, the growth rates of these control cultures are less rapid than in the case of *A. oxydans*. In spite of these differences, however, the total pattern of observations shows that these three species are generically related. A final case in point is that of *A. citreus* (Sacks, 1954). The morphology of this organism, in so far as the publication reveals it, shows it to be very similar to *A. oxydans*. Routine taxonomic tests in the writer's laboratory demonstrate that, unlike *oxydans*, *citreus* does not hydrolyze starch, change litmus milk, enjoy the same pH optima, produce surface growth in liquid media, grow well on potato, potato-glucose, nicotine, nicotine-glucose, glycerol, or asparagine agars. In addition, *citreus* is claimed to have a motile stage.

Growth morphology correlations. The nicotine medium served to provide the best correlating experiment from a time standpoint (figure 7). Within 33 hours, the entire life cycle is completed homogeneously. From the figure it would appear that the greatest period of cell synthesis occurs during the myceloid stage. Since the method employed measures "total cell substance" and since the shaking process augurs against extensive myceloid formation, there is little tendency for sudden changes in the curve due to fragmentation. It is noteworthy that during the lag period, germination proceeds through its initial stages in a manner detectable only by microscopic observation. On the other hand, nutrient broth shake cultures require more than 300 hours before the cystite stage becomes prominent. Even then, homogeneous cultural morphology is lacking. Fragmentation proceeds very slowly. The lag

period is demonstrably shorter than in the case of the nicotine medium, but the log phase maintains the same rate. Static nutrient broth cultures show the same short lag period, but the log phase rate is much reduced; maximum transmission is reached only after some 200 hours (shaken nutrient broth 32 hours; shaken nicotine broth 48 hours). However, at 300 hours a homogeneous culture of cystites is obtained. Static growth allows more extensive myceloid formation; hence, the curve is irregular with a sudden, short rise at the point where the myceloids begin to fragment. The total fragmentation phase, however, requires many hours for completion.

In the various growth cycle studies which have been partly depicted in figures 6 and 7, homogeneous cystite formation has been obtained primarily in three situations: (1) during growth in the semisynthetic nicotine medium, (2) during growth in the static nutrient medium, and (3) during growth in the phase microscopy chamber. It will be noted that in the first instance, the organism was forced to rely heavily upon its synthesizing mechanisms; in the second case, growth proceeded under conditions of probably less than adequate aeration (unpublished results); in the third circumstance, the chamber being a closed system, inadequate aeration and an accumulation of metabolic gas were probably both operative. While the significance of cystite formation is not known, it has been implied, by the use of the synonym "arthrospore", to be a more resistant phase in the life of the organism. Regarding the aforementioned observations speculatively, it seems not remiss to suggest that the above implication may have some justification. Unfortunately, the results of direct experimentation on this important point are lacking at present.

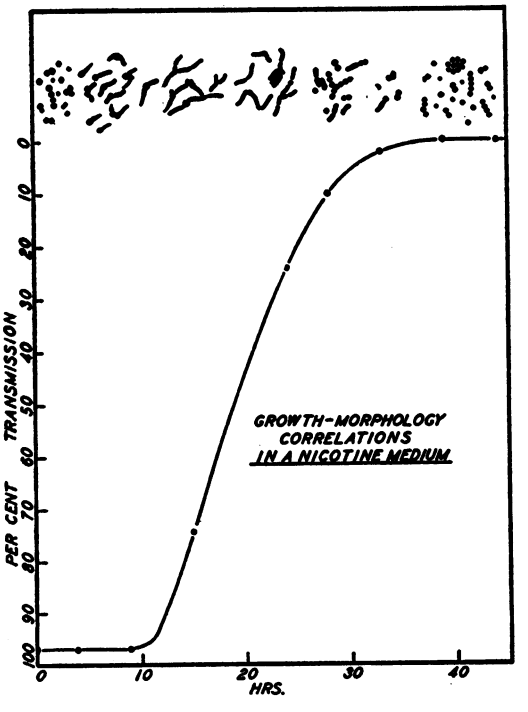

Figure 7. Correlation of morphological progression with turbidimetric growth expression for *Arthrobacter oxydans* in nicotine shake culture.

DISCUSSION

Growth on nicotine would not appear to be a common characteristic among microorganisms. Many bacteria are inhibited by it even in the presence of acceptable substrates. In the author's laboratory it has been strikingly apparent that few, if any, soil or plant molds will grow even suboptimally on nicotine as the source of carbon and nitrogen—strange behavior indeed for this notoriously omnivorous group. On the other hand, there seems to be a far greater tendency for microorganisms, generally, to grow where glucose is the carbon source and nicotine the source of nitrogen. It is not, therefore, possible to speculate that microorganisms have difficulty in opening the ring structure to obtain carbon since, in order to obtain nitrogen, the ring rupture must undoubtedly be accomplished anyway (figure 1). In any event, it is only possible to hypothesize that some of those organisms reported thus far as oxidizing nicotine may fall into a taxonomically feasible group within the genus *Arthrobacter*.

The tenuous nature of the above statement reflects the inadequacy of earlier reports which incompletely describe the cultures involved. Bucherer (1942, 1943) succeeded in describing four species of nicotine decomposing bacteria which he named *Bacterium nicotinobacter*, *B. nicotinophagum*, *B. nicotinovorum*, and *B. nicotianum*. While all species grew on nicotine with the production of a blue pigment, only *B. nicotinovorum* appears to resemble *A. oxydans*. However, while ordinarily complete descriptions were given in all cases, no attempt was made to determine a life cycle in the case of *B. nicotinovorum*. The only morphological clue given is that the culture was

composed of straight and curved rods as well as oval and round forms. It would seem that where a life cycle exists, other cultural characteristics decrease in importance from a determinative standpoint overshadowed as they are by morphological criteria. Bucherer (1942) unqualifiedly states that *B. nicotinovorum* is gram positive and that it grows as well aerobically as anaerobically. The last feature especially serves to differentiate this organism from *A. oxydans*. Whether there are morphological similarities must be left to conjecture. Another organism, designated unofficially as *Corynebacterium nicotinovorum*, has also been described (Abdel-Ghaffar *et al.*, 1952). It was reported as a gram positive, "coccoid bacillus" which was capable of "destroying" nicotine with the production of a blue pigment. It was further characterized as citrate positive, nitrate reducing, and unable to ferment the common sugars. From the brief morphological description, it is very doubtful that the culture is related to *A. oxydans*. Finally, a soil organism, designated as probably belonging to *Pseudomonas* (Wada and Yamasaki, 1953), has been reported to oxidize nicotine with blue pigment production. No cultural description is given. While the taxonomic significance of the production of a blue pigment from nicotine is as yet unknown, it is evident that this is primarily a characteristic of species importance at the present time. Cultures of other *Arthrobacter* and of *Cellulomonas*, *Mycobacterium*, and *Corynebacterium* have failed even to grow in the presence of nicotine in the author's laboratory (unpublished results). Lochhead (1954, *personal communication*) has stated that *A. terregens*, *A. pascens*, and *A. helvolum* grew poorly on the nicotine, isolating medium. No chromogenesis was observed. In addition to these facts, the results of nutritional studies on *A. oxydans* (to be published) provide further areas of difference between the nicotinophile and these *Arthrobacter*. Lesser cultural differences are also prevalent.

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SUMMARY

Seven strains of a microorganism isolated from tobacco leaves as well as from the air of tobacco environs and recently named *Arthrobacter oxydans* have been fully described taxonomically. Two biotypes, recognized as yellow-viscid and pearl-white-butyrous, have been called *A. oxydans* biotype *xanthum* and *A. oxydans* biotype *album*, respectively. The organisms proceed through a life cycle which involves germination from spherical arthrospores or cystites, myceloid formation due to the multiple germination of cystite groups, fragmentation of the myceloids into rods and cocci, and the return of the homogeneous coccoid appearance. The cyclic progression is accompanied by the development and transformation of a gram positive granule in each cell. The organism is gram negative, obligately aerobic, nonmotile, metachromatically granulated, nitrate reducing; indole, methyl red, Voges-Proskauer, and hydrogen sulfide negative but citrate positive; hydrolytic on starch, casein, and gelatin; nonfermentative; catalase positive. Nicotine is vigorously utilized as a sole C and N source with the production of a diffusible, blue pigment. Turbidimetric expressions of growth in various media have been correlated with cultural metamorphosis.

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