MICROBIAL DEGRADATION OF NICOTINE

I. MORPHOLOGY AND PHYSIOLOGY OF Achromobacter nicotinophagum N. SP.

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The destruction of nicotine by microorganisms has been investigated since Batham (1927) observed an increase in the nitrate content of soil to which nicotine had been added. He assumed that this increase was due to the conversion of the alkaloid to nitrate by soil bacteria. Faitelowitz (1927) showed that the nicotine content of nonsterile tobacco extracts decreased on standing, and Weber (1935) obtained a similar effect in bacteriological media free of tobacco and tobacco extracts. The quantitative destruction of nicotine in a synthetic medium by individual species of bacteria was studied by Bucherer and Enders (1942). They isolated three organisms in pure culture and named them Bacterium nicotinophagum, Bacterium nicotinovorum, and Bacterium nicotinobacter. Wada and Yamasaki (1953) reported destruction of nicotine by a microbe said to be a Pseudomonad. Further characterization of this organism and a number of others was performed by Tabuchi (1954), who classified them in the genera Pseudomonas, Xanthomonas, Achromobacter, Bacillus, and Bacterium. Abdel-Ghaffar (1953) isolated several strains of nicotinophiles belonging to the Corynebacteriaceae from cigar tobacco. Sguros (1955) also obtained a number of isolates which he placed in the genus Arthrobacter (Conn and Dimnick, 1947).

This paper describes several organisms which have been isolated in pure culture from tobacco seeds and from soil in which tobacco had grown. Three of these organisms resemble those previously described in the literature, but two others are sufficiently different to warrant a detailed description.

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MATERIALS AND METHODS

The medium used for the isolation of the bacteria consisted of one part tobacco seed washings and two parts of water. The seed washings were prepared by mechanically shaking 5 g seed with 100 ml of tap water for 30 min and then filtering the mixture. Nicotine was added to give a final concentration of 0.01 M and the solution was neutralized to pH 6.8 to 7. This medium and the technique for obtaining heterogenous bacterial cultures from tobacco seed washings were adapted from methods used in the General Cigar laboratories (W. G. Frankenburg, personal communication).

For physiological studies, and as a maintenance medium, a solution was made containing KH₂PO₄, 340 mg; K₂HPO₄, 435 mg; MnSO₄·7H₂O, 200 mg; FeSO₄·7H₂O, 10 mg; and MnSO₄·2H₂O, 10 mg per L. Nicotine and other sources of carbon and nitrogen were added to give a concentration of 0.01 M. This medium was similar to those used by Weber (1935) and by Choman (1954). Two of the organisms studied would not grow in this medium unless vitamin B₁₂ was supplied. This factor was therefore routinely added to the solution at a level of 0.2 μg per L. For plating experiments, 1.5 g agar was added to 100 ml of the above solutions. All salts were C.P. grade conforming to the standards of the American Chemical Society. Nicotine was obtained from Distillation Products, Inc., Rochester, New York, and was redistilled before use. Sterilization was performed by autoclaving at 121°C for 20 min.

Continuous cultivation of the isolated organisms for over a year in the nicotine-salts medium has not detectably diminished their metabolic activity.

The Manual of Methods for the Pure Culture Study of Bacteria (1957) was used for determining taxonomic characteristics. Organisms destined
for staining were grown in nicotine-salts medium for the appropriate length of time at 25 C. Smears from broth were air dried or flame fixed before staining, whereas impression smears were fixed with the vapor of formalin-acetic acid-ethanol (1:5:9). For the study of morphological changes during growth, impression smears were made from agar cultures every 3 hr for 36 hr, and every 12 hr thereafter for 5 days. These smears were Gram stained after fixation.

For growth curve experiments 300 ml Erlenmeyer flasks with colorimeter tube side arms were used. One hundred ml of nicotine-salts medium were inoculated with 1 ml of a suspension of 10-day cells having a turbidity reading of 20 in a Klett colorimeter equipped with a 540 mg filter. The flasks were mechanically shaken at room temperature at a rate of 50 to 60 (3 cm) strokes per min. During the summer months the temperature of the room was maintained at, or slightly below, 25 C by an air conditioner. Periodically the turbidity of the solution was determined with the colorimeter. A 1 ml aliquot of the solution was diluted to a known volume and filtered through a fritted glass funnel of fine porosity. The ultraviolet absorption spectrum of the filtrate was determined in 0.01 N NaOH (pH = 11) and in 0.01 N HCl (pH = 1) with a Beckman model DU spectrophotometer. These pH levels were used to facilitate detection of substances with pH sensitive absorption spectra. When the color of the medium interfered with the turbidity determinations, a 5 ml aliquot of medium was removed and the cells isolated by centrifugation. They were resuspended in fresh medium and the turbidity determined as usual.

RESULTS

The sources of the organisms isolated and some of the results of the physiological experiments are shown in table 1. The organisms listed therein are very similar in that they do not produce indole, are all nonmotile, and give negative methyl-red and Voges-Proskauer tests. Negative results were obtained in tests for capsules, spores, and acid-fastness. Hydrogen sulfide is not pro-

| TABLE 1 |
| A comparison of the morphological and physiological properties of five strains of nicotinophilic organisms |

<table>
<thead>
<tr>
<th>Property</th>
<th>Organism</th>
<th>101</th>
<th>102</th>
<th>104-2</th>
<th>104-5</th>
<th>105</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Soil</td>
<td>Seed washings</td>
<td>Seed washings</td>
<td>Seed washings</td>
<td>Seed washings</td>
<td>Rod</td>
</tr>
<tr>
<td>Morphology</td>
<td>Rod</td>
<td>Rod</td>
<td>Cocci and Rod</td>
<td>Cocci and Rod</td>
<td>0.4 by 1–2</td>
<td></td>
</tr>
<tr>
<td>Size in microns</td>
<td>0.4 by 0.8</td>
<td>0.4 by 0.8</td>
<td>0.7 diam &amp; 0.5</td>
<td>0.8 diam &amp; 0.5</td>
<td>0.4 by 0.8</td>
<td></td>
</tr>
<tr>
<td>Gram reaction</td>
<td>Negative</td>
<td>Negative</td>
<td>Variable</td>
<td>Variable</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Amylase production</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Catalase reaction</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt; required</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Color formed on nicotine broth</td>
<td>Pale yellow</td>
<td>Pale yellow</td>
<td>Brown</td>
<td>Red, violet, and brown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid formed from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
duced on appropriate media and all strains are obligately aerobic, at least when grown on nicotine-containing media. The optimum pH is 6.8 to 7 and the optimum temperature is between 20 and 25 C.

Organisms 104-2 and 104-5 are very similar and appear to be identical to Arthrobacter oxydans Sguros (1954). Both strains produce water soluble pigments when grown on nicotine agar but the colors differ. Strain 104-2 produces a brown pigment exclusively, while strain 104-5 produces first a red, then a blue-violet and finally a brown color. These differences have remained constant during more than two years of repeated culture. Unfortunately no strains of Arthrobacter oxydans were available for comparison with those isolated in this work, so a definite identification could not be made.

Strain 105 is a gram-positive rod throughout its life cycle, and produces a stable blue water-diffusible pigment when grown on nicotine-containing medium. This organism is very similar to Bacterium nicotinovorum described by Bucherer.

The changes in ultraviolet absorption spectrum of a nicotine medium caused by the growth of strains 104-2, 104-5, or 105 are shown in figure 1. At first there is a decrease in the absorption at 260 mµ and the formation of peaks at 230 and 290 mµ. After 36 hr growth these peaks have disappeared and from then on there is a gradual bleaching of the absorption indicating destruction of the aromatic nucleus of the nicotine.

Strains 101 and 102 are physiologically and morphologically different from nicotinophiles previously described in the literature. They are gram-negative rods 0.5 by 0.8 to 1 m. In old cultures the length of the rods may be reduced to the point where they are barely longer than they are wide. Three to 6 hr after inoculating the cells into fresh medium elongated forms, 4 to 7 m in length, are found. These apparently fragment, for examination of slightly older cultures reveals only rods of the characteristic size. These rods divide in a snapping fashion so that the daughter cells come to lie side by side. As the culture ages, the rods become shorter until they resemble the initial inoculum. No club-shaped projections or cystites have been observed in cultures of these organisms.

Growth on nicotine-salts media is a little slower than on nutrient media perhaps due to the toxicity of the alkaloid. These two strains differ from the other organisms in that they require vitamin B₁₂ for growth. This requirement appears to be very specific, for it cannot be replaced by other vitamins, amino acids, or nucleotide bases.

The ultraviolet absorption of the acidified culture filtrates of strains 101 and 102 are seen in figure 2. After 26 hr there is a slight decrease in the absorption at 260 mµ and a partially resolved peak at 295 mµ. A broad peak with a maximum at 275 mµ is evident 18 hr later while the intensity of absorption is slightly decreased. The absorption spectrum observed at 72 hr, and later, is very stable and indicates that some of the nicotine has been transformed into a nonmetabolizable product. This product may be crystallized from a 20-fold concentrated culture filtrate by the addition of concentrated HCl to pH 2. Recrystallization from water yields 6-hydroxy-3-succinoylpyridine, m. 288-290, undepressed on admixture of an authentic specimen. The ultraviolet absorption spectrum and molecular extinction coefficient of the isolated material are identical with those of 6-hydroxy-3-succinoylpyridine, (Tabuchi, 1955).

Figure 1. Typical ultraviolet absorption spectra of diluted culture filtrates of pigment producing nicotinophiles growing on nicotine-salts medium. (pH 1).
A more thorough analysis of the changes in the ultraviolet absorption spectrum of nicotine-containing medium inoculated with strains 101 and 102 will be presented in a second paper.

Heretofore no reports of a gram-negative, nonmotile nicotinophile have appeared in the literature. These organisms are particularly interesting since they require vitamin B₁₂ for growth and produce an ultraviolet absorbing material from nicotine in rather high yields.

Small, nonmotile, gram-negative rods with feeble powers of attacking carbohydrates and litmus milk are assigned to the genus Achromobacter. The name Achromobacter nicotinophagum was originally proposed for strains 101 and 102 (nicotino from Chem. noun nicotine and -phagum from Greek phagaein to eat).

Achromobacter nicotinophagum n. sp.

Morphology: Rods, 0.4 to 0.6 by 0.8 to 1 μ with some elongated forms up to 4 to 7 μ in young cultures, occurring singly, in pairs, short chains, and many cells lying side by side. Nonmotile, gram-negative. Obligately aerobic.

Gelatin colonies: Small punctiform, raised, entire, white translucent. Irridescent under incident light. Slow liquefaction.

Agar colonies: Similar to those on gelatin.

Litmus milk: Slightly alkaline with no peptonization or coagulation. Little acid but no gas formed from glucose, xylose, fructose, sucrose, and glycerol. No acid from lactose and mannitol.

Indole is not produced.

Nitrites may or may not be reduced to nitrites. Methyl-red and Voges-Proskauer tests negative.

Nicotine may be utilized as the sole source of carbon and nitrogen.

Vitamin B₁₂ required for growth.

Optimum temperature: 20 to 25 C.

Optimum pH: 6.8 to 7.

Sources: Tobacco seed and soil in which tobacco has grown.

DISCUSSION

The ability to utilize nicotine as the sole source of carbon and nitrogen seems to be shared by relatively few microorganisms. The very toxicity of this substrate inhibits the adaptation of large numbers of bacteria to its use as a metabolite. The association of colored materials, in particular blue-violet water soluble pigments, with nicotine destruction may indicate similar metabolic pathways. Thus, Bacterium nicotinovorum of Bucherer, Corynebacterium nicotinovorum of Abdel-Ghaffar, the pseudomonads of Wada and Yamasaki, Arthrobacter oxydans of Sguros, and this report would all seem to be related physiologically. Bucherer claimed his organism grew equally well aerobiocally and anaerobiocally whereas the blue pigment producing organisms listed above are all strict aerobes. The similarity of products argues more for a common series of pathways for attack on the alkaloid molecule than it does for an identity of organisms. For the present no conclusions can be made on the identity of these bacterial species.

The organisms described in this paper may be divided into two general types according to the products of nicotine degradation. One group contains strains 105, 104-2, and 104-5. These bacteria oxidize nicotine without the production of an appreciable quantity of residual ultraviolet absorbing material but do produce water soluble pigments. A transient product of their action on nicotine has absorption maxima at 230 and 290
A However, one—the production for growth. They is produced in 6-hydroxy-3-succinoylpyridine.

ACKNOWLEDGMENTS

The work presented herein was performed in the Department of Botany, Columbia University, under the direction and supervision of Professor R. F. Dawson. It was supported by a grant from the General Cigar Company to Columbia University. The helpful suggestions of Drs. W. G. Frankenburg, G. Barber, and Helen B. Funk, and Valia Hylin are gratefully acknowledged.

SUMMARY

Five nicotinophilic microorganisms have been isolated in pure culture from tobacco seed and from soil in which tobacco had grown. These bacteria are able to grow naturally with nicotine as their sole source of carbon, nitrogen, and energy. Three of these strains appear similar to previously described organisms but the remaining two, apparently, have not been observed before. These two gram-negative rods are believed to be variants of Achromobacter nicotinophagum n. sp. They are strictly aerobic, nonmotile, non-sporeforming microbes that require vitamin B12 for growth. They degrade the alkaloid without the production of water diffusible pigments. However, one of the products of this degradation, 6-hydroxy-3-succinooxylypyridine, is produced in rather high yields. The physiological and morphological characteristics of the isolated organisms are described.

REFERENCES


Faitelowitz, A. 1927 Bacterial decomposition of tobacco as leading to the formation of bases in the presence of water. Biochem. J. (London), 21, 262-264.


