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STUDIES ON A NEW OXALATE-DECOMPOSING BACTERIUM, PSEUDOMONAS OXALATICUS

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Since Bhat and Barker (1948) reviewed the existing literature and reported on the isolation and characteristics of an oxalate-decomposing *Vibrio*, only two attempts appear to have been made to study the problem of oxalate decomposition by microorganisms. One of the studied reported (Janota, 1950) relates to the utilization of oxalate by *Pseudomonas extorquens* Bassalik, whereas the other by Müller (1950) pertains to the oxalate-decomposing species of *Proactinomyces* isolated from soils, water, and sheep rumina. The purpose of this paper is to present the details of the isolation and the description of another oxalate-decomposing bacterium isolated from the intestinal tract of common Indian earthworms including the *Pheretima* species.

EXPERIMENTAL METHODS AND RESULTS

Method of obtaining the intestinal contents of the earthworms. Live adult worms collected on different occasions and during the course of two years were washed clean of the adhering particles, chloroform killed, and cleansed once again to free them of the chemical as well as the mucoid substance exuded by them. Thereafter they were surface sterilized by treatment for ten minutes with a disinfectant solution recommended by Fred *et al.* (1932) for the isolation of root nodule bacteria. The worms were washed again, this time in sterile water, and the washings were tested for the efficiency of the surface sterilization by the usual sterility tests. The first 14 segments of each worm were snipped off then with sterile scissors, and the remaining portion constituting the intestines was mashed up with sterile scalpel or scissors in tubes containing 15 ml of sterile physiological saline. After allowing the coarser particles to settle, the supernatant fluid was used as the inoculum for the oxalate enrichments.

Isolation of Pseudomonas oxalaticus. One ml of the suspension from each worm was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of Bhat and Barker's medium (1948) modified by the incorporation of 0.1 per cent yeast autolyzate. Then the flasks were incubated aerobically at room temperature (28 to 30 C). As the oxalate was utilized gradually by the bacteria, the pH of the medium rose to 8.4 and the indicator turned rose red. At this stage a loopful of the culture was examined microscopically and was transferred serially to a second, a third, and a fourth enrichment flask. The last enrichment was streaked out on a solid oxalate medium to which two ml of sterile M/10 calcium chloride per 100 ml of the medium were added to facilitate the detection of the oxalate decomposers. Several of those colonies which showed signs of oxalate utilization were "fished" with a straight sterile needle and transferred into tubes of liquid oxalate medium to verify the utilization of the substrate and also for reisolation. The purified cultures were maintained aerobically on ordinary nutrient agar as well as on oxalate agar at 28 C.

During the investigations we have isolated and studied in detail 6 strains of an oxalate-decomposing pseudomonad from the intestines of 6 different earthworms. They were found to be practically identical in their morphological and cultural characteristics. Two strains, however, differed slightly from the other 4 strains in their inability to utilize certain carbon compounds as well as in their being pathogenic to white mice on intraperitoneal inoculation. Our studies lead us to believe that these isolates are merely different strains of one and the same microorganism for which we propose the name *Pseudomonas oxalaticus* nov spec.

Morphology and staining. The isolated bacterium was observed to be a gram negative short rod (0.3 to 0.4 μ by 0.9 to 1.5 μ), motile, nonsporulating, and noncapsulated. Occasionally large filamentous forms also were observed. Flagella staining revealed 1 to 3 polar flagella.

Cultural characteristics. On nutrient agar, 1 mm

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round, smooth, raised, opaque, slightly fluorescent, entire-edged colonies appear within two days. After 7 days' incubation, the colonies grow up to two mm in diameter and are low convex with a slightly undulating margin. Around this may be seen a scarcely visible, transparent, thin, flat zone with a very irregular margin. This zone is visible only under the low power of the microscope. The organism grows well on nutrient agar at 28 C and 37 C. In nutrient broth, the organism forms a thin pellicle, heavy turbidity, and a viscid sediment. The organism failed to produce any green or yellow water soluble pigment even when cultured on meat infusion media. On Bhat and Barker's oxalate agar, pin-point, transparent to translucent colonies with halo formation develop within 3 days. After 7 days, colonies appear as 1.5 mm round, translucent, with a compact, centrally raised area which is surrounded by a thin, flat, transparent zone. Distinct, large, clear-cut halos are formed round the colonies. The agar plate turns red owing to the utilization of the anions. In liquid oxalate medium, a slight turbidity develops and the medium turns red with the utilization of oxalic acid.

The organism reduces nitrate to nitrite and to ammonia but does not produce indole. All except one strain (Ox_{22}) form hydrogen sulfide. It does not liquefy gelatin and turns milk slightly alkaline accompanied by the reduction of litmus. The methyl red and Voges-Proskauer tests were negative. Catalase is present. Neither acid nor gas is produced in glucose, lactose, sucrose, maltose, starch, dextrin, glycerol, or mannitol.

Pathogenicity. Two strains of the bacterium were observed to be pathogenic to white mice on intraperitoneal inoculation of one ml amounts of thin suspensions made from 24 hr cultures on nutrient agar slopes. The animals died within 24 hr, and the microorganism was found in the peritoneal fluid, heart, liver, and spleen. Two years of laboratory culturing, however, resulted in the loss of virulence of one of the strains.

Substrates utilized for growth. A number of organic compounds were tested as growth substrates by incorporating them in a mineral medium (no. 1) of the following composition: $(NH_4)_2SO_4$, 0.5 g; neutral $Na_2HPO_4 + KH_2PO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $CaSO_4 \cdot 2H_2O$ (saturated solution), 5 ml; phenol red indicator, 5 ml; made up to 1,000 ml with distilled water; pH 7. With the exception of sodium oxalate and sodium formate, the carbon sources were added in one per cent concentration. For oxalate and formate the concentration was restricted to 0.1 per cent due to the toxic nature of these anions. Utilization of the substrate was judged by the observation of increased turbidity over the control with no added substrate. Day to day readings were taken up to 5 days.

TABLE 1

Utilization of carbon sources by different strains of Pseudomonas oxalaticus

STRAIN NO.	SODIUM OXALATE	SODIUM FORMATE	SODIUM ACETATE	SODIUM SUCCINATE	SODIUM LACTATE	SODIUM TARTRATE	SODIUM CITRATE	ETHYL ALCOHOL	GLUCOSE	DEXTRIN	STARCH
Ox_1, Ox_2, and Ox_3	++	++	++++*	++++	++++	+++	+++	+++	++++	+	+
Ox_4 and Ox_6	+	−	++++*	++++	++++	+++	‡	−	−	−	−
Ox_{22}	++	++	++†	+++	+++	+++	+++	−	−	−	−

Key: ++++ = excellent growth; +++ = good growth; ++ = fairly good growth; + = poor growth; − = no growth.

* Growth appears after 3 days.

† Growth appears after 5 days.

‡ Slight growth appears if incubation period is extended to 7 days.

Table 1 reveals that utilization of oxalate, acetate, succinate, lactate, and tartrate is a feature common to all the 6 strains. The strains which are nonpathogenic to white mice differ from the pathogenic strains in their ability to utilize formate and citrate. Acetate and citrate, however, did support growth when the incubation period was extended, suggesting the formation of adaptive enzymes. Some strains do not utilize ethyl alcohol, glucose, dextrin, or starch.

Oxalate tolerance tests revealed that this bacterium could tolerate as much as two per cent oxalate in a mineral salt solution (medium 1) and the maximum growth in the presence of oxalate

took place at a concentration of 0.3 per cent. At this stage permanganate titrations showed that over 80 per cent of oxalate disappeared within 4 days of growth of the organism at 28 C.

For testing the ability of the bacterium to utilize the various nitrogen sources, one per cent of sodium lactate was incorporated in medium 1 in place of the ammonium sulfate. The nitrogen levels were maintained on the basis of 25 mg per 100 ml of the basal solution containing lactate as the source of carbon and energy. Ammonium sulfate, sodium ammonium phosphate, diammonium hydrogen phosphate, ammonium nitrate, potassium nitrate, sodium aspartate, sodium glutamate, and peptone were well utilized. Urea utilization was variable.

Thus, the nutritional pattern of the 6 strains is similar, and the few differences in their ability to utilize certain carbon compounds may be looked upon as strain differences rather than specific differences. This is substantiated further when it is observed that all strains are very much similar in their morphological and cultural characters and also in their utilization of various nitrogen sources. Furthermore, the loss of virulence of strain Ox_4 indicates that pathogenicity is an unstable characteristic.

Taxonomy. The dimensions and morphology of the organism described do not conform with those of either *Bacillus extorquens* (Bassalik, 1913) or *Vibrio oxaliticus*. Unlike *B. extorquens*, this bacterium can grow easily on nutrient agar and is nonchromogenic. Further, this organism utilizes over 80 per cent of oxalate for growth as compared to only 25 per cent of the substrate utilized by the organism isolated by Bassalik (Janota, 1950). The organism also differs from *V. oxaliticus* in its capacity to utilize lactate, succinate, and tartrate although two strains display a slight resemblance to it inasmuch as they were unable to utilize citrate and glucose. What is even more significant, two of the strains were observed to be pathogenic to white mouse on intraperitoneal inoculation.

On the basis of morphology and other characteristics this organism obviously belongs to the genus *Pseudomonas*. *Bergey's Manual* (Breed *et al.*, 1948) describes only one pseudomonad—*Pseudomonas rimaefaciens*—as being able to utilize oxalate. It may be seen readily that these isolates differ from *P. rimaefaciens* in more than one respect. Likewise, *P. aeruginosa* has been known to be supported by oxalates, but it does so only under anaerobic conditions (Robinson, 1932). Our isolates were made and can decompose oxalate under aerobic conditions. Finally, the bacterium could not be identified with any of the pseudomonads described in *Bergey's Manual*. Obviously, we are dealing with a new species of an oxalate-decomposing *Pseudomonas* for which we propose the name *Pseudomonas oxalaticus*.

SUMMARY

The method of isolation, characteristics, and the taxonomy of 6 strains of a new oxalate-decomposing bacterium, *Pseudomonas oxalaticus* nov spec, from the intestine of Indian earthworms are described. All the strains can utilize oxalate, acetate, succinate, lactate, and tartrate in an otherwise basal mineral solution. Utilization of formate, citrate, ethyl alcohol, glucose, dextrin, and starch varies with the strain. The bacterium can tolerate as high as 2 per cent oxalate in a mineral solution even though maximal growth is obtained at a concentration of 0.3 per cent.

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