CHARACTERISTICS OF A YELLOW BACTERIUM ISOLATED FROM THE TISSUE OF JONATHAN APPLE TREES

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ABSTRACT.—A yellow bacterial isolate obtained from a fire blight canker on the Jonathan cultivar was subjected to the various taxonomic microbiological tests for determining its classification. The yellow isolate was nonpathogenic to Jonathan apple trees. It was gram negative, catalase positive, and peritrichously flagellated. It was rod shaped and ranged from 0.6 to 0.9 microns in width and 1.1 to 2.9 microns in length. The optimum temperature for growth was 25° C with minimum and maximum temperatures for growth at 10° C and 35° C, respectively. This bacterium was nonacid fast, hydrolyzed gelatin but not starch, converted nitrate to nitrite, and produced ammonia but no nitrogen gas. Indole and hydrogen sulfide tests were negative. The methyl red-Voges-Proskauer results were variable. Citrate was able to serve as the sole source of carbon for growth. The protein patterns by disc electrophoresis showed the yellow isolate was distinct from *Erwinia amylovora* and *Escherichia coli*.

This yellow isolate appears to belong in the order Eubacteriales, genus *Erwinia*. However, since it is nonpathogenic to Jonathan apple trees, and shows a different protein pattern it should not be considered as belonging to the genus *Erwinia* at this time.

There has been controversy among many workers (Baldwin and Goodman, 1963; Billing and Baker, 1963; and Goodman, 1965) as to the classification of a yellow bacterium that has been commonly associated with *Erwinia amylovora* (Burrill) Winslow et al., in fire blight cankers.

Therefore, it was decided to study the characteristics of this yellow bacterium in detail and attempt to clarify its taxonomic position.

MATERIALS AND METHODS

Since bacteria may vary from one isolate to another within a species (Rosen, 1928), 20 yellow isolates were obtained from different fire blight cankers of the Jonathan cultivar. Each isolate was considered as a separate entity throughout the entire study. Comparisons were made with known species of *Xanthomonas pruni* (Smith) Dowson; *Erwinia amylovora*; *Aerobacter aerogenes* (Kruse) Beijerinck; *Proteus vulgaris* (Hauser); *Escherichia coli* (Migula) Castellani and Chalmers; and *Pseudomonas aeruginosa* (Schroeter) Migula.

The procedures used for characterization of the bacteria were obtained from the "Manual of Microbiological Methods" prepared by the Society of American Bacteriologists and will be described with the results given below.

Protein patterns by disc electrophoresis were obtained from the yellow isolate, *Erwinia amylovora*, and *Escherichia coli*. The disc electrophoresis equipment was obtained from the Canal Industrial Corp., Bethesda, Maryland. Approximately 3.0 g of bacteria of each species were harvested from nutrient broth by centrifugation at 5000 x G for 10 minutes. The pellet was washed twice with 30 ml of 0.1 M phosphate buffer (Na_2HPO_4, 21.5g; KH_2PO_4, 5.4 g in 1 liter of distilled water at pH 7.0), and suspended in 5 ml. The bacteria were then sonicated for 7 minutes with a 18 Kc Mullard sonicator and a ¾ inch

probe. The sonicated solution was centrifuged for 1 hour at 60,000 x G. A protein determination (Lowry et al., 1951) was made on the supernatant. The protein solutions were adjusted to 10,000 μg protein to 1 ml with the above buffer and 0.1 ml was added to 1.9 ml of the standard strength stacking gel. Each column received 0.2 ml of the sample gel. The current was adjusted to 6 ma. per tube. When the tracking dye band had moved about 1 inch into the separating gel the columns were removed immediately and placed in ice water. The starch gels were removed from the glass columns and placed in a dye solution for 1 hour and then destained in 7% acetic acid. The protein patterns of the three bacteria were compared visually (Fig. 1).

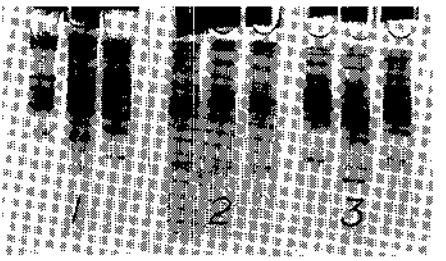

FIGURE 1. — The electrophoretic soluble protein patterns of (1) *E. coli;* (2) *E. amylovora;* (3) yellow isolates.

RESULTS

Pathogenicity tests. — The yellow isolates (hereafter called isolates) were tested for pathogenicity by injecting them as an aqueous suspension into succulent terminal tips of 7-year-old Jonathan apple trees in the field and into 1-year-old Jonathan apple seedlings in the greenhouse. No symptoms developed within 3 weeks after inoculation and thus the isolates were considered nonpathogenic. *E. amylovora,* used as a check, did cause blight symptoms within this period.

Morphology and staining. — All incubations were at 25° C unless noted differently. Cultures were grown on nutrient agar and examined after 24 hours except for the gram stain which was also examined at 12 hours. Heat-fixed films of the isolates were stained by Hucker's modification of the Gram stain and found to be gram negative with some of the bacteria tending to resist decolorization. This phenomenon has been reported by Billing et al. (1961). Size was estimated (using an eyepiece micrometer) by examination of heat-fixed films stained with the methylene blue solution used in the Gram stain. The isolates were rods of irregular length which usually occurred singly, occasionally in pairs and short chains. They varied from 0.6 to 0.9 microns in width and 1.1 to 2.9 microns in length. The isolates were observed as living bacteria in a hanging drop and to demonstrate flagella, Bailey's method as modified by Fisher and Conn was used. All the isolates had 1 to 5 flagella arranged peritrichously. An acid fast stain was made using the Ziehl-Nelsen method and the amended formula of the carbol-fuchsin solution for staining. Isolates were all nonacid fast.

Colony form. — The isolates were incubated on nutrient agar in slanted test tubes and examined after 24 hours and at the end of 1 month. Growth was filiform and the streak a glistening yellow. The margins were thickened and had very fine serrations at the outermost edges. After one month a very viscous slime had accumulated at the bottom of the slant.

Oxygen requirement. — Glucose nutrient agar deeps were made by filling test tubes to a depth of 9 cm with nutrient agar containing 1% glucose. Before the medium solidified, a drop of a nutrient broth suspension from 24-hour-old nutrient broth cultures was added. The tubes were examined after incubation for 1 week. No growth occurred except in the top 1.5 cm of the medium and thus the isolates were considered aerobes.

Optimum temperature. — Each tube was inoculated with one drop from 24-hour-old nutrient broth cultures. The tubes were incubated at 10, 15, 20, 25, 30, and 35° C and the amount of growth was determined by measuring the optical density using a spectrophotometer set at a wave length of 660 millimicrons. Readings were made at 0, 12, 24, and 48 hours after inoculation. The above was repeated twice and the average optical density computed for each temperature and isolate. Maximum growth was at 25° C. At 30° C the growth was less than at 25° C, but considerably better than at 20° C. Some growth occurred at both 10° and 35° C.

Utilization of citrate. — Test tubes containing Koser's citrate broth were inoculated in duplicate with 1 drop of

broth from 24-hour-old nutrient broth cultures. After 7 days of incubation the tubes were checked for cloudiness. The isolates were able to utilize citrate as the sole carbon source.

Hydrolysis of starch. — Cultures were streaked onto 0.2% starch-nutrient agar and incubated for 1 week. Gram's modification of Lugol's iodine solution was used to detect starch hydrolysis. The isolates were not able to hydrolyze starch.

Gelatin liquefaction. — Two methods were used. Stab cultures were made into tubes containing 12% gelatin and incubated at 20° C for 6 weeks. Cultures were also streaked across a 0.4% gelatin-nutrient agar and incubated at 28° C for 2 weeks. After incubation the surface of the medium was flooded with a solution of acid mercuric chloride. The results of both tests indicated all isolates except No. 4 were able to liquefy gelatin.

Catalase production. — About 1 ml of 10% hydrogen peroxide was added to 24-hour-old cultures on nutrient agar. All isolates were catalase positive.

Nitrate reduction. — Two methods were used. Tubes of nitrate broth were inoculated and strips of red litmus paper were placed inside the tubes near the top to check for ammonia production. Ammonia was formed by all isolates. Nitrate agar was also used. Incubation was for 12 days for both procedures. Zinc dust was added to nitrate agar tubes giving negative results to verify that nitrate remained and had not been decomposed beyond the nitrite stage. All isolates except Nos. 4 and 10 formed nitrite within 48 hours. After 12 days of incubation Nos. 4 and 10 were only slightly positive indicating they were very poor nitrate reducers.

Indole production. — Broth composed of 1% trypticase was the medium used. Kovac's test was used to determine the presence of indole after 1, 3, 5, and 11 days. None of the isolates were able to produce indole.

Methyl Red (MR) and Voges-Proskauer (V-P) tests. — The medium contained 7 g trypticase, 5 g K_2HPO_4, and 5 g glucose per liter of distilled water. Duplicate tubes were used and after incubation for 7 days several drops of a methyl red solution were added to one set of tubes. Only isolate Nos. 9, 19, and 20 gave a positive methyl red test. The V-P test for acetoin was performed by adding 0.6 ml of 5% alpha naphthol in absolute ethanol and 0.2 ml of a 40% KOH and 0.3% creatine solution to 1 ml of the 7-day-old broth cultures. After 1 minute of vigorous agitation the tubes were allowed to sit 10 minutes and then observed for development of a pink color. Isolate Nos. 4, 9, 10, 15, and 17 formed acetoin whereas the other 15 isolates could not do so.

Litmus milk test. — Medium was prepared by adding 5 g of litmus to 1 liter of 10% Bacto Skim Milk. Readings were made after 7 days of incubation. The results of this test were extremely variable in regards to pH, oxidation-reduction potential, and the appearance of the milk itself. Isolate Nos. 4, 10, 15, and 17 caused a basic reaction in the medium. Isolate No. 17 also reduced the litmus. Isolate Nos. 5, 7, and 19 were acid in reaction.

A summary of the above tests is shown in Table 1. The results indicated that the 20 yellow isolates were the same species and were treated as such in Table 1.

Electrophoresis. — Soluble protein fractions from *E. coli*, *E. amylovora*, and one of the isolates were separated by electrophoresis. The protein bands were stained and the resulting protein patterns of the various bacteria compared visually (Fig. 1). The results obtained indicate that the isolates and *E. amylovora* are not the same species and both were distinct from *E. coli*.

Discussion

In comparing the test results of the 20 isolates with the report of Farabee and Lockwood (1958) it was noted that their isolates were gram positive and could not reduce nitrate. On this basis the 20 yellow isolates used in this study are not the same as those of Farabee and Lockwood which they classified as *Bacterium*.

The 20 yellow isolates might be the same as those of Goodman (1965) but other than the fact that they were both yellow, gram negative, and had peritrichous flagella, insufficient information was given by him for comparison. Baldwin and Goodman (1963) and Goodman and Shaffer (1963) also showed the yellow bac-

terial cultures sensitive to 2 of 5 *E. amylovora* phages as well as to a 35A-Y phage. Virulent white isolates were sensitive to all 5 of the *E. amylovora* phages but not the 35A-Y phage. From these later results Goodman (1965) decided the relationship between *E. amylovora* and the yellow bacterium was not clear.

Comparison of the results obtained from the authors' 20 yellow isolates with those obtained by Billing and Baker (1963) indicated that both probably are of the same species. To be certain, however, the tests would have to be repeated using the procedures of Billing and Baker (1963) on both the authors' isolates and those of Billing and Baker.

Considerable confusion was found in the older literature concerning yellow gram negative bacteria. James, in 1955, reported "There is little doubt that the yellow chromogen referred to in this report is the

TABLE 1.—A Comparison of the Biochemical Reactions of Yellow Bacteria Isolated from Fire Blight Cankers and Six Known Species of Bacteria. Reaction Symbols are: A, aerobic; +, positive; —, negative; V, variable.

Test	Yellow isolates	*Erwinia amylovora*	*Xanthomonas pruni*	*Proteus vulgaris*	*Aerobacter aerogenes*	*Escherichia coli*	*Pseudomonas aeruginosa*
Stains							
gram	—	—	—	—	—	—	—
acid fast	—	—	—	—	—	—	—
Hydrolysis of							
starch	—	—	+	—	—	—	—
gelatin	+	+	+	—	—	—	+
Oxygen requirement	A	A	A	A	A	A	A
Nitrate test							
NO_3 to NO_2	+	—	—	+	+	+	+
NH_3 formed	+	+	+	+	+	+	+
gas (N_2)	—	—	—	—	—	—	—
Indole	—	—	—	+	—	+	—
H_2S	—	—	—	+	—	—	+
Citrate	+	—	—	—	+	—	+
Methyl red	V	—	—	+	—	+	—
Voges-Proskauer	V	—	—	—	+	—	—
Catalase	+	+	+	+	+	+	+

same as that described by Dugelli, and by Huss and studied in more detail by Mack." He proposed the name *Xanthomonas trifolii* for his yellow bacteria. Billing and Baker (1963), however, have cited reasons for doubting that his cultures were the same.

Hayward and Hodgkiss (1961) have shown that the yellow pigmented bacterium, *Xanthomonas uredovorus*, which originally was thought to possess a single polar flagellum, was found to possess peritrichous flagella. They also proposed that this organism should be transferred to the genus *Erwinia*.

Thus, as a result of this confusion, Billing and Baker (1963) considered their isolates to be members of a poorly defined '*lathyri - herbicola*' group within the genus *Erwinia*.

Bergey's manual (Breed et al., 1957) states that all peritrichously flagellated bacteria are classified in the order Eubacteriales. Further checking of the keys with the test results obtained from the 20 yellow isolates leads to the genus *Erwinia*. With the present classification system the genus *Erwinia* is reserved for organisms which are plant pathogens (Breed et al., 1957). This bacterium, since it is nonpathogenic, can not be properly classified until either a new genus is formed, a host is found, or until the present requirements of the genus *Erwinia* are modified to include nonplant pathogens.

Characterization of bacteria by electrophoretic separation has not been utilized extensively (Rodenberg, 1959). Based on the protein patterns the isolates are definitely not the same species as *E. amylovora* (Fig. 1).

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