Isolation of *Corynebacterium agropyri* from 30- to 40-Year-Old Herbarium Specimens of *Agropyron* Species

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ABSTRACT

Murray, T. D. 1986. Isolation of *Corynebacterium agropyri* from 30- to 40-year-old herbarium specimens of *Agropyron* species. Plant Disease 70:378-380.

Three strains of a yellow-pigmented, gram-positive, coryneform bacterium were isolated from herbarium specimens of *Agropyron smithii, A. trachycaulum,* and *A. riparium* that had been stored for 40, 35, and 30 yr, respectively. The plant samples were diagnosed as having bacterial head blight, caused by *Corynebacterium agropyri*, at the time they were collected, and the strains isolated closely resembled each other and the description of *C. agropyri.* The three strains, designated CA-1, CA-2, and CA-3, are believed to be the only extant cultures of *C. agropyri.* Survival of *C. agropyri* for 40 yr in dried plant material exceeds previous reports of *C. flaccumfaciens* surviving for 24 yr in bean seed.

Yellow gum disease (head blight) of western wheatgrass (*Agropyron smithii* Rydb.) caused by *Corynebacterium agropyri* (O'Gara) Burkh. was first reported in 1915 by O'Gara (11) from samples collected in the Salt Lake Valley of Utah. It was thought to be a new type of bacterial disease in America because of its unusual symptoms, which resembled those of Rathay's disease of orchardgrass (*Dactylis glomerata* L.) (20). According to O'Gara (12), the most prominent symptom of yellow gum disease is "the presence of enormous amounts of surface bacteria which form a lemon-yellow ooze or slime." Ooze typically occurs on upper parts of the plant, forming layers between the stem and leaf sheaths and between glumes of the inflorescence (11–13). The ooze collects on outer parts of the glumes and dries to form hard, brittle, resinous droplets. Infected plants may be slightly stunted and possess varying degrees of distortion on the inflorescence and stem above the uppermost node. Severely affected inflorescences do not produce viable seed. Roots and lower leaves were not affected by the pathogen.

O'Gara described the bacterium as a short rod with rounded ends, 0.4–0.6 × 0.6–1.1 μm, gram-negative, non-spore-forming, nonmotile, and non-acid-fast. Photographs from the original description show cells in V-shaped and end-to-end arrangements. Colonies on nutrient agar were primuline yellow and growth was slow at 25 C, with the optimum temperature of 25–28 C (13).

The bacterium was thought to be closely related to *Aplanobacter rathayi* E. F. Smith and was given the name *A. agropyri* (13). Burkholder (in 2) later transferred *A. agropyri* to the genus *Corynebacterium* and speculated that *C. agropyri* may be identical to *C. rathayi*, cause of head blight of *D. glomerata.*

C. agropyri was not included in the *Approved Lists of Bacterial Names* in 1980 (19) because extant cultures were not available and *C. agropyri* and *C. rathayi* could not be distinguished on the basis of their original descriptions (24). This paper reports the isolation of *C. agropyri* from naturally infected plant samples collected during the period 1945–1955 and stored in the Washington State University Mycological Herbarium. A previous report has been published (9).

MATERIALS AND METHODS

Plant samples and isolation. Isolation of *C. agropyri* was attempted from naturally infected plant samples of *Agropyron smithii*, collected in 1945 at Montpelier, ID; *A. trachycaulum* (Link) Malte, collected in 1950 at Muddy Pass, CO; *A. riparium* Scribn. & Smith, collected in 1955 at Gilmore, ID; and *Stipa speciosa* Trin. & Rupr., collected in 1947 at Johannesburg, CA. All samples were stored in the mycological herbarium under ambient temperature and relative humidity. Original disease diagnoses were based on signs and symptoms present at the time of collection (G. W. Fischer, *personal communication*).

Dried ooze was removed from infected inflorescences with sterile forceps and dissolved in sterile phosphate buffer (0.01 M, pH 6.8). When individual droplets of ooze could not be removed, portions of inflorescences cemented together by the ooze were placed in buffer and the ooze was allowed to dissolve. The suspension was diluted, then 0.1-ml aliquots were spread over the surface of nutrient broth-yeast extract agar (NBY) and incubated at room temperature (22 C). Representative colonies were removed and streaked on NBY to obtain single colonies. This process was repeated three times to ensure pure cultures. Cultures were stored on NBY slants at 5 C and also lyophilized and stored at −20 C.

Identification and pathogenicity tests. Twenty-four-hour cultures were used to determine Gram-stain reaction (16), acid-fast reaction (6), cell size and mode of division, and motility (16). Physiological tests corresponding to those contained in the original description by O'Gara (13) and later by Burkholder (in 2) were conducted. Tests included gelatin liquefaction, nitrate reduction, starch hydrolysis, acid production from carbohydrates, and the catalase test (16).

Pathogenicity tests were conducted on four host species (*A. smithii, A. spicatum* (Pursh) Scribn., *A. intermedium* (Host) Beauv., *A. michnoi*) and one nonhost species (*D. glomerata*). Plants were inoculated with cells (10^7 cfu/ml) from 2- to 4-day-old cultures suspended in phosphate buffer. Inoculation consisted of injecting inoculum into the inflorescence while still enveloped by the uppermost leaf sheath (boot); infiltrating inoculum with a hypodermic needle into the intercellular spaces of leaves until water-soaking appeared; infiltrating intercellular spaces of leaves as before, then wounding the leaf by repeatedly pricking the water-soaked area with a hypodermic needle; or vacuum-infiltrating the inoculum into leaves. Control plants were inoculated in the same manner with either sterile phosphate buffer or a suspension of cells of *Clavibacter michiganense* pv. *michiganense*. All inoculated plants were incubated inside clear-plastic chambers on greenhouse benches at 20–25 C. In addition, tobacco hypersensitivity tests were conducted (16).

RESULTS

Isolation. Yellow-pigmented, gram-positive, coryneform bacteria were isolated in pure culture from samples of *A. smithii, A. trachycaulum,* and *A. riparium.* No isolates were obtained from the collection of *S. speciosa.* The isolates

Scientific Paper 7212, College of Agriculture and Home Economics Research Center, Washington State University, Pullman 99164-6420.

Accepted for publication 24 October 1985 (submitted for electronic processing).

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were designated CA-1, CA-2, and CA-3 from the respective hosts and had survived for 40, 35, and 30 yr, respectively, in the dried samples. Initial growth from dried ooze was slow, but the growth rate increased after subculturing. All three strains reacted the same in physiological tests and were identical to the description of *C. agropyri* with the exception that nitrate was not reduced. Cell sizes of CA-1, CA-2, and CA-3 were within the range originally reported (11), averaging 0.53 × 0.93 μm (range 0.36–0.60 × 0.73–1.23 μm), 0.59 × 1.25 μm (range 0.50–0.67 × 0.83–1.57 μm), and 0.48 × 0.89 μm (range 0.37–0.69 × 0.65–1.20 μm), respectively. Strains CA-1, CA-2, and CA-3 were nonmotile, aerobic, non-acid-fast, and did not liquefy gelatin, reduce nitrate, or hydrolyze starch. Acid was produced from glucose, sucrose, glycerol, and lactose.

All pathogenicity tests and tobacco hypersensitivity tests were negative. Control plants inoculated with sterile phosphate buffer or *C. michiganense* pv. *michiganense* did not develop symptoms.

DISCUSSION

The bacteria isolated from these collections closely resemble each other and the descriptions given for *C. agropyri* by O'Gara (13) and by Burkholder (in 2) and are probably the only extant cultures of *C. agropyri*. The fact that the three strains are similar in morphological and physiological characters and originated from different localities and different hosts, diagnosed by symptoms and signs as having head blight, is further evidence that these are strains of *C. agropyri*.

These isolates differed from the original description of *C. agropyri* only in the Gram-stain reaction and nitrate reduction test. O'Gara (13) originally reported that the intensity of the anilin-gentian-violet stain was never completely retained or lost and concluded the bacterium was gram-negative. However, he worked with 4- to 14-day-old cultures and destained with absolute alcohol for 3 min. In this study, 24-hr cultures were used, as suggested by Vidaver (in 16), and destained for 30 sec with 95% ethanol. It is possible that the use of older cultures and the prolonged destaining time resulted in a false interpretation of the true Gram-stain reaction.

The original report of *C. agropyri* reducing nitrate may have been in error; the same is true for *C. rathayi* (1). Another work has reported the failure of *C. rathayi* and the related *C. iranicum* and *C. tritici* to reduce nitrates (5).

The original description of *C. agropyri* was not detailed enough to distinguish it from *C. rathayi* (1); however, on the basis of host range, *C. agropyri* appears to be distinct from *C. rathayi*. Sprague and Fischer (21) reported the host range for *C. agropyri* included nine species of *Agropyron*, three species of *Hordeum*, three species of *Poa*, two species of *Sitanion*, and one species each of *Sporobolus* and *Stipa*. None of the species reported as hosts for *C. agropyri* are known hosts of *C. rathayi*, which is limited to *D. glomerata, Cynodon dactylon* (L.) Pers., and *Secale cereale* L. in nature (1). The reported geographic distribution of *Corynebacterium agropyri* in the western United States is also much wider than that of *C. rathayi*. *C. agropyri* has been reported from nine states including Washington, Oregon, California, Nevada, Idaho, Montana, Utah, Colorado, and North Dakota, whereas *C. rathayi* is only reported from Oregon and possibly Virginia (14,21,22).

Although pathogenicity was not proven for *C. agropyri*, there is no reason to believe these strains have lost pathogenicity. O'Gara was also unable to prove pathogenicity of *C. agropyri* (13). Others (4,15,23) attempted to prove pathogenicity of *C. tritici* and *C. rathayi* by hypodermic inoculation and failed. Nematodes are required as vectors for both *C. tritici* and *C. rathayi* and may also be required as vectors for *C. agropyri*.

Survival of *C. agropyri* for 40 yr in dried plant material exceeds previous reports of survival of 24 yr for *C. flaccumfaciens* in dried bean seed, 15 yr for *C. flaccumfaciens* var. *aurantiacum, Xanthomonas phaseoli,* and *X. phaseoli* var. *fuscans* in bean seed, and 8 yr for *C. flaccumfaciens* var. *violaceum* in bean seed (3,7,17,18). Long-term survival of bacterial plant pathogens raises important questions about survival mechanisms. Bacterial cells surviving for long periods are in a hypobiotic state in which metabolism is close to a standstill (8). The matrix in which the cells are embedded must be important for survival by providing limited protection against rehydration and perhaps other environmental factors. Nevertheless, it is amazing that a bacterium without specialized survival structures could survive for 40 yr in dried ooze. The importance of long-term survival in the epidemiology of yellow gum is questionable. Bacterial ooze is readily dissolved in water, and under normal conditions of precipitation, the ooze and pathogen would not be expected to survive for more than a few seasons. However, many of the locations from which these collections originated and other reported locations for yellow gum disease (21) are in arid regions. For example, annual precipitation is about 37 cm at Montpelier, ID, and about 26.5 cm at Gilmore, ID (10). Under such arid conditions, the ability to survive in ooze should be a distinct advantage. Survival for 40 yr exemplifies the potential survival capabilities of this and perhaps other plant-pathogenic bacteria.

ACKNOWLEDGMENTS

We wish to thank the Regional Plant Introduction station, Pullman, WA, for supplying seed of *Agropyron* sp. for use in pathogenicity tests and Huazhi Ye for technical assistance.

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