Identification of *Erwinia chrysanthemi* as a Soft-Rot-Inducing Pathogen of Grain Sorghum

S. G. JENSEN, Research Plant Pathologist, USDA, ARS, 406 m Plant Science Hall, University of Nebraska, Lincoln 68583; W. R. MAYBERRY, Microbiologist, Department of Microbiology, College of Medicine, East Tennessee State University, Johnson City 37601; and J. A. OBRIGAWITCH, Research Assistant, Department of Plant Pathology, University of Nebraska, Lincoln 68583-0722

ABSTRACT

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A bacterial pathogen was isolated from pre-boot-stage grain sorghum plants suffering from a soft rot of the stalk. Koch's postulates were satisfied with a bacterium that caused soft rot only at high temperatures (>30 C) but was pathogenic to several commercial sorghum genotypes. The pathogen was identified as an isolate of *Erwinia chrysanthemi* on the basis of comparisons with authentic cultures for standard morphologic-metabolic criteria, composition of soluble cellular proteins (by polyacrylamide gel electrophoresis), and composition of cellular fatty acids (by gas chromatography).

During the summers of 1980 and 1982 in eastern Nebraska, grain sorghum plants *Sorghum bicolor* (L.) Moench. in about the fourth growth stage (17) exhibited a disease syndrome with which we were unfamiliar. In the most severe cases, the top four or five leaves were dead and all lower leaves appeared normal. The dead tissue could be easily lifted from the whorl, revealing a wet mass of necrotic tissue that emitted a putrid odor (5,7,9). The rot was limited at a particular node, and only tissue above that node was rotted (Fig. 1). Other sorghum plants in the area displayed less severe symptoms, the mildest of which were necrotic or pigmented stripes or blotches on the upper leaves with no stalk rot or top necrosis. In 1980, the disease occurred on low-lying, level ground. In 1982, the disease was found on rather level land in upland, rolling country. In certain small, localized areas, disease incidence exceeded 50% of the plant population. The syndrome in certain ways resembled an Erwinia stalk rot of maize occurring in the Philippines (5,6) and in maize sprinkle-irrigated with water from impoundments (9). A soft rot disease of sweet sorghum in Mississippi (19) was also induced by an *Erwinia* sp. This report identifies the pathogen as an isolate of *Erwinia chrysanthemi* and describes and compares three techniques used for identification: standard physiologic and morphologic tests (16), polyacrylamide gel electrophoresis (PAGE) of cellular proteins (1,3), and analysis of cellular fatty acids with gas-liquid chromatography (12–15).

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MATERIALS AND METHODS

Isolation and pathogenicity tests. Bacteria from diseased stalks were isolated by dilution onto potato-dextrose agar. Single colonies were isolated by two to four cycles of serial dilution and transfer to selective media (10). Greenhouse tests for pathogenicity were conducted on the sorghum cultivars Combine Kafir 60, NE 505, RS 626, Asgrow Bugoff, and DeKalb E59+. High humidity was maintained using a mist chamber in which temperature fluctuated diurnally (18–30 C). Bacteria were suspended in 0.1 M phosphate buffer, pH 7.0, to produce inoculum concentrations of $6–8 \times 10^6$ cells per milliliter. The suspensions were injected via hypodermic syringe through leaves into the vicinity of the growing point (7). Erratic infection resulted, and subsequent inoculations and incubations on the same cultivars were done in a controlled-environment chamber held at 35 ± 2 C with humidity maintained at 60–100% (19). For species identification tests, we used several pathogenic isolates as well as non-pathogenic isolates that were similar in colony color and morphology to the pathogenic isolates. Also, several bacteria of established identity were included in these tests.

Physiologic and morphologic tests.

Standard procedures for identifying plant-pathogenic bacteria were followed essentially as described (16).

PAGE of cellular proteins. Total cellular proteins were analyzed by polyacrylamide gel electrophoresis of sodium dodecyl sulfate derivatives (SDS-PAGE) (11). The procedure was that used by Carlson and Vidaver (3) for taxonomic studies of pathogenic *Corynebacterium* spp. Cells were grown to early stationary phase in nutrient-broth yeast-extract broth, then washed three times by centrifugation in 0.2 M sodium phosphate buffer, pH 6.8. The washed pellet was suspended in the same phosphate buffer, and the cells were disrupted with an equal volume of lysozyme (1 mg/ml in 0.05 M Tris [hydroxymethyl] aminomethane-0.02 M methylaminedeamine tetraacetate buffer, pH 8.0). After a 15-min incubation at 37 C, sodium lauryl sulfate (10% w/v) was added to a final concentration of 5%.

The suspensions were boiled 1 min, then stirred to shear the DNA and reduce viscosity. Polyacrylamide slab gels with concentration gradients from 8 to 20% were used for the electrophoretic separation (11). Ten microliters of each sample were layered on the top of each lane, and protein standards of bovine serum albumin (mol wt 66.3), carbonic anhydrase (mol wt 28), and lysozyme (mol wt 14.3) were used for comparison. Electrophoresis was at 60V for 20 hr. Gels then were stained for 1 hr at 50 C with Coomassie brilliant blue R, 0.5 g of stain in methanol, water, and glacial acetic acid in a volume ratio of 5:5:1, and photographed.

Fig. 1. Field symptoms of grain sorghum plants infected with *Erwinia chrysanthemi*. The putrid, wet rot extends into the growing point leading to a necrosis of the upper leaves.

Characterization of cellular fatty acids. Cellular fatty acids were identified according to the techniques of Calhoon et al (2) and Mayberry et al (12–15). Pellets of bacterial cells were suspended in 2 N HCl and hydrolyzed overnight at 100 C. The organic phase was esterified by 1 N HCl in methanol for 1 hr at 100 C. Methylated fatty acids were extracted with chloroform, reduced to dryness, and any hydroxyl groups converted to volatile trimethylsilyl derivatives. The reagents were removed under a stream of dry nitrogen, and the residues were reconstituted in 250 μl of chloroform. Samples (0.5 μl) were injected into a Hewlett-Packard 5840A gas chromotograph equipped with a hydrogen-flame ionization detector. The column was a 10-m tubular glass capillary wall coated with the nonpolar liquid phase SP2100. The instrument was operated in the splitless mode under temperature-programmed conditions such that the relationship of retention time to chain length of fatty acid methyl esters was linear. Esters were identified by their "equivalent chain length" (ECL) values as characterized previously (15). Molar response factors for quantitative analysis were determined empirically. The fatty acid profile of each bacterial isolate was characterized as the calculated mole percent of each fatty acid.

RESULTS

In pathogenicity tests in the greenhouse at 18–30 C, only a small number of plants developed soft rot as seen in the field. Top necrosis symptoms appeared after 5–7 days. When the tests were done in a controlled-environment chamber at 35 C and relatively high humidity (60–100%), typical disease symptoms of top necrosis and a putrid soft rot of the stalk occurred within 2–3 days. More reproducible results were obtained by placing the inoculum into the whorl of the plant (8) than by injection. Several isolates were found (all similar in colony appearance) that induced a high incidence of disease.

All three methods of comparison showed that the newly identified pathogen closely resembles *E. chrysanthemi* RS-61. In biochemical properties, the pathogenic isolate 17-31 showed only three points of difference from the authentic *E. chrysanthemi* RS-61 (Table 1) (4,18). The inability of the pathogenic isolate 17-31 to produce a rot in potatoes may be due to the market potatoes of unknown variety and condition that were used in each of the three separate tests. However, another bacterium, the *Erwinia* sp. isolate from Mississippi, which produces a wet soft rot of sweet sorghum stalks (19), was also marginal in its ability to produce rot of potato in our tests. Both isolates of authentic *E. chrysanthemi* that were used for comparison caused soft rot in potato tubers, but neither induced soft rot in sorghum stalks. Isolates 17-27 and 17-12 were superficially similar in growth and appearance to the pathogenic isolate 17-31, but on the basis of all other tests (Table 1), we concluded that they were different.

Isolates were classified into three groups based on PAGE. The first group included *E. chrysanthemi* RS-61 and other isolates that were very similar to it: isolates 17-7, 17-29, 17-76, and 17-31. All four unknown isolates in this group were pathogenic, with isolates 17-31 and 17-7 being the most virulent. A distinct protein band 185 mm from the origin was characteristic of this group. In a comparison of the five lanes containing that protein, many similarities were apparent (Fig. 2). However, a major

Table 1. Comparison of biochemical properties of eight bacterial species and isolates

Test	*Erwinia* sp. from Mississippi	*E. chrysanthemi* G-129[a]	Isolate 17-31 (pathogenic)	*E. chrysanthemi* RS-61[a]	Isolate 17-27 (nonpathogenic)	Isolate 17-12 (nonpathogenic)	*E. carotovora* subsp. *atroseptica*[a] SR8	*E. carotovora* SR 204[a]
Acid from lactose	+	+	+	+	−	−	+	+
Acid from [2]Me-d glucose	+	+	−	−	+	−	+	+
Acid from palatinose	+	+	−	−	−	−	+	+
Acid from glucose	ND[b]	+	+	+	−	+	ND	ND
Gas from glucose	+	−	+	−	−	ND	ND	ND
Gelatin liquification	−	−	+	+	ND	+	+	+
Potato rot	±	+	−	+	−	−	+	+
Pectate production	−	−	+	+	−	−	+	+
Reducing substances from sucrose	−	−	+	+	−	−	+	−
Phosphatase	+	+	+	+	−	+	−	−
Sensitive to erythromycin	−	−	−	+	+	+	−	−

[a] Obtained from A. K. Vidaver.
[b] Not determined.

protein band about 103 mm from the origin in *E. chrysanthemi* RS-61 was not shared by the other isolates in this group. Other minor differences were also noted.

The second group of organisms, *Erwinia* sp. from Mississippi and *E. chrysanthemi* G-129, resembled each other closely but differed from other isolates in enough ways to set them apart as perhaps a separate subdivision of the larger group. The third group included isolates that differed from the other two groups and from each other in enough significant ways that we discerned no relationships among them.

Data from the determination of cellular fatty acids are presented in Table 2. The mole percent of each fatty acid is listed by ECL. The qualitative and quantitative patterns of fatty acid methyl ester composition also indicated three general groups of cultures identical to the grouping made from the PAGE analysis. Isolates 17-7, 17-29, 17-31, and *E. chrysanthemi* RS-61 were grouped together. The three pathogenic isolates were nearly identical. *E. chrysanthemi* RS-61 was very similar but differed in having higher content of the fatty acid ECLs 15.7 and 17.8 than did the pathogenic isolates.

In the second group, *E. chrysanthemi* G-129 differed from cultures in the first group in the contents of four fatty acids. The contents of fatty acids of ECLs 15.7 and 17.8 were somewhat lower than in the sorghum pathogens, whereas fatty acids with ECLs of 16.0 and 18.9 were more abundant. Again, the Mississippi *Erwinia* sp. and *E. chrysanthemi* G-129 were similar in nearly every respect. The remaining isolates differed from each other and from the other two groups in enough aspects to make them independent. However, there were enough similarities to suggest that some may be part of a larger, less closely related group. First, the isolates *E. carotovora* subsp. *atroseptica* and *E. carotovora* SR 204 resembled the other authentic isolates of *Erwinia* and the pathogenic isolates in having four major fatty acid components with ECLs of 15.7, 16.0, 16.1, and 17.8. Isolate 17-27 may be an *Erwinia* sp., because it contains three of these four key fatty acids; however, it is completely lacking in fatty acid ECL 15.7 and has major acids at 18.6 and 23.8. Isolate 17-12 was completely different from all others both in this and other tests.

DISCUSSION

We have identified a previously undescribed bacterial pathogen associated with a soft rot of sorghum in Nebraska. The effects of this pathogen may vary from complete destruction of a plant to rather minor lesions on the unfurling leaves of the affected plant. We hypothesize that the usual mode of entry into the plant is by the rain splashing contaminated water into the whorl of young, growing plants, a situation that would generally occur only during mild flooding. High temperatures favor pathogenicity of the organism and may be essential for the most severe cases observed. Because of these limitations, we do not believe the disease will become a significant threat except in poorly drained fields where prolonged periods of high temperature may occur during the early stages of seedling development. The disease was observed in different seasons and localities and on several commercial cultivars, which suggests that the pathogen may be widespread, but environmental factors may limit develop-

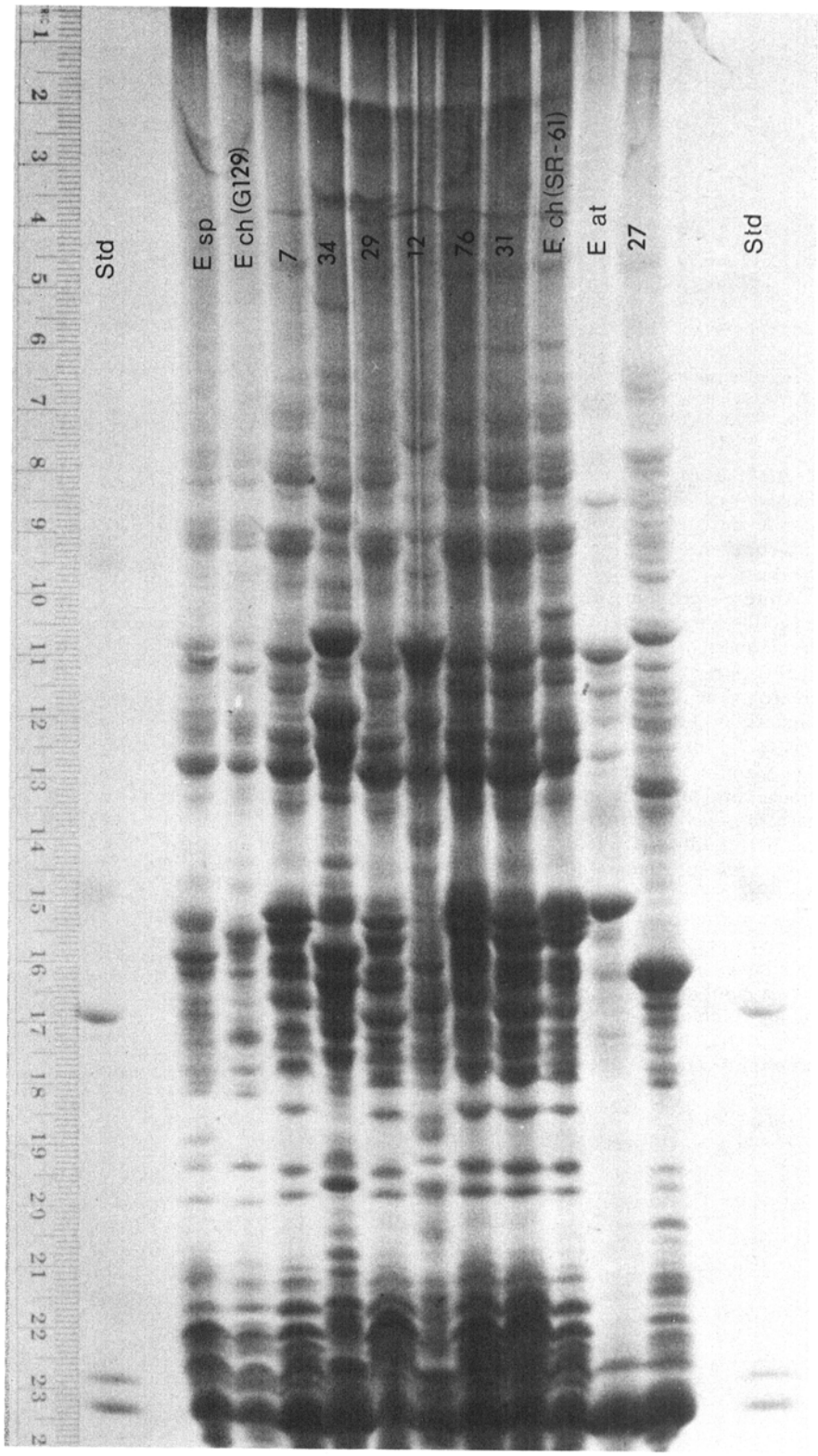

Fig. 2. Cellular proteins separated by polyacrylamide gel electrophoresis from 11 isolates of bacteria. Left to right: protein standards, *Erwinia* sp. from Mississippi, *E. chrysanthemi* G129, 7, 34, 29, 12, 76, 31, *E. chrysanthemi* SR-61, *E. atroseptica*, 27, and protein standards.

Table 2. Comparison of fatty acid methyl esters of 10 bacterial species and isolates

Equivalent chain length	Tentative identification	Fatty acid methyl esters, *O*-trimethylsilyl ethers (mole percent)									
		Erwinia sp. from Mississippi	*E. chrysanthemi* G-129	Isolate 17-7	Isolate 17-29	Isolate 17-31	*E. chrysanthemi* RS-61	Isolate 17-27	Isolate 17-12	*E. carotovora* subsp. *atroseptica* SR8	*E. carotovora* SR204
12.3	h10:0[a]	...	...	2	1	4	2	...	...	...	...
14.0	n14:0	7	2	4	4	4	2	...	...	...	...
14.2	h12:0	...	...	tr[b]	...	tr	...	...	...	...	...
14.7	a15:0	...	...	...	...	...	...	...	62	...	...
15.7	n16:1	3	1	14	17	11	20	...	...	17	31
15.9	16cyc	...	...	1	1	1	3	...	...	...	...
16.0	n16:1	43	46	30	35	28	24	13	29	32	30
16.1	h14:0	14	9	13	4	13	8	10	...	9	16
16.7	a17:0	...	...	...	...	...	...	...	9	...	...
16.8	17cyc	16	10	12	15	12	5	11	...	10	...
17.2	h15:0	...	...	1	1	1	tr	2	...	...	...
17.8	n18:1	5	10	16	16	13	24	22	...	29	23
17.9	?	...	...	1	...	...	1	...	...	...	...
18.0	n18:0	...	...	2	tr	2	tr	3	...	...	...
18.2	h16:0	...	...	1	1	1	tr	1	...	...	...
18.6	?	3	7	1	1	1	...	15	...	3	...
18.9	?	7	10	2	3	5	...	5	...	tr	...
19.1	?	1	2	tr	tr	tr	tr	3	...	tr	...
19.2	?	1	2	tr	tr	tr	...	3	...	tr	...
23.1	?	...	...	tr	tr	tr	...	...	...	...	...
23.8	?	...	...	...	...	...	...	13	...	...	...
24.8	?	...	...	1	1	2	6	1	...	...	...
25.0	?	...	...	1	1	2	5	..	...	...	...

[a] Fatty acids indicated by xN:Y notation. x = chain configuration:n, n = straight chain (normal); a = anteisobranched; i = isobranched, and h = hydroxy. N = chain length, y = number of double bonds, and cyc = cyclopropane.
[b] Trace, less than 0.5 mole percent.

ment of the disease. None of the cultivars that we tested showed resistance to disease development when the proper environmental requirements of the pathogen were met.

All three techniques for identification led to the same general and even specific taxonomic conclusions. The fatty acid methyl ester analysis seemed to have the greatest potential for identifying broad similarities in the *Erwinia* group. PAGE was useful for detecting many points of fine similarity or difference. The biochemical properties are, of course, the basis on which most of the classification has been built. The similarities between the *Erwinia* spp. that were apparent in the biochemical studies were less apparent in the two other methods of comparison. The agreement between the three techniques strengthens our conviction that a combination of procedures using biological, biophysical, and biochemical properties is necessary for accurate and efficient description of taxa.

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