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Genetic Relationships among Strains of *Xanthomonas fragariae* Based on Random Amplified Polymorphic DNA PCR, Repetitive Extragenic Palindromic PCR, and Enterobacterial Repetitive Intergenic Consensus PCR Data and Generation of Multiplexed PCR Primers Useful for the Identification of This Phytopathogen

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Genetic relationships among 25 isolates of *Xanthomonas fragariae* from diverse geographic regions were determined by three PCR methods that rely on different amplification priming strategies: random amplified polymorphic DNA (RAPD) PCR, repetitive extragenic palindromic (REP) PCR, and enterobacterial repetitive intergenic consensus (ERIC) PCR. The results of these assays are mutually consistent and indicate that pathogenic strains are very closely related to each other. RAPD, ERIC, and REP PCR assays identified nine, four, and two genotypes, respectively, within *X. fragariae* isolates. A single nonpathogenic isolate of *X. fragariae* was not distinguishable by these methods. The results of the PCR assays were also fully confirmed by physiological tests. There was no correlation between DNA amplification product patterns and geographic sites of isolation, suggesting that this bacterium has spread largely through exchange of infected plant germ plasm. Sequences identified through the RAPD assays were used to develop three primer pairs for standard PCR assays to identify *X. fragariae*. In addition, we developed a stringent multiplexed PCR assay to identify *X. fragariae* by simultaneously using the three independently derived sets of primers specific for pathogenic strains of the bacteria.

Bacterial angular leafspot disease of strawberry (*Fragaria* × *ananassa* Duch.) has become an increasing impediment to strawberry fruit and plant production worldwide (6). The disease, caused by *Xanthomonas fragariae* (Kennedy and King), was first reported in Minnesota in 1962 (16) and subsequently has been reported in strawberry-growing areas of North America, Europe, Africa, South America, Australia, and New Zealand (21). *X. fragariae* is classified as a quarantine pest by the European and Mediterranean Plant Protection Organization (23). The disease is characterized by small water-soaked lesions on the lower leaf surface which enlarge to become angular spots bounded by small leaf veins. Lesions may coalesce, causing infected leaves to appear scorched or blighted. The bacteria can cause loss of strawberry fruit as a result of infection of the calyx and often become systemic, leading to collapse of the entire plant. Although low concentrations of cupric hydroxide and mancozeb have shown some promise (27), optimal control of the disease is through avoidance of infected plant material and possibly through use of resistant plant germ plasm.

Detailed characterization of the genetic variability among strains of *X. fragariae* has not been reported despite the prevalence and increasing economic importance of the bacterium. Such characterization would be useful for pathogenicity studies and to trace the spread of the disease among continents. In addition to physiological tests, there are several rapid methods used to identify isolates of bacterial pathogens, including serologic testing, fatty acid profiling, and genomic and plasmid DNA restriction fragment length polymorphism analyses (17). Analysis of genomic DNA using PCR-based methods has proven to be a fast, sensitive, and reliable method for determining genetic relationships among pathogenic organisms. For example, the random amplified polymorphic DNA (RAPD) (35) PCR method has been used successfully with a number of microbial species to fingerprint genomes (for examples, see references 1, 4, 18, and 25) and to identify genomic targets for specific PCR assays (4, 22, 26). Consensus sequences derived from highly conserved palindromic inverted repeat regions found in enteric bacteria have also been used to fingerprint gram-negative species (5, 19, 34). These conserved regions have been called repetitive extragenic palindromic (REP) sequences (33) and enterobacterial repetitive intergenic consensus (ERIC) sequences (13).

Detection of *X. fragariae* in asymptomatic infected strawberry plants is the first step in avoiding the disease and controlling its spread, because the pathogen is thought to spread through asymptomatic plants sold as transplants. Current methods for detecting *X. fragariae* rely on isolation, culture, and serological or pathogenicity testing of the organism in question (24). Detection of *X. fragariae* by such methods is complicated by the fact that strains of other yellow-pigmented bacteria (e.g., *Xanthomonas campestris*) are easily isolated from strawberry tissues and are often similar in appearance

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TABLE 1. List of bacterial strains used in this study, their geographic origins, and their pathogenicity for strawberry

Strain	Origin of culture[a]	Angular leafspot on strawberry cv. Dover
Received as *X. fragariae*		
Xf3	North Carolina (DR)	Yes
Xf8	North Carolina (DR)	Yes
Xf10	North Carolina (DR)	Yes
Xf12	North Carolina (DR)	Yes
Xf16	North Carolina (DR)	Yes
Xf40	North Carolina (DR)	Yes
Xf1518	North Carolina (JJ)	Yes
Xf6	California (DR)	Yes
Xf20	California (DR)	Yes
Xf30	California (DR)	Yes
Xf1524	California (JJ)	Yes
Xf1425	Florida (JJ)	Yes
Xf1426	Florida (JJ)	Yes
Xf1429	Florida (JJ)	No
Xf1430	Florida (JJ)	Yes
Xf1431	Florida (JJ)	No
ATCC 33239[b]	Minnesota (ATCC)	Yes
Xf122	Ontario (PR)	Yes
Xf146	Ontario (PR)	Yes
Xf128	Quebec (PR)	Yes
Xf135	Quebec (PR)	Yes
LMG705	Australia (LMG)	Yes
LMG706	Greece (LMG)	Yes
LMG707	Greece (LMG)	No
Xf2510	Spain (INRA)	Yes
Xf1290	Réunion, France (OP)	NT[c]
Xf1238	Réunion, France (OP)	NT
Xf1240	Réunion, France (OP)	NT
Xf1241	Réunion, France (OP)	NT
LG2510	Réunion, France (OP)	NT
Other bacterial species		
X. campestris pv. vesicatoria ATCC 11633, ATCC 736, ATCC 35937, and ATCC 931	New Jersey, Michigan, New Zealand, Michigan (FJL), respectively	NT
36 *X. campestris* strains	Various (JSH) (19 pathovars)	NT
55 Epiphytic strains	Beltsville (field)	NT
7 Epiphytic strains	Beltsville (greenhouse)	NT

[a] DR, D. Ritchie; LMG, LMG Culture Collection, Ghent, Belgium; PR, P. Roberts, University of Florida, Bradenton; JJ, J. Jones, University of Florida, Bradenton; INRA, Institut National de la Recherche Agronomique, Beaucouzé, France; ATCC, American Type Culture Collection, Rockville, Md.; FJL, F. J. Louws, North Carolina State University, Raleigh; OP, Olivier Pruvost, CIRAD FLHOR, Réunion, France; JSH, John Hartung (8).

[b] Three additional isolates of the type strain, obtained from LMG Culture Collection, D. Ritchie, and P. Roberts, were used for initial screening of RAPD marker reproducibility.

[c] NT, not tested.

and growth rate to *X. fragariae*. Recently, the amplification of DNA sequences specific to a particular pathogen by the PCR has been used as a highly sensitive method for the detection of various pathogens in their plant hosts (7, 12).

The objectives of this research were to assess genetic variability among strains of *X. fragariae* isolated from diverse geographic areas by using RAPD, REP, and ERIC PCRs and to develop PCR primers specific to strains of *X. fragariae* for sensitive and specific identification of the pathogen.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. The bacterial strains used in this study and their sources are listed in Table 1. All strains were grown in rotary culture for 2 to 3 days at 23°C in nutrient broth (Difco) supplemented with 10 g of sucrose per liter. Total genomic DNA was extracted from 1.5 ml of culture with the Qiamp Tissue Kit (Qiagen, Chatsworth, Calif.). DNA quantity was estimated by comparison with known standards in ethidium bromide-stained 1% agarose gels.

Nonpathogenic epiphytic bacteria were isolated from field- and greenhouse-grown strawberry varieties Earliglow and Kent which were grown in Beltsville, Md., in 1995 and from numerous varieties in a breeder's trial plot in 1996. Isolations were on sucrose-peptone agar (SPA) (11) or YDC (31) plates supplemented with cycloheximide (100 mg/liter). Isolates were selected to include those with differing growth rates, colony morphologies, and colors. Sixty-two epiphytic isolates from strawberry as well as 36 isolates representing 19 pathovars of *X. campestris* (8) were used to test the specificity of PCR primers.

Pathogenicity tests. Tests were performed at the North Carolina State University Department of Plant Pathology in Raleigh, N.C. Isolates were streaked onto SPA (11) to confirm purity and produce bacterial cells for inoculum. After 5 to 7 days incubation at 28°C, an inoculum was prepared by suspending bacterial colonies in sterile distilled water to a concentration of 10^7 to 10^8 CFU/ml. Just prior to inoculation, a loop of the bacterial suspension was streaked onto SPA to confirm bacterial viability. Strawberry plants of variety Dover were used to test the pathogenicity of the isolates. In preliminary studies, all strains of *X. fragariae* tested produced similar numbers of lesions on this variety (26a). The two most recently expanded leaves were inoculated by rubbing a cotton swab saturated with inoculum two to three times across the abaxial leaf surface. Two plants were tested for each isolate. Plants were individually enclosed in clear plastic bags for 2 weeks and maintained in a chamber at 15°C with a 12-h photoperiod. Control inoculations were performed with water and with lesion-forming strain Xf3. Plants were examined for angular lesions at 2 and 3 weeks after inoculation. The presence of typical water-soaked angular lesions similar to those produced by strain Xf3 indicated pathogenicity of an isolate.

DNA amplification conditions for RAPD, REP, and ERIC PCRs. PCR was performed with 25-μl volumes containing PCR buffer (20 mM NaCl, 50 mM Tris [pH 9.0], 1% Triton X-100, 0.1% gelatin [2]), 3 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTP), 0.2 μM primer (RAPD) or 2.0 μM primer (REP and ERIC), 50 ng of template, and 0.5 U of *Taq* DNA polymerase (Life Technologies, Gaithersburg, Md.). For RAPD analysis, primers with 70 to 80%

TABLE 2. Description of primers, annealing temperatures, sizes of PCR products, and specificity

Primer pair[a]	Sequence 5′-3′[b]	Annealing temp (°C)	Minimum no. of cycles[c]	Approx size of PCR product (bp)	Specificity[d]
300A 300B	GGCTAGGGCGTGATCATC GGCTAGGGCGGATTTGCTG	68[e]	35	Many	No unique product
241A 241B	GCCCGACGCGAGTTGAATC GCCCGACGCGCTACAGACTC	70	35	550	*X. fragariae*
245A 245B	CGCGTGCCAGTGGAGATCC CGCGTGCCAGAACTAGCAG	70	25	300	*X. fragariae*
295A 295B	CGTTCCTGGCCGATTAATAG CGCGTTCCTGCGTTTTTTCG	70	25	615	*X. fragariae*

[a] Primers are named for the UBC 10-base primer that produced the original RAPD product.
[b] Except for primer 295A, the first 10 nucleotides in the sequence (underlined) correspond to the sequence of the original UBC 10-base RAPD primer.
[c] Number of cycles required to observe the amplification product in an ethidium bromide-stained gel.
[d] When using the PCR conditions described in the text.
[e] Amplification results were similar when the annealing temperature was 64°C, although the band intensity was greater.

G+C content were selected from UBC set 3 (University of British Columbia Nucleic Acid-Protein Service Unit, Vancouver, British Columbia, Canada). For REP PCR and ERIC PCR analysis, primers were manufactured as described by de Bruijn (5) by Genosys Biotechnologies (The Woodlands, Tex.). DNA amplification was carried out in a thermal cycler (Perkin-Elmer, Norwalk, Conn.) programmed for either 45 cycles (RAPD) or 30 cycles (REP and ERIC) of 1 min at 94°C; 1 min at 48°C (RAPD), 44°C (REP), or 52°C (ERIC); and 2 min at 72°C. PCRs were analyzed on 1.5% agarose–TAE (0.04 M Tris-acetate, 0.001 M EDTA) gels stained with ethidium bromide. All reactions were repeated at least once.

DNA purification, cloning, and sequencing. RAPD bands of interest were purified from agarose gels with Qiaex resin (Qiagen). Purified DNA was cloned into the TA cloning vector (Invitrogen, San Diego, Calif.) as described in the manufacturer's instructions, and the plasmid was introduced into competent *Escherichia coli* strain DH5α (Life Technologies). Plasmid DNA was isolated from *E. coli* with Wizard columns (Promega Corp., Madison, Wis.), and the inserts were partially sequenced with biotinylated primers by the Sequenase Images (U.S. Biochemical, Cleveland, Ohio) protocol for plasmid DNA templates.

Primers and PCR conditions for specific amplification of *X. fragariae* DNA. Oligonucleotide primers specific for *X. fragariae* were manufactured by Cruachem (Dulles, Va.). All primers except 295A consisted of the original 10-base RAPD primer plus the next 8 to 10 nucleotides as determined by sequence analysis (Table 2).

The PCR was performed with 25-μl volumes containing the buffer described previously, 200 μM dNTP, 0.4 μM primer, and 1.0 U of *Taq* DNA polymerase. Fifty nanograms of bacterial DNA or approximately 10,000 bacterial cells were used as the template. DNA was amplified by lysing bacteria at 94°C for 4 min; this was followed by an amplification profile of 94°C for 1 min, 70°C for 1 min, and 72°C for 1 min for 25 to 35 cycles. PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and photographed with the Eagle Eye Still Imaging Video System (Stratagene, La Jolla, Calif.) processed with the option Invert Image to produce a negative image.

Data analysis. RAPD PCR amplification products were listed as discrete character states per strain (presence or absence of band). Similarity coefficients between each strain were calculated by use of the SIMQUAL program in NTSYS-pc, version 1.7 (29), and these data were subjected to UPGMA cluster analysis by use of the SAHN program of NTSYS. Principal component (PC) analysis was performed on the original data set by the PRINCOMP procedure of SAS (30).

Physiological characterization of strains. Several strains were selected for physiological characterization to confirm that their identification as *X. fragariae* was correct. The methods and media were those described by Schaad (31). Cultural characteristics were determined on YDC plates, while growth at 36°C, gelatin hydrolysis, and the production of urease were observed in yeast-salts broth. Protein hydrolysis was observed on nutrient agar plates overlaid with nutrient agar supplemented with yeast extract and skim milk. Acid production from carbohydrates was observed on media supplemented to 0.5% with filter-sterilized carbohydrate by using bromocresol purple as the pH indicator dye.

RESULTS

Pathogenicity tests. Bacterial strains that caused angular leaf spot lesions on strawberry variety Dover are indicated in Table 1. Strain LMG707 was not pathogenic to strawberry variety Dover; however, we have included it among the *X. fragariae* strains on the basis of colony morphology, DNA fingerprint pattern, and physiological tests. It is possible that this strain is pathogenic to other varieties of strawberry. Strains Xf1429 and Xf1431 also were not pathogenic to strawberry variety Dover and produced anomalous RAPD, REP, and ERIC PCR fingerprints (see below).

RAPD, REP, and ERIC PCRs. To ensure reliable RAPD results, 10-nucleotide primers were tested initially by screening four isolates of the *X. fragariae* type strain (ATCC 33239) obtained from different sources (Table 1). Primers that did not produce identical banding patterns for all four isolates were not used in this study. By using this conservative method of initial screening, 20 of the 35 primers tested were selected for further analysis. A total of 84 bands amplified from twenty 10-nucleotide RAPD primers were scored (for example, see Fig. 1A), of which 39 bands amplified from 19 primers were polymorphic.

Similarity values based on the computer analysis of RAPD markers indicate that the pathogenic strains of *X. fragariae* examined in this study are genetically very similar, despite diverse geographical points of isolation. Similarity values between any two of the 22 pathogenic strains ranged from 0.81 to 1.00, with nine different genotypes identified (Fig. 2). Although the REP and ERIC PCR amplification patterns did not define additional genotypes of *X. fragariae* (Fig. 1B and C), they were consistent with the relationships established by RAPD data (Fig. 1A). REP and ERIC primers defined two and four genotypes of pathogenic *X. fragariae*, respectively, and these genotypes corresponded to major branches on the RAPD-generated cladogram (Fig. 2). Strains received as Xf1429 and Xf1431 which were not pathogenic to strawberry plants produced RAPD, REP, and ERIC fingerprints that were very different from those of the pathogenic strains. In contrast, the nonpathogenic strain LMG707 produced RAPD, REP, and ERIC PCR fingerprints identical to those of pathogenic strains LMG705 and LMG706.

The degree of relatedness among individual strains of *X. fragariae* can also be approximated by PC analysis (14). A plot of the first and second PCs of the nine genotypes by using 84 scored RAPD bands was consistent with the UPGMA-generated phenogram and REP and ERIC PCRs (Fig. 3). The first and second PCs accounted for 51 and 23%, respectively, of the total variation. Four RAPD products (amplified by UBC primers 245, 272, and 284) contributed heavily to PC1, and two

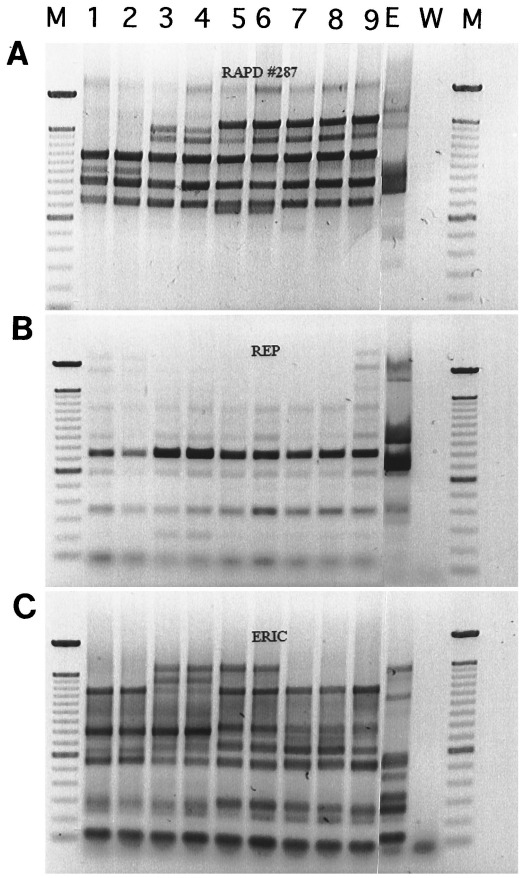

FIG. 1. Amplification of genomic DNA from selected strains of *X. fragariae* by using 10-base RAPD primer UBC 287 (A), REP primers (B), or ERIC primers (C). Lanes: M, 100-bp ladder marker DNA; 1 to 9, pathogenic strains of *X. fragariae* (Xf3, Xf10, LMG705, Xf2510, Xf30, ATCC 33239, Xf16, Xf128, and Xf1425, respectively); E, an epiphytic bacterium isolated from a strawberry leaf; W, water-only PCR negative control.

RAPD products (amplified by UBC primers 230 and 285) contributed heavily to PC2.

Generation of PCR primers specific for *X. fragariae*. Four unique RAPD products, amplified by UBC primers 241, 245, 295, and 300, were chosen on the basis of their amplification size, intensity, and specificity to pathogenic strains of *X. fragariae*. These DNA products were cloned and partially sequenced, and the sequence information was used as the basis for selection of 18- to 20-nucleotide PCR primers specific for pathogenic strains of *X. fragariae* (Table 2). These PCR primers were then tested for specificity by using all of the bacterial strains listed in Table 1 as well as 56 epiphytic strawberry bacteria and 36 strains of *X. campestris*, as described in Materials and Methods. Amplified DNA was detected for all tested pathogenic strains of *X. fragariae* by use of each of the four primer pairs (Fig. 4), while amplified DNA was not detected for any of the *X. campestris* or epiphytic strains tested. The band intensity was dependent on the number of PCR cycles (Table 2). For example, with primers 241A and -B, a PCR product was discernible only after 35 cycles. In contrast, 25 cycles of amplification with primers 245A and -B and 295A and -B resulted in clearly visible DNA products. Results of amplification with primers 300A and -B were poor, and several distinct DNA bands were visible (Fig. 4A), whereas only one 1,300-bp band was produced by using the original 10-nucleotide RAPD primer (data not shown). All of the primers amplified a specific product identical in size to the original cloned RAPD PCR product except for primers 241A and -B. In this case, the original cloned and sequenced RAPD PCR product was 900 bp in length, while the DNA band produced by primers 241A and -B is only 600 bp in length, corresponding to another RAPD band that cosegregated with the 900-bp band (data not shown).

Because they amplified unique products under identical annealing temperatures, three of these primer pairs (241A and -B, 245A and -B, and 295A and -B) made good candidates for multiplexed PCR analysis (3). Thirty-five cycles of amplification of *X. fragariae* DNA with these primers in various combinations resulted in distinct and predictable patterns (Fig. 4B). Identical patterns were consistently produced from a number of arbitrarily selected strains of *X. fragariae* (Fig. 4C).

The limit of detection for each of these primer pairs was determined by visualization of bands in ethidium bromide-stained agarose gels. By using whole cells, approximately 5,000 bacterial cells (as determined by hemacytometer counts) could be detected. Purified bacterial DNA in a PCR could be detected at a level of 50 pg per reaction (data not shown).

Physiological characterization of strains. Colonies of the typical pathogenic strains of *X. fragariae* were medium yellow in color and mucoid on YDC plates, while strain LMG707 was pale yellow and mucoid on YDC plates. The nonpathogenic strains received as Xf1429 and Xf1431 were characterized by dark-yellow mucoid colonies on YDC agar. Strains Xf1429 and Xf1431, which were apparently not *X. fragariae* on the basis of the RAPD, REP, and ERIC PCR data summarized in Fig. 2, and strain LMG707, which was not pathogenic to strawberry variety Dover, were physiologically characterized in parallel

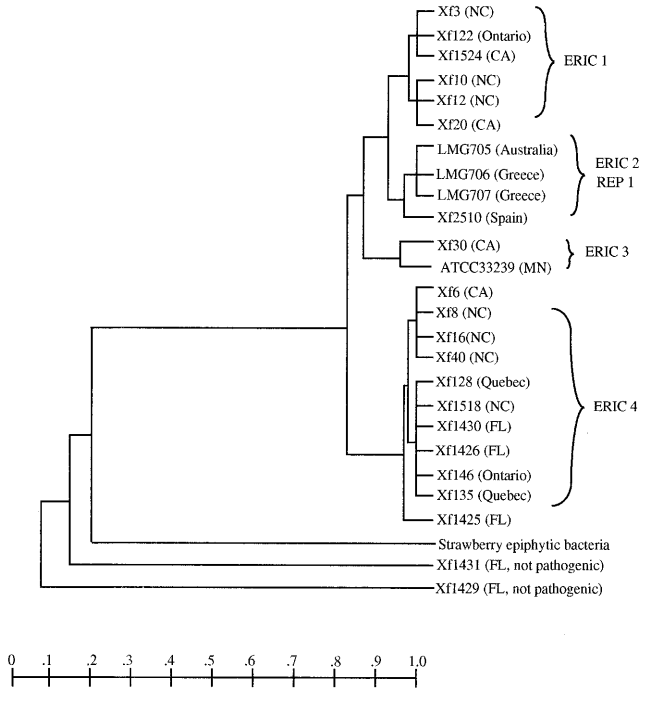

FIG. 2. Phenogram representing computed identity and similarity values among strains of *X. fragariae* based on RAPD data. The nine identical genotypes are indicated by a similarity value of 1.0. REP and ERIC PCR groups, which were not used in constructing the phenogram, are indicated by brackets. REP group 1 corresponds to ERIC group 2. REP group 2 contains ERIC groups 1, 3, and 4.

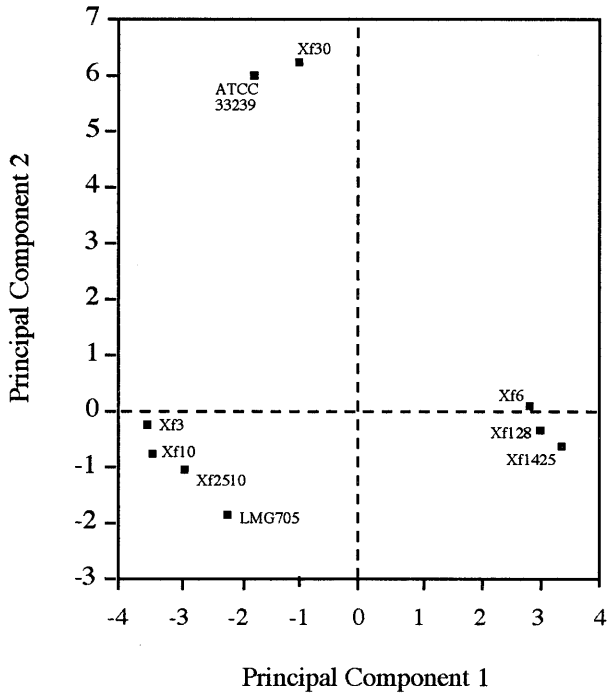

FIG. 3. Plot of first and second PCs from RAPD analysis of pathogenic *X. fragariae*. Symbols represent the nine genotypes defined by RAPDs and are named by one strain from each genotype (see Fig. 2). PC1 and PC2 accounted for 51 and 23% of the total variation, respectively.

with several typical strains of *X. fragariae* and strains of other pathovars of *X. campestris*. The results of the physiological testing were entirely consistent with the RAPD PCR phenogram and the REP and ERIC PCR assays (Table 3). Unlike *X. fragariae*, strains Xf1429 and Xf1431 grew at 36°C, hydrolyzed skim milk protein, and produced acid from arabinose. Strain Xf1429 also liquefied gelatin, and strain Xf1431 produced acid from mannitol, unlike *X. fragariae* (Table 3).

DISCUSSION

Genomic fingerprinting using PCR-based techniques is a reliable, sensitive, and relatively easy method to identify relationships within *X. fragariae* strains and to distinguish this species from similar nonpathogenic species. We have shown previously that estimations of genetic distances within a species of bacteria based on RAPD analysis using carefully selected primers is consistent with RFLP data (25). In the present study, our conservative selection of RAPD primers to enhance the reproducibility of results increased the reliability of the RAPD-generated data.

The phenogram based on RAPD-PCR data presented in Fig. 2 was fully supported by physiological data presented in Table 3. This provides a valuable independent confirmation of our genomic similarity estimates. Significantly, the genomic similarity estimates based on RAPD-PCR data were also fully supported by the REP and ERIC PCR fingerprints, which resolved the pathogenic *X. fragariae* strains into two and four distinct groups, respectively. REP and ERIC PCRs utilize primers derived from consensus sequences of highly conserved repetitive DNA elements from gram-negative bacteria (34). These primers have been useful in discriminating among species and among pathovars within a single species (19, 20). In our study, the four ERIC PCR groups correspond to the four major branches of pathogenic *X. fragariae* on the RAPD PCR-generated similarity phenogram (Fig. 2) and on the PC analysis scattergram (Fig. 3), thereby validating the estimates of genetic relationships based on RAPD PCR. Thus, RAPD, REP, and ERIC PCRs reveal similar genotypic groupings. It is possible that RAPD PCR distinguishes a larger number of genotypes than REP or ERIC PCR does because it employs many more primers and therefore likely samples a larger and more random part of the genome.

Three strains from our collection gave apparently anomalous results in our PCR assays which were resolved by physiological tests. Strain LMG707 was indistinguishable from strains LMG705 and LMG706 on the basis of RAPD, REP, and ERIC PCR fingerprints. However, unlike strains LMG705 and LMG706, strain LMG707 did not cause angular leaf spot lesions on strawberry variety Dover. The identification of LMG707 as *X. fragariae* was confirmed by physiological testing (Table 3). Because of its genetic and physiological similarity to pathogenic strains (Fig. 2; Table 3), LMG707 is possibly pathogenic to other strawberry varieties. In contrast, it is significant that the RAPD, REP, ERIC, and multiplexed PCR assays described herein distinguished strains Xf1429 and Xf1431 from all strains of *X. fragariae*. This result was confirmed by physiological testing. We do not know whether the cultures were incorrect as received by us or if they became contaminated in our hands, but the strains apparently are members of *X. campestris* (Table 3).

The high degree of similarity among pathogenic strains of

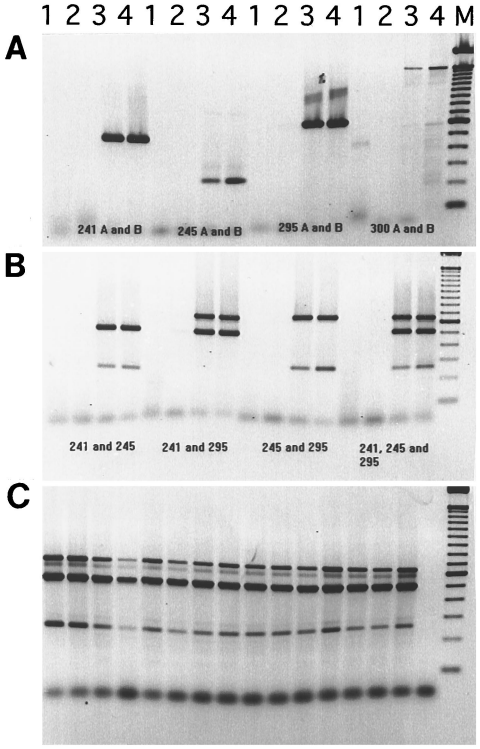

FIG. 4. Amplification of bacterial DNA by using *X. fragariae*-specific primers in a single-primer-pair PCR (A) or multiplex PCR (B and C) for 35 cycles. Primer pairs are indicated on the gel. (A and B) Lanes: 1 to 4, Xf1429, Xf1431, LMG707, and ATCC 33239, respectively. (C) Lanes: left to right, results of multiplexed PCR amplification of *X. fragariae* strains LMG705, LMG706, Xf3, Xf6, Xf8, Xf10, Xf12, Xf16, Xf20, Xf128, Xf1290, Xf1238, Xf1240, Xf1241, LG2510, and the no-template PCR negative control, respectively. Lane M, 100-bp ladder marker DNA.

TABLE 3. Physiological characterization of selected strains used in this study[a]

Strain	Growth at 36°C	Urease production	Protein digestion	Gelatin liquefaction	Acid produced from:			
					Dextrose	Arabinose	Mannose	Mannitol
Strains included in Fig. 2								
LMG705	−	−	−	−	+	−	+	−
LMG706	−	−	−	−	+	−	+	−
LMG707	−	−	−	−	+	−	+	−
Xf3	−	−	−	−	+	−	+	−
ATCC 33239	−	−	−	−	+	−	+	−
Xf1429	+	−	+	+	+	+	+	−
Xf1431	+	−	+	−	+	+	+	+
Other xanthomonads								
XC63	+	−	+	+	−	+	−	−
F1	+	−	+	+	+	+	+	+
X6	+	−	+	+	+	+	+	+
XV907	+	−	+	+	+	+	+	+

[a] Methods were as described by Schaad (31). Results were scored after 10 days of incubation. Strains XC63, F1, X6, and XV907 are *X. campestris* pathovars citri, citrumelo, campestris, and vesicatoria, respectively.

X. fragariae (0.81 to 1.0) has also been observed among *X. campestris* species (9, 32). The similarity among *X. fragariae* strains may be related to clonal propagation, host selection, and the ease with which strains can be spread unintentionally by means of exchange of asymptomatic plant material. The lack of association between the geographic origins of strains with similar or identical fingerprints makes tracing the historical spread of the disease extremely difficult at present. It is possible that further investigation of RAPD PCR fingerprinting would be helpful in this regard.

The three PCR primer pairs 241, 245, and 295 each directed the amplification of specific products from all pathogenic strains of *X. fragariae* tested in standard PCR assays (Fig. 4A). Specificity was preserved with multiplexed PCR (Fig. 4B). Amplified DNA was not detected for any of the 62 epiphytic bacterial strains isolated from strawberry plants or for the 40 tested isolates of *X. campestris*, including one strain of *X. campestris* pv. pelargonii (not shown). A recent preliminary report of *X. fragariae*-specific PCR primers developed from the *hrp* genes indicated that DNA was amplified from a strain of *X. campestris* pv. pelargonii (28) as well as *X. fragariae.*

The limit of PCR detection of *X. fragariae* by using the reaction conditions described was approximately 5,000 bacteria or 50 pg of purified DNA. The level of sensitivity may be a reflection of the high annealing temperature used in the PCR assay or the fact that these primer pairs amplify single- or low-copy-number target sequences. The sensitivity of PCR-based detection assays for bacterial species can be dramatically improved by nested PCR and immunocapture methods (10) or by hybridization capture techniques (15).

In developing PCR primers specific for *X. fragariae*, we encountered several unexpected results. By using primers 300A and -B, several DNA bands were only poorly amplified, whereas only one 1,300-bp DNA fragment was produced by the original 10-nucleotide RAPD primer. Such results may suggest nonspecific annealing of primers 300A and -B and limit the usefulness of this set of primers. Primers 241A and -B amplified a clearly visible DNA product, although this band was approximately 300 bp smaller than the original cloned and sequenced RAPD product.

We have characterized the genetic diversity within *X. fragariae* and developed three sets of PCR primers specific to the limited number of pathogenic strains of *X. fragariae* tested. This provides a first step towards control of the disease by demonstrating the basis for a sensitive and rapid method to identify the pathogen. Because these primers were derived from random points in the genome, each primer pair represents an independent test for *X. fragariae*. Additionally, because the primer pairs use an identical stringent annealing temperature, they can be tested together by use of a single multiplexed PCR assay. Our laboratory is currently investigating protocols for increasing the sensitivity of the PCR assay and applying it to strawberry plant material and testing the assay against additional epiphytic bacteria isolated from strawberry plants. As with any identification test, confirmation of PCR data by independent methods is advisable.

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