Identification of *Xanthomonas fragariae* Field Isolates by rep-PCR Genomic Fingerprinting

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ABSTRACT

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Xanthomonas fragariae, the causal organism of angular leaf spot on cultivated strawberry (*Fragaria* × *ananassa*), is an economically important pathogen of nursery stock in California. The ability to reliably detect this pathogen in a timely manner is crucial for the production and timely distribution of disease-free nursery stock. Pathogenicity testing for this disease requires excessive time, and the bacterium grows slowly on standard culture medium. A medium, similar to that used for culturing *Xylella fastidiosa*, allowed more consistent recovery of *X. fragariae* from infected strawberry plants. Using the polymerase chain reaction (PCR) with primers that anneal to dispersed repetitive bacterial sequences (rep-PCR), we generated genomic fingerprints of reference strains of *X. fragariae* (ATCC 33239 and 33240). These fingerprints were used, in turn, to accurately identify *X. fragariae* field isolates collected over the last 5 years from nurseries in California. The rep-PCR fingerprint results agree with pathogenicity test results, require much less time than the pathogenicity test, and have greater specificity than indirect enzyme-linked immunosorbent assay for identifying *X. fragariae* from field plants. For these reasons, rep-PCR is the fastest and most accurate method for the current identification of *X. fragariae* and it constitutes a useful tool for the production of disease-free strawberry nursery stocks.

Strawberry bacterial leaf-blight, also known as angular leaf spot, is caused by the gram negative bacterium *Xanthomonas fragariae*. This disease was first observed in Utah by Linford in 1927 (13), and the causal agent was determined to be *X. fragariae* in the early 1960s (11,12). Since then, the disease has been observed in several strawberry (*Fragaria* × *ananassa* Duchesne) fruit production areas in the U.S. (9,24) including California nurseries (7), and in other strawberry-producing countries around the world (1,6,20,21,23). Geographical dissemination of the bacterium occurs through contaminated planting stock (19). This can readily occur, because vegetatively propagated plants are trimmed to remove most of the leaves before cold storage and shipment, thereby removing any obvious symptoms. The disease is known to be systemic, invading the vascular system and crown of the plant, and it can cause complete vascular collapse and death of plants (8).

Early-season field diagnosis has been based on angular leaf spot symptoms, which include water-soaked, translucent leaf lesions. In mid- to late-summer, the lesions dry and become brown or black, sometimes displaying red margins. At this stage, angular leaf spot can be confused with common leaf spot caused by *Mycosphaerella fragariae* (Tul.) Lindau or leaf scorch caused by *Diplocarpon earlianum* (Ellis & Everh.) F. A. Wolf (19). Vascular collapse and crown rot symptoms can also be easily confused with symptoms of *Phytophthora cactorum* (Lebert & Cohn) J. Schröt infection (19). Since the strawberry nursery crop has considerable value and most international exportation permits require uninfected plants, this disease is regulated in California through the Nursery Inspection Program (2). This program currently demands a timely and precise identification of *X. fragariae* in suspect planting stock.

Clinical isolation of the pathogen is difficult even from the freshest, early-season, symptomatic plant material. The pathogen is slow-growing (28), does not form colonies easily on most agar-based, solid media (7), and can be quickly overgrown by other contaminating bacteria. Reliable pathogenicity tests generally require 10 to 15 days, as well as a constant supply of disease-free plants. This necessitates consid-erable greenhouse space for propagation of disease-free plants and separate areas for conducting pathogenicity tests. Enzyme-linked immunosorbent assay (ELISA) testing for *X. fragariae* may improve detection and is rapid (26), but ELISA reactions are not completely specific due to cross-reactions of the antisera with some unidentified saprophytic bacteria (14,29). For these reasons, ELISA is not currently used for the detection of *X. fragariae* in the California Nursery Inspection Program. Our research was directed at improving isolation techniques and the specific identification of *X. fragariae* with DNA-based methods, using the polymerase chain reaction (PCR).

All prokaryotes tested to date, including phytopathogenic *Xanthomonas* spp. and *Pseudomonas* spp., have families of repetitive DNA sequences (REP, ERIC, BOX) (31) throughout their genome (18) that can be used as specific primer sites for PCR-based fingerprinting methods (5,15, 30). REP-, ERIC- and BOX-PCR (collectively referred to as rep-PCR) (31) have been successfully employed to identify a variety of plant-associated bacteria including rhizobia, agrobacteria, pseudomonads, xanthomonads, and *Clavibacter* strains (5, 15–17,22,31). In this paper, we report the use of the rep-PCR method with REP and ERIC primers to generate unique genomic fingerprints of *X. fragariae*, which facilitated the rapid and reliable identification of this pathogen.

MATERIALS AND METHODS

Source and maintenance of cultures. All reference bacterial strains are listed in Table 1 and were obtained from the American Type Culture Collection (ATCC) or the California Department of Food and Agriculture (CDFA) collection. All field isolates obtained between 1990 and 1994 originated from nursery-grown plants from various sites in California and were isolated at the University of California, Berkeley, the University of California, Davis, or CDFA. All cultures were preserved in nutrient broth containing 15% (wt/vol) glycerol and were stored at –68°C. Just prior to their use, the stocks were thawed, and the cultures were streaked on solid medium to confirm their purity.

Culture media and isolation of *X. fragariae*. Since this study began, in 1990, several media were evaluated for the culture of known *X. fragariae* strains and for isolation from symptomatic leaf tissues. In each case, visible growth of *X. fragariae* required more than 5 days, and colonies were easily overgrown by other contaminating bacteria. Thus, *X. fragariae* has been considered to be fastidious and may be sensitive to an unknown compound(s) in commercial agar. A solid medium that was developed for culture of *Xylella fastidiosa* (4) and does not contain agar was tested for *X. fragariae* growth, and extremely small, single colonies of *X. fragariae* were observed in 4 days. Further experimentation resulted in the simplification of the Pierce's disease medium (PD) to Pierce's disease modified medium (PDM), containing 5.0 g of dextrose (Sigma, St. Louis MO), 2.5 g of Phytone (BBL Microbiology Systems, Baltimore, MD), and 10 g of Phytagel (BBL Microbiology Systems) per liter. The pH was adjusted to 7.5 with 2 N NaOH before autoclaving. This medium facilitates the growth of *X. fragariae* relative to other media, but it does not select against other bacteria. PDM has been used at CDFA since 1992 for the isolation of *X. fragariae* from early-season strawberry leaves with distinct, angular, water-soaked lesions. To isolate *X. fragariae* from strawberry leaves, the plant tissue was surface sterilized, macerated, and diluted in sterile water, and the macerate was streaked onto fresh PDM. After 4 days, growth was barely discernible and by 7 days light-yellow colonies were observed, which later became mucoid.

Pathogenicity assays. Initial pathogenicity assays were conducted with spray infiltration (7,26) or pressure injection (12). Pressure injection inoculation was more effective and was modified slightly to utilize single-application, plastic syringes, which saved considerable time. Water controls and known pathogenic *X. fragariae* isolates, ATCC 33239 and ATCC 33240, were included in each experiment when unknown bacterial isolates were evaluated. In the first year of testing, water-inoculated negative control plants occasionally developed disease symptoms, possibly due to a previously undetected infection of stock plants. For subsequent pathogenicity tests, test plants were grown from tissue culture stocks and mock inoculations of the plants never developed disease symptoms, thus they were apparently free of bacterial contamination.

Strawberries (cv. Pajaro) were grown in pots in a greenhouse under day lengths ranging from 10 to 12 h at a temperature ranging from 25 to 28°C. A bacterial suspension for inoculation was prepared by suspending cells of a 4- to 5-day-old culture in sterile water to a concentration of $OD_{590} = 0.2$ (1×10^8 CFU/ml) (26). Fully expanded leaves were inoculated at five or more sites by pressure injection with 100

µl of the bacterial suspension. Individual plants were kept in plastic bags for 2 days following inoculation, then plants were kept separated on greenhouse benches. In 2 days, the initial hydrosis caused by the injection method disappeared, but occurred again in 7 to 10 days if isolates were pathogenic. Plants were evaluated for disease at 10 and 15 days for primary and secondary lesions, respectively. The secondary lesions appeared later on inoculated leaves away from the initial sites of inoculation, and *X. fragariae* was reisolated from these lesions.

Bacterial identification using the Biolog GN MicroPlate system. Samples were analyzed according to the manufacturer's protocol (Biolog Inc., Hayward, CA). Bacteria were grown on trypticase soy agar (BBL Microbiology Systems) for 24 h at 25°C. Bacterial cells were removed from plates with sterilized cotton swabs and transferred to a sterile saline solution (0.85% (wt/vol) NaCl). The density of the cell suspension was then adjusted to match the Biolog GN MicroPlate system's turbidity standards. Biolog GN MicroPlates were immediately inoculated with 150 µl of cell suspension per well and incubated for 24 h at 28°C. Plates were read visually and results were analyzed with Biolog GN database version 3.01.

ELISA methods. Polyclonal antibodies prepared against *X. fragariae* that were used in these experiments were obtained from D. Gubler, Department of Plant Pathology, University of California, Davis (25). These antibodies were tested extensively against many different bacterial strains and were shown to be quite specific for *X. fragariae* in that study, which utilized the $F(ab)_2$ ELISA technique (26). Indirect ELISA (I-ELISA) (3) with a commercial anti-rabbit alkaline phosphatase conjugate (Sigma, St. Louis, MO) was used for all ELISAs reported here. Bacterial isolates were grown overnight in liquid nutrient broth; the cells were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS). Bacterial suspensions were adjusted to $OD_{590} = 0.2$ (1×10^8 CFU/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and 100 µl of this bacterial suspension was applied to wells of polyvinyl ELISA plates. The plates were then incubated overnight at 37°C. Plates were washed with PBS and blocked with 1% (wt/vol) bovine serum albumin (Sigma, St. Louis, MO) for 30 min. The polyclonal antibody was diluted to 2 µg/ml (26), applied to the wells and incubated at room temperature for 4 h. After washing with PBS, the anti-rabbit alkaline phosphatase conjugate, diluted

Table 1. Comparison of pathogenicity and indirect (I-) enzyme-linked immunosorbent assay (ELISA) for identification of *Xanthomonas fragariae*, other *Xanthomonas* spp., and isolates obtained from California strawberry nurseries

Bacterial species or field isolates	Source[a]	Strain no.	Pathogenicity[b]	ELISA[c]
X. campestris pv. *translucens*	CDFA	104	Neg	++++
pv. *translucens*	CDFA	103	Neg	+++
pv. pv. *pelargonii*	ATCC	8721	Neg	++
pv. *nerium*	CDFA	278	Neg	+++
pv. *nerium*	CDFA	151	Neg	++
pv. *undulosa*	CDFA	175	Neg	++++
pv. *undulosa*	CDFA	93	Neg	++++
pv. *dieffenbachiae*[d]	CDFA	90-4	Neg	–
pv. *fici*[d]	CDFA	90-9	Neg	+
Xanthomonas sp.[d]	CDFA	90-8	Neg	++
Enterobacter sp.[d]	CDFA	93-5	Neg	–
X. fragariae	ATCC	33240	Pos	+
X. fragariae	ATCC	33239	Pos	++++
X. fragariae	ATCC	29076	Pos	++++
X. fragariae	Field	2062	Pos	+++
X. fragariae	Field	2063	Pos	++++
15 field isolates (1990)	Field	90-1 to 90-15	15: Neg	2: + 13: –
15 field isolates (1991)	Field	91-1 to 91-15	15: Pos	15: +++
15 field isolates (1992)	Field	92-1 to 92-15	15: Pos	15: +++
15 field isolates (1993)	Field	93-1 to 93-15	14: Pos 1: Neg	14: +++ 1: –
72 field isolates (1994)	Field	94-1 to 94-72	72: Pos	72: +++

[a] CDFA, California Department of Food and Agriculture culture collection; ATCC, American Type Culture Collection; Field, sample materials collected from California strawberry nurseries.

[b] Pos = strain pathogenic on strawberry in greenhouse assays; Neg = strain not pathogenic according to this assay.

[c] (+) = I-ELISA absorbance readings at least twice that of water controls, (++) = I-ELISA value greater than four times that of water controls, (+++) = I-ELISA value greater than 8 times that of water controls, (++++) = I-ELISA value greater than 16 times that of water controls.

[d] As determined by the Biolog GN MicroPlate identification system.

1:1,000 in PBS, was applied to the plates and incubated at room temperature for 1.5 h. After a thorough washing with PBS, p-nitro phenol substrate (3) was added and incubated for 15 to 60 min. A positive reaction was determined to be at least twice background, where background was OD_{405} = 0.057. Reactions were rated on the basis of OD_{405}, where + represented values greater than twice background (OD_{405} > 0.114), ++ represented values greater than four times that of background (OD_{405} > 0.228), +++ represented values greater than eight times that of background (OD_{405} > 0.456), and ++++ represented values greater than 16 times that of background (OD_{405} > 0.912).
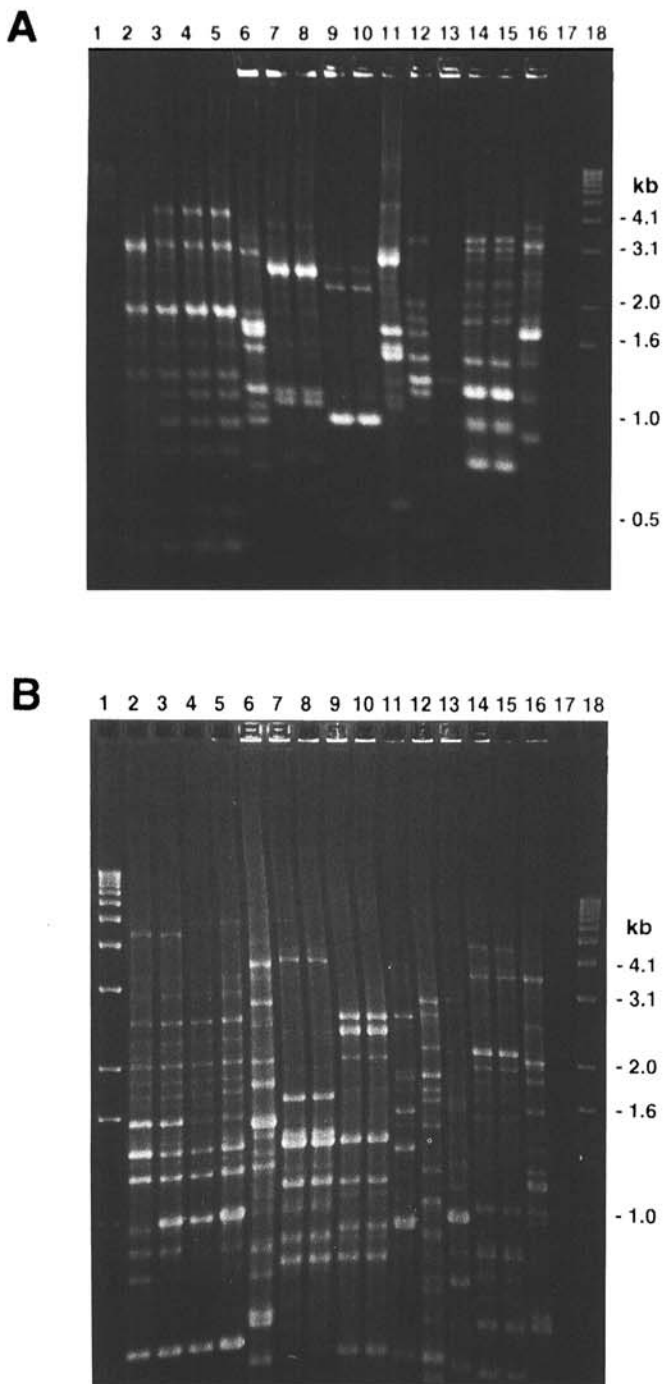
Fig. 1. Genomic fingerprints of *Xanthomonas* species generated from (A) REP-PCR or (B) ERIC-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lanes 1 and 18, molecular size standards; lane 2, *X. fragariae* ATCC 33240; lane 3, *X. fragariae* ATCC 33239; lane 4, *X. fragariae* CDFA 2062; lane 5, *X. fragariae* CDFA 2063; lane 6, *X. campestris* pv. *pelargonii* ATCC 8721; lane 7, *X. campestris* pv. *translucens* CDFA 103; lane 8, *X. campestris* pv. *translucens* CDFA 104; lane 9, *X. campestris* pv. *undulosa* CDFA 93; lane 10, *X. campestris* pv. *undulosa* CDFA 175; lane 11, *X. campestris* pv. *campestris* CDFA 1480; lane 12, *X. campestris* pv. *fici* CDFA 90-9; lane 13, *X. campestris* pv. *juglandis* CDFA 145; lane 14, *X. campestris.* pv. *nerium* CDFA 278; lane 15, *X. campestris* pv. *nerium* CDFA 151; lane 16, *X. campestris* pv. *dieffenbachiae* CDFA 90-4; and lane 17, water control.

PCR assays using rep primers (rep-PCR). The PCR reaction mixture and amplification conditions were essentially the same as those described by Louws et al. (15). REP and ERIC oligonucleotide primers, identical to those used in previous studies (5,15,17,30,31), were purchased either from Michigan State University (East Lansing) or from Oligo's Etc. (Wilsonville, OR). The sequences of the primers were as follows: REP1R-I, 5'-IIIICGI CGICATCIGGC-3'; REP2-I, 5'-ICGICTT ATCIGGCCTAC-3'; ERIC1R, 5'-ATGTA AGCTCCTGGGGATTCAC-3'; ERIC2, 5' -AAGTAAGTGACTGGGGTGAGCG-3'. PCR reactions were performed with 1× reaction buffer (5× reaction buffer contains 83 mM ammonium sulfate, 335 mM Tris HCl pH 8.8, 33.5 mM magnesium chloride, 33.5 μM EDTA, 150 mM 2-mercaptoethanol), 0.85 mg of bovine serum albumin (Boehringer Mannheim Biochemicals, Indianapolis, IN) per ml, 10% (vol/vol) dimethyl sulfoxide, 1.25 mM each dNTP, 62 pmol each primer, and 2 U Ampli*Taq* DNA polymerase (Perkin Elmer Cetus, Branchburg, NJ). Bacterial isolates to be analyzed were taken from streaks on PDM. Bacteria from a representative colony of each isolate were transferred, using a sterile 10-μl pipette tip, to a PCR reaction tube containing 25 μl of the reaction mixture (15). Reaction mixtures were overlaid with silicone oil (Aldrich Chemical Company, Milwaukee, WI) and PCR was performed as described (15) in a DNA Thermal Cycler 480 (Perkin Elmer Cetus). Amplification conditions were as follows: 1 cycle at 95°C for 6 min followed by 35 cycles at 94°C for 1 min, 44°C (REP primers) or 52°C (ERIC primers) for 1 min, 65°C for 8 min. The amplification cycles were followed by a final extension cycle of 68°C for 16 min. A 7-μl portion of the resulting amplification reaction was electrophoresed in a 1.5% (wt/vol) agarose gel at room temperature for 4 h at 5 V/cm in Tris-acetate/EDTA (0.04 M Tris-acetate, 0.001 M EDTA) electrophoresis buffer (27). Amplified DNA fragments were stained with ethidium bromide and photographed on a UV transilluminator with Polaroid type 667 film. Fingerprints generated from different strains were compared visually.

RESULTS

Pathogenicity assays. Strawberry plants (cv. Pajaro) were inoculated with *X. fragariae* reference strains (ATCC 33239 and 33240), a *X. campestris* pv. *pelargonii* reference strain (ATCC 8721), previously characterized CDFA strains, and field isolates of bacteria from strawberry. Inoculated plants were observed for development of angular lesions in 10 to 15 days (Table 1). These symptoms are typical and indicative of *X. fragariae* and do not develop upon inoculation with other bacteria that are not pathogens of strawberry. All of the field isolates from 1990 and one of the

field isolates from 1993 resembled *X. fragariae* colonies in culture, but were not pathogenic on strawberry (Table 1). By means of the Biolog identification system, the isolates from 1990 were determined to be closely related *Xanthomonas* spp., including *X. campestris* pv. *dieffenbachiae* and *X. campestris* pv. *fici*. The field isolate from 1993 that was not pathogenic on strawberry was identified as *Enterobacter sp.* based upon the Biolog identification system (Table 1). None of the *X. fragariae* strains reacted in the Biolog system and therefore could not be identified by this system; this is consistent with previous findings (10).

Detection of *X. fragariae* by I-ELISA. Polyclonal antibodies produced strong positive reactions (greater than 16 times background) in I-ELISA with two of three *X. fragariae* reference isolates, ATCC 33239 and 29076; however, ATCC isolate 33240 did not react as strongly (twice background) (Table 1). When tested by I-ELISA against other species of bacteria, the *X. fragariae* polyclonal antibodies produced false positives with several pathovars of *X. campestris* (Table 1). These antibodies reacted positively with most of the field isolates that were positive in pathogenicity tests. The exceptions were two isolates from 1990, which gave weak positive reactions in I-ELISA (twice background) but were negative according to the pathogenicity assay (Table 1). These two

isolates were later identified as *X. campestris* pv. *fici* and a *Xanthomonas* sp. according to the Biolog identification system.

Identification of *X. fragariae* by REP- and ERIC-PCR. A unique genomic fingerprint for *X. fragariae* was generated by both REP- and ERIC-PCR (Fig. 1). All field isolates that were positive in pathogenicity tests produced very similar REP- or ERIC-PCR banding patterns. These patterns were similar to the patterns from *X. fragariae* reference strains and were distinct from those of all other xanthomonads examined in this work (Fig. 1). However, of the 15 field isolates from 1990 tested, none were pathogenic on strawberry and none produced a fingerprint matching the *X. fragariae* reference strain (ATCC 33239). Upon changing the medium used to isolate from suspect strawberry plants, all of the 15 field isolates from 1991 and 1992 produced genomic fingerprints that matched the reference strain of *X. fragariae*, while 14 of the 15 isolates from 1993 had characteristic *X. fragariae* fingerprints (Fig. 2). In all cases, strains that produced similar REP-PCR patterns also produced ERIC-PCR patterns that were similar to each other. Since each PCR method was equally effective in identifying *X. fragariae*, and a large number of isolates needed to be tested in 1994, only one PCR assay (REP-PCR) was performed during 1994. Of the isolates from 1994 tested, all 72 produced a REP-PCR

genomic fingerprint characteristic for known *X. fragariae* isolates (Fig. 3). *X. campestris* pv. *fici* was included in this experiment to show a contrasting REP-PCR genomic fingerprint (Fig. 3, lane 15).

DISCUSSION

The rapid and accurate identification of *X. fragariae* is important to the strawberry industry. While symptoms are useful for field diagnosis of strawberry angular leaf spot disease, the unambiguous identification of the pathogen is necessary to ensure disease-free nursery stock and to satisfy export requirements. The value of strawberry nursery stock demands the use of an identification method that positively identifies the pathogen, while minimizing the possibility of false positives. Pathogenicity testing can accurately identify *X. fragariae;* however, this method is impractical in some situations because of physical and economic constraints including greenhouse space, time, and labor. Additionally, systemic infections of *X. fragariae* can be asymptomatic initially. If systemically infected but asymptomatic plants are used for pathogenicity testing, this may lead to false positives when symptoms develop later. Therefore, it is crucial that plants used for pathogenicity testing be propagated from disease-free tissue culture stock.

With the I-ELISA procedures described in this study, the polyclonal antiserum was
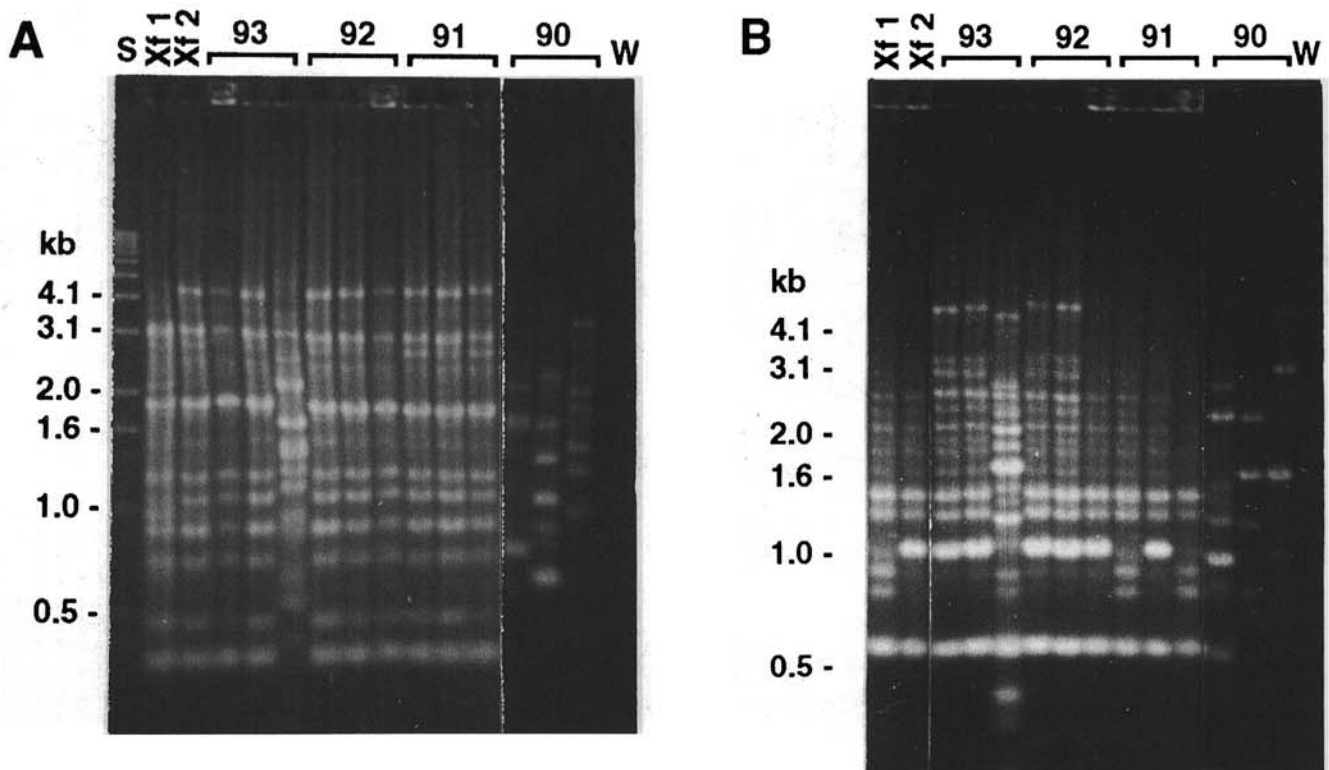
Fig. 2. Genomic fingerprints of field isolates collected from California nurseries in 1990 to 1993 generated from (**A**) REP-PCR or (**B**) ERIC-PCR. Polymerase chain reaction products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane S, molecular size standard; lane Xf1, *X. fragariae* isolate ATCC 33240; lane Xf2, *X. fragariae* isolate ATCC 33239; lane 93, three field strains from 1993; lane 92, three field strains from 1992; lane 91, three field strains from 1991; lane 90, three field strains from 1990; and lane W, water control.

not useful for characterizing *X. fragariae*, because these antibodies cross-reacted with other reference strains of *Xanthomonas* (Table 1). It is not unexpected that species within a genus would have common surface epitopes (29). In addition, two field isolates from 1990, which were weakly positive with the polyclonal antibodies, were nonpathogenic on strawberry. The results reported here differ from those obtained by Rowhani et al. (26) even though the same polyclonal antiserum and some of the same reference bacterial strains were used in both studies. These differences are most likely due to the different type of ELISA procedures used in the two studies. Rowhani et al. used F(ab)₂/Protein A ELISA, while this study used conventional I-ELISA. The F(ab)₂ ELISA system uses F(ab)₂ fragments to selectively trap bacterial cells in the polystyrene well, while bacterial cells are nonspecifically adsorbed onto the wells in I-ELISA. The observed differences in specificity may be due to fewer numbers of heterologous bacterial cells being trapped in the F(ab)₂ system than in the I-ELISA system. If this occurred, then higher heterologous ELISA values would be expected in the I-ELISA because more bacterial cells were present in the well. In addition, differences in the specificity of the Protein A conjugate used in F(ab)₂ ELISA, compared with the anti-rabbit conjugate used in I-ELISA, could also contribute to the observed differences. In this study, rep-PCR gave more reliable identification of *X. fragariae* than I-ELISA.

ERIC- and REP-PCR of known *X. fragariae* isolates generated specific genomic DNA fingerprints of this bacterium (Fig. 1). By comparing known fingerprints with those generated from field isolates (Figs. 2 and 3), it was possible to identify *X. fragariae*. Although there were occasional differences in the intensity of specific amplified fragments (Fig. 2B), the overall genomic fingerprints of field isolates collected from California over 4 years suggests that the *X. fragariae* population in California is homogeneous (Figs. 2 and 3), despite its presence in the state for more than 25 years (8). Some intrapathovar diversity has been identified in *P. syringae* pv. *syringae*, *X. campestris* pv. *campestris*, and *X. campestris* pv. *vesicatoria* by means of this technique (15,17). The genomic fingerprints generated with REP- and ERIC-PCR appeared unique to *X. fragariae* when compared with those of other xanthomonads and nonpathogenic bacteria isolated from strawberry plants in 1990 (Fig. 1). Future studies using REP- and ERIC-PCR on geographically distinct isolates of *X. fragariae* may reveal differences that could provide a means to identify and characterize strains from around the world, as has been shown for *X. campestris* pv. *vesicatoria* (17) and *Rhizobium meliloti* populations (25).

In the first year of this study (1990), none of the field isolates collected were *X. fragariae*, according to pathogenicity tests and rep-PCR (Table 1; Fig. 2). This was due to inexperience in culturing the bacterium and the lack of an appropriate medium for in vitro culture. Improvement of the culture medium and isolation techniques in the second year of the study resulted in the consistent and reliable isolation of *X. fragariae* throughout the course of this study. The isolates from 1990, however, proved to be valuable negative controls for our PCR assays, since these bacteria represent strains that were isolated from field-grown strawberry plants. None of these isolates had the same genomic fingerprint as *X. fragariae*, and none were pathogenic on strawberry according to the greenhouse pathogenicity assay.

All of the field isolates that produced a genomic fingerprint typical of *X. fragariae* were also pathogenic on strawberry. However, the time required for isolation on PDM and PCR testing was approximately 1 week, compared with almost 3 weeks required for pathogenicity testing. Additionally, no false positives were ever identified with REP- or ERIC-PCR, as was the case with I-ELISA (Table 1). Therefore, we feel that this test is the fastest and most accurate identification method for *X. fragariae* in California that is available at this time.

LITERATURE CITED
1. Bradbury, J. F. 1977. *Xanthomonas fragariae*. No. 558 in: Descriptions of Pathogenic Fungi and Bacteria. Commonw. Mycol. Inst. Kew, England.
2. California Code of Regulations. Section 3060.2. CDFA, Sacramento, CA.
3. Clark, M. F., and Bar-Joseph, M. 1984. Enzyme immunosorbent assays in plant virology. Methods Virol. 7:51-85.
4. Davis, M. J., Percell, A. H., and Thompson, S. V. 1978. Pierce's disease of grapevines: Isolation of the causal bacterium. Science 199:75-77.
5. de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58:2180-2187.
6. Dye, D. W., Wilkis, J., and Wilkis. P. 1973. Angular leaf spot of strawberry in New Zealand. N. Z. J. Agric. Res. 16:311-314.
7. Hazel, W. J., Civerolo, E. L., and Bean, G. A. 1980. Procedures for growth and inoculation of *Xanthomonas fragariae*, causal organism of angular leaf spot of strawberry. Plant Dis. 64:178-181.
8. Hildebrand, D. C., Schroth, M. N., and Wilhelm, S. 1967. Systemic invasion of strawberry by *Xanthomonas fragariae* causing vascular collapse. Phytopathology 57:1260-1261.
9. Howard, C. M. 1971. Occurrence of strawberry angular leaf spot, *Xanthomonas fragariae*, in Florida. Plant Dis. Rep. 55:142.
10. Jones, J. B., Chase, A. R., and Harris, G. K. 1993. Evaluation of the Biolog GN MicroPlate system for identification of some plant-pathogenic bacteria. Plant Dis. 77:553-558.
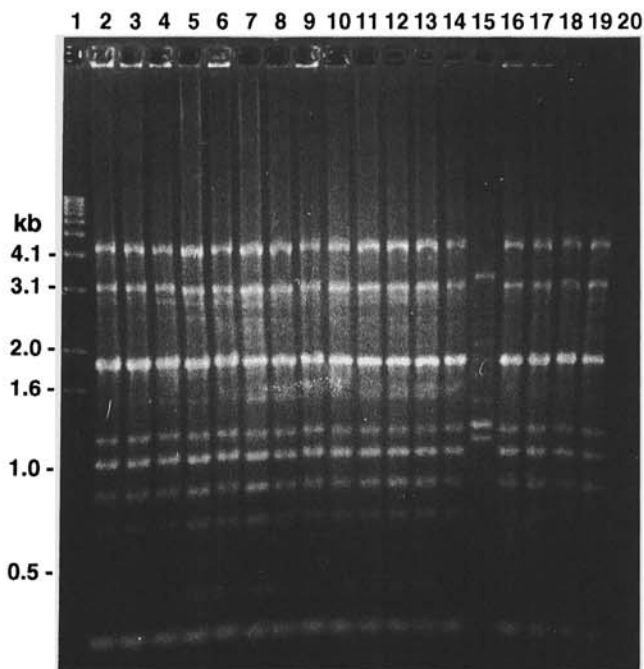11. Kennedy, B. W., and King, T. H. 1960. Angular leaf spot, a new disease of strawberry.

Fig. 3. Genomic fingerprint of field isolates collected in 1994 generated from REP-PCR. Samples were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1, molecular size standard; lane 2, 94-1; lane 3, 94-2; lane 4, 94-3; lane 5, 94-4; lane 6, 94-5; lane 7, 94-6; lane 8, 94-7; lane 9, 94-8; lane 10, 94-9; lane 11, 94-10; lane 12, 94-11; lane 13, 94-12; lane 14, 94-13; lane 15, *X. campestris* pv. *fici*; lane 16, 94-14; lane 17, 94-15; lane 18, 94-16; lane 19, 94-17; and lane 20, water control.

Phytopathology 50:641-642.

12. Kennedy, B. W., and King, T. H. 1962. Angular leaf spot of strawberry caused by *Xanthomonas fragariae* sp. nov. Phytopathology 52:873-875.

13. Linford, M. B. 1927. Plant Diseases in Utah in 1927. Plant Dis. Rep. Supp. #59.

14. Lopez, M. M., Cambra, M., Aramburu, J. M., and Bolinches, J. 1987. Problems of detecting phytopathogenic bacteria by ELISA. Bull. OEPP/EPPO 17:113-117.

15. Louws, F. J., Fulbright, D., Stephens, C., and de Bruijn, F. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.

16. Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 85:528-536.

17. Louws, F. J., Schneider, M., and de Bruijn, F. J. Assessing genetic diversity of microbes using repetitive sequence-based PCR (rep-PCR). In: Nucleic Acid Amplification Methods for the Analysis of Environmental Microbes. G. Toronzos, ed. Technomic Publishing Co., Inc., Lancaster, PA. (In press.)

18. Lupski, J. R., and Weinstock, G. M. 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. J. Bacteriol. 174:4525-4529.

19. Mass. J. L., ed. 1984. Angular leaf spot. Pages 41-42 in: Compendium of Strawberry Diseases. American Phytopathological Society, St. Paul, MN.

20. Mazzucchi, U., Alberghina, A., and Dalli, A. 1973. Occurrence of *Xanthomonas fragariae* Kennedy and King in Italy. Phytopathol. Z. 76:367-370.

21. Neto, J. R., Sugimori, M. H., and Paradela, O. 1978. *Xanthomonas fragariae* Kennedy and King in Brazil. Summa Phytopathol. 4:101-104.

22. Nick, G., and Lindstrom, K. 1994. Use of repetitive sequences and the polymerase reaction to fingerprint genomic DNA of *Rhizobium galegae* strains and to identify the DNA obtained by sonicating the liquid cultures and root nodules. Syst. Appl. Microbiol. 17:265-273.

23. Panogopoulos, C. G., Psallidas, P. G., and Alivizatos, A. 1978. A bacterial leafspot of strawberry in Greece caused by *Xanthomonas fragariae*. Phytopathol. Z. 91:33-38.

24. Powell, D., and Khare, M. N. 1967. Angular leaf spot of strawberry in Kentucky. Plant Dis. Rep. 51:353.

25. Rossbach, S., Rasul, G., Schneider, M., Eardly, B., and de Bruijn, F. J. 1995. Structural and functional conservation of the rhizopine catabolism (*moc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. Mol. Plant-Microbe Interact. 8:549-559.

26. Rowhani, A., Feliciano, A. J., Lips, T., and Gubler, W. D. 1994. Rapid identification of *Xanthomonas fragariae* in infected strawberry leaves by enzyme-Linked immunosorbent assay. Plant Dis. 78:248-250.

27. Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

28. Swings, J. G., Vauterin, L., and Kersters, K. 1993. The bacterium *Xanthomonas*. Pages 121-156 in: *Xanthomonas*. J. G. Swings and E. L. Civerolo, eds. Chapman and Hall, London.

29. Vauterin, L., Hoste, B., Yang, P., Alvarez, A., Kersters, K., and Swings, J. 1993. Taxonomy of the genus *Xanthomonas*. Pages 157-192 in: *Xanthomonas*. J. G. Swings and E. L. Civerolo, eds. Chapman and Hall, London.

30. Versalovic, J., Koeuth, T., and Lupski, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19:6823-6831.

31. Versalovic, J., Schneider, M., de Bruijn, F. J., and Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5:25-40.