

Assessment of the Genetic Diversity among Strains of *Xanthomonas cynarae* by Randomly Amplified Polymorphic DNA Analysis and Development of Specific Characterized Amplified Regions for the Rapid Identification of *X. cynarae*

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The randomly amplified polymorphic DNA (RAPD) method was used to investigate the genetic diversity in *Xanthomonas cynarae*, which causes bacterial bract spot disease of artichoke. This RAPD analysis was also intended to identify molecular markers characteristic of this species, in order to develop PCR-based markers which can be used to detect this pathogenic bacterium in artichoke fields. Among the 340 RAPD primers tested, 40 were selected on their ability to produce reproducible and reliable fingerprints in our genetic background. These 40 primers produced almost similar patterns for the 37 *X. cynarae* strains studied, different from the fingerprints obtained for other *Xanthomonas* species and other xanthomonad-like bacteria isolated from artichoke leaves. Therefore, *X. cynarae* strains form a homogeneous genetic group. However, a little DNA polymorphism within this species was observed and the collection of *X. cynarae* isolates was divided into two groups (one containing three strains, the second one including all other strains). Out of seven RAPD markers characteristic of *X. cynarae* that were cloned, four did not hybridize to the genomic DNA of strains belonging to other *Xanthomonas* species. These four RAPD markers were converted into PCR markers (specific characterized amplified regions [SCARs]); they were sequenced, and a PCR primer pair was designed for each of them. Three derived SCARs are good candidates to develop PCR-based tests to detect *X. cynarae* in artichoke fields.

Water-soaked and dark green spots on capitulum bracts of artichoke (*Cynara scolymus* L.) were observed for the first time in 1954 in Brittany and near Angers (France). Warm and humid periods are favorable for development of this disease, which has been observed in numerous artichoke crops in Brittany and has resulted in substantial economic losses for the last decade. The causal agent of this disease was first isolated by Ridé (25) from such bract spots and was identified as a phytopathogenic bacterium belonging to the genus *Xanthomonas* (25). It was recently classified at the species level as *Xanthomonas cynarae* (33). An identification test of *X. cynarae* was described based on biochemical, physiological, and pathogenicity tests (33). Colonies of *X. cynarae* are yellow and surrounded with a white halo when grown on a Tween medium (17) and produce the typical symptoms of this disease when inoculated to detached scarified bracts of artichoke. A pathogenicity test is required, since other *Xanthomonas* species look like *X. cynarae* when grown on Tween medium. This identification procedure, including pathogenicity tests, was used to monitor bacterial populations in artichoke fields and showed that *X. cynarae* is present on the leaf surface of artichoke before the capitulum development.

A rapid and specific identification test would be very useful to monitor the contamination of artichoke plants in order to develop strategies to control the disease in fields. Since the diagnostic test described above is time consuming and re-

quires, for the pathogenicity test, artichoke bracts which are not available early in the season when the test is required, the aim of this work was to develop a rapid and specific test for the identification of *X. cynarae*, based on the PCR technique (12). To achieve this goal, we used a two-step strategy. First, the genetic diversity in *X. cynarae* was assessed by random amplified polymorphic DNA (RAPD) (36) to determine the population structure of *X. cynarae*, as previously described for many other bacterial species (5, 7, 10, 15, 18, 21, 22, 31). Secondly, we selected RAPD markers specific to *X. cynarae* strains in order to convert them into specific characterized amplified region (SCAR) markers (20) which would be useful in an identification scheme of *X. cynarae*; as they have proven to be with numerous other pathogenic bacteria (4, 9, 16, 23, 24, 26).

MATERIALS AND METHODS

Isolates used. Details on the bacterial strains used in this study are given in Table 1. In addition, 38 strains nonpathogenic to artichoke (*C. scolymus* L.), isolated either from artichoke leaves (28 strains) or from weed leaves (10 strains; these weeds were either *Senebiera* sp. or *Lunaria annua*), were used specifically to assess the specificity to *X. cynarae* of the SCAR markers obtained in this study. All strains were restreaked for colony uniformity on LPGA medium (yeast extract, 7 g; Bacto Peptone, 7 g; glucose, 7 g; agar-agar, 15 g; H₂O, 1,000 ml) and on a Tween medium (17). All strains were grown on Luria-Bertani medium (28) for 24 h at 24°C, except for *Xanthomonas populi*, which was grown at 19°C.

DNA extraction. For RAPD experiments, genomic DNA was extracted following the standard cetyltrimethylammonium bromide (CTAB) methodology described by Ausubel et al. (2). DNA was approximately quantified by electrophoresis in 1.4% (wt/vol) agarose gels in 0.5× Tris-borate-EDTA (28) in comparison with a few DNA samples previously precisely quantified (by using a spectrophotometer). Each DNA concentration was adjusted to 10 ng/μl in sterile distilled water and stored at -20°C. For PCR analyses, crude bacterial DNAs were obtained by a simple boiling method: 20 μl of bacterial suspension (optical

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TABLE 1. List of bacterial strains used in this study

Species	CFBP ^a strain no.	Host	Geographical origin	Yr of isolation
<i>Xanthomonas cynarae</i>				
	18, 19	<i>C. scolymus</i> L.	France	1957
	2044	<i>C. scolymus</i> L.	France	1981
	4182, 4183, 4185, 4186, 4187, 4188 ^{Tb} , 4189, 4190, 4194, 4195, 4196, 4197, 4198, 4199	<i>C. scolymus</i> L.	France	1996
	4200, 4201, 4926, 4927, 4928, 4929, 4930, 4932, 4934, 4935, 4936, 4937, 4938, 4939, 4940, 4941	<i>C. scolymus</i> L.	France	1997
	4719	<i>C. scolymus</i> L.	France	1998
	4209	<i>Senebiera</i> sp.	France	1996
	4943	<i>Senebiera</i> sp.	France	1997
	4942	<i>Lunaria annua</i>	France	1997
Other <i>Xanthomonas</i> species				
<i>X. albilineans</i>	2523 ^T			
<i>X. arboricola</i> pv. <i>juglandis</i>	2528 ^T			
<i>X. arboricola</i> pv. <i>corylina</i>	K100 _{st}			
<i>X. axonopodis</i> pv. <i>phaseoli</i>	2534 ^{PTc}			
<i>X. bromi</i>	3625 ^{PT}			
<i>X. campestris</i> pv. <i>campestris</i>	2350 ^T , 1713, 1869			
<i>X. cassavae</i>	4642 ^T			
<i>X. codiae</i>	4690 ^T			
<i>X. cucurbitae</i>	2542 ^T			
<i>X. fragariae</i>	2057 ^T			
<i>X. hortorum</i> pv. <i>pelargonii</i>	2533 ^{PT}			
<i>X. hyacinthi</i>	1156 ^T			
<i>X. melonis</i>	4644 ^T			
<i>X. oryzae</i> pv. <i>oryzae</i>	2532 ^T			
<i>X. pisi</i>	4643 ^T			
<i>X. populi</i>	1817 ^T			
<i>X. sacchari</i>	4641 ^T			
<i>X. theicola</i>	4691 ^T			
<i>X. translucens</i> pv. <i>translucens</i>	2054 ^T			
<i>X. vasicola</i> pv. <i>holcicola</i>	2543 ^T			
<i>X. vesicatoria</i>	4645 ^T			
<i>Pseudomonas syringae</i> (two strains)				

^a CFBP, Collection Française de Bactéries Phytopathogènes, Station de Pathologie Végétale, Angers, France.

^b T, type strain.

^c PT, pathotype strain.

density at 600 nm, 0.1) was boiled for 5 min and stored at -20°C . *Phytophthora* DNA and artichoke DNA were extracted by a standard CTAB-based procedure.

RAPD analysis. The bacterial RAPD fingerprints were obtained using the procedure described by Williams et al. (36), with minor modifications (3). Briefly, amplifications were carried out in a final volume of 15 μl containing $1\times$ reaction buffer (Promega, Madison, Wis.), 100 μM concentrations of each deoxynucleotide (Eurobio, Les Ulis, France), 1.5 mM MgCl_2 , a 0.5 μM concentration of the primer (Operon Technologies, Alameda, Calif.), 0.06 U of *Taq* DNA polymerase (Promega) per μl , and approximately 10 ng of genomic DNA. RAPD reactions were performed in a 96-well thermal cycler (PTC-100; MJ Research Inc., Watertown, Mass.), with one step at 93°C for 2 min 30 s and 45 cycles at 93°C for 30 s, 35°C for 45 s, and 72°C for 90 s, followed by one step at 72°C for 5 min. Following agarose gel electrophoresis and ethidium bromide staining, amplified patterns were visualized under UV light (300 nm). RAPD amplified bands were scored visually according to their presence or absence for the bacteria studied. Only clear, unambiguous, and reproducible RAPD markers were taken into account. The reproducibility of each scored marker was checked by at least two RAPD experiments. Genetic distances between *X. cynarae* strains were calculated according to the formula established by Nei and Li (19).

Development of specific SCAR markers for *X. cynarae*, from characteristic RAPD markers. (i) **Cloning of DNA fragments from RAPD fingerprints.** Several RAPD fragments common to all *X. cynarae* strains were recovered from agarose gels with a scalpel and purified by using the Ultrafree-DA kit (Millipore, Bedford, Mass.). The extracted DNA fragments were used as templates for another round of amplification using the same RAPD primers with a slightly increased annealing temperature. When such reamplifications resulted in amplicons of the expected sizes, they were cleaned using the Centrifugal Filter Services kit (Millipore) and cloned into the pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.), as recommended by the manufacturer.

(ii) **DNA probes.** DNA probes were made from recombinant plasmids (one for each cloned DNA marker) by PCR amplifications using oligonucleotide primers

T7 and T3 (Invitrogen) flanking the cloning site of the pCR4-TOPO vector and by incorporating digoxigenin-labeled nucleotide (DIG-dUTP) as described by Hoisington (14). Probes were stored at -20°C before hybridization tests.

(iii) **Southern blots and membrane hybridization.** DNAs from three strains of *X. cynarae* (from the Collection Française de Bactéries Phytopathogènes [CFBP], CFBP 19, CFBP 4188^T, and CFBP 4199) and from type strains of 19 of the 20 *Xanthomonas* species described by Vauterin et al. (34) were amplified with the RAPD primer which produced the cloned fragment to be tested. After electrophoresis, DNA patterns were transferred onto a Hybond-N⁺ nylon membrane (Amersham, Arlington Heights, Ill.) by capillary blotting (30). Moreover, *Xanthomonas* strains were grown on nylon membranes according to the method of Sambrook et al. (28) before a colony hybridization step. Prehybridization, hybridization, and DNA hybrid detection were performed as described by Hoisington (14). Autoradiographies were obtained on Kodak X-Omat films after up to 15 min of exposure.

(iv) **DNA sequencing and primer design.** Cloned DNA fragments of interest were sequenced (around 300 nucleotides from both ends of each DNA fragment) by Cybergene (Evry, France). Nucleotide sequence data were compared against the Genbank nucleotide sequence database (BLAST search) and were analyzed with the Primer 3 software in order to design pairs of PCR primers (20-mers) for obtaining SCAR markers characteristic of *X. cynarae*. Oligonucleotides were synthesized by Cybergene.

PCR conditions for the amplification of *X. cynarae*-specific markers (SCARs). PCR amplifications were carried out in a final volume of 40 μl containing $1\times$ buffer (Promega), 1.5 mM MgCl_2 , 200 μM concentrations of each deoxynucleoside triphosphate, 0.2 μM concentrations of each primer, 0.02 U of *Taq* DNA polymerase (Promega) per μl , and 2 μl of a boiled bacterial suspension. The reaction mixtures were subjected to 35 cycles of the following incubations: 45 s at 93°C (except for the first one, 3 min 45 s), 45 s at the appropriate annealing temperature (52°C for primer pair XC800, 55°C for XC420, and 60°C for primer pairs XC1000 and XC850), and 60 s at 72°C (except for the last cycle, 10 min).

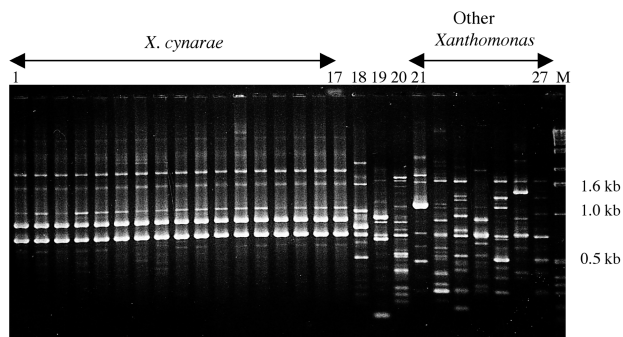


FIG. 1. RAPD fingerprints characteristic of *X. cynarae* species, epiphytic strains nonpathogenic to artichoke, and other *Xanthomonas* species. Lanes 1 to 17, *X. cynarae* strains (CFBP no. 4182, 4183, 4185, 4186, 4188, 4190, 4199, 4200, 4942, 4943, 2044, 4719, 4209, 4930, 4931, 18, and 19) belonging to the three genetic subgroups A1, A2, and B (see text); lanes 18 to 20, epiphytic strains nonpathogenic to artichoke (CFBP no. 4184, 4191, and 4192); lanes 21 to 27, strains belonging to 6 other *Xanthomonas* species (CFBP no. 1713, 1869, 2542, 2532, 2054, K100st, and 2534; Table 1); lane M, molecular weight marker (1-kb ladder; Gibco BRL).

RESULTS

RAPD analysis. In a first trial, 340 RAPD primers (10-mer oligonucleotides with arbitrary sequences) were tested on a few bacterial strains (four strains of *X. cynarae*, two strains of other *Xanthomonas* species, and two xanthomonad-like strains isolated from artichoke leaves but nonpathogenic to this plant) in order to select a set of RAPD primers which produce reliable and reproducible fingerprints for the bacteria studied. Forty primers were retained for assessment of the genetic diversity within the *X. cynarae* species.

The 40 selected RAPD primers were used to analyze genomic DNAs of all 37 *X. cynarae* strains, 7 other *Xanthomonas* bacteria (K 100st, CFBP 2534, CFBP 1713, CFBP 1869, CFBP 2542, CFBP 2532, and CFBP 2054), and 4 epiphytic xanthomonad-like strains isolated from the leaves of artichokes (or weeds [*Senebiera* sp. or *Lunaria annua*]) but nonpathogenic to artichoke plants (Table 1). With each primer, we obtained RAPD patterns which were similar for all *X. cynarae* strains but distinct from those observed for the other bacteria studied (Fig. 1 and 2). Since all *X. cynarae* strains shared one or several common RAPD markers characteristic of this species with each primer used, RAPD results showed that these 37 strains form a single genetic group. Overall, we obtained 112 RAPD markers shared among all *X. cynarae* strains. The reproducibility of all these markers was checked by performing at least two experiments per RAPD primer.

More precisely, 20 RAPD primers provided identical fingerprints for all 37 *X. cynarae* strains (Fig. 1 and 2a). However, the 20 remaining primers revealed a genetic polymorphism within this species (Fig. 2b and c). Therefore, we identified 3 RAPD subgroups for *X. cynarae*, named A1, A2, and B. Table 2 recapitulates these results, in terms of the number of markers that distinguish each subgroup and in terms of genetic distances between subgroups. Subgroups A1 and A2 are very similar, since the distance between them is only 0.007. Both of them are much more distant to group B, since the distances observed between A1 and B and between A2 and B are 0.177 and 0.185, respectively. These results explain our subgroup

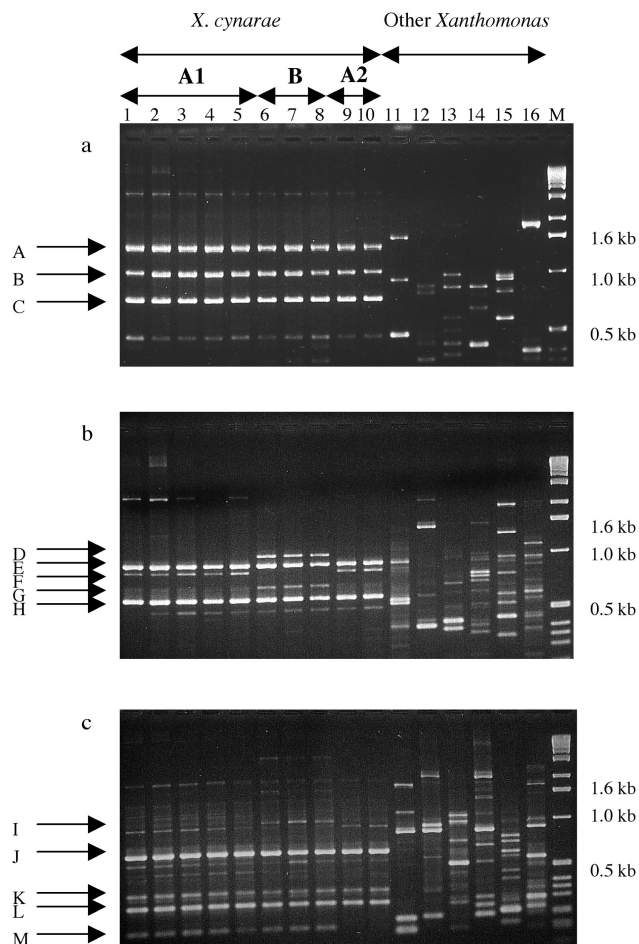


FIG. 2. Examples of RAPD fingerprints revealing the genetic diversity within the *X. cynarae* species. Lanes 1 to 10, *X. cynarae* strains (CFBP no. 2044, 4188, 4199, 4719, 4200, 4209, 4929, 4930, 18, and 19) belonging to RAPD groups A1, A2, or B, as indicated above panel a; lanes 11 to 16, strains belonging to other *Xanthomonas* species (CFBP no. 1713, 2542, 2532, 2054, K100st, and 2534; Table 1); lane M, molecular weight marker (1-kb ladder; Gibco BRL). (a) A, B, and C are RAPD markers characteristic of *X. cynarae*. (b) E and H are RAPD markers shared by all *X. cynarae* strains (A1, A2, and B); D and G are RAPD markers characteristic of group B; F is a RAPD marker characteristic of group A (A1 and A2). (c) I is a faint marker characteristic of group B; J, K, and L are RAPD markers shared by all *X. cynarae* strains (A1, A2, and B); M is a RAPD marker shared by subgroup A1 and group B.

names. The distribution of all 37 *X. cynarae* strains within the three identified RAPD subgroups is presented in Table 3. Most of them belong to subgroup A1, which contains strains isolated in Brittany and in the Loire valley mainly from artichoke leaves, but also from weed leaves (2 strains out of 32). Subgroup A2 contains two strains isolated from artichoke leaves in Brittany in 1957. The three strains which belong to group B were recently isolated in Brittany, either from artichoke leaves or from a weed (*Senebiera* sp.) present in an artichoke field.

Selection of RAPD markers characteristic of *X. cynarae* for development of SCARs. We detected at least one RAPD band common to all *X. cynarae* strains which was absent in all other studied bacteria for each of the 40 RAPD primers used. In

TABLE 2. Genetic distances between the 3 RAPD subgroups identified within the 37 *X. cynarae* strains studied with 40 RAPD primers

RAPD subgroup	No. of RAPD fragments or genetic distance ^a		
	A1	A2	B
A1		2	49
A2	0.007		51
B	0.177	0.185	

^a Numbers of RAPD fragments distinguishing RAPD subgroups are indicated above the diagonal; genetic distances, calculated according to the method of Nei and Li (19), are given below the diagonal.

order to obtain SCARs specific to *X. cynarae*, and therefore usable for the identification of this pathogen, we selected seven of these RAPD fragments for cloning. Our selection criteria were a sufficient DNA length (>400 bp) in order to maximize the availability of convenient sites for designing PCR primers and a strong intensity of the DNA band associated with its location as far as possible from other bands, to increase the chance of cloning the targeted marker. Figure 2a displays three examples (markers A, B, and C) of such selectable RAPD markers.

The lack of DNA sequences homologous to the seven cloned RAPD bands in other *Xanthomonas* genomes was tested by using them as probes in two hybridization experiments: (i) on the RAPD patterns of a representative subset of bacterial strains previously used and obtained with the primer which produced the cloned marker (after Southern blotting) and (ii) on the genomic DNA of 3 *X. cynarae* strains (CFBP 19, CFBP 4188^T, CFBP 4199) and 19 other *Xanthomonas* strains (after dot blotting). In the first experiment, for each of the seven probes no hybridization signal was obtained in the RAPD patterns of non-*X. cynarae* strains, whereas a very strong signal was obtained at the location of the cloned marker in *X. cynarae* RAPD patterns (data not shown). In the second analysis, only four of the seven probes hybridized exclusively to the genomic DNA of the three *X. cynarae* strains (no signal was obtained for other *Xanthomonas* strains). The three other probes produced a strong signal with the three *X. cynarae* strains (high DNA homology) but also a faint one with at least one other *Xanthomonas* strain. Little DNA homology was observed with *Xanthomonas fragariae* DNA in the three cases, with *Xanthomonas bromi* DNA with one probe, and with *Xanthomonas cucurbitae* DNA with another probe. We therefore retained the first four

TABLE 3. Distribution in the three RAPD subgroups and characteristics of the 37 *X. cynarae* strains

RAPD subgroup	No. of strains	Isolated on:	Geographic location	Yr of isolation
A1	28	Artichoke	Brittany, France	1996–97
	2	Weeds	Brittany, France	1997
	2	Artichoke	Loire valley, France	1981–98
A2	2	Artichoke	Brittany, France	1957
B	1	<i>Senebiera</i> sp.	Brittany, France	1996
	2	Artichoke	Brittany, France	1997

TABLE 4. Characteristics of the four primer pairs designed for amplifying PCR markers specific to the *X. cynarae* species

Primer pair	Sequence	%G-C	<i>T_m</i> (°C)
XC800	TAA GGG AAA TGA TCG GCA TC	45	55.3
	CAA GGA ATT GAG CTT CTC CG	50	57.3
XCS6	TGA TAG ATC CCA AGA TGG GC	50	57.3
	AAT GTC TCG CCT TCG TAT GG	50	57.3
XC420	GGC AGG CTG TTC GGT AGG TG	65	63.5
	GGC AGG CTG TAG TTG GTT GA	55	59.4
XC1000	ACT CGC TCG GAG ACT CAT GT	55	59.4
	AGG ATG TCG TGT TGT TGC CC	55	59.4

probes (cloned RAPD markers) as good candidates to design PCR primers specific to the *X. cynarae* species.

Design of *X. cynarae*-specific primers. The DNA sequences of the first 300 bases from both terminal ends of the four selected RAPD fragments were determined. None of these sequences showed a highly significant DNA homology with gene sequences stored in the Genbank database. The analysis of these sequences, using the software Primer 3, allowed us to design a PCR primer pair usable for amplifying each of the four markers. The designed primers are 20-mers with melting temperatures (*T_m*) of around 60°C (usual values for PCR primers; Table 4). We named the four primer pairs XC1000, XC850, XC800, and XC420 based on the expected sizes of the fragments to be amplified.

Only the primer pair XC420 was expected to produce the whole RAPD fragment (full length), since both PCR primers were designed with the RAPD primer sequence at their 5' end (the 10 additional nucleotides of each primer are the ones which directly followed the RAPD primer sequence on both ends). In the three other cases, expected SCARs are shorter than the initial RAPD fragments, since the DNA sequences upon which the primers were designed were internal to the RAPD fragments.

Specificity of the four SCARs for *X. cynarae*. Each of the four designed PCR primer pairs was then tested for its ability to produce an amplicon specific to the *X. cynarae* species, which is a requirement in order to use such PCR primers for identifying this pathogenic species on artichoke leaves. For that purpose, each primer pair was used on a large panel of microorganisms. Each primer pair produced the same PCR fragment for all 37 *X. cynarae* strains, with the expected DNA length. Primer pairs XC1000, XC850, and XC800, when used with a sufficiently high annealing temperature, did not produce any PCR fragment for all other microorganisms studied (including *Phytophthora* and *Pseudomonas* strains) or from artichoke genomic DNA. Figure 3 illustrates the specificity to *X. cynarae* of the SCAR marker obtained using the primer pair XC1000. However, the primer pair XC420 amplified from the DNA of one nonpathogenic xanthomonad-like strain a DNA fragment similar in size and intensity to the one obtained from *X. cynarae* strains, even when the most stringent annealing conditions were applied.

DISCUSSION

The RAPD analysis we performed was intended to assess the genetic diversity within the *X. cynarae* species. RAPD fingerprints allow intraspecific genetic diversity studies but are not very good tools for such analyses at the interspecific level

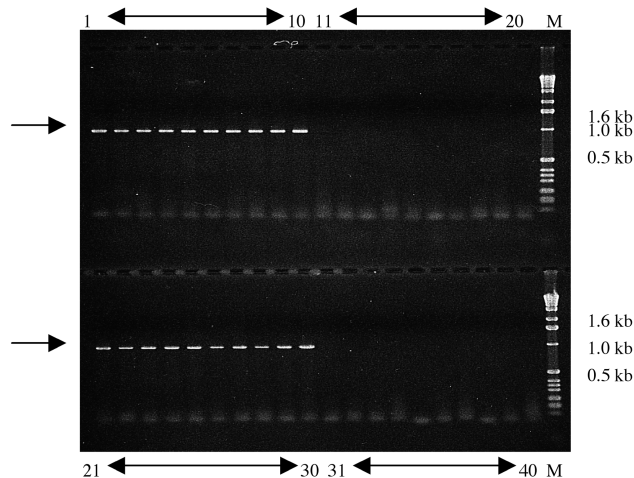


FIG. 3. SCAR marker characteristic of *X. cynarae* obtained by using the primer pair XC1000. Lanes 1 to 10, *X. cynarae* strains (CFBP no. 4182, 4183, 4188, 4190, 4192, 4194, 4199, 4200, 18, and 19); lanes 11 to 20, strains belonging to other *Xanthomonas* species (CFBP no. 2523, 2528, 2534, 3625, 2350, 4642, 4690, 2542, 2057, and 2533; Table 1); lanes 21 to 30, *X. cynarae* strains (CFBP no. 4926, 4927, 4928, 4942, 4943, 2044, 4719, 4209, 4929, and 4930); lanes 31 to 40, epiphytic strains nonpathogenic to artichoke; lane M, molecular weight marker (1-kb ladder; Gibco BRL). The SCAR marker locations are indicated by arrows.

(8). Therefore, we did not use the RAPD technique for a phylogenetic study in the genus *Xanthomonas*. More appropriate technical tools (phenotypical and biochemical tests, DNA-DNA hybridization, and 16S ribosomal DNA sequencing) were used to classify *X. cynarae* in the genus *Xanthomonas* (33).

Before starting the RAPD study within the *X. cynarae* species, we performed a primer-screening step, in order to select a set of RAPD primers which produces reliable fingerprints. Indeed, a bacterial genome is small and therefore may not possess enough sites homologous to a given RAPD primer to amplify only genomic DNA fragments based on good matches of the primer on both ends of each fragment. Nevertheless, in such a case, a RAPD fingerprint is produced from the bacterial DNA, but this pattern is usually nonreproducible, since the selected genomic fragments to be amplified may change from one experiment to another, resulting in different RAPD fingerprints (29). Therefore, by using a set of eight different bacteria (four *X. cynarae*, two xanthomonad-like bacteria isolated from artichoke but nonpathogenic to this plant, and two other *Xanthomonas* species), we selected 40 RAPD primers (out of 340 tested) for their ability to produce reproducible fingerprints as in other studies (7, 18, 21, 31, 32). Moreover, we took into account only the intensive bands that are highly reproducible (6, 9, 13, 21) when analyzing RAPD fingerprints.

With the 40 selected RAPD primers, we obtained many species-specific markers for *X. cynarae*, in agreement with the fact that *X. cynarae* is a genomic species in the genus *Xanthomonas* (33). Moreover, 112 of 163 RAPD markers were common to all *X. cynarae* strains. However, a genetic diversity was observed within *X. cynarae*, since we obtained 51 polymorphic DNA markers within the *X. cynarae* species, which revealed two main genetic groups, A and B. Furthermore, 2 markers of the 163 which were scored distinguished two sub-

groups (A1 and A2) in group A. Subgroup A1 seems widespread in Brittany, since 30 strains of the 35 isolated in this area in 1996 and 1997 belong to this subgroup (Table 3). It is also present in the Loire valley, as indicated by the two studied strains isolated there in 1981 and 1998. Subgroup A2 includes only two *X. cynarae* strains isolated 40 years ago (Table 3). This subgroup A2 is very similar to subgroup A1. If we assume that strains belonging to subgroup A2 are representative of the bacterial population present in Brittany 40 years ago, we can conclude that *X. cynarae* appears to be stable in this region and that group A forms the core of a clonal population.

We only found three strains belonging to group B, all of them recently isolated in Brittany. This group B, although unambiguously composed of *X. cynarae* strains, is quite different from group A (Table 2). We were not able to identify any particular characteristic for the artichoke fields where group B strains were found (random geographical distribution, no rain or wind exposure specificity, no correlation with the artichoke cultivar grown or with the previous crop in the field). However, it may be possible that *X. cynarae* strains belonging to group B were introduced in Brittany by growing artichokes coming from Italy or south-east France. Indeed, Italian artichokes were grown in Brittany during a few years because of an unusual long period of deep frost which destroyed a large part of the local artichoke crop during the winter of 1963-64. Moreover, cultivars of little artichokes, very popular in south-east France, have been grown in Brittany since 1993. Even if Italian artichokes and French little artichokes are not known to be damaged by the bacterial bract spot disease, they may carry a *X. cynarae* population. In order to confirm these hypotheses, it would be interesting to check whether *X. cynarae* is present in south-east France and/or in Italy. In such a case, we would then determine whether those strains belong to group B only.

Three bacterial strains (CFBP 4209, CFBP 4942, and CFBP 4943; Table 1) isolated from the leaf surfaces of weeds which were present in artichoke fields were identified as *X. cynarae* strains based on their phenotypical characteristics and their ability to produce typical symptoms on detached bracts. Two of them belong to subgroup A1, and the third one belongs to group B (Table 3). Therefore, it would be interesting to determine whether *X. cynarae* is able to multiply on such weeds, which would then be identified as sources of contamination in artichoke crops. The three strains we studied may also have been simply washed off from infected artichoke plants.

In the absence of a simple and fast identification procedure of *X. cynarae* strains, our aim was to develop a PCR-based test to identify *X. cynarae*. Indeed, such tests are reported to be sensitive, reliable, and fast, which explains why PCR-based techniques have become more and more widespread for detecting plant pathogens (12). In order to develop PCR markers characteristic of *X. cynarae*, we first identified RAPD markers shared by all *X. cynarae* strains (37 isolates) and not present in the RAPD fingerprints of other *Xanthomonas* species and xanthomonad-like bacteria isolated from artichoke leaves but nonpathogenic to this plant. Such RAPD markers cannot be used to detect *X. cynarae* in a mixture of different bacteria (for example, after a wash of artichoke leaves), since RAPD primers are nonspecific and can amplify DNA fragments from any genome (11, 27, 35). This is the reason why we had to convert RAPD markers characteristic of *X. cynarae* into SCAR mark-

ers (i.e., PCR markers). Nevertheless, the RAPD analysis was useful for studying the population structure of *X. cynarae*.

Of the seven RAPD markers characteristic of *X. cynarae* that were cloned, the four which were homologous only to *X. cynarae* genomic DNA were sequenced. Of the four PCR primer pairs designed, three produced an *X. cynarae*-specific amplicon (XC1000, XC850, and XC800) that is amplified for all three *X. cynarae* genetic subgroups (A1, A2, and B). Therefore, the low genetic diversity observed in the *X. cynarae* species facilitated the development of species-specific SCARs. For a species (or a pathovar) in which the genetic diversity is high, it may be more difficult to develop a single SCAR characteristic of the whole species (or pathovar), and several SCARs may be required to detect all isolates of the targeted bacterial group (1).

A PCR-based test to detect *X. cynarae* on artichoke leaves is now available in our laboratory. Now, we intend to use the primers developed in this study in a quantitative PCR test with a fluorimetric procedure (i.e., the TaqMan technology developed by Perkin-Elmer, Foster City, Calif.). Indeed, a quantitative detection of *X. cynarae* would be necessary to determine whether outbreaks of the disease are correlated to the level of the bacterial population on artichoke leaves. We are also interested in developing SCAR markers characteristic of *X. cynarae* groups A and B, in order to develop epidemiological markers which may be useful for studying the spread of the inoculum in Brittany.

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