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Isolation by Genomic Subtraction of DNA Probes Specific for *Erwinia carotovora* subsp. *atroseptica*

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***Erwinia carotovora* subsp. *atroseptica* is a pathogen of potatoes in Europe because of its ability to induce blackleg symptoms early in the growing season. However, *E. carotovora* subsp. *carotovora* is not able to produce such severe symptoms under the same conditions. On the basis of the technique described by Straus and Ausubel (Proc. Natl. Acad. Sci. USA 87:1889–1893, 1990), we isolated DNA sequences of *E. carotovora* subsp. *atroseptica* 86.20 that were absent from the genomic DNA of *E. carotovora* subsp. *carotovora* CH26. Six DNA fragments ranging from ca. 180 to 400 bp were isolated, cloned, and sequenced. Each fragment was further hybridized with 130 microorganisms including 87 *E. carotovora* strains. One probe was specific for typical *E. carotovora* subsp. *atroseptica* strains, two probes hybridized with all *E. carotovora* subsp. *atroseptica* strains and with a few *E. carotovora* subsp. *carotovora* strains, and two probes recognized only a subset of *E. carotovora* subsp. *atroseptica* strains. The last probe was absent from the genomic DNA of *E. carotovora* subsp. *carotovora* CH26 but was present in the genomes of many strains, including those of other species and genera. This probe is homologous to the *putP* gene of *Escherichia coli*, which encodes a proline carrier. Further use of the probes is discussed.**

Erwinia carotovora has been particularly studied because of its pathogenicity to several crops, of which the potato crop is the most important (22). The *E. carotovora* species has been divided into the four subspecies *atroseptica*, *carotovora*, *betavascularum*, and *wasabiae* on the basis of physiological and biochemical features and pathogenesis (7, 12, 29).

E. carotovora subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. carotovora* subsp. *wasabiae* have narrow host ranges: *E. carotovora* subsp. *betavasculorum* causes soft rot on sugar beets (33), *E. carotovora* subsp. *wasabiae* has been isolated from Japanese horseradish (7), and *E. carotovora* subsp. *atroseptica* is usually restricted to potatoes in cool temperate climates (21). Some *E. carotovora* subsp. *atroseptica* strains not isolated from potatoes have been called atypical, because they exhibit some particular physiological feature, like the ability to grow at 37°C (16, 26). A new subspecies called *odorifera* has been proposed for the atypical *E. carotovora* subsp. *atroseptica* strains which are pathogenic to chicory and produce odorous volatile metabolites (6). The other atypical strains have been identified as *E. carotovora* subsp. *carotovora* strains on the basis of phenotypic and genotypic characteristics (4, 26). *E. carotovora* subsp. *carotovora* is widely distributed in the world and has a broad host range (24, 28), while *E. carotovora* subsp. *carotovora* strains may exhibit variation in pathogenicity to plants (28).

E. carotovora subsp. *atroseptica* is considered the typical blackleg agent in Europe because of its abilities to be pathogenic at low temperatures (<25°C) and to induce disease early in the growing season, which increases decay (24). Most *E. carotovora* subsp. *carotovora* strains cannot produce typical blackleg symptoms at low temperatures (26). Although French potato seed producers consider *E. carotovora* subsp. *carotovora* an opportunist rather than a primary disease causal agent, *E. carotovora* subsp. *carotovora* exhibits increased pathogenicity at temperatures greater than 25°C and seems to be associated with potato blackleg in Arizona and Colorado (30).

Effective control of bacterial diseases consists mainly of prophylactic practices that would benefit from tools allowing detection of a specific pathogen at its minimal level of infectivity (13, 19). Specifically, an identification tool is necessary to discriminate *E. carotovora* subsp. *atroseptica* from *E. carotovora* subsp. *carotovora*. As shown by phenotypic studies, there are differences in the nutritional and ecological features, optimum growth temperatures, host range, and pathogenicity of these two subspecies (12, 23). Such characteristics or other differences should appear at the genomic level.

Genomic subtraction (3, 32) was undertaken between *E. carotovora* subsp. *atroseptica* 86.20 (a source of prospective probes) and *E. carotovora* subsp. *carotovora* CH26 (subtracter DNA). Strain CH26 was isolated from potato and was unable to induce blackleg under controlled conditions (26).

(Part of this work has been patented [4a].)

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used to isolate and clone the specific sequences are described in Table 1. The microorganisms tested in dot blot hybridization experiments with the generated probes are presented in Table 2. *Erwinia* and *Escherichia coli* strains were grown in Luria broth medium (18) at 30 and 37°C, respectively. The following antibiotics were used: ampicillin (50 μg ml^{-1}) and streptomycin (100 μg ml^{-1}). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 40 μg ml^{-1} was used.

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TABLE 1. Bacterial strains and plasmids

	Relevant characteristic(s)	Source or reference
E. coli DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ⁻ *recA1 gyrA* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	Bethesda Research Laboratories
E. carotovora subsp. *atroseptica* 86.20	Wild-type strain isolated from potatoes in France in 1986	B. Jouan[a]
E. carotovora subsp. *carotovora* CH26	Wild-type strain isolated from potatoes in Switzerland in 1985	O. Cazelles[b]
E. chrysanthemi		
3937	Wild-type strain isolated from *Saintpaulia ionantha*	11
PMV4071	3937 *pelE* mutant, Ω fragment with Sm^r and Spc^r from R100.1 in *Bgl*II restriction site	M. Boccara[c]
Plasmids		
pUBS-3	pUC derivative containing pBluescript	G. Murphy[d]
pHP45Ω	pHP45 derivative containing a Sm^r Spc^r fragment from R100.1	25
pTZ19R	pUC derivative with T7 RNA polymerase promoter	17
pPMV174	pUBS-3 derivative containing probe A (404 bp) in *Bam*HI site (Ap^r)	This work
pPMV176	pTZ19R derivative containing probe B (303 bp) in *Bam*HI site (Ap^r)	This work
pPMV177	pTZ19R derivative containing probe C (290 bp) in *Bam*HI site (Ap^r)	This work
pPMV175	pUBS-3 derivative containing probe D (233 bp) in *Bam*HI site (Ap^r)	This work
pPMV178	pTZ19R derivative containing probe E (212 bp) in *Bam*HI site (Ap^r)	This work
pPMV179	pTZ19R derivative containing probe F (183 bp) in *Bam*HI site (Ap^r)	This work

[a] Pathologie Végétale, Institut National de la Recherche Agronomique, Le Rheu, France, personal collection.
[b] Station Fédérale de Recherches Agronomiques, Changins, Switzerland, personal collection.
[c] Pathologie Végétale, Institut National Agronomique Paris-Grignon, Institut National de la Recherche Agronomique, Paris, France.
[d] Institute for Plant Sciences, Norwich, United Kingdom.

DNA preparation. Total genomic DNA was extracted and purified by the method of Klotz and Zimm (10).

The DNA from which specific probes were desired (target DNA) was digested to completion with *Sau*3AI according to the supplier's instructions. After digestion, DNA was extracted once with phenol-chloroform, precipitated with ethanol, washed, and resuspended at 0.1 μg μl^{-1} in EE buffer (pH 8.0) (10 mM *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid, 1 mM EDTA).

Two hundred micrograms of the DNA used to subtract nonspecific sequences (subtracter DNA) was fragmented by sonication in 3 ml of EE buffer in a Vibra-Cell (Sonics and Materials, Danbury, Conn.) in the continuous mode and with an output setting of 5. The average size of DNA fragments was approximately 1 kb for 20 s. The sheared DNA was concentrated with 7.5 ml of 2-butanol, ethanol precipitated, washed, and resuspended at 1 μg μl^{-1} in water.

Genomic DNA for dot blotting was extracted as described above with 10-fold-lower volumes. DNA purity was determined by spectrophotometry and electrophoresis after endonuclease digestion with *Eco*RI.

Large-scale preparations of plasmid DNA were made from clear lysates (15) followed by two centrifugations in a cesium chloride and ethidium bromide density gradient (15). Miniscale preparations were made by the rapid boiling method of Holmes and Quigley (8).

[α-^{32}P]dATP DNA labeling. DNA (50 ng) was labeled by the random primed DNA labeling kit (Boehringer GmbH, Mannheim, Germany) according to the supplier's instructions, except that the incubation was for 4 h at room temperature. Removal of nonincorporated deoxyribonucleotide triphosphates was performed by chromatography on 0.5-ml column (Sepharose CL-6B; Pharmacia, Uppsala, Sweden).

DNA biotinylation. DNA biotinylation was performed in microtitration plates on ice in a dark room. Fifty microliters of sheared DNA at 1 μg μl^{-1} was distributed into each well, and then 50 μl of photobiotin acetate (Sigma) at 2 μg μl^{-1} was added to each well. The mixture was photoactivated by 10 min of illumination (360-nm-wavelength light) from an UV lamp (VL-6LC; Vilbert Fourmat, Marne la Vallée, France) over the plate. After addition of 1 M Tris (pH 9) to a final concentration of 100 mM, biotinylated DNA was extracted four times with water-saturated 1-butanol, ethanol precipitated, washed, and resuspended at 2.5 μg μl^{-1} in 2.5× EE buffer, pH 8.

Genomic subtraction. Ten micrograms of biotinylated subtracter DNA and 250 ng of target DNA were used for the first cycle of genomic subtraction by the protocol of Straus and Ausubel (32). Their protocol was unchanged, except that tracer DNA and yeast tRNA were not added to our reaction mixture. The binding of biotinylated DNA was achieved with a 5% streptavidin solution (Dynabeads M-280; Dynal A.S., Oslo, Norway). After each hybridization cycle, biotinylated subtracter DNA (10 μg) was added to the samples. Four cycles were performed. At the end of the last cycle, the resulting samples were ethanol precipitated and then resuspended in 5 μl of TE buffer (pH 8) (15).

Ligation of adaptors for PCR amplification. The double-strand adaptors used were those described by Straus and Ausubel (32). Subtracted samples (2.5 μl) were ligated for 24 h at 12°C with 150 ng of adaptors, using 4 U of the ligase from Stratagene (La Jolla, Calif.) according to the supplier's instructions. After ligation, the samples were purified by chromatography on Sepharose CL-6B to remove small (<100-bp) DNA fragments, particularly the excess adaptors which could inhibit PCR. The samples were ethanol precipitated and resuspended in water for PCR amplification.

DNA amplification. The PCR medium used was that recommended for *Taq* polymerase from Perkin-Elmer Cetus Corp. (Norwalk, Conn.). DNA amplification occurred in a 50-μl volume topped with 50 μl of mineral oil (Sigma). Samples contained all the ligated DNA and 100 pmol of primer 5'CACTCTCGAGACATCACCG3' (derived from that of Straus and Ausubel [32]). The mixture was subjected to 25 cycles of the following incubations: 1 min at 94°C, 1

min at 65°C, and 1.5 min at 72°C (Pharmacia LKB Gene ATAQ Controller). Ten microliters of each PCR sample was used for a second round of amplification under the same conditions.

Cloning probes from subtracted library. Plasmids used for cloning are described in Table 1. The ligation protocol was the same as the one described above, with an equal concentration of vector and insert. *E. coli* DH5α was transformed by electroporation (1).

DNA blotting. DNAs from a collection of microorganisms (Table 2) were dot blotted by the alkaline method proposed for Hybond N^+ membranes (Amersham International plc, Amersham, England). Genomic DNA for Southern blotting was cleaved by several endonucleases according to the supplier's instructions (Boehringer). Reaction mixtures were run on a 0.8% agarose gel, using 1 μg of 1-kb ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as size markers. Southern blotting was performed on Hybond N^+ membranes according to the supplier's instructions.

Membrane hybridizations. Fifty nanograms of α-^{32}P-labeled DNA was used per hybridization. Membranes were prehybridized for 4 h in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), and 0.01% skim milk (9) at 65°C. Hybridizations were performed in a solution of 6× SSC, 0.1% SDS, and 0.01% skim milk incubated overnight at 65°C. Membranes were washed for 30 min in 3× SSC–0.5% SDS. Membranes were then washed twice in 0.3× SSC–0.5% SDS and incubated at 65°C for 30 min (low-stringency conditions) or washed twice in 0.1× SSC–0.5% SDS and incubated at 68°C for 30 min (high-stringency conditions).

DNA sequence analysis. Plasmid DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (27), using the Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio). Sequences were analyzed with the programs developed by the Genetics Computer Group (5), using GenBank and EMBL data bases for homology searches.

Nucleotide sequence accession numbers. The sequences of the six DNA fragments called probes A to F were deposited in GenBank data base and assigned accession numbers L15412 to L15417, respectively.

RESULTS

Calibration of subtraction. Our subtraction protocol was based on the method described by Straus and Ausubel (32). Because the material used was not identical, some preliminary tests were necessary to adapt the protocol.

After biotinylation of DNA, binding conditions with streptavidin were tested: 1 μg of sheared α-^{32}P-labeled 3937 DNA was mixed with 49 μg of sheared 3937 DNA. The mixture was biotinylated as described in Materials and Methods. Samples (10 μg) of biotinylated DNA were incubated with different concentrations of streptavidin Dynabeads (0.5, 1, 2.5, and 5% solutions). For the 0.5, 1, and 2.5% solutions, some radioactivity remained in the free fraction, while the 5% solution trapped all radioactivity. This concentration was thus determined as the most efficient for binding.

The number of subtraction cycles was chosen to optimize the two following parameters: (i) binding of homologous sequences and (ii) enrichment in nonhomologous sequences. For this purpose, two subtraction experiments were performed with the following pairs: (i) 100 ng of α-^{32}P-labeled *Sau*3AI-digested Ω fragment, a 2-kb Sm^r Spc^r DNA fragment (25), mixed with 150 ng of *E. chrysanthemi* 3937 genomic *Sau*3AI-digested DNA and subtracted with 10 μg of 3937 biotinylated DNA and (ii) 250 ng of α-^{32}P-labeled *Sau*3AI-digested 3937 DNA subtracted with 10 μg of 3937 biotinylated DNA. In the first experiment, recovery at each cycle of the nonhomologous sequences (Ω fragment) was assayed in the free fraction, while in the second experiment, binding of homologous sequences (3937 DNA) was assayed by the radioactivity level in the bound fraction. After four cycles of subtraction, the heterologous sequence/homologous sequence ratio had increased 18-fold and over 99% of homologous sequences had been removed from the solution. However, approximately 40% of heterologous sequences were lost at each cycle, resulting in an 83% loss after four cycles. For these reasons, the best compromise between enrichment in heterologous sequences and losses was four subtraction cycles.

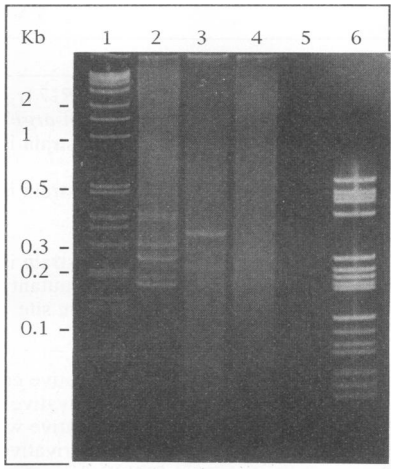

FIG. 1. PCR-amplified fragments selected by genomic subtraction. PCR products were analyzed by electrophoresis (15). Samples (10 μl) of PCR mixture were run on the gel. Lane 1, 1-kb DNA ladder (Bethesda Research Laboratories); lane 2, *E. carotovora* subsp. *atroseptica* 86.20 and *E. carotovora* subsp. *carotovora* CH26 (genomic subtraction); lane 3, *E. chrysanthemi* PMV4071 and *E. chrysanthemi* 3937 (genomic subtraction); lane 4, *E. carotovora* subsp. *carotovora* CH26 and *E. carotovora* subsp. *carotovora* CH26 (genomic subtraction); lane 5, PCR-negative control; lane 6, DNA marker V (Boehringer).

Genomic subtraction of *E. carotovora* subsp. *atroseptica* DNA. Four cycles of subtracting hybridizations were done for each of the following pairs of target and subtracter DNAs: (i) PMV4071 and 3937 strains (differentiated by the Ω fragment) (positive control), (ii) *E. carotovora* subsp. *atroseptica* 86.20 strain and *E. carotovora* subsp. *carotovora* CH26 strain, and (iii) *E. carotovora* subsp. *carotovora* CH26 and *E. carotovora* subsp. *carotovora* CH26 (negative control) (Fig. 1). The positive control yielded only two amplified fragments of approximately 230 and 300 bp. Since *Sau*3AI digestion of the initial Ω fragment generated eight main fragments of 460, 300, 230, 190, 150, 140, 80, and 60 bp, this control indicated that not all of the different sequences in the two DNA sequences were recovered after genomic subtraction. The subtraction between *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* yielded six main fragments (190, 200, 250, 300, 400, and 410 bp) and a double diffuse band around 450 bp. No amplified fragment was

present in the negative control. In all the lanes with genomic subtraction samples, a slight DNA smear was observed. Because this DNA smear was responsible for the background levels in hybridization experiments (using the whole PCR mixture as a probe), cloning of the fragments was undertaken.

Cloning of fragments obtained from subtraction. Samples from PCR were digested with *Sau*3AI before cloning to remove adaptors and obtain *Bam*HI-compatible ends. In one approach, the whole set of fragments was used for shotgun cloning with the *Bam*HI-cleaved pUBS-3 vector. After ligation and electroporation of *E. coli* DH5α cells, colony hybridization was performed by using labeled PCR mixture as a probe. This led to the isolation of clones carrying two different inserts. A second approach was to clone fragments isolated from a 6% polyacrylamide gel (1). The cloning vector used in this case was *Bam*HI-cleaved pTZ-19R. White colonies of electroporated *E. coli* DH5α were selected on medium containing X-Gal. This second strategy yielded clones carrying four additional inserts.

The six different cloned fragments were named probes A, B, C, D, E, and F, respectively, with probe A the largest and probe F the smallest (Table 1).

DNA sequencing and sequence analysis. Probes A, B, C, D, E, and F were sequenced. For each probe, the GenBank and EMBL databases (releases 75.0 and 33.0, respectively) were screened for homologies. No significant homology was observed for the sequences except for probe A, which had significant homology with the sequences of the *putP* genes from *Salmonella typhimurium* (73.8% identity) and *E. coli* (73.2% identity). The *putP* gene encodes a proline permease or carrier (20). This finding agreed with hybridization results that prove the ubiquity of probe A.

Probe specificity assessment. Dot blot membranes were prepared, using a collection of strains of *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *odorifera*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *wasabiae*, and other bacterial species. Hybridizations under high-stringency conditions were performed with each of the six fragments as probes, and hybridizations under low-stringency conditions were performed for probes B, E, and F. For most of the strains, hybridization was achieved at least twice on independent membranes. Table 2 illustrates the results. Each of the probes hybridized to the target DNA (*E. carotovora* subsp. *atroseptica* 86.20) and did not hybridize to the subtracter DNA (*E. carotovora* subsp. *carotovora* CH26). Probe A hybridized to most of the strains tested, including *E. carotovora* subsp. *carotovora* strains, but did not hybridize to a few other *E. carotovora* subsp. *carotovora* strains, such as CH26: this probe was not specific enough to be representative of the *E. carotovora* subsp. *atroseptica* subspecies. Under high-stringency conditions, only the typical *E. carotovora* subsp. *atroseptica* strains hybridized with probe B (data not shown), while under low-stringency conditions, some cross-hybridization occurred with *E. carotovora* subsp. *betavasculorum* strains, as illustrated in Fig. 2. Probe C presented positive signals with all the typical *E. carotovora* subsp. *atroseptica* strains and with some *E. carotovora* subsp. *carotovora* strains. Probe D hybridized with all the *E. carotovora* subsp. *atroseptica* strains and with a few *E. carotovora* subsp. *carotovora* strains. Probes E and F hybridized with only a limited number of *E. carotovora* subsp. *atroseptica* strains (under high- and low-stringency conditions) and thus seemed to be too specific for our purpose. In addition, probe F hybridized with two *E. carotovora* subsp. *wasabiae* strains and one *E. carotovora* subsp. *odorifera* strain. Hybridization of *E. carotovora* subsp. *carotovora* 1489 DNA presented some background noise for all the tested probes (Fig. 2); we later prove that the sample was contaminated by *E. carotovora* subsp. *atroseptica* DNA (data not shown).

Relative localization of the cloned fragments on *E. carotovora* subsp. *atroseptica* genomic DNA. In order to determine whether the cloned fragments corresponded to contiguous sequences, *E. carotovora* subsp. *atroseptica* 86.20 DNA was digested by several endonucleases (*Eco*RI, *Cla*I, and *Sau*3AI) and then blotted for Southern hybridization with each of the probes. As shown in Fig. 3, the absence of an hybridization signal on the control (*Eco*RI-digested *E. carotovora* subsp. *carotovora* CH26 DNA) confirmed that the probes are specific for *E. carotovora* subsp. *atroseptica* 86.20.

Furthermore, the DNA fragments revealed by each probe were different, suggesting that the probes are not clustered on the *E. carotovora* subsp. *atroseptica* 86.20 chromosome.

Surprisingly, probes E and F hybridized to more than one fragment of *E. carotovora* subsp. *atroseptica* DNA (two *Eco*RI fragments and two *Cla*I fragments for probe E; one *Eco*RI fragment and two *Cla*I fragments for probe F). As indicated by their sequence, these probes did not contain an *Eco*RI or *Cla*I site. Therefore, they should correspond to sequences that are duplicated on *E. carotovora* subsp. *atroseptica* 86.20 genome. Moreover, each of these two probes hybridized with a single *Sau*3AI fragment, suggesting a relatively high degree of identity between their two copies.

DISCUSSION

Using genomic subtraction, we isolated six fragments from *E. carotovora* subsp. *atroseptica* 86.20. These fragments corresponded to sequences that were absent from *E. carotovora* subsp. *carotovora* CH26 DNA, as was shown by Southern and dot blot hybridizations. This result was expected because the hybridization conditions of subtraction (1M NaCl at 65°C) were not stringent, and thus, only fully heterologous sequences of *E. carotovora* subsp. *atroseptica* escaped subtraction.

In the positive control of genomic subtraction, only two of eight fragments of the Ω interposon were recovered. By Southern hybridization at low stringency, we showed that this was not due to a partial homology between Ω and subtracter DNA sequences (data not shown). We thus hypothesize that the loss of some of the Ω fragments occurred later, probably at the PCR amplification step. If a fragment (because of its sequence, conformation, or size) is less efficiently replicated than the others, it is bound to be diluted during PCR, especially if the number of cycles is high (two amplifications and 25 cycles per amplification here). This hypothesis is supported by differences in the band patterns, observed for multiplex DNA fragment amplification, like random amplified polymorphic DNA, when small amounts of DNA are used (34).

We conclude from our experiments that only a subset of nonclustered heterologous sequences had been isolated by genomic subtraction.

A possible outcome of this work was to isolate sequences related to pathogenicity in *E. carotovora* subsp. *atroseptica*. Interestingly, probes B, C, and D hybridized to two *E. carotovora* subsp. *atroseptica* strains that were isolated from tomatoes and were also able to induce blackleg on potatoes (2, 26). However, Priou (26) recently showed that

TABLE 2. Strain collection and hybridization results

Strain[a]	Host	Country and/or year of isolation	Dot blot hybridization[b] with probe:						Dot blot position[c]
			A	B	C	D	E	F	
E. carotovora subsp. *atroseptica*									
88.33	Potato	France, 1988[d]	+	+	+	+	+	−	I2
88.45	Potato	France, 1988[d]	+	+	+	+	+	−	NS
88.1	Potato	France, 1988[d]	+	+	+	+	−	−	H3
88.22a	Potato	France, 1988[d]	+	+	+	+	+	+	F3
88.24	Potato	France, 1988[d]	+	+	+	+	−	−	E3
88.30a	Potato	France, 1988[d]	+	+	+	+	+	−	G3
87.7	Potato	France, 1987[d]	+	+	+	+	−	−	B1
87.13	Potato	France, 1987[d]	+	+	+	+	+	+	C1
87.16a	Potato	France, 1987[d]	+	+	+	+	+	−	D3
87.16b	Potato	France, 1987[d]	+	+	+	+	+	−	C3
86.14.11	Potato	France, 1986[d]	+	+	+	+	−	+	D1
86.20	Potato	France, 1986[d]	+	+	+	+	+	+	A1
511	Potato	France, 1964[e]	+	+	+	+	+	−	E1
SF1.1	Potato	Germany[f]	+	+	+	+	+	−	H1
161	Potato	The Netherlands[g]	+	+	+	+	+	+	D2
Cip114	Potato	Peru, 1980[h]	+	+	+	+	+	+	E2
Cip125	Potato	Peru, 1980[h]	+	+	+	+	−	−	F2
Cip131	Potato	Peru, 1980[h]	+	+	+	+	−	−	G2
Cip026	Potato	Peru, 1980[h]	+	+	+	+	+	+	B3
SH164.4	Potato	Réunion, 1988[f]	+	+	+	+	+	+	H2
CH3	Potato	Switzerland, 1985[i]	+	+	+	+	−	−	A3
CH5	Potato	Switzerland, 1985[i]	+	+	+	+	−	−	B2
CH6	Potato	Switzerland, 1985[i]	+	+	+	+	+	−	C2
SF18.296	Potato	Switzerland, 1958[f]	+	+	+	+	+	+	I1
1329	Potato	United Kingdom, 1967[e]	+	+	+	+	−	+	K1
1330	Potato	United Kingdom, 1967[e]	+	+	+	+	−	−	NS
SCRI1043	Potato	United Kingdom, 1985[j]	+	+	+	+	+	+	A2
1526	Potato	United Kingdom, 1957[e]	+	+	+	+	−	+	F1
1527	Potato	United States, 1973[e]	+	+	+	+	+	+	G1
1525	Potato	United States, 1969[e]	+	+	+	+	+	+	K2
1453	Tomato	France, 1973[e]	+	+	+	+	−	−	I3
1546	Tomato	France, 1973[e]	+	+	+	+	−	−	K3
E. carotovora subsp. *betavascularum*									
2121	Sugar beet	United States, 1972[e]	+	−	−	−	−	−	A9
2122	Sugar beet	United States, 1972[e]	+	−	−	−	−	−	B9
1520	Sunflower	Mexico[e]	ND	−	−	ND	ND	ND	C9
E. carotovora subsp. *carotovora*									
89.19	Potato	Argentina, 1989[d]	+	−	−	+	−	−	A4
1H	Water	Spain, 1989[k]	+	−	−	+	−	−	B4
40H	Water	Spain, 1989[k]	+	−	−	+	−	−	C4
SH230.134	Banana	Cuba[f]	+	−	−	−	−	−	C7
CM1	Cabbage	Malawi, 1986[f]	+	−	−	−	−	−	F6
798	Carrot	United States,[e] (ATCC 495)[e]	−	−	−	−	−	−	K6
CH15	Celery	Switzerland, 1988[i]	−	−	−	+	−	−	B8
1489	Chrysanthemum	France, 1971[e]	−	−	−	−	ND	ND	G8
1458	Chrysanthemum	United States, 1971[e]	+	−	+	−	−	−	D8
SH230.115	Corn	Cuba[f]	+	−	−	−	−	−	B7
1350	Cucumber	Italy[e]	+	−	+	−	−	−	E8
1285	Cyclamen	Greece[e]	+	−	−	−	−	−	C8
SE99.1	Witloof chicory	France, 1985[d]	−	−	−	−	−	−	B6
1488	Iris	France, 1973[e]	−	−	−	−	−	−	D6
SB89.7	Leek	France, 1982[f]	−	−	−	−	−	−	C6
2046[T]	Potato	Denmark, 1952[e]	+	−	−	−	−	−	F7
88.22c	Potato	France, 1988[d]	−	−	−	−	−	−	H6
88.29a1	Potato	France, 1988[d]	+	−	−	−	−	−	G5
88.44	Potato	France, 1988[d]	−	−	−	−	−	−	A7
87.25	Potato	France, 1987[d]	−	−	−	−	−	−	F5
86.14.51	Potato	France, 1986[d]	−	−	−	−	−	−	E7
S99	Potato	France, 1977[d]	−	−	−	+	−	−	H8
S101	Potato	France, 1977[d]	−	−	−	−	−	−	I8
76.26	Potato	France, 1976[d]	−	−	−	−	−	−	NS
PM2	Potato	Malawi, 1986[f]	+	−	−	+	−	−	A8

Continued on following page

TABLE 2—*Continued*

Strain[a]	Host	Country and/or year of isolation	Dot blot hybridization[b] with probe:						Dot blot position[c]
			A	B	C	D	E	F	
194	Potato	Morocco, 1963[e]	–	–	–	–	–	–	H5
Cip360	Potato	Peru, 1984[h]	+	–	+	–	–	–	H7
Cip361	Potato	Peru, 1984[h]	+	–	+	–	–	–	I7
Cip009	Potato	Peru, 1977[h]	–	–	+	–	–	–	K7
CH24	Potato	Switzerland, 1987[i]	–	–	–	–	–	–	I6
CH26	Potato	Switzerland, 1985[i]	–	–	–	–	–	–	G7
SCRI193	Potato	United States[j]	+	–	–	–	–	–	K5
1336	Potato	United Kingdom, 1967[e]	–	–	–	–	–	–	I5
Si82.1	Potato	Vietnam, 1989[f]	+	–	–	–	–	–	A6
SG162.6	Sunflower	France, 1987[f]	–	–	–	–	–	–	G6
1403	Sunflower	Yugoslavia, 1969[e]	–	–	–	–	–	–	E6
797	Tobacco	United States, 1951[e]	+	–	+	–	–	–	F8
SG39.1	?	Réunion, 1987[f]	+	–	–	–	–	–	D7
SG39.3	?	Réunion, 1987[f]	+	–	+	–	–	–	K8
E. carotovora subsp. *odorifera*									
1893	Celery	France, 1976[e]	–	–	–	–	–	–	D4
CH11	Celery	Switzerland, 1985[i]	+	–	–	–	–	–	A5
2155	Witloof chicory	France, 1983[e]	+	–	–	–	–	–	C5
2154	Witloof chicory	France, 1982[e]	+	–	–	–	–	–	I4
1892	Witloof chicory	France, 1981[e]	+	–	–	–	–	–	B5
1959	Witloof chicory	France, 1980[e]	+	–	–	–	–	–	H4
1878	Witloof chicory	France, 1979[e]	–	–	–	–	–	–	E4
1879	Witloof chicory	France, 1979[e]	+	–	–	–	–	–	F4
1880	Witloof chicory	France, 1979[e]	–	–	–	–	–	–	G4
1646.2	Leek	France, 1980[f]	+	–	–	–	–	–	E5
1654	Leek	France, 1980[f]	+	–	–	–	–	+	D5
CH4	Lettuce	Switzerland, 1986[i]	–	–	–	–	–	–	K4
E. carotovora subsp. *wasabiae*									
3304	Horseradish	Japan[e]	+	–	–	–	–	+	NS
3308	Horseradish	Japan[e]	+	–	–	–	–	+	NS
E. chrysanthemi									
3716	Kalanchoe	France, 1978[l]	+	–	–	–	–	–	G9
1596	Corn	France, 1978[e]	+	–	–	–	–	–	H9
EP2[2]	Philodendron	Martinique, 1987[m]	+	–	–	–	–	–	I9
1271	Corn	Egypt, 1961[e]	ND	–	–	ND	ND	ND	K9
SH230-C94	Tobacco	Cuba[f]	ND	–	–	ND	ND	ND	A10
3665	Diffenbachia	France, 1974[l]	+	–	–	–	–	–	B10
3937	Saintpaulia	France, 1977[l]	+	–	–	–	–	–	C10
1499	Corn	France, 1973[e]	NC	–	–	ND	ND	ND	D10
1888	Potato	France, 1978[e]	+	–	–	–	–	–	E10
CH29	Potato	Switzerland, 1988[i]	ND	–	–	ND	ND	ND	F10
2267	Potato	Australia, 1978[e]	ND	–	–	ND	ND	ND	G10
1871	Banana	Ivory Coast, 1976[e]	+	–	–	–	–	–	H10
CH36	Potato	Switzerland, 1987[i]	–	–	–	–	–	–	NS
1275	Carnation	United States, 1971[e]	+	–	–	–	–	–	NS
3805	Philodendron	France, 1976[l]	+	–	–	–	–	–	NS
2015	Potato	France, 1975[e]	+	–	–	–	–	–	NS
1236	Parthenium	United States, 1945[e]	+	–	–	–	–	–	NS
B374	Pelargonium	Comoro Islands, 1960[e]	+	–	–	–	–	–	NS
2013	Dahlia	France, 1974[e]	+	–	–	–	–	–	NS
2051	Diffenbachia	United States, 1957[e]	+	–	–	–	–	–	NS
Erwinia amylovora [1]	Hawthorn	France[n]	ND	–	–	ND	ND	ND	F9
Erwinia herbicola [1]	Not pathogenic	France[n]	+	–	–	–	–	–	D9
Erwinia rhapontici [1]	Rhubarb	Switzerland[i]	+	–	–	–	–	–	E9
Pseudomonas fluorescens pv. lomagnae [1]	Garlic	1976[f]	ND	–	–	ND	ND	ND	I10
Pseudomonas marginalis [1]	Potato	United States, 1970[f]	ND	–	–	ND	ND	ND	K10
Pseudomonas solanacearum [1]	Potato	Costa Rica[f]	ND	–	–	ND	ND	ND	A11
Pseudomonas syringae pv. phaseolicola [1]		France[n]	ND	–	–	ND	ND	ND	B11
Pseudomonas sp. [2]		The Netherlands[g]	+	–	–	–	–	–	C-D11
Pseudomonas viridiflava [1]	Chicory	Switzerland[i]	ND	–	–	ND	ND	ND	E11
Rhizobium meliloti [2]		France[n]	ND	–	–	ND	ND	ND	F-G11
Azorhizobium caulinodens [1]	*Sesbania rostrata*	Senegal[m]	ND	–	–	ND	ND	ND	H11
Klebsiella pneumoniae [1]		France[m]	ND	–	–	ND	ND	ND	I11
Agrobacterium tumefaciens [1]		France[n]	ND	–	–	ND	ND	ND	K11

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TABLE 2—*Continued*

Strain[a]	Host	Country and/or year of isolation	Dot blot hybridization[b] with probe:						Dot blot position[c]
			A	B	C	D	E	F	
Xanthomonas campestris [2]		France[n]	ND	–	–	ND	ND	ND	A-B12
Clavibacter michiganensis [1]	Potato	United States, 1976[f]	–	–	–	–	–	–	C12
Brucella melitensis [1]		France[n]	ND	–	–	ND	ND	ND	D12
Yarrovia lipolytica [1]		France[n]	ND	–	–	ND	ND	ND	E12
Yersinia ruckeri [1]	Salmon	France, 1982[n]	ND	–	–	ND	ND	ND	F12
Yersinia pseudotuberculosis [1]	Human pathogen	ATCC 23207-29833	+	–	–	–	–	–	G-H12
Saprophytic bacteria [8]	Potato	Spain[k]	ND	–	–	ND	ND	ND	NS
Escherichia coli [1]			+	–	–	–	–	–	I12
Bacillus polymyxa [1]	Potato	France, 1979[f]	–	–	–	–	–	–	K12

[a] Strains 89.19, 1H, and 40H are atypical *E. carotovora* subsp. *atroseptica* strains recently identifed as *E. carotovora* subsp. *carotovora* strains by using phenotypic and genotypic characteristics (4, 26). Superscript T's indicate the type strain of the subspecies. The numbers in brackets are the numbers of strains tested.

[b] +, hybridization with DNA of the strain; –, no hybridization; ND, data not determined.

[c] Numbers and letters correspond to rows and columns in Fig. 2. NS, data not shown.

[d] Bernard Jouan, Institut National de la Recherche Agronomique, Rennes, France, personal collection.

[e] Collection Française de Bactéries Phytopathogènes, Angers, France.

[f] Régine Samson, Institut National de la Recherche Agronomique, Angers, France, personal collection.

[g] Research Institut for Plant Protection, Wageningen, The Netherlands.

[h] International Potato Center, Lima, Peru.

[i] Olivier Cazelles, Station Fédérale de Recherches Agronomiques, Changins, Switzerland, personal collection.

[j] Scottish Corp Research Institute, Dundee, United Kingdom.

[k] Maria Lopez, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain, personal collection.

[l] Monique Lemattre, Institut National de la Recherche Agronomique, Versailles, France, personal collection.

[m] Claudine Elmerich, Institut Pasteur, Paris, France, personal collection.

[n] Collection Française Informatisée de Souches Microbiennes, Institut National de la Recherche Agronomique, Angers, France.

two *E. carotovora* subsp. *carotovora* strains (88.29a1 and CIP009) are able to induce blackleg symptoms identical to those induced by *E. carotovora* subsp. *atroseptica*. Because probes B, C, and D did not detect these two *E. carotovora* subsp. *carotovora* strains, they may not correspond to sequences involved in this pathogenicity trait. It is therefore likely that the isolated sequences are related to other physiological characteristics specific to *E. carotovora* subsp. *atroseptica*.

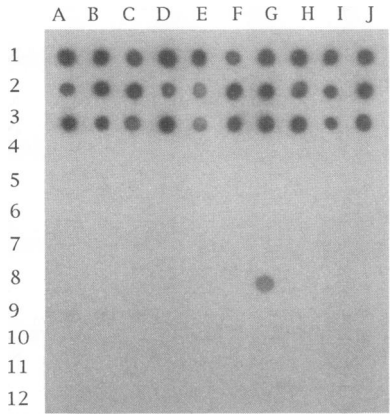

FIG. 2. Bacterial strains revealed by probe B under low-stringency conditions. Dot blot hybridization was performed on the collection of bacterial strains (the strains shown in positions are given in Table 2) under low-stringency conditions, as described in Materials and Methods. Rows 1 to 3 contain typical *E. carotovora* subsp. *atroseptica* strains. Rows 4 to 8 contain *E. carotovora* strains (atypical *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *odorifera*, *E. carotovora* subsp. *carotovora*, and *E. carotovora* subsp. *betavasculorum*). The rest of the membrane is occupied by other bacterial species. The weak signals observed at positions A9, B9, and C9 corresponded to the three *E. carotovora* subsp. *betavasculorum* strains in our collection. The signal at position G8 corresponded to *E. carotovora* subsp. *carotovora* DNA contaminated by *E. carotovora* subsp. *atroseptica* DNA (see Results).

More generally, our data did not show a correlation between the presence of sequences homologous to the probes and any physiological feature of *E. carotovora* subsp. *atroseptica* or *E. carotovora* subsp. *carotovora* strains (26). The exception to this observation was probe A, which corresponded to the proline permease gene. Use of probe A revealed that some *E. carotovora* subsp. *carotovora* strains do not possess a *putP* gene. This raised the question of proline uptake in strains lacking *putP*: i.e., the question of whether such bacteria are unable to efficiently import proline or whether they possess another carrier, as observed in *E. coli* and *S. typhimurium* (14).

The results of dot blot hybridizations showed that the group of *E. carotovora* subsp. *atroseptica* strains was fairly homogeneous, although some heterogeneity was seen with probes E and F. Probe B recognized all *E. carotovora* subsp. *atroseptica* strains, but not the three atypical strains (89.19, 1H, and 40H) that grow at 37°C and do not induce typical blackleg symptoms on potato and were proposed as *E. carotovora* subsp. *carotovora* (4, 26).

On the other hand, the group of *E. carotovora* subsp. *carotovora* strains appeared more heterogeneous. This conclusion is compatible with the results of phylogenic studies applying restriction fragment length polymorphism analysis to *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora pel* genes (4). The homogeneity of the *E. carotovora* subsp. *atroseptica* group may reflect the adaptation of the pathogen to the relatively low genetic diversity of cultivated potatoes. In contrast, *E. carotovora* subsp. *carotovora* is considered an opportunistic pathogen, with a wide host range and geographical distribution, which is consistent with high genetic diversity.

One application of genomic subtraction is to isolate DNA probes for diagnosis, as previously shown for *Rhizobium*

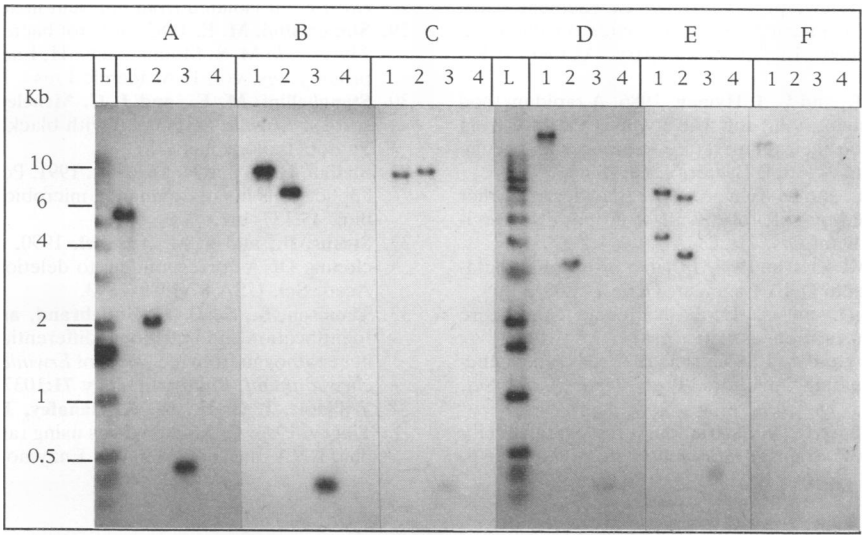

FIG. 3. Autoradiography of Southern blot membranes hybridized with the α-^{32}P-labeled probes. Hybridization results with DNA probes A to F are shown. Lane L, 1-kb DNA ladder. Lanes 1 to 3, *E. carotovora* subsp. *atroseptica* 86.20 DNA digested with *Eco*RI, *Cla*I, and *Sau*3AI, respectively; lane 4, *E. carotovora* subsp. *carotovora* CH26 DNA digested with *Eco*RI.

meliloti (3). Probe B could be used to diagnose the presence of *E. carotovora* subsp. *atroseptica* in soil and plant tissue samples. An improvement in detection sensitivity would be the development of a PCR test based on this sequence (31).

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