A DNA Probe Specific for Serologically Diverse Strains of *Erwinia carotovora*

L. J. Ward and S. H. De Boer

Agriculture Canada, Vancouver Research Station, 6660 N.W. Marine Drive, B.C., Canada, V6T 1X2.
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ABSTRACT

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A DNA probe was developed which was specific for *Erwinia carotovora* and hybridized with all tested strains in the subspecies *atroseptica, carotovora,* and *betavasculorum*, regardless of serogroup specificity. The probe, isolated from a genomic library of *Erwinia,* was selected for its ability to hybridize to genomic DNA of *Erwinia*, but not to genomic DNA from the taxonomically related *Escherichia coli*. On nitrocellulose filters, a single colony of *E. carotovora* could be detected against a background of about 1,000 colonies of soil bacteria. As few as 200 colony-forming units (cfu) of *E. carotovora* subsp. *atroseptica* could be detected in a suspension containing 2×10^3 cfu of soil bacteria per milliliter.

Additional keywords: blackleg, detection, potato, soft rot.

Erwinia carotovora, a gram-negative, facultative anaerobe, secretes pectolytic enzymes and causes diseases of many economically important crops. *Erwinia*-incited soft rot and blackleg diseases of potato have been of particular concern. The bacteria, which survive on potato tubers, serve as a major source of inoculum for these potato diseases (9,12). Indexing seed tubers for the presence of *E. carotovora* would be useful for identifying problem seed lots.

Although serological testing has been quite successful for various phytopathogenic bacteria, serological indexing for *E. carotovora* is hampered by the serological diversity of strains. More than 40 serogroups of *E. carotovora* have been described (3,5). The availability of a specific DNA probe would allow development of tests with DNA hybridization technology, which is independent of the serological reaction of the bacterium.

DNA-DNA hybridization assays already have been used for some time in clinical laboratories for detection of microbial pathogens (20,21). In general, these assays are more rapid, sensitive, and specific than conventional immunological and cultural procedures. To date, however, there has been little application of DNA hybridization technology to detect plant pathogenic bacteria. DNA probes to phytopathogenic bacteria largely have been used for strain identification, although their use for detection and diagnostic purposes has been suggested (8,10,15,19,23).

This report describes the isolation of two DNA probes from chromosomal DNA of *E. carotovora* using two different strategies. Specificity of the probes for strains of *E. carotovora* in different subspecies and serogroups is shown, and preliminary results of using one of the probes for detection of the bacterium in soil are given. A preliminary report of a portion of this work has been presented (7).

MATERIALS AND METHODS

Bacterial cultures. *Erwinia carotovora* subsp. *atroseptica* strains 31, 6, 196, and 198 in serogroups I, XVIII, XX, and XXII, respectively, and *E. c.* subsp. *carotovora* strains 71, 26, 193, and 380 in serogroups III, V, XI, and XXIX, respectively, were used to construct DNA libraries. The bacterial strains were stored in 10% glycerol at −20 C and grown at 23 C in nutrient broth or on nutrient agar as required. Strains of *Erwinia* spp. for testing probe specificity were obtained from various sources (Table 1). Saprophytic bacteria were isolated from potato field soil on crystal violet pectate (CVP) medium to obtain strains that might be isolated along with the pectolytic erwinias.

Extraction of DNA. Bacterial cells grown to mid-log phase were harvested by centrifugation, treated with lysozyme (5 mg/ml) for 30 min, and lysed with 1% sodium dodecyl sulfate (SDS) at 55 C for 30 min. The DNA was sheared by repeated passage through an 18-gauge needle and purified by repeated phenol/chloroform extraction, ethanol precipitation, and cesium chloride-ethidium bromide density gradient centrifugation (13). To negate strain differences, DNA from four strains of *E. c. atroseptica* and four strains of *E. c. carotovora* were used. DNA from *E. c. atroseptica* used for cloning consisted of equal amounts of DNA from strains 6, 31, 196, and 198. DNA from *E. c. carotovora* consisted of equal amounts of DNA from strains 26, 71, 193, and 380. DNA mixtures were digested with *Sau*3A1 (Gibco/BRL, Burlington, Ont.) to an average size of 5 kb or less as determined by agarose gel electrophoresis (13). The reaction was stopped by phenol/chloroform extraction followed by ethanol precipitation.

Construction of libraries. Bluescribe (Vector Cloning Systems, San Diego, CA) was used as the vector for construction of libraries of *E. c. atroseptica* and *E. c. carotovora*. Vector DNA was obtained by the alkaline lysis procedure and cesium chloride-ethidium bromide density gradient centrifugation (13). The DNA was digested with *Bam*HI (GibcoBRL) and treated with calf intestinal phosphatase (Promega, Madison, WI). Separate libraries of *E. c. carotovora* and *E. c. atroseptica* were constructed by combining 150 μg of DNA of *E. c. atroseptica* or *E. c. carotovora* with 300 μg of vector DNA in the presence of T4 DNA ligase (GibcoBRL) for 12 hr at 15 C. Dilutions of this ligation reaction were used to transform competent cells of *Escherichia coli* DH5α according to the supplier's instructions. Transformants were stored at −20 and −80 C in Luria-Bertani medium (LB) containing 20% glycerol.

Selection of DNA probes. Two strategies were used to select potentially specific probes. In the first approach, the *Erwinia* libraries were screened on the selective CVP medium on which untransformed *E. coli* does not grow. For this procedure, an aliquot of transformants from libraries of *E. c. carotovora* and *E. c. atroseptica* was enriched in LB containing ampicillin (100 μg/ml) for 5 hr at 37 C and then spread plated onto CVP medium containing 50 μg/ml of ampicillin. The ampicillin was spread over the CVP medium 24 hr before use. Transformant colonies able to grow on this medium were selected after a 48-hr incubation at 30 C.

The second strategy involved selection of a unique sequence of DNA from *E. carotovora* that did not occur in *E. coli*. Transformants from the libraries of *E. c. carotovora* and *E. c. atroseptica* were combined and enriched as above. Dilutions of the culture were spread on nitrocellulose filters overlaid on LB agar containing ampicillin. Filters with 2,000–3,000 colonies were replica-plated. Filters were treated with alkali, neutralized, and baked at 80 C for 2 hr under vacuum. The filters then were prehybridized with 50 μg/ml denatured DNA from *E. coli* strains HB101 and RR1 (1:1 DNA mixture) containing Bluescribe in 50% formamide and 3× saline sodium citrate (SSC) at 45 C for 24 hr (11). Genomic DNA of *E. c. carotovora* and *E. c. atroseptica* was labeled with α-^{32}P-dATP by a random primer labeling system according to the supplier's instructions (GibcoBRL). Labeled DNA homologous to *E. coli* or Bluescribe was removed by hybridization to a 5-cm^2 piece of Zetaprobe membrane (Bio-Rad Laboratories, Richmond, CA) to which 500 μg of denatured DNA from *E. coli* and Bluescribe had been covalently bound. This membrane was washed with 3× SSC/50% formamide at 50 C and the wash combined with the unbound probe. The mixture was denatured at 75 C for 15 min and hybridized to the filters with transformant colonies for 60 hr at 45 C with agitation in the presence of 50% formamide and total denatured genomic DNA of *E. coli* HB101 and RR1 harboring Bluescribe (50 μg/ml). After hybridization, the filters were washed at 50 and 65 C in 0.1× SSC and 0.5% SDS followed by autoradiography on Kodak X-Omat AR-5 for 24 hr with two intensifying screens at −80 C. Positive colonies were selected from the replicate plate.

Preparation of labeled DNA inserts. Probe DNA was excised from the Bluescribe vector using either *Bam*HI or an *Eco*RI/*Hind*III double digest and followed by gel purification using Geneclean (Bio/Can Scientific Inc., Mississauga, Ont.). Unincorporated nucleotides were removed from α-^{32}P-dATP-labeled probes by ethanol precipitation. The probes were denatured at 75 C for 15 min in the presence of 70% formamide and sonicated salmon sperm DNA (25 μg/ml).

Colony hybridizations. Bacterial strains to be probed were transferred to Zetaprobe membrane by direct transfer of a loopful of cells from colonies on agar medium, by filtration of a broth culture through the membrane in a dot blot vacuum filtration manifold (Schleicher & Schuell, Surrey, UK), or by a colony lift procedure. Alternatively, colonies were grown directly on nitrocellulose filters incubated on nutrient agar or CVP medium. DNA from bacteria on Zetaprobe membranes was released, denatured, and covalently bound by placing the filters on several pads of filter paper saturated with 0.5 M NaOH. DNA from bacteria on nitrocellulose was released with alkali, neutralized, and fixed under vacuum at 80 C for 2 hr. Cell debris subsequently was removed by washing for 2 hr at 60 C in 2× SSC, 0.5% SDS containing 1 μg of proteinase K/milliliter. The filters were prehybridized with denatured salmon sperm DNA (25 μg/ml) for 12 hr at 45 C in 3× SSC, 1% Blotto, 1× Denhardt's solution, 0.5% SDS, and 50% formamide. Denatured labeled probe was added directly to the prehybridization solution to a final concentration of 2–10 ng/ml. Hybridizations were routinely allowed to proceed overnight at 45 C. After hybridization, filters were washed sequentially in 2× SSC, 1× SSC, and 0.1× SSC with 0.5% SDS at 60–65 C. Hybridization was detected as described above.

Detection limits. Strain 31 of *E. c. atroseptica* was mixed with a soil extract to determine the population that could be detected against a background of other bacteria. The soil extract was prepared by shaking 25 g of soil in 10 ml of 0.01 M phosphate-buffered saline for 1 hr and then letting the soil particles settle for 30 min. The top liquid phase was used as the soil extract. Populations of soil bacteria and *E. carotovora* were determined by the plate count procedure on nutrient agar. *E. c. atroseptica* 31 was mixed at several concentrations with soil extract and plated onto nitrocellulose membranes on petri plates containing CVP or nutrient agar. After incubation at 23 C for 24 hr, the filters were treated, probed, and subjected to autoradiography as described above. The *E. c. atroseptica* 31/soil extract mixture also was plated directly on CVP medium and incubated at 23 C for 72 hr for comparison.

In another experiment, 12 serial twofold dilutions of *E. c. atroseptica* 31 were added to a constant concentration of soil extract. After thorough mixing of the suspension, 100 μl was deposited on Zetaprobe membrane in a dot blot vacuum filtration manifold. The membrane was treated as described above. These experiments were repeated twice.

TABLE 1. Strains of *Erwinia carotovora* used in this study

Species or subspecies	No. of strains	Particulars	Source	Reference
atroseptica	4	homologous strains for serogroups	VRS[a]	3,5
carotovora	36	homologous strains for serogroups	VRS	3,5
atroseptica	29	phenon A1	LMG[b]	6
carotovora	15	phenon A2	LMG	6
Intermediate subspecies	13	phenon A3, A4, B, D, and E	LMG	6
betavasculorum	6	phenon C	LMG	6
chrysanthemi	22		IPO[c]	unpublished

[a]Collection at the Vancouver Research Station.
[b]Culture collection of the Laboratorium voor Microbiologie, Rijksuniversiteit, Gent, Belgium.
[c]Instituut voor Plantenziektenkundig Onderzoek, Wageningen, the Netherlands.

RESULTS

Only six colonies were obtained when the transformants of *E. coli* containing DNA of *E. c. carotovora* were selected on CVP medium containing ampicillin. No colonies were obtained with transformants containing DNA of *E. c. atroseptica*. Restriction fragment analysis of the recombinant plasmids in these colonies by agarose gel electrophoresis showed that they all contained a 6-kb insert which could be excised with *Bam*HI. One of the colonies was selected, and the insert designated Probe A for further tests. In an initial screen, Probe A was tested by hybridization against four strains of *E. c. atroseptica* and 36 strains of *E. c. carotovora* representing 40 different serogroups. Only 25 of the strains consistently gave a strong signal (Fig. 1A). Some of the strains gave variable results in repeated tests, and preliminary observations suggested that the reaction of strains was influenced by the presence or absence of pectin in the medium.

The screening of transformants, which was based on hybridization to total labeled DNA of *E. c. carotovora* and *E. c. atroseptica* prehybridized to DNA from *E. coli*, produced 12 positive colonies after a 24-hr exposure. Four of these colonies contained recombinant plasmids with inserts of approximately 1 kb. One of the inserts hybridized uniformly with the 40 serologically different strains (Fig. 1B) and was selected as Probe B for further testing. Probe B hybridized with an additional 63 strains of *E. carotovora* from diverse geographical regions and representing subspecies *atroseptica, carotovora, betavasculorum*, and physiologically intermediate strains (Fig. 2). It hybridized only weakly with one of 21 strains of *Erwinia chrysanthemi*, and two of 35 soil isolates tested as pure cultures. The two soil isolates were tentatively identified as entericlike bacteria on the basis of fatty acid composition (J. Janse and D. Stead, *personal communication*). A summary of the reaction of all bacteria tested with Probe B is given in Table 2.

Single positive colonies could readily be detected with Probe B among about 10^3 colony-forming units (cfu) of soil microorganisms on filters spread with *E. c. atroseptica* 31 cells suspended in the soil extract. No differences were observed

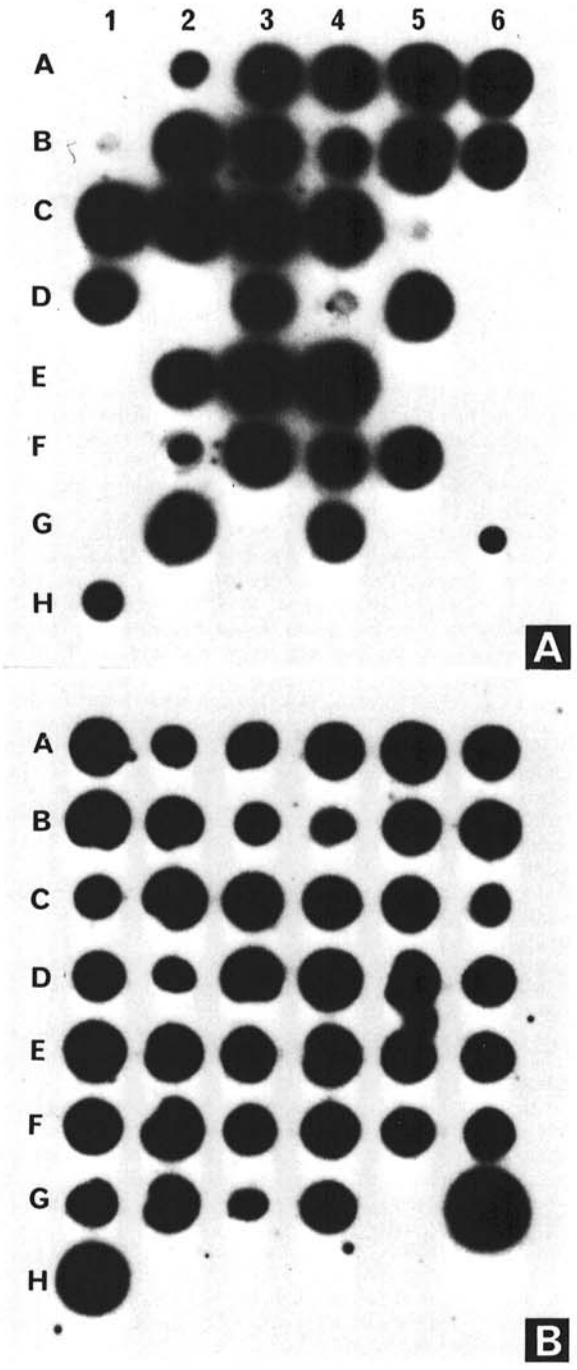

Fig. 1. Dot blot hybridization of DNA probes to purified DNA from the homologous strains for each of 40 serogroups of *E. carotovora*. DNA from *E. carotovora* strains for serogroups I–VI were loaded in position A1–6, serogroups VII–XII in B1–6, serogroups XIII–XVIII in C1–6, serogroups XIX–XXIV in D1–6, serogroups XXV–XXX in E1–6, serogroups XXXI–XXXVI in F1–6, and serogroups XXXVII–XL in G1–4. Bacteriophage λ DNA was loaded in G5, Probe B DNA in G6, and Probe A DNA in H1. Filter A was probed with Probe A, and filter B with Probe B. Note that cross hybridization occurred between probes A and B, which may have been due to small amounts of contaminating vector DNA.

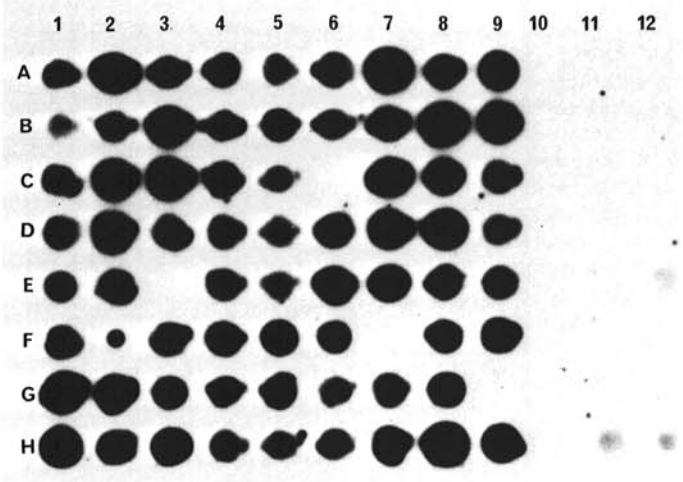

Fig. 2. Dot blot hybridization of Probe B to lysates of various bacterial strains on Zetaprobe membranes. Columns 1–9 in rows A–H were loaded with strains of *E. carotovora* except positions C6, E3, F7, and G9, which were empty. Columns 10–11 in rows A–G, H10, and column 12 in rows A–F were loaded with strains of *E. chrysanthemi*, position H11 and H12 with unidentified soil strains, and position G12 with *E. coli* HB101.

TABLE 2. Reaction of probe B with various bacteria

Bacteria	No. of strains tested	Reaction of strains +	±	−
Erwinia carotovora atroseptica	33	33	0	0
E. c. carotovora	51	51	0	0
E. c. ? subsp.	13	13	0	0
E. c. betavasculorum	6	6	0	0
E. chrysanthemi	21	0	1	20
Escherichia coli	2	0	0	2
Unidentified soil isolates	35	0	2	33

between filters incubated on CVP and nutrient agar. *Erwinia*-like colonies did not grow up on the CVP plates spread with the same suspension. In the experiments in which bacterial suspensions were deposited on filters in the dot blot manifold, as few as 20 cfu's of *E. c. atroseptica* 31 could be detected in 100 μl of soil extract containing 2×10^4 cfu (Fig. 3). The soil extract alone did not hybridize with Probe B.

DISCUSSION

Various strategies have been used to develop genomic DNA probes specific for particular bacterial pathogens. One approach has been to screen the target bacterium plus one or more closely related bacterium with individual or pooled DNA probes (8,21). Another approach has been to use a cloned DNA fragment from a gene known to be involved in the production of a pathogen-specific metabolite (22). In this study, we used two different strategies to develop probes specific to all strains of *E. carotovora*. In the first approach, we attempted to select a DNA fragment which would impart to *E. coli* the ability to grow on CVP medium. *E. coli* normally will not grow on CVP medium, which is selective for the pectolytic *Erwinia*. Although this approach yielded a potential DNA probe (Probe A), further tests of many strains gave variable results (data not shown). Preliminary observations indicated that DNA from *Erwinia* cells preconditioned on pectate-containing medium hybridized best to Probe A. This suggests that Probe A hybridizes to a DNA fragment which may be amplified or rearranged in response to pectate or some other component of CVP medium.

Our second strategy was based on the fact that *Erwinia* and *Escherichia* are related as members of the family Enterobacteriaceae. A sequence of DNA from *E. carotovora* which was absent from the genome of *E. coli* was identified by isolating a fragment of DNA from *E. carotovora* which was not blocked by an excess of DNA from *E. coli*. Probe B, obtained in this manner, reacted with all strains of *E. carotovora* against which it was tested and did not react well with any other bacteria. Weak cross-reactions were observed only with one strain of *E. chrysanthemi* and two soil strains. However, by using shorter exposure time for autoradiography, it should be possible to eliminate all visible cross-reactions while retaining strong positive signals for strains of *E. carotovora*.

Because of its specificity for all strains of *E. carotovora*, Probe B should be useful for detecting soft rot bacteria in ecological and epidemiological studies and for indexing potato tubers for contamination with *E. carotovora*. Probe B hybridized to strains of *E. carotovora*, which had been isolated from various hosts and geographic regions and which differed in serological and physiological characteristics. The lack of hybridization with the closely related *E. chrysanthemi* species and soil bacteria attests to the specificity of the probe. The two soil strains that showed weak cross hybridization have not been fully characterized but may represent nonpectolytic *Erwinia* or a closely related species.

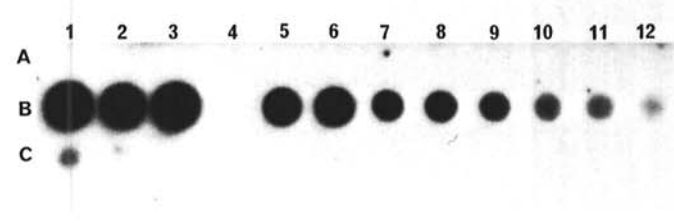

Fig. 3. Dot blot hybridization of Probe B to *E. carotovora* subsp. *atroseptica* and soil bacteria. Soil extract containing 2×10^5 bacterial cfu/ml was loaded in positions A1–12. Soil extract with added *E. carotovora* subsp. *atroseptica* strain 31 at 4×10^4 cfu/ml was loaded in position B5, and the following wells (B6–C12) were loaded with serial twofold dilutions of the *E. c. atroseptica* 31 preparation mixed with undiluted soil extract. A pure culture of *E. c. atroseptica* 31 at 4×10^4 cfu/ml was loaded in positions B1–B3.

Sensitivity of DNA hybridization procedures for detecting *E. carotovora* with Probe B is greater than with other currently available procedures. The only procedure that has been available for detecting all strains of *E. carotovora* has been isolation on a selective medium such as CVP (17). Direct isolation procedures, however, usually are not successful in detecting low numbers of a specific phytopathogen in the presence of high populations of saprophytic bacteria. In preliminary experiments, *E. carotovora* could readily be detected with Probe B in mixtures with soil bacteria in which *Erwinia* could not be detected by direct plating on CVP. In a standard dot blot assay, 200 cfu/ml of *E. carotovora* could be detected in the presence of a 1,000-fold excess of soil bacteria. This sensitivity is considerably greater than that generally accepted for serological tests. The sensitivities of immuno-fluoresence and enzyme-linked immunosorbent assays are in the order of 10^3–10^4 cfu/ml (1,4,16,18). It is anticipated that the sensitivity of detection with the DNA probe can be further enhanced if combined with selective enrichment procedures (14) and with advances in DNA technology (2).

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