# Development and Application of a Plasmid DNA Probe for Detection of Bacteria Causing Common Bacterial Blight of Bean

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This research was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison; by the Agricultural Research Service, USDA; and by a grant from USAID as part of the Bean/Cowpea CRSP.

We thank Steve Vicen for assistance in preparation of figures; Doug Cook for probing *Pseudomonas solanacearum* DNA; Mark Wegmann for technical assistance; and Judy Gosse for processing the manuscript. We also thank S. S. Hirano, P. H. Williams, and N. K. Fritz for critical reading of the manuscript.

Accepted for publication 7 October 1988 (submitted for electronic processing).

## ABSTRACT

Gilbertson, R. L., Maxwell, D. P., Hagedorn, D. J., and Leong, S. A. 1989. Development and application of a plasmid DNA probe for detection of bacteria causing common bacterial blight of bean. Phytopathology 79:518-525.

Total plasmid DNA and cloned plasmid DNA fragments from *Xanthomonas campestris* pv. *phaseoli* were used as probes to detect *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, causal agents of common bacterial blight of bean. Plasmid DNA hybridized extensively to total genomic DNA from 50 strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, less extensively to that from *X. c.* pvs. *alfalfae, carotae, vesicatoria* (races 1 and 2), and *oryzae*, and not at all to that from *X. c.* pvs. *campestris, holcicola*, or *pelargonii*, nonpathogenic xanthomonads from bean debris or other bacterial species. A 3.4-kb *Eco*RI fragment of plasmid DNA, which contains repetitive DNA, was a more specific probe for *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* than total plasmid DNA. The limit of detection of these probes was $10^3$ *X. c. phaseoli* colony-forming units (~10 pg of DNA). A colony hybridization procedure was used to detect colonies of *X. c. phaseoli* recovered from bean leaves and debris, and squash and dot blot hybridization procedures were used to detect *X. c. phaseoli* in bean leaves. Our results indicate that DNA probes are a useful tool for detecting plant pathogenic xanthomonads and may be used in ecological and epidemiological studies.

*Additional keywords*: colony hybridization, *Phaseolus vulgaris*.

Nucleic acid probes are effective diagnostic tools for detection of infectious agents, due to the sequence specificity of the nucleic acid molecule. Conditions that allow the probe to bind only to homologous sequences in a DNA sample prevent cross reaction with DNA from unrelated organisms. Furthermore, DNA structure is relatively consistent, whereas production of other macromolecules such as proteins, carbohydrates, and lipids may change as the result of environmental perturbation, e.g., growth medium.

Tremendous potential exists for the use of nucleic acid probes to detect plant pathogens in plants, plant propagative organs, plant-associated debris, and soil. Considerable progress has already been made in the development of DNA probes for detection of animal and human bacterial pathogens (18,26,31), and DNA probes are currently available for *Legionella* (8,15) and *Salmonella* spp. (9), among others. Nucleic acid probes have been used to detect plant viruses and viroids (2,25,29), *Spiroplasma citri* (24), and the mycoplasmalike organism that causes Western X-disease (17). A DNA probe that includes the phaseolotoxin gene from *Pseudomonas syringae* pv. *phaseolicola*, an important seedborne bacterium that causes halo blight of bean, has been used to detect this bacterium (28).

Identification of plant-pathogenic bacteria is currently based on biochemical, physiological, and pathogenicity tests that are time-consuming, laborious, and often ambiguous and subjective. The species *Xanthomonas campestris* (Pammel 1895) Dowson 1939 is comprised of at least 125 pathovars that are differentiated by their ability to cause disease on a particular plant or plants (4). All members of the genus *Xanthomonas* are thought to be plant pathogens, but there is increasing evidence that nonpathogenic xanthomonads also exist on or in plants and plant debris and in association with pathovars of *X. campestris* (11,13,14,21). Clearly, a need exists to devise rapid and efficient methods to detect pathovars of *X. campestris*.



In this report we describe the development and application of DNA probes for detection of *Xanthomonas campestris* pv. *phaseoli* and *X. c. phaseoli* var. *fuscans*, causal agents of common bacterial blight of bean (*Phaseolus vulgaris* L.). *X. c. phaseoli* var. *fuscans* is a variant of *X. c. phaseoli* that produces a brown melaninlike pigment in culture but causes disease symptoms on beans that are indistinguishable from those caused by *X. c. phaseoli* (32). Plasmid DNA from *X. c. phaseoli* was used as a probe to detect *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, and to differentiate *X. c. phaseoli* from nonpathogenic xanthomonads associated with bean debris. Techniques are described for practical application of DNA probes to the detection of bacteria on and/or in bean plants and in bean debris.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study, their geographical origin, and their sources are listed in Table 1. All strains were obtained from single colonies after two or more single-colony isolations. Xanthomonads were isolated on MXP (6) and subcultured on sucrose peptone agar (SPA, 16). *Erwinia herbicola* and *Escherichia coli* were subcultured on nutrient agar (Difco). Liquid cultures were grown in nutrient broth (Difco). All bacterial strains were stored in 5% $Me_2SO$ at −80 C (27).

**Growth and inoculation of plants.** All *X. c. phaseoli, X. c. phaseoli* var. *fuscans*, and nonpathogenic xanthomonad strains used for DNA extractions were tested for pathogenicity. Beans (*P. vulgaris* 'Topcrop') susceptible to common blight were grown in a controlled environment chamber (16-hr photoperiod, 24 C, 250 $\mu$E $m^{-2}$ $sec^{-1}$). Seeds were germinated in vermiculite, and seedlings were transplanted into 10-cm diameter plastic pots containing a mixture of soil, peat, and sand (2:1:1). Test strains were grown in shake culture in nutrient broth for 24–48 hr at 24 C. Trifoliolate leaves that were one half to three quarters expanded were inoculated on the adaxial surface by the Carborundum cotton swab technique (7). Control plants were inoculated with nutrient broth plus Carborundum. Plants were scored for symptom development 7–10 days after inoculation. Extensive water-soaking

on the under surface of the leaf as well as necrosis and chlorosis were rated as pathogenic responses.

**Isolation of plasmid and total genomic DNA.** Plasmid DNA was extracted from xanthomonads using a modified, small-scale alkaline lysis procedure (22). Cultures were grown to mid-logarithmic phase ($A_{600nm}$ = about 0.7), and cells from 5 ml of medium were used for extraction. Before lysis, cells were washed in 1 ml of 1 M NaCl. Plasmid DNA was resuspended in 15 $\mu$l of TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA [pH 8.0]).

To obtain plasmid DNA for use as a probe, a 1-L culture of *X. c. phaseoli*, strain WT-1, was grown to mid-logarithmic stage and plasmid DNA was extracted, using the alkaline lysis procedure of Maniatis et al (22) after washing cells in 1 M NaCl. Plasmid DNA was separated from chromosomal DNA by CsCl density gradient centrifugation.

Total genomic DNA was extracted from 25 ml of mid-logarithmic phase cultures of xanthomonads, *Erwinia herbicola*, and *Escherichia coli* (3). Cells were washed in 1 M NaCl before DNA extraction. Total genomic DNA was extensively dialyzed in DSB (6 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5), precipitated with ethanol, and resuspended in 0.2–0.8 ml of TE buffer.

TABLE 1. Bacterial strains

| Bacterium | Strain | Location | Source |
|---|---|---|---|
| *Xanthomonas campestris* pathovar | | | |
| *alfalfae* | XA1 | Kansas | D. L. Stuteville |
| *campestris* | XCC1 | Wisconsin | R. L. Gilbertson |
| *carotae* | XCAR1 | Wisconsin | D. K. Willis |
| *holcicola*[a] | XCH429, XCH474 | Kansas | J. L. Leach |
| *pelargonii* | UCRDAC #0782-29 | Ohio | R. L. Clevenstine |
| *phaseoli* | WT1, 45, SH42B, W2–W10, XCPA, H85, H81, W15–W19, XCPGR | Wisconsin | R. L. Gilbertson |
| | XCPM1, XCPM2 | Michigan | A. W. Saettler |
| | BCBI, BCBII | Brazil | R. L. Gilbertson |
| | ES3–ES5, XP15, CNF3, CNF27, CNF30, CNF31, CNF32 | Brazil | J. C. Faria |
| | XCPN | Nebraska | A. K. Vidaver |
| | XCPG1–XCPG4 | Guatemala | R. L. Gilbertson |
| | X34, X45 | Florida | E. L. Civerolo |
| | X47 | Uganda | E. L. Civerolo |
| | X53 | New York | E. L. Civerolo |
| | X50 | Colombia | E. L. Civerolo |
| *phaseoli* var. *fuscans* | WF8, WF9 | Wisconsin | R. L. Gilbertson |
| | MIF1, MIF2, MIF3 | Michigan | A. W. Saettler |
| | XCPFEO, BAT67, ES1 | Brazil | J. C. Faria |
| | XCPFN | Nebraska | A. K. Vidaver |
| | XCPFG1–XCPFG4 | Guatemala | R. L. Gilbertson |
| *oryzae*[a] | XO61, XO86 | Philippines | J. L. Leach |
| *vesicatoria* | XCV1, XCV2, XCV3 | Wisconsin | D. K. Willis |
| *Xanthomonas* spp. | AV1, 23, 23L, D11, D12, D13, D4–D9, D10B, NTL, P3L3, 232, 54, SH42A, D2L, NTD–1 | Wisconsin | R. L. Gilbertson |
| *Pseudomonas syringae* pv. *syringae*[a] | PSS1 | Wisconsin | D. K. Willis |
| *P. syringae*[a] | PS1CIT7 | Wisconsin | D. K. Willis |
| *P. syringae* pv. *phaseolicola*[a] | PSP3100 | Wisconsin | D. K. Willis |
| *Erwinia herbicola* | EH1 | Wisconsin | S. Hirano |
| *Escherichia coli* | JM101 | | (23) |
| *P. solanacearum*[a] | | | |
| race 1 | 26, 30, 25, 134, 154, 90, 130, 143, 147, 152, 8, 27, 119 | Wisconsin | L. Sequeira |
| race 2 | 9, 136, 135, 167, 138, 139, 70, 127, 128 160, 20 | Wisconsin | L. Sequeira |
| race 3 | 23, 19, 80, 81, 145, 150, 153 | Wisconsin | L. Sequeira |
| Mulberry strains | 360, 361, 373 | Wisconsin | L. Sequeira |

[a] Received as total genomic DNA.

**Restriction digests, electrophoresis, blotting, nick translation, and hybridization methods.** Plasmid and total genomic DNA were digested with the restriction endonuclease *Eco*RI according to the manufacturers' recommendations (BRL, Promega Biotech). Digested DNA samples were fractionated by horizontal gel electrophoresis in 0.7% agarose gels in 1/2× Tris-borate buffer (22) and transferred to Zetabind nylon membrane (CUNO, Inc., Meriden, CT). Prehybridization (10 min to 16 hr) and hybridization (16–24 hr) of Southern blots and colony, squash, and dot blot filters were performed in solutions of 50% formamide-0.125 M $Na_2HPO_4$-7% SDS-1 mM EDTA (pH 8.0) at 42 C. All filters were washed twice at 65 C for 20 min with 0.125 M $Na_2HPO_4$-2% SDS-1 mM EDTA and once at 65 C for 20 min with 0.0075 mM $Na_2HPO_4$-1% SDS-1 mM EDTA (1). Filters were blotted dry and exposed at −70 C to Kodak X-Omat AR or OG-1 X-ray film with Cronex Lightning-Plus or Lanex intensifying screens, respectively. Plasmid DNA (0.2–0.5 $\mu$g) was radioisotope-labeled with $^{32}P$ by nick translation (22).

**Cloning of *Eco*RI fragments of plasmid DNA of *X. campestris phaseoli*.** CsCl-purified plasmid DNA from *X. c. phaseoli*, strain WT-1, was digested to completion with *Eco*RI, precipitated with ethanol, and resuspended in TE buffer. One microgram of DNA was ligated with 0.3 $\mu$g of *Eco*RI-treated pBS(+) (Stratagene, La Jolla, CA) in a 10-$\mu$l ligation reaction (22). The ligation mixture was used to transform *Escherichia coli* JM101 to ampicillin resistance. The presence of DNA derived from plasmids of *X. c. phaseoli* in 18 recombinant plasmids was confirmed by Southern hybridization analysis with $^{32}P$-labeled plasmid DNA of *X. c. phaseoli* as a probe.

**Colony blotting of bacteria and evaluation of the plasmid probe.** A colony blot procedure (30) with Whatman 541 filter paper as the transfer membrane was used to screen bacterial colonies. After lysis, the filters were washed for 5 min in 1 M Tris, pH 7.5, then in 2× SSC (22) followed by 95% ethanol. Filters were air-dried and stored or used immediately for hybridization.

To determine if plasmid DNA could distinguish *X. c. phaseoli* from nonpathogenic xanthomonads, *X. c. phaseoli* and a nonpathogenic xanthomonad from bean debris (strain 23) were plated separately and together on SPA or MXP media. These plates were incubated for 3–5 days, and plates with 30–50 colonies were colony blotted. These filters were probed with $^{32}P$-labeled plasmid DNA of *X. c. phaseoli*, strain WT-1.

**Establishment of a bean field plot and recovery and colony blotting of xanthomonads from bean primary leaves and debris.** A field plot was established in Madison, WI, on 15 June 1987 and planted with susceptible (Topcrop) and moderately resistant (Great Northern [GN] Harris) beans (*P. vulgaris*) and resistant tepary beans (*P. acutifolius* Gray) (32). Bean seed was inoculated with dry-leaf inoculum (12) of *X. c. phaseoli* (Wisconsin strain, WT-1) or not inoculated. Treatment plots consisted of two 1.5-m rows spaced 0.9 m apart with 40 seeds per row, and treatments were separated by four border rows of beans. Treatments were replicated four times in a randomized complete block design.

Routine assays have been developed for recovery of xanthomonads from bean leaves (10) and bean debris (R. L. Gilbertson, *unpublished*). These assays involve sonication or grinding of weighed tissue samples in 0.01 M potassium phosphate buffer (pH 7.2), preparing a dilution series from the resultant suspension, and plating 0.1-ml aliquots on MXP. Bacterial counts are made after incubation for 3–5 days at 24 C.

Ten primary leaves were collected from each inoculated and uninoculated treatment replicate at the Madison, WI, field plot on 1 July 1987. Leaves from each replicate were weighed, and total xanthomonad population densities were determined for each treatment. Bean plants were left standing throughout the winter, and the plot was sampled for xanthomonads in bean debris on 6 January, 2 March, and 4 May 1988. Bean debris was separated into leaf, stem, and pod tissues; 20 leaves and 20 2-cm stem and pod sections were weighed; and total xanthomonad population

densities in debris tissues were determined. Colonies from plates with 1–300 xanthomonads recovered from bean primary leaves and debris were replica plated in duplicate onto MXP, and then the original plate and one replica plate were colony blotted and probed with $^{32}$P-labeled plasmid DNA of *X. c. phaseoli*, strain WT-1. The other replica plate was saved for recovering strains for pathogenicity testing. Randomly selected colonies of nonpathogenic xanthomonads and *X. c. phaseoli* were subcultured from plates and tested for pathogenicity. All experiments were conducted at least three times.

**Squash and dot blotting of bean leaves.** Two procedures were used to detect *X. c. phaseoli* in bean leaves. For the squash blot procedure, leaf disks were excised with a sterile No. 4 cork borer; if leaves had a suspected common blight lesion, leaf disks were taken from the lesion margin. These disks were placed on either Whatman 541 paper, nitrocellulose, or Zetabind, and the tissue was squashed onto the paper or membrane with a sterile, round-bottomed glass rod. For the dot blot procedure, three leaf disks were macerated in 0.5 ml of sterile distilled water in a 1.5-ml Eppendorf tube with a sterile plastic pestle (Kontes, Vineland, NJ), and 10-$\mu$l aliquots of these suspensions were spotted onto Whatman 541 paper, nitrocellulose, and Zetabind. Dilution series from these suspensions were prepared in Eppendorf tubes, and 0.1-ml aliquots were plated on MXP to titer the bacteria in leaf tissue. All filters were treated as described for the colony blot filters and probed with $^{32}$P-labeled plasmid DNA of *X. c. phaseoli*, strain WT-1.

**Sensitivity of DNA probes.** To determine the minimum number of colony-forming units of *X. c. phaseoli* that could be detected by total plasmid DNA and a 3.4-kb *Eco*RI plasmid fragment (see Results), shake cultures of *X. c. phaseoli* were grown in nutrient broth to an $A_{600nm}$ of 0.7–0.9. Aliquots (10 $\mu$l) from undiluted and serial dilutions of cultures were spotted onto Zetabind. The filters were treated as described above and probed with either total plasmid DNA of *X. c. phaseoli* or the cloned 3.4-kb *Eco*RI fragment labeled with $^{32}$P ($10^6$ dpm/ml hybridization buffer). From the starting cultures and all dilutions, 100-$\mu$l aliquots were spread on MXP to titer the bacteria. This experiment was conducted three separate times.

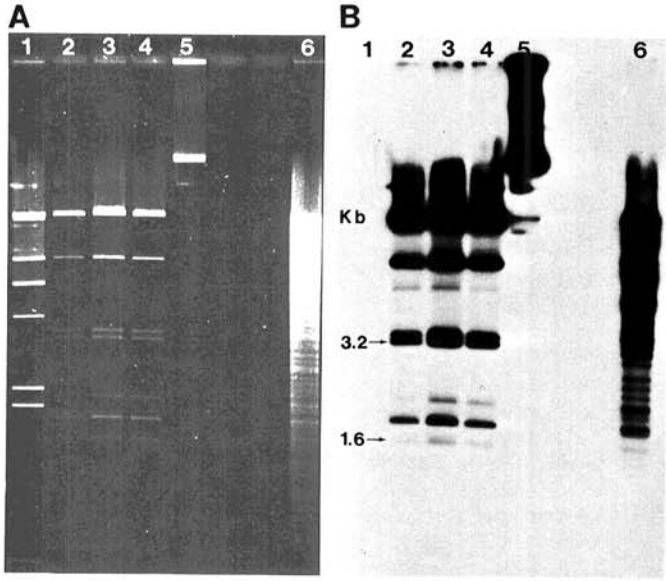


**Fig. 1.** Gel electrophoretic and Southern analysis of plasmid and total genomic DNA of *Xanthomonas campestris* pv. *phaseoli*. DNA was fractionated on a 0.7% agarose gel as described. **A,** Gel stained with ethidium bromide. **B,** Southern transfer of DNA probed with plasmid DNA of *X. c. phaseoli*, strain WT-1. Lane 1, *Hin*dIII-digested lambda marker; lane 2, *Eco*RI-digested plasmid DNA from Wisconsin strain WT-1; lane 3, *Eco*RI-digested plasmid DNA from Wisconsin strain W10; lane 4, *Eco*RI-digested plasmid DNA from Brazilian strain BCBI; lane 5, undigested plasmid DNA from strain WT-1; lane 6, *Eco*RI-digested total genomic DNA from strain WT-1. Arrows indicate *Eco*RI fragments that were cloned and used as probes.

## RESULTS

**Isolation and partial characterization of plasmid DNA from *X. c. phaseoli*, *X. c. phaseoli* var. *fuscans*, and nonpathogenic xanthomonads.** Plasmids were detected in 50 strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* from the United States, Brazil, Colombia, Guatemala, and Uganda but not in 20 strains of nonpathogenic xanthomonads recovered from bean debris collected in Wisconsin. The *Eco*RI restriction pattern of plasmid DNA from 16 strains of *X. c. phaseoli* from Wisconsin (10 strains) and Brazil (six strains) was characteristic (see, for example, Fig. 1); however, nine strains (two from Colombia, five from Brazil, one from Nebraska, and one from Guatemala) had different patterns (see, for example, Fig. 2). Plasmid DNAs from 12 strains of *X. c. phaseoli* var. *fuscans* had different restriction patterns than those of all strains of *X. c. phaseoli* examined (i.e., Fig. 2), regardless of geographical origin; but plasmids from strains of *X. c. phaseoli* var. *fuscans* from Brazil and Guatemala were identical, and all strains of *X. c. phaseoli* var. *fuscans* shared at least three *Eco*RI plasmid fragments (data not shown). All strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* shared a 1.6-kb *Eco*RI plasmid fragment (Figs. 1 and 2).

When CsCl gradient purified total plasmid DNA from *X. c. phaseoli*, strain WT-1, was used to probe Southern blots of *Eco*RI-digested plasmids from strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, all fragments of the plasmids of *X. c. phaseoli* and six

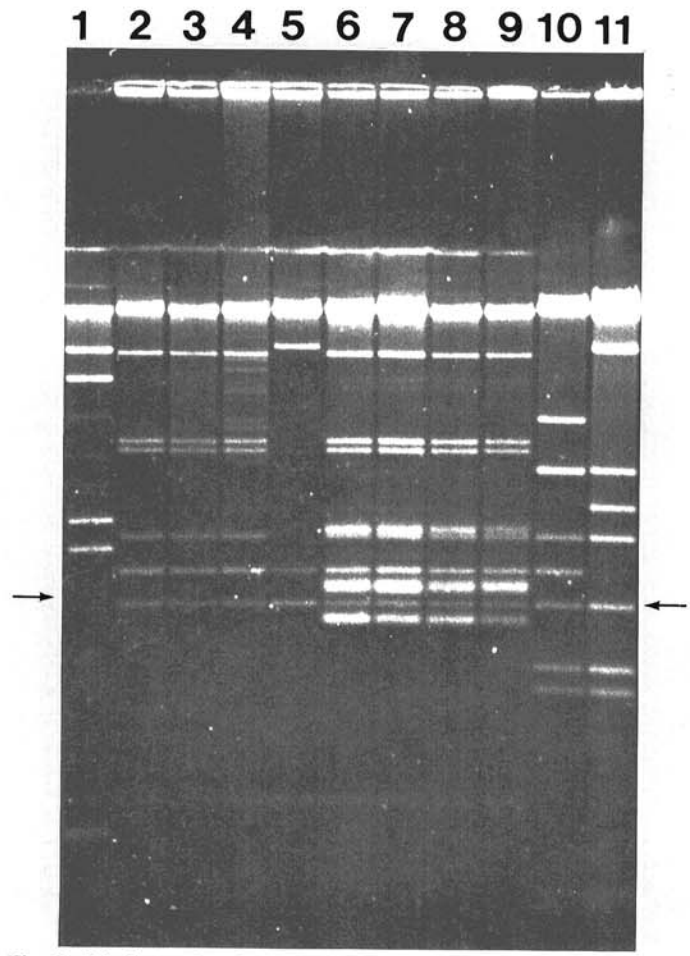


**Fig. 2.** Gel electrophoretic analysis of plasmid DNA of *Xanthomonas campestris* pv. *phaseoli* and *X. c. phaseoli* var. *fuscans*. DNA was digested with *Eco*RI, fractionated on a 0.7% agarose gel as described and stained with ethidium bromide. Lane 1, *Hin*dIII-digested lambda marker; lanes 2–9, plasmid DNA of *X. c. phaseoli* from Wisconsin strains W18, W10, XCPA, Nebraska strain XCPN, and Brazilian strains CNF30, CNF31, ES3, and ES5, respectively; lanes 10 and 11, plasmid DNA of *X. c. phaseoli* var. *fuscans* from Wisconsin strain WF8 and Nebraska strain XCPFN, respectively. Arrow indicates a 1.6-kb *Eco*RI fragment shared in all strains.

of eight or nine fragments of the plasmids of *X. c. phaseoli* var. *fuscans* were found to hybridize (data not shown).

Plasmid DNA from *X. c. phaseoli*, strain WT-1, hybridized to total genomic DNA of all 50 strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, regardless of geographical origin, but not to total genomic DNA of 20 strains of nonpathogenic xanthomonads from bean debris. Interestingly, the pattern of hybridization of plasmid DNA to total genomic DNA of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* was more complex than expected for the *Eco*RI plasmid fragments alone (Fig. 1) and varied among strains (Fig. 3). Plasmid DNA hybridized to total genomic DNA of *X. c.* pvs. *oryzae, vesicatoria* (races 1 and 2), and *carotae*, but the hybridization pattern was less complex than that observed for *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* (Fig. 4). There was weak hybridization of plasmid DNA to total genomic DNA of *X. c.* pv. *alfalfae* (Fig. 4, lane 4) and no hybridization to that from *X. c.* pvs. *campestris, holcicola, vesicatoria* (race 3), or *pelargonii*, or that from *Erwinia herbicola, Escherichia coli, Pseudomonas syringae* pv. *phaseolicola, P. s. syringae*, saprophytic *P. syringae, P. solanacearum*, and healthy bean plants.

Two cloned *Eco*RI plasmid fragments were evaluated as specific probes for *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*. A 1.6-kb fragment, which was shared in all plasmids examined (Figs. 1 and 2), hybridized to a single band in total genomic DNA of *X. c. phaseoli*, *X. c. phaseoli* var. *fuscans*, and *X. c. vesicatoria* race 1, but not to total genomic DNA of *X. c.* pvs. *alfalfae, campestris, carotae, holcicola, oryzae, pelargonii*, or *vesicatoria* races 2 and 3 (data not shown). A 3.4-kb fragment (Fig. 1), when hybridized to total genomic DNA of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, showed a complex pattern of hybridization (Fig. 5) that was similar to the hybridization pattern observed when total plasmid DNA was used as a probe. This 3.4-kb fragment hybridized weakly to total genomic DNA of *X. c.* pvs. *carotae* and *vesicatoria* races 1 and 2 and not at all to total genomic DNA of the other pathovars of *X. campestris* (Fig. 5).

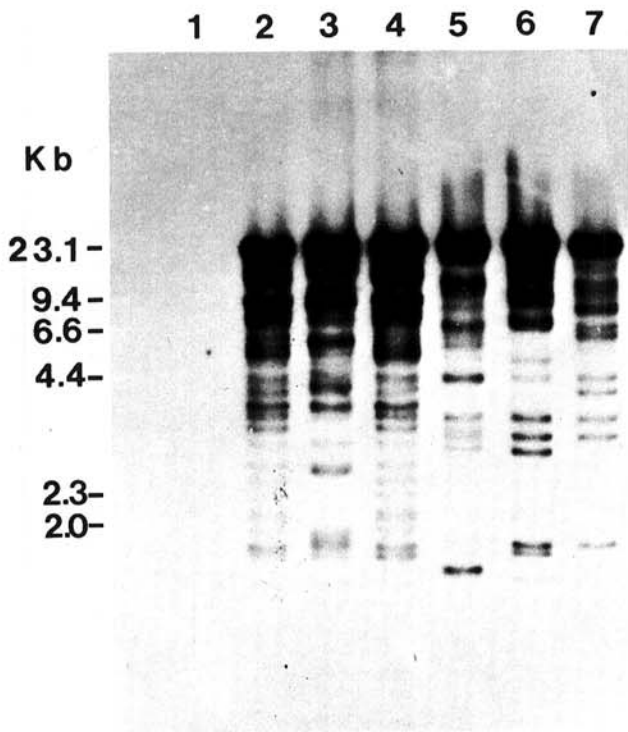


**Fig. 3.** Southern hybridization analysis of total genomic DNA of xanthomonads. DNA was digested with *Eco*RI, fractionated on a 0.7% agarose gel as described, transferred to Zetabind membrane, and probed with plasmid DNA of *X. campestris* pv. *phaseoli*, strain WT-1. Lane 1, nonpathogenic xanthomonad strain from bean debris (232); lanes 2–4, strains of *X. c. phaseoli* from Wisconsin (WT-1) and Brazil (XP-15 and CNF28), respectively; lanes 5–7, strains of *X. c. phaseoli* var. *fuscans* from Wisconsin (WF-8) and Brazil (XCPFEO and BAT67), respectively.

**Development and application of colony blot and squash and dot blot hybridization procedures for detection of *X. c. phaseoli*.** The colony blot procedure (30) was readily adapted to detect xanthomonads, and strong hybridization signals were observed when colonies of *X. c. phaseoli* and/or *X. c. phaseoli* var. *fuscans* were probed with plasmid DNA of *X. c. phaseoli*, whereas no signal was observed from colonies of the nonpathogenic xanthomonads. When *X. c. phaseoli* and nonpathogenic xanthomonads were plated together, plasmid DNA hybridized only to colonies of *X. c. phaseoli* (Fig. 6). In all cases, colonies that hybridized with plasmid DNA were pathogenic on bean, whereas colonies that did not hybridize were not pathogenic on bean.

Plasmid DNA hybridized to all xanthomonad colonies recovered from primary leaves of inoculated bean plants. Strong hybridization signals were observed from colonies blotted from direct platings of bacteria from leaves (Fig. 7) as well as 500 replica-plated colonies. All 20 colonies tested were pathogenic on bean. Population densities of *X. c. phaseoli* on primary leaves of Topcrop, GN Harris, and *P. acutifolius*, based on the number of colonies hybridizing with plasmid DNA in three separate experiments, were calculated to be $6 \times 10^7$, $2 \times 10^5$, and $1 \times 10^2$ xanthomonad colony-forming units per gram of leaf tissue, respectively. These population densities were similar to those determined from dilution plates. No xanthomonads were recovered from control plants.

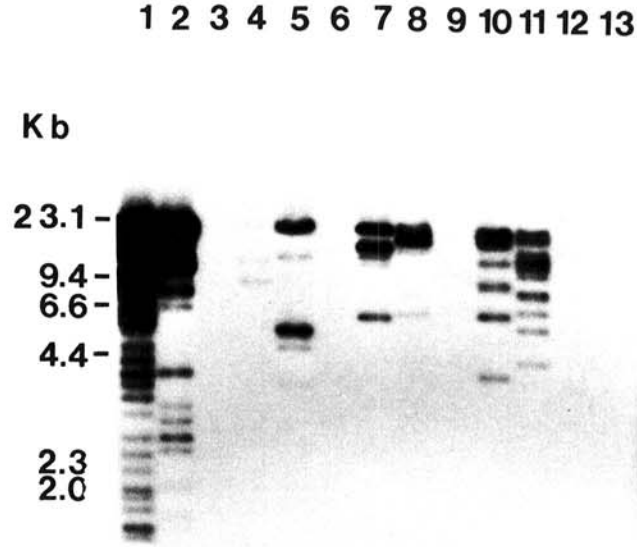


**Fig. 4.** Southern hybridization analysis of total genomic DNA of xanthomonads. DNA was digested with *Eco*RI, fractionated on a 0.7% agarose gel as described, transferred to Zetabind membrane, and probed with plasmid DNA of *X. campestris* pv. *phaseoli*, strain WT-1. Lane 1, *X. c. phaseoli*, strain WT-1; lane 2, *X. c. phaseoli* var. *fuscans*, strain WF-8; lane 3, nonpathogenic xanthomonad strain from bean debris (232); lane 4, *X. c.* pv. *alfalfae*, strain XA-1; lane 5, *X. c.* pv. *carotae*, strain XCAR1; lane 6, *X. c.* pv. *campestris*, strain XCC-1; lanes 7–9, *X. c.* pv. *vesicatoria*, races 1, 2, and 3, respectively (strains XCV1, XCV2, and XCV3, respectively); lanes 10 and 11, *X. c.* pv. *oryzae*, strains XCO61 and XCO86, respectively; lanes 12 and 13, *X. c.* pv. *holcicola*, strains XCH429 and XCH474, respectively.

In the Madison bean plot, plants had severe common bacterial blight symptoms by the end of the growing season. Xanthomonads were consistently recovered from bean leaf, stem, and pod debris throughout the winter (Table 2), with the greatest recovery from stems, leaves, and pods, respectively. Using plasmid DNA as a probe, we detected *X. c. phaseoli* in leaf debris in January, March, and May and in stem debris in January and March. None was found in pod debris. All 13 replica-plated colonies that hybridized with plasmid DNA were pathogenic on bean, whereas all 30 colonies tested that did not hybridize were not pathogenic on bean. In a similar study, 2,000 xanthomonads were recovered from bean debris in no-till plots at Hancock, WI, in May 1987 and screened with the plasmid DNA probe. None of the 2,000 colonies hybridized with the plasmid DNA, and 20 randomly selected colonies were not pathogenic on bean.

Using the squash and dot blot hybridization procedures and the plasmid DNA probe, we detected *X. c. phaseoli* in Topcrop and GN Harris leaves with common bacterial blight symptoms but not in symptomless leaves (Fig. 8). Plasmid DNA hybridized to squash blots of leaves with symptoms on all three support matrixes, but only to dot blots on nitrocellulose and Zetabind. Hybridization signals for squash and dot blots were visualized after 4 hr of exposure and were very strong after 12–16 hr of exposure. There was greater hybridization to squash and dot blots of Topcrop leaves with symptoms than of GN Harris leaves, and this was most apparent with the dot blot procedure (Fig. 8). *X. c. phaseoli* was consistently isolated from leaves with common bacterial blight symptoms, and bacterial populations in diseased leaves ranged from $6 \times 10^8$ to $3 \times 10^9$ cfu $g^{-1}$ and $7 \times 10^5$ to $5 \times 10^6$ cfu $g^{-1}$ in Topcrop and GN Harris, respectively.

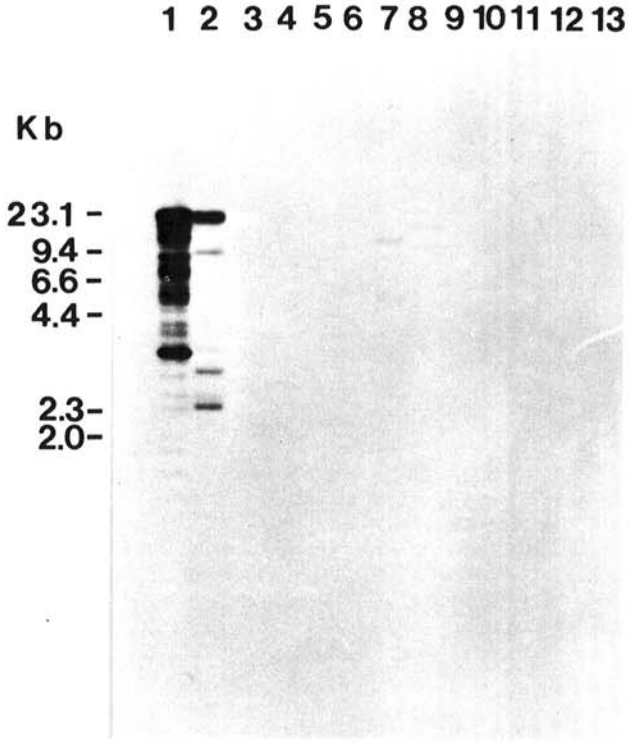


**Fig. 5.** Southern hybridization analysis of total genomic DNA of xanthomonads. DNA was digested with the restriction enzyme *Eco*RI, fractionated on a 0.7% agarose gel as described, transferred to Zetabind membrane, and probed with a 3.4-kb *Eco*RI plasmid fragment from *X. campestris* pv. *phaseoli*, strain WT-1. Lane 1, *X. c. phaseoli*, strain WT-1; lane 2, *X. c. phaseoli* var. *fuscans*, strain WF-8; lane 3, nonpathogenic xanthomonad strain from bean debris (232); lane 4, *X. c.* pv. *alfalfae*, strain XA-1; lane 5, *X. c.* pv. *carotae*, strain XCAR1; lane 6, *X. c.* pv. *campestris*, strain XCC-1; lanes 7–9, *X. c.* pv. *vesicatoria* races 1, 2, and 3, respectively (strains XCV1, XCV2, and XCV3, respectively); lanes 10 and 11, *X. c.* pv. *oryzae*, strains XCO61 and XCO86, respectively; lanes 12 and 13, *X. c.* pv. *holcicola*, strains XCH429 and XCH474, respectively.

**Sensitivity of DNA probes.** The limit of detection for both the total plasmid DNA and the 3.4-kb *Eco*RI plasmid fragment probes was $10^3$ cfu of *X. c. phaseoli*. A 3-day exposure of the filter was necessary for visualization of the signal from $10^3$ cfu, whereas a 12–16-hr exposure was sufficient to visualize signals from greater cell concentrations.

## DISCUSSION

We used plasmid DNA from *X. c. phaseoli* as a probe to detect

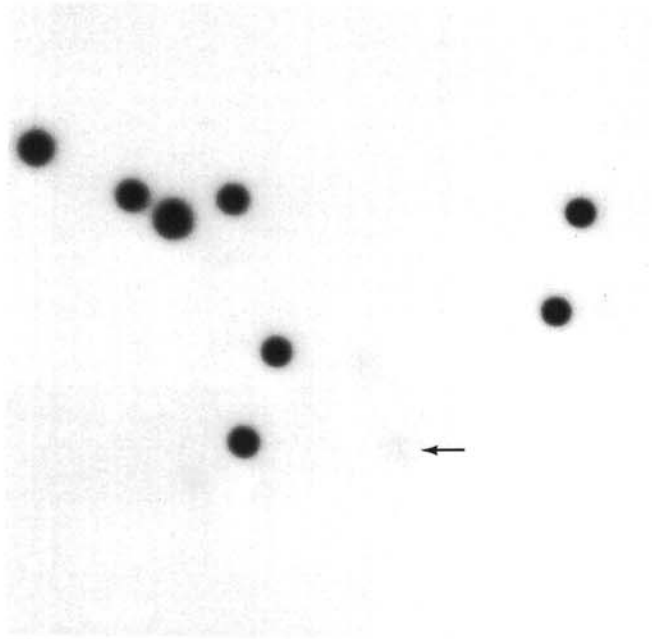

**Fig. 6.** Colony hybridization analysis of DNA from *Xanthomonas campestris* pv. *phaseoli* and nonpathogenic xanthomonad colonies. Colonies were blotted on Whatman 541 filter paper, lysed, and probed with plasmid DNA of *X. c. phaseoli*, strain WT-1. Colonies showing strong signals are *X. c. phaseoli*. Arrow indicates location of a nonpathogenic xanthomonad colony.

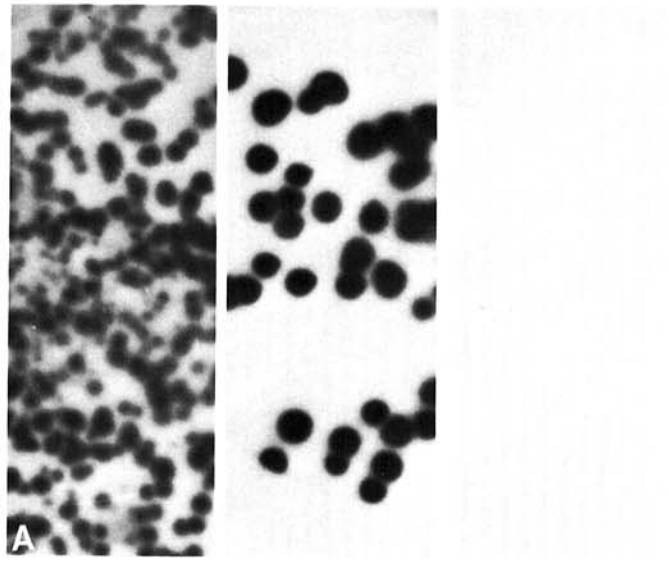


**Fig. 7.** Colony hybridization analysis of DNA from bacterial colonies recovered from primary leaves of beans inoculated with dry-leaf inoculum of *Xanthomonas campestris* pv. *phaseoli*. Colonies were blotted on Whatman 541 filter paper, lysed, and probed with plasmid DNA of *X. c. phaseoli*, strain WT-1. Filters shown are from **A,** susceptible cultivar Topcrop, **B,** moderately resistant cultivar Great Northern Harris, and **C,** resistant *Phaseolus acutifolius*.

*X. c. phaseoli* and *X. c. phaseoli* var. *fuscans*, causal agents of common bacterial blight of bean. Using plasmid DNA as a probe, we detected colonies of *X. c. phaseoli* recovered from bean plants and debris, directly detected *X. c. phaseoli* in bean leaves with common bacterial blight, and differentiated *X. c. phaseoli* from a nonpathogenic xanthomonad associated with bean plants and debris.

We used plasmid isolation procedures and Southern hybridization analysis to show the presence of plasmid DNA and conserved plasmid sequences in 50 strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* from diverse geographical regions that included three continents. Although *Eco*RI restriction enzyme digestion patterns of plasmid DNA from some strains of *X. c. phaseoli* and all strains of *X. c. phaseoli* var. *fuscans* revealed that DNA polymorphisms exist among strains, Southern hybridization analyses with total plasmid DNA and cloned plasmid fragments as probes demonstrated the presence of many shared plasmid sequences among strains. All strains contained a 1.6-kb *Eco*RI fragment. Furthermore, the extensive hybridization of plasmid DNA to total genomic DNA of all strains of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* examined suggests that plasmid sequences are integrated into chromosomal DNA and may encode repetitive DNA sequences. These results agree with the findings of Lazo and Gabriel (19) that plasmid DNA sequences are conserved in *X. c. phaseoli* and may be useful DNA probes for these bacteria. The universal presence of plasmid DNA sequences is not surprising, given the apparent clonal structure of *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* (20) and the seedborne dissemination of these bacteria (32).

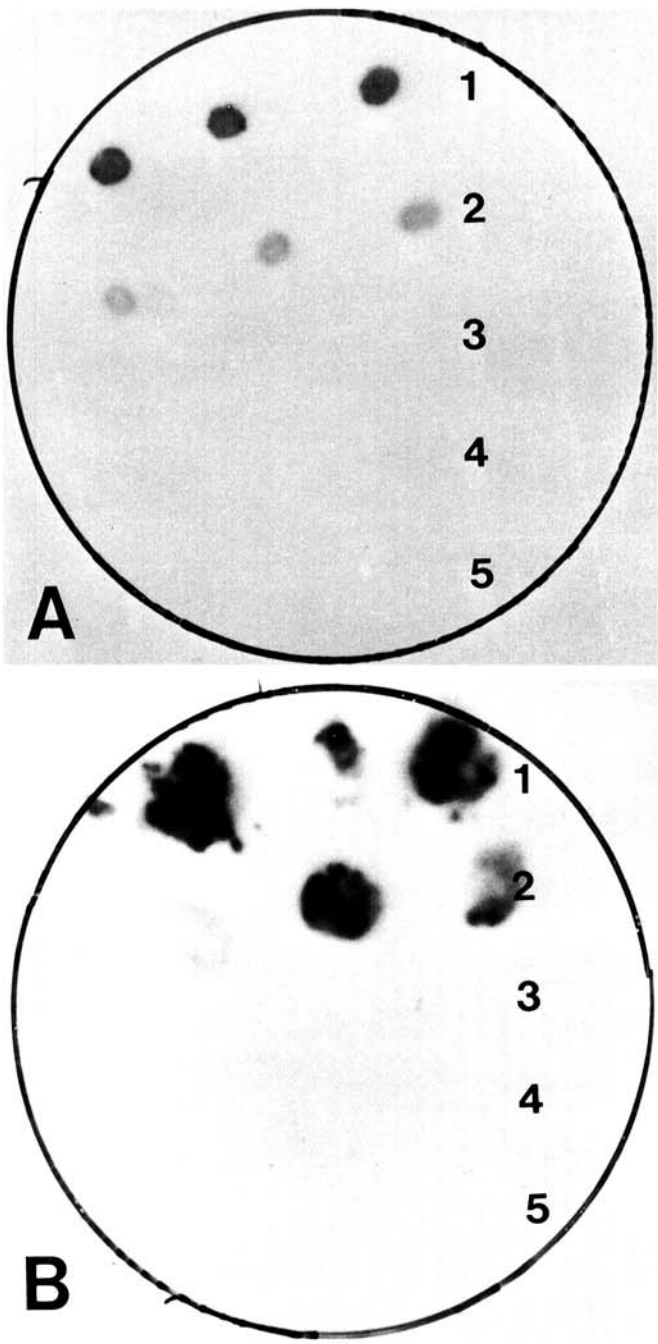


**Fig. 8. A,** Dot and, **B,** squash blot hybridization analysis of bean leaves of susceptible cultivar Topcrop and moderately resistant cultivar Great Northern (GN) Harris with or without common bacterial blight symptoms. Tissue samples were dotted or squashed on Zetabind nylon membrane, lysed, and probed with plasmid DNA of *Xanthomonas campestris* pv. *phaseoli*, strain WT-1. Each row of blots contained three similar samples: row 1, Topcrop leaves with common blight from the field; row 2, GN Harris leaves with common blight from the field; row 3, Topcrop leaves without symptoms from the field; row 4, GN Harris leaves without symptoms from the field; and row 5, Topcrop leaves without symptoms from the growth chamber.

Although total plasmid DNA hybridized to total genomic DNA of some pathovars of *X. campestris*, we found no hybridization to total genomic DNA from nonpathogenic xanthomonads recovered from bean debris nor to that from various other bacterial species, including bacteria that would be frequently isolated from bean plants and/or seeds, such as *Erwinia herbicola* and *P. syringae*. Therefore, plasmid DNA was used to differentiate *X. c. phaseoli* from the nonpathogenic xanthomonads isolated in our study. We assumed that pathovars of *X. campestris* such as *X. c. oryzae*, which also hybridized with the probe, were not colonizing beans or bean debris in Wisconsin. Total plasmid DNA is not *X. c. phaseoli*-specific, but may be useful for differentiating other pathovars of *X. campestris* to which it hybridizes (i.e., *X. c. vesicatoria*) from nonpathogenic xanthomonads (14).

The colony hybridization procedure provided rapid and specific detection of colonies of *X. c. phaseoli* recovered from bean primary leaves and bean debris. Using this procedure, we confirmed that xanthomonad population densities on bean primary leaves, as determined by dilution plating, represented colonization by *X. c. phaseoli* and not by nonpathogenic xanthomonads. Colonization of bean cultivars by *X. c. phaseoli* can be used as a measure of the resistance of a cultivar to common bacterial blight (5), and the colony hybridization procedure can rapidly confirm that xanthomonads recovered from bean leaves are *X. c. phaseoli*. Conversely, we used the colony hybridization procedure to demonstrate that most xanthomonads recovered from bean debris

TABLE 2. Recovery of xanthomonads from bean debris and detection of colonies of *Xanthomonas campestris* pv. *phaseoli* by colony hybridization[a]

| Month | Organ | Number of colonies hybridizing[b] per total colonies tested | |
|---|---|---|---|
| | | Direct isolation[c] | Replica plated[d] |
| January | Leaf | 46/70 | 5/25 |
| | Stem | 12/>400 | 1/25 |
| | Pod | 0/19 | 0/25 |
| March | Leaf | 2/31 | 1/50 |
| | Stem | 2/>400 | 1/100 |
| | Pod | 0/28 | 0/50 |
| May | Leaf | 5/14 | 5/100 |
| | Stem | 0/115 | 0/100 |
| | Pod | 0/9 | 0/50 |

[a] Debris from 10 standing plants was collected each month from a bean plot at Madison, WI. Leaf, stem, and pod debris was separated and ground in sterile 0.01 M potassium phosphate buffer; dilution series were prepared and 0.1-ml aliquots plated on MXP.

[b] Bacterial colonies were blotted on Whatman 541 filter paper and lysed with 0.2 N NaOH; the filters were probed with plasmid DNA of *X. c.* pv. *phaseoli*, strain WT-1.

[c] Counts are means of three plates/organ for each month.

[d] Colonies from plates of various dilutions were replica plated in duplicate; one replica plate was used for colony hybridization, and the other was retained for recovery of colonies for pathogenicity tests.

were not pathogenic on bean, and to detect colonies of *X. c. phaseoli* from among the nonpathogenic xanthomonads in the Madison bean plot. These results agreed with those of a previous *X. c. phaseoli* overwintering study in that most xanthomonads recovered from bean debris in the spring are not pathogenic on bean, but *X. c. phaseoli* can survive the winter at low levels in leaf debris in a no-till system (11; R. L. Gilbertson *unpublished*). Replica plating of colonies allows for confirmation of positive colonies as *X. c. phaseoli* by pathogenicity tests.

Squash and dot blot hybridization of bean leaves with symptoms of common bacterial blight provided a rapid and specific procedure for detection of *X. c. phaseoli* in plant tissue and may be a useful diagnostic tool for this disease. In greenhouse and field situations, it is not unusual to observe symptoms on bean leaves that mimic common bacterial blight, and these hybridization procedures can be used to confirm the presence of *X. c. phaseoli*. Furthermore, the dot blot procedure may provide an indication of the relative susceptibility of a bean cultivar to common blight. Stronger signals were observed from infected leaves of the susceptible cultivar than from the moderately resistant cultivar, indicating that the susceptible leaves contained more bacteria. This observation agreed with the actual numbers of bacteria recovered from these leaves and the greater disease severity observed for the susceptible cultivar. A rapid procedure to measure populations of *X. c. phaseoli* in diseased bean leaves may be a useful tool in breeding for disease resistance to common bacterial blight, as resistant bean cultivars sustain lower populations of *X. c. phaseoli* than susceptible cultivars (5).

These hybridization procedures offer a more rapid detection of plant pathogenic bacteria than is possible with standard pathogenicity tests, and the colony hybridization procedure allows for simultaneous screening of large numbers of colonies. After colonies had grown on a medium for 3–5 days, the colony hybridization procedure was completed in 24 hr, with positive signals visualized after a 4-hr exposure. The squash and dot blot hybridization procedures were completed in 36–48 hr, and positive signals were visualized after a 12–16-hr exposure. Pathogenicity tests can require as long as 2–3 wk to complete, including isolation of bacteria, subculturing, inoculation of plants, and symptom development, and these tests require greenhouse and/or growth chamber facilities. Obviously, hybridization tests cannot eliminate the need for pathogenicity tests, but they can provide a more rapid diagnosis that can be confirmed by pathogenicity tests.

The 3.4-kb *Eco*RI plasmid fragment, which apparently encodes a repetitive sequence that is also present in the bacterial chromosome, hybridized strongly to only *X. c. phaseoli* and *X. c. phaseoli* var. *fuscans* and may be suitable as a specific probe for *X. c. phaseoli*. Nucleic acid probes containing repetitive, chromosomally encoded sequences are desirable because these sequences are amplified in the genome and are unlikely to be lost through recombination or other genetic processes. An ideal probe would contain a species- or pathovar-specific repetitive sequence; these may be present on the 3.4-kb *Eco*RI plasmid fragment from *X. c. phaseoli* and could be identified by using subcloned fragments as probes.

The limit of detection of the two probes tested, total plasmid DNA and the 3.4-kb *Eco*RI plasmid fragment, was $10^3$ cfu of *X. c. phaseoli*. This level of sensitivity should be adequate for the procedures described in this study. For example, in the squash blot procedure, a leaf disk (4 mm in diameter) from the lesion margin of an infected bean leaf contains about $10^5$–$10^8$ cfu, whereas a 10-$\mu$l drop from suspensions used in the dot blot procedure contains $10^4$–$10^7$ cfu.

Nucleic acid probes can be used for detection of infectious agents under many different circumstances and may be particularly useful as diagnostic tools and in ecological and epidemiological studies. Using plasmid DNA of *X. c. phaseoli* as a probe and colony, squash, and dot hybridization procedures, we detected *X. c. phaseoli* on and/or in bean leaves and in bean debris and differentiated *X. c. phaseoli* from nonpathogenic xanthomonads. A plasmid fragment, which contains repetitive DNA, was identified and may be a suitable *X. c. phaseoli*-specific probe. These results indicate that DNA probes can provide a rapid and specific tool for detecting plant-pathogenic bacteria.

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