# Nucleic Acid Probes for Identification of Phytobacteria: Identification of Genus-Specific 16s rRNA Sequences

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## ABSTRACT

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The development of DNA probes for the identification of phytobacteria requires efficient procedures to identify target-specific nucleic acid sequences present in high copy number within the target cells. Probes based on phytobacterial rRNA sequences should provide an extremely sensitive identification method. To establish the potential specificity of this approach, 16s rRNAs of 52 strains were partially sequenced. The approach was based on partially purifying high molecular weight RNAs from crude lysates and selecting the 16s rRNA by hybridization with oligodeoxynucleotide primers to conserved regions. Reverse transcription and dideoxynucleotide chain termination methods allowed the sequencing of 600–800 nucleotides of the approximate total 1,540 comprising 16s rRNA. Although most of the molecule is highly conserved, a region defined by the bases Nos. 1057–1090 (numbered from the cDNA) is variable between the genera assayed. Pathovars or subspecies could not be differentiated based on comparisons within this region. The degree of variation in the conserved molecule is sufficiently high to expect specific discrimination between phytobacterial genera using a synthetic oligodeoxynucleotide probe.

The development of specific, sensitive, and rapid methods to identify phytobacteria is critical for systematic, epidemiological, and control studies. Current assays are based primarily on serological relatedness, infectivity, bacteriophage specificity, fatty acid profiles, or biochemical and physiological parameters (17,18). These assays have proved invaluable but still have significant drawbacks for routine use. Selective media have been developed for many phytobacteria, although where positive confirmation of the target is required, as in seed certification, an additional step is needed to establish target identity.

Genetic relatedness is the foundation for classification and is reflected by DNA homology. Recently, plasmid profiles and restriction fragment length polymorphisms (RFLPs) have been evaluated as a measure of genetic diversity between phytobacteria (6,10,11). However, these procedures require extensive nucleic acid preparation and manipulation and, thus, are unsuited for the routine identification of phytobacteria. Target-specific nucleic acid probes used in colony hybridization procedures retain the inherent advantages of DNA homology assays but also provide a reproducible diagnostic tool (16). In contrast to many identification methods, nucleic acid probes do not depend on the metabolic state of the target organism or the state of gene expression.

We previously developed a DNA probe for *Xanthomonas campestris* pv. *phaseoli* based on random cloning of genomic fragments and subsequent screening of clones for specificity and sensitivity (15). Recently, Gilbertson et al (3) used a 3.4-kb fragment of plasmid DNA as a probe for *X. c. phaseoli*. However, without knowledge of the sequence function or copy number, it is very difficult to identify rapidly and unequivocally an appropriately specific and sensitive fragment to use as a probe. Clearly, the ideal DNA probe would be based on known nucleic acid sequences that taxonomically define the target and are present in high copy number. Schaad et al (19) have used a 2.6-kb *Eco*RI fragment from the phaseolotoxin gene as a probe to identify *Pseudomonas syringae* pv. *phaseolicola*. Unfortunately for the vast majority of phytobacteria such genetic data are not available. An alternative and less restrictive strategy for the development of DNA probes is needed that retains the advantages of nucleic acid comparisons but is broadly applicable for many target phytobacteria and can be developed in a rapid manner.

Group-specific oligodeoxynucleotide probes have been developed that are complementary to unique sequence regions of 16s rRNA (2,4,5,13,14) and 23s rRNA (8). These probes are highly sensitive and can be developed very rapidly. The rRNA molecule was chosen as a foundation for probe development because of its conserved function, ease of obtaining partial sequence information, its size, the presence of diverse sequence regions that are correlated with phylogenetic relatedness, and its abundance (more than 10,000 copies per actively growing cell). The latter attribute significantly increases the inherent sensitivity of a probe derived from unique 16s rRNA sequences. Herein we evaluate the applicability of using 16s rRNA sequences as a foundation for development of specific DNA probes for phytobacteria based on identifying genus-specific sequence regions.

## MATERIALS AND METHODS

**Bacterial strains and materials.** Bacterial strains used in this study are listed in Table 1. All strains were maintained on yeast extract-glucose media (18). Reverse transcriptase, 2′ deoxynucleoside triphosphates and 2′,3′-dideoxynucleoside triphosphates were obtained from Fisher Scientific Co., Pittsburgh, PA. Sequencing primers specific for conserved regions of 16s RNA (9) were purchased from Boehringer Manneheim Biochemicals or were synthesized on an Applied Biosystems (Foster City, CA) 380 A DNA synthesizer from β-cyanoethyl phosphoramidite precursors. Oligodeoxynucleotides were deblocked and released from the support by treatment with concentrated $NH_4OH$ for 8–12 hr at 55 C, desalted by Sephadex G50 chromatography, lyophilized, dissolved in 5 mM Tris-Cl (pH 7.5) containing 0.5 mM EDTA, and examined for purity by electrophoresis on 20% polyacrylamide gels (12). The primer sequences which can be considered universal primers for all 16s rRNAs and their corresponding locations on 16s rRNA are primer A=G-W-A-T-T-A-C-C-G-C-G-G-C-K-G-C-T-G from position 519–536; primer B=C-C-G-T-C-A-A-T-T-C-M-T-T-T-R-A-G-T-T-T from position 907–926; and primer C=A-C-G-G-G-C-C-G-G-T-G-T-G-T-R-C from position 1392–1406, where K = guanine or thymine, M = adenine or cytosine, R = adenine or guanine, and W = adenine or thymine. All other chemicals were from Sigma except where indicated.

**RNA isolation.** Bacterial strains were harvested from log phase cultures by centrifugation at 12,000 *g* for 10 min. The pellet (0.5 g wet weight) was washed twice in ice-cold STE buffer (100 mM

NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspended in 4.5 ml of STE buffer. Cells were broken by passing through a French pressure cell at 20,000 psi. The lysate was collected in a chilled, nuclease-free 15-ml Corex tube and sodium dodecyl sulfate and NaEDTA were added to 1.0% and 20 mM, respectively. The nucleic acid was extracted once with phenol/isoamyl alcohol, twice with phenol/isoamyl alcohol/chlorform and once with chloroform/isoamyl alcohol (12). Nucleic acid was precipitated from the aqueous phase of the last extraction by adding 0.1 volume of 2.0 M sodium acetate and 2.0 volumes of ice-cold ethanol, chilling at −80 C for 30 min and centrifuging at 14,000 *g* for 5 min at 4 C (12). The pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1.0 mM NaEDTA) and reprecipitated as above. The final pellet was resuspended in TE buffer to a concentration of 5 mg/ml. The high molecular weight RNA was precipitated with an equal volume of 4 M NaCl by incubation on ice overnight and was collected by centrifugation at 12,000 *g* for 10 min at 4 C. The pellet was resuspended in TE buffer and residual NaCl removed by precipitating with sodium acetate and ice-cold ethanol as above. The pellet was vacuum dried and resuspended at 2 mg/ml in 10 mM Tris-HCl (pH 7.4) and stored at −20 C.

TABLE 1. Bacterial strains

| Bacterial strains | Number | Location | Source |
|---|---|---|---|
| *Xanthomonas campestris* pv. | | | |
| *campestris* | B4 | Idaho | N. Schaad |
| *carotae* | B819 | Idaho | N. Schaad |
| *citri* | B283 | Idaho | N. Schaad |
| | B728 | Brazil | K. Mohan |
| | B789 | Idaho | N. Schaad |
| | B790 | Thailand | N. Schaad |
| *manihotis* | B468 | Idaho | N. Schaad |
| *nigromaculans* | B966 | Idaho | N. Schaad |
| *oryzae* | KS66 | China | Di Ming |
| | HB13 | China | Di Ming |
| | HB17 | China | Di Ming |
| | HB38 | China | Di Ming |
| | 86 | China | Di Ming |
| | B794 | China | Di Ming |
| | B494 | China | Di Ming |
| *phaseoli* | B702 | Idaho | N. Schaad |
| | Xp5 | Colorado | D. A. Roth |
| | B705 | Idaho | N. Schaad |
| | B706 | Idaho | N. Schaad |
| | B717 | Idaho | N. Schaad |
| | B718 | Idaho | N. Schaad |
| | Xp2 | Wyoming | D. A. Roth |
| | Xp1 | Wyoming | D. A. Roth |
| | Xp4 | Colorado | D. A. Roth |
| *translucens* | B428 | Idaho | N. Schaad |
| *X. fragariae* | B978 | Idaho | N. Schaad |
| *Pseudomonas syringae* pv. | | | |
| *phaseolicola* | Ps1 | Wyoming | D. A. Roth |
| | Ps101 | Wyoming | D. A. Roth |
| | Ps107 | Idaho | N. Schaad |
| | Ps103 | Idaho | N. Schaad |
| | Ps109 | Idaho | N. Schaad |
| *pisi* | PI299 | England | J. Reeves |
| | PI202 | England | J. Reeves |
| | PI974 | England | J. Reeves |
| | PI895 | England | J. Reeves |
| | PI870 | England | J. Reeves |
| | PI1704 | England | J. Reeves |
| *syringae* | Ps127 | Idaho | N. Schaad |
| | Ps113 | Idaho | N. Schaad |
| *tabaci* | Ps111 | Idaho | N. Schaad |
| *tomato* | Ps125 | Florida | J. Jones |
| *viridiflava* | Ps115 | Wyoming | S. Williams |
| *Erwinia amylovora* | 110-B | Wyoming | D. A. Roth |
| | Ea6 | Wyoming | D. A. Roth |
| *Erwinia chrysanthemi* pv. | | | |
| *chrysanthemi* | 30H | Netherlands | J. van Vuurde |
| | 113H | Netherlands | J. van Vuurde |
| | 163H | Netherlands | J. van Vuurde |
| | 702H | Netherlands | J. van Vuurde |
| | 108H | Netherlands | J. van Vuurde |
| | 800H | Netherlands | J. van Vuurde |
| | 139H | Netherlands | J. van Vuurde |
| *Erwinia carotovora* pv. | | | |
| *atroseptica* | J10 | Wisconsin | A. Kelman |

**16s rRNA Sequencing.** Sequencing with base-specific dideoxynucleotide chain termination protocols was adapted from Lane et al (9). Briefly, the DNA primer (0.15 μg) was annealed to the bacterial RNA templates (7 μg) in hybridization buffer (100 mM KCl, 50 mM Tris-HCl, pH 8.5) by heating to 65 C for 5 min and cooling slowly over 20 min to 25 C. The primer-RNA mixture was transferred to a 1.5-μl microcentrifuge tube containing 30 μCi of ($\alpha^{35}$S) dATP (1 Ci = 37 GBq), reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM DTT, 50 mM $MgCl_2$), reverse transcriptase (6.5 U), 250 μM each of dCTP, dGTP, dATP, and dTTP, and one of the following dideoxynucleotides: ddCTP (30 μM), ddGTP (19μM), ddATP (1.25 μM), ddTTP (30 μM), or no dideoxynucleotide. Thus, for each primer, five reactions were done. The reactions were incubated for 5 min at 25 C, then 30 min at 55 C. Chase nucleotide mix (1 mM each of dATP, dCTP, dGTP, and dTTP) containing 1 unit of reverse transcriptase was added, and incubation continued at 55 C for 15 min. The reaction products were precipitated with 2 volumes of ice-cold 95% ethanol, resuspended in 10 μl of stop mix (80% formamide, 10 mM EDTA, 0.08% xylene cyanol blue, 0.08% bromophenol blue) and stored at −20 C until sequencing (no more than a day or two). Mixtures were heated to 65 C for 2 min before loading onto a sequencing gel. Nucleotide sequencing was performed on 45- × 45-cm gels containing 8 M urea and 8% acrylamide. The gels were electrophoresed at 1,700 V for approximately 2.5 hr, at which time the bromophenol blue dye reached the gel bottom. Gels were transferred to Whatman 3MM paper, dried for 2 hr at 80 C and exposed to X-ray film for 4–5 days at room temperature.

## RESULTS

The focus of this study was to determine if short sequence regions of the 16s rRNA from phytobacteria can be identified that are essentially invariant for all members of a particular target group but that differ significantly from all other nontarget groups. With this information, complementary oligodeoxynucleotides could be synthesized, labeled, and used as identification probes. Secondary screening of the probe could be done rapidly by various nucleic acid or modified colony hybridization techniques to assure specificity and sensitivity. Partial nucleotide sequences of the small (16s) subunit of ribosomal RNA were determined from 26 strains of *Xanthomonas*, 10 of *Erwinia*, and 16 of *Pseudomonas*. Approximately 600–800 nucleotide residues were sequenced from a total of approximately 1,540 nucleotides of the 16s rRNA molecule from three oligonucleotide primers homologous to highly conserved regions. In this method the 16s rRNA of the target phytobacteria is selected from the bulk, high molecular weight RNA by hybridization to specific oligodeoxynucleotide primers according to Lane et al (9). This strategy allows rapid preparation of the template followed by reverse transcription and dideoxynucleotide sequencing. Comparison of sequence information obtained by this method with data from sequencing of cloned 16s rRNA genes from *E. coli* (1) indicated that this rapid method is accurate. The process from cell preparation through sequencing takes approximately 9 days (5 days exposure time included). All observed sequence ambiguities were primarily due to compressions and not due to misdirected dideoxynucleotide incorporation. Comparisons with the gene sequence of 16s rRNA from *E. coli* that have been determined by standard sequencing protocols, minimizes incorrect sequence designation due to compressions. Sequences were aligned according to Woese et al (23) where initial

alignment is based on obvious sequence homology and refined by use of the known secondary structural features of the molecule. In cases where apparent base discrepancies were observed, sequencing was repeated to confirm the sequence.

Initial experiments with select strains from the three genera showed that sequence regions accessible from primers B (rRNA hybridization location between bases 907 and 926) and C (positions 1392–1406) were entirely conserved among all strains sequenced and thus, not suitable for development of a probe to differentiate phytobacteria (data not shown). However, significant variability between the phytobacterial genera exists in the rRNA sequence region defined by bases 452 and 485. This region corresponds to the cDNA sequence of the 16s rRNA between positions 1057 and 1090 and is accessible from primer A. Thus, in subsequent experiments only primer A was used to obtain sequence information. Table 2 shows the cDNA sequences derived from this variable region of 16s rRNA. The extent of 16s rRNA sequence variability between phytobacteria is determined by genetic relatedness. As expected, between-genera sequence variation is most significant. The average variation within this region between *Xanthomonas* and *Pseudomonas* is 44% and between *Xanthomonas* and *Erwinia* is 50%. Sequence variation within this region between *Pseudomonas* and *Erwinia* strains is 44%. The 16s rRNA hybridization sequence that defines the genus *Xanthomonas* is from 1064 to 1090 (based on the cDNA sequence) and consists of the following bases: 5′-TAAGGATCGGGTATTAACCGACTGCTT-3′. The primary differences among the strains sequenced occurs at positions 1064, where G is found in the *X. campestris* pvs. *citri, carotae, translucens, nigromaculans,* and *manihotis* and at position 1068 where in *X. c. citri*, a C is substituted for a G. Thus, an oligodeoxynucleotide probe specific for 16s rRNA of the xanthomonads would consist of these sequences or a subset thereof. Within this region, variation among the pathovars is limited and between strains of a pathovar no variation was found. Although fewer strains were assayed within *Pseudomonas* and *Erwinia*, the tentative consensus sequence would be smaller than that available for *Xanthomonas* and consist of: 5′-AGGTGCAAAGCTATTCAACTAGCACTT-3′ for *Pseudomonas* and 5′-ATCAACAAAGGTATTAACCTCATCGCC-3′ for *Erwinia*. Although the sequences from six sero-strains of *P. syringae* pv. *pisi* were identical, they were substantially different from the remaining pseudomonads, suggesting a misidentification or contamination problem. In fact, their 16s rRNA sequences more closely resemble those of the erwiniae. We are obtaining type strains of this pathovar to clarify the situation.

## DISCUSSION

Nucleic acid hybridization probes, based on unique rRNA sequences, have proved to be highly effective in the identification of mammalian pathogenic bacteria (7,20) as well as other prokaryotes (5,8). The key requirement for using this strategy to identify phytobacteria is that unique sequence regions must be defined that can delimit target groups. In addition, it is advantageous if sequence variability for all phytobacteria is generally localized within an easily accessible region of the molecule. Although much of the 16s rRNA molecule is highly conserved among all organisms, definite regions of sequence variability can be identified that are suitable for synthesis of oligonucleotide probes. The primarily variable region for use with phytobacteria exists within the region between bases 1064 and 1090. Therefore, sequencing of the entire molecule, which is very time consuming, is unnecessary. Initial identification of an appropriate sequence for the synthesis of a DNA probe can be made by comparing the sequence from the target bacteria with the library of 16s rRNA from other phytobacteria. We are currently expanding our library of partial 16s rRNA sequences for other genera of phytobacteria. The limited intragenic 16s rRNA sequence variation within this region is not necessarily a limiting drawback to construction of a DNA probe for use as a routine identification probe. These assays must differentiate the target from other organisms within the specific ecosystem. For example, one probe derived from 16s RNA sequences may be used to identify directly any xanthomonads in a bean seed extract containing numerous genera of saprophytic bacteria. For large numbers of samples, this approach would rapidly identify seed lots containing *Xanthomonas* spp. Further differentiation of pathogenic and potentially nonpathogenic strains could then be done with alternative assays. Other assays to delineate phytobacterial general (18) require pure cultures and are not as adaptable as DNA probes for routine clinical use. Sensitive DNA probes may be used to identify target sequences directly in tissue extracts.

Other regions of the molecule may exhibit greater intraspecies variability and, thus, increase the specificity of a DNA probe

TABLE 2. Partial 16s rRNA gene sequences from phytobacteria

| Strain | | 5′-Sequence |
|---|---|---|
| | | 1057 1090 |
| *E. coli* | | AACGTCAATGAGCAAAGGTATTAACTTTACTGCC |
| *X. campestris* pv. | | |
| *campestris* | B4 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| *carotae* | B819 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| *citri* | B283 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| | B728 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| | B789 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| | B790 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| *manihotis* | B468 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| *nigromaculans* | B966 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| *oryzae* | KS 66 | ACCGTCATAACCATCGGGTATTAACCGACTGCTT |
| | HB 13 | ACCGTCATAACCATCGGGTATTAACCGACTGCTT |
| | HB 17 | ACCGTCATAACCATCGGGTATTAACCGACTGCTT |
| | HB 38 | ACCGTCATAACCATCGGGTATTAACCGACTGCTT |
| | B794 | ACCGTCATAACCATCGGGTATTAACCGACTGCTT |
| | 86 | ACCGTCATAACCATCGGGTATTAACCGACTGCTT |
| | B494 | ACCGTCATAAGCATCCGGTATTAACCGACTGCTT |
| *phaseoli* | B702 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | Xp5 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | B705 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | B706 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | B717 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | B718 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | Xp2 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | Xp1 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| | Xp4 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| *translucens* | B 428 | ACCGTCAGAAGGATCGGGTATTAACCGACTGCTT |
| *X. fragariae* | B978 | ACCGTCATAAGGATCGGGTATTAACCGACTGCTT |
| *Erwinia amylovora* | E-266 | AACGTCAATCAACAAAGGTATTAACCTCATCGCC |
| | 110-B | AACGTCAATCAACAAAGGTATTAACCTCATCGCC |
| *E.chrysanthemi* pv. | | |
| *chrysanthemi* | 30-H | AACGTCAATCAACAAAGTTATTAACCTCACCGCC |
| | 113-H | AACGTCAATCAACAAAGTTATTAACCTCACCGCC |
| | 800-H | AACGTCAATCAACAAAGGTATTAACCTCACCGCC |
| | 108-H | AACGTCAATCAACAAAGTTATTAACCTCACCGCC |
| | 139-H | AACGTCAATCAACAAGGTTATTAACCTCACCGCC |
| | 163-H | AACGTCAATCAACAAAGGTATTAACCTCACCGCC |
| | 702-H | AACGTCAATCAACAAAGGTATTAACCTCACCGCC |
| *E. carotovora* pv. | | |
| *atroseptica* | J-10 | AACGTCAATCAACAAAGGTATTAACCTCACCGCC |
| *P. syringae* pv. | | |
| *pisi* | PI-202 | AACGTCAAAACAATCACGTATTAAGTAACCTGCC |
| | PI-299 | AACGTCAAAACAATCACGTATTAAGTAACCTGCC |
| | PI-974 | AACGTCAAAACAATCACGTATTAAGTAACCTGCC |
| | PI-895 | AACGTCAAAACAATCACGTATTAAGTAACCTGCC |
| | PI-870 | AACGTCAAAACAATCACGTATTAAGTAACCTGCC |
| | PI-170 | AACGTCAAAACAATCACGTATTAAGTAACCTGCC |
| *phaseolicola* | PS-1 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| | PS-101 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| | PS-103 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| | PS-107 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| | PS-109 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| *tabaci* | PS-111 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| *syringae* | PS-113 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| *tomato* | PS-125 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |
| *viridiflava* | PS-115 | ACCGTCAAGGTGCAAAGCTATTCAACTAGCACTT |

to differentiate pathovars. To this end, we currently are analyzing other regions of the 16s rRNA molecule as well as the larger and more diverse 23s rRNA. However, for the design of a primary genus-differentiation probe, only sequence information from the region described in this paper appears necessary. Further enhancement of specificity may be possible by selecting a sequence region where individual 16s rRNA base substitutions between target organisms favor maximum destablization during hybridization, i.e., purine for pyrimidine rather than purine for purine substitutions (21,22). A single base change within a 15-mer sequence can be detected with appropriate hybridization conditions and, therefore, is sufficient to establish specificity (21,22).

Further screening of a deoxynucleotide probe derived from 16s rRNA sequence data can be done to insure specificity and sensitivity by colony hybridization procedures modified for RNA. We are now testing a *Xanthomonas*-specific oligonucleotide probe derived from a subset of sequences in the region between 1064 and 1090 in in situ hybridization assays. Initial assays show that only pathogenic pathovars of *Xanthomonas* hybridize with the probe (Di Ming and D. A. Roth, *unpublished*). The inherent sensitivity of probes generated by this method should be an advantage over other gene probes.

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