An Oligonucleotide Array for the Identification and Differentiation of Bacteria Pathogenic on Potato

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ABSTRACT

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Oligonucleotides, 16 to 24 bases long, were selected from the 3′ end of the 16S gene and the 16S-23S intergenic spacer regions of bacteria pathogenic on potato, including *Clavibacter michiganensis* subsp. *sepedonicus*, *Ralstonia solanacearum*, and the pectolytic erwinias, including *Erwinia carotovora* subsp. *atroseptica* and *carotovora* and *E. chrysanthemi.* Oligonucleotides were designed and formatted into an array by pin spotting on nylon membranes. Genomic DNA from bacterial cultures was amplified by polymerase chain reaction using conserved ribosomal primers and labeled simultaneously with digoxigenin-dUTP. Hybridization of amplicons to the array and subsequent serological detection of digoxigenin label revealed different hybridization patterns that were distinct for each species and subspecies tested. Hybridization of amplicons generally was restricted to appropriate homologous oligonucleotides and cross-hybridization with heterologous oligonucleotides was rare. Hybridization patterns were recorded as separate gray values for each hybridized spot and revealed a consistent pattern for multiple strains of each species or subspecies isolated from diverse geographical regions. In preliminary tests, bacteria could be correctly identified and detected by hybridizing to the array amplicons from mixed cultures and inoculated potato tissue.

Additional keywords: blackleg, brown rot, macroarray, microarray, reverse dot blot, ring rot, soft rot.

The gram-positive bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*, and gram-negative bacteria, *Ralstonia solanacearum* and pectolytic erwinias, including *Erwinia carotovora* subsp. *atroseptica* and *carotovora* and *E. chrysanthemi*, are important bacterial pathogens of potato. *E. carotovora* subsp. *carotovora* is ubiquitous in most agricultural environments, surviving in surface water and soil, often in association with decaying crop residues (29). Although bacterial potato pathogens contaminate healthy seed potatoes from infected crop residues and other inoculum sources, the main cause of disease is seed potato tubers that were contaminated with the pathogen during the previous growing season (3). Often, contamination or infection is at a subclinical level, in that no overt symptoms of disease are present. Such latent infections cannot be detected by visual inspection of tuber lots. Consequently, laboratory tests are used to detect *C. michiganensis* subsp. *sepedonicus* and *R. solanacearum* in seed tuber lots to control, respectively, bacterial ring rot disease in Canada and brown rot in western Europe (4,14). Tests for *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* also have been developed, but these have been used to a lesser extent due to the high cost of laboratory testing and the lesser importance of the diseases they incite (28).

The conventional approach to testing for latent bacterial infections in seed potato has been to use serological methods such as enzyme-linked immunosorbent assay and immunofluorescence, sometimes coupled with bioassay or isolation procedures (4,8,28). With the development of species-specific oligonucleotide primers, polymerase chain reaction (PCR) now also can be used as a confirmatory assay. Although the sensitivity of PCR may make its use advantageous, particularly if conducted with one of the real-time technologies (30) which alleviates the need for postamplification identification of PCR product, its widespread use for the potato industry is prohibited by its cost and intensive labor requirements.

DNA array technology, essentially a reverse dot blot technique, is an emerging methodology useful for identification of DNA fragments and may be applicable for rapid identification and detection of plant pathogens associated with plants (20,21,26). An array of species-specific oligonucleotide probes representing the various pathogens of potato, built on a solid support such as a nylon membrane or microscope slide, could be probed readily with labeled PCR products amplified from a potato sample. By using conserved primers to amplify common bacterial genome fragments from extracts of potato tubers that might contain the bacterial pathogens, the presence of DNA sequences indicative of pathogenic species would be revealed by hybridization to species-specific oligonucleotide probes within the array.

This strategy has been used elsewhere. For example, a non-radioactive reverse dot blot method was used initially for the detection of tickborne encephalitis in clinical specimens (11) and, subsequently, for the diagnosis of point mutations in congenital adrenal hyperplasia caused by 21-hydroxylase deficiency (37), and was particularly useful for the identification of retroviral sequences (31). A similar strategy was used to identify lactic acid bacteria in fermented food by targeting specific genomic sequences in the ribosomal gene cluster (7), as well as for diagnosis of *Staphylococcus* spp. by targeting marker genes (13). In plant pathology, array technology targeting the ribosomal gene cluster also was successfully applied to identify oomycete fungi (22) and nematodes (34).

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In this study, we explored the feasibility of using DNA array technology for identification and differentiation of plant pathogenic bacteria by targeting five bacterial pathogens of potato. We identified oligonucleotides in the 16S and the 16S-23S intergenic spacer (IGS) region of the ribosomal gene clusters of the potato bacterial pathogens that were specific for each of the bacteria. Both pure cultures of bacteria and potato extracts were evaluated on arrays established with the specific and heterologous oligonucleotides to determine the discriminatory potential of this technology and application for identification and detection of bacterial pathogens. A preliminary report on a part of this study has been published (10).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Cells of *Erwinia* spp. were cultured in tryptone yeast extract liquid media containing 1.6% (wt/vol) tryptone, 1% (wt/vol) yeast extract, and 0.5% (wt/vol) NaCl (32). Strains of *C. michiganensis* subsp. *sepedonicus* and *R. solanacearum* were grown at ambient temperature in nutrient broth supplemented with yeast extract (30). Stock bacterial cultures were maintained on the same medium supplemented with 15% (wt/vol) glycerol at –70°C.

DNA extraction. Cultures of *Erwinia* spp., *C. michiganensis* subsp. *sepedonicus*, and *R. solanacearum* were harvested in late log phase. Genomic DNA was extracted from 50 to 100 mg fresh weight of cells using a commercial DNA Purification Kit (BIO 101, LaJolla, CA).

PCR amplification, DNA sequencing, and labeling. PCR and sequencing primers were as published in a previous study (9). Amplification and sequencing of 16S rRNA genes was performed to confirm the identity of the strains used in this study as described (9). For digoxigenin-labeling of DNA template, PCR reactions were carried out in a GeneAmp PCR Systems 2400 (Applied Biosystems, Foster City, CA) in 25 µl with 1× Mg^{++}-free PCR buffer (Gibco-BRL, Burlington, Ontario, Canada), 2.0 mM $MgCl_2$, 0.16 mM dNTPs each, 0.4 µM primer each, 2.5 units of Taq DNA polymerase (Gibco/BRL), and 1 µl of genomic DNA (10 to 15 ng/µl) with the following thermal profile: 94°C for 2 min followed by 35 cycles of 94°C for 45 s, 64°C for 45 s, and 72°C for 1.5 min, and a final extension at 72°C for 9 min. Primers 1114f (5′-GCAACGAGCGCAACCCT-3′) (19) and L1r (5′-CA(A/G)GGCATCCACCGT-3′) (12) from conserved sequences flanking the 16S/IGS region were used in PCR to generate digoxigenin-dUTP-labeled amplicons from bacterial DNA templates. Concentration of PCR products was estimated on 1.5% agarose (OMNI PUR, EMScience, Gibbstown, NJ).

TABLE 1. Strain, host, and source information for the bacterial species studied

Bacteria, strains, origin[a]	Host
Erwinia carotovora subsp. *atroseptica*	
UBC E1; B. Copeman, B.C., Canada	*Solanum tuberosum*
31; A. Kelman (SR8), Wisconsin, USA	*S. tuberosum*
196; S. H. De Boer, B.C., Canada	*S. tuberosum*
558; H. Maas Geesteranus, Netherlands	*S. tuberosum*
BBA 9201; F. Niepold, Braunschweig, Germany	*S. tuberosum*
BBA 9204; F. Niepold, Braunschweig, Germany	*S. tuberosum*
BBA 9205; F. Niepold, Braunschweig, Germany	*S. tuberosum*
GSBP 1700; E. Langerfeld, Braunschweig, Germany	*S. tuberosum*
GSPB 1401; DSMZ Braunschweig, Germany	*S. tuberosum*
GSPB 2231; S. H. De Boer, B.C., Canada	*S. tuberosum*
E. carotovora subsp. *odorifera*	
1645-1; R. Samson (16454-1), France	*Cichorium intybus*
1878; R. Samson (1878), France	*C. intybus*
1880; R. Samson (1880), France	*C. intybus*
1893; CFBP 1893/R. Samson, France	*C. intybus*
E. carotovora subsp. *betavasculorum*	
29; H. Maas Geesteranus ex E. Ruppel (DSMB), CO	*Beta vulgaris*
E. carotovora subsp. *wasabiae*	
SR 91; M. Goto (91), Japan	*Armoracia rusticana*
SR 92; M. Goto (92), Japan	*A. rusticana*
SR 93; M. Goto (93), Japan	*A. rusticana*
SR 94; M. Goto (94), Japan	*A. rusticana*
E. carotovora subsp. *carotovora*	
UBC E155; B. Copeman, B.C., Canada	*S. tuberosum*
UBC E161; B. Copeman, B.C., Canada	*S. tuberosum*
21; H. Maas Geesteranus (139), Netherlands	*S. tuberosum*
441; L. Fuckikovsky (G1), Mexico	*Helianthus spp.*
GSPB 2233; S. H. De Boer, Netherlands	*S. tuberosum*
E. chrysanthemi	
340; E. French, Peru	*S. tuberosum*
571; R. Cother (DAR30511), Australia	*S. tuberosum*
572; H. Maas Geesteranus ex R. Dickey (141)	*Musa paradisiaca*
573; H. Maas Geesteranus ex R. Dickey (1015 = NCPPB1186)	*Parthenium argentatum*
580; H. Maas Geesteranus ex R. Dickey (B73)	*Syngonium podophyllum*
582; H. Maas Geesteranus ex R. Dickey (73)	*Dieffenbachia picta*
GSPB 413; W. H. Burkholder, USA	*Chrysanthemum*
GSPB 1406; L. E. Webb, Germany	*Saitpaulia ionantha*
E. stewartii (*Pantoea stewartii* subsp. *stewartii*)	
GSPB 2626; K. Geider, USA	*Zea mays*
E. herbicola (*P. agglomerans*)	
GSPB 450; R. A. Lelliot, England	*Pyrus communis*
LMG 2565; E. Roslycky, Canada	Cereals
GSPB 2695; E. French, Kenya	*L. esculentum*
GSPB 2791; E. French, Peru	*S. tuberosum*
LMG 2300; Z. Volcani, Israel	*S. tuberosum*
LMG 2305; R. Lelliott, Egypt	*S. tuberosum*
Rhizoctonia solanacearum	
PD 511 (=ATCC 11699; NCPPB325)/unknown U.S.	*L. esculentum*
PD 1410; E. French, Sri Lanka	*S. tuberosum*
PD 1424; M. Goto, Japan	*Arachis hypogea*
PD 2763; J. Janse, The Netherlands	*S. tuberosum*
GSPB 2695; E. French, Kenya	*L. esculentum*
GSPB 2791; E. French, Peru	*S. tuberosum*
LMG 2300; Z. Volcani, Isreal	*S. tuberosum*
LMG 2305; R. Lelliott, Egypt	*S. tuberosum*
Pseudomonas fluorescens	
GSPB 1714; Unknown	Unknown
Clavibacter michiganensis subsp. *sepedonicus*	
CS 12; H. Lawrence, Canada	*S. tuberosum*
R10; S. H. De Boer, Canada	*S. tuberosum*
CS 150; 100/N. Gudmestad, United States	*S. tuberosum*
CS 3M; G. A. Nelson, Canada	*S. tuberosum*
CS R3; S. H. De Boer, Canada	*S. tuberosum*
CS R6; S. H. De Boer, United States	*S. tuberosum*
C. michiganensis subsp. *Michiganensis*	
CM 3; A. Nelson, Canada	*L. esculentum*
GSPB 382; A. Mavridis, Greece	*L. esculentum*
GSPB 2315; Ö. Cinar, Turkey	*L. esculentum*
C. michiganensis subsp. *nebraskensis*	
GSPB 2223; M. L. Schuster, United States	*Z. mays*
LMG 5627; M. Schuster, United States	*Z. mays*
C. michiganensis subsp. *tessellarius*	
GSPB 2224; R. R. Carlson, United States	*Triticum aestivum*
LMG 7295; A. Vidaver (78151), USA	*T. aestivum*
C. michiganensis subsp. *insidiousis*	
GSPB 2225; W. H. Burkholder, United States	*Medicago sativa*
Escherichia coli O157:H7	
ATCC 43895; United States	Raw Hamburger
Listeria monocytogenes	
LCDC 81-861; Nova Scotia	*Brassica oleracea*

[a] Strains prefixed LMG, ATCC, BBA, and GSPB were obtained from the Culture Collection of the Laboratoriium voor Microbiologie, Rijsuniversiteit, Gent, Belgium; American Type Culture Collection, Beltsville, United States; Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany; or from the Göttingen Collection of Phytopathogenic Bacteria, Göttingen University, Göttingen Germany, respectively. Other strains were obtained directly from individuals as indicated.

Selection of oligonucleotides. Taxon-specific oligonucleotides were selected from sequences of ribosomal DNA genes, the 16S-23S rDNA IGS regions of pectolytic *Erwinia* spp. (9), and from 16S-23S rDNA sequences of *C. michiganensis* subsp. *sepedonicus* (24) to serve as immobilized oligonucleotides within the array (Table 2). Sequences for species-specific oligonucleotide probes for *R. solanacearum* were based on data available from GenBank (K. H. Pastrik, J. G. Elphinstone, and R. Pukall, *unpublished data*). Alignments were made with Pileup (Genetic Computer Group, Inc., Madison, WI) and unique polymorphisms were found manually. Selected oligonucleotides were designed for optimal and uniform hybridization kinetics using Oligo 6.3 software (1998, Molecular Biology Insights, Inc., West Cascade, CO). C6-amino-terminated oligonucleotides were generated with a DNA synthesizer (Beckman Oligo 1000; Beckman Instruments, Inc., Fullerton, CA).

Preparation of the DNA array. A template of 16- to 24-mer amino-terminated oligonucleotides was prepared in the desired array format. DNA oligonucleotides were diluted from 200 μM stock to 40 μM in sodium bicarbonate buffer (0.4 μM, pH 8.0) in a sterile 384-well microplate. Oligonucleotides were spotted with a VP 384 multi-Blot Replicator (V&P Scientific Inc., San Diego, CA) in three rows of 18 on Immunodyne ABC membranes (PALL Europe Limited, Portsmouth, England) cut into 3-by-9-cm strips. Duplicates of the same oligonucleotides were spotted on the diagonal by repeated printing. Printed membranes were air dried for 10 min and transferred into blocking solution (2× standard saline citrate [SSC] [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate], 0.5% casein [BDH Biochemical, England], and 0.05% Tween 20) and agitated for 15 min. Blots were washed in 2× SSC for 30 min and stored short-term in 2× SSC or air dried and kept in an envelope at room temperature for long-term storage.

During the course of this study, several versions of the array were evaluated but data from only the two final arrays are presented (Fig. 1). The final version of the array included six more oligonucleotides for *E. chrysanthemi* than were used in the penultimate version.

Hybridization to DNA arrays. Before use, membranes were prehybridized for 1.5 h with hybridization buffer (6× SSC, 0.1% *N*-lauroylsarcosine [Sigma, L9150], 0.02% sodium dodecyl sulfate [SDS} [Sigma, L4509], plus 1% casein [BDH Biochemical, Poole, England]). Dig-dUTP-labeled DNA target was denatured in boiling water for 10 min. Overnight hybridization at 54°C with 6 ml of hybridization buffer containing approximately 60 ng of digoxigenin dUTP-labeled DNA target per membrane was followed by two washes with stringency buffer (4× SSC, 0.1% SDS) at hybridization temperature (22). Anti-Dig alkaline phosphatase conjugate (Roche Diagnostics GmbH, Mannheim, Germany) and the chemiluminescent substrate CDP-Star (Roche Diagnostics GmbH) were used following the manufacturer's protocol. Multiple exposures of sealed membranes were done for 15 to 120 min on film (X-Omatic K; Kodak, Rochester, NY) on the day after adding the CDP-Star. The short exposures were enough to see all the strong reactions but, in order to find all possible cross-

TABLE 2. Name, sequence, and origin of amino-terminated 16- to 24-mer oligonucleotides used to configure DNA oligonucleotide arrays

Location	Code	Sequence of oligonucleotides (5′ to 3′)	Origin of sequence[a]
A1	Un1492	TACGGYTACCTTGTTACGACTT	Universal 16S rDNA
A2	Un1518	AAGGAGGTGATCCAACCGC	16S of *Erwinia* spp., *Escherichia coli*
A4	Eca1127A	GCGTAATGGCGGGAAC	16S of *Erwinia carotovora* subsp. *atroseptica*
A5	Eca1127B	GCGTAATGGCGGGAACT	16S of *E. carotovora* subsp. *atroseptica*
A6	Eca1255	GAACTCGCGAGAGCCAG	16S of *E. carotovora* subsp. *atroseptica*
A7	Eca190A	CTGTAAGTAGAGATGGGGCT	Large IGS of *E. carotovora* subsp. *atroseptica*
A8	Eca190B	CTGTAAGTAGAGATGGGGCTA	Large IGS of *E. carotovora* subsp. *atroseptica*
A9	Ech9	ACCTCCTTACCAATAAAGATGT	Small IGS of *E. carotovora* subsp. *atroseptica*
A10	EcaCh361	CCTTAGGGCGAGTTGTGA	Small IGS of *E. carotovora* subsp. *atroseptica*
A11	EcaCh244	AGACATTATCACCGAATATCTTA	Small IGS of *E. carotovora* subsp. *atroseptica*, *wasabiae*
A12	EcaCh245	GACATTATCACCGAATATCTT	Small IGS of *E. carotovora* subsp. *atroseptica*, *wasabiae*
A13	EcaCh370	GAGTTGTGATGAGTCAGTGTG	Small IGS of *E. carotovora* subsp. *atroseptica*
A14	Ecc244A	TTAAGATATTCATGATAATGTCT	Small IGS of most *E. carotovora* subsp. *carotovora*
A15	Ecc244B	TTTAAGATATTCATGATAATGTCT	Small IGS of *E. carotovora* subsp. *carotovora*
A16	Ecc190	GTAAGCAACGATGGGGTTA	Large IGS of *E. carotovora* subsp. *carotovora*, *odorifera*, *betavasculorum*
A17	Ecc191	TAAGCAACGATGGGGTTA	Large IGS of *E. carotovora* subsp. *carotovora*, *odorifera*, *betavasculorum*
B1	EccBe9	ACCTCCTTACCAAGAAGATGT	Large IGS of *E. carotovora* subsp. *carotovora*, *odorifera*, *betavasculorum*
B2	EccBe244	AGACATTATCACGAATATCTTAA	Small IGS of some *E. carotovora* subsp. *carotovora*, *betavasculorum*
B3	EccBe245	GACATTATCACGAATATCTTAA	Small IGS of some *E. carotovora* subsp. *carotovora*, *betavasculorum*
B4	Ebe342	CCGTCATGCATGTTCAAGC	Small IGS of *E. carotovora* subsp. *carotovora* 441, *betavasculorum* 29
B5	EO1128A	GAGTTCCCGACCGAATC	16S of *E. carotovora* subsp. *carotovora*, *odorifera*, *betavasculorum*
B6	EO128B	TGAGTTCCCGACCGAATC	16S of *E. carotovora* subsp. *carotovora*, *odorifera*, *betavasculorum*
B7	EO1255	CTTGCTCTCGCGAGGTC	16S of *E. carotovora* subsp. *carotovora*, *odorifera*, *betavasculorum*, *wasabiae*, *E. chrysanthemi*
B8	Ech171	GACGGTGGGTGAAAGGC	Small IGS of *E. chrysanthemi*
B9	Ech194	AACGCTAACCTAAAACTGATT	Small IGS of *E. chrysanthemi*
B10	Ech165	ATGACTGACGGTGGGTGAA	Small IGS of *E. chrysanthemi*
B11	Ech190	GGTCAACGCTAACCTAAAACT	Small IGS of *E. chrysanthemi*
B14	EchUp333	TCCCGAGACACTTTCGG	Small IGS of *E. chrysanthemi*
B15	Ech333	CCGAAAGTGTCTCGGGA	Small IGS of *E. chrysanthemi*
C2	RaSo41	CGTGCATTCTAGTTAGGCG	IGS of *Ralstonia solanacearum*
C3	RalSo180	ACGGTGGAAGTCTCTGCC	IGS of *R. solanacearum*
C4	RaSo299	CGCAAGCATCGAGTTTTC	IGS of *R. solanacearum*
C5	RaSo333	ATTGCCAAGACGAGTAATAAC	IGS of *R. solanacearum*
C6	RaSo405	ATGAGATGCTCGCAACAAC	IGS of *R. solanacearum*
C7	RaSo460	GAGTGATCGAAAGACCGCT	IGS of *R. solanacearum*
C8	Cms181	GGGTGGGAAAATGGTCTG	IGS of *Clavibacter michiganensis* subsp. *sepedonicus*
C9	Cms182	GGTGGGAAAATGGTCTG	IGS of *C. michiganensis* subsp. *sepedonicus*
C10	Cms250A	ACCAGACACACCAAAAGG	IGS of *C. michiganensis* subsp. *sepedonicus*
C11	Cms250B	AACCAGACACACCAAAAGG	IGS of *C. michiganensis* subsp. *sepedonicus*
C12	Cms250C	AAACCAGACACACCAAAAGG	IGS of *C. michiganensis* subsp. *sepedonicus*

[a] IGS = intergenic spacer region.

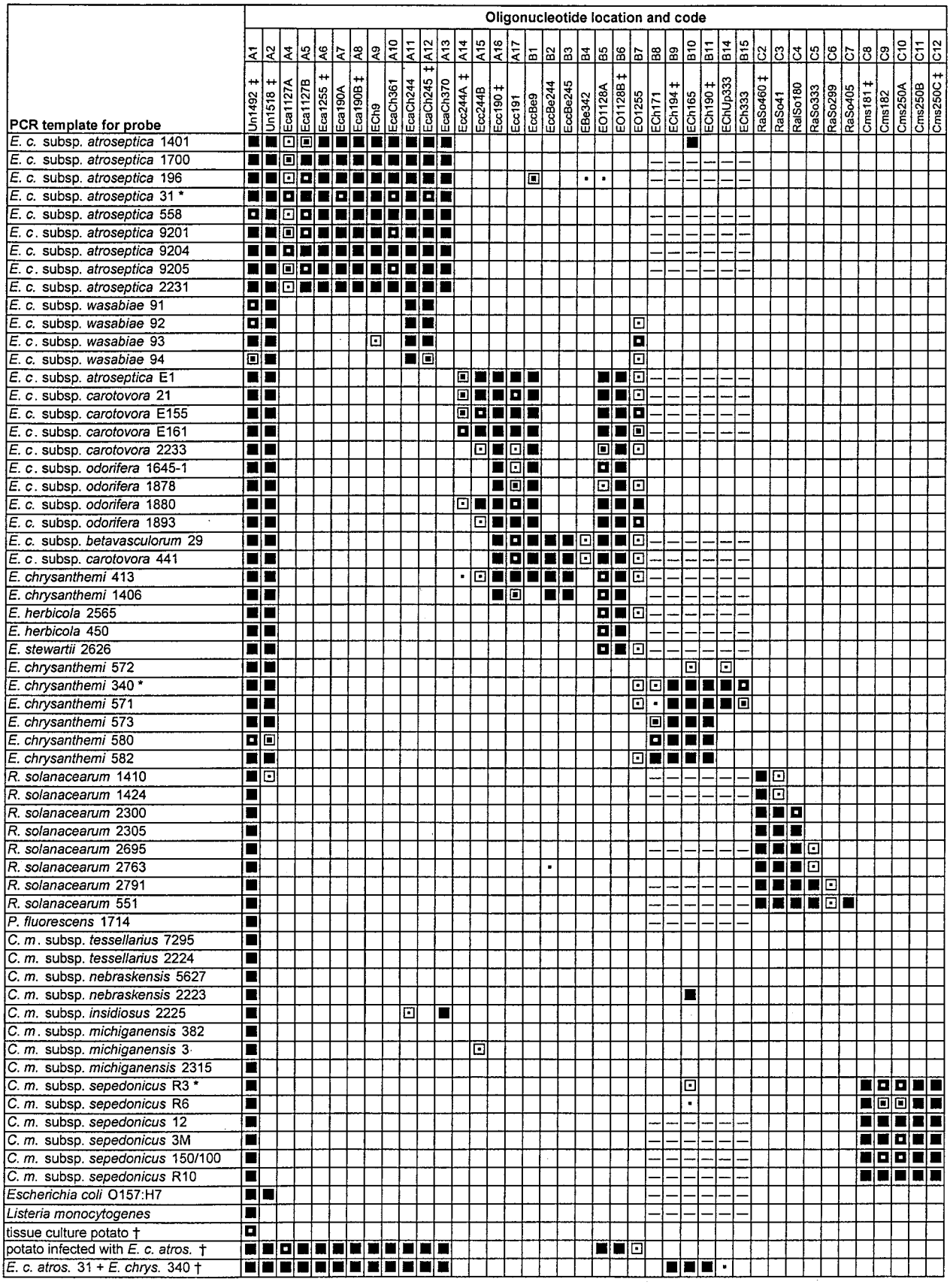

Fig. 1. Hybridization of digoxigenin-labeled polymerase chain reaction amplicons from selected bacterial species and samples to oligonucleotides immobilized on nylon membrane in an array. Hybridization signal strength of each amplicon to an oligonucleotide in the array is expressed in 16-bit grayscale values indicated by the following symbols: □ = <500; ▪ = 500 to 1,000; ⊡ = 1,000 to 10,000; ▣ = 10,000 to 20,000; ◘ = 20,000 to 30,000; ■ = 30,000 to 65, 000; and – = not tested because these six oligonucleotides were present only on a final membrane version. The location of oligonucleotides in the array given at the top of the figure correspond to their location on the membranes in Figures 2 and 3; oligonucleotides are described in Table 2. * = membranes shown in Figure 2. † = membranes shown in Figure 3.

reacting oligonucleotides, the films that gave the maximum number of dots before the background became too dark (i.e., 45 min or more of exposure) were selected for scanning and analyses.

Chemiluminogrammes were scanned at 16-bit gray using Fotolook Ps 2.08 software on an ARCUS II scanner (AGFA, Taiwan, ROC). Gray values for each spot were evaluated using GenePix Pro 3.0.6 (AXON Instruments, Inc., CA).

Membrane arrays were reused after stripping off hybridized DNA with 0.2 M NaOH at 65°C in a hybridization oven for 30 min, rinsing with 2× SSC, and washing with 0.5× SSC for 20 min at 65°C. Stripped membranes were transferred to 2× SSC and agitated for 30 min, and were ready for use or stored as described previously.

Mixed bacteria and potato samples. To test the discriminatory potential of the oligonucleotide array, suspended bacterial cells of *E. carotovora* subsp. *atroseptica* (strain 31) and *E. chrysanthemi* (strain 340) were mixed 1:1 prior to DNA extraction. Genomic DNA mixture combinations of *C. michiganensis* subsp. *sepedonicus* (strains CS 12 and CS 150/100), *E. carotovora* subsp. *atroseptica* (strains 558 and 196), *E. carotovora* subsp. *carotovora* (strains 21 and 441), *E. chrysanthemi* (strains 573 and 340), and *R. solanacearum* (strains 511 and 2791) were mixed 1:1 (10 to 15 ng/µl for each strain) prior to PCR amplification.

DNA extracted from micropropagated noninfected potato plantlets was used as a control for hybridizations to the array. In addition, potato tubers were inoculated with *E. carotovora* subsp. *atroseptica* at approximately 1×10^6 CFU/ml by vacuum infiltration as described (27). DNA from inoculated and uninoculated tubers were amplified and labeled with digoxigenin-11-dUTP by PCR using primer set 1114f/L1r. Labeled DNA was denatured in boiling water for 10 min before hybridizing to arrays using the same procedure as for DNA from pure bacterial colonies.

RESULTS

Development of the oligonucleotide array. Forty specific oligonucleotides were designed for the main potato bacterial pathogens using the 3′ end of the 16S gene or the IGS regions that are bracketed by the 16S and 23S ribosomal genes. The oligonucleotides were designed with comparable melting temperatures. Multiple oligonucleotides were selected on the basis of specificity for each bacterial species (Table 2). For example, for *E. carotovora* subsp. *atroseptica*, three oligonucleotides (Eca1127A, Eca1127B, and Eca1255) were designed from the 16S sequence, two (Eca190A and Eca190B) were from the IGS region of the larger ribosomal operon, and three (Ech9, EcaCh361, and EcaCh370) from the IGS region of the smaller ribosomal operon. Another four oligonucleotides (EcaCh244, EcaCh245, Ecc244A, and Ecc244B) were designed from common IGS sequences within *E. carotovora* subsp. *atroseptica* and *wasabiae* or subsp. *carotovora*, respectively. Seventeen oligonucleotides were designed with specificity to *E. carotovora* subspecies and *E. chrysanthemi*, six with specificity to *R. solanacearum*, and five with specificity to *C. michiganensis* subsp. *sepedonicus* (Table 2). Prior to synthesis and incorporation into the array, oligonucleotide sequences were screened by Blastn searches for homology to bacterial sequences in GenBank.

Two oligonucleotides (Un1492 and Un1518), designed as universal targets, served as the positive controls. Several locations on the array (A3, B12-13, B16-18, C1, and C13-18) not spotted with oligonucleotides served as negative controls. Six additional oligonucleotides (Ech171, Ech194, Ech165, Ech190, EchUp333, and Ech333) were designed for the final version of the array and are shown as missing values for the first data set (Fig. 1).

Evaluation of the specificity of the oligonucleotide array. A fragment of the ribosomal gene cluster was amplified by PCR, using conserved primers, from each bacterial strain used in this study (Table 1). Digoxigenin-labeled PCR amplicons generated from pure cultures of bacteria generally hybridized to the array according to the expected patterns (Fig. 1). Patterns were highly reproducible in the two to three hybridizations conducted with each of the bacterial templates.

Hybridization signal strength, expressed in gray values, ranged from 0 to 65,000 among the oligonucleotide spots on the arrays. Gray values of >500 were arbitrarily considered as positive hybridization signals while lower values, not visible without computer enhancement, were considered to be background signals. Exposures were selected for analysis that gave signal-to-noise ratios, based on positive and negative controls, >4,400:1. Mean gray value for spots regarded as negative was 10.7, whereas the mean of positive signals was 39,460.

Labeled amplicons generated from nine strains of *E. carotovora* subsp. *atroseptica* hybridized to the 10 oligonucleotides designed for the subspecies (Fig. 1). Only two strains (Eca1401 and Eca196) hybridized to oligonucleotides designed for other erwinias (Ech165, EccBe9, Ebe342, and EO128A) but at least in one case (Eca1401), the cross-hybridization could not be duplicated (the membrane that gave a cross reaction is shown in Fig. 1). This was an exception in duplication of results because hybridization with amplicons from the same strain normally showed the same hybridization pattern. The variation in intensity of hybridization to different oligonucleotides is exemplified with strain 31 (Fig. 2A); the amplicon hybridized strongly to oligonucleotide EcaCh370 (location A13) but more weakly with oligonucleotide Eca1127A (location A4). The amplicon generated from strain 31 did not cross-hybridize with heterologous oligonucleotides but did hybridize with the positive control oligonucleotides (Fig. 2, location A1-2). Strain E1, received as *E. carotovora* subsp. *atroseptica*, was an exception in that it did not hybridize to the homologous oligonucleotides but rather presented a hybridization pattern that was the same as other strains of *E. carotovora.* subsp. *carotovora*. This confirms that this strain is likely misnamed, as discussed previously (9). Oligonucleotides EcaCh244 and EcaCh245 were selected as common sequences of *E. carotovora* subsp. *atroseptica* and *wasabiae*, and the unique hybridization of the *E. caroto-*

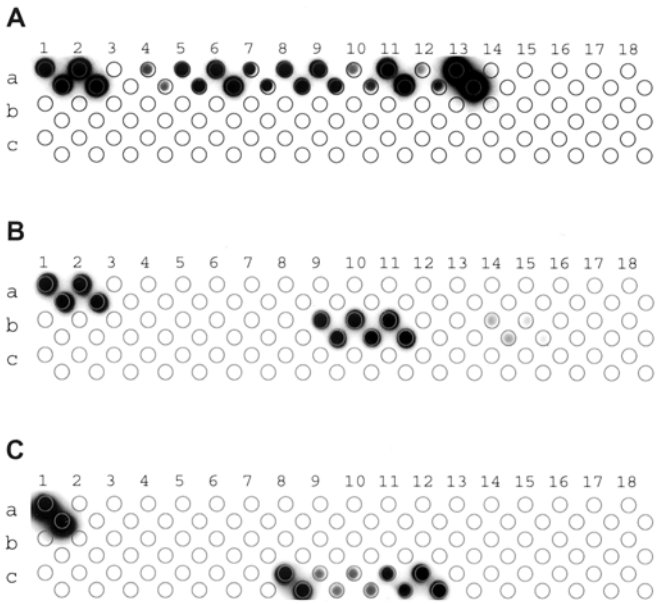

Fig. 2. Hybridization pattern of digoxigenin-labeled polymerase chain reaction amplicons from **A,** *Erwinia carotovora* subsp. *atroseptica* strain 31; **B,** *E. chrysanthemi* strain 340; **C,** and *Clavibacter michiganensis* subsp. *sepedonicus* strain R3 on the oligonucleotide array. Oligonucleotides were spotted, with duplicates on the diagonal, in three rows (a, b, and c) of 18 spots. After hybridization, detection, and film exposure, a template was placed over the membrane to identify spot locations. Positive hybridization signals are visualized as dark spots within template circles and were analyzed on the basis of gray scale values summarized in Figure 1.

vora subsp. *wasabiae* to these oligonucleotides was consistent with the results expected from the sequences (Fig. 1). Strains of *E. carotovora* subsp. *betavasculorum*, *carotovora*, and *odorifera* similarly presented hybridization patterns consistent with the oligonucleotide and rDNA sequence comparisons (Table 2; Fig. 1).

The reaction of *E. chrysanthemi* 340 was typical for the species which hybridized strongly to three oligonucleotides (Ech194, Ech165, and Ech190) (Figs. 1 and 2B locations B9–11, respectively). Weaker hybridizations were noted with oligonucleotides EO1255 (Fig. 2B, not visible at location B7) and EChUp333 and Ech333 (Fig. 2B, location B14-15). Hybridization patterns for *E. chrysanthemi* strains 1406 and 413 were consistent with sequencing data which showed that these strains are likely misnamed (9). These strains were previously shown to be similar to *E. carotovora* subsp. *carotovora* and their hybridization patterns were consistent with this observation (Fig. 1). Strains of *E. herbicola* and *E. stewartii* hybridized with two to three oligonucleotides designed for specificity to *E. carotovora* subspecies but not with eight other oligonucleotides that characterized many *E. carotovora* strains.

Amplicons generated from all six strains of *C. michiganensis* subsp. *sepedonicus* tested hybridized to the five homologous oligonucleotides (Figs. 1 and 2C locations C8–12). Other subspecies of *C. michiganensis*, including *michiganensis*, *insidiosus*, *nebraskensis*, and *tessellarius*, did not hybridize to oligonucleotides designed from the IGS region of *C. michiganensis* subsp. *sepedonicus* (Fig. 1). Only six out of a few hundred heterologous spots probed with Clavibacter DNA produced a signal. These spurious hybridizations did not interfere with bacterial identifications. All eight strains of *R. solanacearum* hybridized to oligonucleotides RaSo460 and RaSo41, but the strains hybridized differentially to the other four *R. solanacearum*-specific oligonucleotides (Fig. 1). *R. solanacearum* amplicons did not hybridize to oligonucleotides designed from sequences of heterologous species except for a single weak heterologous hybridization signal among all the heterologous spots probed with *R. solanacearum* DNA (Fig. 1).

An amplicon from *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Pseudomonas fluorescens* only hybridized with one or both the universal oligonucleotide controls, as expected (Fig. 1).

Discriminatory potential of the oligonucleotide array. Several mixtures of bacterial strains were amplified by PCR with the conserved primers and hybridized to the oligonucleotide array. The mixture of amplicons did not interfere with one another and the expected pattern for each species was obtained. An array probed with amplicons from a mixture of *Erwinia carotovora* subsp. *atroseptica* strain 31 and *E. chrysanthemi* strain 340 is presented in Figure 3A and summarized at the bottom of Figure 1. The hybridization pattern is consistent with that expected for the two *Erwinia* spp.

Bacteria also could be identified directly in potato tissue using the oligonucleotide array. PCR amplicons generated from potato tubers inoculated with *E. carotovora* subsp. *atroseptica* strain 31 produced the same hybridization pattern on the array as pure cultures (Figs. 3B and 1 bottom), again confirming the reproducibility of hybridization pattern. The hybridization to the three oligonucleotides (EO1128A, EO1128B, and EO1255) characteristic of *E. herbiolca* (*Pantoea agglomerans*) and *E. stewartii* (*P. stewartii* subsp. *stewartii*) suggests that there were nonpectolytic enterobacteria among the potato microflora. Bacteria-free potato tissue from microplantlets did not hybridize with any of the oligonucleotides designed for the pathogenic bacteria (Figs. 3C and 1 bottom).

DISCUSSION

Traditional methods for identification of bacteria require the isolation of pure cultures of the organism to be identified. Increasingly, however, molecular methods can be used to identify specific target species within mixtures of microorganisms without prior isolation of pure cultures (25,26). DNA array technology is one such method that has potential for rapid and cost-effective identification of pathogenic bacteria in the complex microbial communities which are normally associated with plants (1,21, 36,38).

In our study, we evaluated the potential usefulness of DNA array technology for identifying bacteria involved in potato pathogenesis and differentiating them from closely related strains. We targeted the ribosomal operon as a source of species- and subspecies-specific oligonucleotides because not only are sequences of this gene region available for numerous bacteria, but they also have been shown to be useful for detection and identification of various plant pathogenic bacteria (16,17,33).

By targeting the ribosomal gene, we were able to use conserved primers for amplifying template DNA across a wide range of bacterial species. The rDNA sequence homology is high among eubacteria; therefore, the 16S and IGS chromosomal regions of any of the plant-pathogenic bacteria could be amplified with a single primer pair (19). Hence, for any field sample, amplicons can be generated without the need for preliminary identification of a target species. The pathogenic species within the sample would be identified by the hybridization pattern on the array. The sensitivity threshold for detecting pathogenic strains among a large number of nonpathogenic bacteria needs to be determined. It is interesting to point out again that nonpathogenic species were detected in a potato sample inoculated with *E. carotovora* subsp. *atroseptica* (Fig. 1).

With the hybridization stringency used in our study, the selected oligonucleotides generally displayed the desired specificity. The oligonucleotides were selected to have at least two base differences from closely related species or subspecies. For example, the oligonucleotides from the IGS region of *C. michiganensis* subsp. *sepedonicus* (Cms181-2; Table 2) differed from the other

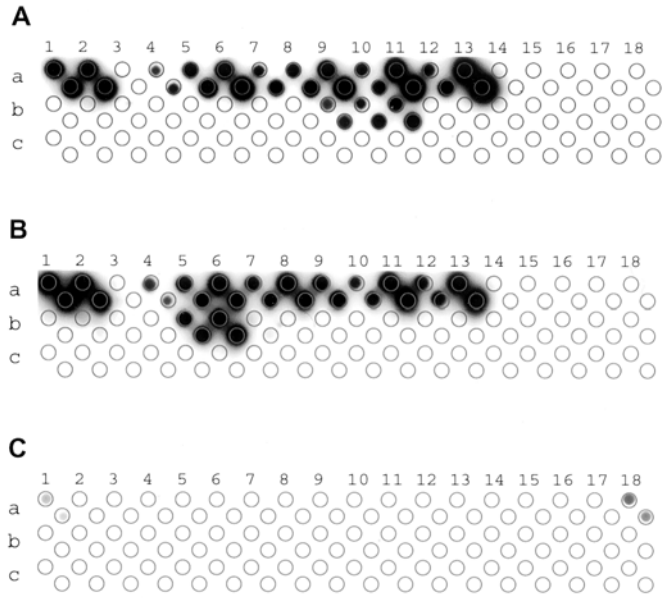

Fig. 3. Hybridization pattern of digoxigenin-labeled polymerase chain reaction amplicons obtained from **A,** a mixture of *Erwinia carotovora* subsp. *atroseptica* strain 31 and *E. chrysanthemi* strain 340 cells; **B,** DNA from *E. carotovora* subsp. *atroseptica*-inoculated potato tuber; and **C,** DNA from potato tissue culture. Oligonucleotides were spotted, with duplicates on the diagonal, in three rows (a, b, and c) of 18 spots. After hybridization, detection, and film exposure, a template was placed over the membrane to identify spot locations. Positive hybridization signals are visualized as dark spots within template circles and were analyzed on the basis of gray scale values summarized in Figure 1.

subspecies *C. michiganensis* subsp. *michiganensis* and *insidiosus* by this minimum of two nucleotides (24). Gray values from hybridization of strains of *C. michiganensis* subsp. *sepedonicus* to oligonucleotide Cms181 varied between 31,000 and 59,000, whereas they varied between 0 and 94 for *C. michiganensis* subsp. *michiganensis* and *insidiosus* (Fig. 1). Unlike primers designed for PCR, discriminatory specificity is greatest when mismatches are in the center of the immobilized oligonucleotide (18); therefore, we had to modify the PCR primer Sp1f (24) into Cms181 and Cms182 for hybridization purposes.

The diversity among the pectolytic *Erwinia* spp. provided special challenges for developing discriminatory oligonucleotides. Whereas *E. carotovora* subsp. *atroseptica* forms a distinct and uniform taxonomic cluster closely related to subsp. *wasabiae* strains (9), it is distinguishable from other *E. carotovora* strains. Differentiation of *E. carotovora* subsp. *carotovora* from subsp. *odorifera* and *betavasculorum* would be challenging using the oligonucleotides selected for our array (Fig. 1). Nevertheless, some apparently misidentified strains, such as strains E1, 413, and 1406, could be placed in the correct taxa on the basis of their hybridization patterns.

The *E. chrysanthemi* strains that cause a serious blackleg-like disease on potato are difficult to differentiate from strains of this species that are pathogenic on other plants (2,15). Despite the heterogeneity within the species, as demonstrated by the work of Dickey (5) and Dickey et al. (6) with phenotypic and serological characteristics, a wide variety of strains in our study showed a distinctive *E. chrysanthemi* hybridization pattern on the array. It is perhaps not surprising that the banana strain 572 did not hybridize with most *E. chrysanthemi*-specific oligonucleotides.

The ease with which species- and subspecies-specific oligonucleotides can be selected depends to a considerable extent on the genetic uniformity of the taxon. *C. michiganensis* subsp. *sepedonicus* is phenotypically very uniform and this was reflected in the consistent hybridization pattern with isolates from regions as diverse as British Columbia, New Brunswick, and North Dakota (Table 1; Fig. 1). In contrast, strains of *R. solanacearum* showed greater diversity in hybridization pattern. Only one strain clearly hybridized with all six oligonucleotides selected, whereas two out of eight strains hybridized to only two oligonucleotides. This is not surprising given the diversity among race 3 strains found on potato in Europe alone (35) and the fact that our study included race 1 and 3 strains from widely separated geographic regions. Testing and sequencing of additional strains will no doubt more clearly delineate signature oligonucleotides of greatest value in an array.

Hybridization of labeled amplicons to our oligonucleotide array resulted in distinct patterns that were sufficient for making reliable presumptive identification of a species or subspecies and discriminated target species from closely related bacteria. However, because a difference in oligonucleotide sequences as small as two bases was sufficient to prevent hybridization, multiple oligonucleotides were required to consistently detect all strains within a species. Variation among strains can result in the failure of hybridization to any one oligonucleotide that is diagnostic for a species or subspecies. Although some bacterial taxons, such as *C. michiganensis* subsp. *sepedonicus*, as already noted, are remarkably uniform, variation in others, such as *E. carotovora* subsp. *carotovora* and *R. solanacearum*, is much greater. We estimate that four to five specific oligonucleotides per bacterial taxon might be required in an array to provide an adequate fingerprint to identify an unknown isolate.

Spurious hybridization of amplicons to oligonucleotides of unrelated taxons can be expected at low frequency due to coincidental similarities in base sequences of unrelated genes amplified at low level, because of similar gene sequences in taxonomically unrelated bacteria or through PCR artifacts. Such spurious hybridizations were minimized by using oligonucleotides of 16 to 24 bases in length. Nevertheless, a small number of bacteria hybridized with heterologous oligonucleotides. These hybridizations were generally weak and in no instance interfered with recognition of taxon-specific hybridization patterns.

We evaluated the ability of the oligonucleotide array to identify bacteria in microbial mixtures and in field samples only in a very preliminary way. The results, however, were encouraging, in that the anticipated hybridization patterns were obtained (Fig. 3). The hybridization pattern of our field sample (Fig. 3B) suggested the presence of a nonpectolytic enterobacteria, probably *E. herbicola*, a common plant epiphyte. Its presence did not interfere with the identification of *E. carotovora* subsp. *atroseptica* in the sample; instead, it illustrated the potential power of arrays for analyzing environmental samples.

Oligonucleotide array technology should be useful in the rapid identification of bacterial pathogens of potato. The utility of such an array would be further enhanced by the addition of oligonucleotides specific for other potato pathogens, including fungi, nematodes, and perhaps even viruses. Several PCR reactions may be required with conserved or degenerate primers, or with a reverse transcription step for RNA viruses, to produce labeled amplicons for the various pathogen groups. Production of the array on alternate platforms such as glass slides or electronic chips would enhance the efficacy and ease of use. In the future, a set of pathogen-specific oligonucleotides could be combined with other oligonucleotides specific to cultivars and genetic transformations to provide a single multipurpose array for the potato industry.

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