

Detection and Genotyping of *Arcobacter* and *Campylobacter* Isolates from Retail Chicken Samples by Use of DNA Oligonucleotide Arrays^{∇†}

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Received 22 December 2006/Accepted 27 March 2007

To explore the use of DNA microarrays for pathogen detection in food, we produced DNA oligonucleotide arrays to simultaneously determine the presence of *Arcobacter* and the presence of *Campylobacter* in retail chicken samples. Probes were selected that target housekeeping and virulence-associated genes in both *Arcobacter butzleri* and thermotolerant *Campylobacter jejuni* and *Campylobacter coli*. These microarrays showed a high level of probe specificity; the signal intensities detected for *A. butzleri*, *C. coli*, or *C. jejuni* probes were at least 10-fold higher than the background levels. Specific identification of *A. butzleri*, *C. coli*, and *C. jejuni* was achieved without the need for a PCR amplification step. By adapting an isolation method that employed membrane filtration and selective media, *C. jejuni* isolates were recovered from package liquid from whole chicken carcasses prior to enrichment. Increasing the time of enrichment resulted in the isolation of *A. butzleri* and increased the recovery of *C. jejuni*. *C. jejuni* isolates were further classified by using an additional subset of probes targeting the lipooligosaccharide (LOS) biosynthesis locus. Our results demonstrated that most of the *C. jejuni* isolates likely possess class B, C, or H LOS. Validation experiments demonstrated that the DNA microarray had a detection sensitivity threshold of approximately 10,000 *C. jejuni* cells. Interestingly, the use of *C. jejuni* sequence-specific primers to label genomic DNA improved the sensitivity of this DNA microarray for detection of *C. jejuni* in whole chicken carcass samples. *C. jejuni* was efficiently detected directly both in package liquid from whole chicken carcasses and in enrichment broths.

Bacterial contamination of food supplies during food harvesting, processing, and preparation is an area of increasing concern in food safety. Infections caused by bacterial food-borne pathogens continue to be a serious health issue, and the prevalence of food-borne illnesses is still substantial in the United States (5). The bacterial agent in the family *Campylobacteraceae* that is the most significant contributor to human gastrointestinal infections is *Campylobacter*; in laboratory-confirmed cases of infection in 2005, *Campylobacter* was second only to *Salmonella* (5). Although the causative agent of these human infections is determined often only at the genus level, the *Campylobacter* spp. that are associated primarily with human gastrointestinal disease are *Campylobacter jejuni* and *Campylobacter coli* (16, 56, 57). These species are often referred to as thermotolerant campylobacters because of their optimal growth at 42°C and are also associated prominently with poultry by colonizing preferentially the avian gastrointestinal tract (8). However, other important infection sources identified are pigs for *C. coli* (59) and untreated water and raw milk for *C. jejuni* (44). Among cases of campylobacteriosis, *C. jejuni* is predominant (16), and in rare instances, these infections may be followed by autoimmune neuropathies such as

Guillain-Barré syndrome and Miller Fisher syndrome (24, 47). It has been postulated that sialylated lipooligosaccharides (LOS) on the cell surface of *C. jejuni* exhibit molecular mimicry with gangliosides on peripheral nerves, resulting in the development of these autoimmune neuropathies.

Arcobacter, another genus in the family *Campylobacteraceae*, also is of importance in human health and food safety (14, 54, 58, 64, 67) and is similar to *Campylobacter* (63). Although four *Arcobacter* species have been associated with animals and humans, *Arcobacter butzleri* is the species isolated mostly from human infections (14, 64). Similar to *Campylobacter*, *Arcobacter* has been isolated more frequently from poultry than from red meats (10, 25, 45), suggesting that poultry may be a major reservoir for this pathogen. However, *Arcobacter* species grow at temperatures much lower (15 to 37°C) than those required for *Campylobacter* growth and lower than the normal avian body temperature (~42°C) (33). These observations suggest that the presence of *Arcobacter* in poultry may be due to contamination from the skin of the birds or through processing and not due to contamination from the birds' gastrointestinal contents (19, 22, 68). Given that contaminated food products are a potential source of *Campylobacter* and *Arcobacter* (14, 44), a key issue in food safety is the development of efficient detection and identification methods for these pathogens as a first step in the control of human infections.

Traditionally, food-borne pathogens have been identified by microbiological culture, followed by immunological methods (4, 38). Most methods for the isolation and identification of campylobacters in foods require enrichment culturing for several days, followed by subculturing on selective media for fur-

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

∇ Published ahead of print on 6 April 2007.

ther phenotypic identification (9). Recently, nucleic acid amplification methods have been developed for specific and efficient detection of these food-borne pathogens (41). For example, multiplex PCR assays have been used to simultaneously identify and discriminate, in a single-step reaction, *C. coli* and *C. jejuni* (6, 7, 28, 42, 46) or *Arcobacter* spp. (3, 20, 23). Although these multiplex PCR assays are more efficient than traditional culturing methods, they are limited by the number of genes that can be detected in a single reaction (4). In addition, certain contaminants that may be present in crude DNA preparations can inhibit the PCRs or can result in non-specificity (46).

The advent of whole-genome-based methods, such as DNA microarrays, offers another means to enhance the detection capabilities and to overcome the limitations of established procedures, such as culturing and PCR (4, 38). The advantage of microarray-based detection is that it can combine the amplification of nucleic acids with its massive screening capability, resulting in sensitivity, specificity, and high-throughput capacity. Previous reports have described the use of microarray-based identification of thermotolerant campylobacters (26, 27, 55, 65). However, these detection assays relied on PCR amplification of a specific region in a few target genes as a first step in pathogen detection.

In the present study, a DNA oligonucleotide array was designed to contain a comprehensive set of 70-mer oligonucleotide probes targeting genes implicated in metabolism or pathogenicity for *A. butzleri*, *C. coli*, and *C. jejuni*. This DNA oligonucleotide array allowed specific identification of *A. butzleri*, *C. coli*, and *C. jejuni* present in retail chicken samples without PCR amplification of a few target genes prior to pathogen detection, as well as genotypic classification of *C. jejuni* isolates based on LOS class. By using *C. jejuni* sequence-specific primers to label genomic DNA, *C. jejuni* was detected directly in package liquid from whole chicken carcasses or in enrichment broths.

MATERIALS AND METHODS

Bacterial reference strains and growth conditions. The bacterial reference strains that were used in this study are listed in Table 1. *C. jejuni* and *C. coli* strains were grown routinely at 42°C under microaerobic conditions (8% CO₂, 4% O₂, 80% N₂, 8% H₂) on Mueller-Hinton (Becton Dickinson Co., Sparks, MD) solid medium supplemented with 0.025% (wt/vol) FeSO₄ · 7H₂O, 0.025% (wt/vol) sodium metabisulfite (anhydrous), and 0.025% (wt/vol) sodium pyruvate (anhydrous). *A. butzleri* strains were grown optimally at 28°C under microaerobic conditions on Oxoid anaerobe basal agar (ABA) (Remel Inc., Lenexa, KS) supplemented with 5% laked horse blood (Hema Resource and Supply, Inc., Aurora, OR).

Isolation of bacteria from retail chicken samples. Ten whole carcasses of chickens that were raised either organically or conventionally were purchased from various local retailers. The liquid from each package was collected and kept at 4°C for 30 min. To promote the growth of *Campylobacter* or *Arcobacter* that may have been present in a food sample, an enrichment culture was prepared by adding 5 ml of the package liquid to 45 ml of Oxoid anaerobe basal broth (Remel Inc., Lenexa, KS) amended with 20 µg/ml amphotericin B and 10 µg/ml trimethoprim (Sigma-Aldrich, St. Louis, MO) in a sterile cell culture flask with a 0.2-µm vented cap, and the flask was incubated at 37°C with gentle shaking (30 rpm) under microaerobic conditions. The antibiotics were added at concentrations that do not inhibit the growth of campylobacteria on the selective media (32, 38a). After incubation of the enrichment broth for 24 h, bacterial isolates were recovered by plating 1 ml of the enrichment broth on ABA. The plates were incubated for 24 to 48 h at 37°C under microaerobic conditions.

A membrane filtration method was adapted (12, 36) for efficient recovery of bacterial isolates from package liquid or from enrichment broths containing

package liquid that were incubated for 6, 12, or 24 h at 37°C. A 250-µl sample of either package liquid or enrichment broth was applied in small drops to a sterile mixed cellulose ester membrane filter that was 47 mm in diameter and had a pore size of 0.65 µm (Millipore Corporation, Billerica, MA), which was placed on the surface of either ABA or Oxoid modified CCDA-Preston (Remel Inc., Lenexa, KS) plates amended with 20 µg/ml amphotericin B and 10 µg/ml trimethoprim. Samples were filtered passively for 30 min at room temperature under the ambient atmosphere. After this incubation period, the filters were removed, and the plates were incubated further for 24 to 48 h at 37°C under microaerobic conditions. The samples were not spread or streaked after the filters were removed. The recovered isolates with a *Campylobacter*-like colony morphology (pale orange colonies on ABA or light gray colonies on modified CCDA-Preston agar) were inspected visually for a very small, curved (S-shaped) single-cell morphology and corkscrew motility by phase-contrast microscopy using a Leica DMR light microscope (Leica Microsystems Inc., Bannockburn, IL).

PCR. PCR reagents were supplied by Epicenter (Madison, WI), and oligonucleotides were purchased from QIAGEN Operon (Alameda, CA) or Sigma-Genosys (The Woodlands, TX). The multiplex PCR assay for simultaneous identification of *Campylobacter* species was adapted from the assay of Klens et al. (28), with the following modifications: 10 pmol/µl forward primers complementary to the *lpxA* nucleotide sequence of *C. coli* and *C. jejuni* and 30 pmol/µl of the *lpxARRK2m* reverse primer were mixed in a 50-µl reaction mixture containing each deoxynucleoside triphosphate at a concentration of 250 µM, 1× MasterAmp *Taq* PCR buffer, 1× MasterAmp *Taq* enhancer, 2.5 mM MgCl₂, and 1 U MasterAmp *Taq* DNA polymerase. Genomic DNA (50 ng) from bacterial isolates, prepared as described below, was then added, and the reaction mixture was placed into an MJ Research thermocycler (Bio-Rad Laboratories, Hercules, CA) with the following settings: 1 min at 94°C, 45 s at 50°C, and 1 min at 72°C for 30 cycles, followed by a final extension of 5 min at 72°C. The multiplex PCR assay for the simultaneous identification of *Arcobacter* species was performed according to the methods described by Houf et al. (23). Conventional PCR for LOS gene-specific amplification was performed as described previously (48). Products for each PCR mixture were examined by agarose gel electrophoresis through 1.5% agarose, and bands were visualized with UV light after ethidium bromide staining. Positive samples were identified based on the presence of bands of anticipated sizes. Images were captured with an AlphaImager gel documentation analysis system (Alpha Innotech, San Leandro, CA).

Construction of the multipathogen oligonucleotide microarray. The 70-mer oligonucleotide probes specific for members of the *Campylobacteraceae* family (see Tables S1 and S2 in the supplemental material) were designed by using the Array Designer 3.0 software (Premier Biosoft International, Palo Alto, CA) with an average melting temperature of 73°C. For printing the DNA microarray, the oligonucleotide probes were dissolved in water to a concentration of 200 µM and were then further diluted in 0.3× saline sodium citrate (SSC)-50% dimethyl sulfoxide to a final DNA concentration of 50 µM (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Each oligonucleotide probe was spotted in triplicate on UltraGaps glass slides (Corning Inc., Corning, NY) coated with amino propyl groups, using an OmniGrid Accent robot (GeneMachines, Ann Arbor, MI) with ChipMaker microspotting pins and printhead (TeleChem International, Inc. Sunnyvale, CA). Immediately after printing, the microarrays were UV cross-linked at 600 mJ by using a GS Gene linker UV chamber (Bio-Rad Laboratories, Hercules, CA) and were stored in a desiccator until further use.

Genomic DNA isolation and labeling. Forty-five milliliters of the package liquid from a whole chicken carcass or 1 ml of an enrichment broth was centrifuged at 14,500 × *g* for 5 min. The pellet was resuspended in 1 ml of anaerobe basal broth, and a 100-µl sample was then centrifuged at 14,500 × *g* for 5 min. Genomic DNA was further isolated from the sample of package liquid, enrichment broth, or bacterial isolates by using a DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA) and following the manufacturer's specifications. Labeling of genomic DNA from the bacterial isolates, reference strains, or 24-h enrichment broth samples was performed by mixing approximately 5 µg of genomic DNA with 5 µl of 10× random octadeoxyribonucleotides (New England Biolabs, Ipswich, MA) and water to obtain a final volume of 41 µl. Labeling of genomic DNA from package liquid or from each 6-, 12-, or 24-h enrichment broth sample was performed by mixing 15 µg of genomic DNA with 2 µM each of the *C. jejuni* sequence-specific primers (see Table S3 in the supplemental material), 5 µl of 10× NEBuffer 2 (New England Biolabs), and water to obtain a final volume of 41 µl. All labeling reaction samples were heated to 95°C for 5 min, cooled for 5 min at 4°C, and then added to the remaining labeling reaction mixture consisting of 5 µl of 10× deoxynucleoside triphosphate labeling mixture (1.2 mM each of dATP, dGTP, dCTP, and 0.5 mM dTTP in 10 mM Tris [pH 8.0]-1 mM EDTA) (Promega Corporation, Madison, WI), 3 µl of Amersham CyDye fluorescent nucleotides, Cy3-dUTP or Cy5-dUTP (GE Healthcare Life

TABLE 1. Bacterial reference strains used in this study

Species	Strain	Other designation	Penner heat-stable serotype/biotype	Clinical or environmental source	Provider (reference) ^a
<i>Arcobacter butzleri</i>	RM3790	29.19		Human	A. Lastovica
	RM4018	ATCC 49616		Human	I. Wesley
	RM4477	NADC 4061		Pig	I. Wesley
	RM4481	NADC 9565		Turkey	I. Wesley
<i>Arcobacter cibarius</i>	RM5244	KH2		Chicken	K. Houf
<i>Arcobacter cryaerophilus</i>	RM1582	ATCC 43158	1A	Cow	I. Wesley
	RM1583	ATCC 49615	1B	Human	I. Wesley
	RM4603	NADC 3144	1A	Pig	I. Wesley
<i>Campylobacter coli</i>	RM1051	ATCC 43479	HS:30	Human	P. Guerry
	RM1166	T21		Chicken	D. Woodward
	RM1505	ATCC 49299	HS:61		W. Johnson (53)
	RM1896	1921		Swine	L. Stanker
	RM2228	72664	HS:34	Chicken	D. Woodward
<i>Campylobacter jejuni</i>	RM1045	ATCC 43429	HS:1	Human	P. Guerry
	RM1048	ATCC 43432	HS:4	Human	P. Guerry
	RM1050	ATCC 43449	HS:23	Human	P. Guerry
	RM1170		HS:31	Chicken	Laboratory collection
	RM1221	ATCC BAA-1062	HS:53	Chicken	Laboratory collection (15)
	RM1285		HS:19	Chicken	Laboratory collection
	RM1503	ATCC 43462	HS:43	Human	W. Johnson
	RM1862	NCTC11168	HS:2	Human	R. Meinersmann (50)
	RM1864	81-176	HS:23,36	Human	R. Meinersmann
RM3407		HS:3		D. Woodward	
<i>Campylobacter lari</i>	RM1890	ATCC 43675		Human	L. Stanker
<i>Campylobacter upsaliensis</i>	RM3195	300.94		Human	A. Lastovica
	RM3777	3.97		Human	A. Lastovica
<i>Escherichia coli</i>	RM2084	ATCC 43895	O157:H7	Raw meat	T. Whittam
<i>Salmonella enterica</i> serovar Typhimurium	RM2967	SARB65		Human	M. Brandl

^a Affiliations of providers: M. Brandl, USDA-ARS, Western Regional Research Center, Albany, CA; P. Guerry, Naval Medical Research Institute, Bethesda, MD; W. Johnson, Laboratory Centre for Disease Control, Winnipeg, Canada; K. Houf, Ghent University, Merelbeke, Belgium; A. Lastovica, University of Cape Town, Cape Town, South Africa; R. Meinersmann, USDA-ARS, R. B. Russell Agricultural Research Center, Athens, GA; L. Stanker, USDA-ARS, Western Regional Research Center, Albany, CA; I. Wesley, USDA-ARS, National Animal Disease Center, Ames, IA; T. Whittam, Michigan State University, East Lansing, MI; D. Woodward, National Microbiology Laboratory, Winnipeg, Canada.

Sciences Corp., Piscataway, NJ), and 5 U of Klenow fragment (3'→5' exo⁻) (New England Biolabs). The mixtures were incubated overnight at 37°C, as previously described (49). Genomic DNA from *C. jejuni* reference strains NCTC11168 and RM1221 were fluorescently labeled with Cy5-dUTP. Genomic DNA from bacterial isolates, from package liquid, and from each enrichment broth were fluorescently labeled with Cy3-UTP. The labeled genomic DNA was purified from unincorporated CyDye fluorescent label by using a QIAquick PCR purification kit (QIAGEN, Valencia, CA) and was eluted finally in 100 µl of 10 mM Tris (pH 8.0). The CyDye-labeled genomic DNA from the tested food sample and from the reference strains were combined and vacuum dried using a Savant ISS110 SpeedVac concentrator (Thermo Electron Corp., Milford, MA).

Microarray hybridization. The CyDye-labeled genomic DNA from the tested food sample and from the reference strains were resuspended in 20 µl of Pronto! Long Oligo/cDNA hybridization solution (Corning Inc., Corning, NY), heated to 95°C for 5 min, and immediately centrifuged at 14,500 × g for 2 min at room temperature. The hybridization mixture was then applied to each DNA microarray and sealed with a HybriSlip plastic coverslip (Sigma-Aldrich, St. Louis, MO). The microarray slide was placed in a hybridization chamber (Corning) and was incubated at 42°C for 18 h, as in previous studies (49). Following hybridization, the slides were transferred to a microarray wash station (TeleChem International, Inc., Sunnyvale, CA) and were washed twice in 2× SSC-0.1% sodium dodecyl sulfate at 42°C for 5 min, twice in 1× SSC at room temperature for 10 min, and finally twice in 0.2× SSC at room temperature for 10 min. The microarray slides were transferred to a slide-drying tray (Evergreen Scientific, Los

Angeles, CA) and were dried by centrifugation at 300 × g for 10 min prior to scanning. At least three replicate hybridization reactions were performed for each food sample. Each replicate hybridization reaction mixture was applied to a microarray on a different slide to account for the slide-to-slide variation.

Microarray data analysis. DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Molecular Devices Corporation, Sunnyvale, CA) at excitation wavelengths of 532 nm (Cy3) and 635 nm (Cy5) with a 10-µm resolution, as previously described (49). For the analysis of the DNA microarray data, the fluorescence signal intensities for each probe spotted in triplicate on each replicate microarray (a total of three replicates) were quantified after subtraction of the local background by using GenePix 4.0 software (Molecular Devices). No significant differences were observed between replicate microarrays. Probes were excluded from further analysis if they had an anomalous spot morphology or were within regions of nonspecific fluorescence. The microarray data were analyzed further with GeneSpring 7.2 software (Agilent Technologies, Santa Clara, CA) for averaging the background-corrected fluorescence signal for the probes on replicate microarrays, for fluorescence signal comparisons, or for average-linkage hierarchical clustering with the standard correlation (49). The average fluorescence signals for probes targeting a pathogen are shown in the figures below using a color scheme, where yellow corresponds to fluorescence signals of >2,000 to 3,000 U, hazel corresponds to fluorescence signals of 1,500 to 2,250 U, blue corresponds to fluorescence signals of 1,000 to 1,500 U, purple corresponds to fluorescence signals of 500 to 750 U, and red corresponds to background levels due to lack of hybridization (<200 to

300 U). Average fluorescence intensities that were at least threefold higher than the background fluorescence (areas on the microarray surrounding the probes) were considered positive signals, as in previous studies (66). A species was considered present in a food sample if positive signals were obtained for a majority of the probes (approximately 70% of the probes) targeting that pathogen.

RESULTS AND DISCUSSION

Development and validation of the DNA oligonucleotide array. To develop DNA microarray technology for the simultaneous detection of multiple pathogens from food, DNA oligonucleotide arrays were designed to contain a comprehensive set of 70-mer oligonucleotide probes specific for members of the *Campylobacteraceae* family (see Table S1 in the supplemental material). The species-specific probes were selected to target genes in *A. butzleri* (W. G. Miller, C. T. Parker, M. Rubenfield, G. L. Mendz, M. M. S. M. Wösten, D. W. Ussery, J. F. Stolz, G. Wang, J. A. Malek, A. Rogosin, L. H. Stanker, and R. E. Mandrell, submitted for publication) and in thermotolerant *C. jejuni* and *C. coli* (15, 50) that encode proteins for housekeeping functions, including biosynthetic and cellular functions, and virulence factors (see Table S1 in the supplemental material). For example, the *C. jejuni*-specific probes targeted the *ciaB*, *cadF*, and *peb2* genes involved in several aspects of bacterial virulence, such as host adherence and invasion (30, 31, 50, 52). As in previous microarray detection studies (26, 27, 65), probes for *C. jejuni* detection targeted *hipO*, a gene encoding hippuricase (37), and *cdtB*, a cytolethal distending toxin subunit gene (2, 13). The *C. coli*-specific probes were designed based on findings of a comparative genome analysis that revealed housekeeping and virulence genes in *C. coli* showing sequence similarity to genes of *C. jejuni* and other *Campylobacter* species (15). Additional probes included in this study targeted genes in *C. jejuni* and *C. coli* encoding novel hypothetical proteins that were identified in the genome sequence analysis of campylobacters to be species specific, having no match to other organisms in the database (15). The recent completion of the genome sequence of *A. butzleri* strain RM4018 provided the needed information for designing species-specific probes targeting genes in *A. butzleri* that showed sequence homology to virulence and housekeeping genes in *Campylobacter* species (Miller et al., submitted). Finally, the Ab0988, Ab2061, and Ab2302 probes were included to target *A. butzleri* genes that were identified to be unique in a pairwise comparative analysis of *A. butzleri* and characterized *Campylobacter* proteome sequences.

To confirm the specificity of the 70-mer oligonucleotide probes, genomic DNA from various reference strains (Table 1) were labeled separately with CyDye with Klenow polymerase and random primers (see Materials and Methods), a method that yields fragment sizes that hybridize efficiently to probes on the microarray (66). Results demonstrated that the fluorescence intensity values were specific for each species (Fig. 1). The fluorescence signal intensities for *C. jejuni* probes were at least 10-fold higher than the background levels when labeled genomic DNA from various *C. jejuni* reference strains was hybridized (Fig. 1). In contrast, the fluorescence signal intensities for the *C. jejuni* probes approached the background levels when genomic DNA from *C. coli* or *A. butzleri* was hybridized (Fig. 1). Fluorescence signals close to background levels were also obtained after testing of genomic DNA from other

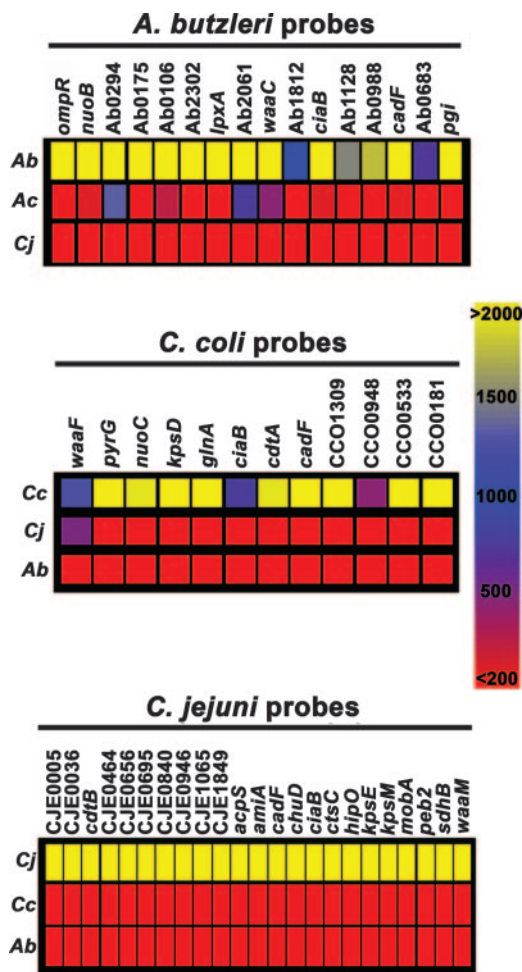


FIG. 1. Probe specificity of the DNA oligonucleotide array. Genomic DNA from *A. butzleri* (Ab), *A. cryaerophilus* (Ac), *C. coli* (Cc), and *C. jejuni* (Cj) reference strains (Table 1) was labeled separately with CyDye and hybridized to the microarray. The color bar shows the average fluorescence signal for each probe, where yellow corresponds to fluorescence signals of >2,000 U, hazel corresponds to fluorescence signals of 1,500 U, blue corresponds to fluorescence signals of 1,000 U, purple corresponds to fluorescence signals of 500 U, and red corresponds to background levels due to lack of hybridization (<200 U). Each row shows the signals for a probe set after labeling of the reference strains, indicated on the left.

Campylobacter spp., such as *Campylobacter lari* and *Campylobacter upsaliensis*, or from other food-borne pathogens, such as *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 reference strains (data not shown). Similarly, a high level of specificity was also observed for probes targeting *C. coli* (Fig. 1). Analysis of the *A. butzleri* probe specificity demonstrated that high fluorescence signal intensities were obtained when several *A. butzleri* reference strains were tested (Fig. 1). In contrast, a lack of hybridization to most *A. butzleri* probes was observed after hybridization of genomic DNA from other closely related *Arcobacter* species, such as *Arcobacter cryaerophilus* (Fig. 1) or *Arcobacter cibarius* (data not shown). Regardless of the pathogen tested, the specific signal for each probe set had a similar range of fluorescence intensity values

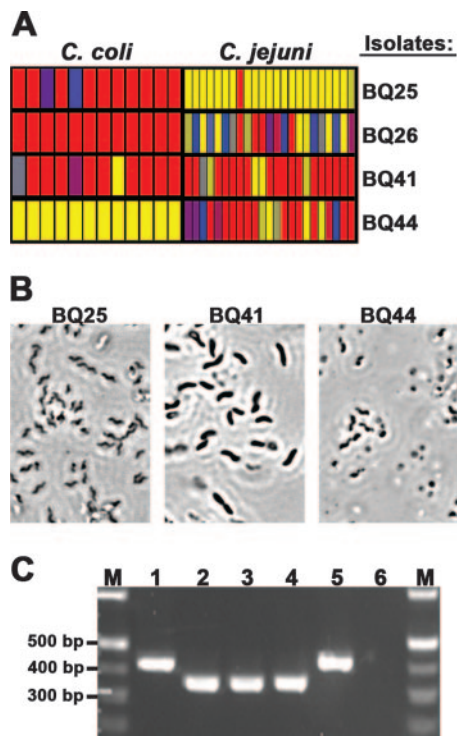


FIG. 2. Validation of the DNA oligonucleotide array for identifying pathogens in food. (A) Genomic DNA from bacterial isolates recovered from a 24-h enrichment broth was labeled with CyDye and hybridized to the microarray. The average fluorescence signal for each *C. jejuni* and *C. coli* probe is color coded, as described in the legend to Fig. 1. (B) Morphology of individual cells as assessed by phase-contrast microscopy at a magnification of $\times 1,000$. (C) Multiplex PCR assay (28) performed to corroborate the microarray data for species identification of *Campylobacter* isolates from retail chicken samples. Lanes M, 100-bp DNA ladder; lane 1, *C. coli* strain RM1166; lane 2, *C. jejuni* strain RM1221; lane 3, isolate BQ25; lane 4, isolate BQ26; lane 5, isolate BQ44; lane 6, isolate BQ41.

that were at least 10-fold higher than the local background levels.

To validate this DNA microarray for the identification of pathogens from food, retail chickens were tested for the presence of *Campylobacter* spp. The package liquid from whole chicken carcasses was collected, added to an enrichment broth, and incubated for 24 h at 37°C (see Materials and Methods). Samples of the enrichment broth were plated on selective media, and the plates were incubated for an additional 48 h. Suspect bacterial colonies were selected, and their genomic DNA was fluorescently labeled and hybridized to the DNA microarray. All probes specific for either *C. jejuni* or *C. coli* yielded significant fluorescence, and the values were at least 10-fold higher than the background levels for isolates BQ25 and BQ44, respectively (Fig. 2A). Although analysis of isolate BQ26 showed that 7/23 of the *C. jejuni* probes (30%) had values that were close to the background values, a large proportion of the probes (70%) had significant fluorescence values. The average fluorescence signal values for 10/23 of the probes were 7.5- to 10-fold higher than the background values, while 6/23 of the probes had average fluorescence values that were 5-fold higher than the background values (Fig. 2A). In

contrast, microarray analysis of isolate BQ41 resulted in no significant fluorescence values for most probes targeting either *C. jejuni* or *C. coli* (Fig. 2A). A lack of hybridization to all of the *A. butzleri* probes was observed when the isolates were analyzed (data not shown). The morphology of individual cells from these bacterial isolates was further assessed by phase-contrast microscopy (Fig. 2B). The cells of isolates BQ25 and BQ44, *Campylobacter*-positive isolates as determined by DNA microarray analysis, were small and spiral shaped (Fig. 2B), a typical single-cell morphology for *Campylobacter* species (62). A similar single-cell morphology was also observed for isolate BQ26 (data not shown). In contrast, isolate BQ41, a *Campylobacter*-negative isolate as determined by DNA microarray analysis, had a different single-cell morphology; the cells were longer and rod shaped (Fig. 2B). Finally, a multiplex PCR assay was conducted to confirm the species of poultry isolates identified by microarray analysis (Fig. 2C). As part of this PCR assay, *C. coli* and *C. jejuni* were discriminated by amplifying the *lpxA* gene using a common primer, LpxAKK2Rm, and species-specific primers (28). Similar to the results obtained with our DNA microarrays, the multiplex PCR assay identified isolates BQ25 and BQ26 as *C. jejuni* since amplification of *lpxA* from these isolates yielded a 330-bp fragment that comigrated with the fragment amplified from the reference strain *C. jejuni* RM1221 (Fig. 2C). Amplification of the isolate BQ44 sequence resulted in a 390-bp fragment that comigrated with the fragment amplified from reference strain *C. coli* RM1166, consistent with the results obtained with DNA microarrays. These results demonstrate that the DNA microarray was highly specific for these species and allowed accurate identification of bacterial isolates from food.

Identification of bacterial isolates from retail chicken samples. To better assess the presence of campylobacteria and related species that could be isolated from retail chicken samples, an efficient isolation protocol (see Materials and Methods) that employs a combination of both membrane filtration and selective media was adapted (12, 36). Samples of either package liquid from whole chicken carcasses or enrichment broths that were incubated for various times were applied to a membrane filter. Small motile bacteria, like most campylobacteria and arcobacteria, would be expected to pass through the membrane filter and be recovered on the selective growth medium after incubation under microaerobic conditions. By performing this isolation procedure, single colonies with phenotypic characteristics of campylobacter colonies were recovered directly from a package liquid sample prior to enrichment, and the estimated yield was approximately 4 to 10 CFU/ml (data not shown). Yields ranging from 12 to 28 CFU/ml were obtained from an enrichment broth that was incubated for 12 h, and similar results were obtained after incubation for 24 h (data not shown). Microarray analysis demonstrated that 80% of the bacterial isolates examined were *C. jejuni*. This conclusion was based on hybridization results indicating significant fluorescence values for all probes specific for this pathogen (Fig. 3). In addition, the microarray analysis demonstrated that three isolates, BQ61, BQ68, and BQ123, were *A. butzleri* since their hybridization signals resulted in significant fluorescence values for 80% of the *A. butzleri* probes, which targeted conserved genes encoding virulence factors and housekeeping functions, such as *lpxA*, *nuoB*, *cadF*,

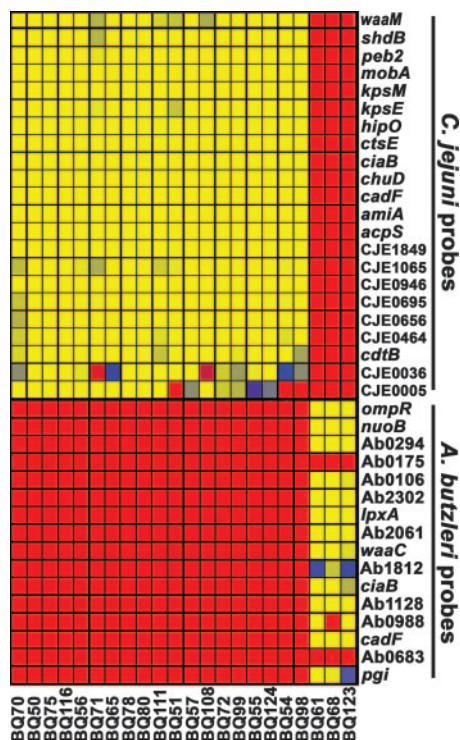


FIG. 3. Identification of bacterial isolates from retail chicken samples recovered by membrane filtration. Genomic DNA from bacterial isolates recovered from retail chicken samples by membrane filtration (see Materials and Methods) was CyDye labeled and hybridized to the microarray. The average fluorescence signals for either *C. jejuni* or *A. butzleri* probes are color coded, where yellow corresponds to fluorescence signals of >3,000 U, hazel corresponds to fluorescence signals of 2,250 U, blue corresponds to fluorescence signals of 1,500 U, purple corresponds to fluorescence signals of 750 U, and red corresponds to background levels due to a lack of hybridization (<300 U). The bacterial isolates are indicated at the bottom.

ciaB, and *waaC*. Although all of the probes were specific for the *A. butzleri* reference strains (Fig. 1), the lack of hybridization with some probes (Ab0175, Ab0988, and Ab0683) suggests that these genes are highly divergent or absent in these *A. butzleri* poultry isolates compared to the reference strains (Fig. 3). To confirm that these *Arcobacter* isolates from retail chicken samples were *A. butzleri*, a multiplex PCR assay that allows simultaneous identification of *Arcobacter* spp. was performed (23). As shown in Fig. 4, the multiplex PCR assay identified isolates BQ61, BQ68, and BQ123 all as *A. butzleri*; amplification with the primer set specific for *A. butzleri* resulted in a 400-bp fragment that comigrated with the fragment amplified from the reference strain *A. butzleri* RM4018. Only *A. cryaerophilus* strain RM1582 amplified a 260-bp fragment with the primer set specific for *A. cryaerophilus* (Fig. 4), an *Arcobacter* species that has been isolated previously from chicken carcasses (1, 21, 25). Isolates BQ61, BQ68, and BQ123 were analyzed further by matrix-assisted laser desorption ionization—time of flight mass spectrometry, an established technique for pathogen identification that detects ribosomal and highly expressed cytosolic proteins (40, 43). The matrix-assisted laser desorption ionization—time of flight mass spectra obtained for *A. butzleri* isolates BQ61, BQ68, and BQ123 were



FIG. 4. Multiplex PCR for species identification of *Arcobacter* isolates. A multiplex PCR assay (23) was performed to validate the microarray data for species identification of *Arcobacter* isolates from retail chicken samples as determined by microarray analysis. Lanes M, 100-bp DNA ladder; lane 1, *A. butzleri* strain RM4018; lane 2, *A. cryaerophilus* strain RM1582; lane 3, isolate BQ61; lane 4, isolate BQ68; lane 5, isolate BQ123.

similar to the spectrum obtained for the reference strain *A. butzleri* RM4018, consistent with the species identification results obtained by microarray and PCR analyses (data not shown). No *C. coli* isolates were identified in this DNA microarray analysis. Possibly, *C. coli* was present in these samples but at much lower concentrations than *C. jejuni*; therefore, an increased number of isolates had to be examined to determine the presence of *C. coli*.

Genotypic classification of *C. jejuni* isolates based on their LOS classes. The *C. jejuni* isolates that were recovered from either package liquid or enrichment broth were classified further using a subset of probes that target the LOS biosynthesis locus, one of the most diverse regions of the *C. jejuni* genome, as demonstrated in previous DNA microarray studies (11, 34, 35, 49, 51, 60). In the present study, probes were designed to target six LOS classes (see Table S2 in the supplemental material) that were shown previously to be represented among *C. jejuni* strains from various sources (48). The specificity of the probes targeting the LOS biosynthesis locus in *C. jejuni* was determined (Fig. 5). Fluorescence signal intensities that were at least 10-fold higher than the background levels were observed for most probes targeting a particular LOS class when reference strains representative of each LOS class were examined. Microarray analysis of *C. jejuni* strains RM1048 and RM1864, strains known to possess class A and B LOS, respectively, hybridized to probes targeting both classes. Only strain RM1864 showed significant fluorescence signals for *cgtAIII*, a probe targeting only class B (Fig. 5). A high level of specificity was also observed when probes targeting LOS class C were tested with reference strain RM1862 (class C) or when a LOS class F probe targeting CJE1279 was tested with reference strain RM1170 (class F). *C. jejuni* strain RM3407, a class H reference strain, hybridized to the class H-specific probe orf39h and to probes targeting both LOS class E and H loci but failed to hybridize to orf28e and orf26e, probes targeting only class E loci (Fig. 5).

To identify differences among *C. jejuni* poultry isolates, the gene content of the LOS biosynthesis locus, a region that contributes to the *C. jejuni* genome diversity, was further examined. As shown in Fig. 5, the microarray analysis demonstrated that *C. jejuni* isolates with the same LOS class clustered together with a distinctive pattern, and most probes for each assigned LOS class had fluorescence signal intensities that were at least 10-fold higher than the background levels. Genomic DNA from seven *C. jejuni* isolates (BQ70, BQ71, BQ50, BQ56, BQ78, BQ108, and BQ65) hybridized to probes

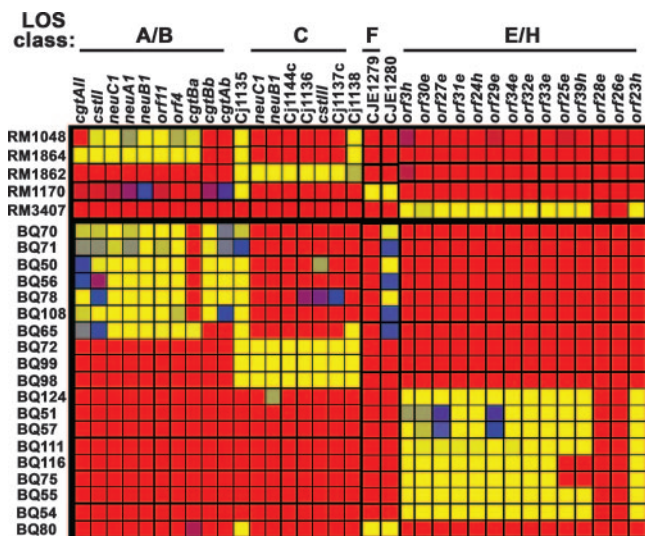


FIG. 5. Genotypic classification of *C. jejuni* isolates based on their LOS classes. Genomic DNA from *C. jejuni* reference strains and from *C. jejuni* isolates recovered from retail chicken samples by membrane filtration (see Materials and Methods) was labeled with CyDye and hybridized to the microarray. The average fluorescence signals for probes targeting the various LOS classes in *C. jejuni* are color coded, as described in the legend to Fig. 3. The reference strains (designations beginning with RM) and bacterial isolates (designations beginning with BQ) are indicated on the left.

specific for LOS classes A and B. The fact that these isolates showed a positive signal for the *cgtAII* probe, a probe that is specific for only class B, indicates that all of these isolates may possess class B LOS (Fig. 5). Furthermore, isolates BQ70, BQ71, BQ50, BQ56, BQ78, and BQ108 also showed a positive signal for the *cgtAb* and *cgtBb* probes, which suggests that these isolates belong to subclass B2, a classification described previously (48). In contrast, *C. jejuni* isolate BQ65 hybridized to the *cgtBa* probe and not to the subclass B2 probes; therefore, this isolate may possess subclass B1 LOS. Another larger group of isolates (BQ124, BQ51, BQ57, BQ111, BQ116, BQ75, BQ55, and BQ54) showed significant fluorescence signals for probes specific for LOS classes E and H. However, the lack of hybridization to probes targeting *orf26e* and *orf28e* indicates that most of the isolates in this group may possess class H LOS. Further analysis of two isolates in this group (BQ75 and BQ116) showed that there was a lack of hybridization to probes targeting *orf25e* and *orf39h*, suggesting that these *C. jejuni* isolates may possess a new class of LOS that may be derived from class H (Fig. 5). Analysis of another small group of *C. jejuni* isolates (BQ72, BQ99, and BQ98) resulted in significant fluorescence signals for all probes specific for LOS class C, while only *C. jejuni* isolate BQ80 hybridized to the probe targeting the LOS class F gene, CJE1279 (Fig. 5). It should be noted that isolates with class B, C, or F LOS hybridized to probe Cj1135, while isolates with class B or F LOS hybridized to probe CJE1280 (Fig. 5; see Table S2 in the supplemental material). To verify the LOS class assignment by this DNA microarray analysis, PCR amplification of genes within the presumed LOS class was performed. Using LOS class-specific primer pairs (48), this PCR assay demonstrated that all *C. jejuni* isolates that were examined had patterns that

TABLE 2. PCR analysis of LOS classes for *C. jejuni* isolates from retail chicken samples

Isolate	Putative LOS class ^a	Specific amplification of open reading frame:					
		<i>cgtAII</i>	<i>cstII</i>	<i>cstIII</i>	CJE1279	<i>orf25e</i>	<i>orf27e</i>
BQ50	B2	+	+	-	-	-	-
BQ51	H	-	-	-	-	+	+
BQ54	H	-	-	-	-	+	+
BQ55	H	-	-	-	-	+	+
BQ56	B2	+	+	-	-	-	-
BQ57	H	-	-	-	-	+	+
BQ65	B1	+	+	-	-	-	-
BQ70	B2	+	+	-	-	-	-
BQ71	B2	+	+	-	-	-	-
BQ72	C	-	-	+	-	-	-
BQ75	Unk	-	-	-	-	-	+
BQ78	B2	+	+	-	-	-	-
BQ80	F	-	-	-	+	-	-
BQ98	C	-	-	+	-	-	-
BQ99	C	-	-	+	-	-	-
BQ108	B2	+	+	-	-	-	-
BQ111	H	-	-	-	-	+	+
BQ116	Unk	-	-	-	-	-	+
BQ124	H	-	-	-	-	+	+

^a Unk, unknown.

were consistent with their class designations (Table 2), correlating with the results obtained with DNA microarrays. Therefore, the pathogen-specific DNA oligonucleotide array proved to be effective for genotypic classification of *C. jejuni* isolates from retail chicken samples.

Efficient identification of *C. jejuni* in package liquid from retail chicken samples. Given that *C. jejuni* is the most common *Campylobacter* species contributing to food-borne human illness and is associated prominently with poultry (44, 57), the sensitivity of our DNA microarray for detection of this species was assessed. Genomic DNA was isolated from *C. jejuni* strain NCTC11168 at various cell concentrations, CyDye labeled with Klenow polymerase and random primers, and hybridized to the microarray (Fig. 6). The results demonstrated that high fluorescence values were obtained for samples containing between 10⁴ and 10⁶ cells with a quantified signal for all *C. jejuni* probes that was at least 10-fold higher than the background levels

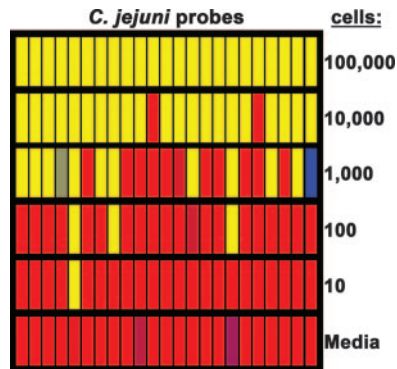


FIG. 6. Threshold sensitivity of the DNA oligonucleotide array. Genomic DNA from various concentrations of *C. jejuni* strain NCTC11168 cells were isolated, CyDye labeled, and hybridized to the microarray. The average fluorescence signal for each *C. jejuni* probe is color coded, as described in the legend to Fig. 1.

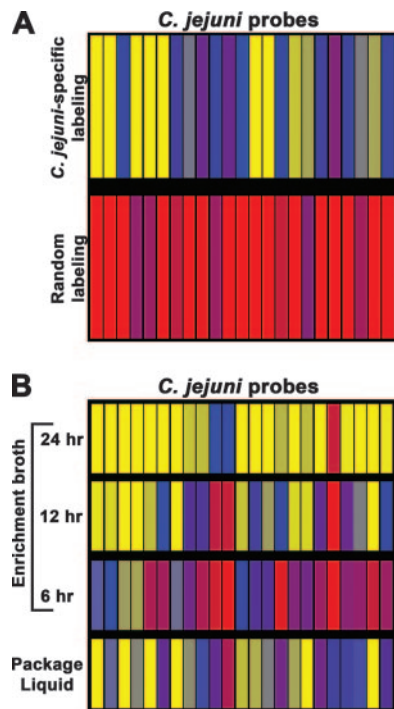


FIG. 7. Detection of *C. jejuni* directly in package liquid from retail chicken samples before and after enrichment. (A) Total genomic DNA (5 μ g) from a 24-h enrichment broth was CyDye labeled with either random primers or *C. jejuni* sequence-specific primers (see Materials and Methods). (B) Total genomic DNA (15 μ g) from package liquid from whole chicken carcasses or from enrichment broths after various incubation times was CyDye labeled with *C. jejuni* sequence-specific primers (see Table S3 in the supplemental material). The CyDye-labeled samples were further hybridized to the microarray. The average fluorescence signal for each *C. jejuni* probe is color coded, as described in the legend to Fig. 1.

(Fig. 6). The fluorescence intensity for the *C. jejuni* probes decreased with a decrease in cell concentration. In samples containing 1,000 cells, approximately 50% of the probes had fluorescence values that were 5- to 10-fold higher than the background values, but only 10% of the probes were significantly fluorescent after analysis of samples representing 100 to 1,000 cells (Fig. 6). No *C. jejuni*-specific signal was detected after microarray analysis of samples with less than 100 cells (Fig. 6). A similar sensitivity threshold was also observed with *C. jejuni* strain RM1221 or after labeling of genomic DNA with Klenow polymerase using *C. jejuni* sequence-specific primers (see Table S3 in the supplementary material) (data not shown). Thus, the detection limit of this DNA microarray for *C. jejuni* was estimated to be approximately 10,000 cells, a cell concentration that is within the range of *C. jejuni*'s infective dose, estimated to be between 500 and 10,000 cells (57).

The feasibility of using our DNA microarrays for the identification of bacterial pathogens in a complex food sample was then examined (Fig. 7). Genomic DNA was isolated from mixed microbial communities from a 24-h enrichment broth and was fluorescently labeled by using Klenow polymerase with random primers. *C. jejuni* was not detected in the 24-h enrichment broth sample; the fluorescence signal intensities that corresponded to *C. jejuni* were the same as the background

levels for most spots (Fig. 7A, bottom panel). However, a traditional culture method confirmed the presence of *C. jejuni* in the sample (data not shown). Since in the microbiota of poultry, other eubacteria, including *Lactobacillus* and *Streptococcus* (39, 69), that may interfere with the detection of *Campylobacter* spp. may be abundant, genomic DNA was labeled with CyDye by using *C. jejuni* sequence-specific primers (see Table S3 in the supplemental material). Interestingly, *C. jejuni*-specific labeling of genomic DNA resulted in identification of this pathogen in this sample (Fig. 7A, top panel). The average fluorescence signals for 8/23 of the *C. jejuni* probes (35%) were 5-fold higher than the background levels, and the average fluorescence signals for 12/23 of the probes (52%) were 7.5- to 10-fold higher than the background levels. Only 3/23 of the probes (13%) had values that approached background levels (Fig. 7A). Fluorescence values that were more than twofold greater than background levels were not detected in spots targeting other pathogens, such as *C. coli* or *A. butzleri* (data not shown). Therefore, these results demonstrate that the *C. jejuni*-specific labeling of total genomic DNA from a 24-h enrichment broth allowed detection of *C. jejuni* in this complex sample.

To assess further whether sequence-specific labeling can detect *C. jejuni* in a food sample without enrichment, total genomic DNA isolated from the microbiota in the package liquid from whole chicken carcasses was labeled and hybridized to the microarray (Fig. 7B). The results of this analysis demonstrated that *C. jejuni* was detectable directly in the poultry package liquid without enrichment (Fig. 7B). The fluorescence signal intensities for 12/23 of the *C. jejuni* probes (52%) were 7.5- to 10-fold higher than the background levels, while the values for 5/23 of the probes (22%) were 5-fold higher than the background levels (Fig. 7B). In contrast, the values for only 6/26 of the probes (26%) approached the background levels (Fig. 7B). Given that 74% of the *C. jejuni* probes had fluorescence values that were more than fivefold higher than the background levels, these results demonstrate that the *C. jejuni*-specific labeling of total genomic DNA from the package liquid allowed detection of *C. jejuni* in this complex sample. As expected, higher *C. jejuni*-specific signal intensities were obtained with increasing times of incubation of the enrichment broth. Less than 10% of the *C. jejuni* spots had significant fluorescence after a 6-h incubation, which resulted in a decrease in detection compared to package liquid without enrichment (Fig. 7B). A possible explanation for the reduced *C. jejuni*-specific hybridization after the 6-h enrichment is the 10-fold dilution of the package liquid in the enrichment broth. When the incubation time for enrichment was increased, at least 70% of the *C. jejuni* spots with fluorescence values that were 5- and 10-fold higher than the background levels were observed for the 12- and 24-h enrichment broth samples, respectively (Fig. 7B). Greater recovery of *C. jejuni* isolates on selective media was obtained with increasing times of incubation, demonstrating that the enrichment broth supported the growth of *C. jejuni* (data not shown). Thus, *C. jejuni*-specific labeling of genomic DNA increased the detection sensitivity of the microarray. Specific identification of *C. jejuni* present in a poultry package liquid sample was possible without an enrichment step.

Conclusions. The present study demonstrated the use of DNA oligonucleotide arrays for the identification of members

of the *Campylobacteraceae* family, specifically *A. butzleri*, *C. coli*, and *C. jejuni*. In the genus *Campylobacter*, *C. jejuni* is the primary cause of human gastrointestinal disease (16, 56, 57, 61). Although less prevalent, *C. coli* is also an important food-borne pathogen, accounting for a significant number of illnesses (61). Due to recent improvements in isolation techniques, *Arcobacter* infections of humans have been recognized to be important in human health and food safety (14, 54, 58, 64, 67). Epidemiological studies demonstrated that *A. butzleri* was the *Arcobacter* species most frequently associated with a persistent and watery diarrhea (64). The potential sources of *Campylobacter* and *Arcobacter* infections include contaminated food products (14, 44); therefore, a major concern in food safety is the development of efficient methods for pathogen detection, identification, and genotypic classification. PCR-based methods, such as multiplex PCR, have been developed to identify and discriminate these pathogens; however, two separate assays have to be performed to identify either *A. butzleri* (3, 20, 23) or *C. jejuni* and *C. coli* (6, 7, 28, 42, 46). The present study demonstrated that in a single assay DNA microarrays can simultaneously detect three species of the *Campylobacteraceae* family, *C. jejuni*, *C. coli*, and *A. butzleri*. Here we also provide the first evidence for the use of DNA microarrays for the detection of *A. butzleri* in food samples.

Results of validation experiments demonstrated that the 70-mer oligonucleotide probes on our DNA microarray were highly specific for each species. The signal intensities corresponding to each pathogen were at least 10-fold higher than the background levels. Previous reports on identification of campylobacters by DNA microarrays indicated that PCR amplification of a few target genes was performed as the first step in the detection of these pathogens (26, 27, 55, 65). The present study was the first study to demonstrate the use of DNA microarrays for identification of *A. butzleri*, *C. coli*, and *C. jejuni* without a PCR amplification step to examine a larger subset of genes, allowing further analysis of the genomic content and diversity of these pathogens.

By adapting a membrane filtration method that was developed initially for the efficient isolation of campylobacteria from human stool samples (12, 32), presumptive campylobacteria were successfully isolated from all retail chicken samples. This method also employed selective media with antibiotics at concentrations shown previously to support the growth of *Campylobacter* spp. (38a). Subsequent identification of these chicken isolates by our DNA microarray revealed that at least some *C. jejuni* isolates could be recovered from package liquid from whole chicken carcasses without enrichment. Nevertheless, increasing the time of enrichment resulted in more efficient recovery of *C. jejuni* and also resulted in the isolation of *A. butzleri*.

Validation experiments demonstrated that our DNA microarray had a detection sensitivity threshold of approximately 10,000 cells, which is within the range of *C. jejuni*'s infectious dose, when genomic DNA from *C. jejuni* was labeled using Klenow polymerase with either random or *C. jejuni* sequence-specific primers. However, the use of random primers did not allow direct detection of low concentrations of *C. jejuni* in a 24-h enrichment broth sample, possibly due to the presence of other more prominent bacterial species that are part of the normal flora of chickens (39, 69). Interestingly, *C. jejuni* se-

quence-specific primers were adequate for labeling *C. jejuni* genomic DNA in a complex sample, thus increasing the sensitivity of the DNA microarray for detection of this pathogen. *C. jejuni* was detected efficiently both in package liquid from whole chicken carcasses and in enrichment broths.

An additional subset of probes was included in the microarray to target the LOS biosynthesis region, a region that contributes to the *C. jejuni* genome diversity (11, 34, 35, 49, 51, 60). The information obtained allowed us to further classify the gene content, to identify differences among the *C. jejuni* poultry isolates, and, potentially, to determine contamination sources. It is noteworthy that a large percentage (53%) of the *C. jejuni* isolates that were examined possessed either class B LOS or class C LOS, classes known for their sialylated ganglioside mimics (17). Sialylated LOS on the cell surface of *C. jejuni* are thought to exhibit molecular mimicry with gangliosides on peripheral nerves, resulting in the development of autoimmune neuropathies, such as Guillain-Barré syndrome and Miller Fisher syndrome (24, 47), and *C. jejuni* infections are considered to be the most frequent antecedent to the development of these neuropathies (24, 47). The finding that the isolates possessed class B LOS is of some importance to food safety and human health since *C. jejuni* strains recovered from Miller Fisher syndrome patients all possessed class B LOS (18, 29). Future work will be aimed at further expanding the probes represented on the microarray to target additional hypervariable regions in *C. jejuni* (11, 34, 35, 49, 51, 60), such as capsule biosynthesis and flagellar modification regions, to better classify *C. jejuni* isolates. Additionally, the DNA microarray will be developed for identification of other members of the *Campylobacteraceae* family, in particular emerging campylobacteria and arcobacteria, and other food-borne pathogens belonging to different genera that are of significant importance to human health to assess the prevalence of these species in either food or environmental samples.

ACKNOWLEDGMENTS

This work was supported by United States Department of Agriculture Agricultural Research Service CRIS project 5325-42000-041 and was part of a United States collaboration with the European Commission Fifth Framework Project QLK1-CT-2002-0220 "CAMPYCHECK."

We thank Sharon T. Horn, Anna H. Bates, Rommel D. Alfonso, and Bradley Gibbs for technical assistance and Magalie R. Guilhabert for critical reading of the manuscript. We especially thank Clifton K. Fagerquist, Leslie A. Harden, and Brandon R. Garbus for the matrix-assisted laser desorption ionization—time of flight data analysis.

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