JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2003, p. 4071–4080 Vol. 41, No. 9
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.9.4071–4080.2003

Microarray-Based Identification of Thermophilic *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*

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Received 9 December 2002/Returned for modification 3 April 2003/Accepted 25 June 2003

DNA microarrays are an excellent potential tool for clinical microbiology, since this technology allows relatively rapid identification and characterization of microbial and viral pathogens. In the present study, an oligonucleotide microarray was developed and used for the analysis of thermophilic *Campylobacter* spp., the primary food-borne pathogen in the United States. We analyzed four *Campylobacter* species: *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*. Our assay relies on the PCR amplification of specific regions in five target genes (*fur*, *glyA*, *cdtABC*, *ceuB-C*, and *fliY*) as a first step, followed by microarray-based analysis of amplified DNAs. Alleles of two genes, *fur* and *glyA*, which are found in all tested thermophilic *Campylobacter* spp., were used for identification and discrimination among four bacterial species, the *ceuB-C* gene was used for discrimination between *C. jejuni* and *C. coli*, and the *fliY* and *cdt* genes were used as additional genetic markers specific either for *C. upsaliensis* and *C. lari* or for *C. jejuni*. The array was developed and validated by using 51 previously characterized *Campylobacter* isolates. All isolates were unambiguously identified on the basis of hybridization patterns with 72 individual species-specific oligoprobes. Microarray identification of *C. jejuni* and *C. coli* was confirmed by PCR amplification of other genes used for identification (*hipO* and *ask*). Our results demonstrate that oligonucleotide microarrays are suitable for rapid and accurate simultaneous differentiation among *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*.

Campylobacter is one of the leading causes of bacterial food-borne diarrheal disease throughout the world (2). Campylobacteriosis is estimated to affect over 2.4 million persons every year in the United States. Although *Campylobacter* does not commonly cause death, the available data suggest that ca. 100 persons with *Campylobacter* infections die each year (19). The genus *Campylobacter* comprises 16 closely related species and 6 subspecies of gram-negative bacteria that primarily colonize the gastrointestinal tracts of a wide variety of host species. Epidemiological data show that the most significant food-borne *Campylobacter* pathogen species is *Campylobacter jejuni* (19).

Conventional methods for detecting and discriminating between *Campylobacter* species are tedious and time-consuming procedures. In addition, some of these assays may yield inconsistent results associated with the genetic divergence among the strains of one species and the presence of closely related genes in other *Campylobacter* species (29, 33). In recent years, numerous molecular diagnostic approaches for detecting and analyzing *Campylobacter* spp. have been developed, including various PCR-based assays (3, 7, 8, 10–12, 14–17, 20–23, 25–27, 29–32, 34, 35, 37–39). These PCR methods have several advantages. In general, they are faster and have higher sensitivity and specificity. However, as with biochemical tests, genetic variability among the isolates of *Campylobacter* species, which has been demonstrated previously (9, 18, 28), can reduce the confidence of bacterial identification by using PCR (24, 29, 33).

In previous studies, we demonstrated that oligonucleotide arrays can be used to characterize *Shigella* spp. and *Escherichia coli* (4) virulence genes involved in bacterial pathogenesis and to identify *Listeria* species (36) and clinically relevant rotavirus G genotypes (5). In the present study, an array containing species-specific oligonucleotide probes for four clinically relevant *Campylobacter* species (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) was developed by using specific regions of five genes (*fur*, *glyA*, *cdt*, *ceuB-C*, and *fliY*). The array readily distinguishes among all four species.

MATERIALS AND METHODS

Bacterial strains. *C. upsalienesis* strains were the generous gift of B. Swaminathan and P. Fields of the National *Salmonella* and *Campylobacter* Reference Laboratories, Centers for Disease Control and Prevention, Atlanta, Ga. Other strains were obtained from R. Thunberg and T. Tran of the Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration (FDA), College Park, Md. Bacterial cultures were grown on brain heart infusion plates (Difco, Detroit, Mich.) under microaerophilic conditions. The bacterial strains used in the present study were as follows.

***C. coli*.** The *C. coli* strains tested were ATCC 33559 (from porcine feces), ATCC 43473 (from human feces), ATCC 43474 (from human feces), ATCC 43475 (from porcine feces), ATCC 43476 (from sheep feces), ATCC 43481 (from turkey feces), ATCC 49941, ATCC 43480 (from porcine feces), ATCC 43478 (from marmoset feces), ATCC 43485 (from human feces), ATCC 43486, and the clinical isolates 3116, 3117, 5100, 6925, 92B4QA, HB37, 7569, 1420, and USDA11.

***C. jejuni*.** The *C. jejuni* strains tested were ATCC 33291 (from human feces), ATCC 35919 (from human feces), ATCC 29428 (from human feces), ATCC 35921 (from human feces), ATCC 35922 (from human feces), ATCC 33560 (from bovine feces), ATCC 43435 (from human feces), ATCC 35918 (from aborted ovine fetus), ATCC 33252 (from human blood), and the clinical isolates DENVER-1, CDC1420, GH18401, GH7493, DENVER-2, and OYSTER-BAY.

***C. lari*.** The *C. lari* strains tested were ATCC 35222 (from dog feces), ATCC 35223 (from child with mild diarrhea), ATCC 35221 (from Herring gull cloacal swab), ATCC 43675 (from human feces), and the clinical isolates 3125, 4899, 4902, 4903, 4906, 4907, and BT9.

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TABLE 1. Primers used for amplification of various *Campylobacter* genes

Target gene	Primer	Nucleotide sequence (5′-3′)	GenBank accession no.	PCR product size(s) (bp)	T_m (°C)	Source or reference
glyA	S1	AAYAAATAMGCWGAAGGWTAT	X53816, AF136501, AF136497, AF136496, AF136495, AF136494, AF136493	640	43–47	1
	S2	TAATACGACTCACTATAGGGATGCATYAAWGGWCCWCCTGG			52–54	
ceuB-ceuC	ceuBCF	ATGCTTTAAATTATATACAAAATATCC	X88849, AL139078, NC_002163	1,229	48	This study
	ceuBCR	TAATACGACTCACTATAGGGTAAGTGATATTTACTACTAAAAGTCC			50	
fur	CmpfurF	AGAAAGCTTATATATGGAAATCAAACAAG	NC_002163, L77075	362, 366, 367, and 370[a]	53	This study
	CmpfurR	TAATACGACTCACTATAGGGAATTYTTTGYTGCTCTAAAATATTATCAAAC			54	
cdt cluster	CmpToxF	CAAATATTTGAAAAAGGGTCATAAATTTGTTGC	AL139074	2,870	56	This study
	CmpToxR	TAATACGACTCACTATAGGGTTTCCATGATAGCGATCATAAAAGACAAGC			58	
hipO	VS-15	GAATGAAATTTTAGAATGGGG	AL139078, X71603	358	47	7
	VS-16	GATATCTATGATTTTATCCTGC			47	
hipO	HIP-F	GAAGAGGGTTTGGGTGGTG	AL139076, Z36940	735	53	7
	HIP-R	AGCTAGCTTCGCATAATAACTTG			52	
ask	CC18-F	GGTATGATTTCTACAAAGCGAG	AF017758	500	51	7
	CC519-R	ATAAAAGACTATCGTCGCGTG			50	

[a] The sizes of the PCR amplicons for *C. lari*, *C. coli*, *C. upsaliensis*, and *C. jejuni*, respectively.

TABLE 2. Oligonucleotide probes for detection and discrimination among *Campylobacter* spp.

Type	Name	Sequence	Length (in nucleotides)	G + C (%)	T_m[a] (°C)
fur specific	FurCJ1	GTAACTTCCATTTCTTTTGG	20	35	46
	FurCJ2	TGGTTCAGCAGGTAAAAAA	19	37	45
	FurCJ3	GCAAAAGAACATGGTTTTAAA	21	29	45
	FurCJ4	GAACCTGATTTAAATGTAGGA	21	33	47
	FurCJ5	GTGTTTGTGGTGATTGTAATAA	22	32	47
	FurCJ6	CAGGGCATTTGATGCAGC	18	56	50
	FurCC1	GTGTGTGTAATAATTGTAATCA	22	27	46
	FurCC2	TTGCTAAAGAGCATGGATTTA	21	33	47
	FurCC3	ATTGCTACGGTTTATAG	17	35	40
	FurCC4	AAAGAGCATGGATTTAA	17	29	37
	FurCC5	TGTGTGTAATAATTGTAA	18	22	37
	FurCC6	TTCGGCTGGAAAAAAAT	17	35	40
	FurCL1	AAAGTTGCGGCGATATTGTA	20	40	48
	FurCL2	TGCTTCAGGGAAGAAATTTG	20	40	48
	FurCL3	TTATCGAACAACAACAAATGTTA	23	26	46
	FurCL4	GCACAATGCAGTAAAAAATAAGG	23	35	50
	FurCL5	AACAAATGTTAATCGCAAAAGAATATAA	28	21	50
	FurCL6	ATGCAGTAAAAAATAAGGTATGTTTA	26	23	49
	FurCU1	GATTTGCAAAGTCTGTGGAAAA	22	36	49
	FurCU2	AATGAGCATCATTTTAAACTCAC	23	30	48
	FurCU3	TATGGAATTTGTAGCGATTGCAA	23	35	50
	FurCU4	AGCGATTGCAATCATAAAACAAAG	24	33	51
	FurCU5	ACGAACTTTCAAACAAGCCTCA	22	41	51
	FurCU6	TCAAACAAGCCTCACCACGACC	22	55	57
glyA specific	GlyCJ1	AGATTGAAACTCTAGCTATTGAA	23	30	48
	GlyCJ2	TTATGCGGCTTTGATTAATCCAGGT	25	40	54
	GlyCJ3	TGTACGAAAGTTGTTTTTACGGCGTAG	27	41	57
	GlyCJ4	AAATTGCTAAAAAAGAAAAACCAAAACTT	29	21	50
	GlyCJ5	GAAATTGCTAATGAAATAGGTGCCTAT	27	33	54
	GlyCJ6	TTTACGGCGTAGAACTTG	18	44	46
	GlyCC1	AGATGAAATCGAAAATTTAGCTATAG	26	27	50
	GlyCC2	GTTTATGCTGCACTTTTAAATCCAG	25	36	53
	GlyCC3	AGCTCTACAACCCACAAAACC	21	48	52
	GlyCC4	GCGGTATCATCATGACTAATGA	22	41	51
	GlyCC5	CGGATGAAGTTGGAGCTTAT	20	45	50
	GlyCC6	GATGGAAGGATAAACTATGAA	21	33	47
	GlyCL1	AAGGTGTGTATATGGCATTGTTAAAT	36	31	52
	GlyCL2	TGAAACGATTGCTATAGAAAGA	22	32	47
	GlyCL3	ACACTTGACTCATGGTTCTAAA	22	36	49
	GlyCL4	GATAGCAAAAGAGATTAAACCAAAA	25	28	49
	GlyCL5	ACTTATTGTTTGTGGTGCTAG	21	38	49
	GlyCL6	GAAATAGCAGATGAGGTTGGT	21	43	50
	GlyCU1	CTAAGGTTAGTAGCTCGGGTAA	22	45	53
	GlyCU2	AACTCATTGTATGCGGGGCAA	21	48	52
	GlyCU3	TATGCTAGGATTATTGATTTTGC	23	30	48
	GlyCU4	AGATAGCCGATGAAGTGGGG	20	55	54
	GlyCU5	GTGGCTGTGAGATTGTTGAT	20	45	50
	GlyCU6	TTTCCCTCACGCACACATCG	20	55	54
ceuB-C specific	CeuCJ1	CTTAATGATTGTAAGCATTATCACTAG	27	30	52
	CeuCJ2	GCTAATTATCCCAAATTTAGTAGCTCTTT	29	31	54
	CeuCJ3	AGCTCTTTATCTAGGTGATAATCTTAGAAA	30	30	55
	CeuCJ4	TTGGTGCGGCTAATTTAAGTGTTTATAAAAAC	32	31	57
	CeuCJ5	ATGCTAATTTTAAGCTTTTTAACACTTAACA	31	23	52
	CeuCJ6	ATCCTTTAAATTTAGGCAAAGATTTAGCGA	30	30	55
	CeuCC1	CTTAATAATAGTTTCTATTATCACTAG	27	22	49
	CeuCC2	TTATCATCCCTAATCTTGTTGCCATTT	22	33	54
	CeuCC3	TGCCATTTATCGCGGGGATAATCTTAAGA	29	41	55
	CeuCC4	TAGGTTCTGCAAATTTGAGTGTATATAGAAAT	32	28	55
	CeuCC5	TTATATCGTAACTTTGCTTAGTTTTAT	27	22	49
	CeuCC6	ACCCTTATAATGGCACTTGTTTTTGTATT	29	31	54
fliY specific	FliY1	TTGATCAATTAGCAAATGATCCT	23	30	48
	FliY2	AAAGGCATAACTTTTTTTGGCTCTGC	26	38	55
	FliY3	TGATCCTTTAGAAATTTTAATAGGTG	26	27	50
	FliY4	TAGCTGATTTAGAAGAAAAAATTTC	25	24	48

Continued on following page

TABLE 2—*Continued*

Type	Name	Sequence	Length (in nucleotides)	G + C (%)	T_m[a] (°C)
	FliY5	TAAGATTTTAACCAAAACTGACT	23	26	46
	FliY6	TAAGAGTGCGTATAGGTAGTAAA	23	35	50
cdtABC specific	CdtA1	CCCCAAATCCAATTTCCTTGTGCTAAAGCCCAAAC	35	46	64
	CdtA2	CGATTAAAGTATAGCCCCAAATCCAATTTCCTTGT	35	37	61
	CdtB1	GCATATTTGCAAAATGTGCATCTACAGCTGTGATA	35	37	61
	CdtB2	CCGCTTGCTTGAGTTGCGCTAGTTGGAAAAACCAC	35	51	67
	CdtC1	GGATTTGTAAATTGCACATAACCAAAAGGAAGTTC	35	34	60
	CdtC2	CATTCATCAGATTCCAAAACTAAAGAACGAATTTG	35	31	59
QC	QCprb	TGGCAGAAGCTATGAAACGATATGGG	27	44	58
	Cy3-QC	CCCATATCGTTTCATAGCTTCTGCCA	26	46	58

[a] The basic melting temperature (T_m) was calculated with the oligonucleotide properties calculator (http://www.basic.nwu.edu/biotools/oligocalc.html).

***C. upsaliensis*.** The *C. upsaliensis* strains tested were clinical isolates D1673, D2237, 5613, 5512, and 5502.

***Arcobacter butzleri*.** The *A. butzleri* strains tested were ATCC 49616 (from human feces) and clinical isolate 5530.

Non-*Campylobacter* species. *Listeria monocytogenes*, *L. innocua*, *Bacillus subtilis*, *B. cereus*, *E. coli*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Yersinia enterocolitica* were used as negative controls in the present study. These bacteria were grown overnight on brain heart infusion plates (Difco, Detroit, Mass.) at 37°C.

Genomic DNA preparation. Freshly grown bacteria were boiled in water (ca. 10^8 cells/ml) for 10 min, followed by centrifugation at 14,000 × *g* for 10 min to remove denatured proteins and bacterial membranes. The presence of genomic DNA in all prepared samples was confirmed by 1% agarose gel electrophoresis, followed by visualization with ethidium bromide.

PCR amplification. Table 1 lists the primers used to amplify the various *Campylobacter* genes in the present study. Reverse PCR primers of each pair contained the T7 RNA polymerase promoter sequence (TAATACGACTCAC TATAGGG) at the 5′ ends. The standard PCR mixture (30 μl) contained 1.5 U of HotStar *Taq* DNA polymerase in the recommended buffer supplemented with 2.5 mM $MgCl_2$ (Qiagen, Chatsworth, Calif.), 600 nM concentrations of each forward and reverse primer, 200 μM concentrations of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), and 1 to 2 μl of DNA template (ca. 0.2 μg of genomic bacterial DNA). The PCR was performed by using a GeneAmp PCR system 9600 thermocycler (PE Applied Biosystems, Foster City, Calif.) with the following cycle conditions: initial activation at 95°C for 15 min; 40 cycles of 94°C for 40 s, 50°C for 1 min, and 72°C extension for 1 min for primers S1-S2 and CmpfurF-CmpfurR or for 3 min for primers ceuBCF-ceuBCR and CmpToxF-CmpToxR; and a final extension at 72°C for 10 min. The presence of

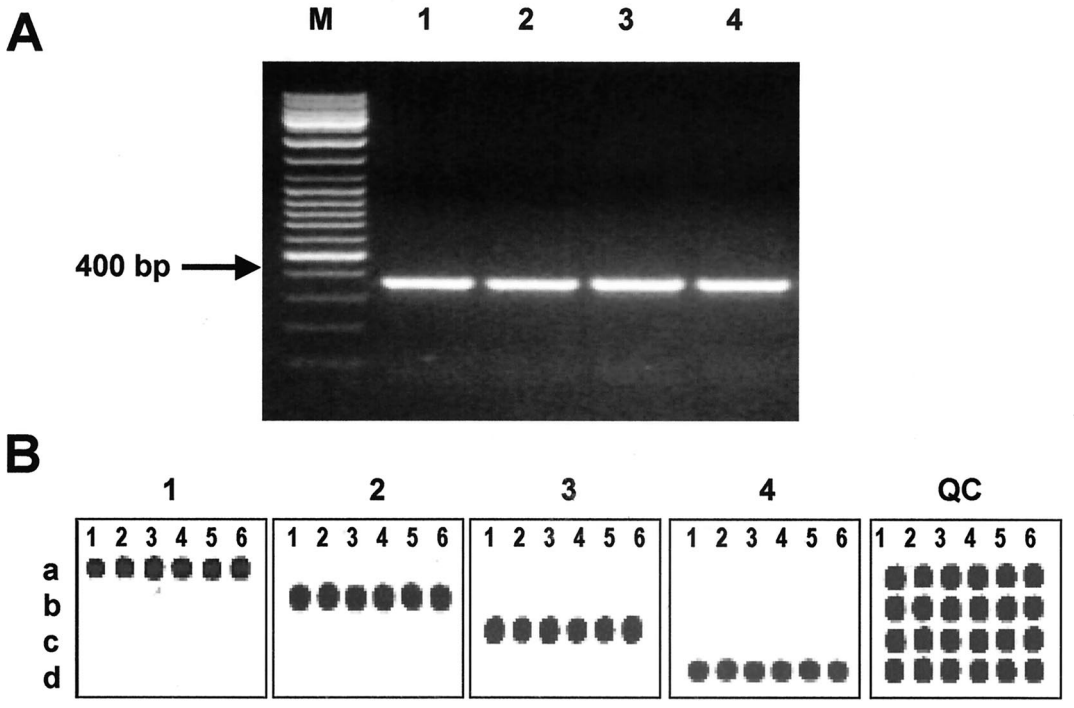

FIG. 1. Microarray-based detection of *Campylobacter* spp. using *fur*-specific oligoprobes. (A) PCR amplification of *fur* gene. Genomic DNAs from four reference strains were amplified by using the universal *fur* gene primers, CmpfurF and CmpfurR (Table 1). The resulting PCR products were separated on a 1.5% agarose gel. Lanes: M, 100-bp DNA Ladder Mix (MBI Fermentas); 1, *C. jejuni* (ATCC 33560); 2, *C. coli* (ATCC 43485); 3, *C. lari* (ATCC 35222); 4, *C. upsaliensis* (D1673). (B) Microarray-based detection of *Campylobacter* spp. by using the *fur*-specific oligoprobes. The *fur*-derived Cy5-labeled ssRNA transcripts were hybridized to the microchip. Each row of the array contains six individual species-specific probes (Table 2) as follows: a, *C. jejuni*; B, *C. coli*; C, *C. lari*; and D, *C. upsaliensis*. The image labeled QC is the microarray QC Cy3 image.

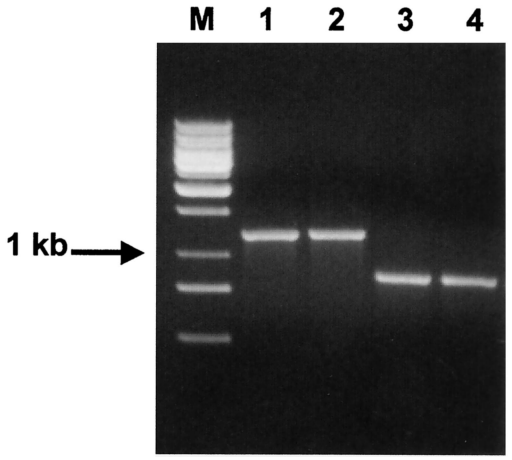

FIG. 2. PCR amplification of *ceuB-C* genes. Genomic DNAs from four reference strains were amplified by using the ceuB-C primers (Table 1). The resulting products were separated by using a 1% agarose gel. Lanes: M, 1-kb DNA ladder mix (MBI Fermentas); 1, *C. jejuni* (ATCC 33560); 2, *C. coli* (ATCC 43485); 3, *C. lari* (ATCC 35222); 4, *C. upsaliensis* (D1673).

amplified PCR products was detected by using a 1% agarose gel, followed by UV visualization after ethidium bromide staining.

In vitro transcription and fluorescent chemical labeling of RNA. Single-stranded RNA (ssRNA) samples for microarray analysis were synthesized by in vitro transcription from the promoter-tagged PCR amplicons by using the MEGAscript T7 high-yield transcription kit (Ambion, Austin, Tex.). The RNA transcription was performed in a 30-μl reaction mixture containing 2 μl of MEGAscript T7 enzyme mix (Ambion); 1× reaction buffer; 5 mM concentrations of ATP, UTP, CTP and GTP; and ca. 0.1 to 0.5 μg of DNA template from the PCR. The reactions were allowed to proceed at 37°C for 1 to 2 h, and then the unincorporated nucleoside triphosphates were removed by purification by using the Centrisep-Spin columns (Princeton Separations, Adelphia, N.J.) according to the manufacturer's protocol.

The Micromax ASAP RNA labeling kit (Perkin-Elmer, Boston, Mass.) was used for Cy5 labeling of the RNA samples for microarray analysis according to the manufacturer's protocol. Fluorescence-labeled ssRNA samples were purified from unincorporated dye by using the Centrisep-Spin columns, dried under vacuum, and solubilized in the Micromax hybridization buffer III at final concentration of 0.3 to 0.5 μM.

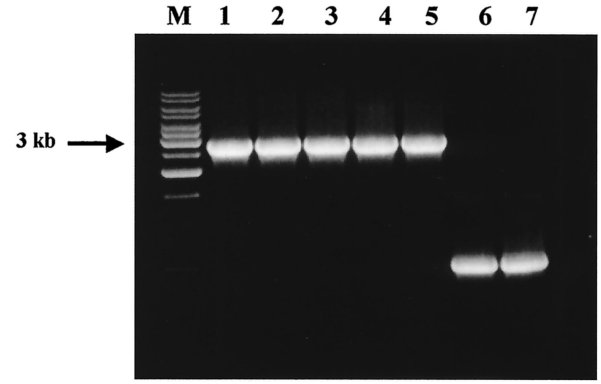

FIG. 3. PCR amplification of the *cdtABC* gene cluster from the *C. jejuni* and the *lctP-cydA* region of *C. coli*. Genomic DNAs from seven reference strains were amplified by using the *cdtABC* primers (Table 1). The resulting products were separated by using a 1% agarose gel. Lanes: M, 1-kb DNA ladder mix (MBI Fermentas); 1, *C. jejuni* (ATCC 33560); 2, *C. jejuni* (ATCC 35918); 3, *C. jejuni* (CDC1420); 4, *C. jejuni* (DENVER-2); 5, *C. jejuni* (GH18401); 6, *C. coli* (ATCC 43485); 7, *C. coli* (ATCC 43473).

Design of oligonucleotide microarray probes. Basic local alignment search tool (BLAST) searching was used to find and retrieve the sequences of homologous target regions of each of the five genes analyzed (Table 2). The retrieved sequences were aligned by using CLUSTALX software (13). The gene-specific oligonucleotide probes were designed to include species-specific variable regions. The selected oligonucleotides are summarized in Table 2. The 5′ end of each oligonucleotide was modified during the synthesis by using the TFA Aminolink CE reagent (PE Applied Biosystems) for immobilization of the oligonucleotides to silylated slides (CEL Associates, Inc., Houston, Tex.).

Microchip design and fabrication. To increase confidence in the microarray analysis and to overcome potential problems of genetic variability among and within species, each analyzed gene was represented by six individual oligoprobes (Table 2) from different parts of the amplified region. To facilitate interpretation of microarray data, all oligonucleotides specific for each gene were placed on separate rows of the array.

Microchips were printed by using a contact microspotting robotic system PIXSYS 5500 (Cartesian Technologies, Inc., Ann Arbor, Mich.) equipped with a microspotting pin (CMP7; ArrayIt, Sunnyvale, Calif.). The average size of spots was 250 μm. The spotting solution contained a mixture of specific oligonucleotide probe (80 μM) and quality control (QC) oligonucleotide (8 μM) in 50% dimethyl sulfoxide. Printed slides were dried for at least 20 min at 80°C and treated for 15 min with a freshly prepared 0.25% $NaBH_4$ solution in water. Slides were washed once for 5 min with 0.2% sodium dodecyl sulfate in water and five times for 1 min each time with distilled water to remove unbound oligonucleotides. Marker spots for array positioning on the slide were made by using 1× spotting solution (ArrayIt) in 0.25 M acetic acid.

Hybridization conditions. Hybridization of the fluorescently labeled ssRNA samples to the microarray was performed in the Micromax hybridization buffer III at 45°C for 30 min. Before hybridization, Cy5-labeled ssRNA sample was mixed with a Cy3-QC probe (Table 2) at molar ratio 10 to 1, followed by denaturing at 95°C for 1 min and chilling to 25°C. Each sample was placed on the microchip and covered with a 5- by 5-mm plastic coverslip to prevent evaporation of the probe during incubation. After hybridization, the slides were washed once for 1 min with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% Tween 20, three times for 1 min with 6× SSC buffer, twice with 2× SSC buffer, and once with 1× SSC buffer and then dried in a stream of air.

Microarray scanning. The fluorescent images of processed microarrays were generated by using ScanArray 5000 (Perkin-Elmer) equipped with two lasers operating at 632 nm (for excitation of Cy5 dye) and 543 nm (for excitation of Cy3 dye). The fluorescent signals from each spot were measured and compared by using QuantArray software (Perkin-Elmer). Fluorescent signals that differed from the average background at a statistically significant level ($P < 0.01$) were considered positive.

Sequencing. In some cases, sequences of the genes from some *Campylobacter* species were determined experimentally. The PCR-amplified DNA fragments were purified by agarose gel electrophoresis, extracted by using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol, and sequenced by using the ABI Prism 310 genetic analyzer system (PE Applied Biosystems).

Nucleotide sequence accession numbers. The GenBank accession numbers of the deposited sequences are AF545662 (strain ATCC 35221), AF545663 (strain ATCC 35222), AF545664 (strain ATCC 35223), and AF545665 (strain ATCC 43675).

RESULTS

Microarray-based identification of four thermophilic *Campylobacter* species by using sequence differences in the *fur* and *glyA* genes. The *fur* gene sequences from *C. jejuni* and *C. upsaliensis* (GenBank AL139075 and L77075) were used to design two primers capable of amplifying any *Campylobacter fur* allele. Degenerate universal primers, CmpfurF and CmpfurR (Table 1), complementary to the semiconserved regions, were shown to produce a 362- to 370-bp PCR product by using genomic bacterial DNA from isolates of *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* as a template (Fig. 1A). However, when DNA from the closely related bacterium *A. butzleri*, or DNAs from other non-*Campylobacter* species were used, no PCR products were observed (data not shown). These primers were

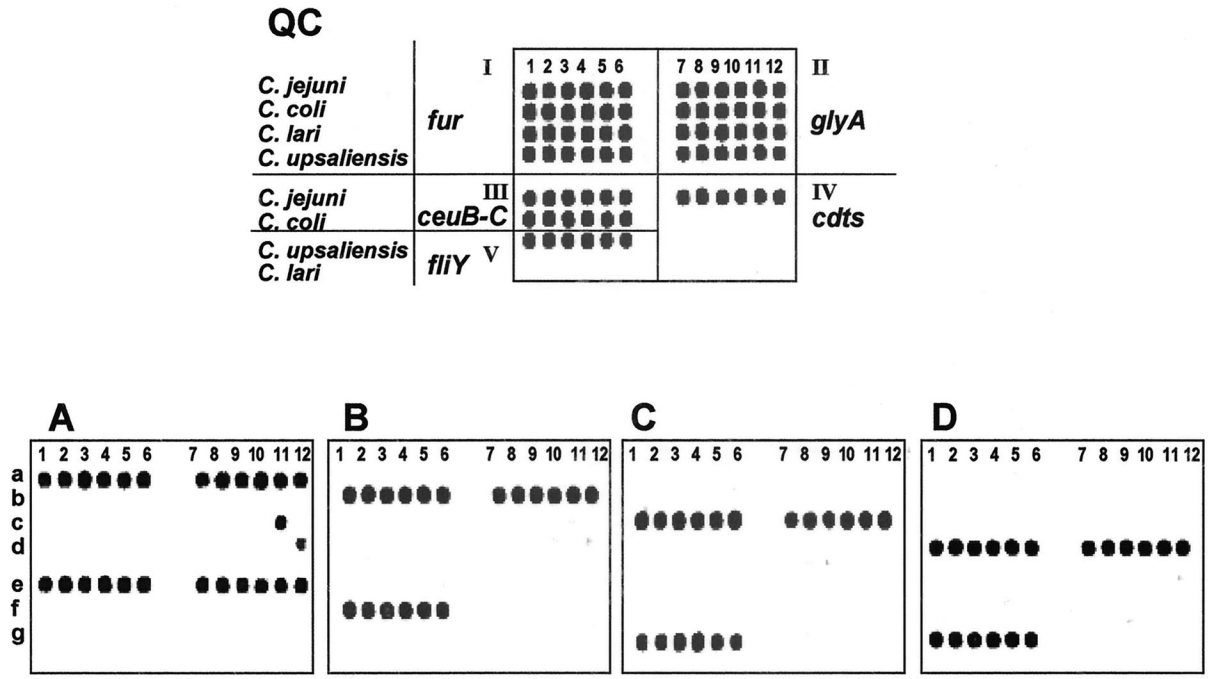

FIG. 4. Composite microarray for *Campylobacter* spp. identification. The QC image shows the layout of the array. The assay was composed of five subarray panels labeled from I to V. Each of four rows (a to d) of the subarray I contains six oligoprobes complementary to species-specific alleles of the *fur* gene. Subarrays from II to V contain oligoprobes for the *glyA*, *ceuB-C*, *cdts*, and *fliY* gene alleles, respectively. Microarray hybridization patterns of each of four *Campylobacter* species—*C. jejuni* (A), *C. coli* (B), *C. lari* (C), and *C. upsaliensis* (D)—are indicated.

also tested with all 51 *Campylobacter* isolates, including *C. jejuni* (n = 15), *C. coli* (n = 20), *C. lari* (n = 11), and *C. upsaliensis* (n = 5), and a fragment of the expected size was amplified from each.

Species-specific *fur* oligoprobes for distinguishing between *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (Table 2) were designed on the basis of comparison of more than 30 different *fur* gene sequences previously determined in our laboratory. Six individual *fur* oligoprobes for each *Campylobacter* species were selected and evaluated in the microarray hybridization with fluorescently labeled ssRNA samples. As shown in Fig. 1B, all species-specific *fur* gene oligoprobes strongly and specifically hybridized to the sample from their respective *Campylobacter* species.

Similar results were observed for the *glyA* gene-based identification. Regions from the *glyA* genes were amplified by using previously described primers S1 and S2 (1) and a set of our newly designed oligoprobes (Table 2). A 640-bp amplified DNA fragment was detected with all 51 *Campylobacter* isolates used in the study, and all species-specific *glyA* gene oligoprobes strongly and specifically hybridized to the *glyA*-derived RNA transcripts (data not shown).

Discrimination between *C. jejuni* and *C. coli* by using regions of the *ceuB-C* genes and detection of the *C. jejuni cdtABC* toxins gene cluster. Primers CeuEF and CeuER were designed for PCR amplification of the target region of the *ceuB-C* genes of *C. jejuni* and *C. coli* and were tested with all *Campylobacter* isolates used. As expected, these primers specifically amplified a 1,229-bp DNA fragment from all *C. jejuni* and *C. coli* strains (Fig. 2, lanes 1 and 2). However, an unexpected 866-bp DNA fragment was amplified from *C. lari* and *C. upsaliensis* (Fig. 2, lanes 3 and 4). Analysis of the amplicon sequences revealed that although these primers amplified the *ceuB-C* genes from *C. jejuni* and *C. coli*, the DNA amplified from *C. lari* and *C. upsaliensis* originated from the putative *fliY* gene, encoding a protein of the flagellar motor switch complex.

To independently confirm the species identification of *C. jejuni*, we used the *cdtABC* gene cluster, since these genes have been found in *C. jejuni* and *C. coli* isolates (3, 10). On the basis of the nucleotide sequence of the complete genome of *C. jejuni* subsp. *jejuni* NCTC 11168 (GenBank AL139074), we designed the CmpToxF and CmpToxR primers for amplification of the *cdtABC* gene cluster from *C. jejuni* (Table 1). The forward and reverse primers include sequences from the flanking *lctP* and *cydA* genes, respectively. The oligoprobes for the detection of the *C. jejuni cdtABC* cluster were designed as described above, and the corresponding sequences are summarized in Table 2. Amplification of a DNA fragment of the predicted size of 2,869 bp (Fig. 3) was observed in all cases when the DNAs from reference ATCC strains and clinical isolates of *C. jejuni* were used as PCR templates. We also observed amplification of a 700-bp PCR product from all *C. coli* strains (Fig. 3, lanes 6 and 7). Direct sequencing of this amplicon showed that it resulted from amplification of the *lctP-cydA* homologous region in the *C. coli* genome. This amplified *C. coli* DNA fragment did not hybridize to any of the *C. jejuni cdtABC*-specific oligoprobes since the *lctP-cydA* region does not include the *cdtABC* toxin gene cluster.

Microarray analysis of four thermophilic *Campylobacter* spp. by using a composite oligonucleotide microchip. To create

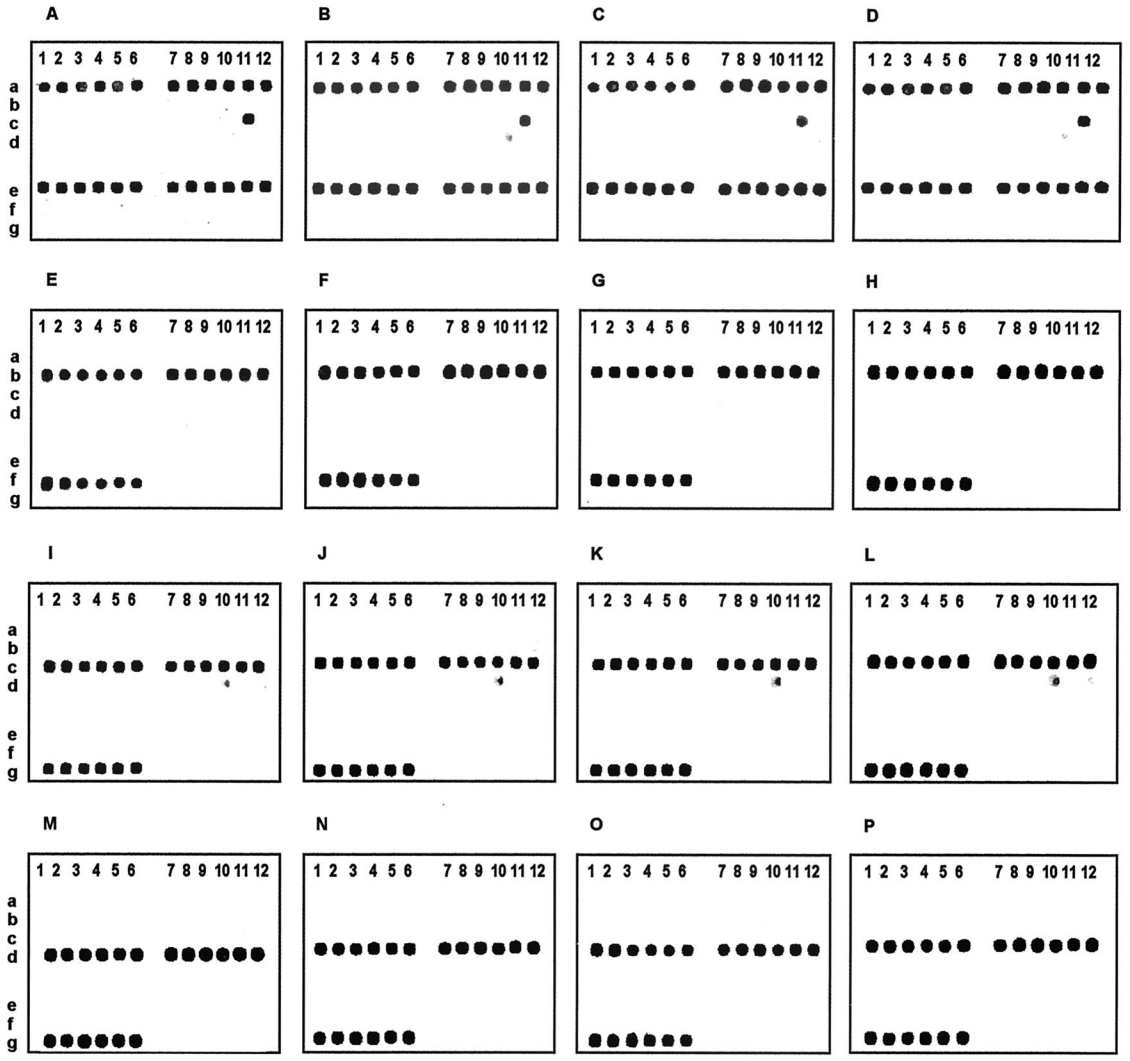

FIG. 5. Hybridization patterns of sixteen *Campylobacter* isolates. The composite microarray (Fig. 4) was used for the analysis of 16 *Campylobacter* isolates: *C. jejuni* (ATCC 35919, ATCC 29428, ATCC 33560, and DENVER-1) (A to D, respectively); *C. coli* (ATCC 33559, ATCC 43481, ATCC 43478, and 92B4QA) (E to H, respectively); *C. lari* (ATCC 35222, ATCC 35221, ATCC 43675, and 3125) (I to L, respectively); and *C. upsaliensis* (D2237, 5613, 5512, and 5502) (M to P, respectively).

a composite microchip for detection and discrimination of the four thermotolerant *Campylobacter* spp. by using specific regions of five target genes, we combined all of the oligoprobes described above (Table 2) into a single microarray with five panels (Fig. 4, QC image, panels I to V). All of the *Campylobacter* isolates listed in Materials and Methods were examined in a four-step procedure: (i) separate amplification of each target gene region, (ii) one-tube synthesis of all ssRNA transcripts from the T7 promoter-tagged PCR amplicons, (iii) fluorescent chemical labeling of ssRNA transcripts, and (iv) hybridization of the RNA probes to the composite microarray.

Using the optimized protocol conditions, we observed efficient and specific hybridization of species-specific oligoprobes with the corresponding *Campylobacter* species (Fig. 4A to D). Some cross-hybridization was observed for the *glyA* probes of *C. jejuni* and *C. lari* (Fig. 4A, spot c-11) and *C. upsaliensis* (Fig. 4A, spot d-12). However, since the *Campylobacter* sp. identification relied on the results of hybridization with six independent oligoprobes for each gene, the cross-hybridization of one or two spots did not affect the species identification.

The specificity of the composite microarray assay was evaluated by analyzing the collection of 51 *Campylobacter* isolates. All of the isolates were unambiguously identified; the results of 16 of these analyses are shown in Fig. 5. The results for *C. jejuni* and *C. coli* were confirmed by a PCR-based species detection method based on the *hipO* and *ask* genes (6). The results of the PCR assays were concordant with those of the microarray-based identification (data not shown).

Microarray identification of *Campylobacter* spp. from mixed samples. To determine the ability of microarray identification to determine *Campylobacter* species in mixed bacterial populations, we prepared and analyzed artificial DNA mixtures of different *Campylobacter* spp. The results showed that the microarray allowed efficient and highly specific detection of each

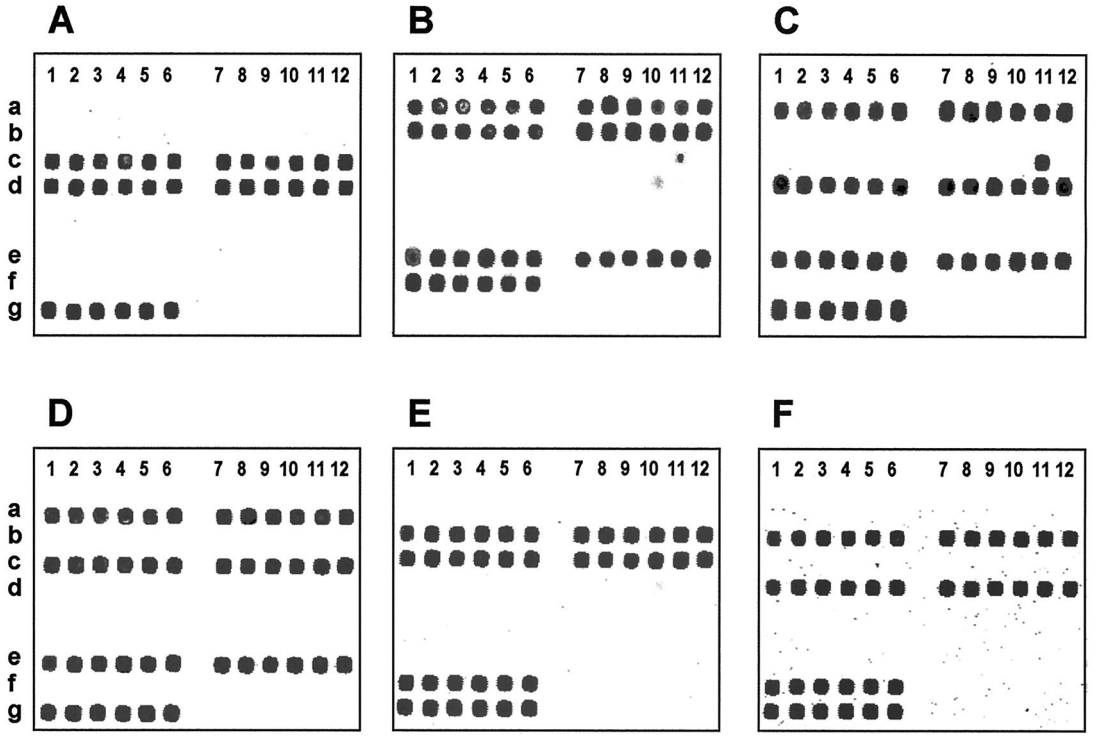

FIG. 6. Microarray hybridization patterns of bacterial samples containing mixtures of different *Campylobacter* species are shown. The composite microarray (Fig. 4) was used for six analyses of mixed *Campylobacter* isolates. Panels A to F show hybridization patterns for mixtures of *C. lari* and *C. upsaliensis* (A), *C. jejuni* and *C. coli* (B), *C. jejuni* and *C. upsaliensis* (C), *C. jejuni* and *C. lari* (D), *C. coli* and *C. lari* (E), and *C. coli* and *C. upsaliensis* (F).

Campylobacter species present in the mixtures (Fig. 6). Moreover, this microarray system allowed unambiguous species identification in the presence of DNA from various non-*Campylobacter* species (data not shown).

DISCUSSION

We describe here an oligonucleotide microarray assay for rapid detection and identification of four *Campylobacter* species of clinical relevance (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*). The approach uses the target regions of five genes: *fur*, *glyA*, *cdtABC* cluster, *ceuB-C*, and *fliY*. The initial microarray identification of each *Campylobacter* species is based on analysis of the *fur* and *glyA* genes, since these genes are well characterized and are found in all *Campylobacter* species and some of them have been used in previous PCR-based analysis methods.

Although the *fur* and *glyA* genes were used to unambiguously identify the four *Campylobacter* species, the *ceuB-C* genes were used to discriminate between *C. jejuni* and *C. coli*, the *fliY* gene to identify both *C. lari* and *C. upsaliensis*, and the *cdtABC* cluster to identify *C. jejuni.* The use of only one set of primers for simultaneous amplification of alleles of the *ceuB-C* genes of *C. jejuni* and *C. coli* and the *fliY* gene of *C. lari* and *C. upsaliensis* allows us to reduce the number of PCRs required for the analysis. The presence of the *cdtABC* gene cluster was used to confirm the identification of *C. jejuni*. Although homologues of these genes are found in some other diarrheagenic bacterial species and some closely related *Campylobacter* spp. such as *C. coli*, the oligonucleotide probes on the array were specific to *C. jejuni* and did not cross-react with other species (Fig. 4).

In our microarray system, we used relatively short oligonucleotides (17 to 35 nucleotides) for two reasons. First, shorter oligoprobe sequences (<25 bp) are often capable of detecting a singe nucleotide mismatch between the template ssRNA and the oligoprobe, thus detecting minor genetic variants in target genes in a bacterial population. Second, the use of multiple oligoprobes allows independent testing of several species-specific regions of each gene. This reduces the probability of misidentification.

We took advantage of the high-density capabilities of the array by analyzing 10 different species on one slide using several sequences per strain, and we performed this analysis simultaneously.

The genetic variability of *Campylobacter* spp., which has been demonstrated previously (9, 18, 28), may be problematic for PCR methods that rely on species-specific primers to identify the bacterial species. To avoid this problem, we deliberately designed degenerate primers for the PCR amplification and replaced the gel-based characterization of PCR products with a sequence-based hybridization method.

By using six spots representing six different sequences of the same gene, we assured detection despite sequence divergence. In addition we used several genes for analysis. This redundancy of sequences within genes and of genes within species will help to overcome the potential problem of sequence divergence and

hybridization specificity. However, the aim of this array was not to distinguish among strains of the same species. Indeed, we deliberately chose conserved sequences found in all strains of a specific species.

Several methods exist for analysis of *Campylobacter* including: nucleic acid hybridization, biochemical reactions, enzyme-linked immunosorbent assay, the combination of enzyme-linked immunosorbent assay and immunomagnetic separation, enzyme-linked fluorescent assay, and PCRs. The combined PCR and microarray analysis we present here has important advantages over these methods. First, it takes advantage of the sensitivity and simplicity of PCR amplification for analyzing even low levels of bacterial contamination in many different samples, including food products, while overcoming the problems of nonspecific products that are often produced in highly sensitive PCR assays. Second, the microarray method enables simultaneous analysis of multiple genetic characteristics of target organism in one experiment. Unlike other nucleic acid hybridization methods, the glass microarray chips analyze several genes, and several sequences for each gene, simultaneously. Thus, identification is made on the basis of multiple genetic characteristics, which limits the probability of both false-positive and false-negative results. In the experiments reported here, the species determination was made based on 72 parameters (the number of spots), increasing the reliability of the results. Third, this method can be used to carry out many analyses simultaneously. We demonstrated that as few as 10 different *Campylobacter* strains could be analyzed on one slide. The PCR-microarray assay can also be scaled up through the use of universal primers for amplification, which reduces the number of primers and the number of reactions needed for analysis of several genes from several species. Finally, microarray analysis can be viewed as a spot pattern recognition assay, which can now be carried out automatically by an increasing number of computerized devices. Thus, the data presented here suggest that microarray analysis is a valuable tool for the identification and characterization of bacterial pathogens and other organisms.

ACKNOWLEDGMENTS

We thank P. Fields and B. Swaminathan of the National *Salmonella* and *Campylobacter* Reference Laboratories, Centers for Disease Control and Prevention, and R. Thunberg and T. Tran of the FDA Center of Food Safety and Applied Nutrition for providing the strains used in the present study. We thank R. Thunberg for technical assistance.

This work was supported in part by USDA grant 0013000 and funding provided by the FDA Office of Science to A.R. and V.C. and by a grant from the U.S. Defense Advanced Research Project Agency to K.C.

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