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Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a Multiplex PCR Developed from the Nucleotide Sequence of the Lipid A Gene *lpxA*

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We describe a multiplex PCR assay to identify and discriminate between isolates of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis*. The *C. jejuni* isolate F38011 *lpxA* gene, encoding a UDP-*N*-acetylglucosamine acyltransferase, was identified by sequence analysis of an expression plasmid that restored wild-type lipopolysaccharide levels in *Escherichia coli* strain SM105 [*lpxA*(Ts)]. With oligonucleotide primers developed to the *C. jejuni lpxA* gene, nearly full-length *lpxA* amplicons were amplified from an additional 11 isolates of *C. jejuni*, 20 isolates of *C. coli*, 16 isolates of *C. lari*, and five isolates of *C. upsaliensis*. The nucleotide sequence of each amplicon was determined, and sequence alignment revealed a high level of species discrimination. Oligonucleotide primers were constructed to exploit species differences, and a multiplex PCR assay was developed to positively identify isolates of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. We characterized an additional set of 41 thermotolerant isolates by partial nucleotide sequence analysis to further demonstrate the uniqueness of each species-specific region. The multiplex PCR assay was validated with 105 genetically defined isolates of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*, 34 strains representing 12 additional *Campylobacter* species, and 24 strains representing 19 non-*Campylobacter* species. Application of the multiplex PCR method to whole-cell lysates obtained from 108 clinical and environmental thermotolerant *Campylobacter* isolates resulted in 100% correlation with biochemical typing methods.

Campylobacteriosis, gastroenteritis caused by the thermotolerant *Campylobacter* spp. (*Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis*), is the most commonly reported gastrointestinal bacterial disease in developed countries (44). *C. jejuni* is most often implicated as the causative agent of campylobacteriosis, followed by *C. coli* (46), *C. upsaliensis* (23), and *C. lari* (35). While *C. lari* and *C. upsaliensis* cause far fewer cases of disease, both species have been recognized as emerging human pathogens (23, 46, 51). The true incidence of disease caused by non-*C. jejuni Campylobacter* spp. is difficult to estimate. This is, in part, due to difficulties in distinguishing between *Campylobacter* species based solely on phenotypic properties commonly used in clinical laboratories (30). In order to better understand the epidemiology of *Campylobacter* spp. and facilitate its control, genetic methods that distinguish between members of this species need to be developed.

Housekeeping genes encode proteins that carry out essential functions in a cell. As such, housekeeping genes make excellent targets for species-specific probes for several reasons. Notably, the products encoded by these genes are functionally constrained; thus, diversity present in the coding region can be used for phylogenetic analysis (37). The gene *lpxA* encodes the enzyme LpxA, a UDP-*N*-acetylglucosamine acyltransferase that catalyzes the first step of lipid A biosynthesis and subsequently lipopolysaccharide biosynthesis (38). Mutations in *lpxA* result in a loss of viability in gram-negative bacteria (38), with the exception of *Neisseria meningitidis* (36). The substrate for LpxA, an (*R*)-3-hydroxy fatty acid, is also used by the enzymatic product of the *fabZ* gene (28). The FabZ protein is involved in the dehydration of β-hydroxylacyl-acyl carrier protein moieties to the corresponding *trans*-2-acyl-acyl carrier protein product, thereby initiating fatty acid biosynthesis (15, 28). The genes *fabZ* and *lpxA* are commonly associated in tandem in gram-negative bacteria, which is also the case for the sequenced *C. jejuni* strain NCTC 11168 (15, 28, 33, 39, 42).

The ability to distinguish between *Campylobacter* species is important in the identification of *Campylobacter* sources and transmission routes (12, 50). Oligodeoxynucleotide primers and PCR assays that distinguish between one or more combinations of the thermotolerant *Campylobacter* species have been described (1, 4, 5, 9, 11, 13, 16, 21, 48). The advantages of PCR-based assays are that they can be performed quickly and relatively cheaply (29). Unfortunately, many of the assays currently available for *Campylobacter* spp. suffer from a lack of sensitivity or specificity (14, 32). Other assays require a combination of PCR amplification, coupled with restriction endonuclease digestion of the PCR amplicon by two to four enzymes, to specifically differentiate the thermotolerant *Campylobacter* spp. (11, 16).

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Recently, species-specific microarrays have been described for the rapid identification of *Campylobacter* spp. (19, 49). These methods have great potential for epidemiological studies, but given the cost of the technology and the lack of microarray facilities in most clinical laboratories, they are currently not practical for routine application. We have developed a multiplex PCR assay, based on nucleotide sequence differences in the *lpxA* gene, to distinguish between *C. coli, C. jejuni*, *C. lari*, and *C. upsaliensis*. This multiplex PCR assay has been shown to be rapid, sensitive, specific, and robust.

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MATERIALS AND METHODS

Reference strains and culture conditions. The reference strains and isolates supplied from individual culture collections used in this study are listed in Table 1. Thermotolerant *Campylobacter* spp. (*C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*) were cultured on Mueller-Hinton medium (Remel, Lenexa, Kans.) supplemented with 5% sheep blood (Invitrogen, Carlsbad, Calif.) and 1.5% agar (Difco, Franklin Lakes, N.J.) at 43°C in a microaerobic atmosphere (5% O_2, 10% CO_2, 10% H_2, 75% N_2).

Campylobacter concisus ATCC 33237, ATCC 43643, ATCC 51561, and ATCC 51562; *Campylobacter curvus* ATCC 35224, ATCC 33237, and RM3269; *Campylobacter fetus* subsp. *fetus* NCTC 10842, RM2086, RM2087, and RM2088; *Campylobacter fetus* subsp. *venerealis* NCTC 10354, RM3224. RM3225, ATCC 19438, ATCC 33561, and ATCC 33226; *Campylobacter gracilis* ATCC 33226; *Campylobacter helveticus* ATCC 51209 and ATCC 51210; *Campylobacter hyointestinalis* ATCC 35217, RM1558, RM1559, RM1561, RM2101, and RM3773; *Campylobacter lanienae* NCTC 13003 and NCTC 13004; *Campylobacter mucosalis* ATCC 43264 and ATCC 43265; *Campylobacter rectus* ATCC 33238 and RM3276; *Campylobacter showae* ATCC 51146; and *Campylobacter sputorum* ATCC 33491 and ATCC 51146 were cultured on triple sugar iron medium at 37°C under anaerobic conditions as described previously (31). *Bacteroides urealyticus* NZRM 2009 was cultured on nonselective sheep blood agar at 37°C under anaerobic conditions. *E. coli* strains SM105 and SM101 were cultured aerobically at 30°C or 43°C on Luria-Bertani (LB) medium (Difco, Franklin Lakes, N.J.) or LB medium supplemented with 1.5% agar.

All other bacterial species (*Arcobacter cryaerophilus* ATCC 43158 and ATCC 49615; *Arcobacter butzleri* ATCC 49616, RM1588, and RM1591; *Arcobacter nitrofigilis* ATCC 33309; *Arcobacter skirrowii* CCUG10374, ATCC 51400, and LMG10234; *Bacillus cereus* NCTC 8035; *Bacillus subtilis* NCTC 3610; *Enterobacter aerogenes* NCTC 10006; *Enterobacter faecalis* NCTC 775; *Escherichia coli* ATCC 25922; *Helicobacter pylori* NZRM2925; *Klebsiella pneumoniae* NCTC 9633; *Listeria innocua* NCTC 11288; *Listeria monocytogenes* NCTC 7073; *Morganella morganii* NCTC 235; *Proteus vulgaris* ATCC 13315; *Pseudomonas aeruginosa* NCTC 10662; *Salmonella enterica* serovar Menston NCTC 7836; and *Shigella flexneri* NCTC 5) were cultured overnight in brain heart infusion broth (Difco) at 35°C. Ampicillin was added at a concentration of 50 μg ml^{-1} as required to maintain expression plasmids.

Nonreference clinical and environmental *Campylobacter* isolates. Additional *Campylobacter* organisms, designated nonreference isolates, were obtained from clinical ($n = 16$) and environmental ($n = 92$) sources in South Canterbury, New Zealand (2, 51). The majority of these isolates were obtained from surface water ($n = 34$), with seven isolates obtained from beef feces, four isolates from beef offal, seven isolates from whole chicken carcasses, 10 isolates from dairy cow feces, five isolates from dog feces, four isolates from duck feces, one isolate from rabbit feces, 13 isolates from sheep feces, and seven isolates from sheep offal.

Phenotypic characterization. Clinical and environmental isolates were examined for darting motility and Gram stained for identification to the genus level. To tentatively establish species identification, conventional methods available for sodium hippurate hydrolysis and nalidixic acid sensitivity were used (24).

Genomic DNA extractions. Validation of the *lpxA* multiplex PCR was conducted with purified genomic DNA for reference strains. Bacterial growth from a single agar plate was harvested with 4 ml of sterile phosphate-buffered saline and a sterilized glass rod. A 1-ml aliquot of cellular growth was transferred to a 1.5-ml microcentrifuge tube, and cells were collected by centrifugation in a benchtop centrifuge at 5,800 × *g*, 4°C, for 5 min. Genomic DNA was extracted with the method of Pospiech and Neumann (34). DNA was quantified with a Hitachi U2000 spectrophotometer. Extracted genomic DNA was stored at −20°C in sterile distilled water supplemented with a final RNase A concentration of 20 μg ml^{-1}.

Campylobacter genomic DNA from clinical and environmental isolates was extracted by heat lysis of bacterial cell cultures (21). For these experiments, bacteria were harvested after 48 h of growth from culture medium and suspended in 2 ml of sterile distilled water with a sterile cotton applicator stick. A 1-ml aliquot of the cell suspension was transferred to a 1.5-ml microcentrifuge tube, and the cell mixture was boiled in a water bath for 10 min. Cell debris was pelleted by centrifugation at 10,300 × *g*, 4°C, for 10 min. The supernatant was transferred to a new 1.5-ml tube and subsequently used as a template for PCR amplification.

Cloning of the *C. jejuni lpxA* gene. An F38011 plasmid expression library (20) was used to transform *E. coli* strain SM105 [*lpxA*(Ts)] to ampicillin resistance (Ap^r). Ampicillin-resistant transformants were patch streaked onto two plates of LB plus agar and ampicillin and incubated at either 30 or 43°C. Plasmids were recovered from transformants capable of growth at the nonpermissive temperature (43°C). The insert from one plasmid, pCI1, was subsequently sequenced and characterized further (17).

PCR. The oligodeoxynucleotide sequencing primers used were obtained from Sigma Genosys (The Woodlands, Tex.). The *lpxA* nucleotide sequence of *C. coli* isolate M275 was determined with primers lpxAF9625 and lpxAR0025 (Table 2). Primers 0301 and 0304 were used to amplify a nearly full-length copy of *lpxA* and the upstream flanking region (793 bp) from isolates of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. The primer pair lpxAF0301 and lpxARKK2m was used to generate a 521-bp PCR amplicon from additional isolates of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. Forward primers complementary to the *lpxA* nucleotide sequence of *C. coli* (lpxA*C. coli*), *C. jejuni* (lpxA*C. jejuni*), *C. lari (*lpxA*C. lari*), and *C. upsaliensis* (lpxA*C. upsaliensis*) were designed with the program Oligo (version 6, MBI Inc, Cascade, Colo.). In combination with the primer lpxARKK2m, amplicons of 391, 331, 233, and 206 bp, respectively, were generated.

For PCR assays, 10 pmol of forward primer μl^{-1} and 10 pmol of reverse primer μl^{-1} were mixed in a 25-μl reaction volume containing 200 μM each deoxynucleoside triphosphate (Invitrogen, Carlsbad, Calif.), 1× reaction buffer without $MgCl_2$ (Qiagen, Valencia, Calif.), an additional 2 mM $MgCl_2$ (Qiagen, Valencia, Calif.), and 1.25 units of *Taq* DNA polymerase (Qiagen). Genomic DNA, 20 to 50 ng, or the equivalent amount of DNA from a whole-cell lysate was then added, and the reaction mixtures were placed into an MJ Research (Waltham, Mass.) thermocycler. The cycling conditions used were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension time of 5 min (35 cycles total). For multiplex PCR assays, 10 pmol of each forward primer μl^{-1} was added to the reaction mixture with 30 pmol of the lpxARKK2m reverse primer μl^{-1}. Each reaction mixture was examined by agarose gel electrophoresis through 3% agarose, and bands were visualized with UV light after staining with ethidium bromide. Images were captured on a Kodak Electrophoresis and Documentation Analysis System 120 (Fisher Scientific, Pittsburgh, Pa.).

Nucleotide sequence analysis. The DNA sequence of the full-length *lpxA* gene from *C. coli* isolate M275, *C. jejuni* isolate F38011, and the remaining *lpxA* PCR amplicons were determined either with an ABI Prism 377 or with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). For nucleotide sequence reactions, dye terminator technology was used, and nucleotide sequence from both strands of the DNA template was determined. Nonincorporated nucleotides and oligonucleotide primers were removed from PCR mixtures with the DyeEx purification kit (Qiagen, Valencia, Calif.). Twofold redundancy of each PCR amplicon was achieved by determining the nucleotide sequence of both strands. The nucleotide sequence of the *lpxA* gene from *C. jejuni* isolate F38011 and *C. coli* isolate M275 were determined in two independent experiments, generating fourfold redundancy for these isolates. Accession numbers for the *lpxA* genes are listed in Table 1.

Phylogenetic analysis of the *lpxA* nucleotide sequence. Nucleotide sequence data were aligned with the program Clustal W (45). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 2.1 (22). Phylogenetic trees were constructed with the unweighted pair group method with arithmetic mean analysis (40). Evolutionary distances were calculated with the method of Jukes and Cantor (18). Bootstrap analysis (10) was performed with 1,000 replicates.

RESULTS

Cloning and sequencing of the *C. jejuni* F38011 *lpxA* gene. The *lpxA* gene from *C. jejuni* isolate F38011 was identified based on its ability to restore lipid A biosynthesis in *E. coli* strain SM105, which contains a temperature-sensitive mutation in *lpxA*, at 43°C (data not shown) (17). In total, 1,059 bp of *lpxA* and flanking DNA sequence was determined. The complete

TABLE 1. Reference bacterial isolates used, and their nucleotide sequence accession numbers, and amplicon sizes in the *lpxA* multiplex PCR assay

Species	Isolate no.	Isolate source[a]	Amplicon size (bp)	Accession no.[b]
Campylobacter coli	M275	Human; Arizona	391	AY531509
	RM1051	Human; Canada	391	AY531503; identical to M275
	RM1166	Chicken	391	AY531502; identical to M275
	RM1505		391	AY531501; identical to M275
	RM1530	Sheep; USA	391	AY531500; identical to M275
	RM1531	Marmoset	391	AY531499; identical to M275
	RM1533	Human; USA	391	AY531497; identical to M275
	RM2225	Chicken; USA	391	AY531508; identical to M275
	RM2228	Chicken; USA	391	AY531507; identical to M275
	RM1532	Human; USA	391	AY531498
	RM1857	Human	391	AY531496
	WA31	Water; NZ	391	AY598971; identical to RM1857
	KLC5104	Human; NZ	391	AY598972; identical to RM1857
	RM1858	Human	391	AY531495
	RM1865	Human	391	AY531494
	RM1878; ATCC 43474	Human	391	AY531493
	RM1896	Swine; USA	391	AY531492
	RM1897	Swine; USA	391	AY531491
	RM3232	Swine	391	AY531504
	RM3230[c]	Swine; Australia	391	AY531506; identical to RM3232
	RM3231[c]	Swine; Australia	391	AY531505; identical to RM3232
	WA27	Water; NZ	391	AY531510
	KLC4366	Human; NZ	391	NS
	KLC5039	Human; NZ	391	NS
	CC39.4	Swine; Missouri	391	NS
	T1631	Human; Arizona	391	NS
	T2144	Human; Arizona	391	NS
	T2232	Human; Arizona	391	NS
Campylobacter jejuni	F38011	Human; Arizona	331	AY531520
	RM1221	Chicken; USA	331	AY531521; identical to F38011
	RM3672	Canada Geese; California	331	AY531512; identical to F38011
	RM3673	Canada Geese; California	331	AY531511; identical to F38011
	S2B	Chicken; USA	331	AY598974; identical to F38011
	FZ917T	Human; NZ	331	AY598976; identical to F38011
	NCTC 11168	Human; UK	331	AL111168
	ANR0493	Human; NZ	331	AY531523; identical to NCTC11168
	YG108S	Human; NZ	331	AY598977; identical to NCTC11168
	YG936T	Human; NZ	331	AY598978; identical to NCTC11168
	RM3664	Canada geese; California	331	AY531519
	RM3665	Canada geese; California	331	AY531518; identical to RM3664
	RM3666	Canada geese; California	331	AY531517; identical to RM3664
	RM3667	Canada geese; California	331	AY531516; identical to RM3664
	ESR 081	Human; NZ	331	AY598979; identical to RM3664
	RM3668	Canada geese; California	331	AY531515
	RM3669	Canada geese; California	331	AY531514; identical to RM3668
	RM3670	Canada geese; California	331	AY531513; identical to RM3668
	KLC2851	Human; NZ	331	AY531522
	M129	Human; Arizona	331	AY598973
	81116		331	AY598975
	KLC4271	Human; NZ	331	AY598980
	ZJ638R	Human; NZ	331	AY598981
	81–176	Human; Minnesota	331	NS
Campylobacter lari	RM1890	Human	233	AY531476
	RM2810		233	AY531488; identical to RM1890
	RM2821	Human; Canada	233	AY531483; identical to RM1890
	RM2100	Human; USA	233	AY531474
	RM2099	Human	233	AY531475; identical to RM2100
	RM2808		233	AY531490; identical to RM2100
	RM2809		233	AY531489; identical to RM2100
	RM2817	Horse; Sweden	233	AY531487; identical to RM2100
	RM2820	Seagull	233	AY531484; identical to RM2100
	RM2826	Human; Belgium	233	AY531478; identical to RM2100
	DRI879	Human; NZ	233	AY598982; identical to RM2100
	RM2819	Seagull; Canada	233	AY531485
	RM2822	Human	233	AY531482
	RM2823	Human; Canada	233	AY531481
	EC007	Water; NZ	233	AY598983; identical to RM2823
	EC009	Water; NZ	233	AY598984
	RM2824	River water; UK	—[d]	AY531480
	RM2825	Human; Canada	—	AY531479
	RM3659	River water; UK	—	AY531477
	NZRM2622		233	NS

Continued on following page

TABLE 1—*Continued*

Species	Isolate no.	Isolate source[a]	Amplicon size (bp)	Accession no.[b]
Campylobacter upsaliensis	ATCC 43954	Canine; Sweden	206	AY598985
	RM1488	Human; Canada	206	AY531471
	ATCC 49815			
	RM2094	Human; USA	206	AY531469; identical to RM1488
	C1137	Canine; Washington	206	AY598988; identical to RM1488
	RM3937	Human; California	206	Identical to RM1488
	RM3939	Human; California	206	AY598996; identical to RM1488
	RM3940	Human, California	206	Identical to RM1488
	RM3941	Human; California	206	Identical to RM1488
	RM3943	Human; California	206	Identical to RM1488
	RM3945	Human; California	206	Identical to RM1488
	RM3946	Canine; California	206	Identical to RM1488
	RM3947	Canine; California	206	Identical to RM1488
	RM3949	Canine; California	206	Identical to RM1488
	RM3950	Canine; California	206	Identical to RM1488
	RM2089	Sheep	206	AY531472
	RM2093	Human; USA	206	AY531470
	RM3195	Human; South Africa	206	AY531473
	RM3777	Human; South Africa	206	AY598986
	C1138	Canine; Washington	206	AY598989
	RM2092	Human	206	AY598987
	RM3779	Human; South Africa	206	AY598990
	RM3778	Human; South Africa	206	Identical to RM3779
	RM3781	Human; South Africa	206	Identical to RM3779
	RM3786	Human; South Africa	206	Identical to RM3779
	RM3780	Human; South Africa	206	AY598991
	RM3776	Human; South Africa	206	Identical to RM3780
	RM3784	Human; South Africa	206	Identical to RM3780
	RM3783	Human; South Africa	206	AY598992
	RM3785	Human; South Africa	206	AY598993
	RM3942	Human; California	206	AY598995
	RM3944	Human; California	206	AY598994
	RM3948	Canine; California	206	Identical to RM3944
	RM3782	Human; South Africa	206	NS

[a] NZ, New Zealand; UK, United Kingdom.
[b] NS, not sequenced.
[c] These isolates were formerly classified as *Campylobacter hyoilei.*
[d] —, no band was amplified during the PCR assay.

nucleotide sequence of the *lpxA* gene from *C. jejuni* F38011 was determined to be 789 bp, with an overall G+C content of 34.2 mol%. The nucleotide sequence of *C. jejuni* F38011 exhibits 98.9% identity with the *lpxA* gene of *C. jejuni* NCTC 11168 (33). The ATG start codon of *C. jejuni* F38011 *lpxA* overlaps the putative TAA stop codon of the upstream gene *fabZ* (Fig. 1). A partial open reading frame with amino acid similarity to that of the translated product of the gene *clpX* was located downstream (33).

Identification of *Campylobacter* species-specific *lpxA* sequences. PCR primers flanking the *C. jejuni lpxA* gene (9625 and 0025) were used to amplify the *lpxA* region from *C. coli*. Comparison of the F38011 *lpxA* gene and flanking region sequences to the same region of the *C. coli* isolate M275 genome revealed 137 changes (12.9%, data not shown), including an 11-bp intergenic spacer separating *fabZ* from *lpxA*. The overall sequence divergence between these two species suggested that the *lpxA* locus might be useful in distinguishing *C. jejuni* from *C. coli* as well as from other thermotolerant *Campylobacter* spp. To determine if the *fabZ-lpxA* sequences could be used to distinguish *Campylobacter* isolates from one another, additional amplicons generated from phenotypically and genetically defined *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* isolates were sequenced. Amplicons, generated with primers lpxAF0301 and lpxAR0304 were obtained from an additional 20 isolates of *C. coli*, 12 isolates of *C. jejuni*, 16 isolates of *C. lari*, and five isolates of *C. upsaliensis* (Table 1).

Phylogenetic analysis of the *lpxA* sequences from the 55 thermotolerant *Campylobacter* isolates clearly separated each species into distinct clades (Fig. 2). Intraspecies variation was also observed. Comparison of the *C. coli* isolates revealed two discrete clusters that possessed 96% identity. The larger of the two clusters consisted of seven highly similar *lpxA* alleles derived from 17 isolates of different geographies and animal hosts. Over the 757-bp sequence, only 5 bp were variable (0.66% difference). Of interest was that two isolates formerly

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)
Forward primers	
lpxAF9625	TGC GTC CTG GAG ATA GGC
0301	CTT AAA GCN ATG ATA GTR GAY AAR
lpxA*C. coli*	AGA CAA ATA AGA GAG AAT CAG
lpxA*C. jejuni*	ACA ACT TGG TGA CGA TGT TGT A
lpxA*C. lari*	TRC CAA ATG TTA AAA TAG GCG A
lpxA*C. upsaliensis*	AAG TCG TAT ATT TTC YTA CGC TTG TGT G
Reverse primers	
lpxAR0025	TAG GCA TTA TTT TTA CCC CTA TAG ACA G
0304	ACA GGR ATT CCR CGY TTT GTY TC
lpxARKK2m	CAA TCA TGD GCD ATA TGA SAA TAH GCC AT

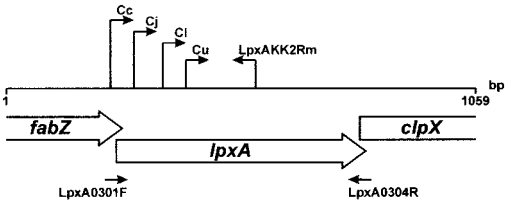

FIG. 1. Arrangement of the *lpxA* locus within the *Campylobacter jejuni* F38011 genome. The *lpxA* gene, flanked at the 5′ end by *fabZ* and at the 3′ end by *clpX* (direction of transcription shown by the arrows), is shown. The numbers indicate the nucleotide sequence obtained from the F38011-derived expression plasmid pCI1. Also indicated (solid arrowheads underneath the gene designations) are the relative positions of the PCR and sequencing primers lpxA0301F and lpxA0304R. The relative position of the species-specific primer positions are indicated as follows: Cc, *C. coli*; Cj, *C. jejuni*; Cl, *C. lari*; and Cu, *C. upsaliensis*, with LpxAKK2Rm representing the universal *lpxA* reverse primer.

identified as *Campylobacter hyoilei* (RM3230 and RM3231) were located in this cluster and were indistinguishable from *C. coli* isolate RM3232. The smaller *C. coli* branch was defined by 48 unique changes over the 757-bp sequence (6.34%). The three isolates constituting the smaller cluster, RM1858, RM1865, and RM1878, were clinical isolates and were over 98% identical (12 variable bp over 757 bp). The *lpxA* gene sequences from the *C. jejuni* isolates were more closely related to those of *C. coli* (less than 7% unique differences) than either *C. lari* or *C. upsaliensis*. The *C. jejuni* isolates were more homogeneous than the isolates of *C. coli* (greater than 98% identity), and five alleles were represented in these 13 isolates.

The clades containing the *C. lari* and *C. upsaliensis lpxA* sequences were distinct from the *C. coli* and *C. jejuni* clades (88% and 86.5% nucleotide identity, respectively). Four of the five isolates of *C. upsaliensis* analyzed contained unique *lpxA* alleles. Too few samples were present to conclusively demonstrate geographic clustering within these samples. Similar to the situation within *C. coli*, analysis of the *C. lari lpxA* sequences revealed a heterogeneous collection of isolates composed of more than one distinctive cluster. The main cluster of 13 isolates had 30 variable nucleotides over 746 bp (4.0% variability) that resulted in five *lpxA* alleles. The second, smaller branch consisted of two isolates (RM2825 and RM3659) that differed at 38 of 746 bp (5.1%) and thus represented two distinct alleles of *C. lari lpxA*. One of these isolates, RM3659, was urease positive. These two isolates differed from the major *C. lari* group at 90 of 746 positions (12.1%). A third branch consisting of the RM2824 *lpxA* sequence was also present. This isolate had the most distinct *lpxA* allele of all the *C. lari* isolates sequenced, differing at 108 of 746 positions (14.5%) from the major *C. lari* group.

A more detailed comparison of the *lpxA* nucleotide sequences from the 55 thermotolerant isolates discussed above revealed several regions that could be used to develop species-specific probes (Fig. 3A to D). A unique feature of the *C. coli lpxA* sequences was an 11-bp intergenic spacer region separating *fabZ* from *lpxA*. *C. upsaliensis* also contained a similar intergenic spacer region of 8 bp, but this sequence differed in composition from that of *C. coli*. A sequence located 41 bp downstream from the *lpxA* start codon was highly conserved between *C. jejuni* isolates but diverged (8 or 9 of 22 bp within *C. coli*, 9 or 11 of 22 bp within *C. lari*, and 12 of 22 bp within *C. upsaliensis*) in other thermotolerant isolates. Similarly, a 20-bp region 141 bp downstream from the putative *lpxA* start site was strongly conserved among the majority of *C. lari* isolates (typified by isolate RM1890) but differed in 7 to 10 of 20 bp from isolates of *C. upsaliensis*, 6 to 8 of 20 bp from *C. coli*, and 6 to 9 of 20 bp between isolates of *C. jejuni*. The three isolates representing the lesser-represented branches of *C. lari* (RM2825, RM3659, and RM2824) were also divergent within this region. The nucleotide region representing *C. upsaliensis* differed from *C. coli* and *C. jejuni* in 9 to 11 positions and *C. lari* in 11 to 14 positions over the 27-bp region.

We also sequenced a variable number of nucleotides (ranging from 176 to 661) from an additional two *C. coli*, nine *C. jejuni*, three *C. lari*, and 27 *C. upsaliensis* isolates from New Zealand, South Africa, and the United States to support our initial sequencing results (Table 1). These sequences revealed identity in the regions selected for the species-specific primers. However, an additional four *C. jejuni*, two *C. upsaliensis*, and one *C. lari lpxA* allele were uncovered. In total, the nucleotide sequences of 10 *C. coli*, 9 *C. jejuni*, 9 *C. lari*, and 14 *C. upsaliensis lpxA* alleles have been deposited in GenBank.

Generation of a multiplex PCR assay. Based on the sequence data above, we designed species-specific primers to the unique regions of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. The species-specific primers were tested against purified chromosomal DNA isolated from reference strains of the thermotolerant *Campylobacter*, nonthermotolerant *Campylobacter*, and non-*Campylobacter* isolates (Table 1 and Materials and Methods). The PCR assay showed almost 100% specificity (the ability of the primer to amplify the targeted species) and 97% sensitivity (the ability of the species-specific primer to amplify all members within the species). All of the *C. coli*, *C. jejuni*, and *C. upsaliensis* isolates reacted with their respective specific primer set to generate a PCR amplicon (Fig. 4, Table 1). Only 3 of the 20 *C. lari* isolates failed to generate a *lari*-specific PCR product; these nonreacting *C. lari* isolates have been shown to be genetically diverse from the main group of isolates (8) (Fig. 3C). Within each of the thermotolerant species, an amplicon was only generated with the specific-species primer set, and no cross-reactivity was observed with any of the species-specific primer sets to any other bacterial species tested with the exception of *Campylobacter helveticus*. The C. *upsaliensis*-specific primer set generated a weaker but detectable band in both isolates of *C. helveticus* (data not shown).

Whole-cell lysates made from environmental and clinical *Campylobacter* isolates were also tested by the multiplex PCR assay; 77 isolates from all sources except dog and rabbit feces were identified as *C. jejuni*, and 20 isolates from all sources except beef offal, whole chicken carcasses, and dog and duck feces were identified as *C. coli*. All five of the *C. upsaliensis* isolates identified were from dog feces, whereas four of the six isolates identified as *C. lari* were from surface water. The remaining two isolates were from clinical samples. The species identification for all isolates correlated with their species designations as determined previously (2, 51). These results indicate that a highly accurate species-specific multiplex PCR assay for the rapid identification of thermotolerant species of *Campylobacter* has been achieved. The PCR worked equally well in our hands with purified genomic DNA and whole-cell lysate material from field isolates.

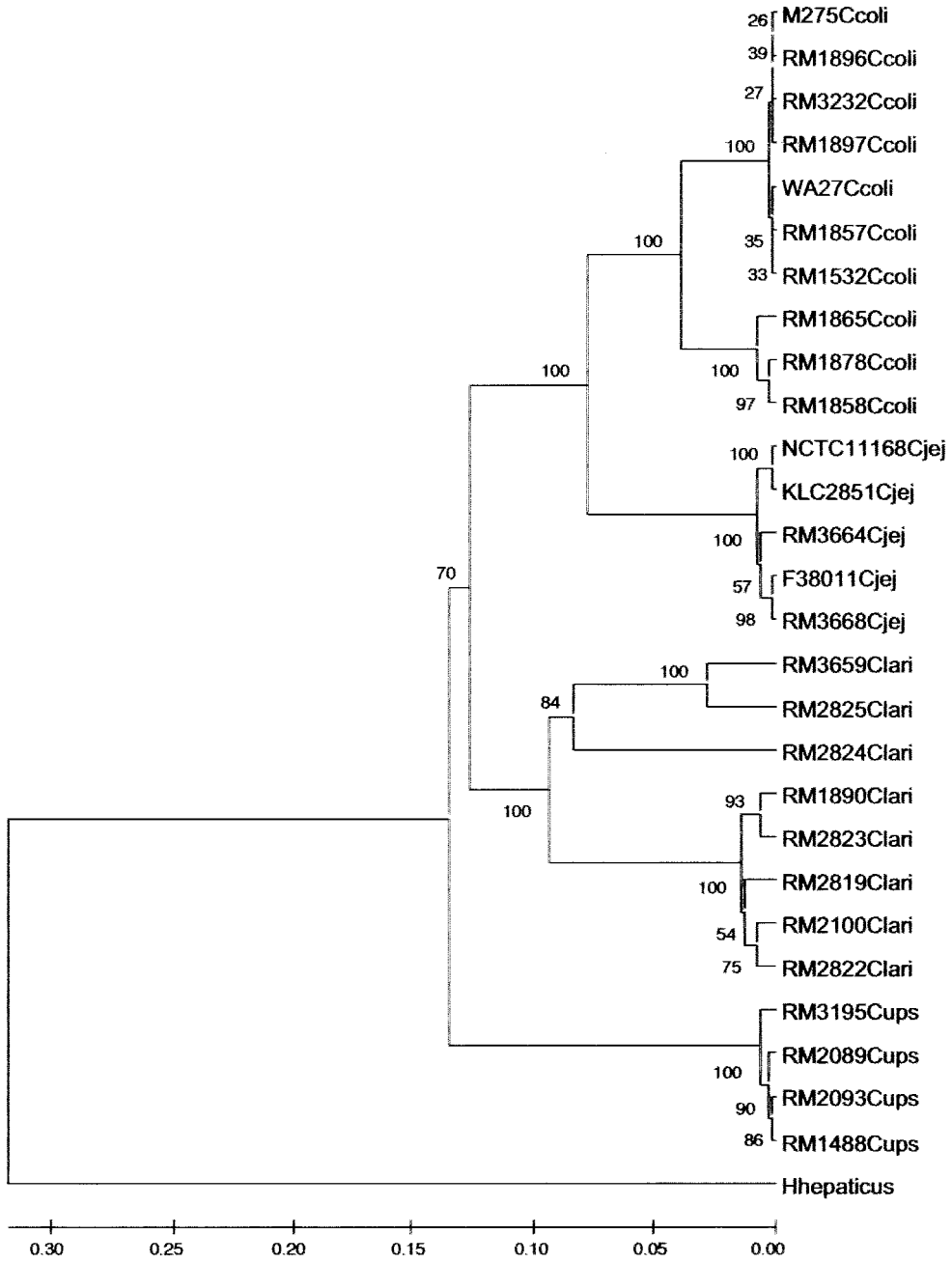

FIG. 2. Phylogenetic analysis of the *lpxA* gene of 53 isolates of thermotolerant *Campylobacter* spp. The *lpxA* gene from isolates of *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* was amplified from genomic DNA by PCR with primers lpxAF0301 and lpxAR0304. Amplicons were sequenced, and the primer regions were removed to facilitate the alignment. In total, 746 bp of *C. jejuni*, and *C. lari* nucleotide sequence, 754 bp of *C. upsaliensis* nucleotide sequence, and 757 bp of *C. coli* nucleotide sequence were aligned. The *lpxA* gene of *Helicobacter hepaticus* (ATCC 51499) was used to root the tree. The scale at the bottom of the figure is a measure of genetic identity. Numbers at the branches indicate the percent bootstrap support for each node. Note that the following isolates were not included in the figure for clarity; the *lpxA* sequences from these isolates were represented as follows. *C. coli* RM3232 also represents isolates RM3230 and RM3231 and M275 also represents isolates RM1051, RM1166, RM1505, RM1530, RM1531, RM1533, RM2225, and RM2228. *C. jejuni* RM3664 also represents isolates RM3665, RM3666, and RM3667; NCTC 11168 also represents ANR0493; RM3668 also represents RM3669 and RM3670; and F38011 also represents isolates RM1221, RM3672, and RM3673. *C. lari* RM2100 also represents RM2099, RM2808, RM2809, RM2817, RM2820, and RM2826 and RM1890 also represents RM2810 and RM2821. *C. upsaliensis*: RM1488 also represents RM2094.

DISCUSSION

The thermotolerant *Campylobacter* species *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* continue to be a major concern with regard to human gastroenteritis. Epidemiological investigations have uncovered numerous point sources for these organisms, but in spite of this, there has been no apparent reduction in reported incidences. Molecular assays, such as the multiplex PCR assay developed in this study, should further expand our

A.

```
            -10         +1                    10
C. jejuni   AGTGGATAAATAA***********TGAAAAAAATTCATCC
C. coli(1)  M-A--C-------GAGAGAATCAG----------------
C. coli(2)  --A--C-------GAGAGAATAAG------G---------
C. coli(s)    AGGCTAAATAAGAGAGAATMAG
C. lari(1)  ------------***********---GT-----------
C. lari(2)  -----------G-***********---GY-----------
C. lari(3)  ------------***********-------T--------
C. upsal    ---------G-G-GAGAATTT***-------G--------
```

B.

```
             40         50         60         70
C. jejuni   GTGCACAACTTGGTGACGATGTTGTARTAGAAGCTTATGC
C. coli(1)  -A--TATTT-A-----T--------TA-T-----------
C. coli(2)  -G--TATTT-A--C--T--------CA-T-----------
C. lari(1)  -R--TAT-A-A-----T--A--AAYYA-----------T-
C. lari(2)  -A--TATTA-A-CW--T---------G-------------
C. lari(3)  -A--TATTA-C-CA--TA-------GA-------------
C. upsal    -A--GATTT-G--C--T--T--GCA-A-T-----------
```

C.

```
            130        140        150        160
C. jejuni   GTGCTCGAATTCTTTCAGATACAACTATAGGYGATCATTC
C. coli(1)  -G--GA----A-----------T--C-----T--------
C. coli(2)  -A--AA----A-----------T--------T--------
C. lari(1)  -Y--AA----CY-RC--A--GTT-AA-----C---G----
C. lari(2)  ----AA-----T-AC-TA--GT---Y--------GA----
C. lari(3)  ----AA-----T-GC--A--GTT-GC-----T--AA----
C. lari(s)              TRCCTAATGTTAAAATAGGCGA
C. upsal    -G---A-----T-AG-C-----G--A-----C---G-AAG
```

D.

```
            160        170        180        190
C. jejuni   GYGATCATTCTCGTGTATTTTCTTATGCHATAGTAGGYGA
C. coli(1)  -T--------------------A-----T--------T--
C. coli(2)  -T---------Y-----------A--C--T-----R--T--
C. lari(1)  -C---G-----AAAA-T-----------T-----T--R--
C. lari(2)  ----GA----AAAA--G-----------C-----W--A--
C. lari(3)  -T--AA-----AAA--T-----------C-----T--G--
C. upsal    -C---G-AAG----A-------Y--C--TTGT--G--T--
```

E.

```
              340        350        360        370
C. jejuni   TTTTATTATGGCTTATTGYCATATTGCTCATGATTGTCTT
C. coli(1)  ------A-----G-----T-----C--A---------C---
C. coli(2)  ------A-----R-----T-----C--A---------C---
C. lari(1)  ---Y--A-----W----CT---------A----------A--
C. lari(2)  ------C----------CT---------G----------AC-
C. lari(3)  ------------A----CT-----A--A---------CA--
C. upsal    C-----A-----A-----T-----A--Y---------CA--
                ATGGCDTATTSTCATATHGCHCATGATTG
```

FIG. 3. Nucleotide sequence alignments of *lpxA* alleles from *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* isolates. The nucleotide sequences shown are composites of all of the sequences of the isolates of each species sequenced with the exception of *C. coli* and *C. lari*. The two major clusters of *C. coli*, designated 1 and 2, are individually displayed, with the species-specific primer shown in bold and underlined below the target species. *C. coli* 1 represent the *C. coli* isolates including M275, while *C. coli* 2 represents isolates such as RM1865. The three major clusters of *C. lari* are designated *C. lari* 1, 2 and 3 and represent isolates including RM1890, RM2825, and RM2824, respectively. An s indicates the species-specific oligonucleotide sequence. The numbering above each panel refers to the nucleotide sequence relative to the *C. jejuni lpxA* start site (indicated as +1 in panel A). Note that the putative start codons for *C. coli* (GTG) and *C. upsaliensis* (TTG) differ from the ATG start codon of *C. jejuni* and *C. lari*. A dash (—) indicates identical nucleotides, and a star (*) indicates the presence of a gap. Divergent bases are indicated with the following nomenclature: R, A or G; Y, C or T; M, A or C; S, C or G; W, A or T; H, A or C or T; and D, A or T or G. Panel A, *C. coli* species-specific primer shown in bold and underlined; panel B, *C. jejuni* species-specific primer shown in bold and underlined; panel C, *C. lari* species-specific primer shown in bold and underlined; panel D, *C. upsaliensis* species-specific primer shown in bold and underlined; panel E; universal *lpxA* reverse primer LpxAKK2Rm is shown in bold and underlined.

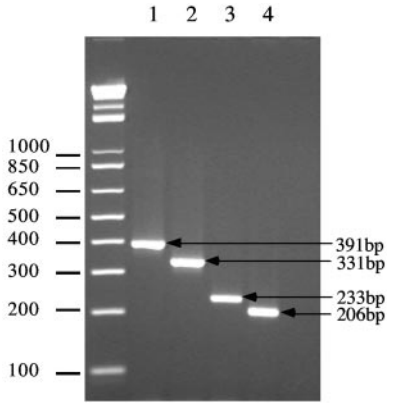

FIG. 4. Multiplex PCR analysis of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis*. Species-specific PCR amplicons were resolved after electrophoresis through a 3% agarose gel. Lane 1, *C. coli* (391 bp); lane 2, *C. jejuni* (331 bp); lane 3, *C. lari* (233 bp); and lane 4, *C. upsaliensis* (206 bp). A DNA ladder (in base pairs) is shown on the left-hand edge of the gel.

knowledge of *Campylobacter* epidemiology by allowing multiple species identification and detection from numerous potential point sources. The *lpxA* sequences that we obtained represent isolates from a range of habitats and geographies, mainly from the United States and New Zealand. Further genotypic data collected by pulsed-field gel electrophoresis support the groupings obtained with the *lpxA* sequence data (data not shown). The multiplex PCR assay described in this report was able to identify *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* strains isolated from both human and environmental sources with either purified genomic DNA or DNA obtained after heat lysis.

The multiplex PCR primers used in this study were based on nucleotide sequences generated from a relatively large sample of thermotolerant isolates. Nucleotide sequence analysis of the *lpxA* gene from *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* strains was readily able to discriminate these bacterial isolates into individual species. Within the *lpxA* sequence, regions of divergence and conservation were located to generate a robust, specific and sensitive PCR assay. Nucleotide sequence analysis also revealed a limited number of *lpxA* alleles within each species. In *C. coli*, an 11-nucleotide intergenic spacer separates the genes *fabZ* and *lpxA*. This spacer is completely absent from *C. jejuni* and *C. lari* isolates, and a modified version is present in *C. upsaliensis*. There are an additional 111 nucleotide changes over the length of the *C. coli* and *C. jejuni lpxA* genes

(14%). Comparatively, the genes encoding heptosyl transferase I, an enzyme necessary for lipopolysaccharide core biosynthesis, and *Campylobacter* adhesion to fibronectin protein (CadF), have 19.8% and 12.6% nucleotide differences, respectively, between *C. coli* and *C. jejuni* (20, 21). Similarly, unique regions were located in the *lpxA* sequences of *C. lari* and *C. upsaliensis*, while overall the sequences were distinct from that of *C. jejuni*.

Recently, the ability of DNA sequences to distinguish between strains of *C. jejuni* and other organisms (6, 7, 43) has been reported. Given the variability in the *lpxA* nucleotide sequence observed within the thermotolerant species in this study, future nucleotide sequence analysis of *lpxA* alleles may also be a useful component in strain discrimination. Unlike the *flaA* gene, which has been analyzed in a similar manner (26), the *lpxA* gene is less likely to be influenced by recombination and is capable of discriminating between the closely related species *C. coli* and *C. jejuni*. While nucleotide sequence data have been used in the design of many of the existing species-specific *Campylobacter* primer sets, there are only a few examples in which the extent of sequence variability among isolates within or across species has been determined (26, 27, 48). Analysis of an increased number of isolates from a single species in the primer target region should facilitate the development of more accurate and precise primer sets.

An unanticipated finding of our study was the apparent heterogeneity that exists at a genetic level between two *C. coli* clusters. Others have argued that bacterial strains that are less than 97% identical at the 16S ribosomal DNA gene level will not give a DNA reassociation of greater than 60%, defining them as possibly new species (41). Clearly caution must be used in making this interpretation based solely on a single genetic marker such as the *lpxA* DNA sequence (47). We are currently in the process of extending our genetic and phenotypic analyses of *C. coli* in order to support our initial findings with the *lpxA* allele.

Taxonomically, urease-positive thermophilic *Campylobacter* spp. have been classified as *C. lari* (3). Several investigators have found that isolates within the *C. lari* taxon are very heterogeneous (8, 25). With multilocus enzyme electrophoresis, Matsuda et al. (25) showed that urease-positive thermophilic campylobacters are genetically hypervariable and form a cluster separate from the main *C. lari* cluster, while Duim et al. (8) used fragment length polymorphism, whole-cell protein profiles, and limited DNA-DNA hybridization analyses to reach a similar conclusion. In fact, Duim et al. suggest that there may be as many as five genogroups.

While our single-gene data do not permit as extensive a phylogenetic analysis as multilocus enzyme electrophoresis or fragment length polymorphism, it may be possible, with the *lpxA* sequence, to rapidly identify members of each genogroup. It is therefore of interest to note that with the eight overlapping strains used in this study and that of Duim et al. (8), identical classification results were obtained (i.e., RM2817, RM2819, RM2821, RM2822, RM2823, and RM2826 are all group 1, RM2825 is a group III isolate, and RM2824 is a group IV isolate). We are in the process of validating this initial observation.

In summary, we have described a multiplex PCR method that rapidly identifies thermotolerant campylobacters as *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*. We are currently expanding the *Campylobacter* taxa distinguished by the *lpxA* gene as well as investigating the relationship among isolates of *C. coli* and *C. lari* as revealed by their *lpxA* nucleotide sequences.

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