Identification of Distinct *Campylobacter lari* Genogroups by Amplified Fragment Length Polymorphism and Protein Electrophoretic Profiles

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Campylobacter lari **is a phenotypically and genotypically diverse species that comprises the classical nalidixic acid-resistant thermophilic campylobacters (NARTC) and the biochemical *C. lari* variants, including the urease-positive campylobacters (UPTC), the nalidixic acid-susceptible campylobacters (NASC), and the urease-producing nalidixic acid-susceptible campylobacters. To study the taxonomic and epidemiological relationships among strains of the *C. lari* variants, amplified fragment length polymorphism (AFLP) profiling and whole-cell protein profile analysis were performed with 55 *C. lari* strains. Great genetic heterogeneity in AFLP and protein profiles was observed. Numerical analysis of AFLP profiles and of partial protein profiles allowed discrimination of four distinct genogroups. AFLP cluster I included nearly homogeneous patterns for *C. lari* NARTC strains (genogroup I). UPTC strains together with non-urease-producing NASC strains produced highly diverse patterns and were placed in genogroup II. The genogroup III strains had the NASC phenotype and produced more homogeneous patterns. Finally, genogroup IV strains had the classical NARTC phenotype and produced AFLP patterns that were very distinct from those of other genogroups. One UPTC strain had aberrant patterns and clustered separately, which may indicate that there is an additional genogroup. Preliminary DNA-DNA hybridization experiments suggested that genogroups I and III represent a single genomic species and that genogroup IV represents a distinct species. The detection of moderate levels of DNA-DNA hybridization between a genogroup II reference strain and genogroup I and III reference strains highlights the need for further DNA-DNA hybridization experiments to clarify the taxonomic status of the former group. No correlation of genogroups with different sources of strains was identified. These data show that UPTC strains are genetically diverse and distinct from NARTC strains. In addition, they indicate that the classical NARTC phenotype encompasses at least two genogroups.**

The most important *Campylobacter* species found in human infections are the thermophilic species *C. jejuni*, *C. coli*, *C. upsaliensis*, and *C. lari* (31). In humans, *C. lari* has been associated with diarrhea (6, 7, 31), with bacteremia in immunocompromised and immunocompetent patients (18, 21, 23), with urinary tract infections (3), with reactive arthritis (15), and, recently, with a prosthetic joint infection (35). Infections after consumption of contaminated shellfish, as well as a large outbreak due to a common waterborne source, have been reported (1, 23). Outbreaks caused by *C. lari* have been incidentally reported, and it is assumed that the number of *C. lari* infections is greatly underreported (12). This may result from the fact that *C. lari* is phenotypically difficult to distinguish from some other *Campylobacter* species, particularly *C. jejuni* and *C. coli*. As molecular typing methods are not always used in clinical diagnostic laboratories, identification is generally limited to the genus level. *C. lari* could easily be distinguished from other thermophilic *Campylobacter* species based on resis-

tance to nalidixic acid until in the 1990s nalidixic acid-resistant *C. jejuni* and *C. coli* strains emerged (10, 22).

C. lari is widely distributed in the environment and can be isolated from a variety of sources, including water and animals. Seagulls have been shown to be a reservoir for *C. lari*, and it has been proposed that they contribute to contamination of water storage reservoirs and of mussels and oyster banks (5, 11, 25).

C. lari variants that differ from the primary nalidixic acid-resistant thermophilic *Campylobacter* (NARTC) strains have emerged, and these strains have been referred to as nalidixic acid-susceptible (NASC) strains, urease-producing (UPTC) strains, and urease-producing nalidixic acid-susceptible strains (19, 25, 33). UPTC strains differ from *C. lari* in producing urease and have been isolated worldwide from water, mussels, and oysters banks (5, 11, 25), and human infections (19). The clinical relevance of the UPTC strains, as well as of other *C. lari* variants, is not yet fully understood. Moreover, differences in virulence among the biochemical *C. lari* variants have not been identified yet.

C. lari can be isolated from several environmental sources and is an infrequent, but possibly underrecognized, cause of human infection. Whether strains associated with human infections have specific characteristics which differ from those of strains from environmental sources is still unknown. In this

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TABLE 1. *C. lari* strains used in this study

Genogroup	Strain[a]	Other designation[a]	Source	Country
I	LMG 9887	CCUG 15035	Seagull	Canada
	LMG 9889	CCUG 19528	Human	Canada
	LMG 7929	CCUG 23948	Dog, feces	United Kingdom
	LMG 9913	CCUG 20581	Human	
	LMG 8845	CCUG 23949	Child, feces	United Kingdom
	LMG 8846[T]	NCTC 1352[T]	Gull	United Kingdom
	LMG 9152	CCUG 24266	Horse, intestine	Sweden
	LMG 9914	CCUG 20593	Human	Canada
	LMG 14338	CA 53	Human, feces	Belgium
	LMG 19175	M595b	Human, feces	Belgium
	R3341	Brug 425	Human, feces	Belgium
II	LMG 19453	CCUG 22396	Human	France
	LMG 7791	CCUG 18267	Water	United Kingdom
	R-11304	CDC D5450	Human	United States
	4870-41		Shellfish	The Netherlands
	2315BVA		Shellfish	The Netherlands
	3363-41		Shellfish	The Netherlands
	LMG 17563	2767A1VAP	Shellfish	The Netherlands
	3365-41		Shellfish	The Netherlands
	LMG 17562	2576PW	Shellfish	The Netherlands
	LMG 17560	2316A3	Shellfish	The Netherlands
	2665PW		Shellfish	The Netherlands
	LMG 17558	2314RG	Shellfish	The Netherlands
	3437-41		Shellfish	The Netherlands
	4698A2		Shellfish	The Netherlands
	LMG 17559	2315RG	Shellfish	The Netherlands
	LMG 17561	2323BVA	Shellfish	The Netherlands
	3363-36		Shellfish	The Netherlands
	2352PW		Shellfish	The Netherlands
	2897P		Shellfish	The Netherlands
	M595a		Human, feces	Belgium
	4207		Shellfish	The Netherlands
	3439-36		Shellfish	The Netherlands
	2352BVA		Shellfish	The Netherlands
	4870-36		Shellfish	The Netherlands
III	LMG 8844	NCTC 11844	Seawater	United Kingdom
	LMG 11760	LCDC 12273	Human	Canada
	R-1189	CA6022	Human, feces	Belgium
	2897R		Shellfish	The Netherlands
	2664BVA		Shellfish	The Netherlands
	2575PW		Shellfish	The Netherlands
IV	LMG 9253	D-411	Human, feces	United Kingdom
	LMG 11251	CA 4129	Human, feces	Belgium
	R-1638	Brug 251	Dialysis fluid	Belgium
	R-4681	3968	Human feces	Belgium
	4604BVA		Shellfish	The Netherlands
	2898R		Shellfish	The Netherlands
	2314BVA		Shellfish	The Netherlands
	3365-36		Shellfish	The Netherlands
	3437-36		Shellfish	The Netherlands
	4604A3		Shellfish	The Netherlands
	2663BVA		Shellfish	The Netherlands
	LMG 17564	2665BVA	Shellfish	The Netherlands
	3364-36		Shellfish	The Netherlands
None	4698PW[b]		Shellfish	The Netherlands

[a] LMG, BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University of Ghent, Ghent, Belgium; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden.
[b] Strain 4698PW does not belong to any of the groups.

study the highly discriminating genotyping method amplified fragment length polymorphism analysis (AFLP) (16, 30), analysis of whole-cell protein profiles (34), and DNA-DNA hybridization studies were combined to establish the phylogeny of *C. lari* variants from several sources.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 55 *C. lari* strains were analyzed. The strain designations and available information about the organisms are shown in Table 1. Fourteen reference strains of *C. lari*, three UPTC strains (LMG 7791, LMG 19453, and R-11304) and five clinical isolates (R-3341,

M595a, R-1189, R-1638, and R-4681) were obtained from the BCCM/LMG Culture Collection (Ghent, Belgium). Thirty-three strains were isolated from oysters and mussels (11).

Bacteria were grown on blood agar plates supplemented with 5% sheep blood at 37°C for 2 to 3 days under microaerobic conditions. An Anoxomat system was used to prepare a final gas mixture consisting of 6% O_2, 7% CO_2, 80% N_2, and 7% H_2 (Mart B.V., Lichtenvoorde, The Netherlands). Bacterial cultures were stored at −80°C in 15% glycerol in heart infusion broth. Routine biochemical tests were performed to determine the hydrolysis of urease and the susceptibility to nalidixic acid (2).

AFLP analysis. The AFLP analysis was performed by using the previously described AFLP method for *Campylobacter* genotyping, which is a protocol adapted from the AFLP microbial fingerprinting method of PE Applied Biosystems (9). Briefly, isolated chromosomal DNA was digested with *Hin*dIII and *Hha*I, and in a simultaneous reaction the fragments were ligated to restriction site-specific adapters for 2 h at 37°C. Then a preselective PCR with the adapter-specific *Hin*dIII primer (5′GACTGCGTACCAGCTT) and *Hha*I primer (5′GATGAGTCCTGATCGC) was performed. Next, an aliquot was subjected to a selective PCR in which both primers contained an additional A at the 3′ end (*Hin*dIII primer, 5′GACTGCGTACCAGCTTA; and *Hha*I primer, 5′ GATGA GTCCTGATCGCA). The 5′ end of the *Hin*dIII primer contained a fluorescent label. The final products were electrophoresed on a 7.3% denaturing acrylamide sequencing gel for 5 h by using an ABI 373A automated DNA sequencer.

Data processing. After electrophoresis, the banding pattern data were collected with the ABI Genescan software (PE Applied Biosystems). Each gel track was then imported into the GelCompar 4.2 software package (Applied Maths, Kortrijk, Belgium) with the program ABICON (Applied Maths). Gels were normalized by using an internal standard (Genescan-500, labeled with the red fluorophore 6-carboxy-x-rhodamine [ROX]; PE Applied Biosystems) that was added to each sample. After normalization of the gels, the levels of genetic similarity between AFLP patterns were calculated with the Pearson product-moment correlation coefficient. For cluster analysis of AFLP banding patterns the unweighted pair group method using average linkages was used.

Polyacrylamide gel electrophoresis of whole-cell proteins. The whole-cell protein profiles of the 14 *C. lari* reference strains and the 33 oyster and mussel isolates have been reported in previous studies (11, 33, 34). In the present study, whole-cell protein extracts were prepared from the eight additional isolates. Extract preparation and polyacrylamide gel electrophoresis were performed as described previously (28). However, only the 20,100- to 36,000-molecular-weight region of the whole-cell protein profile (see Fig. 2) was used for numerical analysis with the GelCompar 4.2 software (Applied Maths).

Determination of the DNA base composition. DNA was enzymatically degraded into nucleosides as described by Mesbah et al. (20). The nucleoside mixture obtained was then separated by high-performance liquid chromatography by using a Waters SymmetryShield C_8 column kept at 37°C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5% acetonitrile. Nonmethylated lambda phage (Sigma, St. Louis, Mo.) was used as the calibration reference.

DNA-DNA hybridization. The levels of DNA-DNA hybridization for strains LMG 8846 (type strain of *C. lari*), R-11304, LMG 11760, and LMG 9253, representatives of genogroups I through IV, respectively, were determined. Chromosomal DNA was isolated by the method described by Pitcher et al. (27). One milligram of DNA was used for DNA-DNA reassociation in a microtiter well by the method described by Ezaki et al. (13). The hybridization temperature was 30°C.

RESULTS

AFLP analysis with restriction endonucleases *Hin*dIII and *Hha*I revealed a considerable degree of genomic heterogeneity among the 55 *C. lari* strains tested. The dendrogram in Fig. 1 shows the genotypic similarities among the AFLP banding patterns obtained, which contained clearly separated bands that varied in size from 100 to 500 bp. Numerical analysis differentiated the AFLP patterns into four distinct clusters, representing genogroups I to IV, and grouped one strain (4698PW) separately. The latter strain may belong to an additional genogroup. The banding patterns of different clusters showed less than 20% similarity, and the pattern of AFLP cluster IV consisted of a significantly lower number of bands (Fig. 1). The reproducibility of the AFLP analysis was deter-

mined by performing the whole assay, starting with isolation of chromosomal DNA, four times for each sample. The similarity between patterns obtained repeatedly was at least 90%, as determined by the Pearson product-moment correlation, and the results correlated perfectly with previously described reproducibility values (9, 24).

The classical *C. lari* strains, which are nalidixic acid resistant and urease negative, clustered in AFLP cluster I. The AFLP banding patterns of these strains were homogeneous, exhibiting more than 60% similarity. Two strains, R-3341 and LMG 7929, had patterns which were 90% similar. The patterns of AFLP cluster II strains were highly diverse, and the UPTC reference strains LMG 19453, LMG 7791, and R-11304 grouped in this cluster. The strains in cluster II were all nalidixic acid sensitive, whereas strains that were both positive and negative for urease activity were present. AFLP cluster III consisted of six additional strains that were nalidixic acid sensitive and urease negative. The banding patterns in this cluster were more homogeneous than the patterns in cluster II, and cluster III was distinguished from cluster II by patterns that exhibited less than 20% similarity (Fig. 1). Strain LMG 8844 produced a highly divergent banding pattern that exhibited only 39% similarity with the other patterns in cluster III. This similarity level corresponded with the intraspecies diversity demonstrated in cluster analysis when AFLP patterns from other *Campylobacter* species were used (8).

The AFLP banding patterns for cluster IV were considerably different from the patterns obtained for the other clusters. The AFLP profiles had only 16 to 24 bands and revealed interstrain genetic diversity (Fig. 1). Biochemical analysis identified these strains as members of a group of non-urease-producing classical NARTC *C. lari* strains.

The whole-cell protein profiles of all of the strains examined were visually very similar but included a variable dense band region, as described previously for *C. lari* and other thermotolerant *Campylobacter* species (4, 25, 34). When this variable dense band region was omitted from the numerical analysis, all isolates examined formed a single tight cluster, as reported previously (34). However, the low-molecular-mass region of the profile (Fig. 2) had additional heterogeneity. If numerical analysis was used only for this region, it resulted in delineation of the same four groups that were observed by AFLP analysis. A dendrogram illustrating this for a subset of the isolates (five randomly chosen strains per cluster) is shown in Fig. 3. The protein patterns of two representative strains for each of the four genogroups are shown in Fig. 2. Strain 4698PW clustered separately as determined by AFLP analysis (Fig. 1) and was identified as *C. lari* by using a whole-cell protein profile without the variable dense band region; however, subsequent analysis of the low-molecular-mass region excluded this organism from the four main genogroups (data not shown). Whole-cell protein electrophoresis and AFLP confirmed that this strain was slightly aberrant and may have indicated that this strain belongs to an additional unidentified distinct genogroup of *C. lari*.

The AFLP and protein profiles revealed no substantial differences between *C. lari* strains isolated from humans and *C. lari* strains isolated from birds, oysters, and mussels. Strains from these sources were present in all AFLP and protein profile groups identified.

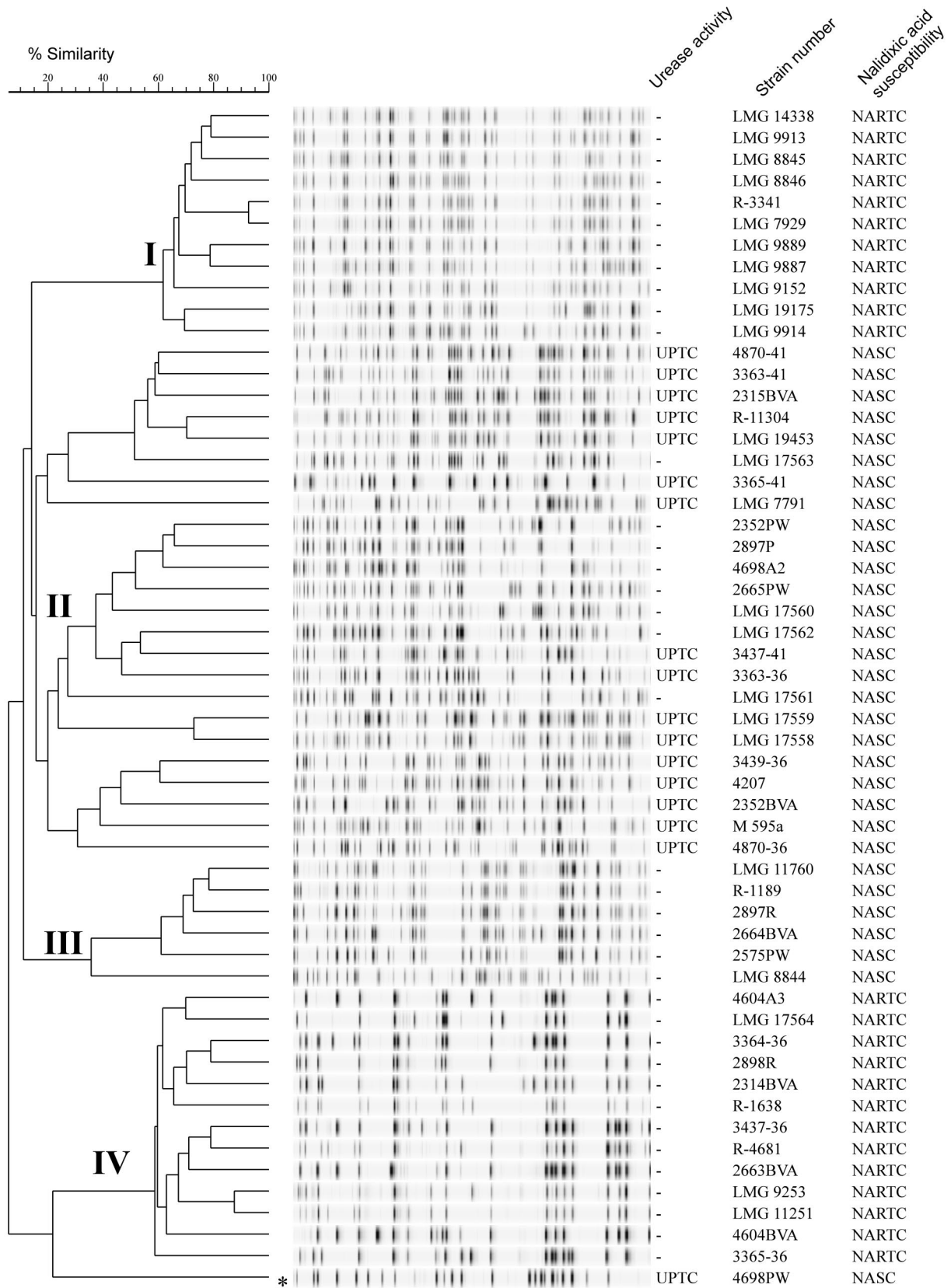|	Urease activity	Strain number	Nalidixic acid susceptibility
	–	LMG 14338	NARTC
	–	LMG 9913	NARTC
	–	LMG 8845	NARTC
	–	LMG 8846	NARTC
	–	R-3341	NARTC
	–	LMG 7929	NARTC
	–	LMG 9889	NARTC
	–	LMG 9887	NARTC
	–	LMG 9152	NARTC
	–	LMG 19175	NARTC
	–	LMG 9914	NARTC
	UPTC	4870-41	NASC
	UPTC	3363-41	NASC
	UPTC	2315BVA	NASC
	UPTC	R-11304	NASC
	UPTC	LMG 19453	NASC
	–	LMG 17563	NASC
	UPTC	3365-41	NASC
	UPTC	LMG 7791	NASC
	–	2352PW	NASC
	–	2897P	NASC
	–	4698A2	NASC
	–	2665PW	NASC
	–	LMG 17560	NASC
	–	LMG 17562	NASC
	UPTC	3437-41	NASC
	UPTC	3363-36	NASC
	–	LMG 17561	NASC
	UPTC	LMG 17559	NASC
	UPTC	LMG 17558	NASC
	UPTC	3439-36	NASC
	UPTC	4207	NASC
	UPTC	2352BVA	NASC
	UPTC	M 595a	NASC
	UPTC	4870-36	NASC
	–	LMG 11760	NASC
	–	R-1189	NASC
	–	2897R	NASC
	–	2664BVA	NASC
	–	2575PW	NASC
	–	LMG 8844	NASC
	–	4604A3	NARTC
	–	LMG 17564	NARTC
	–	3364-36	NARTC
	–	2898R	NARTC
	–	2314BVA	NARTC
	–	R-1638	NARTC
	–	3437-36	NARTC
	–	R-4681	NARTC
	–	2663BVA	NARTC
	–	LMG 9253	NARTC
	–	LMG 11251	NARTC
	–	4604BVA	NARTC
	–	3365-36	NARTC
*	UPTC	4698PW	NASC

FIG. 1. Dendrogram derived from unweighted pair group method using average linkage cluster analysis of AFLP fingerprints of *C. lari* strains analyzed in this study. The scale bar indicates levels of linkage between patterns. The roman numerals indicate the distinct clusters to which the strains belong. The asterisk indicates a highly diverse AFLP pattern that clusters separately.
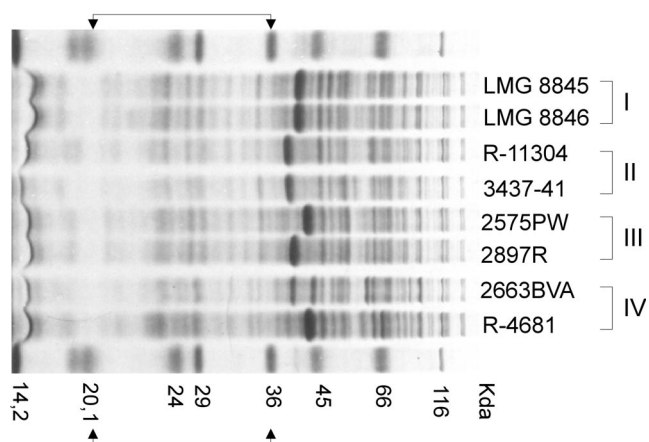
FIG. 2. Whole-cell protein profiles of two representative strains for each *C. lari* genogroup. The roman numerals are AFLP cluster numbers, as explained in the text. The molecular mass markers used (top and bottom lanes) were β-galactosidase (116 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) (observed as a double band), and lysozyme (14.2 kDa). The brackets indicate the region used in the numerical analysis.

A preliminary DNA-DNA hybridization study was performed in order to assess the genomic divergence of the four genogroups. The levels of hybridization among four strains representing the four genogroups are shown in Table 2. A high level of hybridization (80%) between the genogroup I and III
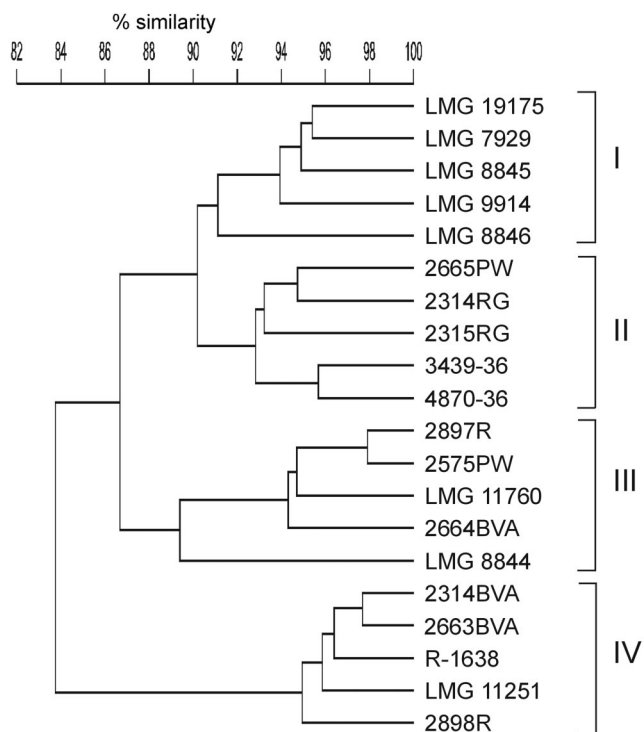

FIG. 3. Dendrogram derived from numerical analysis of partial protein profiles (20,100- to 36,000-molecular-weight region) of five randomly chosen strains from each *C. lari* genogroup. The roman numerals are AFLP cluster numbers, as explained in the text.

TABLE 2. DNA-DNA binding values for strains examined

Genogroup	Strain	DNA binding values with:			
		Genogroup I strain	Genogroup II strain	Genogroup III strain	Genogroup IV strain
I	LMG 8846^T	100			
II	R-11304	62	100		
III	LMG 11760	80	76	100	
IV	LMG 9253	55	59	62	100

isolates was observed. Both of these isolates exhibited low levels of hybridization (55 and 62%) with the representative of genogroup IV. The levels of hybridization of the genogroup II isolate with the other isolates were low to intermediate (59 to 76%). The average G+C content of the strains was determined to be 29%.

DISCUSSION

Genotypic and phenotypic analyses in which numerical analysis of AFLP and whole-cell protein profiles of a set of biochemical variants of *C. lari* (NARTC, NASC, and UPTC strains) was used showed that the variants were separated in four groups and a single separate strain with a unique profile. The NARTC strains were divided into two clusters (clusters I and IV). The second cluster (cluster II) contained UPTC and non-urease-producing strains that were nalidixic acid sensitive (NASC strains). Cluster III contained only non-urease-producing NASC strains.

It has been shown previously that there is extensive heterogeneity at the genetic and protein levels among *C. lari* strains and between different biochemical variants (11, 25, 26). AFLP analysis of *C. lari* detected considerably more diversity in this species than was observed with AFLP analysis of a range of other *Campylobacter* species (8, 24). By using the low-molecular-weight region of the protein profiles the same four groups that were observed with AFLP were observed. Although AFLP genomic fingerprinting analysis has recently been shown to be an accurate approach for determining bacterial taxonomy and the phylogenetic structure of bacteria, no general threshold levels for delineation of bacterial species have been established (29). Therefore, we performed a preliminary DNA-DNA hybridization experiment to quantify the degrees of genomic divergence among the four taxa. The results suggested that genogroups I and III represent a single genomic species and that genogroup IV represents a distinct species. Detection of low to moderate levels of DNA-DNA hybridization between a genogroup II reference strain and genogroup I and III reference strains highlights the need for further DNA-DNA hybridization experiments to clarify the taxonomic status of the former genogroup.

UPTC *C. lari* strains can hydrolyze urea. Whether the UPTC *C. lari* strains deserve separate taxonomic status, either as a distinct species closely related to *C. lari* or as a subspecies or biovar of *C. lari*, is unclear. By using semiquantitative DNA-DNA hybridization, three UPTC isolates have been classified as *C. lari* (19). Whole-cell protein electrophoresis based on analysis of complete protein profiles identified UPTC strains as *C. lari*, and therefore it was proposed that these organisms

should be considered distinct biovars of *C. lari* (25). Recently, On and Harrington (24) showed that there are substantial differences between the AFLP patterns of UPTC and NARTC strains. Our data support this observation. However, these authors also found that the cluster comprising most of the UPTC isolates (cluster II) also comprised urease-negative isolates, indicating that urease activity is not uniformly present in this taxon. In addition, one of the UPTC strains, 4698PW, represented another genogroup within *C. lari*, demonstrating that the capacity to produce urease activity is present in multiple *C. lari*-like campylobacters. These results indicate that the UPTC phenotype is not confined to a single taxon and that it is a variable characteristic within the taxon.

The AFLP patterns of UPTC strains were extremely heterogeneous and confirmed the previously observed variations in randomly amplified polymorphic DNA patterns and GTPase gene sequences of these strains (11, 32). The previously described indistinguishable pulsed-field gel electrophoresis patterns obtained for three UPTC strains from seagulls are inconsistent with these results (17), although this finding may have been affected by sampling bias and the low number of strains tested.

A remarkable observation is the uniform distribution of nalidixic acid susceptibility among the genogroups (Fig. 1). However, as nalidixic acid resistance results from reversible single DNA point mutations in the *gyrA* or *parC* genes (14), this characteristic may be inappropriate for use in classification of *C. lari* strains (23). In this case, it seems likely that the identical nalidixic acid susceptibilities of strains within a genogroup are due to coincidence.

The epidemiology and natural habitat of *C. lari* are largely unknown. Initially, most strains were isolated from gulls, but *C. lari* has now been isolated from a variety of environmental and animal sources. *C. lari* is infrequently isolated from humans, but it is associated with severe diseases in both immunocompetent and immunocompromised hosts. It is still not known whether strains from these sources contain determinants that may contribute to specialization for a niche or whether hydrolysis of urease is an important determinant for human infection. In the present study no correlation between AFLP or protein profiles and a source (human, shellfish, animal) could be identified.

In conclusion, *C. lari* shows extensive genetic diversity. By using AFLP and whole-cell protein analysis, the *C. lari* population studied could be divided into four clearly distinct genogroups and an additional unique isolate. Preliminary DNA-DNA hybridization experiments indicated that two genogroups (genogroups I and III) represent a single genomic species and suggested that one of the genogroups (genogroup IV) deserves separate species rank. The taxonomic relationships within genogroup II and between genogroup II and the other taxa require further analysis by DNA-DNA hybridization. We obtained no evidence for host specificity of the genogroups. Molecular characterization of more *C. lari* isolates from various sources is needed to elucidate the epidemiology of *C. lari* genogroups in the environment and in human infection.

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