

Multilocus Sequence Typing System for *Campylobacter jejuni*

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The gram-negative bacterium *Campylobacter jejuni* has extensive reservoirs in livestock and the environment and is a frequent cause of gastroenteritis in humans. To date, the lack of (i) methods suitable for population genetic analysis and (ii) a universally accepted nomenclature has hindered studies of the epidemiology and population biology of this organism. Here, a multilocus sequence typing (MLST) system for this organism is described, which exploits the genetic variation present in seven housekeeping loci to determine the genetic relationships among isolates. The MLST system was established using 194 *C. jejuni* isolates of diverse origins, from humans, animals, and the environment. The allelic profiles, or sequence types (STs), of these isolates were deposited on the Internet (<http://mlst.zoo.ox.ac.uk>), forming a virtual isolate collection which could be continually expanded. These data indicated that *C. jejuni* is genetically diverse, with a weakly clonal population structure, and that intra- and interspecies horizontal genetic exchange was common. Of the 155 STs observed, 51 (26% of the isolate collection) were unique, with the remainder of the collection being categorized into 11 lineages or clonal complexes of related STs with between 2 and 56 members. In some cases membership in a given lineage or ST correlated with the possession of a particular Penner HS serotype. Application of this approach to further isolate collections will enable an integrated global picture of *C. jejuni* epidemiology to be established and will permit more detailed studies of the population genetics of this organism.

Campylobacter jejuni is the most common causative agent of human enterocolitis in many industrialized countries, representing a substantial drain on public health resources. Typically, infection is associated with sudden onset of fever, abdominal cramps, and diarrhea containing blood and leukocytes (16, 30, 34). Sequelae occur occasionally, and links have been made between infection by particular *C. jejuni* serotypes and Guillain-Barré syndrome (24). The gram-negative bacterium is widespread in the environment (13), forming part of the natural intestinal flora of birds and mammals (17). The handling or consumption of raw or undercooked meat products, particularly chicken contaminated during slaughter, is often implicated in disease (3). However, the majority of *C. jejuni* infections are considered to be sporadic, with the source of infection remaining unidentified (2). Occasional larger-scale outbreaks of campylobacteriosis have been linked with consumption of contaminated water or raw milk (17, 27).

A plethora of methods have been developed to discriminate *C. jejuni* isolates for the investigation of epidemiology and infections. However, the lack of widely available reagents for methods such as serotyping has limited their general use. Genotyping methods have been developed (36), but the techniques and their interpretation have not been standardized or

broadly accepted. This, coupled with the lack of a universal nomenclature system for isolate profiles, has prevented the development of an international campylobacter typing database.

Multilocus sequence typing (MLST) (20) has been successful in the characterization of several other bacteria (1, 8, 20, 33). This technique employs the same philosophy as multilocus enzyme electrophoresis (29), in that neutral genetic variation from multiple chromosomal locations is indexed, but exploits nucleotide sequence determination to identify this variation. In MLST studies of other bacteria, stretches of nucleotide sequence of ~500 bp from 7 loci provided discrimination approximately equivalent to that obtained with 15 to 20 loci in multilocus enzyme electrophoresis analyses (20). Sequence data are readily compared among laboratories and lend themselves to electronic storage and distribution. Furthermore, MLST can reduce the need to transport live bacteria, since nucleotide sequence determination from PCR products can be achieved from killed-cell suspensions, purified DNA, or clinical material. A World Wide Web site for the storage and exchange of data and protocols for MLST has been established (<http://mlst.zoo.ox.ac.uk>). While MLST is particularly suited to long-term and global epidemiology, as it identifies variation which is accumulating slowly within a population (20), the data can be used in the investigation of individual outbreaks, especially when MLST data are combined with other data, such as the nucleotide sequences of genes encoding antigens (6, 9).

Here, an MLST scheme for *C. jejuni* is described. The system is based on the nucleotide sequences of seven housekeeping loci and was established by the examination of 194 isolates obtained from a variety of sources. A total of 155 distinct

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sequence types (STs) were identified, which were resolved into 62 clonal lineages or complexes. There was evidence for extensive horizontal genetic exchange, including import of alleles from at least two other *Campylobacter* species, including *C. coli*. Further analysis indicated that *C. jejuni* had a weakly clonal population structure and that some complexes were associated with particular Penner HS serotypes. These results provide a basis for further investigations of the epidemiology and population genetics of *C. jejuni* by MLST.

MATERIALS AND METHODS

Campylobacter isolates. *C. jejuni* isolates were obtained from the collection held at Preston Public Health Laboratory, Preston, United Kingdom (35), and included isolates from cases of human campylobacteriosis, livestock, and the environment. Reference isolates for the Penner heat-stable antigen serotyping scheme (28) were donated to the Preston Public Health Laboratory culture collection by J. Penner and are available from the National Collection of Type Cultures and American Type Culture Collection. Eleven further isolates were obtained from a collection held in The Netherlands at the Research Laboratory for Infectious Diseases, at the National Institute of Public Health and the Environment, and at the Department of Bacteriology, Institute for Animal Science and Health. Of these, the nonhuman isolates were obtained from geographically dispersed farms in The Netherlands (11), and human isolates were obtained from a case-control study among general practitioners in The Netherlands. The total of 194 isolates comprised 79 from human cases of campylobacteriosis, 38 from livestock, 34 from environmental locations, 3 from milk, and 40 of the reference isolates for the Penner serotyping scheme. Of the human isolates, 75 were from cases which occurred in the United Kingdom during 1990 and 1991, 3 were isolated in the Netherlands during 1997 and 1998, and 1 was obtained from a case in Australia during 1998. The livestock isolates included 34 from chickens, 3 from cattle, and 1 from a duck (including 24 isolates from the United Kingdom, 5 from The Netherlands, and 5 from New Zealand, all isolated during the 1990s). The three isolates from milk were obtained in the United Kingdom during 1991, and all of the environmental isolates were from the sand of bathing beaches (United Kingdom, 1994 and 5), with the exception of one, which was from water (United Kingdom, 1991).

Culture of isolates and preparation of chromosomal DNA. All of the bacterial isolates included had been maintained with minimal passages at -70°C in 20% (vol/vol) glycerol in brain heart infusion broth, and consequently none of the genetic variation studied was likely to have been introduced during storage. Prior to DNA extraction, cultures were removed from storage and allowed to thaw at room temperature. For each isolate, a blood agar plate was spread for discrete colonies and incubated at 37°C for 72 h under microaerobic conditions (5). Chromosomal DNA was extracted using an Isoquick kit (Microprobe Corporation) or Wizard genomic DNA purification kit (Promega, Madison, Wis.).

Choice of loci. A number of candidate loci, encoding enzymes responsible for intermediary metabolism, were identified by searching the *C. jejuni* genome database (http://www.sanger.ac.uk/Projects/C_jejuni/) (26) with gene sequences from other bacteria. Suitable genes were then chosen on the basis of a number of criteria, including chromosomal location, suitability for primer design, and sequence diversity in pilot studies. The following seven loci were chosen for the MLST scheme (protein products are shown in parentheses): *aspA* (aspartase A), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), and *uncA* (ATP synthase α subunit). The chromosomal locations of these housekeeping loci suggested that it was unlikely for any of them to be co-inherited in the same recombination event, as the minimum distance between loci was 70 kb (Fig. 1).

Amplification and nucleotide sequence determination. PCR products were amplified with oligonucleotide primer pairs designed from the published *C. jejuni* sequence (26). A range of primers were tested, with those shown in Table 1 providing reliable amplification from a diverse range of samples (additional dideoxynucleotide primers are described at <http://mlst.zoo.ox.ac.uk>). Each 50- μl amplification reaction mixture comprised ~ 10 ng of campylobacter chromosomal DNA, 1 μM each PCR primer, 1 \times PCR buffer (Perkin-Elmer Corp.), 1.5 mM MgCl_2 , 0.8 mM deoxynucleoside triphosphates, and 1.25 U of Amplitaq polymerase (Perkin-Elmer Corp.). The reaction conditions were denaturation at 94°C for 2 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min, for 35 cycles. The amplification products were purified by precipitation with 20% polyethylene glycol-2.5 M NaCl (7), and their nucleotide sequences were determined at least once on each DNA strand using internal nested primers

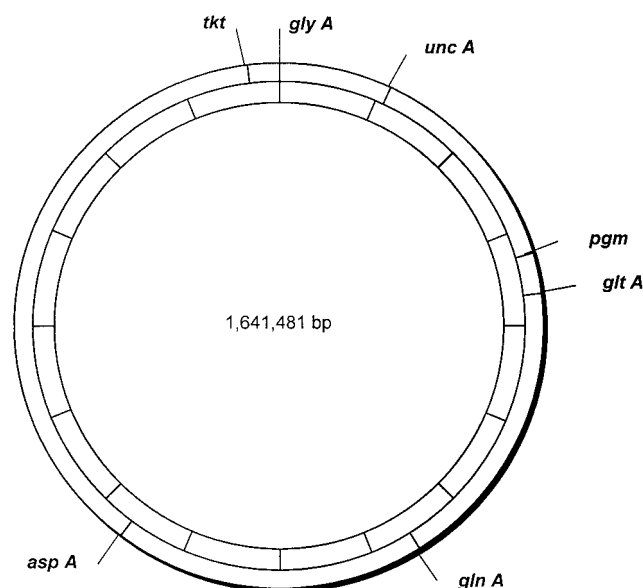


FIG. 1. Chromosomal locations of MLST loci. The positions of the seven loci are shown on a map of the *C. jejuni* chromosome derived from the genome sequence of isolate NCTC 11168 (http://www.sanger.ac.uk/Projects/C_jejuni/). The 1,641,481-bp genome is divided into 10 segments (indicated on the inner circle), with each segment representing 164,148 bp.

(Table 1) and BigDye Ready Reaction Mix (PE Biosystems) in accordance with the manufacturer's instructions. Unincorporated dye terminators were removed by precipitation of the termination products with 95% ethanol, and the reaction products were separated and detected with an ABI Prism 3700 or an ABI Prism 377 automated DNA sequencer (PE Biosystems). Sequences were assembled from the resultant chromatograms with the STADEN suite of computer programs (32).

Allele and ST assignment. For each locus, distinct allele sequences were assigned arbitrary allele numbers in order of identification; these were in-frame internal fragments of the gene which contained an exact number of codons. Each isolate was therefore designated by seven numbers, constituting an allelic profile or ST. The data were deposited in a database accessible on the Internet at <http://mlst.zoo.ox.ac.uk/>. The STs were identified by arbitrary numbers assigned in order of description (e.g., ST-1). New sequences were assigned allele numbers and isolates were assigned their STs by interrogating the database. Allele numbers for new sequences and ST numbers for new allelic profiles are available by submission to the database.

The STs were grouped into lineages or clonal complexes using the program BURST (E. J. Feil and M.-S. Chan, available at <http://mlst.zoo.ox.ac.uk>). The members of a lineage were defined as groups of two or more independent isolates with an ST that shared identical alleles at four or more loci. Each lineage was named after the ST identified as the putative founder of the group by BURST, followed by the word "complex" (e.g., ST-21 complex).

Phylogenetic analyses. The degree of clonality within the data set was estimated by measuring the index of association (I_a) and was calculated for all STs and for a subset of STs representative of each lineage with a program written by J. Maynard Smith (23). The relationships among the STs in a given complex were investigated by constructing a distance matrix of allelic mismatches with the program MLD DISTANCE MATRIX (K. A. Jolley). Each locus difference was treated identically in that no relationships among the different alleles were assumed. The distance matrix was then visualized by Split decomposition analysis using SPLITSTREE version 3.1 (14, 21). Where necessary, higher resolution of the splits graph was obtained by progressively pruning resolved branches, and the graphs were annotated by reference to the allelic profiles. Other data analyses, including calculation of d_N/d_S , were performed using the MEGA suite of programs (18). All of the programs were available for electronic downloading (<http://mlst.zoo.ox.ac.uk>, <http://bibserv.techfak.uni-bielefeld.de/splits>, and <http://evolgen.biol.metro-u.ac.jp/MEGA/>).

TABLE 1. Oligonucleotide primers for *Campylobacter* MLST

Locus	Function	Dideoxyoligonucleotide primer		Amplicon size (bp)
		Name and sequence		
		Forward	Reverse	
<i>asp</i>	Amplification	asp-A9, 5'-AGT ACT AAT GAT GCT TAT CC-3'	asp-A10, 5'-ATT TCA TCA ATT TGT TCT TTG C-3'	899
	Sequencing	asp-S3, 5'-CCA ACT GCA AGA TGC TGT ACC-3'	asp-S6, 5'-TTA ATT TGC GGT AAT ACC ATC-3'	
<i>gln</i>	Amplification	gln-A1, 5'-TAG GAA CTT GGC ATC ATA TTA CC-3'	gln-A2, 5'-TTG GAC GAG CTT CTA CTG GC-3'	1,262
	Sequencing	gln-S3, 5'-CAT GCA ATC AAT GAA GAA AC-3'	gln-S6, 5'-TTC CAT AAG CTC ATA TGA AC-3'	
<i>glt</i>	Amplification	glt-A1, 5'-GGG CTT GAC TTC TAC AGC TAC TTG-3'	glt-A2, 5'-CCA AAT AAA GTT GTC TTG GAC GG-3'	1,012
	Sequencing	glt-S1, 5'-GTG GCT ATC CTA TAG AGT GGC-3'	glt-S6, 5'-CCA AAG CGC ACC AAT ACC TG-3'	
<i>gly</i>	Amplification	gly-A1, 5'-GAG TTA GAG CGT CAA TGT GAA GG-3'	gly-A2, 5'-AAA CCT CTG GCA GTA AGG GC-3'	816
	Sequencing	gly-S3, 5'-AGC TAA TCA AGG TGT TTA TGC GG-3'	gly-S4, 5'-AGG TGA TTA TCC GTT CCA TCG C-3'	
<i>pgm</i>	Amplification	pgm-A7, 5'-TAC TAA TAA TAT CTT AGT AGG-3'	pgm-A8, 5'-CAC AAC ATT TTT CAT TTC TTT TTC-3'	1,150
	Sequencing	pgm-S5, 5'-GGT TTT AGA TGT GGC TCA TG-3'	pgm-S2, 3'-TCC AGA ATA GCG AAA TAA GG-3'	
<i>tkl</i>	Amplification	tkl-A3, 5'-GCA AAC TCA GGA CAC CCA GG-3'	tkl-A6, 5'-AAA GCA TTG TTA ATG GCT GC-3'	1,102
	Sequencing	tkl-S5, 5'-GCT TAG CAG ATA TTT TAA GTG-3'	tkl-S4, 5'-ACT TCT TCA CCC AAA GGT GCG-3'	
<i>unc</i>	Amplification	unc-A7, 5'-ATG GAC TTA AGA ATA TTA TGG C-3'	unc-A2, 5'-GCT AAG CGG AGA ATA AGG TGG-3'	1,120
	Sequencing	unc-S5, 5'-TGT TGC AAT TGG TCA AAA GC-3'	unc-S4, 5'-TGC CTC ATC TAA ATC ACT AGC-3'	

RESULTS

Diversity of housekeeping genes. The alleles defined for the MLST scheme were between 402 bp (*gltA*) and 507 bp (*glyA*) in length, and between 27 (*gltA* and *unc*) and 46 (*pgm*) alleles were present per locus. The proportion of variable sites present in the MLST alleles ranged from 9.2% (*pgm*) to 21.2% (*tkl*). In part, this was due to the polymorphisms present in a minority of alleles which were divergent (11 to 15% nucleotide sequence difference) from all other allele sequences. Such alleles were observed at least once for each of the MLST loci and were present in a total of 11 isolates, which had divergent alleles at between one and six loci. Searches of the GenBank database established that two of these, at the *gltA* locus, were very similar (97% nucleotide sequence identity) to the sequence of this gene from *C. coli*. When the divergent alleles were removed from analysis, the remaining allele sequences had between 5.2% (*aspA*) and 11.8% (*pgm*) variable sites (Table 2).

The proportion of nucleotide changes which changed the amino acid sequence was calculated and indicated by d_N (non-synonymous base substitutions), and the proportion of nucleotide changes which did not change the amino acid sequence was indicated by d_S (synonymous base substitutions). The d_N/d_S ratios were calculated for all seven loci and were much less than 1 whether or not the more divergent alleles were included in the analysis (Table 2). The frequency that each allele occurred in the sample population is shown in Fig. 2; in

each case several alleles predominated, with the remainder observed in one or two isolates.

STs and lineages. There were a total of 155 STs among the 194 isolates examined, 140 (90%) of which were present only once, with the most common ST (ST-21) occurring eight times in the dataset. Assignment of the STs to lineages established that 51 STs were both unique and unrelated to any others (data are available at <http://mlst.zoo.ox.ac.uk/>). The remaining isolates were assigned to 11 complexes: the ST-21 complex was the largest, with 56 members; the ST-45 complex had 23 members; and the ST-179 complex comprised 7 STs. There were two lineages with three member STs (two lineages) and six lineages with two members (Table 3). The I_A for the complete data set was 2.016, with a value of 0.5671 obtained when only one representative of each lineage was included.

Interrelationships of members of the ST-21 complex. The relationships among members of the ST-21 complex were visualized by an annotated splits graph of a distance matrix generated by pairwise comparisons of the allelic profiles (Fig. 3). The unresolved splits graph including all members of the complex is shown at the top left of Fig. 3. The outer branches were then pruned to show a partially resolved network, which was further pruned to resolve completely the center of the graph. Consistent with the lineage assignment by BURST, this placed the predicted founder ST at a central position of the split graph. The relationships among other members of the group were assessed by examining the number of nodes (representing the number of changes) between two isolates. For example, in the central region of the graph, ST-21 and ST-19 are separated by one node, representing one allele change between the two. Their STs are 2-1-1-3-2-1-5 and 2-1-5-3-2-1-5, respectively. ST-21 and ST-31 were separated by two nodes and differed by two alleles (ST-31 is 2-20-12-3-2-1-5).

Relationships of lineage, source, and serotype. Isolates belonging to the two largest lineages present in the data set, the ST-21 complex and ST-45 complex, had originated from a diversity of sources (Table 3). The ST-21 complex included 59 of the 79 human isolates studied (75%), 14 of the 34 chicken isolates (41%), 7 of the 33 sand isolates (21%), 3 of the 3 cattle isolates (100%), and 3 of the 3 milk isolates (100%). The ST-45

TABLE 2. Genetic diversity at *C. jejuni* MLST loci^a

Locus	Fragment size (bp)	No. of alleles	No. of variable sites	% Variable sites	d_N/d_S
<i>aspA</i>	477	37 (35)	67 (25)	14 (5.2)	0.055 (0.049)
<i>glnA</i>	477	39 (36)	69 (30)	14.4 (6.3)	0.045 (0.071)
<i>gltA</i>	402	27 (25)	63 (32)	15.7 (8)	0.059 (0.057)
<i>glyA</i>	507	37 (35)	107 (59)	21.1 (11.6)	0.058 (0.057)
<i>pgm</i>	498	46 (45)	108 (59)	21.7 (11.8)	0.048 (0.038)
<i>tkl</i>	459	37 (32)	98 (48)	21.3 (10.5)	0.033 (0.037)
<i>uncA</i>	489	27 (25)	91 (41)	18.6 (8.4)	0.028 (0.036)

^a The values in parentheses exclude alleles which were likely to have originated in other species.

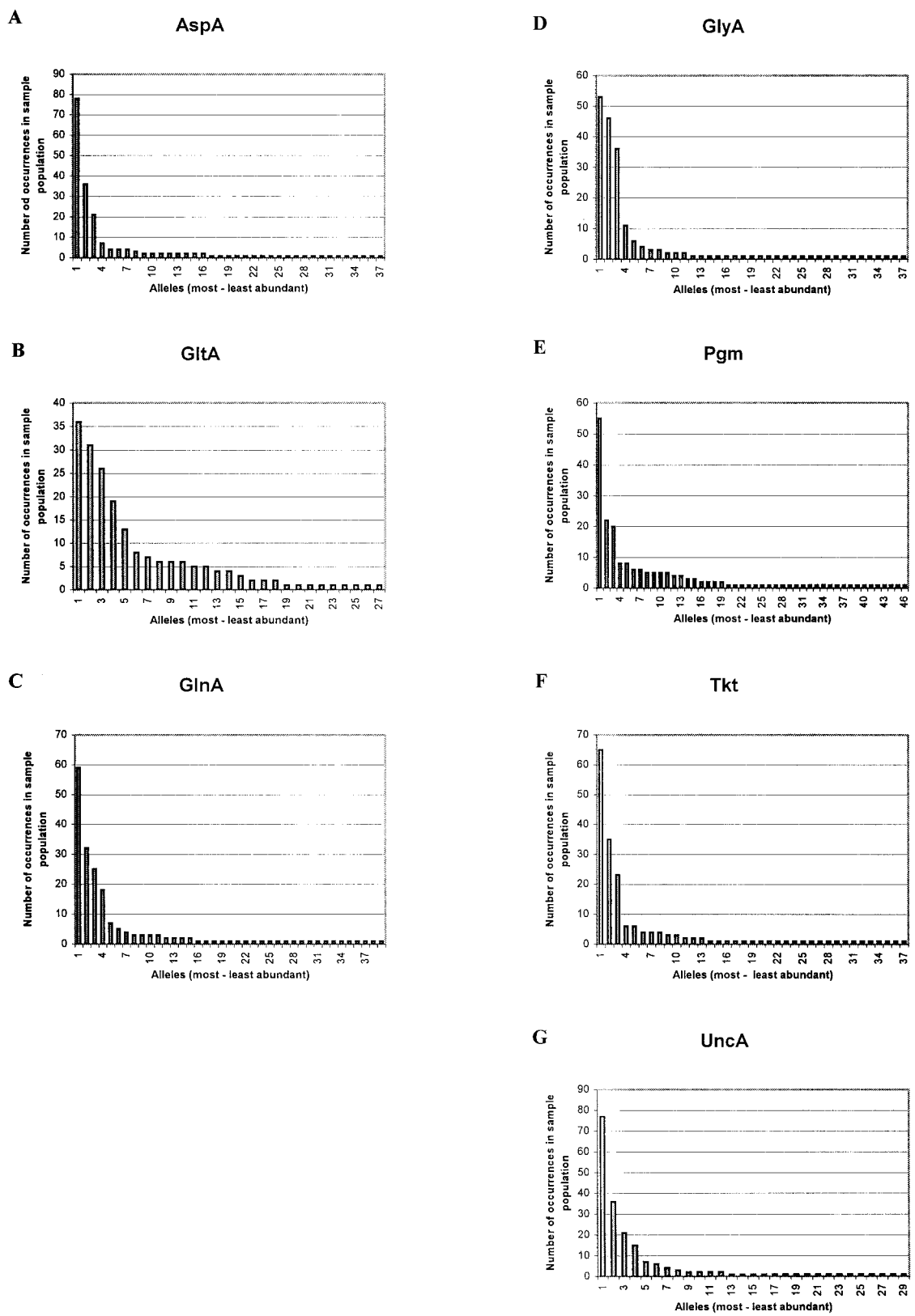


FIG. 2. Allele frequencies in the sample population. For each of the seven loci (A to G), the number of times that each allele occurs in the isolate collection is shown. The frequencies are shown in the order of most to least abundant.

TABLE 3. *C. jejuni* lineages

Lineage	ST	Isolate				
		Name ^a	Source	Yr isolated	Country	Penner serotype ^c
ST-21 complex	13	P02 (ATCC 43430)	Calf			2
		2692	Human	1991	United Kingdom	2
	18	313	Human	1990	United Kingdom	1
	19	2167	Human	1991	United Kingdom	1
		304	Chicken	1990	United Kingdom	1
		307	Human	1990	United Kingdom	1
		3907	Human	1991	United Kingdom	1
		319	Human	1990	United Kingdom	1
		316	Human	1990	United Kingdom	1
	20	3618	Human	1991	United Kingdom	2
	21	2248	Human	1991	United Kingdom	2
		3616	Milk	1991	United Kingdom	2
		2567	Human	1991	United Kingdom	2
		2836	Human	1991	United Kingdom	NT ^d
		3175	Human	1991	United Kingdom	2
		3617	Milk	1991	United Kingdom	2
		2269	Human	1991	United Kingdom	2
		1576	Human	1991	United Kingdom	NT
	31	321	Human	1990	United Kingdom	1
	32	322	Human	1991	United Kingdom	1
	33	333	Human	1990	United Kingdom	1
	35	327	Human	1990	United Kingdom	1
	36	1741	Human	1992	United Kingdom	4c
	38	1835	Human	1992	United Kingdom	NT
	43	NCTC 11168 ^b	Human	1977	United Kingdom	2
	44	161H ^f	Chicken	1998	The Netherlands	1, 44
	47	79203	Sand	1994–1995	United Kingdom	10
		79202	Sand	1994–1995	United Kingdom	10
		79204	Sand	1994–1995	United Kingdom	10
	48	Cy6412	Cattle	1998	The Netherlands	
	50	2817	Water	1991	United Kingdom	2
		314	Human	1991	United Kingdom	1
		1951	Chicken	1990	United Kingdom	1
		309	Chicken	1990	United Kingdom	1
	53	2399	Human	1991	United Kingdom	2
		3281	Human	1991	United Kingdom	2
		2457	Human	1991	United Kingdom	2
		C356	Chicken	1990	The Netherlands	2
	61	1589	Cattle	1991	The Netherlands	13
		2018	Human	1992	United Kingdom	4c
		1739	Human	1992	United Kingdom	4c
		2019	Human	1992	United Kingdom	4c
		2037	Human	1992	United Kingdom	4c
	67	2473	Chicken	1991	United Kingdom	1
	69	79201	Sand	1994–1995	United Kingdom	1
	72	1441	Cattle	1993	New Zealand	13, 50
	75	3615	Milk	1991	United Kingdom	2
	76	1434	Chicken	1993	New Zealand	2
	79	3748	Human	1991	United Kingdom	4
	86	P4 (NCTC 12561)				4
		1939	Chicken	1990	United Kingdom	1
	90	3897	Human	1991	United Kingdom	2
	91	79178	Sand	1994–1995	United Kingdom	4
	93	1529	Human	1993	United Kingdom	NT
		1564	Human	1992	United Kingdom	NT
		1715	Human	1992	United Kingdom	4c
		2017	Human	1992	United Kingdom	4c
		2035	Human	1992	United Kingdom	4c
		3222	Human	1991	United Kingdom	4
	98	79238	Sand	1994–1995	United Kingdom	4
	102	337	Human	1990	United Kingdom	1
	103	1656	Human	1992	United Kingdom	
	104	3782	Human	1991	United Kingdom	4
	105	3174	Human	1991	United Kingdom	2
	107	2945	Human	1991	United Kingdom	2
	108	2879	Human	1991	United Kingdom	2

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TABLE 3—Continued

Lineage	ST	Isolate				
		Name ^a	Source	Yr isolated	Country	Penner serotype ^c
	110	2582	Human	1991	United Kingdom	2
	111	2546	Human	1991	United Kingdom	2
	112	2255	Human	1991	United Kingdom	2
	114	2160	Human	1991	United Kingdom	2
	118	1950	Chicken	1990	United Kingdom	1
	119	2241	Human	1991	United Kingdom	2
	120	2856	Human	1991	United Kingdom	44
	124	317	Chicken	1990	United Kingdom	1
	125	326	Human	1990	United Kingdom	1
	135	2386	Chicken	1991	United Kingdom	1
	136	79205	Sand	1994–1995	United Kingdom	10
	141	2272	Human	1991	United Kingdom	2
	142	2325	Human	1991	United Kingdom	4
	156	P50 (ATCC 43465)	Human	1983		50
	157	330	Human	1990	United Kingdom	1
	159	1827	Human	1992	United Kingdom	NT
	161	3827	Human	1991	United Kingdom	4
	164	3550	Human	1991	United Kingdom	2
	165	1953	Chicken	1991	United Kingdom	1
	167	2529	Chicken	1991	United Kingdom	2
	169	2844	Human	1991	United Kingdom	2
	170	2987	Human	1991	United Kingdom	2
ST-45 complex	1	P9 (ATCC 43437)	Goat			9
	2	P12 (ATCC 43440)	Human			12
	6	P27 (ATCC 43450)	Human			27
	8	P33 (ATCC 43454)	Human			33
	10	P55 (ATCC 43468)	Human			55
	25	1429	Chicken	1991		9
	45	3057	Chicken	1991	United Kingdom	60
		P7 (ATCC 43435)	Human			7
	66	3109	Chicken	1991	United Kingdom	6
	68	3105	Chicken	1991	United Kingdom	4, 16, 50
	70	79228	Sand	1994–1995	United Kingdom	38
	77	2656	Chicken	1991	United Kingdom	27
	88	P42 (ATCC 43461)	Human		Canada	42
	94	3110	Chicken	1991	United Kingdom	60
	95	3188	Chicken	1991	United Kingdom	NT
	97	1436	Chicken	1993	New Zealand	NT
	109	2809	Human	1991	United Kingdom	55
	128	P38 (ATCC 43458)	Human			38
	137	P45 (ATCC 43464)	Human			45
	146	87034	Sand	1994–1995	United Kingdom	NT
	163	2924	Chicken	1991	United Kingdom	4, 13, 50
	168	2897	Human	1991	United Kingdom	3, 37
	171	3108	Chicken	1991	United Kingdom	6
	173	3052	Chicken	1991	United Kingdom	4, 16, 50
ST-179 complex	80	79125	Sand	1994–1995	United Kingdom	2
	99	79129	Sand	1994–1995	United Kingdom	5
	100	79207	Sand	1994–1995	United Kingdom	2
	117	79045	Sand	1994–1995	United Kingdom	5
	152	79371	Sand	1994–1995	United Kingdom	2
	153	79372	Sand	1994–1995	United Kingdom	2
	179	78972	Sand	1994–1995	United Kingdom	5
ST-22 complex	16	P19 (ATCC 43446)	Human			19
	22	3201	Human	1991	United Kingdom	19
		1997–1591	Human	1997	The Netherlands	19
	78	3779	Human	1991	United Kingdom	19
ST-177 complex	81	79260	Sand	1994–1995	United Kingdom	55
	144	79308	Sand	1994–1995	United Kingdom	NT
	177	79309	Sand	1994–1995	United Kingdom	NT
ST-17 complex	14	P11 (ATCC 43439)	Human		Canada	11

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TABLE 3—Continued

Lineage	ST	Isolate				
		Name ^a	Source	Yr isolated	Country	Penner serotype ^c
	17	3157	Human	1991	United Kingdom	11
		2475	Human	1991	United Kingdom	11
ST-51 complex	27	P37 (ATCC 43457)	Human			37
	51	160H	Chicken	1998	The Netherlands	
ST-65 complex	34	335	Human	1990	United Kingdom	1
	65	323	Chicken	1990	United Kingdom	1
ST-52 complex	52	c2143	Chicken	1991	The Netherlands	10
	172	2320	Human	1991	United Kingdom	
ST-125 complex	125	326	Human	1990	United Kingdom	1
	135	2386	Chicken	1991	United Kingdom	1
ST-130 complex	130	P64 (ATCC 49302)	Human			64
	162	P65 (ATCC 49303)	Not stated			65

^a Where appropriate the American Type Culture Collection (ATCC) or National Collection of Type Cultures (NCTC) designation is also given. Names beginning with P indicate reference isolates for the Penner serotyping scheme.

^b *Campylobacter* isolate for which the complete genome sequence is available at http://www.sanger.ac.uk/Projects/C_jejuni/.

^c From reference 28.

^d NT, nontypeable.

complex included 10 of the 79 human isolates (13%), 11 of the 34 chicken isolates (32%), and 2 of the 33 sand isolates (6%). Three Penner HS serotypes predominated in the ST-21 complex (HS1, 25%; HS2, 33%; and HS4, 8%), and some of the STs forming this lineage were homogenous for serotype (e.g., the six ST-19 isolates were all HS1, while the eight ST-21 isolates were all HS2 or non typeable), although these serotypes were also present in isolates exhibiting different STs. Conversely, the ST-45 complex contained a wide variety of serotypes and a number of cross-reactive isolates, but the two most common serotypes observed in the ST-21 complex, HS1 and HS2, were not present in the ST-45 complex. The remaining complexes comprised small numbers in the isolate collection and these were homogenous for serotype, with the exception of the seven ST-179 complex isolates which originated in sand and were HS2 or HS5 and two members of the ST-130 complex (Table 3).

DISCUSSION

Unambiguous, discriminatory isolate characterization schemes are essential for epidemiological, population genetic, and evolutionary studies. Ideally, these schemes generate data that are relevant to all of these areas, but before the recent advent of high-throughput nucleotide sequence determination technology, this goal had proved elusive (19). There is a particular need for appropriate typing schemes for *C. jejuni*, as this common human pathogen (3) has extensive animal and environmental reservoirs and the relationships between disease-associated and animal or environmental populations remain to be fully elucidated. This study demonstrates that MLST (i) discriminates among *C. jejuni* isolates effectively and (ii) generates data that can be applied to the investigation of the population structure and evolutionary mechanisms in this organism. The advantages of MLST include high discrimina-

tion, reproducibility, simplicity of interpretation by using one technique rather than a combination of techniques, and the generation of data which are directly comparable among laboratories via the Internet (20). The ease with which the system can be transferred among laboratories was exploited in this study, with the sequence determinations being performed in two separate locations (Oxford and Bilthoven).

The seven loci chosen were a suitable basis for an MLST typing scheme, as they could be amplified and sequenced from isolates obtained from a wide variety of sources, were unlinked on the *C. jejuni* chromosome (Fig. 1), exhibited sufficient diversity to provide a high degree of resolution, and were not subject to positive selection, as demonstrated by the d_N/d_S ratios calculated for each locus (Table 2). The fact that the d_N/d_S ratios were much less than 1 demonstrates that there is selection against amino acid change (a d_N/d_S ratio of greater than 1 implies selection for amino acid changes). The nucleotide sequence determination for these loci was consistent with the results obtained previously from a number of phenotypic and genotypic studies of *C. jejuni*, which suggested that populations of this organism are highly diverse (35, 36). Some of this diversity was likely to have been imported recently from related species, with a potential donor, *C. coli*, identified for some of the more diverse sequences. When these likely importation events were excluded (Table 2), the *C. jejuni* sample exhibited nucleotide sequence diversity similar to that observed in *Neisseria meningitidis* (12, 15a, 20) and substantially greater than that seen in *Streptococcus pneumoniae* (8).

The housekeeping gene sequences provided evidence that horizontal genetic exchange has a major influence on the structure and evolution of *Campylobacter* populations, which was itself consistent with previous findings for the antigen genes of *C. jejuni* (10). The fall in the I_A from 2, for the whole data set, to 0.57, when only one example of each lineage was included, was indicative of a weakly clonal population (22) which con-

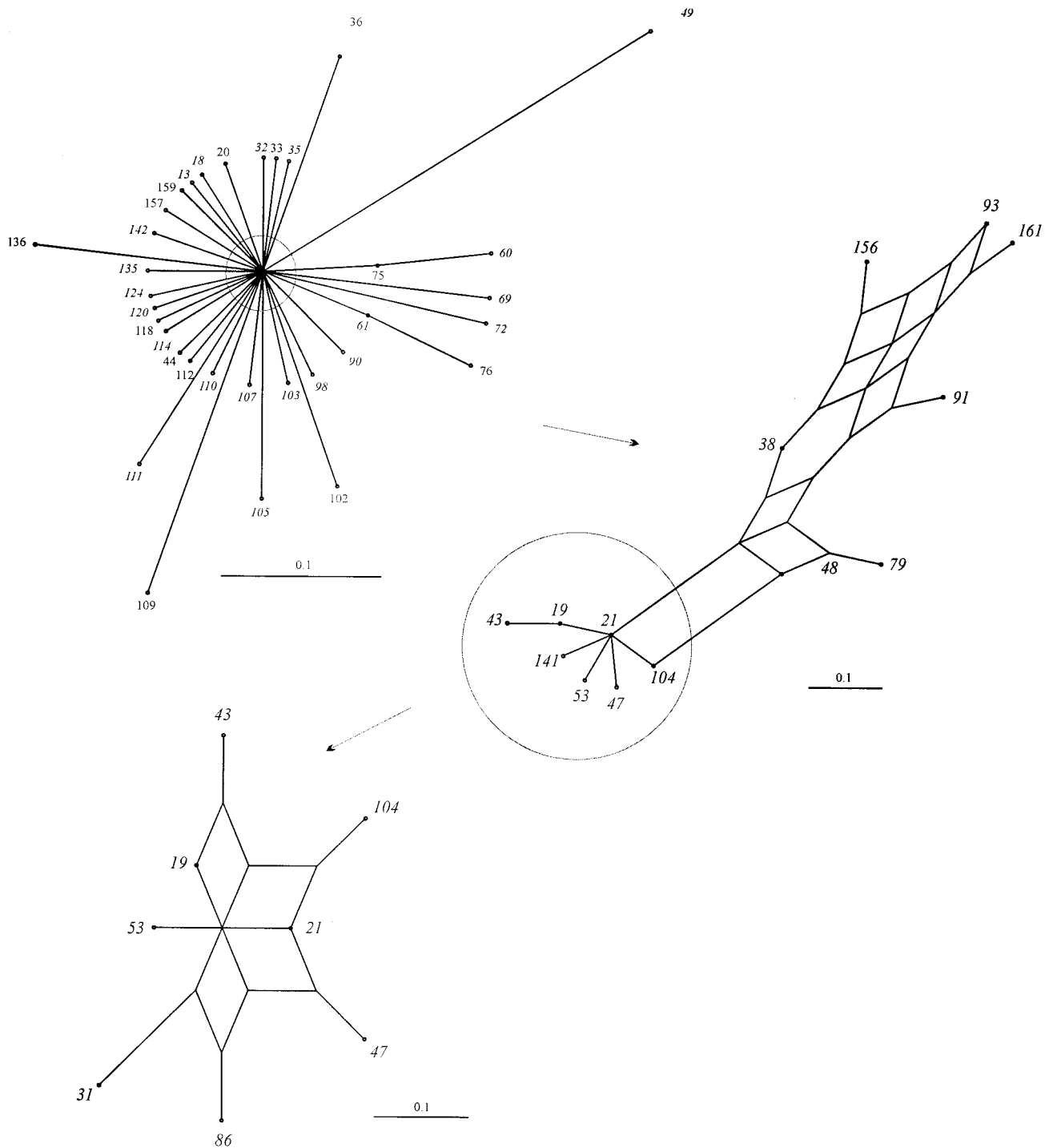


FIG. 3. Splits graphs showing the interrelationships of members of ST-21 complex. Progressive pruning of branches to improve resolution is indicated by the circles and arrows. The completely resolved region (bottom left) derived from the center of the unpruned graph contains the most abundant members of the complex and the predicted founder of the lineage, ST-21.

tained a number of clonal complexes of relatively recent evolutionary origin with no tree-like phylogenetic relationship with each other (12). The presence of the same allele in isolates of diverse origins and different lineages (for example, *aspA* allele number 2 in isolates from humans, sand, and poultry) supported this view, as did the fact that the majority of

changes within the most common lineages were likely to be due to recombinational replacement rather than mutation.

The presence of apparent lineages in isolate collections of weakly clonal organisms may be amplified by sampling (23); for example, the presence of many isolates belonging to the ST-21 complex could have been a consequence of members of this

complex being more likely to be associated with human infection. Alternatively, certain lineages might be associated with a particular niche, for example, the ST-179 complex, which contained environmental isolates (from sand of United Kingdom bathing beaches) (4). Further MLST analyses of appropriate isolate collections are necessary to address these questions more fully. However, the finding that human isolates cluster predominantly in the ST-21 complex, while chicken isolates have a broader distribution, suggests that it may be unlikely that the majority of the human strains come from chickens but that human strains may come from different sources like cattle, although the number of strains studied from cattle were low. A more detailed nucleotide sequence-based investigation of the relationships of organisms classified as different *Campylobacter* species is also warranted given the evidence for genetic exchange among these organisms.

A number of typing techniques have been applied to *C. jejuni*, with Penner HS typing, which is based on the lipopolysaccharide component of the outer membrane (28), being favored by many laboratories. The data presented here demonstrated that the Penner HS serotype was consistent and conserved in some lineages, (Table 3); for example, there was a correlation between Penner HS type and ST among some members of the ST-21 complex. However, the members of the ST-45 complex were highly diverse for Penner HS serotype (Table 3). These data are consistent with those reported earlier (25, 31, 35) and suggested the existence of a number of *C. jejuni* strains which were genetically and antigenically stable over the sampling period.

This data set provides a basis for the exploitation of MLST in the study of *C. jejuni*. The MLST approach is in principle applicable to any bacterial species, and while the oligonucleotide primers described here were not designed for characterization of other *Campylobacter* species, the evidence for interspecies horizontal genetic exchange between *C. jejuni* and at least one other *Campylobacter* species strongly suggests that this MLST system will be directly applicable to other *Campylobacter* species. The identification of alleles with gene sequences that were diverse from the majority of *C. jejuni* sequences may indicate disparity between microbiological and nucleotide sequence-based species classifications of these organisms, but additional data and analyses will be required to address these issues.

The MLST scheme provides a means for the investigation of disease outbreaks in both global and local contexts, permitting confirmation of suspected routes of transmission from the environment and livestock to humans. In addition, MLST data will assist in resolving broader issues such as the relationship of environmental to disease isolates at the population level, the structure of *Campylobacter* populations, the existence or otherwise of widely distributed lineages, and the extent of intra- and interspecies recombination. The *Campylobacter* MLST website is a freely accessible resource available to the community as a whole for the investigation of this important and as yet incompletely understood pathogen. Submission of data from other laboratories is welcomed.

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