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Epidemiological Association of Different *Campylobacter jejuni* Groups with Metabolism-Associated Genetic Markers▽†

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In this study, multilocus sequence typing (MLST) was combined with the genetic detection of six genetic markers, *ansB*, *dmsA*, *ggt*, *cj1585c*, *cjj81176-1367/71* (*cj1365c*), and the two-gene marker *tlp7* (*cj0951c* plus *cj0952c*), to assess if their presence correlated with different *C. jejuni* clonal groups. Using a collection of 266 *C. jejuni* isolates from (in decreasing order of sample size) humans, chickens, cattle, and turkeys, it was further investigated whether the resulting genotypes correlated with the isolation source. We found combinations of the six marker genes to be mutually exclusive, and their patterns of presence or absence correlated to some degree with animal source. Together with MLST results, the obtained genotypes could be segregated into six groups. An association was identified for *ansB*, *dmsA*, and *ggt* with the MLST-clonal complexes (MLST-CC) 22, 42, 45, and 283, which formed the most prominent group, in which chickens were the most prevalent animal source. Two other groups, characterized by the presence of *cj1585c*, *cjj81176-1367/71*, and the two-gene marker *tlp7*, associated with either MLST-CC 21 or 61, were overrepresented in isolates of bovine origin. Mutually exclusive marker gene combinations were observed for *ansB*, *dmsA*, and *ggt*, typically found in CC 45 and the related CC 22, 42, and 283, whereas the other three marker genes were found mostly in CC 21, 48, and 206. The presence of the two-gene marker *tlp7*, which is typical for MLST 21 and 53 as well as for MLST-CC 61, strongly correlates with a bovine host; this is interpreted as an example of host adaptation. In cases of *C. jejuni* outbreaks, these genetic markers could be helpful for more effective source tracking.

Campylobacteriosis is the most common form of bacterial food-borne enteritis in both the developed and developing worlds (35). *Campylobacter jejuni*, the most prevalent microbial species leading to enteritis, has a wide range of hosts, varying from chickens, turkeys, cattle, and other mammals to humans (35).

Comparison of different *C. jejuni* genomes (10, 15, 26) led to the identification of numerous nonubiquitous genes. The presence or absence of four nonubiquitous genes was found to correlate with the source of the isolate (11). The authors of that study tested the presence of (i) *dmsA*, encoding a subunit of the putative tripartite anaerobic dimethyl sulfoxide oxidoreductase (DMSO/trimethylamine *N*-oxide reductase); (ii) *cj1585c*, encoding another oxidoreductase and replacing *dmsA* to *-D* in strain NCTC 11168; (iii) the serine protease gene *cjj81176-1367/1371*; and (iv) the γ-glutamyl-transpeptidase gene *ggt* (11). They identified that *ggt* and *dmsA* are present more frequently in isolates from humans and chickens, whereas *cjj81176-1367/1371* and *cj1581c* are more common in bovine isolates.

Because the enzyme phosphofructokinase is absent, *C. jejuni* is unable to metabolize glucose. Instead, *C. jejuni* metabolizes free amino and keto acids originating mainly from the host or the normal flora of the hosts' intestine (22). Compounds such as succinate, D-lactate, malate, and formate serve as electron donors instead of glucose (14, 19, 26, 28, 32). In addition to the nonubiquitous *ggt* gene, the well-characterized strain 81-176 possesses a nonubiquitous accessory *sec* signal upstream of the asparaginase-encoding gene *ansB*, resulting in a periplasmic localization of the enzyme (16). This enables the strain to deaminate asparagine to aspartate, while no homolog of an asparagine transporter was detected in any of the known *C. jejuni* genome sequences (16). The periplasmic γ-glutamyl-transpeptidase seems to play a pivotal role in colonization of the chicken intestine. Knockout of *ggt* results in increased cell invasiveness (1). Because of this potentially important genetic determinant, we were interested in assessing the presence of *ggt* in combination with other genetic markers and investigating any possible association with host specificity.

Besides hydrogen and α-ketoglutarate, formic acid is a possible electron donor in the *C. jejuni* electron transport chain (33). Recently, we demonstrated that TLP7 (transducer-like protein 7), one of the group A receptors with structural similarity to methyl-accepting chemotaxis proteins (MCPs) of *Escherichia coli*, mediates chemotaxis to formic acid and plays a role in host cell invasion and motility (31). In the strain huB2, like in NCTC 11168, the TLP7 receptor is a heterodimeric protein, and its gene is split into two parts: *cj0951c*, encoding the putative MCP domain-containing signal transduction protein, and *cj0952c*, encoding a HAMP-containing membrane protein and a CXXCH motif, indicating that it may be involved in the formate signaling pathway and/or formate respiration (GU799572). In contrast to the case for strains NCTC 11168

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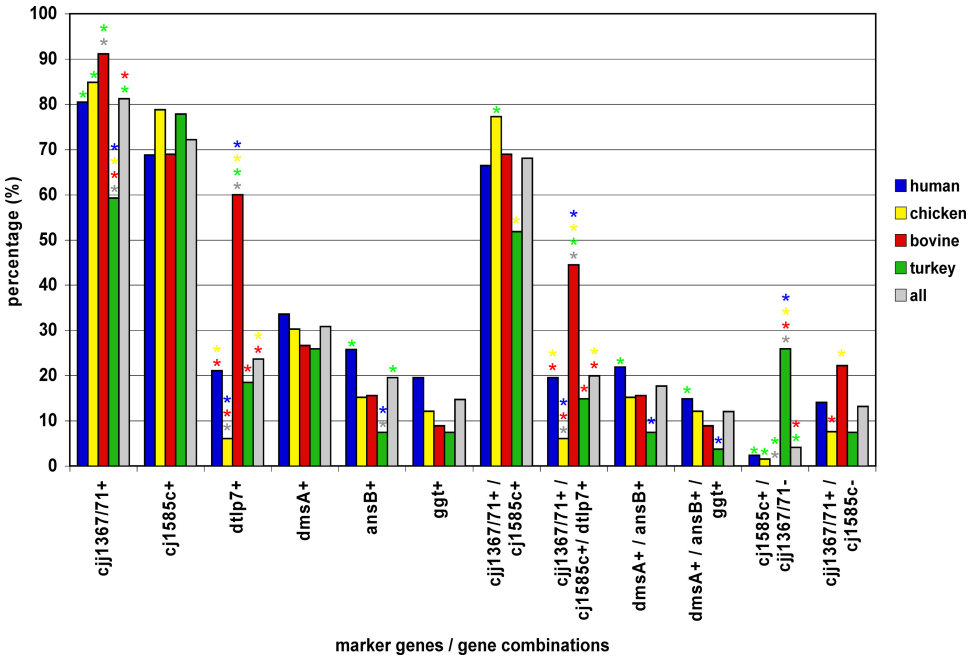


FIG. 1. Frequency distribution of marker genes and marker gene combinations within *C. jejuni* isolates from different host species. *cjj1367*, *cjj81176-1367/1371* gene (*cj1365c*), encoding a serine protease; *cj1585c*, gene encoding an oxidoreductase and replacing *dmsA* to *-D* in NCTC 11168; *dtlp7*, *cj0951c* and *cj0952c* genes, encoding the heterodimeric form of transducer-like protein 7; *dmsA*, gene encoding the dimethyl sulfoxide oxidoreductase subunit A; *ansB*, gene encoding an asparaginase with an accessory N-terminal *sec*-dependent secretion signal for periplasmic localization of the enzyme; *ggt*, gene encoding the γ-glutamyl-transpeptidase. Significant differences ($P < 0.05$) in values are marked by stars in the colors of the comparator values. A missing star indicates no significant ($P > 0.05$) difference.

and huB2, both domains of TLP7 are encoded by a single gene in strain 81-176 (31). Because of these findings, we assumed that Cj0952c might even function on its own and that the introduction of a stop codon between *cj0951c* and *cj0952c* may be an expression of some sort of metabolic host adaptation. In order to discriminate between the single gene (*tlp7*) and the two-gene variant, we named the two-gene variant resulting in a heterodimeric receptor *dtlp7* (*cj0951c* plus *cj0952c*).

In this study, the mutual relationships of *ansB*, *ggt*, *dmsA*, *cj1585c*, *cjj81176-1367/1371* (*cj1365c*), and *dtlp7* within isolates of human, chicken, bovine, and turkey origin and their association with multilocus sequence types-clonal complexes (MLST-CC) were investigated in order to reveal a possible relationship between clonal groups and metabolic adaptation indicative of host tropism.

MATERIALS AND METHODS

In the present study, 266 *C. jejuni* isolates from humans (128 isolates), chickens (66 isolates), cattle (45 isolates), and turkeys (27 isolates) were typed by MLST and screened for the six genetic markers mentioned above. For PCR detection of *dtlp7* (*cj0951c* plus *cj0952c*), we used the primers *tlp7*-F01 (5′-AGG-TTT-CTG-CTG-CAA-TTT-TTG-TGG-TG-3′) and *tlp7*-R01 (5′-AGC-AAG-TTC-TCC-AAG-TTC-ATT-GCC-A-3′), followed by AseI restriction of the amplicon. AseI cuts the PCR fragment directly at the additional stop codon. Detection of *ansB* was performed using the primers *ansB*-F01 (5′-GGG-GAA-TGG-TAA-CTC-CAC-AA-3′) and *ansA*/*B*-R01 (5′-GCA-CTT-ATA-GCA-GTT-GAT-GGA-CG-3′), followed by NspI digestion of the PCR product. The NspI cutting site was localized within the sequence of the accessory *sec* signal. The primer pairs for the detection of *dtlp7* and *ansB* were deduced from common regions of the published sequences of strains NCTC 11168 (26) and 81-176 (15). For PCR detection of *dmsA*, *ggt*, *cjj81176-1367/1371*, and *cj1585c*, we used primers that have already been described by Gonzalez et al. (11). MLST was conducted by using established primers (6). In order to construct a phylogenetic tree from the resulting sequences, using the unweighted-pair group method using average linkages (UPGMA), MEGA4 software was used (20). One thousand replicates of bootstrap analyses were carried out to fulfill confidence estimates for phylogenetic tree topology. For this study, we used the *C. jejuni* MLST website (http://pubmlst.org/campylobacter/) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford (18). MLST profiles and data for all isolates used in this work were submitted to the PubMLST database.

RESULTS

In the 266 isolates investigated, the marker gene *cjj81176-1367/1371* was the most prevalent, detected in 216 isolates (81.2%). The *cj1585c* gene was found in 72.2% (192 isolates) of isolates, followed by *dmsA* (30.8%; 82 isolates), *dtlp7* (23.7%; 63 isolates), *ansB* (19.5%; 52 isolates), and *ggt* (14.7%; 39 isolates). Figure 1 shows the distribution of these marker genes and of common combinations, split up according to the source of the isolates. This figure also demonstrates that *cjj81176-1367/1371* and *cj1585c* were associated with each other in 68.0% (181/266 isolates) of the isolates. The marker gene *dtlp7* seemed to be associated with bovine isolates: 60.0% (27/45 isolates) of these were *dtlp7* positive. Only a minor proportion of the isolates were *ggt* positive. The frequency of *ggt* decreased from 19.5% (25/128 isolates) in human isolates to 12.1% (8/66 isolates) in chicken isolates, 8.9% (4/45 isolates) in bovine isolates, and 7.4% (2/27 isolates) in turkey isolates. This sequential finding (human > chicken > bovine > turkey), already described by Gonzalez and coworkers (7), could also be found for *dmsA* (33.6% [43/128 isolates] > 30.3% [20/66 isolates] > 26.7% [12/45 isolates] ≈ 25.9% [7/27 isolates]) and, to

TABLE 1. Distribution of marker genes, MLST-CC, and isolate origins within the six determined groups and nine subgroups

Group or subgroup	Most prevalent marker genes[a]						CC	Origin(s)[b]
	cjj1367	*cj1585c*	*dtlp7*	*dmsA*	*ansB*	*ggt*		
1a	X	X	X				21	b
1b	X	X					21, 48, 49, 206, 446	h, c, b, t
2a	X			X	X	X	22, 42	h, c, b
2b				X	X	X	45, 283	h, c
3a	X	X					52, 353, 354, 443, 658, 828	h, c, t
3b	X	X	X				61	b
4				X			1034, 1332	t
5	X	X					None	c
6	X			X	X		257	h, t

[a] *cjj1367*, *cjj81176-1367/1371* gene (*cj1365c*), encoding a serine protease; *cj1585c*, gene encoding an oxidoreductase and replacing *dmsA* to *-D* in NCTC 11168; *dtlp7*, *cj0951c* and *cj0952c* genes, encoding the heterodimeric form of transducer-like protein 7; *dmsA*, gene encoding the dimethyl sulfoxide oxidoreductase subunit A; *ansB*, gene encoding an asparaginase with an accessory N-terminal *sec*-dependent secretion signal for periplasmic localization of the enzyme; *ggt*, gene encoding the γ-glutamyl-transpeptidase.

[b] h, human; c, chicken; b, bovine; t, turkey.

a lesser extent, *ansB* (25.8% [33/128 isolates] > 15.2% [10/66 isolates] ≈ 15.6% [7/45 isolates] > 7.4% [2/27 isolates]). Similar trends could be demonstrated for the combinations *dmsA-ansB* (21.9% [28/128 isolates] > 15.2% [10/66 isolates] ≈ 15.6% [7/45 isolates] > 7.4% [2/27 isolates]) and *dmsA-ansB-ggt* (14.8% [19/128 isolates] > 12.1% [8/66 isolates] > 8.9% [4/45 isolates] > 3.7% [1/27 isolates]). However, most of these frequency differences between the several host species were not significant, as indicated in Fig. 1. The presence of *ggt* coincided with that of *ansB* in 92% (36/39 isolates) of isolates and with that of *dmsA* in 90% (35/39 isolates) of isolates, whereas only 43% (35/82 isolates) of the *dmsA*-positive isolates and 69% (36/52 isolates) of the *ansB*-positive isolates were *ggt* positive. Furthermore, 90% (47/52 isolates) of *ansB*-positive isolates were *dmsA* positive, but only 57% (47/82 isolates) of *dmsA*-positive isolates showed the presence of *ansB*. There was no *dtlp7*-positive isolate showing the presence of *ansB* or *ggt*, and vice versa.

Another group of isolates (4.1% [11/266 isolates]), mostly originating from turkeys (63.6% [7/11 isolates]), was *cj1585c* positive but *cjj81176-1367/1371* negative. The reversed finding, of *cj1585c*-negative but *cjj81176-1367/1371*-positive isolates, was found more frequently in humans (51.4% [18/35 isolates]) and cattle (28.6% [10/35 isolates]).

Additional MLST characterization of all 266 isolates led to the identification of six groups and nine subgroups, as can be seen in Table 1 as well as in Fig. 2 and 3 and in Fig. S1 in the supplemental material.

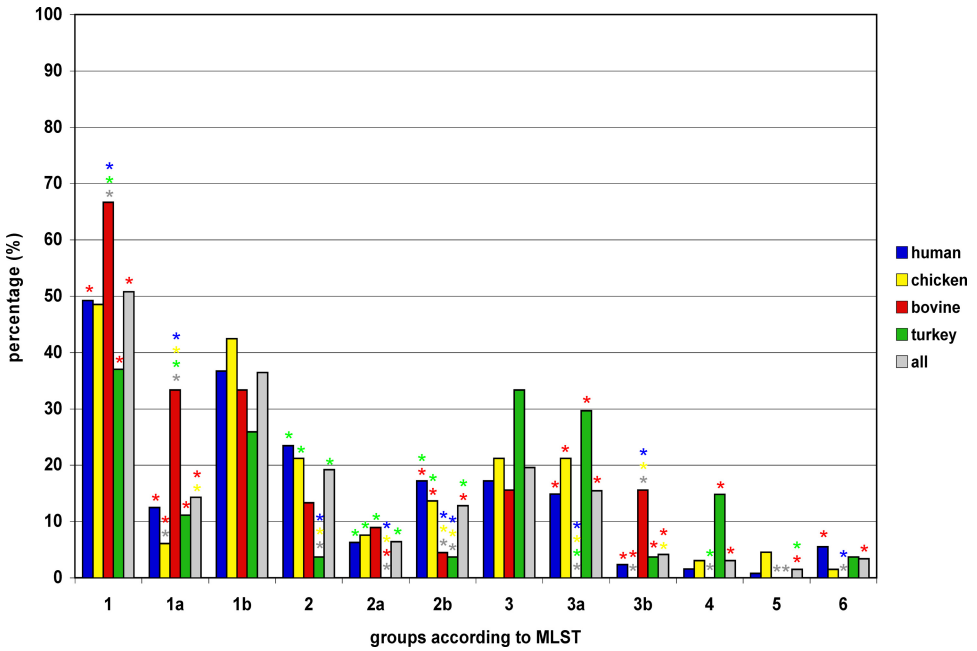
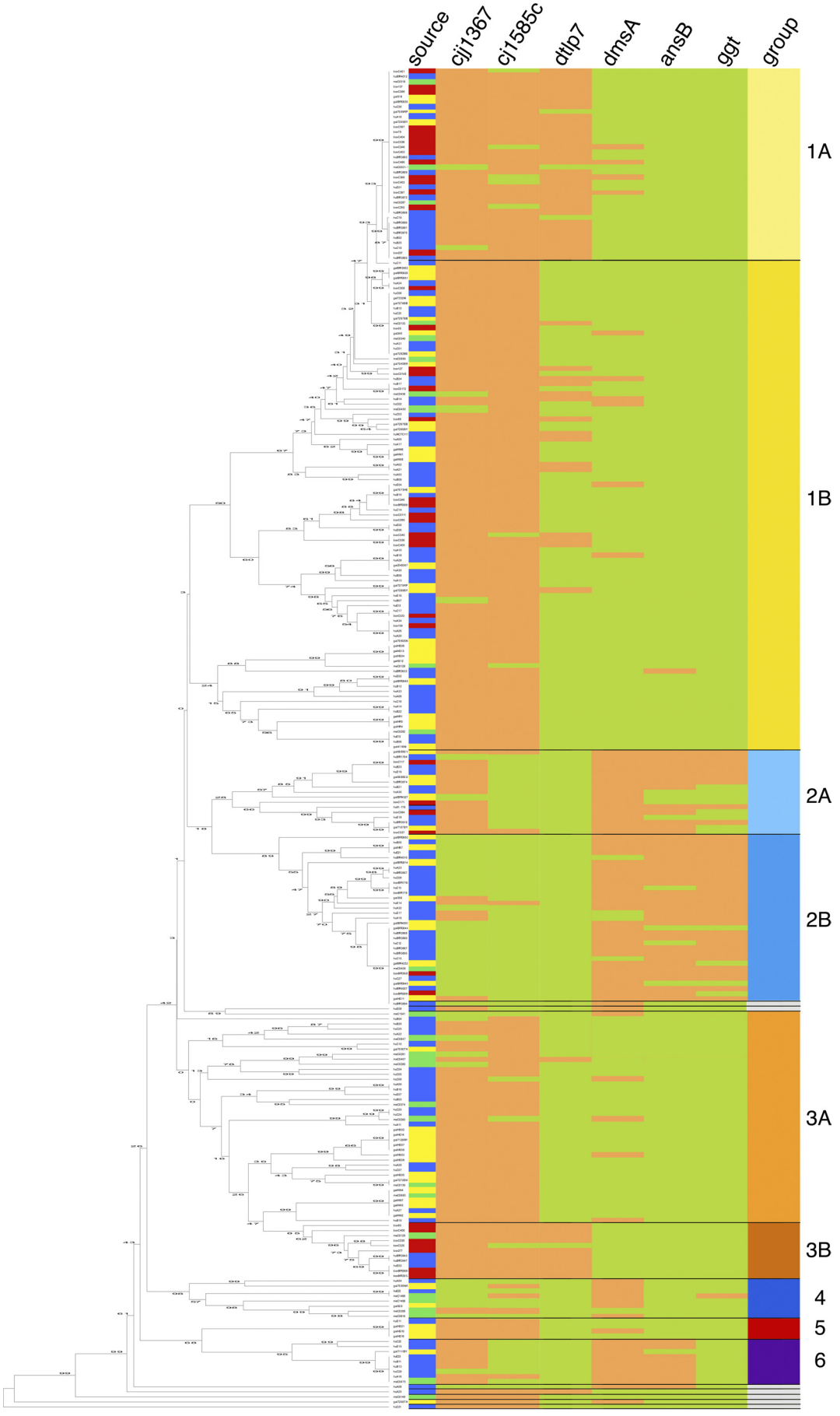


FIG. 2. Frequency distribution of defined MLST-CC and marker gene-associated groups within *C. jejuni* isolates from different host species. Groups: 1, consisting of subgroups 1a and 1b; 2, consisting of subgroups 2a and 2b; 3, consisting of subgroups 3a and 3b; 4; 5; and 6. Significant differences ($P < 0.05$) in values are marked by stars in the colors of the comparator values. A missing star indicates no significant ($P > 0.05$) difference.


source
cjj1367
cj1585c
dtlp7
dmsA
ansB
ggt
group
1A
1B
2A
2B
3A
3B
4
5
6

Major group 1 consisted of 50.8% (135/266 isolates) of all isolates and was characterized by the presence of *cjj81176-1367/1371* and *cj1585c*. Isolates of this group originated from all four host species and belonged to the MLST-CC 21, 48, 49, 206, and 446. Within this group, subgroup 1a (14.3% of isolates [38/266 isolates]), of predominantly bovine isolates, was additionally positive for *dtlp7*.

The second group encompassed 19.2% (51/266 isolates) of the isolates and was predominantly positive for *dmsA*, *ansB*, and *ggt*. Within this group, subgroup 2a (6.4% of isolates [17/266 isolates]) was additionally positive for *cjj81176-1367/1371*. *cjj81176-1367/1371*-positive group 2a isolates belonged to the MLST-CC 22 and 42. *cjj81176-1367/1371*-negative group 2b isolates belonged to the MLST-CC 45 and 283. In group 2, isolates from chickens and humans were clearly overrepresented.

Similar to group 1, group 3 consisted of *cjj81176-1367/1371*- and *cj1585c*-positive isolates originating from all four host species and belonged to the MLST-CC 52, 353, 354, 443, 658, and 828. In this group, a *dtlp7*-positive subgroup (4.1% of isolates [11/266 isolates]) could be found, belonging exclusively to MLST-CC 61, with a large proportion of bovine isolates.

The majority of the group 4 isolates belonged to the MLST-CC 1034 or 1332 and tested positive for *dmsA*. Group 4 isolates were isolated mostly from turkeys.

Group 5 isolates, like those of groups 1b and 3a, tested positive for *cjj81176-1367/1371* and *cj1585c*, and in most cases, they were isolated from chickens.

Finally, group 6 isolates of MLST-CC 257, originating from chickens and humans, were positive for *dmsA*, *ansB*, and *cjj81176-1367/1371*, comparable to group 2a isolates. In contrast to group 2a isolates, not one isolate in group 6 was positive for *ggt*. Thus, the higher prevalence of *ansB* than of *ggt* is due to group 6 isolates, and the higher prevalence of *dmsA* than of *ggt* and *ansB* is due to group 4 and 6 isolates.

DISCUSSION

The genotypic characterization performed here, based on the presence or absence of six metabolism-associated genetic markers in combination with MLST, divided the 266 isolates into six separate groups, of which three could be split into subgroups. These groups and subgroups showed moderate correlations with host origin. The observed host association was weaker than that observed for *Campylobacter coli*, which produces a more significant correlation between MLST-CC or amplified fragment length polymorphism (AFLP) clusters and isolate source (17, 21, 24). The population of *C. coli* may display a higher degree of clonality than that of *C. jejuni*.

We identified three *C. jejuni* groups, groups 1, 3, and 5, in which the *cjj81176-1367/1371* and *cj1585c* genes were associated. Within these groups, two subgroups of different clonality, 1a and 3b, were identified as having a two-gene organization for the TLP7 receptor, as described by Tareen and coworkers (31). Remarkably, this receptor variant was detected significantly ($P < 0.001$) more often in isolates from cattle. Epidemiological surveillance data collected in the year 2008 demonstrated that 6.7% of cattle herds, 4.7% of fresh bovine meat, and 1.3% of raw milk in Germany were contaminated with *Campylobacter* spp. and that more than 83% of bovine *Campylobacter* isolates belonged to the species *C. jejuni* (8). It has been assumed from different models that between 5% and 35% of all *C. jejuni* isolates from humans were transmitted via cattle or contaminated cattle products (5, 25, 27, 34). While 60% of the bovine isolates and about 20% of the human isolates harbored the heterodimeric receptor variant, it can be extrapolated that in accordance with the above-mentioned models, about one-third of all human *C. jejuni* isolates in our study originated in some way from cattle. This may suggest the use of *dtlp7* as a marker for isolates of bovine origin. It is likely that *cj0951c*, encoding a putative MCP domain-containing signal transduction protein, does not interact exclusively with *cj0952c*; alternative and as yet unknown mechanisms may be responsible for adaptation to the bovine host. It has been demonstrated that MLST-CC 21 correlates with lipooligosaccharide (LOS) class C and that these isolates display more invasiveness in Caco-2 cells (13). Additionally, it is known that most hyperinvasive strains belong to MLST-CC 21 (9). Since subgroup 1a in particular is associated with MLST-CC 21, it can be deduced that this group represents *C. jejuni* strains with increased invasiveness.

Our data also revealed an association of *ansB* and *dmsA* in groups 2 and 6. In group 2, an additional association with *ggt* and also, for subgroup 2a, with *cjj81176-1367/1371* could be identified. It seems that isolates with an extended amino acid metabolism are more prevalent in humans than previously recognized (11). The most likely transmission route of group 2 isolates, from chickens to humans, is consistent with the role of *ggt* in the persistent colonization of the avian intestine (1). Moreover, we identified a *C. jejuni* subpopulation of isolates belonging to MLST-CC 1034 or 1332 (group 4) that seems to circulate primarily within turkey populations and is encountered less frequently in humans. A similar finding was recently demonstrated for CC 403, which seems to represent a *C. jejuni*

FIG. 3. MLST-based UPGMA tree and arrangement of the six different marker genes within the six defined groups (nine subgroups). The MLST-based UPGMA tree for 266 *C. jejuni* isolates is depicted on the left. The numbers shown on the branches of the tree indicate the linkage distances. The right side shows a table of all isolates in the order of the UPGMA tree depicting the source of the isolate, the presence or absence of the six marker genes, and their association with one of the groups defined in Table 1. Sources: blue, human isolates; yellow, chicken isolates; red, bovine isolates; green, turkey isolates. The presence of a genetic marker is marked with light red, and its absence is marked with light green. The genetic markers, from left to right, are as follows: *cjj1367*, *cjj81176-1367/1371* gene (*cj1365c*), encoding a serine protease; *cj1585c*, gene encoding an oxidoreductase and replacing *dmsA* to *-D* in NCTC 11168; *dtlp7*, *cj0951c* and *cj0952c* genes, encoding the heterodimeric form of transducer-like protein 7; *dmsA*, gene encoding the dimethyl sulfoxide oxidoreductase subunit A; *ansB*, gene encoding an asparaginase with an accessory N-terminal *sec*-dependent secretion signal for periplasmic localization of the enzyme; *ggt*, gene encoding the γ-glutamyl-transpeptidase. Groups: light yellow, 1a; intense yellow, 1b; cyan blue, 2a; bondi blue, 2b; carrot orange, 3a; orange-red, 3b; blue, 4; red, 5; purple, 6; white, singletons.

clone adapted to swine (23). Our results underscore the findings of Manning and coworkers showing that CC 21, 61, and 48 are overrepresented within bovine isolates and that CC 45, 257, and 283 originated from poultry, especially chicken (23). Due to their negativity for *cjj81176-1367/71* (*cj1365c*), group 2b and 4 isolates may have originated from environmental sources, especially water (2; see below). Thus, poultry may function merely as an intermediate host for a minor proportion of this group, while a human-environment (water)-human transmission route is also possible.

There is also growing evidence for different *C. jejuni* subpopulations originating from different hosts and following different transmission routes. The most frequent MLST-CC in *C. jejuni* isolates from humans are MLST-CC 21 and 45 (7, 30), belonging to groups 1(a) and 2b, respectively. Habib and coworkers observed differences in the stress responses of the isolates belonging to these two MLST-CC (12): CC 21 strains were more tolerant to extreme temperatures, whereas CC 45 isolates showed increased survival in oxidative and freeze stress models. Thus, besides metabolic differences and preferences in host tropism, variation in stress responses may contribute to the establishment of certain *C. jejuni* lineages in defined environments. This is supported by the finding that campylobacteriosis cases caused by CC 21 or CC 45 isolates show different peaks in their temporal distribution (30). CC 45 isolates are more prevalent during the early summer months and seem to follow an environmental transmission route. In contrast, CC 21 cases are reported more or less consistently throughout the whole year, with a peak during late summer months (29) and with a clear association with infected cattle (3, 4).

Up to now, there are few genetic markers that can be used for effective source tracking. Champion and coworkers used genomotyping to identify a cluster of six genes, *cj1321* to *cj1326*, within the O-linked flagellin glycosylation locus, which is absent in most nonlivestock isolates but present in almost every chicken/livestock-associated strain (2). These nonlivestock strains are potentially nonpathogenic and isolated mostly from asymptomatic carriers or from environmental sources. Additionally, Champion et al. demonstrated that the potential secreted serine protease gene *cj1365c*, which is the homologous gene to *cjj81176-1367/71* in strain NCTC 11168, is absent in most isolates from environmental sources but present in clinical and livestock isolates. Thus, they hypothesized that it could be a marker for strains following an environmental transmission route, which could be the case for group 2b (CC 45) and group 4 (CC 1034 and 1332) isolates.

According to the data presented here, the mutually related marker genes *ansB*, *dmsA*, and *ggt* can be used to distinguish CC 45 and the related CC 283 from CC 22 and 42, which are additionally positive for *cjj81176-1367/71* (*cj1365c*), and from CC 21 and its related CC 48 and 206, which are exclusively positive for *cj1585c* and *cjj81176-1367/71* (*cj1365c*). The two-gene marker *tlp7*, typical for MLST 21 and 53 as well as MLST-CC 61, strongly indicates a host adaptation to cattle. This leads to the conclusion that the presence of *dtlp7* in an outbreak strain is a significant indicator of a bovine source.

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