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Survival of Clinical and Poultry-Derived Isolates of *Campylobacter jejuni* at a Low Temperature (4°C)

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***Campylobacter jejuni* is a leading cause of bacterial gastroenteritis in humans, and contamination of poultry has been implicated in illness. The bacteria are fastidious in terms of their temperature requirements, being unable to grow below ca. 31°C, but have been found to be physiologically active at lower temperatures and to tolerate exposure to low temperatures in a strain-dependent manner. In this study, 19 field isolates of *C. jejuni* (10 of clinical and 9 of poultry origin) were studied for their ability to tolerate prolonged exposure to low temperature (4°C). Although substantial variability was found among different strains, clinical isolates tended to be significantly more likely to remain viable following cold exposure than poultry-derived strains. In contrast, the relative degree of tolerance of the bacteria to freezing at −20°C and freeze-thawing was strain specific but independent of strain source (poultry versus clinical) and degree of cold (4°C) tolerance.**

Campylobacter jejuni is currently a leading cause of bacterial gastroenteritis in humans (1, 20, 30). Infection by *C. jejuni* is also the most common antecedent to Guillain-Barré syndrome, an autoimmune disorder of the peripheral nervous system (19). *C. jejuni* and related campylobacters are unique among human food-borne pathogens in being obligate microaerophiles and in their narrow and rather unusual temperature range for growth. *C. jejuni* and other "thermophilic campylobacters" grow optimally at a relatively high temperature (42°C), but their minimal growth temperature is in the range of 31 to 36°C (3, 5, 8), and growth ceases abruptly around 30°C (8).

C. jejuni is a commensal microbe in avian species, including poultry (13, 36), and epidemiological studies have frequently implicated raw and undercooked poultry in human campylobacteriosis (1, 20, 30). A substantial portion (as much as 98%) of poultry at retail is contaminated with the pathogen (1, 29). Other meat products can also be contaminated with *Campylobacter* and can contribute to human illness, along with untreated water, raw milk, and exposure to live birds and to pets with diarrhea (1, 20).

Several studies suggest that, in spite of fastidious requirements for growth, *C. jejuni* has the potential for remarkable survival under conditions nonpermissive to growth. In surface waters and water microcosms, survival was shown to be limited to a few days at ambient temperatures of ca. 20°C but was noticeably enhanced (up to several weeks) at 4°C (2, 22, 31). Rollins and Colwell (26) showed that at 4°C *C. jejuni* could survive and remain at the viable but nonculturable stage for about 4 months. Oxygen consumption, catalase activity, ATP generation, chemotaxis, and protein synthesis were also observed at 4°C (8). Furthermore, Lee et al. (15) showed that *C. jejuni* remained viable on raw chicken skin fragments at −20 and −70°C for 14 and 56 days, respectively. In the same study, *C. jejuni* was also able to persist on the chicken skin fragments at 4°C (15).

The ability of *C. jejuni* to survive refrigeration and freezing is of obvious relevance to food safety and public health. Currently, however, survival of this pathogen in the cold remains poorly understood. As a species, *C. jejuni* exhibits pronounced genotypic and phenotypic variability (21, 34), and survival of the pathogen in water has been shown to vary markedly among different strains (11, 31). Studies on *Campylobacter* cold and freezing tolerance have commonly involved single isolates, and the impact of strain variability in cold and freezing tolerance has not been investigated. In this study, a number of distinct *C. jejuni* strains of both clinical and poultry origin were characterized for viability at 4 and −20°C. Our results indicate substantial variability among strains in cold survival, with human clinical isolates appearing to be significantly more capable of prolonged survival at 4°C than poultry-derived strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Campylobacter* strains used in this study are listed in Table 1. Poultry-derived strains were isolated as described below, at the Environmental Microbiology Laboratory, Hawaii State Department of Health, during the 1998–1999 surveillance for *Campylobacter* contamination of poultry. These poultry strains were obtained from different brands of poultry, purchased from different supermarkets. With the exception of CJ33, CJ35, and CJ38, which were isolated from the same poultry sample, all strains were from different products. All poultry strains (including CJ33, CJ35, and CJ38) were found to have distinct genotypes (12; K. F. Chan, H. L. Tran, and S. Kathariou, unpublished results). Human clinical strains were derived from clinically confirmed cases of *Campylobacter* infections during the same time period and were provided by the State of Hawaii Medical Microbiology Laboratory. These clinical isolates were also found to represent distinct genotypes (12; Chan et al., unpublished). All strains were passaged minimally and were preserved in brain heart infusion broth (Difco) with 20% sterile glycerol at −70°C. *Campylobacter* strains were grown in Mueller-Hinton broth (MHB; Difco) or on Mueller-Hinton agar (MHA; Difco) at 42°C for 40 h under microaerobic conditions (CampyPak; BBL). To ensure optimal growth, agar plates were kept from overdrying.

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TABLE 1. Ranking of *C. jejuni* isolates used in this study in terms of survival following prolonged incubation in the cold (4°C)

Isolate	Origin	Log_{10} CFU/ml on:		Rate of viability loss[a]
		Day 0	Day 14	
CJ63	Clinical	7.89	7.30	0.0513
CJ22	Clinical	7.70	6.92	0.0513
CJ45	Clinical	7.00	6.09	0.0650
CJ67	Clinical	8.07	7.06	0.0697
CJ19	Clinical	9.26	7.74	0.1136
CJ17	Clinical	9.64	7.71	0.1171
CJ35	Poultry	7.70	6.03	0.1195
CJ25	Clinical	8.09	6.04	0.1319
CJ26	Clinical	7.87	5.44	0.1659
CJ52	Poultry	7.62	5.17	0.1867
CJ38	Poultry	6.83	3.25[b]	0.2232
CJ37	Poultry	7.53	4.41	0.2236
CJ1	Poultry	8.73	3.72	0.2734
CJ5	Poultry	9.14	2.94	0.4091
CJ41	Clinical	6.70	<1[b]	0.4110
CJ7	Poultry	9.03	2.56	0.4230
CJ33	Poultry	7.96	1.60	0.5007
CJ14	Clinical	9.75	3.27	0.5212
CJ3	Poultry	8.14	<1	0.6710

[a] Absolute value of the slope of the linear best fit of the respective cold survival curve. The linear best fit was generated by Microsoft Excel.
[b] Represents data on day 15.

Isolation of poultry strains of *C. jejuni*. Raw poultry (from refrigerated display cases of local supermarkets) was purchased, transported on ice to the Hawaii State Department of Health laboratory, and processed within 72 h of purchase. The poultry was placed in a sterile stomacher bag and rinsed with a rocking motion for 2 min in Butterfield's phosphate buffer (pH 7.2). The resulting chicken rinse (CR) was used for selective enrichments and isolation of *Campylobacter* following established protocols (10, 24). Presumptive isolates were further examined with the Campy Index latex agglutination kit (Integrated Diagnostics) and bacteriologically confirmed by Gram stain, observation of cell shape and characteristic motility, determination of oxidase, catalase, and hippuricase activities, and other standard biochemical markers. Isolates with ambiguous hippuricase assay results were further tested by PCR using previously described primers and conditions for hippuricase gene detection (16).

Assessment of viability following cold (4°C) storage. Following growth in MHB at 42°C for 40 h under microaerobic conditions, the liquid cultures were placed in a 4°C incubator. Viable cells in the cultures were enumerated by serial dilution using MHB as the diluent and plating in duplicate immediately before the 4°C storage. Unless otherwise indicated, viable cell counts of the 4°C-stored cultures were subsequently determined at 2-day intervals. All cell enumerations were done using colonies grown for 40 h at 42°C microaerobically. Each strain was tested at least twice.

Survival of bacteria during cold storage in CR. Bacterial cells were grown to confluence on MHA plates (40 h, 42°C under microaerobic conditions). Half of the confluent culture from the plate was resuspended in 30 ml of MHB in a culture flask, whereas the other half was resuspended in 30 ml of autoclaved CR liquid (obtained as described above) in another culture flask. The flasks were swirled to homogenize the cell suspensions and placed in a 4°C incubator. Viable cells in MHB and CR were enumerated by serial dilution using MHB as a diluent and plating in duplicate immediately before the 4°C storage, and viable counts of the 4°C-stored suspensions were determined every 7 days as described above.

Survival during frozen storage. Cell suspensions in MHB and CR were obtained as described above, and 1-ml volumes were distributed into sterile Eppendorf tubes which were then stored at −20°C. Viable cells in the suspensions were enumerated by serial dilution using MHB as a diluent and plating in duplicate immediately before freezing. At 2-day intervals, tubes were removed from frozen storage, thawed in an ambient-temperature water bath, used immediately for viable cell count determinations as described above, and then discarded.

Statistical analysis. The general linear models procedure of SAS (SAS Institute, Cary, N.C.) was utilized to compute all statistical inferences. The slopes of curves within each figure were calculated and compared to determine statistically significant differences. Fisher's exact test (35) was employed to calculate the exact probability of obtaining the observed data set.

RESULTS

***C. jejuni* strains vary noticeably in terms of their cold tolerance.** Nine poultry-derived and 10 clinical isolates of *C. jejuni* were chosen for investigation of their cold and freezing tolerance. Plate count monitoring of the viability profile of different isolates over 14 days at 4°C revealed significant differences among strains (Fig. 1 and Table 1). Viability of certain strains (e.g., CJ22) showed no appreciable decrease following 14 days of storage at 4°C, whereas viable counts of others (e.g., CJ26 and CJ52) had declined by a factor of ca. 10 to 100 by day 10. On the other hand, plate counts of certain strains (e.g., CJ3) declined precipitously following 4°C storage, by factors of ca. 100 and 10^5 at 4 and 8 days, respectively. These differences in viability among strains were reproducibly observed in independent experiments, suggesting that the phenotypes were a strain-specific property with a genetic basis.

In Fig. 1, CJ1, CJ17, and CJ19 exhibit a rather unusual survival curve. During the first 2 days, the viable count declined by a factor of ca. 100. The viability then increased steadily by a factor of ca. 10 until day 6 and slowly decreased afterwards. The rise in viable count from day 2 to day 6 may reflect an increase in the number of culturable *C. jejuni* cells, possibly resulting from the adaptation to low temperature.

The 19 *C. jejuni* strains were ranked in terms of their rates of viability loss following a 14-day exposure at 4°C, as shown in Table 1.

Cold tolerance is more pronounced among clinical isolates of *C. jejuni* than among poultry-derived strains. The data in Table 1 suggest that poultry-derived and clinical strains differ in terms of their rates of viability loss at 4°C. Survival curves with slopes less negative than the median (median represented by CJ52, with a slope of −0.1867) were obtained primarily by clinical isolates, whereas the survival curves of most poultry-derived strains had slopes more negative than the median.

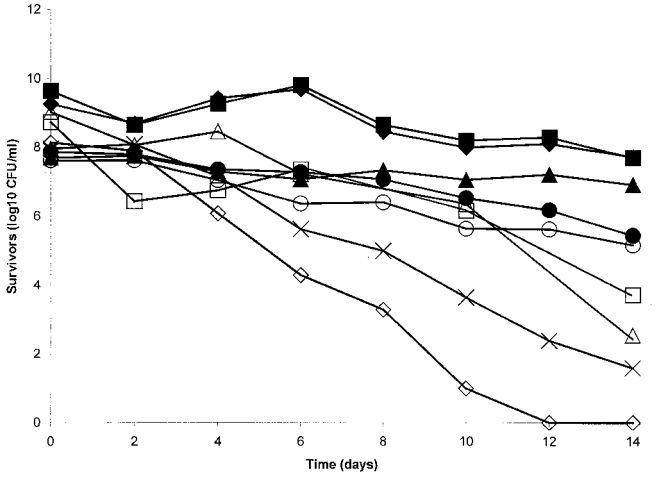

FIG. 1. Survival curves of representative clinical and poultry-derived *C. jejuni* strains at 4°C. Poultry isolates were CJ1 (□), CJ3 (◇), CJ7 (△), CJ33 (×), and CJ52 (○). Clinical isolates were CJ17 (■), CJ19 (◆), CJ22 (▲), and CJ26 (●). Results for CJ3 on days 12 and 14 were below the limit of detection, 10 CFU/ml (1 in log_{10} scale). Data shown are means from duplicate plates in a representative experiment and were collected as described in Materials and Methods.

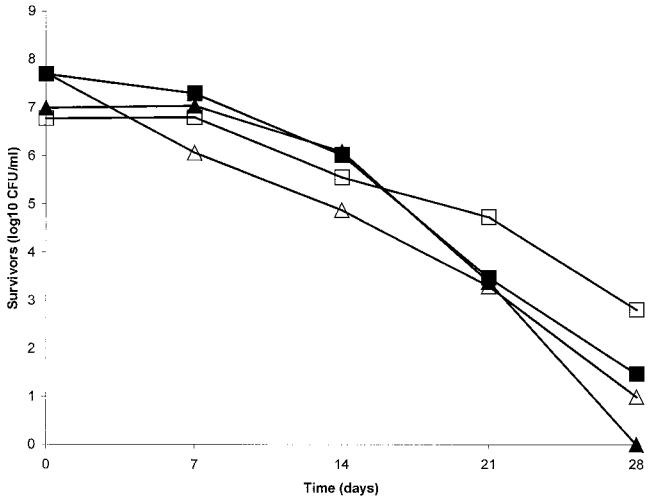

FIG. 2. Cold (4°C) tolerance of *C. jejuni* in MHB and CR. CJ35 (squares) and CJ45 (triangles) were stored in MHB (closed symbols) or in CR (open symbols). The result for CJ45 in MHB on day 28 was below the limit of detection (10 CFU/ml). Data shown are means from duplicate plates in a representative experiment and were collected as described in Materials and Methods. The general linear models procedure was utilized to compute all statistical inferences. The slope of each data curve was calculated and compared. No significant differences were found among these four sets of data ($P > 0.05$).

Using Fisher's exact test (35), the higher incidence of clinical isolates above the median was statistically significant ($P < 0.05$). Of the 10 clinical isolates which we screened, 6 had only limited viability loss during the surveyed period ($<10^2$), 2 declined by a factor of 10^2 to 10^3, and only 2 (CJ14 and CJ41) were found to rapidly lose viability in the cold (by a factor of 10^5 to 10^6). Conversely, among the 9 screened poultry-derived strains, 5 had marked loss of viability (by a factor of 10^5 to 10^6) and 4 declined at intermediate rates (by a factor of 10^2 to 10^3) (Table 1).

The cold tolerance phenotype is maintained in CR-derived storage medium. To obtain an estimate of the relevance of cold tolerance in MHB to survival in the actual food product (refrigerated poultry), we examined 4°C survival in MHB versus autoclaved CR-derived storage medium. Comparative survival in MHB and CR over 14 days at 4°C was examined with three poultry strains (CJ7, CJ35, and CJ38) and three clinical strains (CJ17, CJ41, CJ45). Survival appeared overall similar in MHB and in CR (Fig. 2 and data not shown). Strains which lost viability rapidly in MHB had similarly rapid rates of viability loss in CR, and conversely those with low rates of CFU decline in MHB behaved similarly in CR. The results suggest that cold survival in our model system (MHB) may simulate survival in refrigerated poultry. In addition, these results indicated that the rate of viability loss is a strain-specific property that is not affected by differences in medium composition likely to be present between MHB and CR.

Viability of *C. jejuni* strains is reduced markedly by freezing, regardless of the relative ability of the isolates to remain viable at 4°C. Six *C. jejuni* strains (CJ1, CJ7, CJ17, CJ19, CJ35, and CJ45) with different rates of viability loss at 4°C (Table 1) were examined in terms of their survival following freezing at −20°C in MHB and CR. The isolates were maintained in the frozen state and thawed only once, immediately before assessment of viability by plate counts. Freezing in MHB or CR resulted in a marked reduction (by a factor of 10^3 or greater) in viability of all strains (Table 2). Interestingly, freezing in CR enhanced survival in four of the six strains (two strains, CJ17 and CJ19, were not affected) (Table 2). Further investigation of freezing tolerance of two strains (CJ35 and CJ45) over a longer time period (32 days) confirmed the impact of CR in enhancing survival over the entire period, in comparison to cells frozen in MHB (Fig. 3). In CR, viable counts also dropped noticeably after freezing, albeit to a lesser extent than in MHB, and remained relatively stable thereafter, until ca. 26 days of frozen storage (Fig. 3). Since each of the monitored samples was thawed only once, the results suggest that the reduction in viability was mostly in response to the freezing and/or thawing of the frozen suspensions and that the duration of freezing was not of significant impact over the first 3 to 4 weeks. In conclusion, all screened strains were found to be highly sensitive to freeze-thawing, regardless of their rate of viability loss at 4°C. Certain strains, nonetheless, retained significant viability upon prolonged freezing at −20°C (and thawing), and survival was enhanced when CR was used as the freeze-thawing medium.

Cell morphology at 4°C (spiral versus coccoid) is not strongly correlated with survival at 4°C or with viability following freeze-thawing. *C. jejuni* is well-known for its transition from a spiral to a coccoid morphotype during exposure to adverse environmental conditions (1, 7). We examined, therefore, whether strains with different rates of CFU decline at 4°C differed in the timing and extent of this morphological transition.

Microscopic examination of a number of cultures stored at 4°C failed to reveal a strong correlation between cell morphology at any given time during the 4°C storage and the number of CFU. Although cells from all isolates were spiral and motile when examined immediately before storage at 4°C, some isolates remained spiral even when few or no culturable cells were

TABLE 2. Effect of medium on freezing (−20°C) tolerance and cell morphology of *C. jejuni* strains[a]

Isolate	Origin	Broth	No. of CFU/ml on:		Day 12 cell morphology[b]
			Day 0	Day 12	
CJ1	Poultry	MH	7.40×10^6	<10	Coccoid
		CR	7.90×10^6	1.67×10^3	Coccoid
CJ7	Poultry	MH	8.50×10^6	<10	Coccoid
		CR	1.30×10^7	8.00×10^1	Coccoid
CJ35	Poultry	MH	3.00×10^7	3.50×10^1	Coccoid
		CR	5.00×10^6	1.63×10^4	Coccoid
CJ17	Clinical	MH	7.60×10^7	6.20×10^2	Coccoid
		CR	6.45×10^7	9.80×10^2	Coccoid
CJ19	Clinical	MH	2.00×10^7	<10	Spiral
		CR	1.20×10^7	<10	Spiral
CJ45	Clinical	MH	8.00×10^7	3.06×10^3	Coccoid
		CR	7.00×10^7	3.46×10^4	Coccoid

[a] *C. jejuni* cultures were kept in MHB or CR at −20°C for 12 days, and viability was assessed as described in Materials and Methods.
[b] Spiral, at least one-fourth of the cells were spiral; Coccoid, almost all of the cells were coccoid.

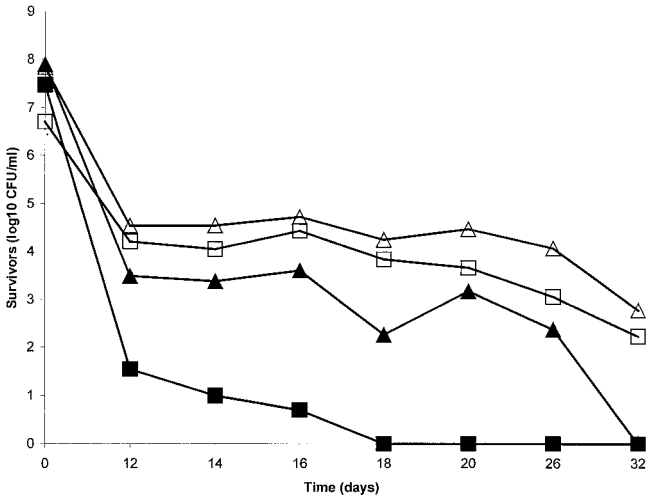

FIG. 3. Freezing tolerance of *C. jejuni* in MHB and CR. CJ35 (squares) and CJ45 (triangles) were stored at −20°C in MHB (closed symbols) or in CR (open symbols). Frozen cultures were thawed only once for assessment of survival. Results for CJ35 in MHB on days 18, 20, 26, and 32, and CJ45 in MHB on day 32 were below the limit of detection (10 CFU/ml). Data shown are means from duplicate plates in a representative experiment and were collected as described in Materials and Methods. The general linear models procedure was utilized to compute all statistical inferences. The slope of each data curve was calculated and compared. The difference between CJ35 in MHB and CJ35 in CR was statistically significant ($P < 0.005$). A significant difference was also observed between CJ45 in MHB and CJ45 in CR ($P < 0.05$).

present, while others became primarily coccoid but maintained 10^3 to 10^4 CFU/ml (Table 3). The results appeared to be strain specific.

The impact of freeze-thawing on cell morphology also appeared to be strain dependent and unrelated to degree of freeze-thaw tolerance or to freezing medium (MHB or CR) (Table 2).

DISCUSSION

In the United States and many other industrialized nations, raw poultry products are commonly exposed to refrigeration or freezing for variable lengths of time before they reach the consumer. Since raw or undercooked chicken is considered to be an important risk factor for human campylobacteriosis (20, 30), one would expect that the pathogen must have the ability to tolerate refrigeration and freeze-thawing. The ability of *C. jejuni* to tolerate these and other conditions inhospitable for bacterial survival in food is currently poorly understood. In addition, *C. jejuni* is genetically remarkably diverse (21, 34), and it is therefore important to characterize the degree to which aspects of the adaptive physiology of the pathogen (including tolerance to cold and to freeze-thawing) may differ among different strains.

The results from this study suggest that even though the rate of CFU decline varied markedly among different strains of *C. jejuni*, the strains with lowest rates of viability loss over 14 days of storage at 4°C were predominantly of clinical origin. This was initially surprising, since all poultry-derived isolates in this study were isolated from refrigerated material and might therefore be more likely to be cold tolerant. On the other hand, if poultry is contaminated by diverse strains which vary in cold tolerance, refrigeration (which is often prolonged) may constitute a powerful selection for cold tolerance in poultry-derived strains that enter the pool of human clinical isolates. Strains that survive in relatively high numbers in the refrigerated food (such as CJ35 and CJ52) may constitute the majority of the inoculum that reaches consumers and that is relevant in terms of human infection.

In this study we also identified two clinical isolates (CJ14 and CJ41) whose CFU numbers declined rapidly at 4°C. If they are indeed transmitted through contaminated poultry, such isolates may have become implicated in illness because their viable counts in the product may have been high (e.g., due to consumption of the contaminated poultry following only a short refrigeration period). Alternatively, such strains may be of enhanced virulence to humans, with lower than average infectious doses. It is also possible that such clinical isolates were transmitted via a route other than contaminated poultry. Contaminated water, raw milk, and contact with live birds and pets have also been implicated in human infections by *C. jejuni* (1, 20, 30).

At this time, the mechanisms which underlie the observed differences in cold tolerance are not known. We failed to observe a strong correlation between viability of the bacteria at 4°C (as determined by plate counts) and cell morphology (spiral versus coccoid). These results suggest that the transition to the coccoid morphotype at 4°C is a strain-specific response that does not readily reflect loss of viability and are in agreement with results recently described by other investigators (7, 14). In addition, the viability estimates which we obtained should be regarded as minimal estimates, since cells may remain viable substantially longer than can be cultured. Fluorescence with the respiratory dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride) is one way to detect the presence of viable cells (25). Preliminary data from our laboratory suggest that strains which had lower rates of CFU decline remained viable for a longer period of time when detected by CTC. These data also showed that fluorescence was present even if the cells had become

TABLE 3. Cell morphology and cold (4°C) tolerance of *C. jejuni* strains[a]

Isolate	Origin	Cell morphology	No. of CFU/ml
CJ3	Poultry	Coccoid[d]	1.00×10^1
CJ7	Poultry	Spiral[b]	2.85×10^5
CJ33	Poultry	Coccoid[d]	4.50×10^3
CJ37	Poultry	Spiral[c]	1.15×10^5
CJ38	Poultry	Coccoid[d]	1.22×10^4
CJ52	Poultry	Coccoid[d]	4.30×10^5
CJ17	Clinical	Coccoid[d]	6.38×10^6
CJ22	Clinical	Spiral[b]	1.13×10^7
CJ25	Clinical	Spiral[b]	8.25×10^6
CJ26	Clinical	Spiral[b]	3.30×10^6
CJ41	Clinical	Spiral[b]	3.09×10^3
CJ63	Clinical	Spiral[b]	1.66×10^7
CJ67	Clinical	Coccoid[d]	2.08×10^7

[a] Cell morphology and survival were determined following 10 days of incubation at 4°C in MHB.
[b] At least 1/4 of the cells were spiral.
[c] At least 1/10 of the cells were spiral.
[d] Almost all of the cells were coccoid.

nonculturable. Thus, the differences which we observed on the basis of plate counts may also reflect differences in viability assessed by other criteria. In this study we opted to concentrate on CFU-based viability assessments in order to obtain readily interpretable estimates of potential inoculum levels of the bacteria following exposure at 4°C or −20°C for time periods relevant to poultry at retail. Although the viable-but-nonculturable state has been recognized and studied in *C. jejuni* (14, 26, 32), conflicting results have been obtained concerning the infectivity of the putative viable but noncultural forms in animal models (18, 28).

All strains in this study were found to be markedly sensitive to freezing and/or freeze-thawing, in agreement with previous findings (9). Although certain strains survived at modest levels (CFU decline by ca. 10^2 to 10^5 following 10 to 30 days at −20°C and one thawing), such survival could not be readily correlated with rates of viability loss of the strains at 4°C. Indeed, freeze-thaw injury is mediated by unique processes, such as ice nucleation and dehydration (17), not commonly encountered during cold (4°C) stress. Our results suggest that the observed loss of viability reflected mostly death of cells in response to freezing and/or thawing (and depended less on the length of the frozen storage). Recent work with *Campylobacter coli* also showed similar sensitivity to freezing and thawing and identified the major role of superoxide anions in freeze-thawing injury (27). It is not yet known whether oxidative damage is implicated in cold tolerance of *C. jejuni* (or *C. coli*).

The strain-specific differences in CFU decline at 4°C suggest a genetic basis. We have applied several molecular subtyping tools to analyze the strains, including restriction fragment length polymorphism (RFLPs) analysis with a probe derived from the chemotaxis-related gene *tlpA* (6), multiplex PCR-RFLPs utilizing products from two distinct genomic regions of *C. jejuni* encoding gyrase and lipopolysaccharide biosynthesis functions (4, 23, 33), and pulsed-field gel electrophoresis. Our data suggest that strains with lower rates of viability loss at 4°C were of diverse genotypes (as were those that lost viability rapidly) (Chan et al., unpublished). Interestingly, strain CJ41, which is of clinical origin but rapidly lost viability at 4°C (Table 1), was quite distinct from other clinical strains on the basis of *tlpA*-RFLPs and PCR-RFLP data.

There is currently a clear need to further investigate the mechanisms underlying the ability of *C. jejuni* to survive following prolonged exposure to low temperatures. Strains with markedly different rates of CFU decline at 4°C, such as those identified here, together with the recent availability of the *C. jejuni* genome sequence (21) are expected to facilitate these further studies.

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