

IN VITRO SURVIVAL AT LOW pH AND ACID ADAPTATION RESPONSE OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI*

BASHAR W. SHAHEEN¹, MICHAEL E. MILLER² and
OMAR A. OYARZABAL^{1,3}

¹*Department of Poultry Science*

²*Department of Biological Sciences*
Auburn University, Auburn, AL 36849

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ABSTRACT

The survival of Campylobacter jejuni and Campylobacter coli at pH 7.0, 6.0, 5.0 and 4.0 for up to 24 h, and the induction of an acid adaptation response in 12 C. jejuni and 10 C. coli strains in early exponential or late stationary phases in tryptic soy broth (TSB) and Brucella broth were investigated. C. coli strains were more sensitive than C. jejuni in media adjusted to pH 5.0 or 4.0 with hydrochloric acid. Five log₁₀ cfu/mL of C. coli were inhibited at 12 h in pH 5.0, but only 2.5 log₁₀ cfu/mL of C. jejuni cells were inhibited under the same conditions. No viable cells were detected at 4 h in pH 4.0 from an inoculum of 6 log₁₀ cfu/mL of C. coli. Late stationary phase cells of C. jejuni exposed to pH 5.0 for 4 h in TSB exhibited a significant (P ≤ 0.05) acid tolerance response when compared to nonexposed cells. Late stationary phase cells of C. coli exposed to pH 5.0 for 3 h in TSB and late stationary and early exponential phase cells exposed to pH 5.0 for 3 h in Brucella broth showed a significant (P ≤ 0.05) acid tolerance response when compared to nonexposed cells. The acid tolerance responses observed for C. jejuni and C. coli conferred adapted cells no more than 1 log₁₀ cfu/mL survival advantage over nonadapted cells. Brucella broth appeared to be more protective than TSB for the survival of C. jejuni and C. coli at pH 4.0.

PRACTICAL APPLICATIONS

This research can help us understand the extent of *Campylobacter* reduction on meat surfaces by decontamination strategies based on low pH. If the

³ Corresponding author. TEL: 334-844-2608; FAX: 334-844-2641; EMAIL: oyarzoa@auburn.edu

protection by acid adaptation response is minimal, the surface pH of poultry meat could be decreased temporarily to significantly reduce the contamination with *Campylobacter*. The reduction effect of lemon-based marinades on artificially inoculated and naturally occurring *Campylobacter* could be quantified and used to develop low-pH marinades for the reduction of *Campylobacter* spp. Studies could also focus on the development of new processing and preservation techniques that could reduce *Campylobacter* spp. in highly contaminated food products. In addition, research on the human health implications of *Campylobacter* cells that survive other sublethal stressors, such as low- or high-temperature shock or starvation, could elucidate if any cross-protection effect is expressed by *Campylobacter* spp.

INTRODUCTION

The ability of some bacterial foodborne pathogens to adapt to mildly acidic environments results in an increased survival at lethal low pH (Goodson and Rowbury 1989; Benjamin and Datta 1995; Gahan *et al.* 1996). Understanding the mechanisms for survival is important to food processors, who aim at reducing the growth of bacterial pathogens in foods, and to physicians, because the acidic environment of the stomach is an important barrier to defend the host against colonization by foodborne pathogens (Hill *et al.* 1995; Audia *et al.* 2001). Studies with *Salmonella enterica* serotype Typhimurium (Foster and Hall 1990; Foster 1991; Audia *et al.* 2001), *Escherichia coli* (Hersh *et al.* 1996; Lin *et al.* 1996; Paul and Hirshfield 2003) and *Listeria monocytogenes* (Hill *et al.* 1995; Gahan *et al.* 1996) have demonstrated that exposure to mild pH (~4.8–5.5) stimulates the production of preshock proteins that protect cells against otherwise lethal acid shocks (pH 3.0 for *Listeria* and *Salmonella*, and pH 2.0 for *E. coli*). The growth phase of the culture appears to define the kind of stress response expected at low pH (Audia *et al.* 2001). For instance, stationary phase and starved cells of *E. coli* O157:H7 are more acid tolerant than mid-exponential phase cells (Hengge-Aronis 1993; Arnold and Kaspar 1995). The adaptation to mild acidic conditions can also result in the induction of resistance to other stresses, as it is the case with adapted cells of *Salmonella* Typhimurium that exhibit resistance toward heat, salt, activated lactoperoxidase system and polymyxin B (Kelly *et al.* 2001).

There is a large body of research on the survival strategies used by *E. coli* and *Salmonella* to cope with low pH. For *Salmonella*, the acid-shock proteins (ASPs) involved in the acid tolerance response that is triggered during the exponential phase of growth are different from the ASPs activated when cells reach the stationary phase (Audia *et al.* 2001). For *E. coli*, three

independent acid resistance systems (ARS) with overlapping controls have been revealed, which may also be present in *E. coli* O157:H7. The ARS that is triggered in the stationary phase is responsible for the survival in acid conditions above pH 3.0, while the other ARS account for the survival below pH 3.0 (Hersh *et al.* 1996; Lin *et al.* 1996). However, in contrast with other gram-negative foodborne bacteria, little is known about the physiological mechanisms used by *Campylobacter* spp. to survive at a low pH (Park 2002).

Recent findings support the theory that *Campylobacter jejuni* responds to stress in unique ways. Exponential phase cells of *C. jejuni* appear to be more resistant to mild heat stress (50C) and oxidative stress caused by aeration (Kelly *et al.* 2001). Furthermore, the absence of *rpoS* homologues in the *Campylobacter* genome (Parkhill *et al.* 2000) suggests that the entry into stationary phase does not induce a known mechanism of resistance (Kelly *et al.* 2001). *C. jejuni* grows within a pH range of 6.5–7.5, and it is inhibited at pH 4.9 or less in broth (Doyle and Roman 1981) and at pH 5.1 in plate media (Gill and Harris 1983). However, Fletcher *et al.* (1983) determined that the growth of *C. jejuni* in *Brucella* supplemented with yeast extract (Doyle and Roman 1982) and adjusted to pH 5.5 was statistically similar to the growth at pH 7.5. Wu *et al.* (1994) found a significant increase in protein induction by *C. jejuni* that underwent an acidic shock at pH 3.0, but not by cells that were acid shocked at pH 4.0 or 5.0. Murphy *et al.* (2003a,b) also reported an adaptive tolerance response to acid and/or aerobic conditions in *C. jejuni* in exponential and stationary phases. All studies to date have reported a similar behavior at low pH between *C. jejuni* and *Campylobacter coli*.

The purposes of these studies were to investigate the survival of *C. jejuni* and *C. coli* at pH 7.0, 6.0, 5.0 and 4.0 for up to 24 h, and to determine if early exponential or late stationary phase cells of *C. jejuni* and *C. coli* exhibit an acid adaptation response that protects the cells from the inhibitory effects of pH 4.0, when exposed to tryptic soy broth (TSB) or *Brucella* broth at pH 5.0.

MATERIALS AND METHODS

Strain Characterization

The *C. jejuni* and *C. coli* strains used in this study are listed in Table 1. Strains were maintained at –80C in TSB (Difco, Detroit, MI) supplemented with 20% glycerol (v/v). Stock cultures were transferred at least twice to modified Campy-Cefex (Oyarzabal *et al.* 2005) and grown at 42C for 48 h under a microaerophilic gas mixture containing 10% CO₂, 5% O₂ and 85%

TABLE 1.
STRAINS USED IN THE STUDIES AND THEIR ANTIBIOTIC SUSCEPTIBILITY

Species	Strain ID	Source	Origin	Antimicrobial Susceptibility*					
				AZ	CI	CM	EM	GM	TC
<i>Campylobacter jejuni</i>	2002-341	CDC†	Human	S	R	S	S	S	S
	2002-348	CDC	Human	S	R	S	S	S	R
	2002-351	CDC	Human	S	R	S	S	S	S
	2002-353	CDC	Human	S	R	S	S	S	S
	2002-370	CDC	Human	S	R	S	S	S	S
	2002-404	CDC	Human	S	R	S	S	S	S
	2002-409	CDC	Human	S	R	S	S	S	R
	2002-410	CDC	Human	S	R	S	S	S	S
	2002-420	CDC	Human	S	R	S	S	S	S
	2002-439	CDC	Human	S	R	S	I	S	S
	33560	ATCC‡	Bovine feces	S	S	S	I	I	S
	Post 5	AU§	Broiler chicken	S	I	I	I	I	S
	<i>Campylobacter coli</i>	43473	ATCC	Human feces	R	S	I	R	S
49941		ATCC	Human	R	S	I	R	S	S
43133		ATCC	Pig	R	S	R	R	I	R
43481		ATCC	Turkey feces	R	S	I	R	S	R
BAA-371		ATCC	Human feces	R	R	R	R	I	R
51798		ATCC	Pig	R	S	R	R	S	R
43484		ATCC	Human	S	S	S	S	S	S
33559		ATCC	Pig Feces	S	S	S	S	S	I
mCC 66		AU	Broiler chicken	I	S	R	I	S	S
801		ADPH¶	Human	S	S	I	I	S	S

* S, sensitive; I, intermediate; R, resistant; AZ, azithromycin; CI, ciprofloxacin; CM, chloramphenicol; EM, erythromycin; GM, gentamicin; TC, tetracycline.

† Centers for Disease Control and Prevention, Atlanta, GA.

‡ American Type Culture Collection, Manassas, VA.

§ Department of Poultry Science, Auburn University, Auburn, AL.

¶ Alabama Department of Public Health, Montgomery, AL.

N₂ (BOC Gases, Hixson, TN) in sealed plastic bags (Oyarzabal *et al.* 2005) or in anaerobic jars gassed with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD). Bacterial DNA was extracted using PrepMan Ultra (Applied Biosystems, Foster City, CA) and isolates were identified to the species level using a multiplex polymerase chain reaction assay (Cloak and Fratamico 2002; Oyarzabal *et al.* 2005). The identity of the isolates was confirmed with a RiboPrinter (Qualicon, Wilmington, DE) (Fig. 1). This DNA profile was also made to assure that each strain used in the experiment has a unique DNA profile. We also identified the antimicrobial resistance profile for each isolate with E-Test strips (AB Biodisk, Piscataway, NJ) to ensure that we have included strains with different profiles.

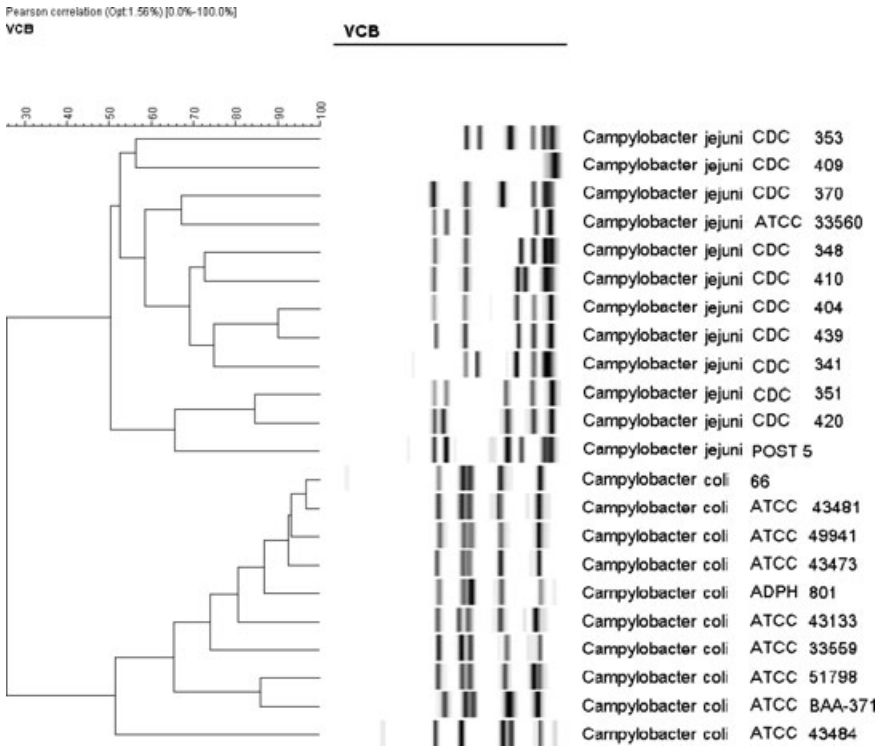


FIG. 1. RIBOTYPING RESULTS OF *CAMPYLOBACTER* STRAINS

Restriction was done with PstI according to the manufacturer's instructions. Bands were detected and analyzed with bionumerics (Applied-Math, Inc., Austin, TX) using the coefficient of Pearson's clustering to determine profile relatedness.

The identity of all *C. coli* isolates was also confirmed with API Campy tests (bioMérieux, Hazelwood, MO).

Preparation of Inocula

To prepare late stationary phase cultures, cells were collected from modified Campy-Cefex plates incubated for 48 h at 42°C. Cells were resuspended into sterile phosphate-buffered saline (PBS) (pH 7.0) to an optical density at 600 nm (OD_{600}) of 0.2 (approximately 10^6 – 10^7 cfu/mL) using a Spectronic GENESYS 20 Vis (Thermo Electron Co., Waltham, MA). To create early exponential phase cultures, 1 mL of the inoculated PBS was transferred to 10 mL of TSB (pH 7.0) and incubated for 4 h under microaerophilic conditions at 42°C. This bacterial suspension was transferred to TSB tubes at different pH values for survival studies.

Scanning Electron Microscopy (SEM)

To determine the signs of stress induced at pH 5.0 and 4.0, SEM photographs were collected for *C. jejuni* 2002-353 and *C. coli* ATCC 51798 and mCC 66. After 2–3 h exposure to pH 7.0, 5.0 or 4.0, 1 mL of the culture was fixed in 2% glutaraldehyde, 1% osmium tetroxide and 0.1 M cacodylate buffer for 20 min. Samples were washed twice in 0.1 M cacodylate buffer (pH 7.2) before final suspension in deionized, distilled water. Cells were dehydrated using air drying for 3 h. Cultures were mounted on aluminum stubs with ventral surface upward and sputter coated with gold/palladium. Specimens were examined in a Zeiss DSM 940 scanning electron microscope (Thornwood, NY) operated at 15 kV.

Survival of Stationary Phase Cells of *C. jejuni* and *C. coli* in TSB at pH 7.0, 6.0, 5.0 and 4.0

In the first set of studies, tubes with 10 mL TSB adjusted to pH 7.0, 6.0, 5.0 and 4.0 with 1 N HCl were inoculated at 42C under microaerophilic conditions with 0.1–1.0 mL of PBS containing stationary phase cultures of *C. jejuni* ATCC 33560 or *C. coli* ATCC 43133. Counts were determined immediately after the inoculation of each pH tube and at 4, 8, 12 and 24 h. The survivals in TSB at pH 4.0 were also enumerated at 0.5, 1 and 2 h. Counts were evaluated by serial dilutions in PBS with spread plating in duplicate on *Campylobacter* blood free agar plates (mCCDA, Acumedia, Baltimore, MD) and modified Campy-Cefex (Oyarzabal *et al.* 2005) plates that were incubated at 42C under microaerophilic conditions for 48 h.

Induction of an Acid Tolerance Response in Early Exponential and Late Stationary Phase Cells of *C. jejuni* and *C. coli*

Experiments were conducted with *C. jejuni* and *C. coli* strains to induce an acid adaptation response at pH 5.0. Forty-eight-hour cultures were used as stationary phase cells or to induce early exponential phase cells as described (Murphy *et al.* 2003a,b, 2005). Cultures in PBS (pH 7.0) were transferred (1 mL) to TSB tubes adjusted to pH 5.0 (final pH 5.1–5.2) and incubated at 42C under microaerophilic conditions for 4 h. *C. coli* strains were incubated at pH 5.0 for 3 h. Cultures were then transferred (1 mL) to TSB tubes adjusted to pH 4.0 (final pH 4.0). Control cells were collected as described and transferred (0.1 mL) to pH 4.0 (final pH 4.0). Initial counts were determined immediately after the transfer to pH 4.0, for both control and pH 5.0-exposed cells, and final counts were determined at 2 h of incubation at 42C under microaerophilic conditions. Initial counts varied from 4.9 to 6.4 (average 5.8) log₁₀ cfu/mL. Similar experiments were done using *Brucella* broth (pH 7.0; Acumedia), but

the final counts in pH 4.0 were determined after 3 h of incubation at 42C under microaerophilic conditions. Counts were determined by serial dilution and spread plating on mCCDA plates, which were incubated at 42C under microaerophilic conditions.

Statistical Analysis

A minimum of three independent replicates was performed for each experiment. Results were converted to \log_{10} cfu/mL, and the average of the replicates for each strain and for each phase growth was used for analysis. To assess the survival of early exponential and late stationary phase cells of *C. jejuni* and *C. coli* at pH 4.0 after exposure to pH 5.0, the reduction between initial counts and final counts was determined. Data were examined with the UNIVARIATE procedure of SAS (release 9.1, SAS Institute, Inc., Cary, NC) for descriptive statistics and goodness-of-fit tests for normal distribution. If the data sets fit a normal distribution, the means between the control and the exposed groups were analyzed for differences ($P \leq 0.05$) using Duncan's test (GLM procedure of SAS). Data sets that did not fit into a normal distribution were analyzed by the Wilcoxon two-sample test using the NPAR1WAY procedure of SAS.

RESULTS AND DISCUSSION

Survival of *C. jejuni* and *C. coli* in TSB at pH 7.0, 6.0, 5.0 and 4.0

The first sets of experiments were intended to determine the survival of two ATCC strains of *Campylobacter* at low pH. Because there were no differences between mCCDA and Campy-Cefex plates, we decided to use only mCCDA plates throughout the rest of the experiments. At pH 7.0 and 6.0, no changes were observed in the number of *C. jejuni* and *C. coli* cells over time. The decline in surviving cells was greater for *C. coli* than for *C. jejuni* at pH 5.0 and 4.0. At pH 5.0, the decrease in surviving cells between 4 and 8 h was approximately $2 \log_{10}$ cfu/mL for *C. coli* and *C. jejuni*, which was considerably higher than the reduction seen for the first 4 h (Fig. 2A). At 12 h at pH 5.0, the number of *C. jejuni* cells decreased to approximately $2.5 \log_{10}$ cfu/mL, which represents approximately a 1,000-fold decrease from the original inoculation, while *C. coli* exhibited a $6 \log_{10}$ cfu/mL decrease of the original inoculation. No *C. jejuni* cells survived at 8 h in pH 4.0. *C. coli* cells were not detected at 4 h in pH 4.0, even after the enrichment of the samples in Bolton broth (Oxoid, New York, NY) without antibiotics. No cells of either *C. jejuni* or *C. coli* were recovered at 24 h in either pH 5.0 or 4.0. The different survival patterns of *C. jejuni* and *C. coli* at low pH were also observed with similar

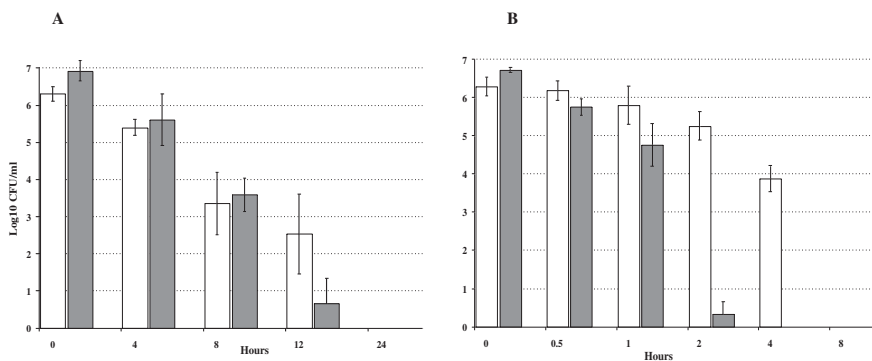


FIG. 2. DECREASE IN STATIONARY PHASE CELLS OF *CAMPYLOBACTER JEJUNI* ATCC 33560 (□) AND *CAMPYLOBACTER COLI* ATCC 43133 (■) IN TRYPTIC SOY BROTH AT pH 5.0 (A) AND pH 4.0 (B)

All strains were unrecoverable at 24 h in pH 5.0 and at 8 h in pH 4.0. Error bars represent ± 1 standard error of the mean.

experiments involving *C. coli* strain mCC 66 and *C. jejuni* strains Post 5, 2002-351, 2002-370, 2002-409, 2002-420 and 2002-439 (data not shown).

The decline in the surviving cells of *C. jejuni* appeared to be in agreement with previous findings that reported a decline of 3.2 log₁₀ cfu/mL of *Campylobacter* in a mixture of water (250 mL) and commercial broiler feed (6.25 g) acidified at pH 4.0 with HCl (Chaveerach *et al.* 2002). However, with the use of Mueller–Hinton broth adjusted to pH 4.0 with formic acid, 10 strains of *C. jejuni* rapidly died (1 h) at 37°C, and no cells were recovered at 2 h of incubation even when enrichment broth was used (Chaveerach *et al.* 2003). In Luria–Bertani broth acidified to pH 4.0 with HCl, *C. jejuni* was recovered at a rate of 0.1% of the original inoculation after 2-h exposure (Waterman and Small 1998). In addition, ascorbic acid at concentrations equal or above 0.5 mg/mL (Fletcher *et al.* 1983) and acetic, formic and lactic acids (Waterman and Small 1998; Chaveerach *et al.* 2002) have been shown to inhibit *C. jejuni* growth. It appears that *Campylobacter* cells may be more sensitive to organic acids than HCl, as reported with *L. monocytogenes* (Phan-Thanh *et al.* 2000). A short-term exposure of less than 1 h yielded a minimal decrease in the population of *C. jejuni* cells. Similar results were found with an exposure of 30 min to pH 3.6 (HCl) and for three *C. jejuni* isolates inoculated at 7 log₁₀ cfu/mL (Blaser *et al.* 1980).

It has been reported that the survival patterns of *C. jejuni* and *C. coli* at pH 4.0 are the same (Chaveerach *et al.* 2002). However, we found a striking difference between the survival patterns of these two *Campylobacter* spp. The experiments with an ATCC strain of *C. coli* showed that this strain was highly

sensitive to pH 4.0 (TSB adjusted with HCl), a sensitivity that has not been previously described. More than $5 \log_{10}$ cfu/mL of *C. coli* were inhibited in 2 h and no survivals were detected at 4 h at pH 4.0. Similar results were found with mCC 66, a strain isolated from processed broilers. This sensitivity to HCl by *C. coli* appears to be a physiological trend unique for this *Campylobacter* species and which warrants further studies.

Induction of an Acid Tolerance Response in Early Exponential and Late Stationary Phase Cells of *C. jejuni* and *C. coli*

The survival of *C. jejuni* and *C. coli* at pH 5.0 and 4.0 allowed us to design experiments to induce an acid tolerance response by these bacteria at pH 5.0. The choice of a 4-h adaptation to pH 5.0 was based on reports suggesting that no increase in any induction to adaptation was found after 5 h with a *C. jejuni* strain (Murphy *et al.* 2003a). To test if this time/pH combination was lethal, cells exposed to pH 5.0 in TSB were transferred to TSB tubes at pH 7.0 and were then incubated at 42C for up to 24 h under microaerophilic conditions. This experiment was performed with all 12 *C. jejuni* strains and for early exponential and late stationary phase cells in TSB. The same numbers of cells were observed for each growth phase at 2 h, while an increase in the number of cells at 24 h, with final concentrations above $7 \log_{10}$ cfu/mL, was observed with early exponential cells transferred to pH 7.0 (Table 2). Therefore, the acid shock induced under these conditions was not lethal for *C. jejuni*. The decision to use a 2-h exposure time at pH 4.0 for the final experiment was based on the results found when doing the death curve at pH 5.0 and 4.0 (Fig. 2).

TABLE 2.
SURVIVAL OF *CAMPYLOBACTER JEJUNI* IN TRYPTIC SOY
BROTH (TSB) AT pH 7.0 AFTER EXPOSURE TO TSB pH 5.0
EXPRESSED AS \log_{10} cfu/mL*

Phase	Time (h)		
	0	2	24
Exponential†	6.1 ^A	6.3 ^A	7.8 ^B
Stationary‡	5.3 ^A	5.5 ^A	NA

* Means of 12 strains in triplicates were analyzed for differences ($P < 0.05$) using Duncan's test (GLM procedure of SAS). Different letters within the same row means significant difference.

† Pooled standard error of the mean (SEM) = 0.09.

‡ Pooled SEM = 0.16.

NA, data not collected.

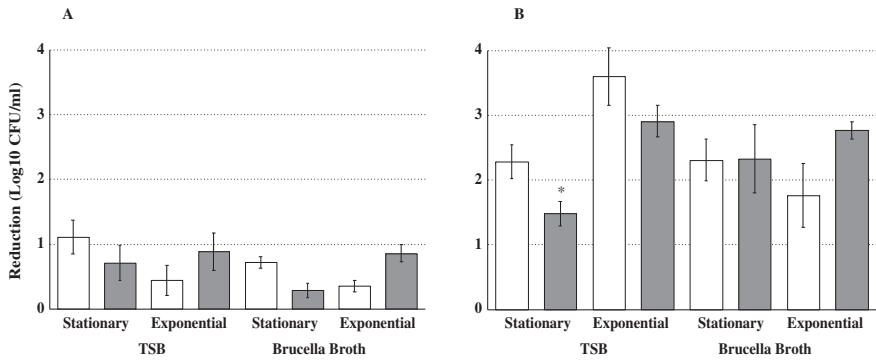


FIG. 3. INHIBITION OF *CAMPYLOBACTER JEJUNI* STRAINS 2002-353 (A) AND 2002-439 (B) AT 4 h IN TRYPTIC SOY BROTH AT pH 4.0 AND 3 h IN *BRUCELLA* BROTH AT pH 4.0 AFTER 5-h EXPOSURE TO pH 5.0

□, control cells; ■, pH 5.0-exposed cells. Experiments were replicated three to five times. Error bars represent ± 1 standard error of the mean. *, means of control and pH 5.0-exposed cells are different ($P \leq 0.05$).

The experiments were based on survivals on agar plates. These experiments usually yield results with large variability and data that may not be normally distributed. To compensate for this variability, the average of three experiments for each stain was used for statistical analysis. After exposure to pH 4.0, cells were immediately plated out for enumeration using PBS at pH 7.0 to neutralize any carryover of low pH and to measure any transient response that might appear (Yousef and Courtney 2002). Data from *C. jejuni* experiments gave a normal distribution under the UNIVARIATE procedure of SAS.

A large variability in the results was observed among the 12 *C. jejuni* strains. The strains that were the most sensitive (2002-439) and the most resistant (2002-353) to low pH were two fluoroquinolone-resistant (FQR) strains (Table 1) isolated from humans (Anon 2004). The subject of fluoroquinolone resistance among *C. jejuni* isolates has raised some concerns about the properties of these isolates and their survival in the food supply (Smith *et al.* 1999). A recent report (Luo *et al.* 2005) suggested that FQR strains had an advantage to colonize chickens when FQR and nonresistant strains were inoculated concurrently. These *in vitro* results revealed that the acid adaptation response observed with stationary phase cells in TSB was present only in the most sensitive strain (Fig. 3).

An acid tolerance response was observed in *C. jejuni* cells in late stationary phase in TSB at pH 5.0 for 4 h. Adapted cells survived more than control cells ($P \leq 0.05$) at pH 4.0. However, this response conferred a survival advantage of less than 1 log₁₀ cfu/mL to adapted cells. No response to acid adapta-

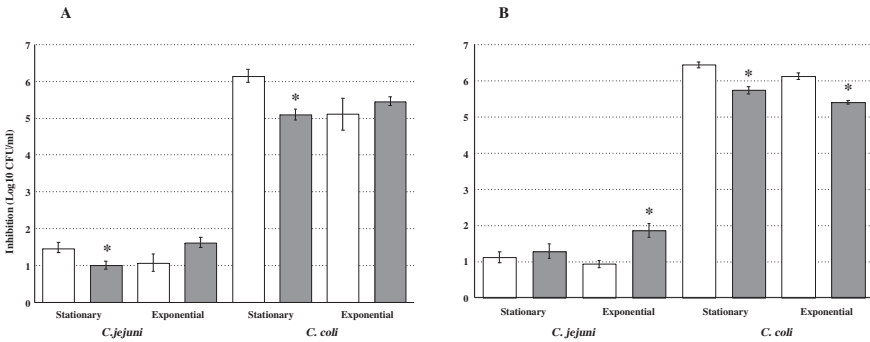


FIG. 4. INHIBITION OF EXPONENTIAL AND STATIONARY PHASE CELLS OF 12 STRAINS OF *CAMPYLOBACTER JEJUNI* AND 10 STRAINS OF *CAMPYLOBACTER COLI* IN TRYPTIC SOY BROTH AT pH 4.0 FOR 2 h (A) OR *BRUCELLA* BROTH AT pH 4.0 FOR 3 h (B). *C. jejuni* cells were exposed to pH 5.0 for 4 h, while *C. coli* cells were exposed to pH 5.0 for 3 h. □, control cells; ■, pH 5.0-exposed cells. Error bars represent ± 1 standard error of the mean. *, means of control and pH 5.0-exposed cells are different ($P \leq 0.05$).

tion was found in early exponential phase cells in TSB or early exponential or late stationary cells in *Brucella* broth (Fig. 4). In general, early exponential phase cells of *C. jejuni* exposed to pH 5.0 for 4 h in TSB and *Brucella* broth were more sensitive to pH 4.0 ($P \leq 0.05$) than nonexposed cells. Murphy *et al.* (2003a,b) reported that early stationary phase cells of a *C. jejuni* strain adapted aerobically at pH 5.5 induced an acid tolerance response that provided these cells with greater survival advantages than nonadapted cells as early as 1.7 h after exposure to pH 4.4. The adapted *C. jejuni* strain had a 3 log₁₀ cfu survival advantage over control cells at pH 4.4 at approximately 4.6 h (Murphy *et al.* 2005). Similar to the results, mid-exponential and late stationary phase cells did not induce any acid tolerance response, and cells exposed to pH 5.5 for 5 h exhibited similar or increased death rate when compared to nonexposed cells (Murphy *et al.* 2003a). It may appear that the variability in the physiological properties of different *C. jejuni* accounts for these discrepancies. It may also be possible that the adaptation under aerobic conditions promotes an acid resistance that we could not induce when we performed the experiments under microaerophilic conditions. Furthermore, Wu *et al.* (1994) failed to find any stress-induced protein in a *C. jejuni* strain subjected to pH 5.0 or 4.0 for 1 h, but were able to find protein homologues of *E. coli* GroEL and GroES at pH 8.6. The findings suggest that stationary phase cells of *C. jejuni* may exhibit an acid tolerance response that can be described as moderate when compared to the exponential phase and stationary phase responses described for *Salmonella* (Foster and Hall 1990; Audia *et al.* 2001), *E. coli* (Lin *et al.* 1995, 1996; Hersh *et al.* 1996) and *L. monocytogenes* (Gahan and Hill 1999; Phan-Thanh *et al.*

2000). Furthermore, we do not know if this acid tolerance response expressed by *C. jejuni* in media adjusted to low pH with HCl will protect the cells against volatile fatty acids, as it has been described for *Salmonella* serotype Typhimurium (Baik *et al.* 1996). More research is required to understand how *C. jejuni* cells at different growth phases react with different stressors. It is also important to note that in opposition to results found at low pH, stationary phase cells of *C. jejuni* have been reported to be more sensitive to heat and aeration than mid-exponential phase cells (Kelly *et al.* 2001), and that the known RpoS-mediated acid tolerance response triggered in the stationary phase cells of *E. coli* and *Salmonella* (Goodson and Rowbury 1989; Slonczewski and Foster 1996; Foster 1999) does not appear to be present in *C. jejuni* (Parkhill *et al.* 2000; Kelly *et al.* 2001).

Data from *C. coli* experiments gave a non-normal distribution even after the logarithmic transformation of the data. Therefore, these data were analyzed with a nonparametric test. Many experiments resulted in no surviving cells at 2 h in TSB pH 4.0, which explained in part the variability in the collected data and confirmed the sensitivity of *C. coli* to low pH. In preliminary experiments using a 4-h exposure at pH 5.0, we found that *C. coli* cells died quickly when transferred to pH 4.0. For this reason, we used a 3-h adaptation at pH 5.0 for all *C. coli* strains. Although in early experiment, we realized that *C. coli* was almost completely inhibited at pH 4.0 for 2 h, we decided to keep this final time to compare results with data obtained using *C. jejuni* strains. *C. coli* strains exhibited an acid adaptation response ($P \leq 0.05$) in late stationary phase in TSB and both early exponential and late stationary phases in *Brucella* broth. Similarly to the adaptation observed in *C. jejuni*, *C. coli* cells exposed to pH 5.0 had a survival advantage of no more than 1 log₁₀ cfu/mL when compared to nonexposed cells. More than 5 log₁₀ cfu/mL reductions for all *C. coli* experiments, regardless of the medium and the growth phase, were observed (Fig. 4). These findings revealed a unique response to pH stress by *C. coli*, which warrants further studies if we are to understand the survival at low pH by *Campylobacter* spp. In the U.S.A., it is assumed that the contribution to human campylobacteriosis by non-*jejuni* *Campylobacter* is minimal (Mead *et al.* 1999) and therefore *C. coli* has been less studied than *C. jejuni*. However, recent studies have suggested that *C. coli* and other species may play a more significant role in human campylobacteriosis because the methodology in the U.S.A. favors recovery of *C. jejuni* isolates. In Germany, a total of 18.6% of *Campylobacter* isolates from humans from November 2002 to October 2003 were identified as *C. coli* (Gurtler *et al.* 2005), which suggests that in other countries *C. coli* may be an important source of human campylobacteriosis.

When the final counts were taken at 2 h of exposure to pH 4.0 in *Brucella* broth, a consistent survival rate of more than 90% was found. When the final

counts were collected at 3 h of exposure to pH 4.0, the reduction values for *C. jejuni* and *C. coli* were similar to the reduction observed in TSB at 2 h (Fig. 4). *Brucella* broth at pH 4.0 was more protective for *Campylobacter* than TSB at pH 4.0. Similar variations in the survival of one *C. jejuni* strain in different broths, including three *Brucella* broths from different manufacturers, have been recently described, with an adaptive tolerance response that could be induced only in three broths (two *Brucella* broths) out of seven broths tested (Murphy *et al.* 2005). This information highlights the complexity of selecting a medium and/or a standard strain for stress studies with *Campylobacter*. Variations in experimental protocols (e.g., phase growth, minimal versus complex media, etc.) and the inconsistent results obtained with different media limit the comparisons that can be made among results obtained in different laboratories (Bearson *et al.* 1997). Perhaps the development of a defined minimal medium suitable for *Campylobacter* growth will help the scientific community standardize the experimental protocols for stress studies and further identify the survival strategies used by *Campylobacter* spp.

Stress Response Observed Under SEM

Signs of degenerative stress were observed under SEM for both *C. jejuni* and *C. coli* at pH 5.0 and 4.0 (Fig. 5). These changes confirmed that the cells were undergoing a stress response at low pH. Acidic stress, particularly at pH 4.0, will alter the pH homeostasis and allow for the accumulation of lethal levels of H⁺ in the cytoplasm (Hill *et al.* 1995; Bearson *et al.* 1997). Cells subjected to pH 5.0 for 4 h were transferred to TSB pH 7.0 and then incubated at 42C for 24 h under microaerophilic conditions. In all these cases, cells were able to recover and grow to approximately 10⁷ cfu/mL. Similar changes involving the loss of integrity of the cytoplasmic membranes have been reported for *Campylobacter* subjected to low pH (Chaveerach *et al.* 2002). According to the decline in surviving cells seen in the experiments, cells of *C. jejuni* and *C. coli* may suffer irreversible stress after 8 h of exposure at pH 5.0.

In summary, we have identified a unique sensitivity to low pH by *C. coli* and acid adaptation responses by *C. jejuni* and *C. coli* exposed to pH 5.0. This is the first description of a high sensitivity of *C. coli* to broth adjusted to pH 4.0 with HCl and of an acid adaptation response by *C. coli*. The adaptation responses were induced primarily in late stationary phases and were not consistent in all *C. jejuni* or *C. coli* strains. Furthermore, the adaptation induced at pH 5.0 provided adapted cells with no more than 1 log₁₀ cfu/mL survival advantage. *Brucella* broth appeared to be more protective than TSB at pH 4.0 for *C. jejuni* and *C. coli*, although the protection could be described as marginal.

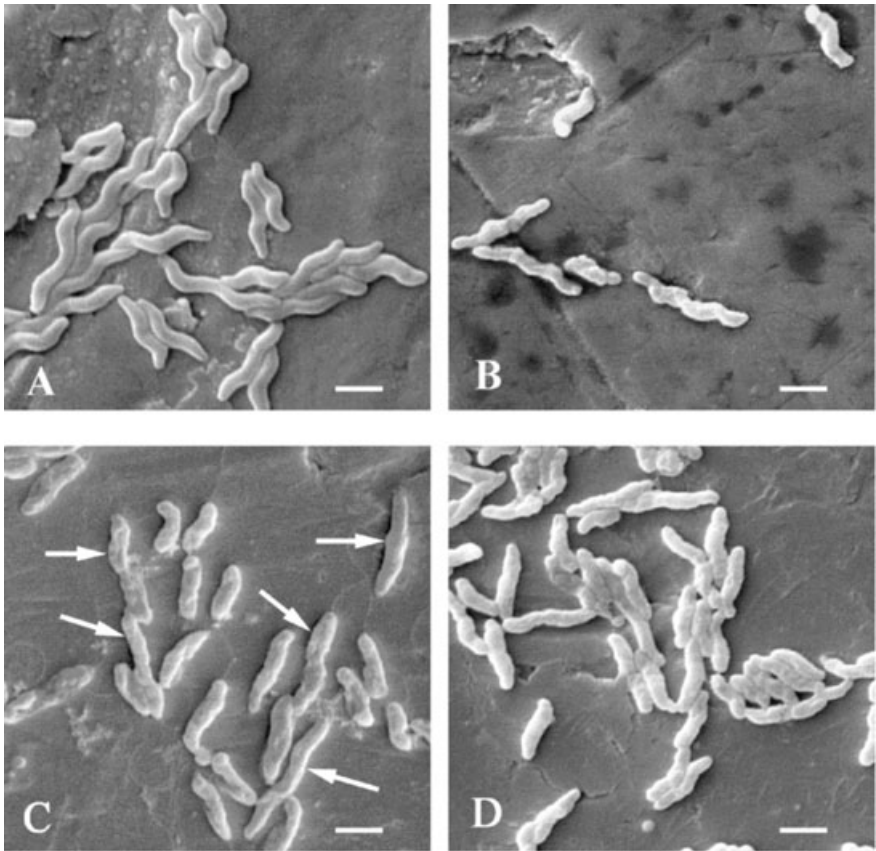


FIG. 5. MORPHOLOGICAL CHANGES OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* CELLS UNDER SCANNING ELECTRON MICROSCOPY

C. jejuni cells with integral cytoplasmic membranes at pH 7 (A) and cells with disruption in the membranes at pH 4.0 (B). *C. coli* ATCC 51798 cells at pH 5.0 (C) and pH 4.0 (D) showing irregularities on their surfaces and disruption of the cytoplasmic membranes. Photographs were collected after 3-h exposure to pH 7.0, 5.0 or 4.0. One milliliter of the culture was fixed in 2% glutaraldehyde, 1% osmium tetroxide and 0.1 M cacodylate buffer (pH 7.2) for 20 min. Specimens were examined in a Zeiss DSM 940 scanning electron microscope operated at 15 kV. Bars = 1 μ m.

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