IN VITRO SURVIVAL OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* AT LOW PH

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THESIS ABSTRACT

IN VITRO SURVIVAL OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* AT LOW PH

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A series of experiments were performed to investigate the effect of pre-exposure to acidic conditions of early exponential and stationary-phase cells of *Campylobacter jejuni* and *C. coli*. The sublethal acid exposure to protect *C. jejuni* and *C. coli* against low pH treatment was also studied. Twelve *C. jejuni* strains, including ten fluoroquinolone-resistant *C. jejuni* isolated from humans, and nine *C. coli* strains, were grown at 42^{o}C under microaerophilic conditionss (85% N_2, 10% CO_2, 5% and O_2) in modified Campy Cefex (mCC) agar to create stationary-phase cells. The colonies were then suspended in Tryptic Soya broth (TSB, at final pH 7), and grown to the appropriate early exponential-phase under microaerophilic conditions. The cells in both phases were exposed to TSB at

pH 5 for 4 h before the final challenge (TSB at final pH 4). The same procedures were performed utilizing Brucella broth. Survival was determined after 2-3 h of the final challenge by using viable cell counts and utilizing Campylobacter blood free selective media (mCCDA). Pre-exposure of the early exponential-phase and the late stationary-phase cells to moderate pH 5 shows no induction of resistance to the pH 4, where the control cells show better survival. Moreover, the survival time increased from 2 to 3 h when using Brucella broth instead of TSB. *Campylobacter coli* were very sensitive to the lethal acidic treatments. The results showed that *Campylobacter jejuni* and *C. coli* failed to produce an acid adaptation response in early-exponential or stationary-phase in Tryptic Soya broth or Brucella broth (pH 5). The images obtained from scanning electron microscopy also indicated that cells displayed different morphological changes at different pH values.

The traditionally used liquid broth and chemically defined medium M 199 was also evaluated for *Campylobacter jejuni* in vitro. The liquid media compared were M 199, Brucella broth (BB), Brain Heart Infusion broth supplemented with yeast extract (BHIYE), and Tryptone Soya broth supplemented with yeast extract (TSBYE). An initial inoculum of 10^3 to 10^4 CFU/ml resulted in greater than or equal to 10^6 CFU/ml in BB and M 199 after incubation for up to 72 h, while lower growth rate was observed in both TSBYE and BHIYE. Among all liquid media compared, M 199 exhibited the least percent of coccoid cell formation. On M 199, *C. jejuni* grew over a pH range of 5 to 8.

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I. LITERATURE REVIEW

The Bacterial Growth Curve

In batch culture and under favorable conditions, a growing bacterial population doubles at regular intervals, and this is called exponential growth. When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over time, plotting the data will yield bacterial growth curve (Figure 1 below).

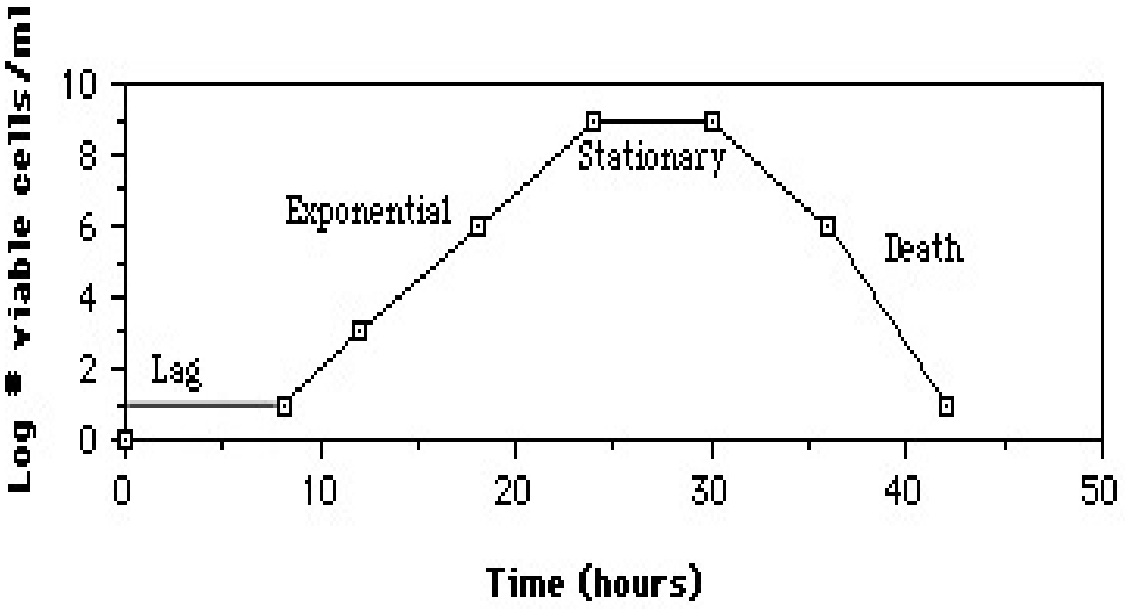

Figure 1. The bacterial growth curve. When bacteria are grown in a closed system (also called a batch culture), like a test tube, a prog ression through a series of phases can observed : no net growth occurs (lag phase). This is followed by a phase of exponential growth, where the cell mass increases exponentially with time. When their growth

bcomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs time.

Phases of bacterial growth curve:

1. Lag Phase.

When cells in the stationary phase of growth are transferred to fresh medium, a lag phase occurs. Although there is no apparent cell division occurring, the cells may be growing in volume or mass. The lag phase is due to the time required for the physiological adaptation of stationary phase cells in preparation for growth. The lag phase can be due the time required for the recovery of cells from toxic products of metabolism that may accumulate in the external medium, such as acid, bases, alcohols, or solvents. Sometimes, new enzymes or coenzymes, proteins, RNA, etc. must be synthesized before growth resumes especially if the fresh media is different from the inoculum medium. The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

2. **Exponential (log) Phase**.

In exponential phase, all cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the

composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as generation time.

3. Stationary Phase.

Exponential growth cannot be continued forever in a batch culture (e.g. a closed system such as a test tube or flask). It may be because of exhaustion of available nutrients, limitation of oxygen, or the accumulation of inhibitory metabolites or end products and exhaustion of space, in this case called a lack of "biological space". The stationary phase, like the lag phase, is not necessarily a period of quiescence.

4. Death Phase.

If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. Common causes of death include the depletion of cellular energy and the activity of autolytic (self-destructive) enzymes. Some bacteria can remain viable for along time. For example, some bacteria sporulate or form cysts or form coccoid form when exponential growth ceases. Some bacteria can adapt to nutrient depletion and remain viable for along periods in stationary phase.

pH homeostasis in gram-negative bacteria.

Enteric pathogens try to keep the internal pH close to 7.6 to 7.8 during growth (3). The difference between internal pH and outside pH is known as delta pH (Δ pH). The internal pH is maintained by pumping the protons into the cell at alkaline outside pH, or by extruding the protons in an acidic environment (18). Two systems are critical to

maintaining the internal pH homeostasis in gram negative bacteria. These systems are potassium-proton antiporters (21) and sodium-proton antiporters (4). Briefly, shifting to acidic condition causes the potassium-proton antiporter system to alkalinize the cytoplasm, whereas shifting to alkaline media result in acidification of the cytoplasm through sodium-proton antiporter (10, 46).

In addition to the above mentioned systems, adaptive acid stress response systems are "emergency" systems which enable the enteric pathogens to induce certain regulatory pathways to cope with the damage caused by severe acidic conditions. Inducible acid stress responses in some gram negative bacteria are provided in this review.

Acid resistance properties of *Escherichia coli*.

The human stomach maintains the acidic environment around pH 2, with an approximate emptying time of 2 h (41). *Escherichia coli* possess remarkable acid resistance systems that enable it to cause disease at low inocula (10 – 100 CFU/ml). These bacteria are also able to withstand a pH-2 challenge for at least 2 h (24). Four *E. coli* inducible acid resistance systems in stationary-phase have been reported that provide protection to severe acidic environments. The first system is acid resistance system one, AR1: which relies directly on the stationary-phase sigma factor, and protects cells at pH 2·5 in minimal media (22). There is a need for activation (not induction) by brief exposure to glutamate prior to challenge at pH 2.5. The cyclic adenosine monophosphate (cAMP) receptor protein (CRP) appears to control whether or not the sigma factor-dependent induction mechanism will operate. In rapidly growing cells in complex media,

the high value of CRP appears to prevent sigma factor-dependent transcription of the *gad* genes. In stationary-phase cells (growing on glucose), cAMP values are low, thereby allowing sigma factor -dependent *gad* expression (7). The second acid resistance system, AR2, requires inducible glutamate decarboxylase, an enzyme that convert intracellular glutamate to gamma amino butyric acid (GABA) and glutamate/GABA antiporter *gad C* (6). Two glutamate decarboxylase (GAD) genes, *gadA* and *gadBC, encode* two proteins, GadA and GadB which provide resistance at pH 2.5 as long as GadC antiporter is present (9). Inducible amino acid decarboxylase systems provide protection to low pH by consumption of intracellular protons. In this system, protons leak across the cell membrane at low pH challenge, and then are consumed by amino acid decaboxylation reactions (e.g., glutamate decarboxylase GAD to form GABA). To consume protons efficiently, the decarboxylation product should be carried out by specific substrate/product antiporters (GadC antiporter) (35). The third AR system requires inducible arginine decarboxylase (*AdiA*) and the AdiC antiporter, that convert intracellular arginine to agmatine with consumtion of protons in the process (15, 19). The optimum expression of AR3 occurs at low pH 2 in complex media. A fourth system was recently described as lysine dependent, and involves the inducible lysine decarboxylase (19). In these described systems, protons entering the cell are consumed by the decarboxylase reaction via exchange with the amino acid α-carboxyl group. The product of decarboxylation is then returned to the exterior by antiport in exchange for more substrate (27). Consuming protons by decarboxylation would produce a less acidic internal pH and generate an inside positive potential that could help repel protons and/or prevent excessive proton motive force. This acidophilic strategy developed by *E. coli*

help to convert membrane potential from an inside negative charge to positive charge, appears to be an important system used to survive extreme acidic environments (34).

In contrast, the adaptation of *E. coli* during the exponential-phase has been determined and called acid habituation (AH). This occurs when *E. coli* are adapted in nutrient broth at pH 5, with the survival measured when the cells are challenged to pH 3, and involves a short exposure time of 7 minutes (16). Many inducing-agents such as glutamate, aspartate, proline, glucose, and KCL can be added to the log-phase cells for 60 minutes that cause induction at a neutral pH (36). Phosphate and the phosphate-specific porin PhoE have been shown to play a major role in AH during the exponential-phase. Thus, high values of phosphate in the medium are proposed to block access of H^+ to the PhoE pore and thereby interfere with signal transduction (2, 37). Extracellular proteins made at pH 5 are thought to be important to induce acid habituation in log-phase cells grown at neutral pH (2, 38, 39).

Another system that provides protection to log-phase cells is called acid tolerance response (ATR). In an ATR system, log-phase cells are protected from severe acid stress for several hours at pH 3, unlike the previously described AH which provides protection for only 7 minutes at pH 3 in complex media. Cyclopropane fatty acid (CFA) formation in the membrane is critical for ATR in *E. coli* (8). The potential roles of CFAs during the ATR in *E. coli* include interaction with membrane proteins that influence proton's traffic. Unsaturated fatty acids are converted to their cyclopropyl derivatives by the action of a cyclopropane fatty acid (CFA) synthase encoded by the *cfa* gene (17).

Acid tolerance response of *Salmonella* serovar Typhimurium.

Salmonella typhimurium has both a log-phase and a stationary-phase acid tolerance system called acid tolerance response (ATR). In both phases, cells grown in moderately acidic environment trigger the synthesis of certain proteins which protect the cells from severe acidic condition. This occurs when exponential cells are grown at pH 7.7 in minimal media, then, exposed to moderately acidic environment at pH 5.8. This treatment enhances the synthesis of pre-acidic proteins that allow adapted bacteria to survive severe acidic shock (13). Adaptation to pH 4.5 enhances the synthesis of other sets of proteins called acidic shock proteins (ASPs) (11). About 50 ASPs, which differ from those of pre-acidic proteins, are believed to be produced due to the lowering of the internal pH as the outer pH decreases (12).

In log-phase ATR, three regulatory genes are involved in controlling the expression of subsets of ASPs. The regulatory genes include the alternate sigma factor sigma factor, which control the expression of 10 ASPs; the iron regulator Fur for 5 ASPs; and the two components signal transduction system PhoPQ for four ASPs. In addition to the ASPs, the Ada protein, which is also known as transcriptional regulator, is required to survive in low pH condition but does not appear to be induced by acid or to regulate ASPs (2).

The acid tolerance of stationary-phase of *Salmonella typhimurium* involves both sigma factor-dependent and sigma factor-independent systems (23). The σ^S-dependent stationary-phase is not induced by acid, as indicated in the log phase dependent system, but the entrance of cells into the stationary-phase was enough to induce the sigma factor system and the acid tolerance. For the acid tolerance sigma factor-independent system,

the only regulator found to affect this system is the OmpR response regulator. It has been shown that low pH conditions appear to induce the production of OmpR, which, in its phosphorylated state, can trigger expression of genes needed for acid-induced stationary-phase tolerance (1).

There is a link between the survival at low pH and virulence. *Salmonella typhimurium* is able to survive and replicate in the severe acidic condition of the macrophage for 60 min (32). Other studies also indicate that mutants defective in acid tolerance attenuate the pathogenecity of the microorganism (14, 45).

Helicobacter pylori **acid tolerance.**

H. pylori colonizes the stomach and causes gastroduodenal diseases, such as gastritis, peptic ulcer disease and gastric carcinoma in humans (5). This organism has exceptional acid tolerance mechanisms that enable it to survive the severe acidic condition of the stomach. The most important mechanism involves the production of urease, which converts urea to carbon dioxide and ammonia an alkaline product (25). One model suggested for urease protection is that the external urease provides a "cloud" of less acidic conditions around the organism, and the internal urease plays major role in supporting the internal pH homeostasis (40). Furthermore, by using urease inhibitor (fluorofamide), which poorly penetrates the cell, external urease has been shown to be unimportant for internal pH homeostasis (40). The above mentioned results are also supported by the fact that internal urease does not become active until the pH outside is below 6 and remains active through an outside pH 2.5, and that the external urease is inactivated at pH below 4.5. Considering the optimum pH for urease activity is 7.5, how

do the cells protect themselves from alkalinizing their internal pH when the cells grow at neutral pH outside in the presence of urea? This question has been solved by the identification of UreI which is an inner membrane proton-gated, urea-specific channel (43). The UreI pore opens as pH drops below 6.5 and the cytoplasmic urease gains access to the outside urea. The enzymatic activity neutralizes the acidic condition, and the pore closes and urea transport ceases. It has been determined that at least six periplasmic amino acid residues are important for activation of the UreI transport activity (44, 33). Protonation of these amino acid residues causes a conformation change that opens the UreI pore.

***Campylobacter jejuni* acid tolerance.**

Campylobacter spp. are a common causes of food foodborn illness in humans (20). Due to the fastidious nature of the organism, *Campylobacter* are very sensitive to oxygen, unable to grown at temperature below 29^{o}C, and at pH below 4.9 or at sodium chloride values above 2.5% (42). Unlike other food borne pathogens, *Campylobacter* lacks many regulatory pathways in stationary-phase that enable them to cope with the stresses (31, 30). Apparently, the lack of *rpoS* increases the sensitivity of *Campylobacter* to many stress conditions. Previous studies indicated that the resistance of *C. jejuni* NCTC 11351 to different kind of stresses, such as heat or oxidative stress, is not induced upon the entry to the stationary-phase (20). This sensitivity is also described by acidic challenge of *C. jejuni* CI 120, in which acid adaptation did not provide a protection to mid exponential or the stationary-phase cells (29). More recently, by utilizing different media such as Brucella broth (BB), Tryptic Soya broth supplemented with yeast extract,

Mueller Hinton broth, and Brain Heart Infusion supplemented with yeast extract, no ATR was induced (28).

Interestingly, upon the entry to the stationary-phase, *C. jejuni* displayed a change in membrane fatty acid composition, particularly a decrease of the proportion of unsaturated fatty acid and increase in the content of cyclopropane and short-chain fatty acids (26). This change in membrane composition coincided with an increase in the cellular pressure resistance, but no change in acid or heat treatment was observed. In contrast to *E. coli,* where cyclopropane fatty acids in the membrane play a major role to increase resistance to protect against acid stress (8, 17), no such role is reported for acid resistance in *C. jejuni* (26)

REFERENCES

1. **Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park.** 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**2245-52.
2. **Bearson, B. L., Wilson, and J. W. Foster.** 1998. A low pH inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. J. Bacteriol. **180:**2409-2417.
3. **Bijlsma, J. J., M. M. G. Gerrits, R. Imamdi, C. M. Vandenbrouche-Grauls, and J. G. Kusters.** 1998. Urease-positive, acid-sensitive mutants of *Helicobacter pylori*: urease-independent acid resistance involved in growth at low pH. FEMS Microbiol. Lett. **167:**309-313.
4. **Brey, R. N., B. P. Rosen, and E. N. Sorensen.** 1979. Cation/proton antiporter systems in *Escherichia coli*. J. Biol. Chem. **255:**39-44.
5. **Buck, G. E.** 1990. *Campylobacter pylori* and gastroduodenal disease. Clin. Microbiol. Rev. **3:**1-12.
6. **Castanie-Cornet, M. P., and J. W. Foster.** 2001. *Escherichia coli* acid resistance: cAMP receptor protein and a 20 bp cis-acting sequence control pH and stationary-phase expression of the *gadA* and *gadBC* glutamate decarboxylase genes. Microbiol. **147:**709-715.
7. **Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster.** 1999. Control of acid resistance in *Escherichia coli*. J. Bacteriol. **181:**3525-3535.
8. **Chang, Y. Y., and J. E. Cronan.** 1999. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol. Microbiol. **33:**249-259.

9. **De Biase, D., A. Tramonti, F. Bossa, and P. Visca.** 1999. The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. Mol. Microbiol. **32:**1198-1211.

10. **Dibrov, P. A.** 1991. The role of sodium ion transport in *Escherichia coli* genetics. Biochim. Biophys. Acta **1056:**209-224.

11. **Foster, J. W.** 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **173:**1981-1987.

12. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for adaptive acid tolerance response. J. Bacteriol.**173:** 6896-6902.

13. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129-5135.

14. **Garcia-del Portillo, F., J. W. Foster, and B. B. Finlay.** 1993. The role of acid tolerance response genes in *Salmonella typhimurium* virulence. Infect. Immun. **61:**4489-4492.

15. **Gong, S., H. Richard, and J. W. Foster.** 2003. YjdE (AdiC) is the arginine:agmatine antiporter essential for arginine-dependent acid resistance in *Escherichia coli*. J. Bacteriol. **185:**4402-4409.

16. **Goodson, M., and R. J. Rowbury.** 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. Lett. Appl. Microbiol. **8:**77–79.

17. **Grogan, D. W., and J. E. Cronan**. 1997. Cyclopropane ring formation in membrane lipids of bacteria. Microbiol. Mol. Biol. Rev. **61:**429–441.

18. **Hengge-Aronis**, **R.** 2000. Microbial responses to acid stress. pp. 99–115. In: G. Storz

and R. Hengge-Aronis, (eds.) *Bacterial Stress Responses*. ASM Press, Washington, DC.

19. **Lange, R., and Hengge-Aronis, R.** 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5:**49-59.
20. **Iyer, R., C. Williams, and C. Miller.** 2003. Arginine-agmatine antiporter in extreme acid resistance in *Escherichia coli*. J. Bacteriol. **185:**6556-61
21. **Kelly, A. F., S. F. Park, R. Bovill, and B. M. Mackey.** 2001. Survival of *Campylobacter jejuni* during stationary-phase: evidence for the absence of a phenotypic stationary-phase response. Appl. Environ. Microbiol. **67:**2248-54.
22. **Kroll, R. G., and I. R. Booth.** 1983. The relation between intracellular pH, the pH gradient and potassium transport in *Escherichia coli.* J. Biochem**. 216:**706-719.
23. **Lee, I. S., J. L. Slonczewski, and J. W. Foster.** 1994. A low pH inducible stationary-phase acid tolerance response in *Salmonella typhimurium.* J. Bacteriol. **176:**1422-1426.
24. **Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster.** 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. **62:**3094-100.
25. **Marchall, B. J., L. J. Barrett, C. Prakash, R. W. McCallum, and R. Guerrant.** 1990. Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effects of acid. Gastroenterology **99:** 697-702.
26. **Martínez-Rodriguez, A., and B. M. Mackey.** 2005. Physiological changes in *Campylobacter jejuni* on entry into stationary-phase. Int. J. Food Microbiol. **101:**1-8.
27. **Molenaar, D., J. S. Bosscher, B. ten Brink, A. J. Driessen, and W. N. Konings.**

1993. Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. J. Bacteriol. **175:**2864-2870.

28. **Murphy, C., C. Carroll, and K. N. Jordan.** 2005. The effect of different media on the survival and induction of stress responses by *Campylobacter jejuni*. J. Microbiol. Methods. **62**:161-166.
29. **Murphy, C., C. Carroll, and K. N. Jordan.** 2003. Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. FEMS Microbiol. Lett. **223:**89-93.
30. **Park, S. F.** 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. Int. J. Food Microbiol. **74:**177–188.
31. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. M. van Vliet, S. Whitehead, and B.G. Barrell**. 2000. The genome sequence of the foodborne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature. **403:**665–668.
32. **Rathman, M., M. D. Sjaastad, and S. Falkow.** 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. **64:** 2765-2773.
33. **Rektorsachek, M., A. Buhmann, D. Weeks, D. Schwan, K. W. Bensch, S. Eskandari, D. Scott, G. Sachs, and K. Melchers.** 2000. Acid resistance of *Helicobacter pylori* depends on the Urel membrane protein and inner membrane

proton barrier. Mol. Microbiol. **36:**141-152.

34. **Richard, H. T., and Foster J. W.** 2004. *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J. Bacteriol. **186:**6032-41.
35. **Richard, H. T., and J. W. Foster.** 2003. Acid resistance in *Escherichia coli*. Adv. Appl. Microbiol. **52:**167-186.
36. **Rowbury, R. J.** 1999. Acid tolerance induced by metabolites and secreted proteins, and how tolerance can be counteracted, p. 93-106. In J. C. Derek and G. Gardew (ed.), *Bacterial responses to pH*. John Wiley & Sons, Ltd., Chichester, England.
37. **Rowbury, R. J., M. Goodson, and D. A. Wallace.** 1992. The PhoE porin and transmission of the chemical stimulus for induction of acid resistance (acid habituation) in *Escherichia coli*. J. Appl. Bacteriol. **72:**233–243.
38. **Rowbury, R. J., T. J. Humphrey, and M. Goodson.** 1999. Properties of L-glutamate-induced acid tolerance response which involves the functioning of extracellular induction components. J. Appl. Microbiol. **86:**325-330.
39. **Rowbury, R. J., N. H. Hussain, and M. Goodson.** 1998. Extracellular proteins and other components as obligate intermediates in the induction of a range of acid tolerance and sensitisation responses in *Escherichia coli*. FEMS Microbiol. Lett. **166:**283-288.
40. **Scott, D. R., D. L. Weeks, C. Hong, S. Postius, K, Melchers, and G. Sachs.** 1998. The role of internal urease in acid resistance of *Helicobacter pylori.* Gastroenterology **114:**58-70.
41. **Smith, J. L.** 2003. The role of gastric acid in preventing foodborne disease and how

bacteria overcome acid conditions. J. Food Prot. **66:**1292-1303.

42. **Solomon, E. B., and D. G. Hoover**. 1999. *Campylobacter jejuni*: a bacterial paradox. J. Food Safety. **19:**121–136.
43. **Weeks, D. L., S. Eskandari, D. R. Scott, and G. Sachs.** 2000. A H^+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. Science**. 287:** 482-485.
44. **Weeks, D. L., G. Sachs.** 2001. Sites of pH regulation of urea channel of *Helicobacter pylori.* Mol. Microbiol. **40:**1249-1259.
45. **Wilmes-Riesenberg, M. R., B. Bearson, J. W. Foster, and R. Curtiss III.** 1996. Role of the acid tolerance response in virulence of *Salmonella typhimurium.* Infect. Immun. **64:** 6220-6228.
46. **Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan.** 1984. The sodium/proton antiporter is part of the pH homeostasis mechanism in *Escherichia coli.* J. Biol. Chem. **257:**3687-3691.

II. THE EFFECTS OF DIFFERENT PH VALUES ON THE SURVIVAL OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI*

INTRODUCTION

Campylobacter jejuni and *C. coli* are important causative agents of gastrointestinal infections in humans (15), with an estimated 2.5 million cases each year in the United States (5).*Campylobacter* is a normal inhabitant of the gastrointestinal tract of broiler chickens (20). Many studies linked the occurrence of *Campylobacter* in chickens and the increased incidence of campylobacteriosis in humans. Deming et al. (4) identified that humans could be infected through eating re-contaminated fully-cooked chicken, eating chicken that was reported to be raw or undercooked, or eating chicken meat that was in contact with cats. Another study indicated that consuming unpasteurized milk was associated with an outbreak of campylobacteriosis in Hungary (13).

Some important factors involved in the epidemiology of *Campylobacter* in humans are: handling raw chicken, which causes sporadic *Campylobacter* infection (7), eating pork (19), living or working on a farm (19), barbequing (1, 19) and traveling. Traveling is proposed to be an important factor in the acquisition of bacteria resistant to antibiotics (6, 18).

There are many factors that influence stress tolerance of bacteria. The value of inherent stress tolerance varies among natural isolates. It has been proposed that *Salmonella entritidis* PT4 isolated from clinical cases had better inherent tolerance to heat, acidity, oxidative stress and surface stress than non-clinical ones (8). Those clinical isolates also showed more resistance to the host defenses systems and displayed enhanced virulence (9). Another factor playing an important role in inherent stress tolerance and survival of bacteria in foods is the growth phase of contaminating organism. In general, the stress tolerance is at the lowest in mid log-phase and increases in late log-phase and the stationary-phase. RpoS play a major role to induce the tolerance response during the stationary-phase to different kind of stresses such as heat, H_2O_2 (10), osmotic stress (11), and irradiation (16).

The purpose of this study was to investigate the survival of *C. jejuni* and *C. coli* at pH 6, 5 and 4 during the early exponential and late stationary-phase. Furthermore, scanning electron microscopy (SEM) was utilized to determine the nature of morphological changes that occur upon the exposure of *C. coli* and *C. jejuni* to different pH values.

MATERIALS AND METHODS

Bacterial Strains

C. jejuni ATCC 33560, CDC 2002-370 and Post 5, and *C. coli* ATCC 43473, 43133 and mCC 66 were used in this study. All strains were maintained at -80^{o} C. Cultures were transferred from frozen stocks to modified Campy Cefex (mCC) and grown at 42^{o}C under microaerophilic conditions (BOC Gas, 5% O_2, 10% CO_2 and 85%

N_2). The cultures were incubated for 48 h before starting each experiment.

Preparation of inoculum

A loop of the culture was obtained from mCC plates and suspended into 9 ml sterile phosphate buffer saline (PBS). The suspension was adjusted to MacFarland No. 0.5 turbidity to achieve a concentration of 10^6-10^8 CFU/ml and adjusting them to an optical density at 600 of 0.1 to 0.2 (approximately $1x10^8$ CFU/ml), measured with spectrophotometer (Spectronic GENESYS 20 Vis; Thermo Electron Co, Waltham, MA). From each PBS tube, one ml was transferred to 10 ml tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) and incubated for 4 h under microaerophilic conditions at 42^{o}C to create early exponential-phase cultures.

To maintain the stationary-phase cells; mCC culture plates were incubated under microaerophilic conditions for 48 h. The cells were then harvested in PBS and concentrated to MacFarland No. 0.5 turbidity (~10^6-10^8 CFU/ml)**.**

Acid resistance of Campylobacter spp. in exponential and stationary-phase

Preliminary studies were done to determine the survival of *Campylobacter jejuni* and *C. coli* at different pH values and over time. Tubes with 10 ml TSB adjusted to pH 7, 6, 5 and 4 with 1N HCL were inoculated with 0.1 to 1 ml of PBS containing exponential or stationary-phase cultures. Survival (percentage) was determined at 0.5, 1, 4 and 24 h at 42^{o}C under microaerophilic conditions. Initial and final counts were determined on *Campylobacter* blood free agar plates (mCCDA, Acumedia, Baltimore, MD) incubated at 42^{o}C under microaerophilic conditions for 48 h.

Scanning electron microscopy (SEM)

To determine the value of stress induced at pH 5 and pH 4, SEM photographs were collected for two *C. coli* cultures (mCC 66 and ATCC 51798), and one *C. jejuni* (CDC 2002-353). After 2–3 h exposure to pH 4, one ml of the culture was fixed in 2% Glutaraldehyde, 1% Osmium tetroxide and 0.1 M Cacodylate buffer (pH 7.2) for 20 minutes. Cultures were mounted on aluminum stubs with ventral surface upwards and sputter–coated with gold/plalladium. Specimens were examined in a Zeiss DSM 940 SEM operated at 15 kV.

Statistical analysis

All experiments were performed in triplicates. Results were converted to $\log_{10}$ CFU/ml and expressed as percent survival after each test. Data were analyzed using GLM procedures (SAS Institute Incorporated, Cary, NC) and means analyzed for differences by Duncan's test (SAS Program) with significance set at $P \leq 0.05$.

RESULTS

Tolerance of Campylobacter spp. to different pH value

The survival percentage of *Campylobacter* spp. indicated that there is no significant difference after exposure to pH 6 for 1 h and 4 h and at both of exponential and stationary-phases. Under moderate acidic condition (pH 6), *Campylobacter* survival was slightly decreased after 24 h ($P > 0.05$). At pH 5, the survival of *C. jejuni* declined with time and at 4 h only 74 to 75% of the original population was recovered. The

survival of *C. jejuni* decreased pronouncedly at pH 4, with only 24% surviving after 4 h. *C. coli* strains were quickly inactivated by pH 4. After 1 h, only 55 and 42% of *C. coli* were recoverable at pH 4 at the exponential and stationary phases, respectively. No *C. coli* were recovered after 4 h, even after the enrichment of the samples in Bolton broth (Oxoid) without antibiotics.

Morphological changes of Campylobacter spp. upon exposure to acid stress

The morphological change and topography due to acidic stress were examined by SEM (Fig. 1, 2, 3). The control *C. coli* and *C. jejuni* cells have a normal spiral shape with smooth surface (Fig. 1 A, 2 A) and in a large magnification (Fig. 3 A, C). Cells stressed at pH 4.5 completely lost their spiral shape, resulting in roughness and amorphous collapsed bodies (Fig. 1 B, D, 2 B) and in a large magnification (Fig. 3 B, D). These results indicate the possibility that the outer membrane of the cells or the cytoplasmic membrane partially perturbed. *Campylobacter* cells exposed to pH 5 showed ruffling and craters on the surface (Fig. 1 C) and in a large magnification (Fig. 2 F) compared with control cells (Fig. 2 E).

DISCUSSION

Our results are in agreement with what has been reported previously by Kelly et al. (14) who indicated that the resistance of the cells to heat and aeration was greater during exponential-phase, and that there is a fluctuation in resistance during the early and late stationary-phases. These results correlate with the absence of *RpoS* in the genome of *Campylobacter jejuni* NCTC 1135, the strain used in their experiment**.**

Unexpectedly, our results pointed out that all *C. coli* strains lost their viability

quickly in TSB broth at pH 4. This observation was noted in both the exponential and the stationary-phases with a better survival for the control cells compared with the exposed ones, and unlike the experiments by Kelly et al. (14) in which the *C. coli* strain UA585 showed a pattern of survival similar to that observed in *C. jejuni* following entry in the stationary-phase. Chaveerach et al. (2) found that ten strains of *C. jejuni* and *C. coli* were equally inhibited by a mixture of water and commercial broiler feed acidified at pH 4.0 with formic, acetic, propionic, or hydrochloric acid. Those ten strains also lost completely their culturability in Mueller-Hinton broth adjusted to pH 4 with formic acid and could not be recovered even when enrichment media was used (2). Variation in strains, media and methodologies used may explain the discrepancy between our results and the results obtained by previous studies.

Whereas, the viable cell counts decreased upon exposure to acidic conditions, this decrease paralleled with a gradual change in *Campylobacter* cell morphology (Figure 1 B, D; Figure 2 B) and in a large magnification (Fig. 3 B, D). Stress-induced morphology has been also reported with *E. coli*, which occurs as a result of inducing gene *bolA* belonging to the rpoS regulon upon exposure to different stresses during the stationary-phase (17). It is apparent that *Campylobacter* spp. respond to the change in the environment, especially the change in pH, and these responses are reflected as altered cell morphology. These results are in agreement with previously reported morphological changes of two Gram-negative pathogens, *E. coli* O157:H7 and *S. typhimurium*, after exposure to hydrostatic pressure and bacteriocin mixture (nisin and pediocin) (3, 12). The results presented above demonstrated that acidic stress, particularly at pH 4 and 5, could be caused obviously greater morphological changes on cell surfaces of *Campylobacter*,

allowed H^+ to gain access to cytoplasmic components of the cell. Such changes in the cell envelope could subsequently induce lysis and an obvious reduction in viable cell counts especially in *C. coli.* In conclusion, these results indicate that *Campylobacter,* especially *C. coli,* are sensitive to low pH. The influence of media on acid sensitivity should be investigated.

REFERENCES

1. **Allerberger, F., N. Al-Jazrawi, P. Kreidl, M. P. Dierich, G. Feierl, I. Hein, and M. Wagner.** 2003. Barbecued chicken causing a multi-state outbreak of *Campylobacter jejuni* enteritis. Infection. **31:**19-23.
2. **Chaveerach, P., A. A. H. M. ter Huurne, L. J. A. Lipman, and F. van Knapen.** 2003. Survival and Resuscitation of Ten Strains of *Campylobacter jejuni* and *Campylobacter coli* under Acid Conditions. Appl. Environ. Microbiol. **69**:711-714.
3. **Chaveerach, P., D. A. Keuzenkamp, H. A. Urlings, L. J. Lipman, and F. van Knapen.** 2002. In vitro study on the effect of organic acids on *Campylobacter jejuni*/*coli* populations in mixtures of water and feed. Poult. Sci. **81:**621-628.
4. **Deming, M., R. Tauxe, P. Blake, S. Dixon, B. Fowler, S. Jones, E. Lockamy, C. Patton, and R. Sikes.** 1987. *Campylobacter* enteritis at a university: transmission from eating chicken and from cats. Am. J. Epidemiol. **126:** 526-534.
5. **Friedman, C. R., J. Neimann, H. G. Wegener, and R. V. Tauxe**. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121–39. In I. Nachamkin, M. J. Blaser, (ed.), *Campylobacter.* Washington: ASM Press.
6. **Gaunt, P. N., and L. J. V. Piddock.** 1996. Ciprofloxacin resistant *Campylobacter spp.* in humans: an epidemiological and laboratory study. J. Antimicrob. Chemother. **37:**747-757.
7. **Hopkins, R. S., and A. S. Scott.** 1983. Handling raw chicken as a source for sporadic *Campylobacter jejuni* infections.J. Infect. Dis. **148:**770.
8. **Humphrey, T. J., E. Slater, K. McAlpine, R. J. Rowbury, and R. J. Gilbert.** 1995.

Salmonella enteritidis phage type 4 isolates more tolerant of heat, acid, or hydrogen peroxide also survive longer on surfaces. Appl. Environ. Microbiol. **61:**3161-4.

9. **Humphrey, T. J., A. Williams, K. McAlpine, M. S. Lever, J. Guard-Petter, and J. M. Cox.** 1996. Isolates *of Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. Epidemiol. Infect. **117:**79-88.
10. **Jenkins, D. E., J. E. Schulz, and A. Martin.** 1988. Starvation-induced cross-protection against heat or H_2O_2 –challenge in *Escherichia coli.* J. Bacteriol. **170:**3910-3914.
11. **Jenkins, D. E., S. A. Chaisson, and A. Martin.** 1990. Starvation-induced cross-protection against osmotic challenge in *Escherichia coli.* J. Bacteriol. **172:**2779-2781.
12. **Kalchayanand, N., P. Dunne, A. Sikes and B. Ray.** 2004. Viability loss and morphological change of foodborne pathogens following exposure to hydrostatic pressures in the presence and absence of bacteriocins. Int. J. Food Microbiol. **91:**91–98.
13. **Kalman, M., E. Szollosi, B. Czermann, M. Zimanyi, S. Szekeres, and M. Kalman.** 2000. Milkborne *Campylobacter* infection in Hungary. J. Food Prot. **63:** 1426-1429.
14. **Kelly, A. F., S. F. Park, R. Bovill, and B. M. Mackey.** 2001. Survival of *Campylobacter jejuni* during stationary-phase: evidence for the absence of a phenotypic stationary-phase response. Appl. Environ. Microbiol. **67:**2248-54.
15. **Nachamkin, I., J. Engberg, and F. M**. **Aaestrup.** 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species. pp. 45-66. In: I. Nachamkin and M. J. Blaser

eds., *Campylobacter* 2ed. (). ASM Press, Washington, DC.

16. **Rowburry, R. J.** 1972. Observation on starvation-induced resistance enhancement (SIRE) in *Salmonella typhimurium*. Int. J. Radiat. Biol. **21:**297-302.
17. **Santos, J. M., P. Freire, M. Vicente, and C. M. Arraiano.** 1999. The stationary-phase morphogene bolA from *Escherichia coli* is induced by stress during early stages of growth. Mol. Microbiol. **32:**789-98.
18. **Schorr, D., H. Schmid, , H. Rieder, A. Baumgartner, H. Vorkauf, and A. Burnens.** 1994. Risk factors for *Campylobacter enteritis* in Switzerland. Zbl. Hyg. **196:** 327-337.
19. **Studahl, A., and Y. Andersson.** 2000. Risk factors for indigenous *Campylobacter* infection: A Swedish case-control study. Epidemiol. Infect. **125:** 269-275.
20. **Zimmer, M., H. Barnhart, U. Idris, and M. D. Lee.** 2003. Detection of *Campylobacter jejuni* strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. Avian Dis. **47:**101-7.

Species	Phase	pH 6			pH 5		pH 4		
		1 h	4 h	24 h	1 h	4 h	0.5 h	1 h	4 h
C. coli	Exponential	88	87	79	82^{A}	68	76^{A}	55^{A}	0
	Stationary	86	86	79	76^{B}	64	63^{B}	42^{B}	0
	SEM	1.0	0.9	2.2	1.3	2.0	3.9	3.6	0
C. jejuni	Exponential	88	88	75	86	75	83	77	24
	Stationary	88	87	75	85	74	83	75	24
	SEM	0.5	0.5	3.0	0.9	1.1	1.2	2.0	4.5

Table 1. Survival (% of initial inoculum) of exponential and stationary-phase cells of *C. jejuni* (ATCC 33560 and 43473, CDC 2002-370 and Post 5) and *C. coli* (ATCC 43473, 43133 and mCC 66) at pH 6, 5 and 4. All strains were unrecoverable at 24 h in pH 5 and pH 4. SEM: standard error of the mean. Means with different superscripts are statistically significant ($P \leq 0.05$) for the same pH and time. Comparison made between exponential and stationary-phase cells.

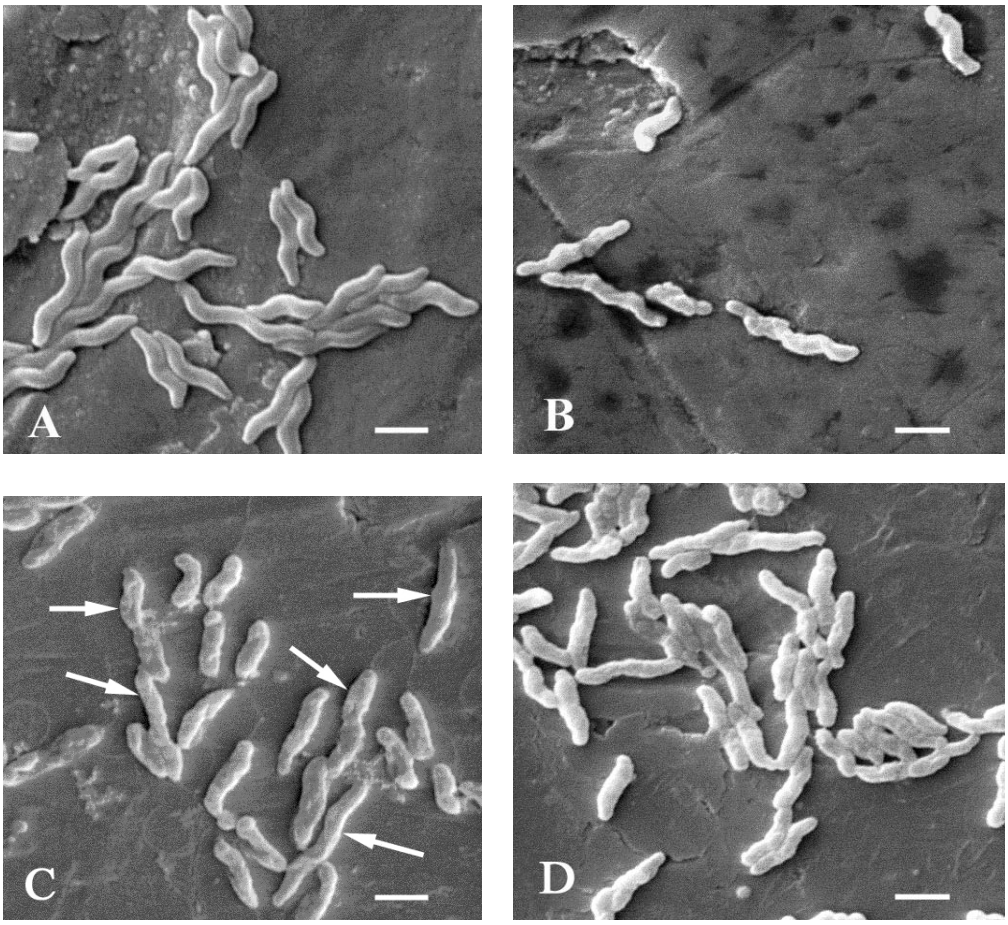

Figure 1. Morphological changes of *C. coli* ATCC 51798 and mCC 66 cells under SEM. mCC 66 cells with integral cytoplasmic membranes at pH 7 (A) and cells with disruption in the membranes at pH 4 (B). ATCC 51798 cells at pH 5, with arrows showed ruffling and craters on the surface (C) and pH 4 (D). Photographs were collected after 2–3 h exposure to pH 7, 5 or 4. One ml of the culture was fixed in 2% glutaraldehyde, 1% osmium tetroxide and 0.1 M cacodylate buffer (pH 7.2) for 20 minutes. Specimens were examined in a Zeiss DSM 940 scanning electron microscope operated at 15 kV. Bars= 1μm.

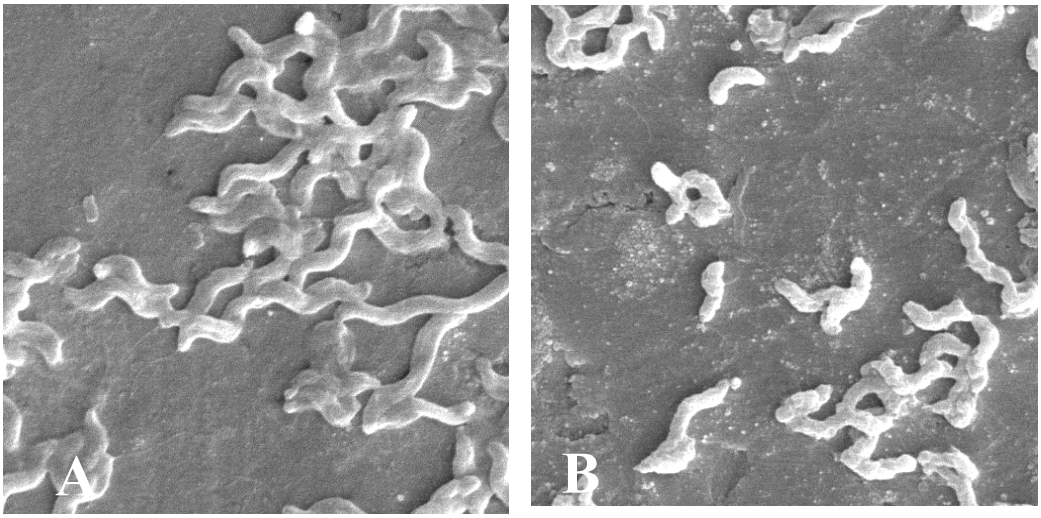

Figure 2. Morphological changes of *C. jejuni* CDC 2002-353 cells under scanning electron microscopy. CDC 2002-353 cells with integral cytoplasmic membranes at pH 7 (A) and cells with disruption in the membranes at pH 4 (B).

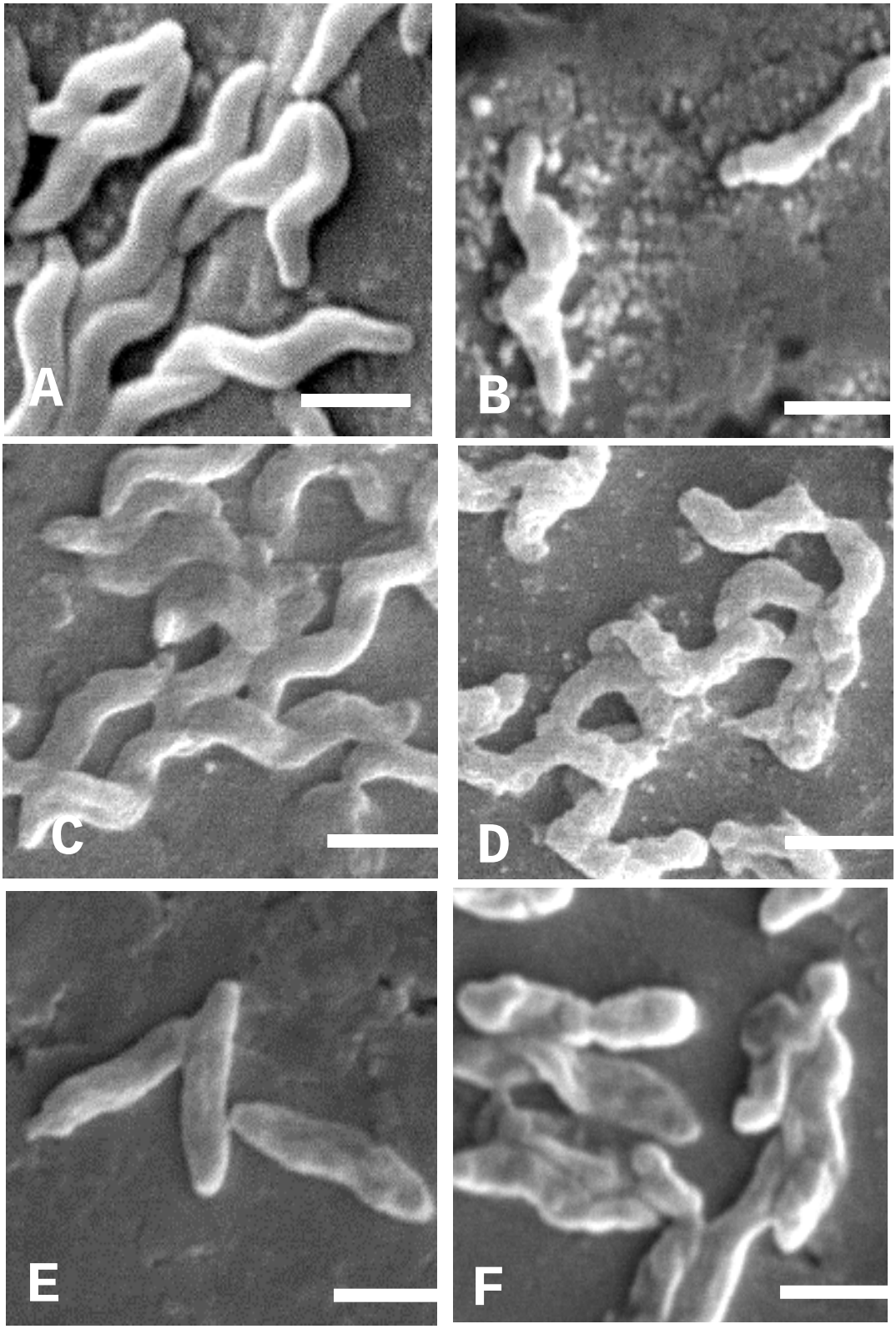
A
B
C
D
E
F

Figure 3. Morphological changes of and *C. coli* mCC 66, *C. jejuni* CDC 2002-353, and *C. coli* ATCC 51798 cells under SEM. mCC 66 cells with integral cytoplasmic membranes at pH 7 (A) and cells with disruption in the membranes at pH 4 (B). Similar cell morphology has been observed for CDC 2002-353 in control cells (C) and stressed cells at pH 4 (D). ATCC 51798 cells at pH 5 showed ruffling and craters on the surface (F) compared with control cells at pH 7 (E). Photographs were collected after 2–3 h exposure to pH 7, 5 or 4. One ml of the culture was fixed in 2% glutaraldehyde, 1% osmium tetroxide and 0.1 M cacodylate buffer (pH 7.2) for 20 minutes. Specimens were examined in a Zeiss DSM 940 scanning electron microscope operated at 15 kV. Bars= 1µm.

III. EXPOSURE TO SUBLETHAL LOW PH BY FLUOROQUINOLONE-RESISTANT *CAMPYLOBACTER JEJUNI* AND *C. COLI*

INTRODUCTION

The mechanisms of acid tolerance have been discovered for many Gram positive and Gram negative bacteria (6). However, few studies have been conducted on the stress response properties of *Campylobacter* spp. Kelly et al. (10) indicated that the resistance of *Campylobacter jejuni* to heat and aeration was greater during the exponential-phase and that there are fluctuations in the resistance during the early and late stationary-phases. This study also indicated that one *C. coli* strain had a pattern of survival similar to that observed in *C. jejuni* following entry to the stationary-phase.

More recently, a study was conducted to identify the adaptive tolerance response to acid and/or aerobic conditions in exponential and stationary-phases using one *C. jejuni* strain (13). The survival of *C. jejuni* CI 120 adapted cells during the early stationary-phase was better than the mid-exponential-phase cells after adaptation to mild pH, and ATR was induced under aerobic + acidic conditions in both early and late stationary-phases. Under acidic adaptation, the late stationary-phase showed inconsistent survival rates at different times and at low pH challenge.

Fluoroquinolone resistance (FQ^R) in *Campylobacter* has increased rapidly in

many countries (5). Up to 80% of *Campylobacter* isolates are fluoroquinolone-resistant in Thailand and Spain (9). However, in many parts of the world fluoroquinolone resistance values remain low (5). In the US, data collected by the Centers for Disease Control and Prevention (CDC) through the National Antimicrobial Resistance Monitoring System (NARMS) showed that 14.2% of the *C. jejuni* isolates tested in 2000 were fluoroquinolone resistant (3). In vivo fitness of FQ^R *Campylobacter jejuni* in chickens indicates that acquisition of FQ^R in *Campylobacter* does not impair its colonization in the absence of antibiotic selective pressure (11)**.** This finding indicates that FQ^R *Campylobacter* is able to stay in the flock until the slaughter age, with no obvious history of using FQ antimicrobial as treatment. Furthermore, the enhanced fitness in FQ^R *Campylobacter* was not due to compensatory mutation in the genes targeted by FQ, but was directly linked to the resistance-conferring mutation in *gyr A*. The previous study also proposed that other etiological factors, such as bacterial virulence, host immune system, and environmental condition and management practice play a role to increase the prevalence of FQ^R in *Campylobacter* isolates.

The purpose of this study was to determine if early-exponential or stationary-phase cells of 10 fluoroquinolone-resistant *C. jejuni* isolated from humans, two fluoroquinolone-sensitive *C. jejuni* strains and nine *C. coli* strains that showed an acid adaptation response when subjected to pH 5 for 4 h. Furthermore, the influence of different broth media in the response to low pH by *C. jejuni* and *C. coli* was also assessed.

MATERIALS AND METHODS

Bacterial strains

C. jejuni strains used in the studies included one chicken isolate (Post 5), ATCC 33560, and ten fluoroquinolone-resistant (FQ^R) strains (CDC 2002-341, -348, -351, -353, -370, -404, -409, -410, -420 and -439) from the Centers for Disease Control and Prevention (2). The *C. coli* strains included a chicken isolate (mCC 66), and ATCC numbers 33559, 43133, 43473, 43481, 43484, 49941, 51798 and BAA-371. All strains were maintained at -80^o C in glycerol (20-30%). Cultures were transferred from frozen stocks to modified Campy-Cefex (mCC) (14) and grown at 42^{o}C under microaerophilic conditions (BOC Gas, 5% O_2, 10% CO_2 and 85% N_2). Cultures were incubated for 48 h before starting each experiment. Table 1 shows the antibiotic resistance profile of 10 FQ^R strains of *C. jejuni* (CDC 2002). The identity of the isolates was also confirmed with an automated ribotyping system (Qualicon, Wilmington, DE) (Figure 1).

Preparation of inoculum

A loop of the culture was harvested from mCC plates and suspended into 9 ml sterile phosphate buffer saline (PBS). The suspension was adjusted to MacFarland No. 0.5 turbidity to achieve a concentration of 10^6-10^8 CFU/ml and adjusting them to an optical density at 600 of 0.1 to 0.2 (approximately 1x10^8 CFU/ml), measured with spectrophotometer (Spectronic GENESYS 20 Vis; Thermo Electron Co, Waltham, MA). To create early exponential-phase cultures, one ml of the inoculated PBS was transferred to 10 ml tryptic soy broth (TSB pH 7, Difco Laboratories, Detroit, MI) and incubated for 4 h under microaerophilic conditions at 42^{o}C.

To obtain stationary-phase cells, mCC culture plates were incubated under microaerophilic conditions for 48 h. The cells were then harvested and adjusted to MacFarland No. 0.5 turbidity before each experiment**.**

Studies to induce acid stress

Forty-eight hour cultures were used as stationary-phase cells or used to induce early exponential-phase cells as described under ***Preparation of Inoculum***. Cultures were transferred to TSB adjusted to pH 5 that was incubated at 42^{o}C under microaerophilic conditions for 4 h. Cultures were then transferred to TSB adjusted to pH 4. Initial counts were determined immediately after the transfer to pH 4 for both control and pH-5-exposed cells. Final counts were determined after 2 h of incubation at 42^{o}C under microaerophilic conditions. Similar experiments were done using Brucella broth (BB pH 7, Acumedia) but the final counts in pH 4 were determined at 3 h incubation at 42^{o}C under microaerophilic conditions.

Automated ribotyping

Automated ribotyping was performed by using RiboPrinter (Qualicon, Wilmington, DE) with the restriction enzyme *Pst*I and according to the manufacturer' instructions. Briefly, single colonies from a 24-48h cultures on blood agar plate (mCC) were suspended in a sample buffer and heated at 80°C for 15 min. The cell suspension was lysed and the chromosomal DNA was isolated, digested with *Pst*I, electrophoresed, and simultaneously blotted in an automated manner. Bands were detected and analyzed

with Bionumerics software using the band matching coefficient of Pearson's clustering to determine profile relatedness.

Statistical analysis

All experiments were performed in triplicates. Results were converted to $\log_{10}$ CFU/ml and expressed as percent survival after each test. Data were analyzed using GLM procedures (SAS Institute Incorporated, Cary, NC) and means analyzed for differences by Duncan's test (SAS Program) with significance set at $P \leq 0.05$.

RESULTS

The results of susceptibility testing among *Campylobacter* CDC 2002 isolates to fluoroquinolone are shown in Table 1. FQ^R in CDC strains are noticed particularly against Nalidixic Acid (MIC>256) followed by Ciprofloxacin (MIC>32). The multi-drug resistance pattern (≥2 drugs) has been observed in all *Campylobacter* CDC 2002 isolates, involving two drugs: Ciprofloxacin and Nalidixic Acid.

Acid tolerance response of Campylobacter jejuni and C. coli

During the exponential-phase, the non-exposed cells showed a pronounced survival advantage over the exposed cells in all *C. jejuni* strains used (Table 2). This was statistically significant in almost 50% of *C. jejuni* strains in TSB experiment. The survival of *C. jejuni* in Brucella broth was extended to 3 h; because at 2 h a higher percent of survival was ascend for both exposed and control cells in both phases (data not shown). Similar results were obtained by using Brucella broth during the exponential-

phase, in which the non-exposed cells showed a better survival compared with the exposed ones. However, in BB, the exposed cells showed a remarkable decrease at low pH, and this was significant in all *C. jejuni* strains (Table 2). CDC 2002-439 showed a very low percent survival at low pH in both exposed and control cells, with no differences for both media and growth phases (Table 2). It is clear from this data that as the time increases, survivals decrease, especially in the exposed cells during the exponential-phase.

C. jejuni strains showed a fluctuation in the survival at low pH during the stationary-phase, and this was strain specific. In the TSB experiments, the non-exposed cells gave a better survival than the exposed cells. However; this was only statistically significant for CDC 2002-410, -351, post 5, and ATCC 33560. It should be noted that as time increased to 3 h in Brucella broth stationary-phase experiment, more strains displayed a larger survival rate in the non-exposed cells (Table 2).

In contrast to *C. jejuni*, *C. coli* strains were very sensitive to the low pH treatment (Table 3). Statistically, the mean survival of non-exposed cells for *C. coli* ATCC 44941, -43133, and -43473 was significantly larger than the exposed cells, especially during the exponential-phase. Furthermore, *C. coli*-stationary-phase experiments showed a remarkable sensitivity to low pH treatment compared with *C. jejuni* and for both exposed and non-exposed cells. In the TSB experiment, the control cells showed very low survival with almost no counts observed for the exposed cells in the stationary-phase. In Brucella broth experiments cells died quickly and almost no bacterial counts detected in the plates in the non-exposed and exposed groups (data not shown).

Interestingly, a group analysis for all strains also indicated a better survival of the

control cells compared with the exposed cells (Table 4). The survival advantage of the control cells was noticed in both exponential and stationary-phases.

DISCUSSION

The increase in fluoroquinolone-resistant among *C. jejuni* strains in the United States is not well understood. Smith et al. (1999) suggested that foreign travel was the main contributing factor for Minnesota residents (18). Furthermore, the use of a fluoroquinolone antibiotic within a month before the collection of the stool samples appeared to be associated with increase fluoroquinolone resistance (18). Many studies focused on the great possibility acquiring antimicrobial resistance from animals (i.e. poultry) to humans (4). Other studies suggested the relationship between the use of fluoroquinolones in animals and the resistance among human isolates in the 1980s in European countries (5). Because of the complex transmission cycle, studying the transmission of antimicrobial resistance from animals to humans has become very difficult (8). In both TSB and BB experiments, the sublethal exposure to low pH during the early exponential-phase at pH 5 for 4 h caused no acid adaptation in fluoroquinolone-resistance *C. jejuni* cells (Table 2). Probably this is correlated with the absence of regulatory mechanisms in *Campylobacter* especially an alternative sigma factor, which provides protection to different environmental stresses upon the entry to the stationary-phase. On the other hand, our results demonstrated that survival advantage of fluoroquinolone-resistance *C. jejuni* during the stationary-phase in TSB or BB was not induced upon exposure to pH 5. The data (Table 2) showed better survival ($P \leq 0.05$) of control cells over the exposed ones. Wu et al. (19) also failed to find any stress-induced

protein in *Campylobacter jejuni* subjected to pH 5 or 4 for 1 h, but were able to find proteins homologue of *E. coli* GroEL and GroES at pH 8.6.

ATCC *C. coli* strains quickly lost their viability in TSB or BB after being challenged to low pH 4 (Table 3). This observation was noted both in the exponential or the stationary-phases with a better survival for the non-exposed cells. Our finding disagree with that reported by Kelly et al. (10) in which a *C. coli* strain had a pattern of survival similar to that observed in *C. jejuni* following entry in the stationary-phase (10). Different kinds of stresses, strains variations, different media and methodologies used may explain the discrepancy between our result and results obtained by the previous study.

Our conclusion about the survival of exposed cells during early exponential-phase is consistent with what has been reported by Murphy et al. (2003), although their study investigated the mid exponential-phase rather than early exponential-phase (12). This study indicated inconsistencies with the survival of late stationary-phase cells over time and at low pH. Our findings revealed a variation in the survival among different strains studied during the late stationary-phase, with no induction observed at pH 5. Additionally, the non-exposed cells always showed better survival than the exposed cells in both TSB and Brucella Broth. The survival of non-exposed cells was statistically significant, and strain specific (Table 2 and 3). Group analysis also indicated that the survival of non-exposed was statistically significant in both experiments (Table 4).

Murphy et al (2003) experiment also indicated that ATR was induced under aerobic + acidic conditions in both the early and the late stationary-phases. It can be concluded that the exposure to mild acidic treatment does not provide protection to sever

acidic shock in *Campylobacter*, and that those other stresses such as aerobic conditions as reported previously (12) could play a major role in cross protection. *Campylobacter* in many food systems is readily exposed to oxygen that leads to the formation of antioxidant enzymes which play a major role in protecting against oxidative stress and indirectly against low pH. Superoxide dismutase (SOD) (15, 16, 17), alkyl hydroperoxidase reductase (Ahp) (1), and catalase (KatA) (7), play a key role as an oxidative defense mechanism in *Campylobacter*. Those enzymes play an important role in the survival of *Campylobacter* in many foods such as milk, poultry, and meat, and also have a role in the survival to freezing (16, 18). SOD is believed to enhance *Campylobacter* colonization in animal models (16), and invasion of mammalian cells in vitro (15). Because our experiments were done completely under microaerophilic conditions, further studies are required to investigate the role of antioxidant enzyme during the exposure to low pH and their role to provide a cross protection to low pH.

Ribotyping results for all *Campylobacter* strains indicated that two different species of *C. jejuni* and *C. coli* strains separate themselves into two distinct groups with very low similarity (25.54%) (Figure 1). If ATCC 43133 is not included, the three very closely related *C. coli* strains are 92.53% similar.

In our attempt to investigate the role of different media to induce the survival of *Campylobacter* spp. against sever acidic condition, we found that when *Campylobacter* spp. are cultured in a rich medium such as Brucella broth, the bacterial survival in pH 4 is greater than that observed in TSB. In both phases, the data showed a close similarity in the results between TSB and BB for both exposed and non-exposed cells (Table 4). Time is a critical factor in determining the survival advantage for each exposed and non-

exposed cells, and time variation between TSB and BB maybe correlated with the different components of the two media. These results illustrated no difference between two media to induce acid adaptation to severe acidic environment. Murphy et al. (12) have recently described a variation in the survival of one *C. jejuni* strain in different broths and in three Brucella broths from different manufacturers. The strain showed similar results to ours, with exposed cells surviving less than non-exposed cells in one Brucella broth from one manufacturer. However, the same strain exhibited a tolerance response at pH 4.5 in the other two Brucella broths. This information highlights the complexity of selecting a standard strain and or broth for stress studies with *Campylobacter*.

It is apparent that the survival of FQ^R *Campylobacter jejuni* to low pH was not enhanced upon exposure to moderate acidic conditions. Also our findings indicated that cells grown at neutral pH showed a better survival than the exposed cells in both exponential and stationary-phase. The lack of a defined minimal medium suitable for *Campylobacter* growth prevented clear understanding of the nutrient requirements of the organism, and the environmental condition that play an important role in the survival of *Campylobacter* cells under stress conditions. Due to the growth media complexity, the effects of pH on the growth survival of *Campylobacter* should be assessed in a defined medium.

REFERENCES

1. **Baillon, M. L., A. H. van Vliet, J. M. Ketley, C. Constantinidou, and C.W. Penn**. 1999. An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. J. Bacteriol. **181:**4798–4804.
2. **CDC.** 2004. National Antimicrobial Resistance Monitoring System for Enteric Bacteria: 2002. Human isolates final. Atlanta, GA: U. S. Department of Health and Human services, Centers for Disease Control and Prevention. [Online.] http://www.cdc.gov/narms/annual/2002/2002ANNUALREPORTFINAL.pdf
3. **CDC.** 2000. National Antimicrobial Resistance Monitoring System for Enteric Bacteria: 2000. Annual report. NARMS. Atlanta: Center for Disease Control and Prevention.
4. **Endtz, H. P., G. J. Ruijs, B. van Klingeren, W. H. Jansen, T. van der Reyden, and R. P. Mouton.** 1991. Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J. Antimicrobioe. Chemother. **27:**199-208.
5. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin.** 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. Emerg. Infect. Dis. **7:**24–34.
6. **Foster, J.W.** 2000. Microbial responses to acid stress, pp. 99–115. In G. Storz and R. Hengge-Aronis, *Bacterial Stress Responses* (ed.). ASM Press, Washington, DC.
7. **Grant, K. A., and S. F. Park**. 1995. Molecular characterization of *katA* from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter*

coli by intraspecific allelic exchange. Microbiol. **141:**1369–1376.

8. **Guerrant, R. L., T. Van Gilder, T. S. Steiner, N. M. Thielman, L. Slutsker, R. V. Tauxe, T. Hennessy, P. M. Griffin, H. DuPont, R. B. Sack, P. Tarr, M. Neill, I. Nachamkin, L. B. Reller, M. T. Osterholm, M. L. Bennish, and L. K. Pickering.** 2001. Practice guidelines for the management of infectious diarrhea. Clin. Infect. Dis. **32:**331-351.
9. **Hoge, C. W., J. M. Gambel, A. Srijan, C. Pitarangsi, and P. Echeverria**. 1998. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clin. Infect. Dis. **26:**341–5.
10. **Kelly, A. F., S. F. Park, R. Bovill, and B. M. Mackey.** 2001. Survival of c*ampylobacter jejuni* during stationary-phase: evidence for the absence of a phenotypic stationary-phase response. Appl. Environ. Microbiol. **67:**2248-54.
11. **Luo, N., S. Pereira, O. Sahin, J. Lin, S. Huang, L. Michel, and Q. Zhang.** 2005. Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc. Natl. Acad. Sci. USA **102**:541-546.
12. **Murphy, C., C. Carroll, and K. N. Jordan.** 2005. The effect of different media on the survival and induction of stress responses by *Campylobacter jejuni*. J. Microbiol. Methods. **62**:161-166.
13. **Murphy, C., C. Carroll, and K. N. Jordan.** 2003. Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. FEMS Microbiol. Lett. **223:**89-93.

14. **Oyarzabal, O. A., K. S. Macklin, J. M. Barbaree, and R. S. Miller.** 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. Appl. Environ. Microbiol. **71**:3351-3354.

15. **Pesci, E. C., D. L. Cottle, and C.L. Picket**. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect. Immun. **62:**2687–2695.

16. **Purdy, D., S. Cawthraw, J. H. Dickinson, D. G. Newell, and S. F. Park.** 1999. Generation of a superoxide dismutase-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonization. Appl. Environ. Microbiol. **65:**2540–2546.

17. **Purdy, D., and S. F. Park**. 1994. Cloning, nucleotide sequence, and characterisation of a gene encoding superoxide dismutase from *Campylobacter jejuni* and *Campylobacter coli*. Microbiology. **140:**1203–1208.

18. **Stead, D., and S. F. Park**. 2000. Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze–thaw stress. Appl. Environ. Microbiol. **66:** 3110–3112.

19. **Wu, Y. L., L. H. Lee, D. M. Rollins, and W. M. Ching.** 1994. Heat shock- and alkaline pH-Induced proteins of *Campylobacter jejuni*: Characterization and immunological properties. Infect. Immun. **62**:4256-4260.

	Antimicrobial (MIC Values)							
Strain	NA	CM	CL	CI	EM	AZ	GM	TC
CDC 2002 341	>256	0.25	1.0	>32	0.50	0.094	0.250	0.064
CDC 2002 348	>256	0.19	0.5	>32	0.75	0.125	0.190	32
CDC 2002 351	>256	0.75	3.0	>32	1.00	0.125	0.250	0.25
CDC 2002 353	>256	0.50	1.5	>32	1.00	0.094	0.125	0.19
CDC 2002 370	>256	0.75	4.0	>32	1.00	0.190	0.190	0.5
CDC 2002 404	>256	0.50	2.0	>32	0.50	0.047	0.250	0.25
CDC 2002 409	>256	0.38	6.0	>32	0.75	0.094	0.250	>256
CDC 2002 410	>256	0.38	1.5	>32	1.00	0.125	0.250	0.125
CDC 2002 420	>256	0.25	1.5	>32	0.75	0.125	0.250	0.19
CDC 2002 439	>256	0.38	6.0	>32	1.50	0.125	0.250	0.25

Table 1. Antibiotic resistance profile of *Campylobacter jejuni* strains.

NA = nalidixic acid, CM = chloramphenicol, CL = clindamycin, CI = ciprofloxacin, EM = erythromycin, AZ = azithromycin, GM = gentamicin, TC = tetracycline.

		Survival (%)							
		TSB pH 4 (2 h)				Brucella broth pH 4 (3 h)			
Strain	**Treatment**	**Expo.**	**SEM**	**Statio.**	**SEM**	**Expo.**	**SEM**	**Statio.**	**SEM**
CDC 2002-341	Exposed (pH 5)	63.6^{B}		69.7		75.0^{B}		80.2	
	Control (pH 7)	88.6^{A}	6.26	77.4	4.65	79.8^{A}	0.90	85.8	2.66
CDC 2002-348	Exposed (pH 5)	77.6^{B}		90.6		54.7^{B}		84.4	
	Control (pH 7)	94.3^{A}	3.86	94.0	1.79	82.7^{A}	1.29	88.8	1.57
CDC 2002-351	Exposed (pH 5)	78.6^{B}		87.3		59.7^{B}		93.4	
	Control (pH 7)	96.3^{A}	1.39	95.6	1.85*	81.6^{A}	2.00	88.0	1.80
CDC 2002-353	Exposed (pH 5)	85.6		87.0		83.0^{B}		59.8^{B}	
	Control (pH 7)	92.3	4.05	91.8	4.87	93.6^{A}	2.16	74.2^{A}	2.85
CDC 2002-370	Exposed (pH 5)	65.6^{B}		78.4		48.6^{B}		48.9^{B}	
	Control (pH 7)	91.3^{A}	3.55	80.0	1.43	86.2^{A}	2.23	65.9^{A}	3.14
CDC 2002-409	Exposed (pH 5)	74.0		81.3		79.6^{B}		76.3^{B}	
	Control (pH 7)	93.6	6.18	84.2	1.50	89.7^{A}	2.70	81.1^{A}	1.10

	Exposed								
CDC	(pH 5)	61.3^{B}		65.0		63.2^{B}		69.1^{B}	
2002-	Control								
404	(pH 7)	89.3^{A}	5.23	70.2	4.99	83.5^{A}	2.25	88.9^{A}	2.22
	Exposed								
CDC	(pH 5)	62.3		62.9^{B}		68.3^{B}		71.9	
2002-	Control								
410	(pH 7)	70.6	2.47	77.1^{A}	1.40	86.0^{A}	1.88	75.0	3.28
	Exposed								
CDC	(pH 5)	72.3^{B}		80.4		70.7^{B}		75.7	
2002-	Control								
420	(pH 7)	85.3^{A}	1.81	84.5	2.09	87.0^{A}	2.02	83.1	2.07
	Exposed								
CDC	(pH 5)	58.3		59.6		46.2		62.0	
2002-	Control								
439	(pH 7)	56.6	2.50	71.0	6.05	51.6	2.28	65.7	5.61
	Exposed								
	(pH 5)	85.3		69.4^{B}		52.6^{B}		79.9	
	Control								
Post 5	(pH 7)	87.6	2.18	80.6^{A}	1.93	82.1^{A}	2.54	80.2	4.26
	Exposed								
	(pH 5)	78.6		79.6^{B}		77.5^{B}		60.6^{B}	
ATCC	Control								
33560	(pH 7)	83.0	5.90	92.6^{A}	1.71	87.3^{A}	1.78	85.4^{A}	2.82

Table 2. Survival (%) of *Campylobacter jejuni* at pH 4 on TSB and Brucella broth. SEM: standard error of the mean. Means (for the same strain and phase) with different

superscripts are statistically significant ($P \leq 0.05$). Comparison made between exposed and non-exposed (control) cells

Strain	Treatment	Survival (%) in TSB pH 4 (2 h)			
		Exponential	SEM	Stationary	SEM
	Exposed (pH 5)	0.0		0.0	
mCC 66	Control (pH 7)	20.0	14.14	4.6	3.30
	Exposed (pH 5)	0.0^{B}		0.0	
ATCC 43473	Control (pH 7)	40.2^{A}	5.07	0.0	0.0
	Exposed (pH 5)	0.0^{B}		0.0	
ATCC 44941	Control (pH 7)	45.6^{A}	1.56	5.1	3.60
	Exposed (pH 5)	0.0^{B}		0.0	
ATCC 43133	Control (pH 7)	45.7^{A}	2.23	0.0	0.0
	Exposed (pH 5)	0.0		2.9	
ATCC 43481	Control (pH 7)	0.0	0.0	3.5	3.28
	Exposed (pH 5)	0.0		0.0	
ATCC BAA-371	Control (pH 7)	2.6	1.8	6.4	4.52

Table 3. Percentage survival of *Campylobacter coli* at pH 4 on TSB and Brucella Broth. ATCC 51798, 33559 and 43484 were unrecoverable in TSB and Brucella. ATCC 43133 and BAA-371 gave recoverable cells (10% or less) only in Brucella broth, exponential-phase and for non-exposed cells. SEM: standard error of the mean. Means with different superscripts are statistically significant ($P \leq 0.05$) for the same strain and phase. Comparison made between exposed and non-exposed cells.

Media	Phase	Treatment	Survival (%)	
			C. coli	*C. jejuni*
TSB pH 4 (2 h)	Exponential	Control (pH 7)	17.1^{A}	85.4^{A}
		Exposed (pH 5)	0.0^{B}	72.3^{B}
		SEM	3.1	2.0
	Stationary	Control (pH 7)	2.1	81.7
		Exposed (pH 5)	0.3	77.4
		SEM	0.8	1.8
Brucella broth pH 4 (3 h)	Exponential	Control (pH 7)	1.5^{A}	82.6^{A}
		Exposed (pH 5)	0.0^{B}	64.9^{B}
		SEM	0.5	1.9
	Stationary	Control (pH 7)	0.0	80.1^{A}
		Exposed (pH 5)	0.0	71.8^{B}
		SEM	0.0	1.8

Table 4. Survival (%) of *C. jejuni* and *C. coli* strains analyzed for survival in TSB or Brucella broth. SEM: standard error of the mean. Means (for the same strain and phase) with different superscripts are statistically significant ($P \leq 0.05$). Comparison made between exposed and non-exposed (control) cells.

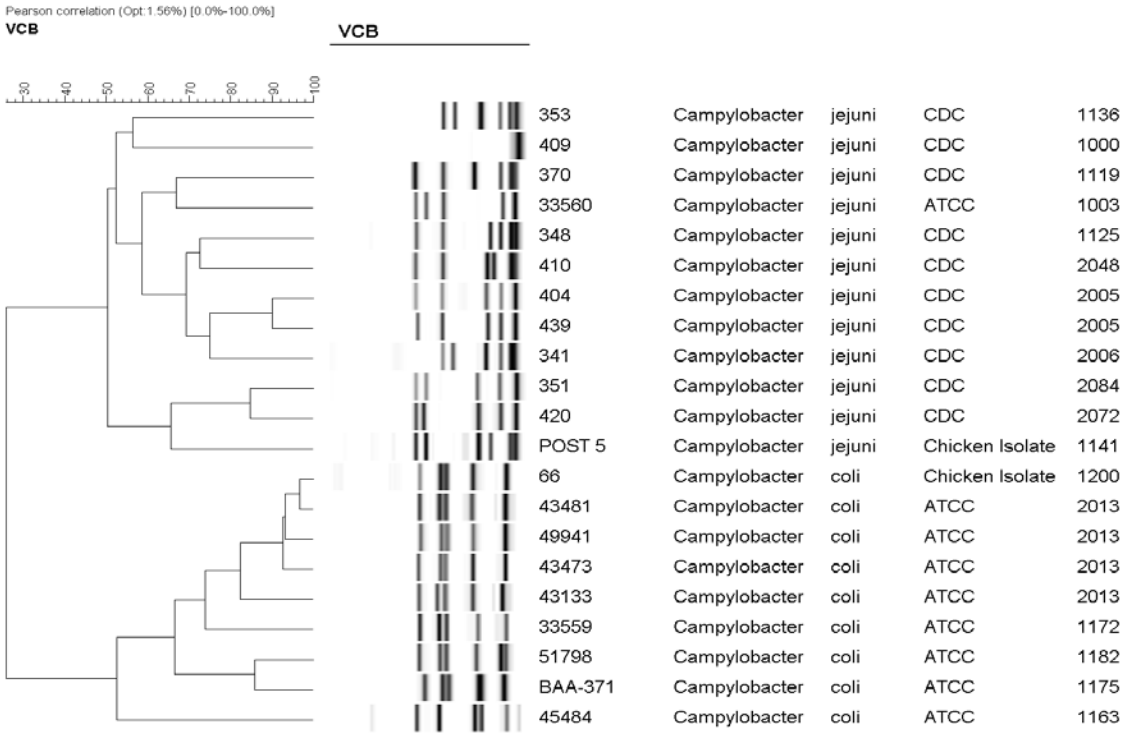

Figure 1. Dendorogram of *Campylobacter* strains. Restriction was done with *Pst*I according to manufacturer's instructions. Bands were detected and analyzed with BioNumerics (Applied-Math, Inc., Austin, TX) using the coefficient of Pearson's clustering to determine profile relatedness.

IV. GROWTH AND SURVIVAL OF CAMPYLOBACTER JEJUNI IN CHEMICALLY DEFINED MEDIUM M 199

INTRODUCTION

Many selective media have been designed to isolate *C. jejuni* from foods. These media utilize mixture of antibiotics in a rich base and are usually incubated at 42^{o}C under microaerophilic conditions for 24 h (7, 8). Due to the microaerophilic nature of the organism, it is important to incorporate ingredients to neutralize the toxic effect of the substances that form in the presence of oxygen and light.

For most plate media, the incorporation of blood at values of 5% to 15% is common (9). Other supplements are also incorporated as a replacement of blood, primarily a combination of ferrous sulphate, sodium metabisulphite, and sodium pysulphate (FBP) at different concentrations (12, 13). Blood and FBP are believed to neutralize the toxic effect of oxygen derivatives (14, 15, 23). Haematin (16) and charcoal (3, 16) are also used to quench oxygen derivatives.

Campylobacter enrichment broths have been developed to isolate the organism from samples where the numbers of *Campylobacter* are relatively low (5, 4). The most commonly used media for isolation of *Campylobacter* from a food sample are Preston and *Campylobacter* blood free agar plates (mCCD) media of Bolton. Both of those are

utilized as enrichment media (5, 9).

For stress response studies, the use of complex media does not necessary give results that mimic in vivo survival of *C. jejuni.* Furthermore, certain nutrient requirements for *Campylobacter* growth can not be evaluated by using complex media, and their role are still poorly understood. A chemically defined medium has been developed previously by Dickgiesser et al. (10) for cultivation of *C. jejuni* strains of human origin. About 57.7% of the strains investigated required amino acid L-methionine. However, *C. intestinalis* were unable to grow in the previous described media. Therefore, the use chemically defined media must be investigated further to better understand the physiology of *Campylobacter*, and the regulatory pathway required for stress response.

The purpose of the study was to evaluate the growth of *C. jejuni* and *C. coli* in a medium in which all components were chemically defined and to determine the effect of different pH values on cell viability. Furthermore, the difference in terms of cell viability, cell morphology, and cell motility of *Campylobacter* between the chemically defined media M 199 and the traditional media used for isolation was also evaluated.

MATERIALS AND METHODS

Strains used

C. jejuni strains used in the studies included one human strain (CDC 2002-353), and one *C. coli* (ATCC 49941). Three *C. jejuni* strains Post 5, ATCC 700819, and CDC 2002-353 were used in the comparative study between minimal medium and the traditional media.

Preparation of inoculum

Cultures were transferred from frozen stocks to modified Campy Cefex (mCC) plates and grown at 42^{o}C under microaerophilic conditions (BOC Gas, 5% O_2, 10% CO_2 and 85% N_2). The cultures were incubated for 48 h before starting each experiment. The media used in this study are as follow: chemically defined medium M 199 (Gibco) (21); Brucella broth (BB; Acumedia); Brain Heart Infusion broth (Oxoid) supplemented with 0.6% (w/v) yeast extract (BHIYE) and Tryptone Soya broth (Oxoid) supplemented with 0.6% (w/v) yeast extract (TSBYE). No antibiotics or other supplements, such as blood or FBP, were added to the media.

Survival of Campylobacter at different pH values utilizing M 199

C. jejuni (CDC 2002-353), and *C. coli* (ATCC 49941) were streaked on mCC and incubated for 48 h under micoaerophilic conditions. The pH of the defined medium was adjusted to different pH values (pH 8, pH 7, pH 6, pH 5, and pH 4) by direct addition of 1 M HCl and confirming with a pH Meter (AB 15, Fisher Scientific). The cell cultures obtained from mCC plates were harvested and suspended into 10 ml of sterile phosphate buffer saline (PBS). The suspension was adjusted to MacFarland No. 0.5 turbidity to achieve a concentration of 10^6-10^8 CFU/ml and adjusting them to an optical density at 600 of 0.1 to 0.2 (approximately 1x10^8 CFU/ml), measured with spectrophotometer (Spectronic GENESYS 20 Vis; Thermo Electron Co, Waltham, MA). From both PBS tubes, 0.1 ml was transferred into 10 ml M 199 tubes adjusted to different pH values. The initial counts were performed immediately after transfer to different pH values. Cultures

tubes were tested for survival at 0, 4 and 24 h after incubation at 42^{o}C under microaerophilic conditions. Viable cell counts at different times were determined on *Campylobacter* blood free agar plates (mCCDA, Acumedia, Baltimore, MD), and incubated at 42^{o}C under microaerophilic conditions for 48 h.

Comparison between M 199, BB, BHIYE, and TSBYE

Each *C. jejuni* isolate was harvested from mCC plates and suspended into 9 ml sterile phosphate buffer saline (PBS). The suspension was adjusted to MacFarland No. 0.5 turbidity. Utilizing phosphate buffer saline (PBS), another serial dilution (1:10) was performed from the previous concentration to obtain a concentration of 10^{5}-10^{6} CFU/ml. From each diluted PBS tube, 0.1 ml was transferred to 10 ml of the four media used to achieve a further dilution of 1:1000-1:10,000. The initial count for each strain was determined on *Campylobacter* blood free agar plates (mCCDA, Acumedia, Baltimore, MD) incubated at 42^{o} C under microaerophilic conditions for 48 h. All broth cultures were incubated at 42^{o} C under microaerophilic conditions and the survival for each strain in different broth was determined at 24, 48, and 72 h. Bacterial counts were performed by utilizing Petroff Hausser counting chamber (Hausser Scientific). The pH for different broth cultures was also determined at different times during the experiment.

RESULTS AND DISCUSSION

Although the fastidious nature of the organism requires special culture conditions in vitro (22), good growth is now achieved in many complex media (9) and in a

chemically defined media for *C. jejuni* described by Dickgiesser and Czylwik (10). In this study, we describe the growth in chemically defined medium M 199 without supplements of both *C. jejuni* and *C. coli*. Good growth was achieved with overnight incubation in our available defined medium. *C. jejuni* grew over the range of pH 5 to pH 8, whereas *C. coli* became more sensitive to moderate pH 5 especially after 24 h incubation (Figure 1, 2). Both organisms, however, are very sensitive to high acidic condition of pH 4, and readily inactivated after 4 h in the defined medium. These results are in agreement with the previous study indicating that the organism is unable to growth below pH 4.9 and is killed rapidly at pH below this value (2, 16).

The value of growth provided by BB and in a defined media was about the same in all *C. jejuni* strains examined (Figure 3). However, the difference in BHIYE and TSBYE to support growth of *C. jejuni* indicates that some growth factors. The growth-limiting factor could be the difference in protein content between the different broth cultures. However, supplements such as yeast extracts has been shown to support the growth of *C. jejuni* at moderate pH value (11).

In this study, each experiment has initiated with a low inoculum (10^3-10^4 CFU/ml), and this was satisfactory for achieving a high growth rate after 24 h. This also allowed a continual culture system that sustained growth beyond 48 h. The highest value of cells concentration (< 7 $\log_{10}$ CFU/ml) was achieved by using two different isolates after 24 h (Fig. 3). Other studies have achieved peak concentrations of *Helicobacter pylori* resulted in ≥ 10^8 CFU/ml after using different initial inoculums of 10^5, 10^3 to 10^4, or 10^2 CFU/ml after incubation for 24 h, 48 h, or 72 h, respectively (18). Fetal calf serum supplemented to BB provided a maximum growth for *H. pylori* (18). No supplementation

was used in this study. Antibiotics utilized in most selective media and enrichment broths to inhibit the competitive microflora (7, 16) were not used in this study.

The cell motility was significantly greater on BB than on any of the other three liquid media used to culture *C. jejuni* (Table 1). However, the conversion to coccoid form was greater on BB than on any of the other media. This was particularly noticed after incubation for 48 h and extended incubation for 72 h in *C. jejuni* ATCC 700819 and Post 5. In contrast, the defined medium M 199 showed the lowest rate of coccoid cell formation compared with other three media, even after 72 h in *C. jejuni* CDC 2002-353 and Post 5. The prolonged incubation of *Campylobacter* for more than 48 h stimulated the conversion to the coccoid form. This maybe easily induced following exposure to oxygen, changes in temperature, and starvation. (20). The coccoid is degenerative form that has a decreased values of nucleic acids and peptides and lacks of cellular integrity (1, 6, 17).

Our previous results indicated that BB does not provide any induction to low pH in *C. jejuni* and *C. coli.* More recently, Murphy et al. (19) indicated that several media such as BHIYE, TSBYE, MH, and BB can not induce adaptation for *C. jejuni* to low pH treatment. The defined medium M 199 exhibited a particular advantage over all the non-selective broth cultures that have been used in this study to culture *C. jejuni*. Cultures of *C. jejuni* in defined medium showed an improvement in cell morphology and decrease in coccal cells compared with other broths. Additionally, the improvement in the growth viability using low inoculum indicated that the defined medium may be an alternative use for future studies.

In conclusion, our results indicate that studies of the growth of *C. jejuni* in a medium in which all components are chemically defined is possible. This defined medium provides enhancement in cell viability and cell morphology and could provides additional information on nutrient requirement, and new approach to study *Campylobacter* acid tolerance.

REFERENCES

1. **Beumer, R. R., J. de Vries, and F.M. Rombouts.** 1992. *Campylobacter jejuni* non-culturable coccoid cells. Int. J. Food Microbiol. **15:**153–163.
2. **Blaser, M. J., H. L. Hardesty, B. Powers, and W. L. L.Wang.** 1980. Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. J. Clin. Microbiol. **11:**309–313.
3. **Bolton, F. J., D. N. Hutchinson, and D. Coates.** 1984. A blood-free selective medium for the isolation of *Campylobacter jejuni* from faeces. J. Clin. Microbiol. **19:**169–171.
4. **Bolton, F. J., and D. Coates.** 1983. Development of a blood-free *Campylobacter* medium: screening tests on basal media and supplements, and the ability of selected supplements to facilitate aerotolerance. J. Appl. Bacteriol. **54:**115–125.
5. **Bolton, F. J., and L. Robertson.** 1982. A selective medium for isolating *Campylobacter jejuni/coli*. J. Clin. Pathol. **35:**462–467.
6. **Boucher, S. N., E. R. Slater, A. H. Chamberlain, and M.R. Adams.** 1994. Production and viability of coccoid forms of *Campylobacter jejuni*. J. Appl. Bacteriol. **77:**303–307.
7. **Butzler, J. P., and M. B. Skirrow,** 1979. *Campylobacter enteritis*. Clin. Gastroenterol. **8**:737–765.
8. **Butzler, J. P., M., De Boeck, and H. Goossens.** 1983. New selective medium for isolation of *Campylobacter jejuni* from faecal specimens. Lancet. **i:**818.
9. **Corry, J. E., D. E. Post, P. Colin, and M. J. Laisney.** 1995. Culture media for the isolation of campylobacters. Int. J. Food Microbiol. **26:**43-76.

10. **Dickgiesser, N., and D. Czylwik.** 1985. Chemically defined media for auxotyping of *Campylobacter jejuni*. Zentralbl. Bakteriol. Mikrobiol. **260:**57-64.

11. **Fletcher, R. D., A. C. Albers, A. K. Chen, and J. N. Albertson, Jr.** 1983. Ascorbic acid inhibition of *Campylobacter jejuni* growth. Appl. Environ. Microbiol**. 45:**792-795.

12. **George, H. A., P. S. Hoffman, R. M. Smibert, and N.R. Krieg,** 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. J. Clin. Microbiol. **8**:36–41.

13. **Hoffman, H. A., N. R. Krieg, and R. M. Smibert**. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni.* I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. **25:**1–7.

14. **Hoffman, P.S., H.A. George, N.R. Krieg, and R.M. Smibert.** 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni* II. Role of exogenous Superoxide anions and hydrogen peroxide. Can. J. Microbiol. **25:**8–16.

15. **Juven, B. J., and I. Rosenthal.** 1985. Effect of free-radical and oxygen scavengers on photochemically generated oxygen toxicity and on the aerotolerance of *Campylobacter jejuni*. J. Appl. Bacteriol. **59:**413–419.

16. **Karmali, M. A., A. E. Simor, M. Roscoe, P. C. Fleming, S. S. Smith, and J. Lane.** 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from faeces. J. Clin. Microbiol. **23:**456–459.

17. **Moran, A. P., and M. E. Upton**. 1987. Factors affecting production of coccoid forms by *Campylobacter jejuni* on solid media during incubation. J. Appl. Bacteriol. **62:**527–537.

18. **Morgan, D. R., R. Freedman, C. E. Depew, and W. G. Kraft.** 1987. Growth of *Campylobacter pylori* in liquid media. J Clin Microbiol. **25:**2123–2125.

19. **Murphy, C., C. Carroll, and K. N. Jordan.** 2005. The effect of different media on the survival and induction of stress responses by *Campylobacter jejuni*. J. Microbiol. Methods. **62**:161-166.

20. **Park, S. F.** 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens**.** . Int. J. Food Microbiol. **74:**177-188.

21. **Testerman., T. L., D. J. McGee, H. L. Mobley.** 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. J. Clin. Microbiol. **39:**3842-50.

22. **Skirrow, M. B., J. Benjamin, M. H. H. Razi, and S. Waterman.** 1982. Isolation, cultivation and identification of *Campylobacter jejuni* and *Campylobacter coli*, p. 313–328. In J.E.L. Corry, D. Roberts, and F. A. Skinner (ed.), *Isolation and Identification Methods for Food Poisoning Organisms*, Academic Press, London.

23. **Weinrich, K., K. Winkler, and E. Heberer.** 1990. Untersuchungen zur Eignung ausgewählter Humin-saurenchargen als Nahbodenzusatz für Medien sur Isolierung thermophiler Campylobacterspecies. Deusche Tierarztliche Wochenschrift. **97:**511–515.

	time	initial		24 h		48 h		72 h	
Media	**strain**	**(%) coccoid**	**Motility**	**(%) coccoid**	**Motility**	**(%) coccoid**	**Motility**	**(%) coccoid**	**Motility**
M199	**ATCC 700819**	**0**	**+ +**	**0**	**+**	**1**	**-**	**40**	**- -**
TSBYE		**0**	**+ +**	**0**	**- -**	**2**	**- -**	**90**	**- -**
BHIYE		**0**	**+ +**	**3**	**- -**	**20**	**- -**	**0**	**+ +**
BB		**0**	**+ +**	**0**	**50% + +**	**40**	**50% +**	**40**	**-**
M199	**Post 5**	**0**	**+ +**	**0**	**-**	**0**	**-**	**3**	**-**
TSBYE		**0**	**+ +**	**0**	**-**	**1**	**80% + +**	**20**	**80% + +**
BHIYE		**0**	**+ +**	**4**	**- -**	**1**	**-**	**9**	**- -**
BB		**0**	**+ +**	**0**	**95% + +**	**40**	**50% + +**	**95**	**- -**
M199	**CDC 353**	**0**	**+ +**	**0**	**-**	**0**	**+**	**1-2**	**-**
TSBYE		**0**	**+ +**	**0**	**-**	**2-3**	**-**	**1-2**	**- -**
BHIYE		**0**	**+ +**	**3**	**- -**	**50**	**- -**	**70**	**- -**
BB		**0**	**+ +**	**0**	**-**	**0**	**+**	**1**	**+**

Table 1. Evaluation the survival of different *C. jejuni* isolates in BB, TSBYE, BHIYE, and M 199 based on the motility and percent of coccoid cell formation at 24 h, 48 h, and 72 h. The coccoid cell formation was measured by taking the % of coccal cells among 100 cells counted in Triplicate. The motility test was evaluated upon microscopic examination of the sample utilizing Petroff Hausser counting champer. Motilility standard includes: (+ +) very fast, (+) fast, (-) slow, and (- -) very slow.

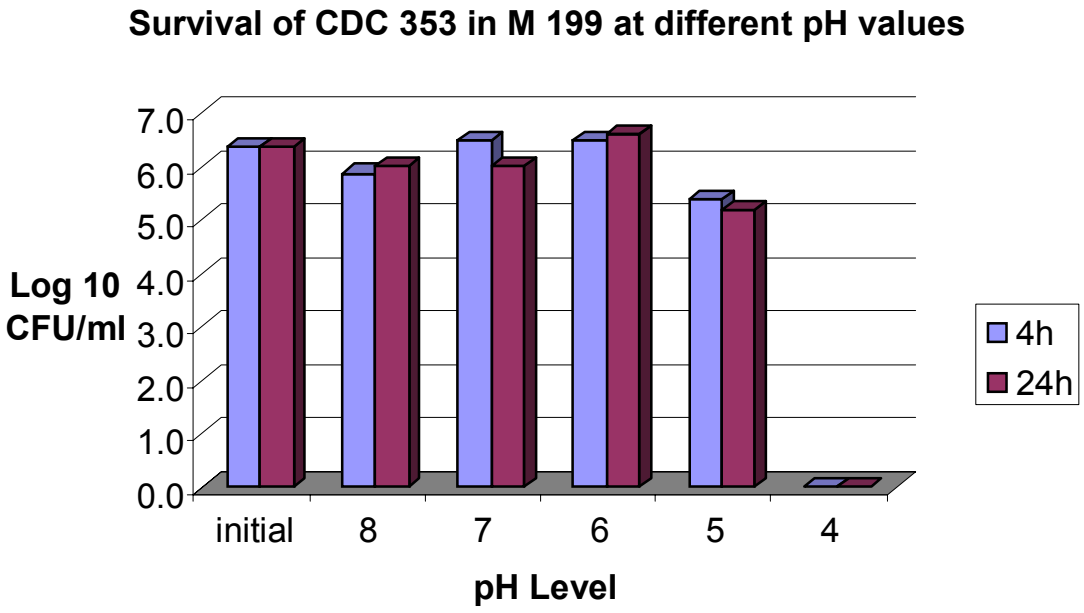

Figure. 1. Viability after incubation of *C. jejuni* CDC 2002-353 in a defined medium at different pH values: after 4h, and 24 h.

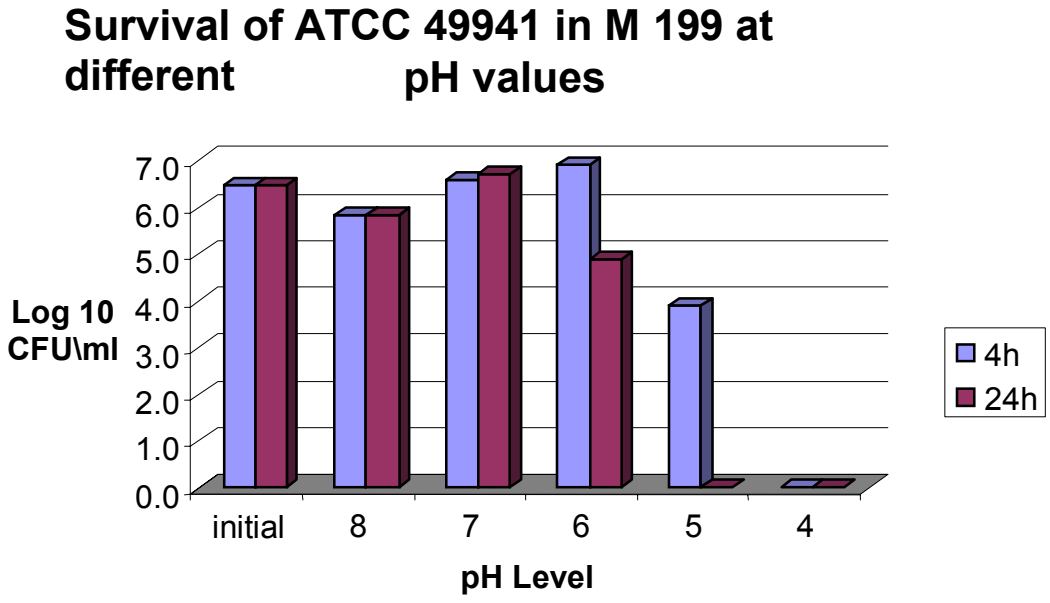

Figure. 2. Viability after incubation of *C. coli* ATCC 49941 in a defined medium at different pH values: after 4h, and 24 h.

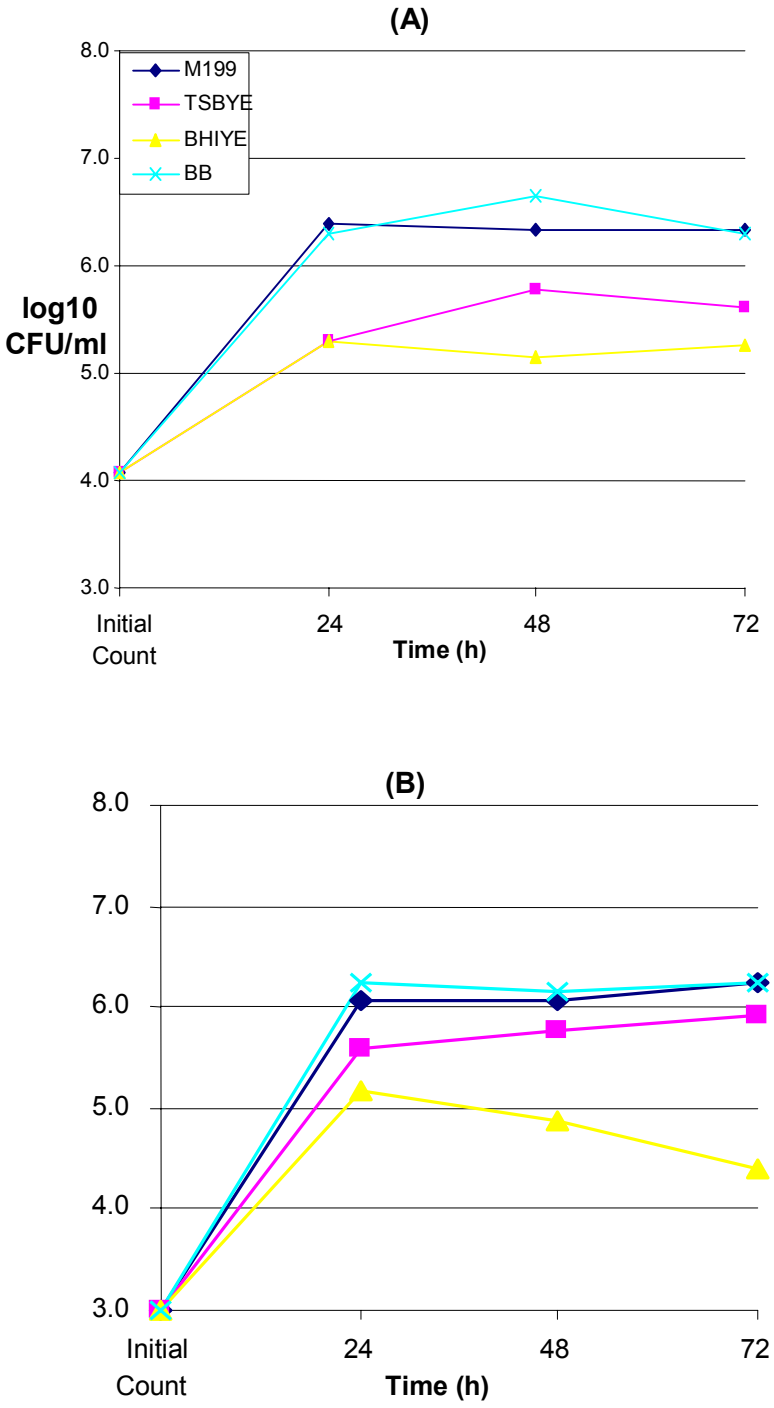

Figure. 3. Growth of *C. jejuni* strains in different broth cultures. Broth cultures of *C. jejuni* strains grown in mCC were diluted 1:1000 or 1:10,000 into fresh M 199, TSBYE, BHIYE, and BB broth and incubated at 42°C in a microaerophilic conditions containing

5% O_2, 10% CO_2 and 85% N_2. At different times, portions were analyzed using Petroff Hausser Counting Chamber. (A) represent *C. jejuni* (ATCC 700819), and (B) *C. jejuni* human isolate (CDC 2002-353).

V. REFERENCES

Allerberger, F., N. Al-Jazrawi, P. Kreidl, M. P. Dierich, G. Feierl, I. Hein, and M. Wagner. 2003. Barbecued chicken causing a multi-state outbreak of *Campylobacter jejuni* enteritis. Infection. **31:** 19-23.

Baillon, M. L., A. H. van Vliet, J. M. Ketley, C. Constantinidou, and C.W. Penn. 1999. An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. J. Bacteriol. **181:**4798–4804.

Bang, I. S., B. H. Kim, J. W. Foster, and YK. Park. 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**2245-52.

Bearson, B. L., Wilson, and J. W. Foster. 1998. A low pH inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. J. Bacteriol. **180:**2409-2417.

Beumer, R. R., J. de Vries, and F.M. Rombouts. 1992. *Campylobacter jejuni* non-culturable coccoid cells. Int. J. Food Microbiol. **15:**153–163.

Bijlsma, J. J., M. M. G. Gerrits, R. Imamdi, C. M. Vandenbrouche-Grauls, and J. G. Kusters. 1998. Urease-positive, acid-sensitive mutants of *Helicobacter pylori*:

urease-independent acid resistance involved in growth at low pH. FEMS Microbiol. Lett. **167:**309-313.

Blaser, M. J., H. L. Hardesty, B. Powers, and W. L. L.Wang. 1980. Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. J. Clin. Microbiol. **11:**309–313.

Bolton, F. J., D. N. Hutchinson, and D. Coates. 1984. A blood-free selective medium for the isolation of *Campylobacter jejuni* from faeces. J. Clin. Microbiol. **19:**169–171.

Bolton, F. J., and D. Coates. 1983. Development of a blood-free Campylobacter medium: screening tests on basal media and supplements, and the ability of selected supplements to facilitate aerotolerance. J. Appl. Bacteriol. **54:**115–125.

Bolton, F. J., and L. Robertson. 1982. A selective medium for isolating *Campylobacter jejuni/coli*. J. Clin. Pathol. **35:**462–467.

Boucher, S. N., E. R. Slater, A. H. Chamberlain, and M.R. Adams. 1994. Production and viability of coccoid forms of *Campylobacter jejuni*. J. Appl. Bacteriol. **77:**303–307.

Brey, R. N., B. P. Rosen, and E. N. Sorensen. 1979. Cation/proton antiporter systems in *Escherichia coli*. J. Biol. Chem. **255:**39-44.

Buck, G. E. 1990. *Campylobacter pylori* and gastroduodenal disease. Clin. Microbiol. Rev. **3:**1-12.

Butzler, J. P., and M. B. Skirrow, 1979.Campylobacter enteritis. Clin. Gastroenterol. **8**:737–765.

Butzler, J. P., M., De Boeck, and H. Goossens. 1983. New selective medium for

isolation of *Campylobacter jejuni* from faecal specimens. Lancet. **i:**818

Castanie-Cornet, M. P., and J. W. Foster. 2001. *Escherichia coli* acid resistance: cAMP receptor protein and a 20 bp cis-acting sequence control pH and stationary-phase expression of the *gadA* and *gadBC* glutamate decarboxylase genes. Microbiology **147:**709-715.

Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster. 1999. Control of acid resistance in *Escherichia coli*. J. Bacteriol. **181:**3525-3535.

CDC, 2004. National Antimicrobial Resistance Monitoring System for Enteric Bacteria: 2002. Human isolates final. Atlanta, GA: U. S. Department of Health and Human services, Centers for Disease Control and Prevention. [Online.] http://www.cdc.gov/narms/annual/2002/2002ANNUALREPORTFINAL.pdf

CDC, 2000. National Antimicrobial Resistance Monitoring System for Enteric Bacteria: 2000. Annual report. NARMS. Atlanta: The Center for disease control and prevention.

Chang, Y. Y., and J. E. Cronan. 1999. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol. Microbiol. **33:**249-259.

Chaveerach, P., A. A. H. M. ter Huurne, L. J. A. Lipman, and F. van Knapen. 2003. Survival and Resuscitation of Ten Strains of *Campylobacter jejuni* and *Campylobacter coli* under Acid Conditions. Appl. Environ. Microbiol. **69**:711-714.

Chaveerach, P., D. A. Keuzenkamp, H. A. Urlings, L. J. Lipman, and F. van Knapen. 2002. In vitro study on the effect of organic acids on *Campylobacter jejuni*/*coli* populations in mixtures of water and feed. Poult. Sci. **81:**621-628.

Cheng, H. Y., and C. C. Chou. 2001. Acid adaptation and temperature effect on the survival of *Escherichia coli* O157:H7 in acidic fruit juice and lactic fermented milk product. Int. J. of Food Microbiol. **70**:189–195.

Corry, J. E., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of campylobacters. Int. J. Food Microbiol. **26:**43-76.

Deming, M., R. Tauxe, P. Blake, S. Dixon, B. Fowler, S. Jones, E. Lockamy, C. Patton, and R. Sikes. 1987. *Campylobacter enteritis* at a university: transmission from eating chicken and from cats. Am. J. Epidemiol. **126:** 526-534.

De Biase, D., A. Tramonti, F. Bossa, and P. Visca. 1999. The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. Mol. Microbiol. **32:**1198-1211.

Dibrov, P. A. 1991. The role of sodium ion transport in *Escherichia coli* genetics. Biochim. Biophys. Acta **1056:**209-224.

Dickgiesser, N., and D. Czyhwik. 1985. Chemically defined media for auxotyping of *Campylobacter jejuni*. Zentralbl. Bakteriol. Mikrobiol. **260:**57-64.

Endtz, H. P., G. J. Ruijs, B. van Klingeren, W. H. Jansen, T. van der Reyden, and R. P. Mouton. 1991. Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J. Antimicrob. Chemother. **27:**199-208.

Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin. 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. Emerg. Infect. Dis. **7:**24–34.

Fletcher, R. D., A. C. Albers, A. K. Chen, and J. N. Albertson, Jr. 1983. Ascorbic acid inhibition of *Campylobacter jejuni* growth. Appl. Environ. Microbiol**. 45:**792-795.

Friedman, C. R., J. Neimann, H. G. Wegener, and R. V. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In: I. Nachamkin, M. J. Blaser, eds., *Campylobacter.* Washington: ASM Press. p. 121–39.

Foster, J.W. 2000. Microbial responses to acid stress, p. 99–115. In G. Storz and R. Hengge-Aronis, eds., *Bacterial Stress Responses* (eds.). ASM Press, Washington, DC.

Foster, J. W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **173:**1981-1987.

Foster, J. W. 1991. *Salmonella* acid shock proteins are required for adaptive acid tolerance response. J. Bacteriol.**173:** 6896-6902.

Foster, J. W., and H. K. Hall. 1991. Inducible pH homeostasis and acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129-5135.

Gahan C. G. M., B. O'Driscoll, and C. Hill. Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. Appl. Environ Microbiol. **62**:3128–3132.

Garcia-del Portillo, F., J. W. Foster, and B. B. Finlay. 1993. The role of acid tolerance response genes in *Salmonella typhimurium* virulence. Infect. Immun. **61:**4489-4492.

Gaunt, P. N., and L. J. V. Piddock. 1996. Ciprofloxacin resistant *Campylobacter spp.*

in humans: an epidemiological and laboratory study. J. Antimicrob. Chemother. **37:** 747-757.

George, H. A., P. S. Hoffman, R. M. Smibert, and N.R. Krieg, 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. J. Clin. Microbiol. **8**:36–41.

Gong, S., H. Richard, and J. W. Foster. 2003. YjdE (AdiC) is the arginine:agmatine antiporter essential for arginine-dependent acid resistance in *Escherichia coli*. J. Bacteriol. **185:**4402-4409.

Goodson, M., and R. J. Rowbury. 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. Lett. Appl. Microbiol. **8:**77–79.

Grant, K. A., and S. F. Park. 1995. Molecular characterization of *katA* from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by intraspecific allelic exchange. Microbiol. **141:**1369–

Grogan, D. W., and J. E. Cronan. 1997. Cyclopropane ring formation in membrane lipids of bacteria. Microbiol. Mol. Biol. Rev. **61:**429–441.1376.

Guerrant, R. L., T. Van Gilder, T. S. Steiner, N. M. Thielman, L. Slutsker, R. V. Tauxe, T. Hennessy, P. M. Griffin, H. DuPont, R. B. Sack, P. Tarr, M. Neill, I. Nachamkin, L. B. Reller, M. T. Osterholm, M. L. Bennish, and L. K. Pickering. 2001. Practice guidelines for the management of infectious diarrhea. Clin. Infect. Dis. **32:**331-351.

Hengge-Aronis, **R.** 2000. Microbial responses to acid stress, p. 99–115. In G. Storz and R. Hengge-Aronis, (eds.). *Bacterial Stress Responses*. ASM Press, Washington, DC.

Hoffman, H. A., N. R. Krieg, and R. M. Smibert. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. **25:**1–7.

Hoffman, P.S., H.A. George, N.R. Krieg, and R.M. Smibert. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni* II. Role of exogenous Superoxide anions and hydrogen peroxide. Can. J. Microbiol. **25:**8–16.

Hoge, C. W., J. M. Gambel, A. Srijan, C. Pitarangsi, and P. Echeverria. 1998. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clin. Infect. Di.s. **26:**341–5.

Hopkins, R. S., and A. S. Scott. 1983. Handling raw chicken as a source for sporadic *Campylobacter jejuni* infections.J. Infect. Dis. **148:**770.

Humphrey, T. J., E. Slater, K. McAlpine, R. J. Rowbury, and R. J. Gilbert. 1995. *Salmonella enteritidis* phage type 4 isolates more tolerant of heat, acid, or hydrogen peroxide also survive longer on surfaces. Appl. Environ. Microbiol. **61:**3161-4.

Humphrey, T. J., A. Williams, K. McAlpine, M. S. Lever, J. Guard-Petter, and J. M. Cox. 1996. Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. Epidemiol. Infect. **117:**79-88.

Humphrey, T. J., E. Slater, K. McAlpine, R. J. Rowbury, and R. J. Gilbert. 1995. *Salmonella enteritidis* phage type 4 isolates more tolerant of heat, acid, or hydrogen peroxide also survive longer on surfaces. Appl. Environ. Microbiol. **61:**3161-4.

Lange, R., and Hengge-Aronis, R. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5:**49-59.

Iyer, R., C. Williams, and C. Miller. 2003. Arginine-agmatine antiporter in extreme acid resistance in *Escherichia coli*. J. Bacteriol. **185:**6556-61.

Jenkins, D. E., J. E. Schulz, and A. Martin. 1988. Starvation-induced cross-protection against heat or H_2O_2 –challenge in *Escherichia coli.* J. Bacteriol. **170:**3910-3914.

Jenkins, D. E., S. A. Chaisson, and A. Martin. 1990. Starvation-induced cross-protection against osmotic challenge in *Escherichia coli.* J. Bacteriol. **172:**2779-2781.

Juven, B. J., and I. Rosenthal. 1985. Effect of free-radical and oxygen scavengers on photochemically generated oxygen toxicity and on the aerotolerance of *Campylobacter jejuni*. J. Appl. Bacteriol. **59:**413–419.

Kalman, M., E. Szollosi, B. Czermann, M. Zimanyi, S. Szekeres, and M. Kalman. 2000. Milkborne *Campylobacter* infection in Hungary. J. Food Prot. **63:**1426-1429.

Karmali, M. A., A. E. Simor, M. Roscoe, P. C. Fleming, S. S. Smith, and J. Lane. 1986. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from faeces. J. Clin. Microbiol. **23:**456–459.

Kelly, A. F., S. F. Park, R. Bovill, and B. M. Mackey. 2001. Survival of *Campylobacter jejuni* during stationary-phase: evidence for the absence of a phenotypic stationary-phase response. Appl. Environ. Microbiol. **67:**2248-54.

Kramer, J. M., J. A. Frost, F. J. Bolton, and D. R. Wareing. 2000. Campylobacter contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. J. Food Prot. **63:**1654-1659.

Kroll, R. G., and I. R. Booth. 1983. The relation between intracellular pH, the pH gradient and potassium transport in *Escherichia coli.* J. Biochem**. 216:**706-719.

Lee, I. S., J. L. Slonczewski, and J. W. Foster. 1994. A low pH inducible stationary-phase acid tolerance response in *Salmonella typhimurium.* J. Bacteriol. **176:**1422-1426.

Leyer, G. J., and E.A. Johnson 1992. Acid adaptation promotes survival of *Salmonella* spp in cheese. Appl. Environ Microbiol. **58**:2075–2080.

Leyer, G. J., L. L. Wang, and E.A. Johnson. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Environ Microbiol. **61:**3752–3755.

Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. **62:**3094-100.

Luo, N., S. Pereira, O. Sahin, J. Lin, S. Huang, L. Michel, and Q. Zhang. 2005. Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc. Natl. Acad. Sci. USA **102**:541-546.

Marchall, B. J., L. J. Barrett, C. Prakash, R. W. McCallum, and R. Guerrant. 1990. Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effects of acid. Gastroenterol. **99:** 697-702.

Martínez-Rodriguez, A., and B. M. Mackey. 2005. Physiological changes in *Campylobacter jejuni* on entry into stationary-phase. Int. J. Food Microbiol. **101:**1-8.

Molenaar, D., J. S. Bosscher, B. ten Brink, A. J. Driessen, and W. N. Konings. 1993. Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. J. Bacteriol. **175:**2864-2870.

Moran, A. P., and M. E. Upton. 1987. Factors affecting production of coccoid forms by *Campylobacter jejuni* on solid media during incubation. J. Appl. Bacteriol. **62:**527–537.

Morgan, D. R., R. Freedman, C. E. Depew, and W. G. Kraft. 1987. Growth of *Campylobacter pylori* in liquid media. J Clin Microbiol. **25:**2123–2125.

Murphy, C., C. Carroll, and K. N. Jordan. 2005. The effect of different media on the survival and induction of stress responses by *Campylobacter jejuni*. J. Microbiol. Methods. **62:**161-166.

Murphy, C., C. Carroll, and K. N. Jordan. 2005. The effect of different media on the survival and induction of stress responses by *Campylobacter jejuni*. J. Microbiol. Methods. **62:**161-166.

Murphy, C., C. Carroll, and K. N. Jordan. 2003. Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. FEMS Microbiol. Lett. **223:**89-93.

Nachamkin, I., H. Ung, and M. Li. 2002. Increasing fluoroquinolone resistance in *Campylobacter jejuni*, Pennsylvania, USA, 1982-2001. Emerg. Infect. Dis. **8:**1501-1503.

Nachamkin, I., J. Engberg, and F. M. **Aaestrup.** 2000. Diagnosis and antimicrobial susceptibility of *Campylobacter* species, pp. 45-66. In I. Nachamkin and M. J. Blaser (ed.). *Campylobacter* 2nd. ASM Press, Washington, DC.

Oyarzabal, O. A., K. S. Macklin, J. M. Barbaree, and R. S. Miller. 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. Appl. Environ. Microbiol. **71**:3351-3354.

Park, S. F. 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. Int. J. Food Microbiol. **74:**177–188.

Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. M. van Vliet, S. Whitehead, and B.G. Barrell. 2000. The genome sequence of the foodborne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature. **403:**665–668.

Pesci, E. C., D. L. Cottle, and C.L. Picket. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect. Immun. **62:**2687–2695.

Purdy, D., S. Cawthraw, J. H. Dickinson, D. G. Newell, and S. F. Park. 1999. Generation of a superoxide dismutase-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonization.

Appl. Environ. Microbiol. **65:**2540–2546.

Purdy, D., and S. F. Park. 1994. Cloning, nucleotide sequence, and characterisation of a gene encoding superoxide dismutase from *Campylobacter jejuni* and *Campylobacter coli*. Microbiol. **140:**1203–1208.

Rathman, M., M. D. Sjaastad, and S. Falkow. 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. **64:** 2765-2773.

Rektorsachek, M., A. Buhmann, D. Weeks, D. Schwan, K. W. Bensch, S. Eskandari, D. Scott, G. Sachs, and K. Melchers. 2000. Acid resistance of *Helicobacter pylori* depends on the Urel membrane protein and inner membrane proton barrier. Mol. Microbiol. **36:**141-152.

Richard, H. T., and Foster J. W. 2004. *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J. Bacteriol. **186:**6032-41.

Richard, H. T., and J. W. Foster. 2003. Acid resistance in *Escherichia coli*. Adv. Appl. Microbiol. **52:**167-186.

Rowbury, R. J. 1999. Acid tolerance induced by metabolites and secreted proteins, and how tolerance can be counteracted, p. 93-106. In J. C. Derek and G. Gardew (ed.), *Bacterial responses to pH*. John Wiley & Sons, Ltd., Chichester, England.

Rowburry, R. J. 1972. Observation on starvation-induced resistance enhancement (SIRE) in *Salmonella typhimurium*. Int. J. Radiat. Biol. **21:**297-302.

Rowbury, R. J., M. Goodson, and D. A. Wallace. 1992. The PhoE porin and transmission of the chemical stimulus for induction of acid resistance (acid

habituation) in *Escherichia coli*. J. Appl. Bacteriol. **72:**233–243.

Rowbury, R. J., T. J. Humphrey, and M. Goodson. 1999. Properties of L- glutamate-induced acid tolerance response which involves the functioning of extracellular induction components. J. Appl. Microbiol. **86:**325-330.

Rowbury, R. J., N. H. Hussain, and M. Goodson. 1998. Extracellular proteins and other components as obligate intermediates in the induction of a range of acid tolerance and sensitisation responses in *Escherichia coli*. FEMS Micobiol. Lett. **166:**283-288.

Ruiz, J., P. Goni, F. Marco, F. Gallardo, B. Mirelis, T. Jimenez De Anta, and J. Vila. 1998. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyrA* gene mutations in quinolone-resistant clinical isolates. Microbiol. Immunol. **42:**223–6.

Santos, J. M., P. Freire, M. Vicente, and C. M. Arraiano. 1999. The stationary-phase morphogene bolA from *Escherichia coli* is induced by stress during early stages of growth. Mol. Microbiol. **32:**789-98.

Scott, D. R., D. L. Weeks, C. Hong, S. Postius, K, Melchers, and G. Sachs. 1998. The role of internal urease in acid resistance of *Helicobacter pylori.* Gastroenterol. **114:**58-70.

Schorr, D., H. Schmid, , H. Rieder, A. Baumgartner, H. Vorkauf, and A. Burnens. 1994. Risk factors for *Campylobacter enteritis* in Switzerland. Zbl. Hyg. **196:**327-337.

Skirrow, M. B., J. Benjamin, M. H. H. Razi, and S. Waterman. 1982. Isolation, cultivation and identification of *Campylobacter jejuni* and *Campylobacter coli*, p.

313–328. In J.E.L. Corry, D. Roberts, and F. A. Skinner, (eds.). *Isolation and Identification Methods for Food Poisoning Organisms*, Academic Press, London.

Smith, J. L. 2003. The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions. J. Food Prot. **66:**1292-1303.

Solomon, E. B., and D. G. Hoover. 1999. *Campylobacter jejuni*: a bacterial paradox. J. Food Safety. **19:**121–136.

Stead, D., and S. F. Park. 2000. Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze–thaw stress. Appl. Environ. Microbiol. **66:**3110–3112.

Studahl, A., and Y. Andersson. 2000. Risk factors for indigenous *Campylobacter* infection: A Swedish case-control study. Epidemiol. Infect. **125:**269-275.

Testerman., T. L., D. J. McGee, H. L. Mobley. 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. J. Clin. Microbiol. **39:**3842-50.

Wang, Y., W. M. Huang, and D. E. Taylor DE. 1993. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. Antimicrob. Agents Chemother. **37:**457-63.

Weeks, D. L., S. Eskandari, D. R. Scott, and G. Sachs. 2000. A H^+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. Science**.** **287:**482-485.

Weeks, D. L., G. Sachs. 2001. Sites of pH regulation of urea channel of *Helicobacter pylori.* Mol. Microbiol. **40:**1249-1259.

Weinrich, K., K. Winkler, and E. Heberer. 1990. Untersuchungen zur Eignung

ausgewählter Humin-saurenchargen als Nahbodenzusatz für Medien sur Isolierung thermophiler Campylobacterspecies. Deusche Tierarztliche Wochenschrift. **97:**511–515.

Wilmes-Riesenberg, M. R., B. Bearson, J. W. Foster, and R. Curtiss III. 1996. Role of the acid tolerance response in virulence of *Salmonella typhimurium.* Infect. Immun. **64:**6220-6228.

Wu, Y. L., L. H. Lee, D. M. Rollins, and W. M. Ching. 1994. Heat shock- and alkaline pH-Induced proteins of *Campylobacter jejuni*: Characterization and immunological properties. Infect. Immun. **62:**4256-4260.

Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan. 1984. The sodium/proton antiporter is part of the pH homeostasis mechanism in *Escherichia coli.* J. Biol. Chem. **257:**3687-3691.

Zimmer, M., H. Barnhart, U. Idris, and M. D. Lee. 2003. Detection of *Campylobacter jejuni* strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. Avian Dis. **47:**101-7.