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Effects of Low Temperature, Starvation and Oxidative Stress on the Physiology of *Campylobacter jejuni* Cells

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Campylobacter jejuni is a food-borne pathogen that causes diarrheal diseases in humans and animals. Still unknown mechanisms allow the cells to overcome significant stresses despite the absence of several traditional stress response genes. *C. jejuni* is problematic because of its ability to adapt and survive in various conditions of extraintestinal environment, common to food production and supply chain. The authors demonstrated that preliminary exposure to 25 °C did not influence the heat resistance of *C. jejuni* cells at 55 °C. In addition, changes of morphology, culturability and viability were noticeable for fed and starved cultures exposed to low temperature and oxidative stresses. All physiology changes were influenced by starvation and, to a lesser extent, by other stresses. Starvation was the most significant factor in *C. jejuni* survival, coccoid cell formation and especially culturability. Temperature downshift, oxidative stress and starvation, usually in combination with other factors, can trigger transition of bacteria to a viable but nonculturable state. Results showed substantial variability in responses to stresses applied, indicating no correlation between low temperature and oxidative stress response of campylobacters. Understanding of microbial physiology after exposure to various stresses is essential for food safety.

Keywords
Campylobacter jejuni
stress response
survival
starvation
low temperature
oxidative stress

INTRODUCTION

Campylobacter jejuni, a gram-negative food-borne pathogen, is a common bacterial cause of human gastrointestinal infection.[1] It is known to be uncommon in terms of its ecological features and sensitive to different environmental conditions. Its physiology, including stress response, is poorly understood and different from other food-transmitted pathogenic bacteria.[2,3] However, stress response is expected to affect the resistance and survival of pathogens throughout the food production chain.[4]

Minimal growth temperature of thermophilic *C. jejuni* is in the range of 31 to 36 °C. Lack of stress adaptive response factors, such as cold stress proteins, may partly explain the failure to replicate and grow below 30 °C, as described by many authors.[3,5–7] Nevertheless, *C. jejuni* is still metabolically active at temperatures below its minimal growth.[5] As reported, *C. jejuni* survives better at 4 °C than at 10 or 25 °C.[8,9] Mechanisms that allow low-temperature growth and survival involve membrane modifications maintaining membrane fluidity (including nutrient uptake) and maintenance of the structural integrity of macromolecules and macromolecule assemblies such as proteins and ribosomes. Activity of ribosomes seems to play a central role in the cold adaptation process.[4,10–11]

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Bacterial ribosomes are believed to play a central role also in heat-shock regulatory pathways. However, the cells contain several targets for the action of heat; hence, it can be proposed that the basal heat resistance of microorganisms may be due to the stability of macromolecules inside the cell and the membrane, *i.e.,* ribosomes, nucleic acids, enzymes and proteins.[4,12] The major adaptive response to temperature elevation is the heat shock response, which involves induction of special proteins – called heat shock proteins (HSPs).[13,14]

The response to oxidative stress in many enteric gram-negative bacteria is mediated by the key regulators SoxRS and OxyR, which are not present in *C. jejuni*, but an alternative regulator termed PerR has been identified.[15] As reported by Dodd *et al*.,[16] sublethal injury results in production of free radicals, which could cause oxidative damage to the cell.

Under low nutrient conditions, the cells maintain viability by entering into a slow growth state marked by reduction in cell volume, physiological changes and *de novo* protein synthesis.[17–19] Stress resistance of bacteria is time, genus, specium and even strain dependent,[20] but *Campylobacter* spp. seems to often survive through transformation into the more resistant viable but non-culturable (VBNC) state.[21,22]

Considering the confirmed importance of sublethal pre-stress starvation,[23] we aimed to estimate the influence of short-term low temperature incubation on the resistance and survival of *C. jejuni.* In addition, we focused on long-term (more than 42 days) exposure to low temperature (25 °C) and oxidative stress at the atmospheric concentration of oxygen for 80 hours. During that time, we were observing the morphology, culturability and viability of cells and we estimated the likeness between the two stresses, since free radicals should be responsible for cell damage, rather than stress *per se*.[16,24]

EXPERIMENTAL

Bacterial Strain, Culture Media and Growth Conditions

C. jejuni K 49/4 was isolated from poultry meat and was previously identified phenotypically and by the multiplex polymerase chain reaction (mPCR).[25] The culture was stored at –80 °C in brain heart infusion broth supplemented with blood and glycerol[26] and subcultured prior to being subjected to test conditions. The growth occurred microaerobically (5 % O_2, 10 % CO_2, 85 % N_2) at 42 °C for 9 h in Preston broth (Oxoid, Hampshire, UK) to produce exponential phase cells, or for 15 h to produce stationary phase cells, as determined previously.[23]

To prepare starved culture, cells were harvested from the exponential or stationary growth phases by centrifugation at 10000 rpm for 5 min at 4 °C, washed, resuspended in Ringer solution and exposed to starvation for 5 h. For fed culture, exponential and stationary phase cells were further incubated in Preston broth.

Culturability Testing

Culturability was assessed on charcoal cefoperazone deoxycholate (CCDA, Oxoid, UK) or Karmali (Biokar Diagnostics, Beauvais, France) agar plates after serial sample dilutions and microaerobic incubation at 42 °C for 24 or 48 h. All experiments were independently repeated three or more times and the mean values as well as standard deviations were calculated.

Viability Assay

Total and viable counts were performed by the LIVE/DEAD® *Bac*Light™ viability kit (L-7012, Molecular Probes, Eugene, Oregon, USA) using SYTO 9, penetrating intact plasma membranes, and propidium iodide, penetrating only compromised membranes, as previously described[23] and observed using a fluorescence microscope (Eclipse TE300, Nikon, super high pressure mercury lamp power supply, x-60 oil immersion fluorescent objective, Nikon digital camera DXM 1200, Nikon Programming Equipment Laboratory Imaging Ltd., LUCIA 4.60, Tokyo, Japan) using blue excitation light. All experiments were independently repeated three or more times. Number of surviving cells expressed in percents as well as standard deviations were calculated.

Morphology Assay

Morphology was assessed using a transmission electron microscope (PHILIPS CM 100, Philips Electronics N.V. Eindhoven, The Netherlands). The cells were applied to formwar/carboned, 400 mesh copper grids and then negatively stained with 3 % phosphorus tungsten acid, pH = 4.0–5.0.

Low Temperature and Starvation

Fed and starved cells from the exponential growth phase were shifted from 42 °C to 25 °C for 1–42 days. The relationship between fed cultures incubated at 25 °C and at 42 °C represented the influence of low temperature incubation. The relationship between starved culture incubated at 25 °C and fed culture incubated at 25 °C represented the influence of the medium and thereby starvation.

Oxidative Stress and Starvation

Fed and starved cells from the exponential growth phase were incubated at 42 °C at atmospheric oxygen concentration for 1–80 h. The relationship between fed cultures incubated at atmospheric oxygen concentration and under microaerophilic conditions represented the influence of oxidative stress. The relationship between starved and fed culture incubated at atmospheric oxygen concentration represented the influence of the medium and thereby starvation.

Chloramphenicol and Heat Stress

Chloramphenicol (5 mg ml^{-1}) was added as an inhibitor of protein synthesis 1, 2, 5 and 24 h before low temperature incubation and subsequent heat stress at 55 °C for 3 min. After heating, the samples were cooled on ice before analysis.

RESULTS AND DISCUSSION

The Effect of Low Temperature as Prestress on Heat Resistance

Many bacteria can respond to several sublethal stress factors by increasing their heat tolerance, but literature data on the effect of low temperature on subsequent stress heat protection are scarce. Van Bogelen and Neidhardt[27] proposed that the ribosome might be the major temperature sensor in *Escherichia coli*. The effect of cold shock reducing the thermal tolerance of *Listeria monocytogenes* illustrates the use of imposed stress in order to make the microorganism more susceptible to a subsequent processing step.[28,29] The cells showed a reduction in thermal tolerance after exposure to cold shock, even if they were returned to optimal growth prior to thermal challenge.[30]

Different prestresses might be applied to induce higher consequent stress resistance. In our previous work, we compared the viability of cells after exposure to heat stress (55 °C for 3 min) where the cells were previously starved for 1–24 h at 42 °C with or without chloramphenicol added. The highest degree of protection was observed after 5 h of starvation, while 24 h of starvation displayed no difference in the level of resistance to heat stress.[31] Similar relations were also obtained when starvation occurred at 4 °C with a stronger effect of chloramphenicol added on cells survival. To assess the role of low temperature pre-incubation for subsequent heat resistance, we added chloramphenicol, as a protein synthesis inhibitor, before incubation of cells at low temperature for 1, 2, 5 and 24 h. Bars in Figure 1 represent cell viability after heat shock, with or without chloramphenicol added. Differences in cells viability were noticeable, indicating that chloramphenicol actually inhibited proteins after 1 to 2 h of low temperature incubation. In contrast to pre-starved culture, we could not confirm the significant role of low temperature pre-incubation. The cells exposed to low temperature did not survive better than those without adaptive treatment. We observed a reduction in the survival

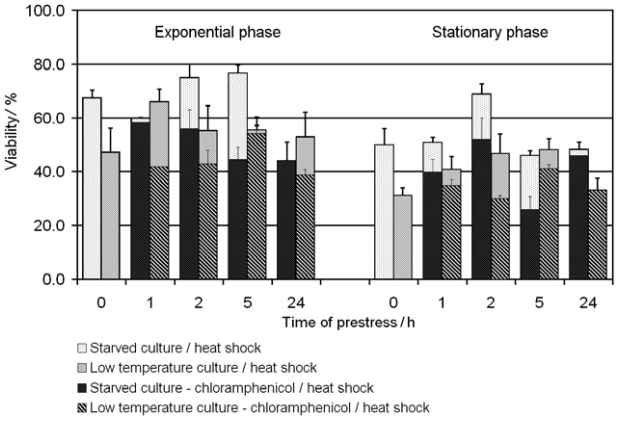

Figure 1. Viability of *C. jejuni* K49/4 cells exposed to prestress for 1, 2, 5 and 24 h at low temperature or starvation and subsequent heat shock (55 °C, 3 min,) with (lower part) or without (upper part) chloramphenicol added. The cells originated from exponential and stationary growth phases. Error bars indicate standard deviations.

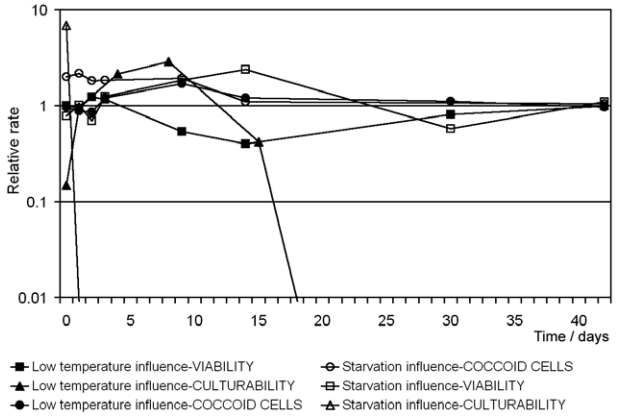

Figure 2. Relative rate of viability, coccoid cell formation and culturability of fed and starved cells from the exponential growth phase of *C. jejuni* K49/4 cells incubated at low temperature (25 °C). The relationship between fed culture incubated at 25 °C and fed culture incubated at 42 °C represents the influence of low temperature incubation. The relationship between starved and fed cultures incubated at 25 °C represents the influence of starvation. Maximal standard deviation was ± 0.03.

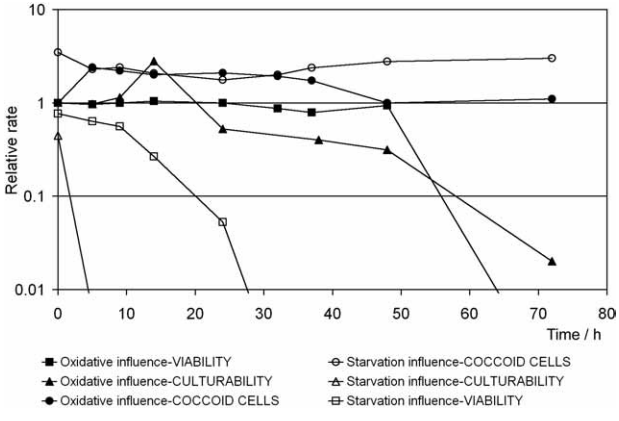

Figure 3. Relative rate of viability, coccoid cell formation and culturability of fed and starved cells from the exponential growth phase of *C. jejuni* K49/4 cells incubated at the atmospheric concentration of oxygen. The relationship between fed culture incubated at the atmospheric concentration of oxygen and fed culture incubated at microaerophilic conditions represents the influence of oxygen concentration. The relationship between starved and fed cultures incubated at atmospheric oxygen concentration represents the influence of starvation. Maximal standard deviation was ± 0.03.

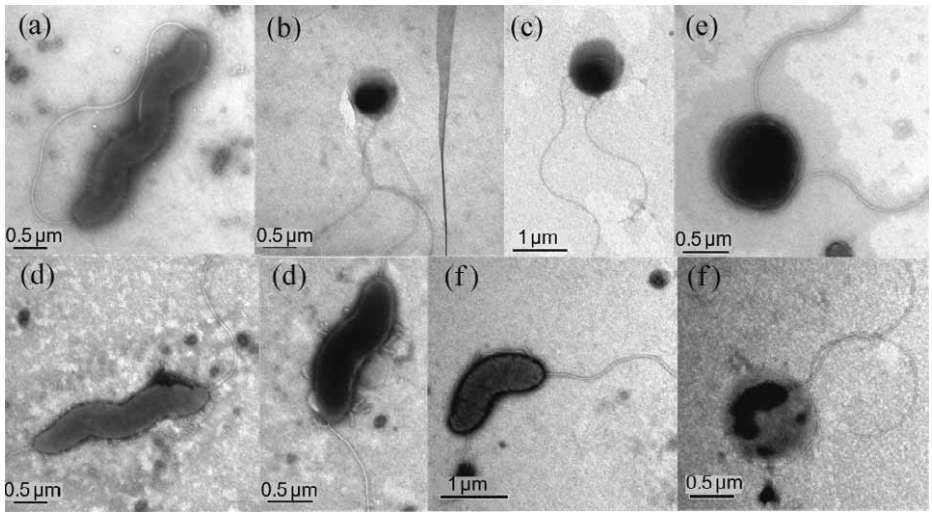

Figure 4. *C. jejuni* K49/4 morphology of fed culture at 42 °C for 9 hours (a); fed culture at 25 °C for 30 days (b); starved culture at 25 °C for 30 days (c); fed culture at the atmospheric concentration of oxygen for 10 h (d); starved culture at the atmospheric concentration of oxygen for 10 h (e); and fed culture at atmospheric oxygen concentration for 30 h (f) as observed by the electron microscopy method. Bars: 0.5 µm or 1.0 µm.

of cells exposed to heat stress if the culture was pre-exposed to low temperature. Cells in the exponential phase had better heat resistance than in the stationary growth phase. This confirms that exponential phase cells are more active and capable of synthesizing protective factors. Therefore, in further stress response studies, we focused on 5 h pre-starved culture (starved culture) and fed culture from the exponential growth phase.

Culturability and Viability Changes upon Long-term Low Temperature Incubation

Relatively little is known about the growth, structural and metabolic changes of campylobacters at low temperatures.[32] Viable counts determined simultaneously with culturability showed that the cellular integrity was maintained longer than culturability. In fact, the cells survived 42 days of the experiment, based on viability signs other than culturability (Figure 2). Additional pre-starvation slightly increased the rate of cell survival in the first days and accelerated their transformation into non-culturable cells. The number of culturable starved cells exposed to low temperature was reduced to under a detectable level already in the first few days. In fed culture, reduction of culturability was visible in 20 days with a previous increase during the first 10 days. Thus, we can conclude from the relative rate of culturability that the influence of starvation was much stronger than the influence of low temperature.

Culturability and Viability Changes upon Oxidative Stress

Decrease in the biomass of cultures as a consequence of the application of oxidative stress as compared to low nutrition factors was reported by Harvey and Leach.[33] They demonstrated subsequent cell ability to survive at a sustained oxygen concentration, although at reduced growth rate, with maintenance of a new steady state. In our previous experiment,[23] cell starvation did not improve the survival of *Campylobacter* cells, as it was the case of the temperature stress response cited.[33] Comparing stress survival of starved and fed *Campylobacter* cells in this work, oxygen exposure was found to be a great stress for starved culture with no additional oxidative resistance produced during starvation (Figure 3). Relative rates of viable cells declined quickly, as clearly visible in starved culture. Relative rate of culturability of fed culture exposed to oxygen slightly increased in the first hours and then started to decline. Rapid decline in culturability of starved culture and slower decline in viability suggest that changes from culturable to VBNC state were taking place in starved cells (Figure 3). Exponential growth phase cells (starved or non-starved) survived oxidative stress better than stationary growth cells; at the same time, coccoid cell formation was lower (data not shown).

Morphological Changes

C. jejuni is well known for its transition from a spiral to a coccoid morphotype during exposure to adverse environmental conditions,[35,36] especially starvation.[23] Coccoid form could be a dormant state, nonculturable but metabolically active and recoverable in animal host conditions,[35] or a nonviable, degenerative form.[3,36,37]

We observed that at the beginning of the incubation period, *C. jejuni* cells from the mid-exponential phase remained spiral. Changes in cell morphology from spiral to coccoid forms were detected under stress conditions presented in this study – low temperature and oxidative exposure (Figures 2–4) and coincided with survival decline.

The formation of coccoid cells examined was mostly influenced by starvation and to a lesser extent by low temperature, which was visible in the increased relative rate of coccoid cells of starved culture (Figure 2). Coccoid cells accounted for 40 % of cells in nonculturable starved culture at low temperature (data not shown). Morphological cell transformation was most significant in the first 17 days of low temperature exposure and in the first 25 days of starvation and low temperature incubation, when coccoid cells accounted for more than 80 % of the culture (data not shown). This was clearly visible in coccoid morphology of cells of fed or starved cultures, with cell volume reducing after incubation at 25 °C for 30 days (Figure 4).

Also under oxidative stress conditions, starvation was essential for formation of coccoid cells. This was clear from the higher relative rate of coccoid cells of starved culture (Figure 3). Under aerobic conditions, nonculturable cells were spiral, shorter spiral or coccoid cells. Coccoid cells were mostly present in starved culture exposed to the atmospheric concentration of oxygen (Figure 4). Therefore, coccoid cell formation could be a component of starvation response.

The formation of more resistant VBNC form is usually accompanied by the change in cell size and transformation from the spiral to coccoid cell form.[21,36] It has been reported that *C. jejuni* can survive in the VBNC form in aqueous microcosms for a period longer than 130 days.[37] Transformation of cells into the VBNC state in our work was observable as a relative decline in culturability but not viability of cells (Figures 2–3). According to our results, low temperature, oxidative stress and especially exposure to low-nutrient conditions are the stimuli for cells to enter the viable but nonculturable state.

CONCLUSIONS

The function of ribosomes seems to be the sensor in bacteria that plays a central role in the temperature adaptation process.[27] Understanding the mechanism of thermal tolerance is an important approach that could reveal strategies to increase the thermal sensitivity of C. *jejuni* in food production and food supply chain. Improved thermal resistance of starved cells has been reported.[23,31] Significant resistance of 5-hour starved *C. jejuni* cells, which gained temperature and peroxide but not higher oxygen cross-protection, was also previously established.[23] In this experiment, the heat resistance of pre-stressed (starvation, low temperature) *C. jejuni* culture was studied when treated at 55 °C for 3 min. We conclude that preliminary exposure to low temperature at 25 °C did not influence the heat resistance of *C. jejuni* cells.

Low temperature, oxidative and especially starvation conditions affected *C. jejuni* cells, which reacted with various morphological and physiological changes. Starvation was the most significant factor in *C. jejuni* survival, coccoid cell formation and especially culturability. Therefore, relative rate declines in culturability of starved cells exposed to stress conditions were significant compared to fed cells exposed to the same stresses. Temperature shift downward, oxidative stress and starvation, usually in combination with other factors, can trigger off transition of bacteria to the viable but nonculturable state. Results showed substantial variability among the stresses applied, indicating no correlation between low temperature and oxidative stress responses of campylobacters. Understanding of microbial physiology after exposure to various stresses clarifies the microbial stress response, which could be helpful in increasing food safety.

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REFERENCES

1. R. V. Tauxe, *Int. J. Food Microbiol.* **78** (2002) 31–41.
2. *Campylobacter*, in: T. A. Roberts, A. C. Baird-Parker, and R. B. Tompkin (Eds.), *Microorganisms in Foods 5*, *Microbiological Specifications of Food Pathogens,* ICMSF, Blackie Academic and Professional, London, 1996, pp. 45–65.
3. S. Park, *Int. J. Food Microbiol.* **74** (2002) 177–188.
4. T. Abee and J. A. Wouters, *Int. J. Food Microbiol.* **50** (1999) 65–91.
5. W. C. Hazeleger, J. A. Wouters, F. M. Rombouts, and T. Abee, *Appl. Environ. Microbiol.* **64** (1998) 3917–3922.
6. J. Parkhill, 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, *Nature* **403** (2000) 665–668.
7. C. Murphy, C. Carroll, and K. N. Jordan, *J. Appl. Microbiol.* **100** (2006) 623–632.
8. M. J. Blaser, H. L. Hardesty, B. Powers, and W. L. L. Wang, *J. Clin. Microbiol.* **11** (1980) 309–313.
9. C. M. Buswell, Y. M. Kerlihy, L. M. Lawtrence, J. T. M. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach, *Appl. Environ. Microbiol.* **64** (1998) 733–741.
10. R. Jaenicke, *Eur. J. Biochem.* **202** (1991) 715–728.
11. E. D. Berry and P. M. Foegeding, *J. Food Prot.* **60** (1997) 1583–1594.
12. R. G. Earnshaw, J. Appleyard, and R. M. Hurst, *Int. J. Food Microbiol.* **28** (1995) 197–219.
13. S. Lindquist and E. A. Craig, *Annu. Rev. Genet.* **22** (1988) 631–77.
14. F. Arsene, T. Tomoyasu, and B. Bukau, *Int. J. Food Microbiol.* **55** (2000) 1–3.
15. A. H. M. van Vliet, M. L. A. Baillon, C. W. Penn, and J. M. Ketley, *J. Bacteriol.* **181** (1999) 6371–6376.

16. C. E. R. Dodd, R. L. Sharman, S. F. Bloomfield, I. R. Booth, and G. S. A. B. Stewart, *Trends Food Sci. Technol.* **8** (1997) 238–241.
17. A. Matin, *FEMS Microbiol. Ecol.* **74** (1990) 185–196.
18. J. E. Moore, *Int. J. Food Sci. Technol.* **36** (2001) 593–600.
19. S. Kjelleberg, N. Albertson, K. Flardh, L. Holmquist, A. Jouper-Jaan, R. Marouga, J. Ostling, B. Svenblad, and D. Weichart, *Antonie van Leeuwenhoek*, **63** (1993) 333–341.
20. J. C. Giard, A. Hartke, S. Flahaut, A. Benachour, P. Boutibonnes, and Y. Auffray, *Curr. Microbiol.* **32** (1996) 264–271.
21. D. M. Rollins and R. R. Colwell, *Appl. Environ. Microbiol.* ***52*** (1986) 531–538.
22. J. D. Oliver, *J. Microbiol.* **43** (2005) 93–100.
23. A. Klančnik, N. Botteldoorn, L. Herman, and S. Smole Možina, *Int. J. Food Microbiol.* **112** (2006) 200–207.
24. T. G. Aldsworth, R. L. Sharman, and C. E. R. Dodd, *Cell. Mol. Life Sci.* **56** (1999) 378–383.
25. T. Zorman and S. Smole Možina, *Food Technol. Biotechnol.* **40** (2002) 177–184.
26. L. Herman, M. Heyndrickx, K. Grijspeerdt, D. Vandekerchove, I. Rollier, and L. De Zutter, *Epidemiol. Infect.* **131** (2003) 1169–1180.
27. R. A. van Bogelen and F. C. Neidhardt, *Proc. Natl. Acad. Sci. USA.* **87** (1990) 5589–5593.
28. D. O. Bayles, M. H. Tunick, A. Thomas, T. A. Foglia, and A. J. Miller, *Appl. Environ. Microbiol.* **66** (2000) 4351–4355.
29. A. J. Miller, D. O. Bayles, and B. S. Elben, *Appl. Environ. Microbiol.* **66** (2000) 4345–4350.
30. P. Graumann and M. A. Marahiel, *Arch. Microbiol.* **166** (1996) 293–300.
31. A. Klančnik, B. Brumec, P. Jamnik, and S. Smole Možina, unpublished paper.
32. C. Höller, D. Witthuhn, and B. Janzen-Blunck, *Appl. Environ. Microbiol.* **64** (1998) 581–587.
33. P. Harvey and S. Leach, *J. Appl. Microbiol.* **85** (1998) 398–404.
34. W. C. Hazeleger, J. D. Janse, P. M. F. J. Koenraad, R. R. Beumer, F. M. Rombouts, and T. Abee, *Appl. Environ. Microbiol.* **61** (1995) 2713–2719.
35. D. McDougald, S. A. Rice, D. Weichart, and S. Kjelleberg, *FEMS Microbiol. Ecol.* **25** (1998) 1–9.
36. A. P. Moran and M. E. Upton, *J. Appl. Bacteriol.* **60** (1986) 103–110.
37. W. Baffone, A. Casaroli, B. Citterio, L. Pierfelici, R. Campana, E. Vittoria, E. Guaglianone, and G. Donelli, *Int. J. Food Microbiol.* **107** (2006) 83–91.

SAŽETAK

Utjecaj niske temperature, gladovanja i oksidativnog stresa na fiziologiju stanica *Campylobacter jejuni*

Anja Klančnik, Tina Zorman i Sonja Smole Možina

Campylobacter jejuni je patogen iz hrane i uzročnik bolesti diareje u ljudi i životinja. Nepoznatim mehanizmima njegove stanice mogu podnijeti značajan stres unatoč nedostatku poznatih gena uključenih u odgovaranju na stres. Problem s *C. jejuni* je njegova sposobnost adaptacije i što može preživjeti u izvancrijevnom prostoru, a često se pojavljuje i u proizvodnji i distribuciji hrane. Pokazali smo da preliminarno izlaganje *C. jejuni* na 25 °C ne utječe na njegovu otpornost na temperaturni stres od 55 °C. K tome, uočene su promjene u morfologiji, u preživljavanju i vijabilnosti hranjenih ili izgladnjivanih kultura kada su one izložene niskoj temperaturi i oksidativnom stresu. Sve fiziološke promjene su povezane s gladovanjem dok je utjecaj drugih stresova bio manji. Najznačajniji faktor za preživljenje stanica *C. jejuni*, stvaranje kokoidnih stanica i posebice za sposobnost rasta u kulturi bilo je gladovanje. Smanjena temperatura, oksidativni stres i gladovanje, obično u kombinaciji s drugim faktorima, mogu prouzročiti tranziciju bakterija u vijabilno stanje bez sposobnosti rasta u kulturi. Rezultati su pokazali znatnu varijabilnost u odgovoru na dani stres bez korelacije između niske temperature i oksidativnog odgovora kampilobaktera. Poznavanje mikrobne fiziologije nakon izlaganja različitim stresovima osnovno je za povećanje sigurnosti hrane.