

Research Note

Altered Hydrophobicity and Membrane Composition in Stress-Adapted *Listeria innocua*

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ABSTRACT

Exposure of *Listeria innocua* to acid and starvation stress decreases sensitivity to the quaternary ammonium compound cetrимide, whereas exposure to cold and heat stress increases sensitivity to this compound. Changes in membrane lipids occur in response to certain types of stress, and these changes likely impact cell sensitivity to chemical sanitizers. The present study included an assessment of the effects of acid, starvation, cold, and heat stress on net cell hydrophobicity and fatty acid composition in *L. innocua*. Net cell hydrophobicity was determined by measuring absorbance of stress-adapted cell suspensions after partitioning with the nonpolar solvent *n*-hexadecane. Free fatty acids extracted from stress-adapted suspensions were analyzed by gas chromatography. Adaptation to acid and starvation increased net cell hydrophobicity and decreased membrane fluidity, which was correlated with reductions in anteiso fatty acids and in ratios of anteiso to iso fatty acids. Conversely, cold-stressed populations exhibited decreased net cell hydrophobicity and increased membrane fluidity with a corresponding increase in C₁₅:C₁₇ and anteiso:iso ratios and in C₁₈ unsaturated fatty acids. No significant changes in net cell hydrophobicity or membrane fluidity were observed in heat-stressed cells, which exhibited increased sensitivity to cetrимide, suggesting another mechanism for altered cell sensitivity. These findings indicate that the efficacy of cetrимide against *Listeria* is partially dependent on the physiological state of the organism following exposure to various environmental stresses.

Cell membrane composition dictates both hydrophobicity and membrane fluidity of bacterial cells. A membrane composed of long-chain (C₁₇) fatty acids is tightly packed and nonfluid, whereas membranes containing lower melting point (C₁₅) unsaturated fatty acids are more fluid (10, 25, 26), and the ratio of short- to long-chain fatty acids (C₁₅:C₁₇) is used as a measure of membrane fluidity (10). Membrane fluidity also is impacted by fatty acid structure; an increase in the levels of highly branched anteiso fatty acids enhances membrane fluidity compared with the less branched iso forms (1). Fatty acids found in bacterial cell membranes can change in length, saturation, and branching in response to stress, and these changes alter membrane fluidity and the cell's ability to interact with and survive in the surrounding environment (25). For example, a change in temperature can alter acyl chain length, the degree of saturation, and the branch position of fatty acids (13). In *Listeria monocytogenes*, cell membrane-bound fatty acids are altered in response to a change in temperature, but alterations in the fatty acid head group are generally minor (13). The transition temperature of the lipid bilayer increases with chain length and degree of saturation of its component fatty acid residues (25) such that higher temperatures are required for mobility of saturated membranes.

Net hydrophobicity of a microbial cell can be altered by fatty acid and protein compositional changes that occur in response to stress (5). An increase in short-chain hydrophilic fatty acids decreases cell hydrophobicity, whereas an increase in long-chain hydrophobic fatty acids increases cell hydrophobicity. Membrane hydrophobicity also affects bacterial attachment to surfaces (2, 24) and the ability of metabolites to transit the cell membrane.

Various stresses encountered by microorganisms in food-processing facilities may induce cellular changes that result in altered sensitivity to commonly used chemical sanitizers. The biocidal mode of action of benzalkonium chloride (cetrимide) and other quaternary ammonium compounds (QACs) follows a six-step process of (i) adsorption of the molecule to the cell surface, (ii) diffusion through the cell wall, (iii) binding to the cytoplasmic membrane, (iv) disruption of the cytoplasmic membrane, (v) release of potassium ions and other cytoplasmic constituents, and (vi) precipitation of cell contents followed by cell death (18). Although QACs act on all bacteria, gram-negative organisms are more sensitive (9). The critical factor for biocidal efficacy is the hydrophilic-lipophilic balance of the QAC (18). Fatty acid and protein compositional changes in the cell membrane can occur in response to stress, and these factors likely influence the ability of cetrимide to disrupt the cytoplasmic membrane through changes in net cell hydrophobicity (2, 12). The cell membrane of *Listeria inno-*

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cua, a commonly encountered indicator organism for the potential presence of *L. monocytogenes*, also is likely influenced by changes in fatty acid composition upon adaptation to stress, and these changes alter net hydrophobicity and the ability of QACs such as cetrимide to penetrate and inactivate the organism.

We previously demonstrated that the sensitivity of *L. innocua* to cetrимide was diminished by prior exposure to acid and starvation and increased by cold and heat stress (19). To maintain intracellular homeostasis, bacteria respond to stress by changing fatty acid and protein composition (1, 12, 22). Thus, we hypothesized that altered cetrимide sensitivity is related to net cell hydrophobicity and cell membrane fluidity. The objective of this study was to evaluate correlations between changes in net hydrophobicity and lipid composition of acid-, starvation-, cold-, and heat-adapted cells of *L. innocua* and the previously observed differences in cetrимide sensitivity.

MATERIALS AND METHODS

***L. innocua* strain.** *L. innocua* ATCC 33090 (Microbiologics, St. Cloud, Minn.) was maintained at -80°C in tryptic soy broth containing 0.6% (wt/vol) yeast extract (TSBYE; Difco, Becton Dickinson, Sparks, Md.) and 20% glycerol and then subcultured twice in TSBYE for 24 h at 35°C before use.

Stress adaptation. Cultures were acid adapted as described by Buchanan and Edelson (3). *L. innocua* was inoculated into TSB supplemented with 1% (wt/vol) glucose (EM Science, Gibbstown, N.J.). The culture was incubated for 18 to 20 h at 35°C , and then the pH was decreased from 5.5 to 4.7, at which point the *L. innocua* was classified as acid adapted. A non-acid-adapted control culture (pH 7.0) was prepared by adding 0.15 ml of 0.25 M But-terfield phosphate water (BPW) (23) to 1 ml of the acid-adapted culture and incubating this culture for 1 h at 37°C .

Using the nutrient starvation method described by Rowe and Kirk (21), overnight (18 to 20 h at 35°C) TSBYE cultures (9 ml) of *L. innocua* were pelleted by centrifugation ($5,000 \times g$ for 10 min) and resuspended in 1 ml of TSBYE ($\sim 10^8$ CFU/ml), and 0.2 ml of this culture was added to 20 ml of sterile distilled water. These cells were then starved for 24 h at 37°C (21). The control culture was similarly prepared by resuspending 0.2 ml of a pelleted ($5,000 \times g$ for 10 min) overnight TSBYE culture in 20 ml of sterile distilled water.

Cold-adapted cells were obtained using the method of Leenan and Drake (11). Overnight (18 to 20 h at 35°C) TSBYE cultures of *L. innocua* were pelleted by centrifugation ($5,000 \times g$ for 10 min), resuspended in 1 ml of TSBYE to contain $\sim 10^8$ CFU/ml (11), and then incubated at 10°C for 5 days. The control culture was similarly prepared by suspending a pelleted overnight *L. innocua* culture in 1 ml of TSBYE to contain 10^8 CFU/ml.

For the heat adaptation method of Lou and Yousef (15), a 5-ml TSBYE culture of *L. innocua* in log phase (8 h at 37°C) containing $\sim 10^6$ CFU/ml was pelleted by centrifugation ($5,000 \times g$ for 10 min), washed twice in 0.25 M BPW, and resuspended in 5 ml of BPW (23). The control culture was prepared by adding 0.5 ml of TSBYE to 1 ml of washed cells and then incubating this culture for 1 h at 45°C in a static waterbath (heat adapted) or at room temperature (22°C).

Net cell hydrophobicity. Net cell hydrophobicity was determined by measuring the absorbance of the cell suspension after partitioning the culture with *n*-hexadecane (Sigma-Aldrich Corp.,

St Louis, Mo.) (20). An equal volume of *n*-hexadecane was added to adapted and control cultures, and the tube contents were mixed by vortexing for 20 s and then allowed to partition into the polar (water) or nonpolar (*n*-hexadecane) phases for 10 min at 22°C . Absorbance of the aqueous phase at 650 nm was measured with a spectrophotometer before (Abs T_0) and after (Abs T_F) partitioning, and net cell hydrophobicity was calculated as follows:

$$\text{net cell hydrophobicity} = [1 - (\text{Abs } T_F / \text{Abs } T_0)] \times 100$$

Fatty acid analysis. Total lipids were extracted according to the method of Folch et al. (7) with several modifications. The adapted and nonadapted control cultures were pelleted by centrifugation ($10,000 \times g$ for 10 min at 22°C), washed twice in BPW, and then extracted using 2 ml of chloroform and then 2 ml of methanol and 1 ml of a saturated sodium chloride solution in double-distilled water. After centrifugation ($1,500 \times g$ for 10 min at 22°C), the bottom chloroform layer was transferred to a second test tube. The top methanol-water layer was extracted again with chloroform and centrifuged, and the new chloroform layer was combined with the previous chloroform extract. Chloroform was removed from the lipid extract by gently passing a stream of nitrogen over the chloroform extract. The lipids were then dissolved in 1.0 ml of analytical grade heptane (VWR International, West Chester, Pa.) and 2.0 ml of analytical grade 7% (wt/vol) boron trifluoride (Sigma-Aldrich) in methanol. The test tube was then flushed with nitrogen, tightly capped, incubated at 100°C for 45 min, and subsequently cooled to room temperature. After cooling, 2 ml of water was added, and the top heptane layer containing fatty acid methyl esters was concentrated under nitrogen.

Fatty acid methyl esters were separated and quantified with an HP 5890 gas chromatograph equipped with an flame ionization detector and a DB-1 column (30 m by 0.32 mm inside diameter, film thickness 1.0 μm ; Agilent J&W, Wilmington, Del.). Helium was the carrier gas, and the inlet and flame ionization detector temperatures were 250 and 280°C , respectively. The gas chromatograph oven temperature was initially maintained at 150°C for 4 min and then increased to 250°C at a rate of $4^{\circ}\text{C}/\text{min}$ with a final holding time of 18 min. Fatty acid methyl esters were identified using standards obtained from Metreya, Inc. (Pleasant Gap, Pa.).

Statistical analyses. Hydrophobicity and fatty acid experiments were conducted in duplicate or triplicate and replicated three times. Differences between these paired data sets were analyzed using a mixed model analysis of variance with culture as a fixed factor and date of analysis as a random factor ($P < 0.05$). Mean test and control values were calculated using the least squares means method, which adjusted the means for unbalanced data when either the adapted or nonadapted cultures had an unequal number of observations on the same day.

RESULTS AND DISCUSSION

Cell surface properties including net cell hydrophobicity and membrane fluidity as impacted by fatty acid composition can be altered through exposure to various environmental stresses, and these alterations in turn can affect the efficacy of various sanitizers, including cetrимide. Net cell hydrophobicity, which reflects the aggregate charge of the fatty acids and proteins at the cellular membrane, likely affects the ability of cetrимide to adhere to and intercalate into the cell membrane. In addition, the ability of bacteria to change their membrane fluidity in response to different environmental stresses affects critical biochemical reac-

TABLE 1. Percentage of C_{15} , C_{17} , and C_{18} fatty acids in the total fatty acids recovered from the cell membranes of stress-adapted and nonadapted *L. innocua*

Fatty acid	Acid		Starvation		Cold		Heat	
	Control (%) ^a	Adapted (%)	Control (%)	Adapted (%)	Control (%)	Adapted (%)	Control (%)	Adapted (%)
C_{15}	37.4 ± 1.3	32.6 ± 2.7 ^a	36.7 ± 2.8	33.4 ± 1.8	36.0 ± 3.8	38.2 ± 2.9 ^a	40.0 ± 1.9	36.8 ± 2.4 ^a
C_{17}	23.4 ± 0.9	21.8 ± 1.7	22.1 ± 1.3	20.6 ± 0.7	24.0 ± 1.7	20.9 ± 1.4 ^a	26.1 ± 1.8	22.8 ± 0.9 ^a
$C_{18:0}$	3.6 ± 0.5	4.3 ± 0.9	3.5 ± 0.8	1.9 ± 0.2 ^a	2.4 ± 0.4	1.3 ± 0.1 ^a	4.3 ± 1.2	5.5 ± 1.4 ^a
C_{18} unsaturated	8.4 ± 1.3	8.7 ± 2.1	7.2 ± 1.5	6.8 ± 0.8	4.1 ± 1.8	6.2 ± 2.3 ^a	1.9 ± 0.4	2.6 ± 0.7 ^a
Anteiso	44.4 ± 1.5	38.9 ± 3.5 ^a	40.9 ± 2.9	32.0 ± 2.7 ^a	38.8 ± 3.5	40.4 ± 3.0	42.7 ± 2.7	40.1 ± 2.9 ^a
Iso	18.8 ± 0.8	18.4 ± 1.2	23.6 ± 1.4	23.5 ± 1.1	24.1 ± 2.1	22.7 ± 1.7	25.6 ± 0.3	23.8 ± 0.5 ^a
Fatty acid ratios								
$C_{15}:C_{17}$	1.6 ± 0.0	1.5 ± 0.0 ^a	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.8 ± 0.0 ^a	1.6 ± 0.0	1.6 ± 0.0
Anteiso:iso	2.4 ± 0.1	1.9 ± 0.2 ^a	1.7 ± 0.1	1.3 ± 0.1 ^a	1.6 ± 0.1	1.8 ± 0.0 ^a	1.7 ± 0.1	1.7 ± 0.1

^a Significantly different from the respective control population ($P < 0.05$).

tions, transport systems, protein secretion, and ultimately survival (13, 26). In this study, fatty acid profiles (Table 1) and net cell hydrophobicity (Table 2) were determined for control and stress-adapted *L. innocua*. However, use of different culture conditions and stress protocols precluded any direct statistical comparisons between the different stresses.

The primary means by which bacteria maintain constant membrane fluidity at different growth temperatures is by adjusting their fatty acid composition (13), a process termed homeoviscous adaptation (26). The fatty acids identified by gas chromatography (Table 1) comprised the majority of fatty acids (50 to 70%) present and were typical of the genus *Listeria* (1, 4, 10, 13, 17, 22). The C_{18} fatty acids included stearic ($C_{18:0}$), unsaturated oleic ($C_{18:1}$ 9c, $C_{18:1}$ 9t), and linoleic ($C_{18:2}$ 9c, 12c) acids.

Hydrophobicity of the cell membrane affects bacterial attachment to surfaces (2) and the ability of metabolites to transit the membrane. Net cell hydrophobicity of acid-adapted *L. innocua* was significantly higher ($P < 0.05$) (Table 2) and the membrane was less fluid, as indicated by decreases in the C_{15} anteiso fatty acids and by the $C_{15}:C_{17}$ and anteiso:iso ratios (Table 1). In other studies, *L. monocytogenes* Scott A exhibited increased hydrophobicity at a reduced pH when grown in TSBYE supplemented with either glucose or lactic acid (16), and acid-adapted *Salmonella* Typhimurium also displayed both increased cell surface hydrophobicity and increased resistance to certain surface-active agents (12). Hence, increased hydrophobicity and/or decreased membrane fluidity of acid-adapted *L. in-*

nocua cells might diminish the ability of cetrимide to adhere to or intercalate into the cellular membrane.

Starvation likewise increased net cell hydrophobicity (Table 2) and lowered membrane fluidity of *L. innocua*, as indicated by a significant reduction in anteiso fatty acids and the anteiso:iso ratio (Table 1). In another study, starved cells of *Escherichia coli* O157:H7 were more resistant to chlorine and deoxycholate, a membrane-active detergent (14). Similar to the acid tolerance response, the starvation response is characterized by cell membrane proteins scavenging nutrients (6), and these cell membrane proteins likely alter net cell hydrophobicity and thus influence the ability of cetrимide to bind and interact with the cell membrane.

In contrast to acid- and starvation-adapted *L. innocua*, cold-adapted populations exhibited decreased hydrophobicity (Table 2) and increased membrane fluidity, as indicated by increases in C_{18} unsaturated fatty acids and in the $C_{15}:C_{17}$ ratio (Table 1), as has also been reported for *L. monocytogenes* at lower temperatures (4). Thus, the cold-adapted population is likely maintaining membrane fluidity by shifting to shorter chain fatty acids with lower melting or transition points.

Increased cell membrane fluidity may enhance chemical sanitizer affinity for or ability to intercalate into the cell membrane. This finding is consistent with the mechanism proposed for nisin sensitivity of cold-adapted cultures of *L. monocytogenes*. The bactericidal activity of nisin is due to pore formation in the bacterial membrane, which is preceded by three steps: binding, insertion, and aggregation (5). Cold-adapted *L. monocytogenes* cells exhibited diminished survival when exposed to nisin (13), similar to the diminished survival of cold-adapted *L. innocua* exposed to cetrимide (19).

In prior research, diminished survival was observed when heat-adapted cells of *L. innocua* were exposed to cetrимide (19). However, no significant changes in cell hydrophobicity or indicators of membrane fluidity ($C_{15}:C_{17}$ or anteiso:iso ratios) were observed except for the increase in C_{18} unsaturated fatty acids in heat-adapted *L. innocua*. Sur-

TABLE 2. Net cell hydrophobicity of control and stress-adapted *L. innocua*

Condition	Control (%)	Adapted (%)	% change
Acid	32.6 ± 1.8	36.4 ± 1.9 ^a	3.8
Starvation	9.7 ± 1.1	18.3 ± 6.2 ^a	8.6
Cold	6.3 ± 1.6	3.1 ± 0.5 ^a	-3.2
Heat	2.9 ± 0.4	4.3 ± 0.5 ^a	1.4

^a Significantly different from the respective control population.

vival of heat-adapted *L. innocua* in contrast to acid-, starvation-, and cold-adapted *L. innocua*, following exposure to cetrimide was poorly correlated with changes in cell hydrophobicity and membrane fluidity. The microbial heat shock response results in a large increase in non-membrane-associated intracellular heat shock proteins. Because neither net cell hydrophobicity nor membrane fluidity were altered after heat adaptation, another mechanism involving possible changes in heat shock proteins is likely responsible for enhanced sensitivity of heat-adapted *L. innocua* to cetrimide.

Many food-processing environments are known to harbor diverse groups of *Listeria* species, and some strains become endemic and persist for many years (8). These persistent strains most often reside in specific niches that are less accessible to cleaning and sanitizing or within biofilms that diminish sanitizer efficacy. Some stressful conditions including cold, heat, acid, and starvation within food-manufacturing facilities also could enhance resistance to QACs, particularly when used at subrecommended levels (19). The intrinsic stress adaptation mechanisms identified in this study may enable *Listeria* to evade the lethal effects of sanitizers and persist in food-processing environments. However, other modifications within these environment can stress-adapt pathogens and concurrently enhance their sensitivity to QACs. The results of these modifications may in turn lead to the development of alternative intervention strategies for enhancing sanitizer efficacy.

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