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Morphological changes of temperature- and pH-stressed *Salmonella* following exposure to cetylpyridinium chloride and nisin

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Abstract

The outer membrane of Gram-negative bacteria such as *Salmonella*, act as a permeability barrier, preventing nisin gaining access to the cytoplasmic membrane. If the outer membrane permeability is reduced, however, Gram-negative bacteria can show nisin sensitivity. In this study, temperature stresses (heating, chilling and freezing), pH stress (pH 4.5, 5.0, 6.0) and cetylpyridinium chloride (CPC)-nisin treatment were used to alter the outer membrane permeability of *Salmonella*, producing a loss of barrier function and reduced resistance to nisin. The morphological changes in *Salmonella* were examined using scanning electron microscopy. Temperature and pH-stressed *S. typhimurium* cells, untreated and treated with CPC-nisin had perturbed cell morphology, including apparent indentations and craters in the cell surfaces and collapsed amorphous bodies.

Keywords: *Salmonella*; Nisin; Cetylpyridinium chloride; Outer membrane

1. Introduction

Nisin is a 35-amino acid cationic peptide antimicrobial bacteriocin, produced by *Lactococcus lactis* subs. *lactis* (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996) and has been accepted as a food additive by both the FDA and WHO. As a result to date it has found several applications in food (Schillinger, Geisen, & Holzapfel, 1996). Nisin is active against almost all Gram-positive bacteria and their germinated spores, but not against Gram-negative bacteria or fungi (Stevens, Klapes, Sheldon, & Klaenhammer, 1992; Delves-Broughton et al., 1996). The sensitivity of some Gram-negative bacteria to nisin has been reported (Stevens, Sheldon, Klapes, & Klaenhammer, 1991). The interference of nisin in the cytoplasmic membrane of susceptible species leads to pore formation and dissipation of the proton motive force. The consequence is the efflux of low-molecular-weight solutes, such as amino acids and K^+ which are involved in the maintenance of the cells; turgor pressure, enzyme activation, and regulation intracellular pH homeostasis, together with depletion of intracellular ATP (Moll, Roberts, Konings, & Driessen, 1996; Montville, Chung, Chikindas, & Chen, 1999). In some bacteria, this interference of nisin with lipid II (C55 bactoprenol pyrophosphate—a carrier involved in cell wall biosynthesis) in the membrane has been proved to be the cause of death due to loss of cell integrity (Brotz et al., 1998; Chung & Hancock, 2000).

In general, the protective outer membrane, surrounding the cytoplasmic membrane and peptidoglycan layer of Gram-negative cells cannot be damaged by nisin. The inner and outer membranes are composed of glycerophospholipids and lipopolysaccharides, respectively. Lipopolysaccharides, which are composed of a lipid part and heteropolysaccharide, partly show an anionic characteristic which is an essential property of the

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hydrophilic surface (Nikaido, 1996). Since outer membrane is a permeability barrier of hydrophobic substances and macromolecules, nisin, as a hydrophobic macromolecule ($M_r = 3353$), cannot, reach its site of action (Helander & Mattila-Sandholm, 2000). To use nisin to act as an antimicrobial reagent for Gram-negative bacteria, outer membrane of Gram-negative should be disrupted to allow the reaching of nisin to its site of target into its cell. Outer membrane can be damaged by various methods; e.g. by using chelating agents including EDTA, or by pretreatment with cell membrane disrupters such as trisodium phosphate or by physical treatments (hydrostatic pressure, heat, freezing and thawing) (Kalchayanand, Hanlin, & Ray, 1992; Cutter & Siragura, 1995; Hauben, Wuytack, Soontjens, & Michiels, 1996; Boziaris, Humpheson, & Adams, 1998; Carneiro de Melo, Cassar, & Miles, 1998). These disruption mechanisms result in the changes of morphologies and structures of outer membrane such as blebs, vesiculation and damage or release of lipopolysaccharides. These changes can alter the permeability barrier of the outer membrane, resulting in the efflux of periplasmic enzymes, and sensitivity to hydrophobic compounds, dyes and surfactants (Katsui et al., 1982; Tsuchido, Katsui, Takeuchi, Takano, & Shibasaki, 1985). However, such injury can be subsequently repaired if the cell is not severely disrupted (Boziaris & Adams, 2001).

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound having antimicrobial properties against many bacteria such as *Esherichia coli* O157: H7, coliforms, *Salmonella typhimurium*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Staphylococcus aureus* as well as viruses (FDA, 1998; Polhman, Stivarius, McElyea, & Waldroup, 2002). It is classified as a cationic surface active agent and can therefore absorb phosphates in negatively charged bacteria cell membranes, resulting in cell wall disruption (Randford, Beighton, Nugent, & Jackson, 1997).

Salmonella, a Gram-negative bacterium, is one of the most important causes of foodborne disease. Its contamination in food products brings significant public health concern (Jung et al., 2003). Generally, during food processing, the outer membrane of Gram-negative bacteria including *Salmonella* could be disrupted by stress conditions such as temperature and pH stress occurring during food processing. Accordingly, nisin could be applied as biopreservative to inhibit an activity of these Gram-negative bacteria in food production under these stress conditions. In addition, the use of disruptive agents such as CPC to increase the damage of a outer membrane could be a significant alternative. Therefore, this study aimed to investigate the effect of temperature- and pH-stress following a CPC-nisin treatment on *Salmonella typhimurium* by determining of morphological changes of cells using scanning electron microscopy (SEM), and viability of cells.

2. Materials and methods

2.1. Bacterial strain

S. typhimurium S36 used throughout this study containing the mini-Tn5 plasmid containing the complete *lux* (CDABE) with kanamycin resistance gene cassette. It was grown in 100 ml of brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) added with kanamycin (30 μg/ml), at 37 °C and 150 rpm for 12–14 h. Culture was transferred to a sterile tube and centrifuged at 4000*g* for 10 min, 4 °C to sediment bacterial cells. Cell pellets were washed once in sterile maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) and resuspended in MRD. The populations ca. 10^7 cfu/ml (A_{600} 0.2) of cells in the suspensions were used for following study.

2.2. Cetylpyridinium chloride (CPC) and nisin

Cetylpyridinium chloride (CPC) (Sigma, UK) solution was prepared at concentration of 0.05 g in 100 ml distilled water. Nisin (Sigma, UK) solution was prepared at concentration of 100 μg nisin in 1 ml of 0.02 mol/l HCl. Each solution was filter sterilized (0.45 μm, Millipore, France) and stored at 4 °C until used.

2.3. Temperature stress

The temperature stresses (heating, chilling, and freezing/thawing) of 10 ml (10^7 cfu/ml) of *S. typhimurium* S36 were conducted in universal bottles containing MRD. For heating, the bottles containing *Salmonella* suspensions were heated at 55 ± 1 °C for 10 min in water-bath and immediately placed in cold water at 4 °C for 5 min before moving to room temperature (22 °C) for 5 min. For chilling, the cell suspensions were chilled in an ice water-bath (~0.5 °C) for 30 min and then let to room temperature for 5 min. Freezing/thawing treatment was operated by placing the cell suspensions in a freezer at −20 °C for 24 h, then placing in an incubator at 37 °C for 30 min to let its thaw before moving to room temperature for 5 min. The control cells were placed in a stationary incubator at 37 °C for 30 min and then left for 5 min at room temperature.

The viability of temperature stressed cells was determined. All suspensions of temperature stresses were serially diluted in MRD and were spread onto BHI agar. Plates were incubated at 37 °C for 16–18 h