Letters in Applied Microbiology 2000, **31**, 233–237

Transient sensitivity to nisin in cold-shocked Gram negatives

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116/2000: received 2 June 2000 and accepted 6 June 2000

I.S. BOZIARIS AND M.R. ADAMS. 2000. Rapid chilling in the presence of nisin caused a dose-dependent reduction in the populations of several Gram-negative bacteria, despite the fact that appreciable structural injury to the outer membrane was not detected. *Pseudomonas aeruginosa* was most affected, followed by *Pseudomonas fragi*, *Salmonella enteritidis* PT4, PT7 and *Escherichia coli*, respectively. Addition of nisin after the chilling treatment had no effect. The results are ascribed to a transient susceptibility caused by phase changes in the lipids associated with the outer membrane, which are rapidly reversed when the cells return to higher temperatures. Combinations of chilling shock, nisin and EDTA gave much lower reductions of *Salmonella* and *Pseudomonas* on chicken skin in comparison with broths. This is attributed to a buffering of the temperature shock experienced by adherent bacteria and binding of the nisin by food particles.

INTRODUCTION

Nisin is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis,* which is active against Gram-positive organisms (Delves-Broughton 1990). It is not generally active against Gram-negative bacteria, yeasts and fungi, though Gram negatives show nisin sensitivity when their outer membrane (OM) permeability is altered by chelators or some physical treatments (Stevens *et al*. 1991; Kalchayanand *et al*. 1992, , 1994; Boziaris *et al*. 1998). Cold shock has been shown to result in the leakage of cations and u.v.-absorbing material, as well as damage of the permeability barrier (Strange and Ness 1963; Strange and Postgate 1964), and may therefore allow nisin to penetrate to the cytoplasmic membrane where it can exert its effect.

In this work, the effect of rapid and slow chilling on nisin sensitivity was studied in a number of Gram negatives in broth and on chicken skin.

MATERIALS AND METHODS

Organisms

Salmonella enteritidis PT4, P167807 and PT7, P469815 were supplied by The Division of Enteric pathogens, Central Public Health Laboratory, London, UK. *Salmonella enteritidis* PT7 is a strain with shorter lipopolysaccharide chains on its outer membrane than PT4 (Chart *et al*. 1989). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* USCC 2186 and *Pseudomonas fragi* ATCC 4973 were from The University of Surrey collection. All bacteria were stored frozen on beads (Protect; Technical Service Consultants Ltd, Heywood, UK) at −70 °C. For resuscitation, one bead was added to 10 ml Nutrient Broth and incubated at 37 °C or 25 °C (*Ps. fragi*) for 24 h.

Microbiological media

Microbiological media were supplied by Oxoid. Maximum Recovery Diluent (MRD) was used for serial dilutions. Nutrient Agar (NA) supplemented with 0·15% w/v sodium dodecyl sulphate (NA/SDS) was prepared by adding filter-sterilized (0·45 μm filters, Minisart, NML, Sartorious, USA) solution of SDS to molten autoclaved NA to give the correct final concentration of nutrients and SDS.

Chemicals

Purified nisin (5×10^7 IU g^{-1}) was supplied by Aplin and Barrett Ltd, Dorset, UK. Solutions of nisin were prepared in 0·02 N HCl (pH 2), filter-sterilized (0·45 μm Minisart) and stored at 4 °C.

SDS was provided by Sigma and disodium EDTA was supplied by Fisons. Concentrated solutions of SDS and EDTA in deionized water were sterilized by filtration through a 0·45-μm filter (Minisart).

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Experimental design

Experiments were designed to minimize variances by using a single culture for each series of replicates. In such a case, paired observations between the control and each of the treated samples could be carried out and evaluated using *t*-tests.

Chilling treatments

Chilling of bacterial populations was performed in MacCartney bottles containing nutrient broth (NB) or phosphate-buffered saline (PBS), with or without nisin as specified. Stationary phase cultures (50 ml) were harvested after 18–24 h incubation at 37 °C or 25 °C (*Ps. fragi*) by centrifugation (1500 *g* for 15 min at 20 °C). The pellets were washed in saline and resuspended in 1 ml NB or PBS. The suspension (0·1 ml) was then added to 9·9 ml NB or PBS, pre-chilled to 0·5 °C, and left for 10 min. The bottles were then warmed to 30 °C in a water-bath for 5 min. For slow cooling, the suspension was added to the treatment medium before its transfer to the ice-water bath and left until the temperature reached 0·5–1·0 °C. Temperatures were recorded with an NAMAS-certified probe and digital indicator (Pt 100 probe and Series 268 indicator, Anville Instruments, Camberley, UK).

After treatments, 5 ml were centrifuged at 1500 *g* for 15 min. The pellets were washed in 5 ml saline to remove any remaining nisin and re-centrifuged. The bacterial pellet was resuspended in 5 ml MRD, serially-diluted, and plated as 0·1 ml spread plates on NA and NA supplemented with 0·15% w/v SDS. The plates were incubated at 37 °C or 25 °C for 48 h. All the trials were carried out at least in triplicate

Decontamination of chicken skin

Whole chicken was purchased locally and the skin carefully removed. Circular pieces, approximately 2 cm^2, were cut using a sharp cork borer. The pieces were placed in open sterile Petri dishes under a u.v. lamp irradiating at a maximum wavelength of 254 nm, and left for 10 min. The pieces were inverted and left for 10 min, then stored at −20 °C until use (< 4 weeks).

From the concentrated bacterial suspension in saline, 1 ml was added to 9 ml MRD containing 0·01% v/v Tween-20 to give a population of approximately 10^8 cfu ml^{-1}; 0·02 ml were spread on each chicken piece and left to dry for 30 min at room temperature.

Universal bottles were prepared as follows. Control (tap water only), nisin 500 IU ml^{-1}, EDTA 20 mmol $l^{-1}$and nisin plus EDTA (500 IU ml^{-1} and 20 mmol l^{-1}, respectively) were placed in a 37 °C water-bath. A duplicate set was prepared and chilled down to 0·5 °C in an iced water-bath. The pH was adjusted to 7·0 with concentrated NaOH. Pieces of chicken skin were immersed, using sterile forceps, in one of the solutions and left for 10 min. After 10 min, the temperature of the chilled suspensions was raised to 30 °C by transferring to a 30 °C water-bath for 5 min. Each piece was then placed into a sterile stomacher bag with 20 ml saline and treated for 2 min in a Stomacher (Seward Medical Ltd, London, UK). Decimal serial dilutions were prepared in MRD and 0·1 ml plated on NA. The results expressed as cfu cm^{-2} of skin surface. All trials were carried out at least in triplicate.

RESULTS AND DISCUSSION

Chilling treatments and nisin

The temperature profiles of the two chilling treatments used are shown in Fig. 1. In the case of rapid chilling, the bacterial suspension reached 0·5 °C almost instantly, and remained there for 10 min before being warmed to 30 °C. In the slow chilling protocol, it took more than 15 min to reach 0·5 °C.

The number of survivors and the extent of injury in the surviving populations was determined using counts on non-selective (NA) and selective media (NA with SDS). Outer membrane injury can allow surface-active compounds, like SDS, access to the plasma membrane where it exerts its inhibitory effect (Ray 1979).

Figure 2 shows the effect of rapid chilling on the bacterial populations in the presence and absence of nisin. Rapid chilling generally gave reductions of much less than 0·5

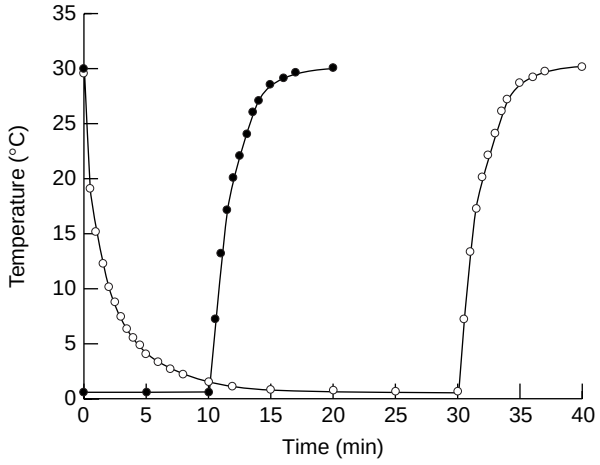

Fig. 1 Temperature profiles of rapid (●) and slow (○) chilling treatments in NB

logs. No significant differences between NA and NA/SDS counts ($P > 0{\cdot}05$) after treatment were seen for the Enterobacteriaceae, indicating no outer membrane injury in the survivors. With the pseudomonads, OM injury was detected ($P < 0{\cdot}05$) in less than 50% of the survivors. Slow chilling had a negligible effect on survival and injury for all the micro-organisms tested, giving virtually identical counts on NA and NA/SDS.

When nisin was present in the medium, no effect was seen with slow chilling and this protocol not used in subsequent work. During rapid chilling, however, a dose-dependent increase in lethality was observed (Fig. 2). *Pseudomonas aeruginosa* was the most sensitive of the isolates examined, showing a 1·5–2·5 log reduction. *Pseudomonas fragi*, and *Salm. enteritidis* PT4 and PT7 were less sensitive but also showed significant reductions (0·5–1 logs, $P < 0{\cdot}05$). *Escherichia coli* showed non-significant reductions for low nisin concentrations, and only slight sensitivity ($< 0{\cdot}5$ logs) at nisin concentrations of 2500 IU ml^{-1}. Unstressed cells were not sensitive to nisin; numbers were unaffected by exposure to 2500 IU ml^{-1} for 30 min.

The reduction of counts after cold shock in the presence of nisin was greater than the extent of injury recorded after cold shock alone. If nisin inactivated only those cells showing injury after treatment, then its presence would have reduced counts by less than 50% (0·3 log) in all cases and

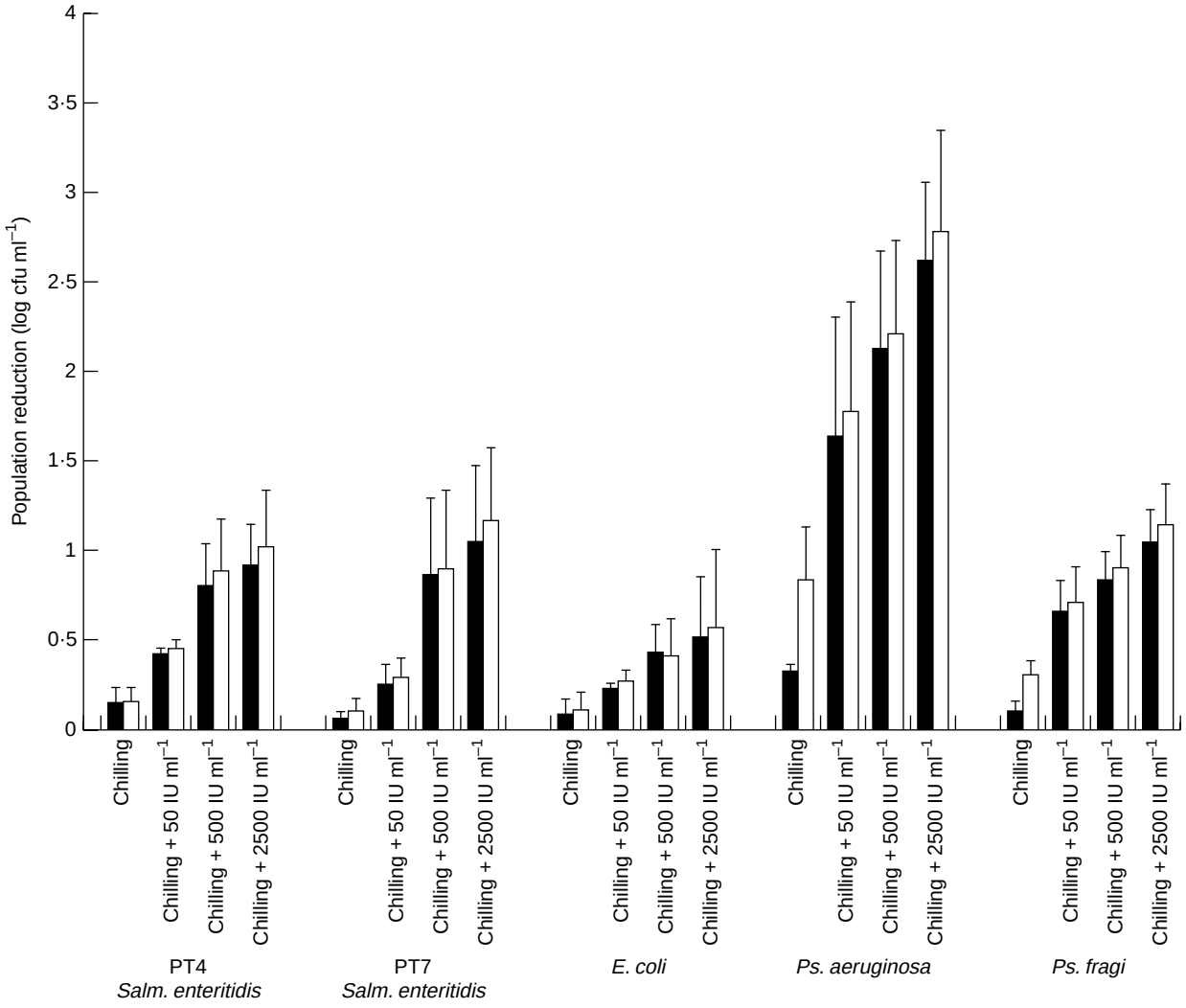

Fig. 2 Effect of chilling with and without the presence of nisin on injury and inactivation of Gram negatives in NB. The error bars show the standard deviation of three replicates. (■), NA; (□), NA/SDS

produced no reduction in the Enterobacteriaceae counts. This indicates that nisin was not simply inactivating the cells displaying injury after treatment. In fact, nisin added after the chilling treatment had only a slight effect on bacterial numbers, regardless of whether or not they displayed any injury (sensitivity to SDS in nutrient agar). Pseudomonads showed slight sensitivity to high nisin concentrations (2500 IU ml^{-1}), with a reduction of 0·6 logs for *Ps. aeruginosa*, but the Enterobacteriaceae were virtually unaffected. These results suggests that transient injury during processing had made the cells nisin-sensitive, and that all cells injured in this way had either been inactivated by nisin, or had recovered rapidly after treatment. The fact that similar transient susceptibility was seen when PBS was used instead of NB implies that recovery was not due to active repair of bacteria.

It seems that the transient susceptibility was related to a rapid physical recovery or restoration of the outer membrane barrier when the stress factor was removed. This is similar to the transient susceptibility to nisin recorded during high pressure treatment of Gram negatives (Hauben *et al.* 1996). The OM is an asymmetric bilayer where the LPS occupy the upper layer and phosholipids the layer below (Nikaido and Vaara 1985). Cold shock causes crystallization of the liquid-like lipids within membranes, creating channels (Haest *et al.* 1972). When the chilling is slow, the relative mobility of the lipid chains permits them to rearrange and maintain the integrity of the membrane (Leder 1972). These effects are normally associated with the cytoplasmic membrane. However, rapid solidification of the phospholipids in the OM might also cause defects, weakening the anchoring of LPS and OM proteins. Slow chilling allowed the cell to retain integrity of the OM and hence, nisin resistance. The temperature of the phase transition varies with the membrane fatty acid composition (Steim *et al.* 1969), so sensitivity will vary between different strains and species, as demonstrated here. The transience of susceptibility may be due to a restoration and reorganization of the structures when the temperature increases, allowing re-melting of the fatty acids and re-stabilization of the bonds in the OM structure.

Decontamination of chicken skin

A practical application of a combination of cold shock and nisin could be the decontamination of chicken carcasses, since chilling by carcass immersion in cold water is a common post-slaughter practice (Bolder 1998). Decontamination of carcasses using various food grade chemicals, including nisin, has been tried with varying degrees of success (Shefet *et al.* 1995; Carneiro de Melo *et al.* 1997). Results from using combinations of cold shock, EDTA and nisin on *Salm. enteritidis* PT4 and *Ps. aeruginosa* attached to chicken skin are presented in Fig. 3.

Reductions resulting from chilling and chilling/nisin were less than those seen in broth experiments, and were not significant in the case of *Salmonella* ($P > 0·05$). The addition of EDTA produced further significant reductions

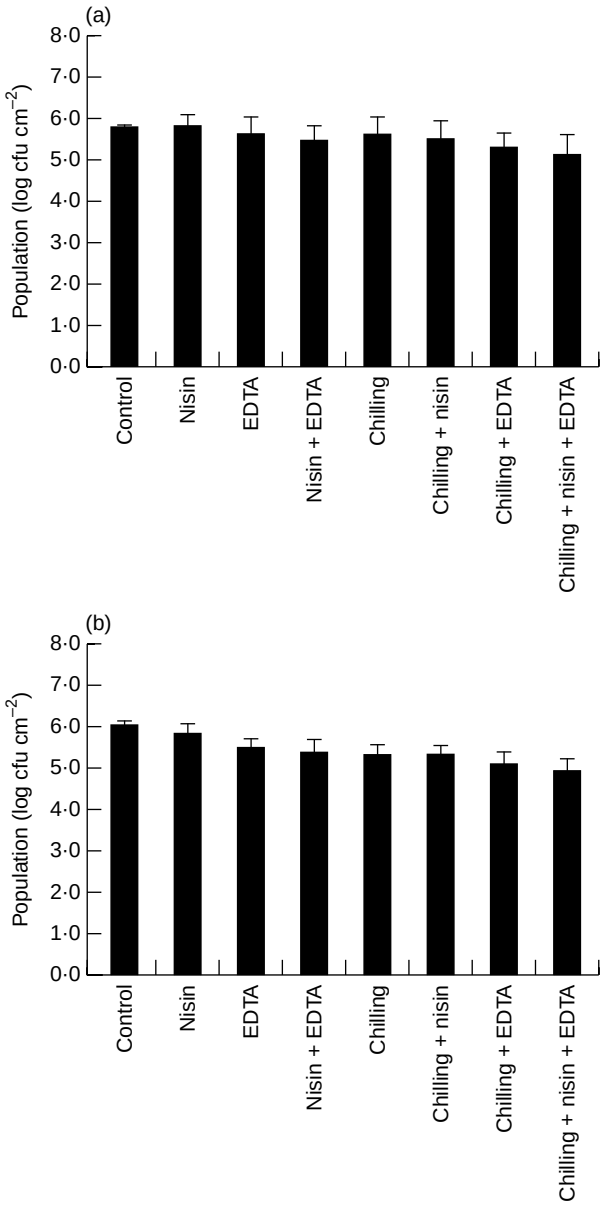

Fig. 3 Effect of combinations of chilling, EDTA nisin on the decontamination of chicken surface from (a) *Salmonella enteritidis* PT4 and (b) *Pseudomonas aeruginosa*. The error bars show the standard deviation of three replicate experiments

($P < 0{\cdot}05$), though these were still small. The combined effect of chilling, nisin and EDTA reduced skin populations of *Salmonella* by 0·7 log and *Pseudomonas* by 1·3 log cfu. In the case of *Pseudomonas,* the reduction appears to be due more to the chilling effect than to the effect of chilling/nisin and/or EDTA (Fig. 3b).

In previous studies with combinations of nisin and chelators, reduction in the numbers of attached bacteria was less than that seen with cells suspended in broth (Cutter and Siragusa 1995; Carneiro de Melo *et al.* 1997). It is generally accepted that bacteria attached to surfaces are less accessible to antimicrobials, and nisin in particular is known to bind to food materials, hence becoming less available. Also, the cold shock is less severe for attached bacteria as the substratum will act as a thermal buffer as the chicken slowly cools.

In the protocol used here, the chilling shock did not prove sufficient to produce a useful transient susceptibility to nisin in attached bacteria. It has been shown recently that prolonged chill storage in liquids at 6·5 °C sensitize the Gram negatives to nisin (Elliason and Tatini 1999). Refinement of the chilling system to produce a more rapid cold shock, followed by prolonged storage at chill temperatures, could provide an effective way of sensitizing Gram negatives to nisin in a wide range of foods.

ACKNOWLEDGEMENTS

The authors would like to thank the State Scholarship Foundation of (I.K.Y.) of the Hellenic Republic for financial support of I.S. Boziaris.

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