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REVIEW ARTICLE

Mechanisms of nisin resistance in Gram-positive bacteria

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Abstract Nisin is the most prominent lantibiotic and is used as a food preservative due to its high potency against certain Gram-positive bacteria. However, the effectiveness of nisin is often affected by environmental factors such as pH, temperature, food composition, structure, as well as food microbiota. The development of nisin resistance has been seen among various Gram-positive bacteria. The mechanisms under the acquisition of nisin resistance are complicated and may differ among strains. This paper presents a brief review of possible mechanisms of the development of resistance to nisin among Gram-positive bacteria.

Keywords Nisin-resistance · Cell wall · Membrane phospholipid · Two-component system

Introduction

Nowadays, food safety has become an important issue globally due to increasing foodborne diseases and changes in food habits. The occurrence of illness due to the consumption of foods contaminated by bacteria has a great impact on public health worldwide. Therefore, the development of food preservatives, especially biological food preservatives, is attracting more attention. Bacteriocins, which are ribosomally synthesized by several lactic acid bacteria (LAB), exhibit antimicrobial activity and offer potential applications in food preservation. Until now, the only bacteriocin licensed as a food preservative has been nisin.

Nisin is a lanthionine-containing peptide produced by certain strains of *Lactococcus lactis* (Lubelski et al. 2008) and is widely used in the food industry as a safe and natural preservative. Nisin has an antimicrobial activity against a broad range of Gram-positive bacteria, including many foodborne pathogens and spoilage bacteria. Studies have shown that nisin kills bacteria primarily by pore formation in the cytoplasmic membrane and by inhibiting peptidoglycan synthesis (Breukink et al. 1999; Breukink and de Kruijff 2006; Brötz et al. 1998). In addition, nisin can induce cell autolysis and inhibit the outgrowth of bacterial spores (Gut et al. 2008; Montville et al. 2006).

However, the bactericidal efficacy of nisin in foods has been compromised by the occurrence of nisin resistance in various bacteria, making it the main concern of nisin application in food preservation. In foods with a long shelf-life, even a small number of these resistant bacterial cells can multiply to a very large number and then may cause to foodborne outbreaks and food spoilage. Thus, understanding the mechanisms of the development of nisin resistance is essential for the application of nisin in the food industry.

Some Gram-positive bacteria that are repeatedly exposed to increasing nisin concentrations can acquire nisin resistance (Harris et al. 1991; Ming and Daeschel 1993; Mazzotta and Montville 1997; Garde et al. 2004). Until now, nisin resistance has been reported in several species of bacteria, including *Lactobacillus casei* (Breuer and Radler 1996), *Streptococcus thermophilus* (Alifax and Chevalier 1962; Garde et al. 2004), *Pediococcus acidilactici* (Goulhen et al. 1998), *Streptococcus bovis* (Mantovani and Russell 2001), *Listeria monocytogenes* (Harris et al. 1991; Davies and Adams 1994; Mazzotta and Montville 1997; Collins et al. 2010), *Listeria innocua* (Maisnier-Patin and Richard 1996), *Bacillus cereus* (Jarvis 1967), *Staphylococcus aureus* (Blake et al. 2011), and *Clostridium botulinum* (Mazzotta et al. 1997; Mazzotta and Montville 1999) (Table 1). The present work reviews the proposed physiological and molecular mechanisms of nisin resistance development among Gram-positive bacteria.

Cell wall modification

Alterations in the cell wall have been proposed as the main mechanism for bacteriocin resistance in bacteria. In the presence of nisin, the nisin-resistant variants of *Listeria innocua*

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Table 1 The main mechnisms involved in nisin resistance in Gram-positive bacteria

Main mechanism		Strain	Reference
Cell wall modification	Increased positive charges in cell wall	*Listeria innocua*	Maisnier-Patin and Richard 1996
		Lactococcus lactis	Kramer et al. 2008
		Staphylococcus aureus	Peschel et al. 1999
		Streptococcus pneumoniae	Kovács et al. 2006
		Streptococcus bovis	Mantovani and Russell 2001
		Streptococcus agalactiae	Poyart et al. 2001
		Bacillus cereus	Abi Khattar et al. 2009
		Clostridium difficile	McBride and Sonenshein 2011
	Penicillin binding protein	*Listeria monocytogenes*	Gravesen et al. 2001; Collins et al. 2010
		Lactococcus lactis	Kramer et al. 2006
Modifications of membrane phospholipid composition	Produce more phosphatidylglycerol and less diphosphatidylglycerol	*Listeria monocytogenes*	Verheul et al. 1997
	Decrease anionic phospholipid Reduced fluidity and stabilization	*Listeria monocytogenes*	Crandall and Montville 1998; Ming and Daeschel 1993; Mazzotta and Montville 1997
	Lower amount of saturated fatty acid and less elongated fatty acids	*Lactococcus lactis*	Kramer et al. 2006
Nisin inactivation via enzymatic action	Nisinase	*Streptococcus thermophilus*	Alifax and Chevalier 1962
		Lactobacillus plantarum	Kooy 1952
		Bacillus species	Jarvis 1967
	Nisin resistance protein	*Lactococcus lactis*	Liu et al. 1997; Froseth and McKay 1991; Tang et al. 2001
ABC transporter	*ysaBCD*	*Lactococcus lactis*	Kramer et al. 2006
	AnrAB	*Listeria monocytogenes*	Collins et al. 2010
	VraDE	*Staphylococcus aureus*	Hiron et al. 2011
	VraFG	*Staphylococcus aureus*	Herbert et al. 2007; Falord et al. 2011
	NsaAB	*Staphylococcus aureus*	Kolar et al. 2011
Regulatory networks	BraSR	*Staphylococcus aureus*	Hiron et al. 2011
	LiaRS	*Bacillus subtilis*	Mascher et al. 2004
	GraSR	*Staphylococcus aureus*	Herbert et al. 2007; Falord et al. 2011
	NsaSR	*Staphylococcus aureus*	Blake et al. 2011; Kolar et al. 2011
	LiaFSR	*Streptococcus mutans*	Suntharalingam et al. 2009
	LiaSR	*Streptococcus gordonii*	McCormick et al. 2011
	DltRS	*Streptococcus agalactiae*	Poyart et al. 2001
	LisRK; VirRS	*Listeria monocytogenes*	Cotter et al. 2002; Collins et al. 2010
	σ factor	*Listeria monocytogenes*	Palmer et al. 2009

showed a thickened cell wall and increased cell wall hydrophobicity (Maisnier-Patin and Richard 1996) (Fig. 1b, e). In addition, the removal of cell wall from nisin-resistant *Listeria monocytogenes* F6861 resulted in the loss of nisin resistance, suggesting that changes, such as the loss of positively charged wall teichoic acids (WTA), are responsible for nisin resistance (Davies and Adams 1994; Davies et al. 1996) (Fig. 1a).

Previous studies on the mechanism of the anti-bacterial function of nisin have shown that nisin kills bacteria primarily by the formation of pores in the cytoplasmic membrane via binding to lipid II, an essential bacterial cell wall precursor (Brötz et al. 1998; Breukink et al. 1999). Because of the importance of lipid II for the activity of nisin, decreasing the amount of lipid II would be a simple mechanism for generation of bacterial resistance by bacteria for generation resistance to nisin. But in the study conducted by Kramer et al. (2004), no differences in lipid II levels were detectable in nisin-resistant *Listeria monocytogenes* or *Micrococcus flavus* strains compared to their control parent strains. Further studies found that the cell wall composition of the resistant strains was changed compared with the sensitive parental strains (Davies et al. 1996; Verheul et al. 1997; Crandall and Montville 1998; Mantovani and Russell 2001). It is well accepted that the cell wall of Gram-positive bacteria is highly negatively charged, which is primarily due to lipoteichoic acids (LTA) and WTA. LTA and WTA can carry different substituents on the polyol group, one of which is D-alanine. Coupling of D-alanine to teichoic acid results in positive charges being incorporated

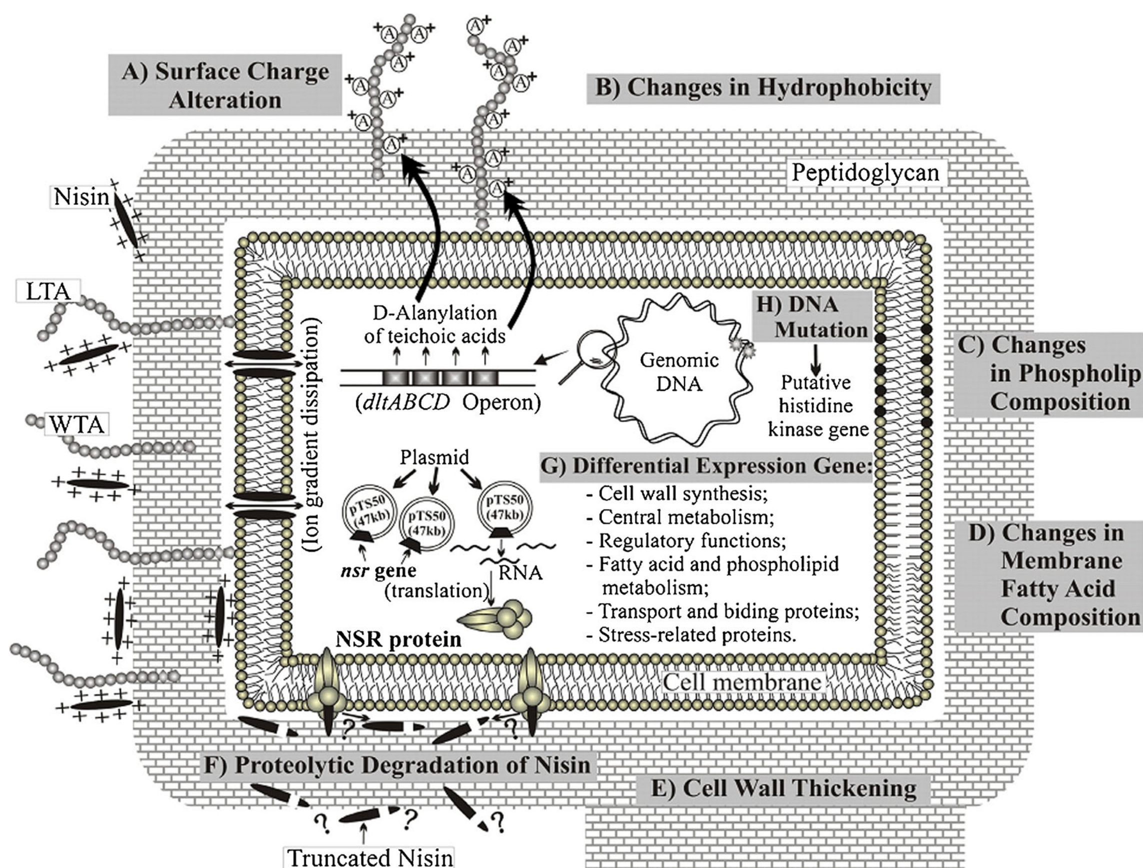

Fig. 1 Mechanisms of nisin resistance in bacteria. In some cases, more than one kind of alteration has been reported for the same bacterium. **a** net surface charge alteration; **b** changes in hydrophobicity; **c** change in phospholipid composition; **d** change in membrane fatty acid composition; **e** cell wall thickening; **f** proteolytic degradation of nisin; *question mark* indicates that the mechanism of nisin degradation *in vivo* by NSR is not known; **g** differential expression genes; **h** DNA mutation. *WTA* wall teichoic acid, *LTA* lipoteichoic acid. See text for details. The figure has been redrawed from Mantovani et al. (2011)

into the mostly negatively charged cell wall (Delcour et al. 1999; Peschel et al. 1999; Mantovani and Russell 2001; Neuhaus and Baddiley 2003), and the increased positive charges in the cell wall probably hamper nisin from reaching lipid II in the cytoplasmic membrane. In addition, the alteration of the amount of D-alanine in LTA has been shown to be a major cause of nisin resistance in non-producing *Lactococcus lactis* (Kramer et al. 2008). Incorporation of D-alanine into cell wall teichoic acids is mediated by proteins encoded by the *dltABCD* operon, and the *dlt* mutant of *Staphylococcus aureus* that was defective in D-alanine incorporation into LTA was more sensitive to nisin than wild-type cells, indicating that the positively charged D-alanine residues exclude the positively charged nisin molecules (Kramer et al. 2006; Peschel et al. 1999). The role of *dlt* operon in the resistance to nisin and other antimicrobial peptides was also investigated in *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Bacillus cereus* and *Clostridium difficile* (Kovács et al. 2006; Poyart et al. 2001; Abi Khattar et al. 2009; McBride and Sonenshein 2011), showing that the D-alanylation of teichoic acids provides protection against nisin and other antimicrobial peptides (Fig 1).

In the study conducted by Gravesen et al. (2001), the expression level of a putative penicillin binding protein (PBP) of a nisin-resistant strain of *Listeria monocytogenes* was significantly increased. PBPs are membrane-associated proteins that are involved in the second stage of peptidoglycan biosynthesis, following the formation of lipid II. PBP2A, a member of class B of PBPs, has been found to play unique roles in the septation and regulation of the morphology of bacterial cells (Höltje 1998). In the study conducted by Kramer et al. (2006), PBP2A expression was twice as high in the nisin-resistant strain relative to *Lactococcus lactis* IL1403. Thus, the expression of PBP may affect the composition of the bacterial cell wall, thereby altering the sensitivity of the bacteria to nisin by preventing nisin from reaching the lipid II molecules.

Modifications of membrane phospholipid composition

The bactericidal activity of nisin is due to pore formation in the cytoplasmic membrane (Demel et al. 1996), and the cell's

sensitivity to nisin is influenced by the membrane lipid composition (Mazzotta and Montville 1997).

Ming and Daeschel (1995) noted that the total phospholipids of nisin-resistant cells were significantly decreased and these resistant cells contained a greater proportion of straight-chain fatty acids whereas the parental cells contained more branched-chain fatty acids. Verheul et al. (1997) found that the nisin-resistant strain of *Listeria monocytogenes* produces relatively more phosphatidylglycerol (PG) and less diphosphatidylglycerol (DPG), and their findings demonstrated that the phospholipid head group alterations, particularly the content of DPG, were the basis of a nisin-resistant variant of *Listeria monocytogenes* Scott A. Previously, it has been reported that nisin penetrates more deeply into lipid monolayers of DPG than into other lipids including PG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG) (Demel et al. 1996). In the study conducted by Crandall and Montville (1998), nisin-resistant strains of *Listeria monocytogenes* ATCC 700302 exhibited decreased anionic phospholipid (cardiolipin and PG) and increased PE in the cell membrane, which resulted in a decreased net negative charge. There is no doubt that the anionic phospholipids in the cell membrane play an important role in nisin interaction with cell membranes (Driessen et al. 1995; Martin et al. 1996), and the decreased net negative charge could hinder the binding of cationic compounds such as nisin. In addition to the *dlt* operon, a *mprF*-dependent lysylination of PG also increases net cell surface charge and prevents cationic antimicrobial peptides (AMP) binding (Peschel et al. 2001; Thedieck et al. 2006).

In addition, the cell membranes of nisin-resistant cells exhibited increased long-chain fatty acids and reduced the ratios of C15/C17 fatty acids, suggesting that the reduced fluidity can contribute to a more rigid membrane (Ming and Daeschel 1993; Mazzotta and Montville 1997) (Fig. 1c, d).

In the *Lactococcus lactis* nisin-resistant strain, a lower expression of the *fab* operon, which is involved in the saturation and elongation of fatty acids, was observed, which might lead to a lower amount of saturated fatty acids and less elongated fatty acids in the membrane, making it less densely packed (Kramer et al. 2006). Unlike *Lactococcus lactis*, a previous report has shown that the cells of *Listeria monocytogenes* Scott A, when grown at 10 °C, had increased amounts of shorter, branched-chain fatty acids, increased fluidity of the cytoplasmic membrane, and were more sensitive to nisin than cells grown at 30 °C (Li et al. 2002).

Nisinase and nisin-resistance protein

Multiple studies demonstrated that *Streptococcus thermophilus*, *Lactobacillus plantarum*, and certain *Bacillus* species produce the enzyme nisinase to neutralize the antimicrobial activity of the nisin (Alifax and Chevalier 1962; Kooy 1952; Jarvis 1967; Jarvis and Farr 1971). The nisinase isolated from several *Bacillus* spp. was shown to be a dehydropeptide reductase since it specifically reduced the C-terminal dehydroalanyl-lysine of nisin to alanyl-lysine (Jarvis 1970; Hurst 1981).

Nisin resistance in the nisin nonproducer *Lactococcus lactis* subsp. *diacetylactis* DRC3 was reported to be conferred by a specific nisin-resistance gene (*nsr*), which is located onto a 60-kb plasmid and encodes a 35-kDa nisin resistance protein (NSR) (Froseth and McKay 1991). Thereafter, several *nsr* genes located on plasmids have been characterized (Liu et al. 1997; Tang et al. 2001). In the study conducted by Sun et al. (2009), it has been demonstrated that the purified NSRSD (NSR without the predicted N-terminal signal peptide sequence) could proteolytically inactivate nisin *in vitro* by cleaving the peptide bond between β-methyllanthionine^{28} and Ser^{29}, and the truncated nisin produced from the cleavage of nisin by NSR displays a markedly decreased affinity for the cell membrane and showed a 100-fold reduced inhibitory activity against *Lactococcus lactis* MG1363. However, the exact mechanism of nisin resistance by NSR *in vivo* is still poorly understood (Fig. 1f).

ABC transporters

One of the approaches most frequently employed by bacteria to survive exposure to antimicrobials is through the removal of such compounds from the cell envelope via ATP-binding cassette (ABC) transporters (van Veen et al. 1996; Lubelski et al. 2006; Velamakanni et al. 2008). The genes *ysaBC* in *Lactococcus lactis* IL-1403, which are orthologs of genes *mbrB* and *mbrA* for encoding ABC transporters, were expressed at a 10-fold higher level in nisin-resistant cells (Tsuda et al. 2002; Kramer et al. 2006). In the study conducted by Collins et al. (2010), the mutant of the permease component of an ABC transporter (*anrB*) exhibited increased sensitivity to lantibiotics and its expression was positively regulated by the VirRS two-component system. The roles of ABC transporters in nisin resistance have also been reported in *Bacillus subtilis*, *Streptococcus pneumoniae* and other bacteria, and the results indicated that ABC transporters are very important for the defense of bacterial pathogens against multiple antimicrobial compounds. These transporters can be used as targets for the development of new antimicrobials (Hansen et al. 2009; Majchrzykiewicz et al. 2010).

Two-component system

Two-component signal-transducing systems (TCS) consist of a histidine kinase (HK) that senses a specific

environmental stimulus, and a congnate response regulator (RR) that mediates the cellular response. A prototypical Gram-positive TCS that orchestrates cell envelope stress response is the LiaRS system. The LiaRS of *B. subtilis* is one of the several antibiotic-sensing systems that coordinate the genetic response to cell wall antibiotics and is functionally and genetically linked to a third protein, LiaF, which acts as a strong inhibitor of LiaR-dependent gene expression. The *lia* locus consists of six genes, *liaIH-liaGFSR*, which are induced by nisin, bacitracin, ramoplanin and vancomycin (Mascher et al. 2004). Moreover, the LiaRS-LiaF three-component systems are widespread amongst the *Firmicutes* bacteria (Jordan et al. 2006). In *Streptococcus mutans*, the *liaFSR* system was shown to upregulate gene products involved in cell wall PG matrix biosynthesis and membrane protein biogenesis. The expression levels of *liaR* remained elevated at high concentrations of nisin and vancomycin, but not all the *lia* mutants were sensitive to nisin (Suntharalingam et al. 2009). Previously, it has been reported that the *liaRS* homologs in both *Lactococcus lactis* and *Staphylococcus aureus* are transcriptionally induced by lipid II cycle inhibitors (Kuroda et al. 2003; Martínez et al. 2007). In the study conducted by McCormick et al. (2011), the LiaSR in *S. gordonii* regulates the expression of the *dlt* operon, which is responsible for D-alanylation of LTA, and the *liaS* and *liaR* mutants showed an increased in *dlt* expression over the parental strain.

The expression of ABC transporters involved in the resistance to lantibiotics are often regulated by a TCS. The regulatory relationship between TCS and ABC transporters has been demonstrated in *B. subtilis* (Joseph et al. 2002). The BceRS TCS specifically responds to the extracellular presence of bacitracin and its activation resulting in a strong up-regulation of *bceAB* (ABC transporter) expression, which is an efficient bacitracin resistance determinant (Mascher et al. 2003). The BceS HK belongs to the so-called 'intramembrane-sensing histidine kinase' (IM-HK) family, defined as conserved in low G+C% Gram-positive bacteria and characterized by a very short amino-terminal sensing domain, composed of two transmembrane helices separated by a small loop of only a few amino acids, which is thought to be buried in the cytoplasmic membrane (Mascher 2006). *Staphylococcus aureus* possesses 16 TCSs, two of which (BraSR and GraSR) belong to the IM-HK family. In the BraS/BraR system, two ABC transporters, named VraDE and BraDE, which play distinct and original roles in antibiotic resistance, were regulated by BraSR (named NsaRS or BceRS). The VraDE transporter acts specifically as a detoxification module and is sufficient to confer bacitracin and nisin resistance, and the BraDE is only involved in bacitracin sensing and signaling through BraSR (Hiron et al. 2011; Dintner et al. 2011). The expression of *BraSR* is upregulated by a variety of cell envelope-damaging antibiotics and during nisin-induced stress, and the microarray analysis reveals that the majority of BraSR-regulated genes are involved in transport, drug resistance, cell envelope synthesis and virulence (Blake et al. 2011; Kolar et al. 2011).

The main regulatory TCS controlling cationic antimicrobial peptide (CAMP) resistance in staphylococci is the GraSR, which has been extensively studied over the past 5 years, and GraSR was shown to be required for resistance of *S. aureus* and *Staphylococcus epidermidis* to several CAMPs, by controlling expression of *dlt*, *mprF* and transporter *vraFG* (Herbert et al. 2007; Falord et al. 2011). This TCS is also in fact a five-component system, requiring the accessory regulatory protein GraX as well as the VraFG ABC transporter in order to function. The further study on the role of GraSR have found that a 9-amino-acid extracellular loop of GraS with a high density of negative charges is responsible for AMP binding and induction (Li et al. 2007a, b; Sass and Bierbaum 2009; Falord et al. 2011). But the model of CAMP signaling and resistance through the GraSR pathway in *Staphylococcus aureus* proposed by Falord et al. (2012) showed that CAMPs would first be sensed by the VraFG ABC transporter, and the stimulus is sensed either by the VraFG ABC transporter and then transferred to GraS, which in turn activates GraR, or through CAMP interaction with both VraFG and the extracellular loop of GraS. However, the mechanism of how the VraFG ABC transporter senses CAMPs is still not fully understood.

An additional TCS LisRK in *Listeria monocytogenes* plays a significant role in stress responses and nisin resistance. The *lisK* mutant (lacking the LisK histidine-kinase sensor component) displays significantly enhanced resistance to nisin and reduced expression of *pbp2229*, which encodes a putative penicillin-binding protein 1 and may mediate enhanced nisin resistance by shielding lipid II and possibly also by reducing the extracellular lipid II concentration (Cotter et al. 2002; Gravesen et al. 2004).

Besides the TCSs, Palmer et al. (2009) noted that the sigma (B) and sigma (L) both affect *Listeria monocytogenes* sensitivity to nisin, and the *sigB* null mutant is more resistant to nisin than the parental strain. Therefore, a complex regulatory network contributes to nisin resistance in bacteria and regulates the expression of genes involved in the cell wall biosynthesis, energy metabolism, fatty acid and phospholipid metabolism, regulatory functions, and stress-related proteins (Kramer et al. 2006) (Fig. 1g).

Conclusion

In the past few years, a large number of bacteriocins from LAB have been characterized. Due to their broad spectra of inhibition, the use of bacteriocins in the food industry can

help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods that are more naturally preserved. Until now, only nisin and pediocin PA-1 have been sufficiently well characterized to be used in the food indunstry as biopreservatives.

Field et al. (2012) reported for the first time that altering residue 29 of nisin A can result in the generation of variants with enhanced antimicrobial activity against both Gram-postive and Gram-negative bacteria, but the mechanistic basis for the enhanced activity of derivatives relative to nisin A is unclear. The further study on the structural variants of nisin will provide more information on its structure, properties, and function, and more nisin mutants with enhanced efficacy against pathogenic or food-spoiling bacteria can be obtained by site-directed mutagenesis and chemical modification.

The effectiveness of nisin is often controlled by environmental factors, such as pH, temperature, food composition, and structure, as well as the food microbiota. The development of highly tolerant or resistant pathogenic microorganisms, which decrease the efficiency of nisin as a biopreservative, remains the main concern of its application.

As shown in this review, the mechanisms controlling the acquisition of nisin resistance are complicated and may differ among strains. Most studies have indicated that some patterns of nisin resistance also participate in the resistance to other antimicrobials or antibiotics. Therefore, an undesirable consequence of an extended use of nisin in food might be cross-resistance to other antimicrobials and clinically used antibiotics to control foodborne pathogens such as *Listeria monocytogenes*. The consumption of food contaminated by nisin-resistant variants of pathogens increases the risk of contracting diseases. It is likely that the clear correlation between resistance to nisin and to antibiotics worldwide used to treat pathogenic bacteria will be established by further studies.

In view of the specific degradation of nisin, NSR have received more attention in the last few years, and several *nsr* genes located on plasmids have been characterized. However, the molecular mechanism of proteolytic degradation *in vivo* and whether the proteolytic mechanism could be applied to the occurrence of other structure-related lantibiotic resistance mechanisms remains to be elucidated. On the other hand, compared to the transfer of antibiotic resistance marker genes between LAB and other bacteria, there are very few studies that have investigated whether the NSR-encoding plasmids transfer occurs among bacteria. Further studies will be required to evaluate the potential of transfer of the *nsr* gene from LAB to other bacteria, such as the pathogenic bacteria.

In recent years, several research groups have successfully used mutagenesis to achieve more evidence of the mechanism of nisin resistance in certain strains; however, the comprehensive mechanism involved is not yet fully explained. In future studies, multiple modern molecular biology techniques, such as the microarray technology, which offers a new opportunity to gain insight into global gene expression profiles in nisin-resistant variants, will improve our understanding of the strategies that bacterial cells employ. Further study on nisin resistance will not only lead to our increased understanding of the characteristics of nisin but will also help us to improve the optimal conditions for the application of nisin in foods and to minimize the emergence of nisin resistance.

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