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The transcriptional response of virulence genes in *Listeria monocytogenes* during inactivation by nisin

Hui Shi [a], Quoclinh Trinh [a], Wentao Xu [a,b,*], Yunbo Luo [a,b], Wenying Tian [b], Kunlun Huang [a,b,*]

[a] Laboratory of Food Safety, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Supervision & Testing Center of Agricultural Products Quality, Ministry of Agriculture, Beijing 100083, China

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ABSTRACT

The influence of preservatives on the expression of the virulence genes in *Listeria monocytogenes* (*L. monocytogenes*) is significant in assessing their functions and effectiveness against pathogens. In this study, the transcriptional response of the virulence genes (*inlA*, *prfA*, *plcA*, and *hlyA*) in *L. monocytogenes* during inactivation by nisin was investigated at 37 °C and 4 °C. The expression of these virulence genes decreased rapidly and remained at a constant level when nisin was added both at 37 °C and 4 °C. The decrease in the expression of the virulence genes caused by nisin at 37 °C was higher than that at 4 °C; thus, the ability of nisin to inhibit virulence genes expression at 37 °C is more effective than that at 4 °C. Regarding the effect of the nisin concentration on transcriptional inhibition, 500 IU/mL nisin caused significantly lower expression of the virulence genes than 100 IU/mL nisin at both temperatures. With the cell count increasing after the early bactericidal activity of nisin, the expression of the virulence genes also began to increase. Interestingly, the expression of these virulence genes all responded uniformly to nisin of different concentrations at different temperatures. A comprehensive understanding of the transcriptional response of the virulence genes in *L. monocytogenes* during inactivation by nisin could contribute to the effective application of nisin in the food industry.

1. Introduction

Listeria monocytogenes (*L. monocytogenes*) is a common food-borne pathogen, and its virulence is of great concern, especially because of its ability to grow under refrigeration (Gandhi & Chikindas, 2007). *L. monocytogenes* can cause serious diseases, such as septicemia, meningitis and meningoencephalitis in immunocompromised individuals, newborns and the elderly, and miscarriage and stillbirth in pregnant women (Vazquez-Boland et al., 2001).

L. monocytogenes can produce a variety of toxins that contribute to its ability to colonize and cause diseases. Most of the chromosomal genes encoding these virulence products are found on the 9-kb pathogenic island known as *Listeria* pathogenicity island 1 (LIPI-1), but some genes are also found in other places on the chromosome (Kastbjerg, Larsen, Gram, & Ingmer, 2010). Among these genes, *prfA* encodes the 27-kDa polypeptide PrfA that is the major transcriptional factor regulating the expression of *prfA* and other virulence genes. Internalin, encoded by *inl*, is an essential factor for *L. monocytogenes* to adhere to and invade the host cell. *HlyA* encodes the 60-kDa hemolysin protein, which allows *L. monocytogenes* to escape from vacuoles by dissolving phagosome membranes. The phosphatidylinositol-specific phospholipase C protein encoded by *plcA* has a function similar to the hemolysin protein, and these two products can function cooperatively. The expression of these virulence genes can be induced or repressed by several environmental factors and, consequently, affect the virulence of *L. monocytogenes* (Böckmann, Dickneite, Middendorf, Goebel, & Sokolovic, 1996; Ermolaeva et al., 2004; Kastbjerg et al., 2010; Milenbachs, Brown, Moors, & Youngman, 1997).

To control *L. monocytogenes* in the food industry, natural antimicrobials have been widely used. The use of these antimicrobials can reduce the addition of chemical preservatives and offers an alternative to satisfy the increasing consumer demand for safe, fresh tasting, and minimally processed foods (Arqués, Rodríguez, Nuñez, & Medina, 2011). Nisin, an antimicrobial peptide derived from lactic acid bacteria, is authorized as a food preservative in over 50 countries worldwide. Nisin has a broad inhibitory spectrum against Gram-positive bacteria and has been applied in a variety of

* Corresponding authors. Laboratory of Food Safety, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China. Tel.: +86 10 6273 6479; fax: +86 10 6273 7786.
E-mail addresses: cauxwt@yahoo.cn, xuwentaoboy@sina.com (W. Xu), huangkl009@sina.com (K. Huang).

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foods to control *L. mono*cytogenes (O'Sullivan, Ross, & Hill, 2002; Rodríguez, Arqués, Gaya, Nuñez, & Medina, 2001). The target for nisin action against vegetative cells is the cytoplasmic membrane. A major breakthrough on the mode of action of nisin against vegetative cells was the discovery that the cell wall peptidoglycan precursor lipid II acts as a docking molecule for nisin, and it is the nisin-lipid II complex that inserts itself into the cytoplasmic membrane forming transient pores that cause leakage of essential cellular material (Breukink et al., 1999; Wiedemann et al., 2001). A further mode of action of nisin is that it also inhibits peptidoglycan synthesis, a component of bacteria cell walls (Hasper et al., 2006).

In assessing the functions and effectiveness of preservatives against *L. monocytogenes*, their ability to inhibit cell growth had been investigated (Arqués et al., 2011; Silva Malheiros, Daroit, Silveira, & Brandelli, 2010; Tokarskyy & Marshall, 2008). Recently, research has focused on how preservatives affect the expression of the virulence genes in *L. monocytogenes* in order to gain a comprehensive and precise understanding of preservatives, such as common chemical preservatives and organic acids (Kastbjerg et al., 2010; Stasiewicz, Wiedmann, & Bergholz, 2011). In this study, the transcriptional response of the virulence genes in *L. monocytogenes* (*inlA*, *prfA*, *plcA*, and *hlyA*) during inactivation by nisin was investigated at 37 °C and 4 °C. A comprehensive understanding of the transcriptional response of the virulence genes caused by nisin may contribute to the effective application of nisin in the food industry.

2. Materials and methods

2.1. Bacterial strains, media, and cultivation

The *L. monocytogenes* strain CMCC 55004, which was obtained from Institute of Veterinary Drug Control, China, was used in this study. Cultures were started from freezer stocks and grown on trypticase soy agar (TSA) medium (trypticase soy broth (YSB) plus 18 g/L agar) with an overnight incubation at 37 °C. Then, a single colony was selected and inoculated into 20 mL of YSB for 12 h (37 °C, 200 rpm), when the optical density at 600 nm (OD_{600}) was approximately 1.0.

2.2. Design of PCR primers

Specific primers used for quantitative PCR (qPCR) (Table 1) of the virulence genes (*inlA*, *prfA*, *plcA*, and *hlyA*) and internal control genes (*rpoB* and *gap*) were designed using Primer Blast from the National Center for Biotechnology Information. The complementation and hairpin structure of primers were controlled and especially avoided at the 3′ end. Moreover, a BLAST analysis and PCR amplification were performed to check the primer specificity. The primer efficiency (E) was controlled using sample standard curves by qPCR, generated by 10-fold serial dilutions and calculated using the formula $E = 10^{-1/\text{slope}} - 1$.

2.3. Nisin treatment

The *L. monocytogenes* cultures described above were incubated at room temperature (RT) for 30 min and then used to inoculate 20 mL of YSB at a final inoculum of approximately 8×10^7 cfu/mL. This point was set as the initial time (0 h), and the expression level of the virulence genes was used as the calibrator. Nisin (Sigma, USA) was resuspended in 0.02 M HCl to 10,000 IU/mL and added immediately after inoculation at a final activity of 100 IU/mL or 500 IU/mL. The sample with 100 IU/mL nisin, the sample with 500 IU/mL nisin, and the control sample without nisin were incubated at 37 °C or 4 °C. For samples incubated at 37 °C, the cell counts (determined on TSA plates) and the transcriptional level of the virulence genes (determined by reverse transcription and qPCR) were investigated at1 h, 2 h, 4 h, 8 h, and 12 h, until the cells resumed growth. For samples incubated at 4 °C, the cell counts and the transcriptional level of the virulence genes were investigated at 1 h, 2 h, 4 h, 8 h, 12 h, 2 d, and 4 d, until the cells resumed growth.

2.4. RNA extraction

RNA was extracted from *L. monocytogenes* using an RNAprep pure Cell/Bacteria Kit (Tiangen Inc.), following the manufacturer's instructions. The total RNA was then resuspended in 50 μl of diethyl pyrocarbonate (DEPC)-treated water. The RNA purity (represented by the A_{260}/A_{230} and A_{260}/A_{280} ratios) and integrity (RNA integrity number) were assessed according to a previous study (Stasiewicz et al., 2011) and used in the next step.

2.5. Reverse transcription and qPCR (RT-qPCR)

cDNA was reverse transcribed from 5 μl of total RNA using a transcript first-strand cDNA synthesis supermix (Transgen Biotech, Beijing, China) according to the manufacturer's instructions. qPCR and data analysis were performed on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). All qPCR reactions were performed in a total volume of 20 μL containing 12 μL of Sybrgreen Mix (Takara Bio, Inc., Kyoto, Japan), 1.0 μL of 0.5 μM forward primer, 1.0 μL of 0.5 μM reverse primer, 2 μL of cDNA and 14 μL of deionized water. Cycling parameters for qPCR included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s and primer extension at 72 °C for 30 s. The fluorescent products were detected after the extension step of each cycle. RT-qPCR data were log10 transformed and normalized to the geometric means for the housekeeping genes *rpoB* and *gap* as previously described

Table 1
The sequence of oligonucleotide primers.

Locus	Primer name	Sequence	Product size (bp)	Efficiency	r^2	Reference
rpoB	rpoB-F	TCGTCGTCTTCGTTCTGTTG	221	100	0.999	This study
	rpoB-R	GTTCGCCAAGTGGATTTGTT				
gap	gap-F	GAACTGGAACACGTTGAGCA	140	92	0.998	
	gap-R	TCCAAAAGGTGACTTCCGTC				
inlA	inlA-F	ATAGGCACATTGGCGAGTTT	160	100	0.999	
	inlA-R	GTGCGGTTAAACCTGCTAGG				
prfA	prfA-F	TGAACGCTCAAGCAGAAGAA	287	93	0.999	
	prfA-R	AACGTATGCGGTAGCCTGTT				
plcA	plcA-F	CAAAATTTACCATGGGCCAA	245	96	0.999	
	plcA-R	TTCCGCGGACATCTTTTAAT				
hlyA	hlyA-F	CTTTTAACCGGGAAACACCA	302	95	0.999	
	hlyA-R	TCTTGCGTTACCTGGCAAAT				