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Transcriptomic study on persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin

Shuyan Wu[a], Pak-Lam Yu[b], Dave Wheeler[c], Steve Flint[a,*]

[a] Massey Institute of Food Science and Technology, Massey University, Riddet Road, 4442 Palmerston North, New Zealand
[b] School of Engineering & Advanced Technology, Massey University, Riddet Road, 4442 Palmerston North, New Zealand
[c] Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

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ABSTRACT

Objectives: The aim of this study was to determine gene expression associated with the persistence of a *Listeria monocytogenes* stationary-phase population when facing lethal nisin treatment.
Methods: RNA-Seq analysis was used for gene expression profiling of persister cells in nutrient-rich medium (persister TN) compared with untreated cells (non-persister). The results were confirmed using reverse transcription quantitative PCR (RT-qPCR).
Results: Functional genes associated with the persister population were identified in multiple systems, such as heat-shock-related stress response, cell wall synthesis, ATP-binding cassette (ABC) transport system, phosphotransferase system (PTS) and SOS/DNA repair.
Conclusions: This study pointed to genetic regulation of persister cells exposed to lethal nisin concentrations and provides some insight into possible mechanisms of impeding bacterial persistence.

1. Introduction

Persistence of microbial contamination in a food manufacturing environment can result in long-term survival of a proportion of a pathogen population despite repeated treatments with antimicrobials [1]. Following sanitation and disinfection, different natural antimicrobials used as preservatives in food processing and final products can control the growth bacteria but cannot eradicate and kill persister cells. How *Listeria monocytogenes* persists and survives in food environments is poorly understood and it is important given the low infective dose and high mortality rate associated with this pathogenic bacterium.

Information on the mechanism of *L. monocytogenes* persistence is limited. Studies investigating persistent *L. monocytogenes* have focused on sampling *L. monocytogenes* isolates from food environments or food products over a long period of time. Transcriptomic analysis of *L. monocytogenes* isolates from food environments demonstrated that gene regulation associated with carbohydrate metabolism and flagella-mediated motility occurred in cells adapted to stress [2]. Persisters are defined as a subpopulation of bacteria that can survive under prolonged exposure to antimicrobials, and in an antimicrobial-free environment persisters can switch back to a growing state and generate a new population that is as susceptible to antimicrobial treatment as the parental strain [3]. However, persistence studies of *L. monocytogenes* using populations that have re-grown from the persistent *L. monocytogenes* population are not a true reflection of the physiological state of persisters. Transcriptomic analyses of *L. monocytogenes* are mainly focused on tolerance to sublethal concentrations of treatments with disinfectants and preservatives, and these are not a true reflection of a persister population following exposure to what are accepted to be lethal concentrations of antimicrobials. Nisin is an antimicrobial peptide used commercially as a natural preservative in food. This study focused on the lethal antimicrobial impact of nisin on persister formation of *L. monocytogenes*.

Persister cells represent a subpopulation of cells that are 'temporarily' resistant to antimicrobials. The persister phenotype is believed to be due to distinct changes in gene expression following antimicrobial challenge, hence differing from resistant cells [4]. Our previous study [5] collected persister cells from *L. monocytogenes* stationary cells exposed to lethal concentrations of nisin (75 μg/mL).

Studies that have reported on the possible mechanisms involved in persister adaption [6] use low concentrations of antimicrobial agents, however we are not aware of any transcriptional-level studies that have attempted to explore gene expression associated with *L. monocytogenes* persisters under what are

* Corresponding author.
E-mail address: S.H.Flint@massey.ac.nz (S. Flint).

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considered lethal concentrations of nisin. The aim of this study was to assess *L. monocytogenes* persisters following exposure to lethal concentrations of nisin using transcriptome sequencing and subsequent RNA-Seq analysis.

2. Methods

2.1. Bacterial strains and growth conditions

The *L. monocytogenes* strain used in this study was isolated from a food manufacturing environment by AsureQuality Limited, New Zealand (labelled as strain A1 in our earlier publication) [5]. It has been identified as serotype O1/2 and deposited as NZRM 4734 by the Environmental and Scientific Research Ltd. (ESR) Special Bacteriology and Culture Collection Laboratory (Porirua, New Zealand).

A planktonic culture of *L. monocytogenes* NZRM 4734 was obtained from a single colony inoculated into 20 mL of BD™ tryptic soy broth (TSB) (Becton Dickinson, USA) and was incubated at 30 °C for 18 h. Then, 5 mL of the overnight culture was centrifuged, was re-suspended into fresh TSB medium and was treated with 75 μg/mL nisin (Nisaplin®; DuPont, Macquarie Park, NSW, Australia) at 30 °C for 90 min. Surviving cells from the treated re-suspended culture were labelled as 'persister TN'. Culture that had not been exposed to nisin treatment provided control cells to compare with the persister population [5].

2.2. Total RNA extraction

RNA was extracted from the persister TN cells and from the untreated control cells in three independent preparations (triplicates) using the following procedure. The culture (4 mL) was centrifuged for 10 min at 4000 × *g* and the resultant pellet was re-suspended in 800 μL of RNase-free water containing 5 mg/mL lysozyme (Sigma-Aldrich) for 10 min of incubation at 37 °C. The re-suspension was mixed by vortexing with acid-washed glass beads (Sigma-Aldrich) for 10 min and was then centrifuged for 1 min at 5000 × *g* to obtain the supernatant. Total RNA was isolated and purified from the supernatant using a NucleoSpin® RNA II Kit (Macherey-Nagel, Germany). RNA purity was assessed by NanoDrop (Titertek-Berthold, Germany), whilst RNA integrity was checked by electrophoresis (Invitrogen, USA).

2.3. Transcriptome sequencing

With three biological replicates for each group, six RNA samples in total were sent for sequencing on the Illumina sequencing platform. The Otago Genomics & Bioinformatics Facility at the University of Otago (Dunedin, New Zealand) prepared the cDNA libraries for the treatment and control groups and performed the sequencing of cDNA libraries with the Illumina MiSeq system (Illumina Inc.). Output data were sequencing adapter-cleaned and base quality-trimmed at an error probability of <0.01 for subsequent analysis. Mapping of reads to the reference genome *L. monocytogenes* EGD-e [7] was performed with Bowtie2 v.2.3.0. Gene-based read counts were generated for each treatment and control group with HTSeq-count using 'union' model.

2.4. Differential expression analyses

DESeq2 v.1.8.2 was used to identify differential expression between persister and control cells as described in the package's vignette. The false discovery rate (FDR) method was applied to reduce the effect of multiple testing. Significant differential expression was based on a $\log_2$-fold change of >1.5 and an FDR-adjusted *P*-value of <0.05.

2.5. Reverse transcription quantitative PCR (RT-qPCR) validation

Ten differentially expressed genes were selected for RT-qPCR analysis to verify gene expression of RNA-Seq data. Total RNA was isolated by the same method mentioned earlier and was tested using one-step RT-qPCR (Luna® Universal One-Step RT-qPCR Kit; New England Biolabs Inc.). Quantitation of each transcript was performed using a Light Cycler 480 platform (Roche Diagnostics) in triplicate. The 16S rRNA gene of *L. monocytogenes* was used as the housekeeping gene. The primers used are listed in Table 1. The specificity of primers was assessed using the dissociation curve method, and relative gene expression was calculated by the comparative $-\Delta\Delta C_T$ method.

3. Results

3.1. RNA-Seq analysis

On average, 2.08 million high-quality reads were obtained for each library. An average of 91.05% of the sequenced reads successfully aligned to the reference genome of *L. monocytogenes* EGD-e (serotype 1/2a; GenBank accession no. **NC_003210**). The closely related *L. monocytogenes* EGD-e genome was used as a reference since the genome of the tested food isolate has not been sequenced. This was justified as this investigation was looking for relative changes in gene expression between the persister TN and control populations [11]. RNA-Seq analysis identified a total of 356 significantly differential expressed genes above a $\log_2$-fold change cut-off of 1.5 in persister TN compared with control cells (Fig. 1), of which 182 had known functions with 85 genes upregulated and 97 genes downregulated. Table 2 summarises 55 differentially expressed genes in persister TN compared with untreated cells. There were complex gene expression differences in persister TN, and functional groups of genes were identified with involvement in multiple systems such as heat-shock-related stress response and cell wall synthesis (Table 2). A large number of single nucleotide polymorphisms (SNPs) in the reads were detected in the study strain versus the reference, which may indicate that the food isolate used in this study is somewhat divergent from the reference strain in the analysis. There is a limitation that some mechanisms specific to the food isolate may not have been examined by the transcriptomic study because the reference strain chosen may have some genetic differences from the test strain. Using the food isolate's own fully sequenced genome as a reference would produce a more comprehensive image of how this *L. monocytogenes* food environmental isolate survived under lethal nisin exposure. However, this study provides some insight into a number of pathways possibly influenced in the persister population produced following exposure to a lethal dose of nisin. A selection of the pathways identified in the persister populations is scrutinised in the discussion.

3.2. Reverse transcription quantitative PCR (RT-qPCR) validation

As shown in Fig. 2, RT-qPCR data correlated well with the RNA-Seq data ($R^2 = 0.8715$).

4. Discussion

4.1. Stress response

Downregulation of the *clp* gene (*lmo1138*) was detected, indicating the low transcription level of protein metabolism within persisters of *L. monocytogenes*. Stress proteins Usp, Fri and Csp are important for bacteria to survive under different stress conditions. Interestingly, the persister TN showed downregulation