International Journal of Food Microbiology 283 (2018) 14–21

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Gene profiling-based phenotyping for identification of cellular parameters that contribute to fitness, stress-tolerance and virulence of *Listeria monocytogenes* variants

Jeroen Koomen[a], Heidy M.W. den Besten[a], Karin I. Metselaar[a,1], Marcel H. Tempelaars[a], Lucas M. Wijnands[b], Marcel H. Zwietering[a], Tjakko Abee[a,*]

[a] Food Microbiology, Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[b] National Institute of Public Health and the Environment P.O. Box 1, 3720 BA Bilthoven, The Netherlands

ARTICLE INFO

Keywords:
SigB
rpsU
Listeria monocytogenes
Stress resistant variants

ABSTRACT

Microbial population heterogeneity allows for a differential microbial response to environmental stresses and can lead to the selection of stress resistant variants. In this study, we have used two different stress resistant variants of *Listeria monocytogenes* LO28 with mutations in the *rpsU* gene encoding ribosomal protein S21, to elucidate features that can contribute to fitness, stress-tolerance and host interaction using a comparative gene profiling and phenotyping approach. Transcriptome analysis showed that 116 genes were upregulated and 114 genes were downregulated in both *rpsU* variants. Upregulated genes included a major contribution of SigB-controlled genes such as intracellular acid resistance-associated glutamate decarboxylase (GAD) (*gad*3), genes involved in compatible solute uptake (*opuC*), glycerol metabolism (*glpF*, *glpK*, *glpD*), and virulence (*inlA*, *inlB*). Downregulated genes in the two variants involved mainly genes involved in flagella synthesis and motility. Phenotyping results of the two *rpsU* variants matched the gene profiling data including enhanced freezing resistance conceivably linked to compatible solute accumulation, higher glycerol utilisation rates, and better adhesion to Caco 2 cells presumably linked to higher expression of internalins. Also, bright field and electron microscopy analysis confirmed reduced flagellation of the variants. The activation of SigB-mediated stress defence offers an explanation for the multiple-stress resistant phenotype in *rpsU* variants.

1. Introduction

Listeria monocytogenes is a ubiquitous Gram positive foodborne pathogen that can cause the rare but severe disease listeriosis (Toledo-Arana et al., 2009). Due to its ubiquitous nature, *L. monocytogenes* needs to be able to adapt to environmental stresses in its transition from the environment to the human gastro-intestinal tract. Population heterogeneity is an inherent feature of microorganisms and heterogeneity in stress response between individual cells of a population can result in survival of a small fraction of the population when subjected to (food-relevant) lethal stresses such as heat or low pH. This type of non-uniform killing leads to non-linear inactivation kinetics and tailing of the inactivation curve (Avery, 2006). Tailing leads to higher than expected number of cells surviving an inactivation treatment, which can be problematic for the accurate modelling of inactivation procedures. Moreover, non-homogeneous killing can lead to the selection of stress resistant variants from a population. The fraction of stress resistant cells in a population has been shown to be comprised of both cells that show a transient phenotypic resistance, and cells that show a stable genotypic resistance (Metselaar et al., 2013; Van Boeijen et al., 2011; Van Boeijen et al., 2008). Indeed, from the tail of the inactivation curve, stable stress resistant variants have been isolated for *L. monocytogenes* EGDe, LO28, and ScottA when exposed to either heat, low pH or high hydrostatic pressure (HHP) (Karatzas and Bennik, 2002; Metselaar et al., 2013; Metselaar et al., 2015; Van Boeijen et al., 2011; Van Boeijen et al., 2008). However, the specific mechanism of resistance in these stable stress resistant variants is still poorly understood. For variants selected by HHP treatment, a mutation in the class III heat shock repressor *ctsR* was shown to be responsible for the increased stress resistance in some of the variants (Van Boeijen et al., 2010). Interestingly, these HHP selected variants showed cross resistance to other stresses including heat and acid stress. In 2013, Metselaar et al. could isolate 23 stable stress resistant variants upon acid treatment. Although phenotypic characteristics such as heat and acid resistance and impaired growth rate

* Corresponding author.
E-mail address: tjakko.abee@wur.nl (T. Abee).
[1] Present address, Corbion, P.O. Box 21, 4200 AA Gorinchem, The Netherlands

https://doi.org/10.1016/j.ijfoodmicro.2018.06.003
Received 27 February 2018; Received in revised form 31 May 2018; Accepted 6 June 2018
Available online 07 June 2018

were observed in both the HHP selected and the acid stress selected variants, a whole genome sequencing and Structural Variation (SV) analysis on the acid stress selected variants of *L. monocytogenes* LO28 revealed no mutations in the *ctsR* region in any of the 23 variants. The SV analysis revealed that 11 of the 23 acid stress selected variants that shared similar phenotypes all had a mutation in the *rpsU* gene locus. Our current study focuses on two of these *rpsU* variants, namely, variant 14, which has a deletion encompassing *rpsU*, *yqeY and* half of *phoH*, and variant 15 that carries a single point mutation resulting in an amino acid substitution, changing an arginine into a proline. In previous work (Metselaar et al., 2015) RT-PCR analysis revealed significantly lower expression of the *rpsU* gene in variant 15, and as expected, no transcript in variant 14. For these variants, protection from lethal acid stress seems to be correlated (Metselaar et al., 2015) with increased activity of the glutamate decarboxylase (GAD) system (Cotter et al., 2001; Feehily and Karatzas, 2013; Karatzas et al., 2012), but complementary mechanisms contributing to the observed multiple stress-resistant phenotype of the variants are unknown. Therefore, in the current study we investigated the differential transcriptomic and phenotypic responses of *L. monocytogenes* LO28 variants 14 and 15 in comparison to the wild type to further characterize the variants and to elucidate features that can contribute to fitness, stress-tolerance, and virulence.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Listeria monocytogenes LO28 wild type (WT) strain (Wageningen Food & Biobased Research, The Netherlands) and stress resistant variants 14 and 15 (Metselaar et al., 2013) were used in this study. All bacterial cultures were cultured as described elsewhere (Metselaar et al., 2013). Briefly, cells from −80 °C stock were grown at 30 °C for 48 h on brain heart infusion (BHI, Oxoid, Hampshire) agar (1.5% [w/w], bacteriological agar no. 1 Oxoid, Hampshire) plates. A single colony was then used to inoculate 20 ml of BHI broth in a 100 ml Erlenmeyer flask (Fisher, USA). After overnight (ON) culturing at 30 °C under shaking at 160 rpm, (Innova 42; New Brunswick Scientific, Edison, NJ) 0.5% (v/v) inoculum was added to fresh BHI broth. Cells were grown under shaking at 160 rpm in BHI at 30 °C until the late-exponential growth phase (OD_{600} = 0.4–0.5).

2.2. RNA isolation, cDNA synthesis and labelling

RNA was isolated from late-exponentially growing cultures of the WT and variants 14 and 15. Cultures (20 ml) were centrifuged in 50 ml Falcon tubes for 1 min at room temperature (11.000 × *g*). Immediately after centrifugation the pellet was resuspended in 1 ml TRI reagent (Ambion) by vortexing, snap frozen in liquid nitrogen and stored at −80 °C until use. RNA was extracted according to the RNAwiz (Ambion) protocol. Residual DNA was enzymatically removed using the TURBO DNA-free kit (Ambion) according to manufacturer's instructions. The quality of the extracted RNA was checked by using the Bioanalyzer (Agilent) with the Agilent RNA 6000 Nano kit, according to manufacturer's instructions. RIN scores were between 8.5 and 10. Complementary DNA (cDNA) with amino-allyl-labelled dUTP (Ambion) was synthesized from RNA by using Superscript III reverse transcriptase (Invitrogen). Labelling and hybridization were performed as described elsewhere (Mols et al., 2013).

2.3. Microarray design and data analysis

A custom-made array design for *L. monocytogenes* LO28 was based on the 8 × 15 K platform of Agilent Technologies (GEO accession number: GSE114672, on the GPL25009 platform) and the genome sequence of *L. monocytogenes* EGDe (NCBI accession number NC_003210.1). Two biological replicates of variant 14, and three biological replicates of variant 15 were used. Microarrays were scanned with an Agilent G2505C scanner. Image analysis and processing were performed with the Agilent Feature Extraction software (version 10.7.3.1). Transcriptome profiles were normalized using LOWESS normalization (Yang et al., 2002) as implemented in MicroPreP (van Hijum et al., 2003). The data were corrected for inter-slide differences based on total signal intensity per slide using Postprep (Yang et al., 2002) and median intensity of the different probes per gene was selected as the gene expression intensity. CyberT software was used to compare the different transcriptomes (Baldi and Long, 2001) resulting in gene expression ratios and false discovery rates (FDR) for each gene. The gene was considered significantly differentially expressed when FDR-adjusted *P*-value was < 0.05 and expression fold change was higher than 3 ($\log_2$ ratio > 1.58 for upregulation, and < −1.58 for downregulation) (Hayrapetyan et al., 2015). FunRich version 2.1.2 (Pathan et al., 2015) was used for functional enrichment analysis.

2.4. Freeze-thaw resistance

100 µl of late exponential phase cultures of the WT strain and variants 14 and 15 were each transferred into 10 ml of fresh BHI and BHI supplemented with 100 µg/ml chloramphenicol as an inhibitor of protein synthesis. For each culture, 1.5 ml of inoculated BHI was transferred into a 2.0 ml Eppendorf tube, after which the Eppendorf tubes were collected in a water bath floater and placed in a tray containing a coolant mixture of 50% (v/v) glycerol (Fluca, Buchs) and deionized water pre-cooled to −20 °C to ensure an even rate of freezing of the three cultures. After freezing for 2 h, all samples were thawed for 15 min in a water bath (Julabo JW II, Germany) set to 25 °C. Appropriate dilutions of the first sample were prepared in Peptone Physiological Salt (PPS) solution, 0.1% w/v peptone, and 0.9% w/v NaCl (Tritium Microbiologie, The Netherlands) and spiral plated on BHI agar plates (Eddy Jet, IUL Instruments) in duplicate. Samples for the second and third round of freezing and thawing were frozen again, after which the samples of the second round were thawed and plated. This process was repeated for the third round. Plates were counted after 3–4 days to allow recovery of the cells. Experiments were done with independent biological triplicates.

2.5. Glycerol consumption

100 ml cultures of the WT strain and variants 14 and 15 were grown in BHI medium in 500 ml Erlenmeyer flasks. Late-exponential phase cells were harvested by centrifuging 2 × 50 ml of cell suspension for 5 min at 2880 × *g*. Pellets were resuspended in phosphate buffered saline, pH 7.4 (PBS, KH_2PO_4 1.06 mM; NaCL 155.17 mM; Na_2HPO_4-$7H_2O$ 2.97 mM) (Gibco, Life Technologies, Scotland), and centrifuged again for 5 min at 2880 × *g* to remove all traces of BHI medium. The pellet was resuspended in 20 ml of nutrient broth (NB) (Oxoid, Hampshire) supplemented with 25 mM glycerol, 100 µg of chloramphenicol as an inhibitor of protein synthesis per ml and incubated in a 100 ml Erlenmeyer flask (Fisher, USA) at 30 °C. A 1 ml sample was taken directly after resuspension in NB as time point zero, followed by sampling after 60, 120 and 180 min of incubation. Samples were centrifuged for 5 min at 17.000 × *g* to remove cells. The supernatant was filter sterilized using a 0.2 µm syringe filter (Minisart NML, Sartorius Stedim Biotech GmbH, Germany). 0.5 ml of supernatant was deproteinized by the Carrez AB method. Briefly, 0.25 ml of cold Carrez A (42.20 g/l $K_4FE(CN)_6.3H_2O$) was added to 0.5 ml of sample. After thorough mixing with a MS 2 minishaker (IKA, Staufen, Germany) 0.25 ml of Carrez B (57.50 g/l $ZnSO_4.7H_2O$) was added, and the sample was centrifuged at 17.000 × *g* for 5 min. 10 µl of supernatant was analysed using an Ultimate 3000 HPLC (Dionex, USA) equipped with a 300 × 7.8 mm Aminex HPX 87-H ion exclusion column (Biorad, USA), kept at 40 °C with 0.05 M H_2SO_4 as eluent at a flow of 0.6 ml/min. Glycerol was detected by a Shodex R-101 refractive index detector