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

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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

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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

(Shodex, USA). A standard curve was constructed by serial dilutions of glycerol in Milli-Q water (Millipore, USA). Peaks were annotated and integrated using Chromelion version 7.2 SR4 analysis software.

Differences in glycerol consumption between the WT and variants were evaluated by a Student *t*-test using Microsoft Excel. Differences were considered significantly different when $p < 0.05$.

2.6. Flagella imaging

Late-exponential phase cells of the WT strain and variants 14 and 15, grown at 30 °C were visualized using either the Ryu protocol (Kodaka et al., 1982; Ryu, 1937) or transmission electron microscopy (TEM). In the Ryu protocol, a wet mount of a cell suspension was made using a glass microscope slide with a coverslip. Then, a solution of one-part dye (saturated crystal violet in ethanol absolute) was added to 10 parts of mordant solution (aluminum potassium sulfate ($KAl(SO_4)_2{\cdot}12H_2O$) 57 g/l; Phenol (C_6H_5OH) 25 g/l; Tannic Acid ($C_{76}H_{52}O_{46}$) 20 g/l). A drop of this dye-mordant solution was placed on the side of the coverslip, allowing the dye-mordant solution to enter the wet mount via capillary action. Images were taken at phase contrast settings using an Olympus BX 41 microscope with an Olympus UIS-2 PLAN-C 100× PH3 oil immersion lens coupled to an Olympus XC 30 digital camera via a 0.63× magnifier tube. Olympus CellB version 3.5 software running under Windows 7 (Microsoft, USA) was used for image acquisition. Contrast was enhanced over the entire image using ImageJ (version 1.5f National Institutes of Health, Maryland, USA). For TEM, cells were pelleted at 2880 ×*g* and washed with phosphate buffered saline to remove traces of BHI and applied to copper TEM grids and stained with 2% uranyl acetate for 30s. The samples were visualized using a JEOL 1100, (Wageningen Electron Microscopy Centre, Wageningen University & Research, The Netherlands) operated at 100 kV. Experiments were performed with independent biological triplicates.

2.7. Caco-2 adhesion and invasion assay

Caco-2 human intestine epithelial cells were obtained from the American Type Culture Collection (Caco-2, ATCC HTB-37) and cells at passage (41) were used for all experiments. Cells were routinely cultured in Tissue culture medium (TCM), containing Dulbecco's Modified Eagle's Medium (DMEM, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Integro, The Netherlands), 1% (200 mM) glutamine (Gibco), Non-Essential Amino Acids (10 mM/amino acid, Gibco) and 0.1% w/v gentamycin (50.0 mg/ml, Gibco) in 75 cm^2 flasks (Corning Incorporated, NY, USA).

The cells were grown to confluence in 12-well tissue culture plates (Corning Incorporated, NY, USA) following the procedure described previously (Oliveira et al., 2011) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. 12-well plates were seeded in each well with 1.6×10^5 cells/ml. Medium was replaced every 2–3 days. Inoculated 12-well plates were incubated for 12–14 days at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for full cell differentiation. Prior to all experiments, Caco-2 cells were washed three times with TCM without gentamycin and FBS pre-warmed to 37 °C. A final inoculum concentration of 6.7 log cfu/ml was obtained by adding 40 µl of late exponential phase cells of *L. monocytogenes* WT and variants 14 and 15 to the monolayers. After inoculation, the 12 well plates were centrifuged (Hettich Rotina 420R, with 4784A swing-out rotor, Hettich Benelux, The Netherlands) for 1 min at 175 ×*g* to create a proximity between the Caco-2 and *L. monocytogenes* cells. The bacteria suspension was removed after 1 h of incubation at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Then, the Caco-2 monolayers were washed 3 times with 1 ml of pre-warmed PPS. To quantify the number of cells adhered and/or invaded to the Caco-2 cells, the Caco-2 cells were lysed with 1 ml of 1% v/v Triton X-100 (Sigma–Aldrich, Steinheim, Germany) in PPS and serially diluted in PPS. Appropriate dilutions were plated on BHI agar in duplicate, and colonies were enumerated after 2 days of incubation at 37 °C. The ratio of percentage recovery (defined as number of cells (cfu/ml) attached and/or invaded divided by number of cells (cfu/ml) inoculated) in the variants over percentage recovery in the WT was reported. Two technical replicates were used with three wells per replicate.

3. Results

3.1. General transcriptome response of variants 14 and 15

Microarray analysis showed that gene expression was different between the WT and variants 14 and 15. The number of differentially expressed genes in variants 14 and 15 is shown in Fig. 1. There was a clear overlap in the expression profiles of both variants, as 116 and 114 genes were upregulated and downregulated in both variants, respectively. To provide a more detailed insight into the transcriptomic responses of variants 14 and 15, the COG classes of the overlapping set of genes are shown in Fig. 1c (for a complete list of genes in the overlapping set and their corresponding COG classes, see Supplementary Tables S1 and S2). Notable shifts in expression in the variants were seen in COG classes related to metabolism and energy conversion (G: carbohydrate transport and metabolism, E: Amino acid transport and metabolism, C: Energy production and conversion), cell motility (N), signal transduction mechanisms (T), and transcription (K). Based on the previously described multiple-stress resistant phenotype of the *rpsU* variants, we first focused on parameters involved in stress response.

3.2. General stress response

The alternative transcription factor SigB and its regulon are known to play a key role in general stress adaptation in *L. monocytogenes* (Guldimann et al., 2016; NicAogain and O'Byrne, 2016). The SigB regulon has been investigated in several studies using different methods, including DNA microarrays and RNAseq (Mujahid et al., 2013; Oliver et al., 2010; Ollinger et al., 2009; Raengpradub et al., 2008), see Guldimann et al. (2016) for a recent overview of SigB in relation to resilience. Here we used the SigB regulon described by Mujahid et al., 2013 based on a gene expression dataset obtained by DNA microarrays and RNAseq, and compared the differential expression of these SigB-regulated genes in variants 14 and 15. The transcriptome analysis of the variants showed that the majority (ca. 70%) of the SigB regulon, consisting in total of around 145 genes, was upregulated. Additionally, the SigB-controlled *ctc* gene encoding ribosomal protein L25 (previously referred to as general stress protein Ctc) (Gardan et al., 2003), was found to be higher expressed in the *rpsU* variants (Fig. 2a/b, Supplementary Table S15). This pointed to an important role of the SigB regulon in acquired stress resistance of the variants, although the differential expression of the gene coding for the alternative transcription factor *sigB* (Sigma B, lmo0895) was significant (1.47 and 1.08 $\log_2$ fold change respectively for variant 14 and 15 with FDR < 0.005), but just below the stringent cut-off that was used (i.e. 1.58 $\log_2$ fold change). A selection of the SigB-regulated genes with known impact on *L. monocytogenes* stress-tolerance and host interaction is presented in Fig. 2c. The SigB-regulated *gad-D3* gene (lmo2434) (Kazmierczak et al., 2003) is responsible for intracellular conversion of glutamate into GABA (Feehily and Karatzas, 2013) and was upregulated in both variants (Fig. 2c). Additionally, genes involved in the SigB and PrfA controlled arginine deiminase (ADI) system (Ryan et al., 2009) were upregulated (see Supplementary Table S8). Upregulation of these genes aligns with the previously reported increased acid resistance of these variants (Metselaar et al., 2015). In the genome of *L. monocytogenes* various SigB-controlled genes encoding compatible solute transporters have been identified (Sleator and Hill, 2010; Sleator et al., 2001), and genes encoding for the compatible solute transporter OpuC (encoded by the *opuC* operon) were among the highest upregulated genes in the variants (Fig. 2c). Also, SigB-regulated genes involved in

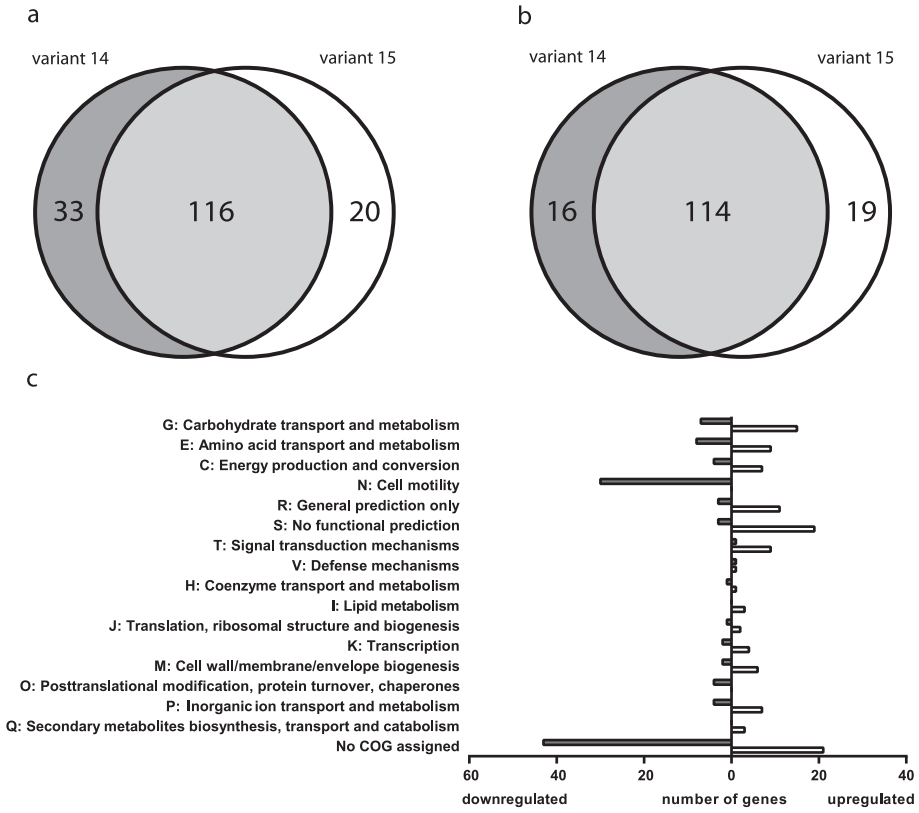

Fig. 1. Differentially expressed genes in *L. monocytogenes* LO28 variants 14 and 15 compared to the wild type. Panel (a) represents genes that were upregulated in the variants compared to the wild type, panel (b) represents genes that were downregulated in the variants compared to the wild type. The dark grey shaded circle part represents genes only up- or downregulated in variant 14, the white circle part represents genes only up- or downregulated in variant 15. The light grey circle part represents the overlap in expression between the variants, with 116 and 114 genes up- and downregulated, respectively. (c) COG assignment of the number of upregulated (open bars) and downregulated genes (shaded bars) in both variants 14 and 15 compared to the wild type. Expression of individual genes is listed in Tables S1 to S6.

glycerol metabolism (represented by *glpF/K/D, dhaK/L*) were upregulated, pointing to a shift in metabolism compared to the WT, as well as SigB-regulated genes known to be involved in initial attachment to epithelium cells (represented by *inlA* in Fig. 2c). Based on these observations a range of experiments was designed to determine corresponding relevant phenotypes of the two variants.

3.3. Freeze-thaw resistance

Listeria monocytogenes may be exposed to freezing-thawing stress in natural environments, as well as during storage and transport of foods. Compatible solutes are known to have a role in resistance to freezing-thawing stress (Sleator and Hill, 2010; Wemekamp-Kamphuis et al., 2004a), and the observed upregulation of the SigB-regulated *opuC* operon in variants 14 and 15 conceivably results in higher intracellular concentrations of compatible solutes such as carnitine present in BHI leading to improved freezing-thawing resistance. Therefore, *L. monocytogenes* LO28 WT and variants 14 and 15 were exposed to consecutive cycles of freezing and thawing. Indeed, while the WT decreases up to 3 to 4 log cfu/ml after three rounds of freezing and thawing, the cell counts of both variants did not decline, indicating enhanced stress-tolerance (Fig. 3). Experiments with chloramphenicol as inhibitor of protein synthesis showed a similar trend with slightly higher variation between the data points (data not shown) indicating that de novo protein synthesis was not required to sustain enhanced stress-tolerance of the variants. In addition to protection against freezing and thawing stress, compatible solutes are involved in osmoprotection. Therefore, *L. monocytogenes* LO28 WT and variants 14 and 15 were also exposed to a 24% w/v solution of NaCl in PPS for 16 h, but no killing was observed for WT or variants 14 and 15 (data not shown).

3.4. Glycerol metabolism

Increased expression of glycerol metabolism associated genes (Fig. 4a and Supplementary Table S10) indicates an increased production of glycerol metabolic enzymes in the variants. Glycerol catabolism in *L. monocytogenes* is strongly linked to the expression of *sigB* (Abram et al., 2008), while simultaneous upregulation of *prfA* has been reported in glycerol grown cultures (Joseph et al., 2008). The transcriptome analysis showed that indeed *prfA* was upregulated in both variants as well as the SigB-regulated gene encoding the putative glycerol uptake facilitator protein $GlpF_1$ (lmo1539), while the second non SigB-regulated gene encoding the putative glycerol uptake facilitator protein $GlpF_2$ (lmo1167) was not differentially expressed. After facilitated diffusion of glycerol into the cell via $GlpF_1$, glycerol can be metabolized into dihydroxyacetone phosphate (DHA-P) via glycerol-3-phosphate (Fig. 4a). The glycerol kinase gene (*glpK,* lmo1538) was upregulated in both variants and is suspected to catalyse the ATP-dependent phosphorylation of glycerol to yield glycerol-3-phosphate (Joseph et al., 2008). The *glpD* (lmo1293) gene coding for GlpD, which catalyzes the conversion of glycerol-3-phosphate into DHA-P was also upregulated in variants 14 and 15. Previous work on glycerol metabolism in *Listeria innocua* (Monniot et al., 2012) described the *golD* operon in *L. innocua* (lin0359-lin0369), and a homologous operon (*golD*) is present in *L. monocytogenes* (lmo0341-lmo0351). This *gol* operon is part of the second glycerol utilisation pathway that depends on GolD for the conversion of glycerol into dihydroxyacetone (DHA). While the *golD* gene (lmo0344) was not upregulated in variants 14 and 15, the genes needed to perform the subsequent utilisation steps in this pathway (*dhaK/L*) were upregulated. Consequently, glycerol consumption was assessed in exponentially growing cells of *L. monocytogenes* LO28 WT and variants 14 and 15. Indeed, glycerol utilisation was increased in the variants compared to the WT after 3 h of incubation in glycerol supplemented medium (Fig. 4b). This pattern was observed while the cells were incubated with chloramphenicol, indicating that de novo protein synthesis did not contribute to this phenotype. Notably, the genes lmo0722 and lmo1381 encoding pyruvate oxidase and acylphosphatase, respectively, were also higher expressed (Supplementary Table S11). This points to downstream utilisation of pyruvate generated from glycerol via acetyl phosphate leading to the

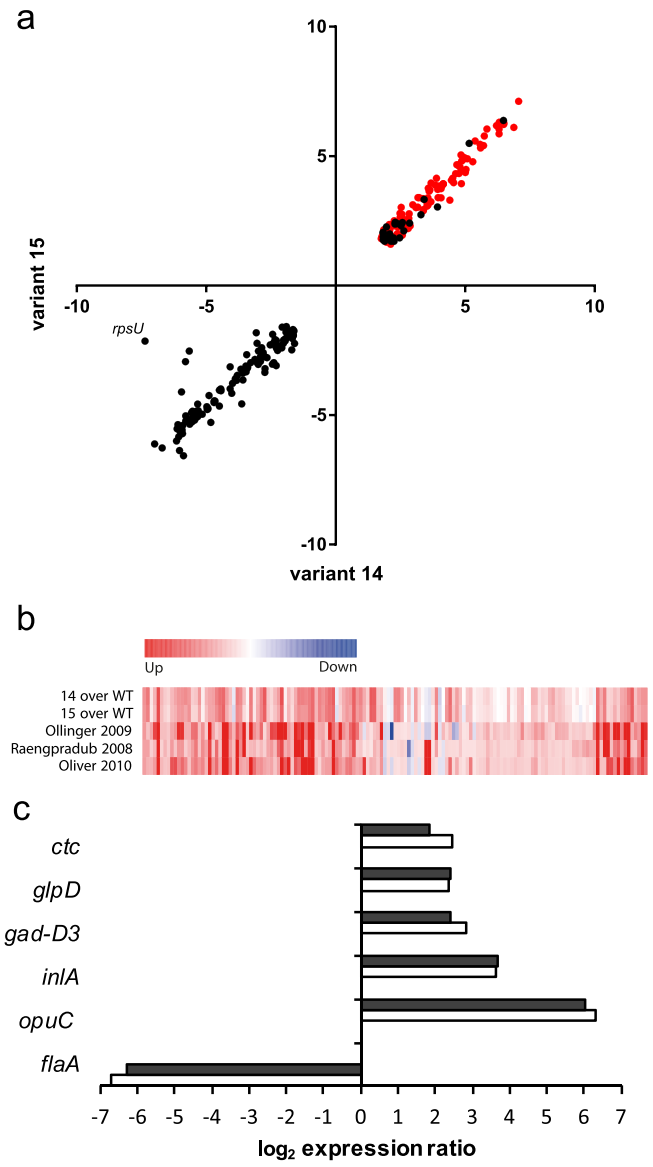

Fig. 2. Expression of representative SigB-regulated genes in *L. monocytogenes* variants compared to the wild type. (a) Regression of all genes that were significantly up- or downregulated in both variants 14 and 15. Genes shown in red are part of the SigB operon as described by Mujahid et al., 2013 (b) The heatmap displays relative expression of SigB-related genes in variants 14 and 15 compared to the wild type, and relative expression of these SigB-regulated genes as reported by Ollinger et al. (2009), Raengpradub et al. (2008) and Oliver et al. (2010). (c) Relative expression of selected SigB-regulated genes. The filled bars represent expression of genes in variant 15, the open bars represent expression of genes in variant 14. Expression of individual genes is listed in Table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

production of acetate. HPLC analysis of samples obtained after 3 h incubation demonstrated that glycerol was indeed preferentially converted into acetate in the variants (data not shown).

3.5. Motility

In our study, both flagella cluster 1 and putative flagella cluster 2 were downregulated in variants 14 and 15 (Supplementary Fig. S1 and Supplementary Table S11), and therefore flagella staining was used to analyse the presence or absence of flagella in WT and variants 14 and 15. Fig. 5 shows the reduced presence of flagella (no flagella for 30 observed cells) in both variants, while flagella were clearly observed for approximately 50% of the cells in the WT strain.

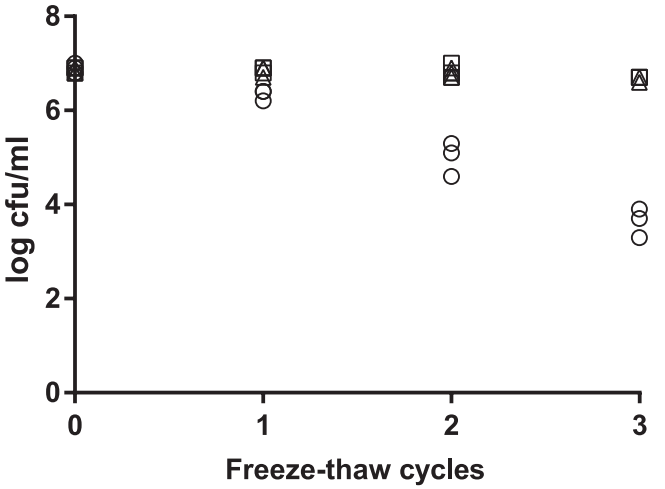

Fig. 3. Survival of *L. monocytogenes* LO28 wild type and variants after, 0 (equals the initial concentration), 1, 2, and 3 cycles of freezing and thawing. The wild type is represented by circles, variant 14 by squares, and variant 15 by triangles.

3.6. Caco-2 attachment and invasion

After stomach passage and crossing of the intestinal barrier, *L. monocytogenes* induces internalisation by non-phagocytal host cells using the cell surface proteins internalin A (*inlA*) and B (*inlB*). The InlA protein mediates the infection of human enterocyte like cells lines such as Caco-2 via the human E-cadherin receptor (Bonazzi et al., 2009) while InlB is specific for the hepatocyte growth factor (HGF) receptor Met (Pizarro-Cerda et al., 2012). In vitro, expression of either *inlA* or *inlB* is sufficient for attachment to and internalisation in non-phagocytic cells (Pizarro-Cerda et al., 2012). In variants 14 and 15, both *inlA* and *inlB* were upregulated (see Fig. 2 and Supplementary Table S14). Therefore the attachment and invasion of *L. monocytogenes* LO28 WT strain and variants 14 and 15 during incubation with Caco-2 cells was determined. The recovery ratio in Caco-2 cells of both variants 14 and 15 was eight-fold higher in comparison to the WT (Fig. 6), indicating that the variants performed better in attachment and/or invasion than the WT strain.

4. Discussion

Genotypic heterogeneity within a bacterial population may allow for elevated survival when a population of bacteria is subjected to food relevant stresses. In this study we focused on the transcriptomic behaviour of two multiple-stress resistant *rpsU* variants of *L. monocytogenes* LO28 that were previously isolated from a heterogeneous population. Notably, the transcriptomic response of variant 14, harbouring a large deletion that spans the ribosomal *rpsU* gene, as well as *yqeY* and half of *phoH*, was highly similar to the transcriptomic response of variant 15, harbouring a single point mutation in *rpsU,* that resulted in an amino acid change from arginine to proline. Despite the mutation in *rpsU*, and the strikingly lower expression of *rpsU* in variant 15 both variants apparently possessed functional ribosomes, and were highly stress resistant. Using a comparative gene-profiling and phenotyping approach, we now provided evidence that the multiple stress resistant phenotype could be explained by the activation of the SigB regulon. Both variants show an upregulation of about 70% of the 145 genes of the SigB regulon included in the analysis, although no mutations in *sigB* or its regulatory sequences were found (Metselaar et al., 2015). Whether additional

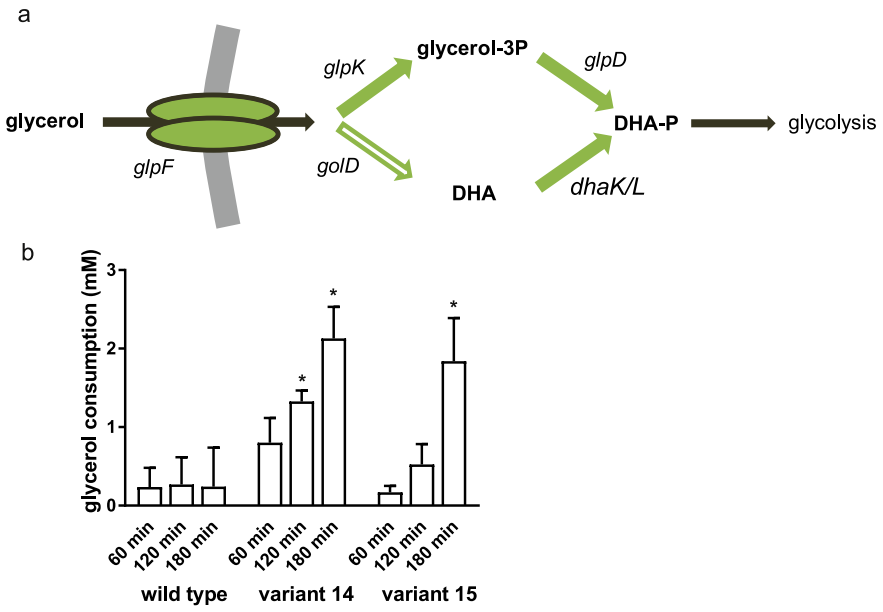

Fig. 4. Glycerol uptake and consumption in *L. monocytogenes* LO28 wild type and variants. (a) Glycerol is imported in the cell via GlpF and can be converted to glycerol-3 phosphate by GlpK or to dihydroxyacetone (DHA) by GolD, before entering glycolysis as dihydroxyacetone phosphate (DHA-P). Closed arrows represent upregulated genes in both variants 14 and 15 relative to the wild type, open arrows represent no differential expression in both variants. *glpK* is upregulated in variant 14, however expression in variant 15 falls just below the stringent cut-off used here (see Table S10). (b) Glycerol usage by *Listeria monocytogenes* LO28 WT and variants. Late exponential cells were concentrated and incubated in nutrient broth supplemented with glycerol for 60, 120 and 180 min. Error bars indicate standard errors. *Indicates significant difference over the same time point in the WT.

factors are contributing to the observed multiple stress resistance phenotype of the variants remains to be elucidated.

One of the primary systems to overcome acid stress in *L. monocytogenes* is the partially SigB-regulated GAD system. This system exchanges extracellular glutamate for intracellularly produced gamma-aminobutyrate ($GABA_i$) under acidic conditions using the gadT1 and gadT2 antiporters. Intracellular glutamate is decarboxylated into $GABA_i$ by gadD1, gadD2, or gadD3 while consuming a proton, thereby increasing the pH of the cytoplasm (Karatzas et al., 2010). As in previous work, (Metselaar et al., 2015), we did not find an elevated transcription of antiporter/decarboxylase pair *gadT1D1* or gad*T2D2* of the external GAD system. However, the SigB-regulated *gadD3* of the internal GAD system, operating without a glutamate/GABA antiporter was upregulated. GadD3 is hypothesised to play an important role in acid resistance by mediating the conversion of glutamate into $GABA_i$ with concomitant consumption (removal) of protons in the cytoplasm (Karatzas et al., 2010; Wemekamp-Kamphuis et al., 2004b), and indeed elevated accumulation of $GABA_i$ has been previously found in variants 14 and 15 in response to acid stress (Metselaar et al., 2015).

In *L. monocytogenes*, transcription of genes involved in glycerol catabolism was shown to be SigB dependent (Abram et al., 2008). In variants 14 and 15, increased transcription of the SigB-regulated $GlpF_1$ (lmo1539) gene pointed to increased glycerol catabolism in variants 14 and 15. Indeed, during exponential growth in BHI we found upregulation of a specific set of genes in the variants involved in glycerol uptake: $glpF_1$ (lmo1539), $glpK_1$ (lm1538) and *glpD* (lmo1293) but not of $glpF_2$ (lmo1167) and $glpK_2$ (lmo1034). During growth, *L. monocytogenes* preferentially uses sugars that are taken up by the phosphoenol-pyruvate (PEP): phosphotransferase systems (PTS) such as glucose (Joseph et al., 2008). The main carbon source in BHI is glucose, and the presence of PTS sugars in the medium normally inhibits the catabolism of non-PTS carbon sources such as glycerol via carbon catabolite repression (CCR) (Gilbreth et al., 2004; Joseph et al., 2008; Milenbachs et al., 1997; Park and Kroll, 1993). However, our variants 14 and 15, when grown in BHI display a pattern of gene expression like WT cells of *L. monocytogenes* EGD-e grown to OD_{600} 0.5 in defined minimal medium with glycerol (Joseph et al., 2008) suggesting mitigation of catabolite repression.

Another study reported that cells grown in the presence of a non-PTS carbon source such as glycerol, show a high activity of *prfA* (Joseph

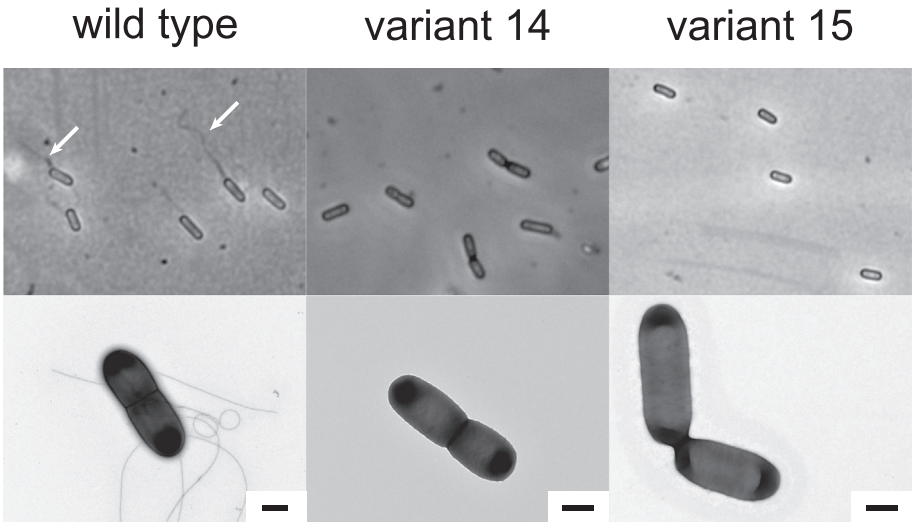

Fig. 5. Flagella imaging of *L. monocytogenes* LO28 WT and variants. Top row, *L. monocytogenes* flagella staining with crystal violet as described in Ryu et al. (1937). White arrows indicate flagella in WT. Bottom row, TEM image of *L. monocytogenes* cells. Scale bar indicates 500 nm.

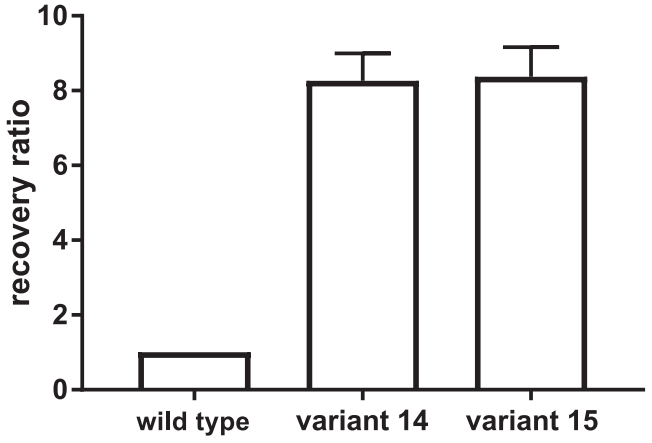

Fig. 6. Recovery ratio of *Listeria monocytogenes* LO28 wild type compared to variants 14 and 15 from Caco-2 cells. Recovery ratio is defined as N recovered (attached and invaded) over N inoculated. Wild type recovery ratio is set at 1. Error bars represent standard error of three technical replicates.

and Goebel, 2007; Mertins et al., 2007) and experiments with Δ*glpk* and Δ*glpD* mutants indicated that components related to glycerol metabolism may modulate the transcription of *prfA*. Notably, glycerol has been reported as one of the main carbon sources for *L. monocytogenes* during cytosolic growth (Bruno Jr. and Freitag, 2010; Fuchs et al., 2012). Therefore, the observation that both *prfA* and the genes for glycerol metabolism are constitutively expressed in the variants suggests that variants 14 and 15 are metabolically primed for replication in eukaryotic cells (Bruno Jr. and Freitag, 2010), conceivably affecting their virulence potential. Indeed, in variants 14 and 15, with upregulated *glpK* and *glpD*, we see upregulation of the PrfA/SigB-regulated *inlA* and *inlB*. InlA is essential for attachment and invasion of enterocytes and enterocyte cell lines such as Caco-2, while InlB mediates the attachment of *L. monocytogenes* to fibroblasts and hepatocytes. Although *L. monocytogenes* LO28 carries a premature stopcodon in *inlA*, truncating the InlA protein at 63 kDa as opposed to the 80 kDa InlA of epidemic strains, infection studies show that *L. monocytogenes* LO28 is still able to adhere to and invade Caco-2 cells (Olier et al., 2003). In variants 14 and 15, we observed an eight-fold increase over the WT in attachment and invasion to a Caco-2 cell line, indicating a higher potential for adhesion and invasion in the variants.

Both variants 14 and 15 showed a clear reduction in expression of motility associated genes, including flagellar biosynthesis genes (*fliN, fliP, fliQ, fliR, flhB, flhA, flhF*, and *flgG*) and motor control genes (*motA, motB*). In variants 14 and 15, there was no significant difference in expression of the motility gene repressor *mogR* over the wild type (see table S13). In line with the downregulation of the flagellar biosynthesis genes, the *gmaR* gene encoding the MogR-anti-repressor GmaR, was strongly downregulated in both variants (see table S11), conceivably allowing the MogR protein to repress expression of the flagella operon (Lebreton and Cossart, 2016). Whether the previously described SigB-activated long antisense RNA Anti0677 (Lebreton and Cossart, 2016; Schultze et al., 2014; Toledo-Arana et al., 2009) plays an additional role in the observed downregulation of motility genes in the flagella operon in the two *L. monocytogenes* variants remains to be elucidated.

Activation of a systemic stress defence response via SigB is energetically costly, and shutdown of the energy consuming flagella synthesis apparatus can reduce some of the energetic costs of SigB activation. Indeed, *rpsU* variants have been described to have reduced fitness relative to the WT, showing reduced growth rates at 7 °C and 30 °C (Metselaar et al., 2015). Notably, Metselaar et al. (2013) described growth rate differences that were a function of media pH for *rpsU* variants, suggesting that stress resistance and growth rate are growth condition dependent and mechanically linked. This could provide a clue to elucidate the correlation between the *rpsU* mutations and activation of the SigB regulon reported in the current paper. SigB activity is controlled both translationally and posttranslationally in *Listeria*, allowing the bacterial cells to rapidly respond to changes in environmental conditions. The posttranslational control of SigB activity involves a phosphorylation cascade that is highly conserved in species containing *sigB*, including *L. monocytogenes* (Ferreira et al., 2004) and is governed by the "stressosome", a signal relay hub that integrates multiple environmental (stress) signals to regulate SigB activity. A published overview of network motifs in *L. monocytogenes* (Guariglia-Oropeza et al., 2014) underlines the role of *sigB* as a central hub in the stress response of *L. monocytogenes*. In variants 14 and 15, we found strong upregulation of genes that were under the direct control of SigB (e.g. *uspL*1–3, *inlAB, bsh*) in these regulatory networks, but not of genes that were co-regulated by other regulators. However, additional network effects remain to be elucidated.

In conclusion, the activation of SigB-mediated stress defence offers an explanation for the multiple-stress resistant phenotype observed in *rpsU* variants. Strikingly, our DNA microarray analysis showed that expression of upregulated or downregulated genes largely overlaps between variants 14 and 15, while variant 14 carries a deletion of the *rpsU* and *yqeY* gene, and a partial deletion of *phoH*, whereas variant 15 carries only an amino acid substitution in the *rpsU* gene which may affect functionality of the RpsU protein. The exact mechanism of SigB induction via *rpsU* and stressosome associated genes remains to be elucidated. Moreover, a better mechanistic understanding of *rpsU* associated multi-stress resistance will provide valuable insights into the generation of genotypic heterogeneity within bacterial populations.

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