Food Control 161 (2024) 110393

Contents lists available at ScienceDirect

Food Control

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

Listeria monocytogenes uses *de novo* purine synthesis to enhance fitness in Lyoner-type sausage

Philipp-Michael Beindorf [a,*], Jule Anna Horlbog [b], Irene Esteban Cuesta [a], Claudia Guldimann [a], Irmak Şah [a]

[a] Food Safety and Analytics, Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich (LMU), Schönleutnerstrasse 8, 85764, Oberschleissheim, Germany
[b] Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland

ARTICLE INFO

Keywords:
Listeria monocytogenes
Transposon mutagenesis
Deli meat
Lyoner
PurB
Purine synthesis

ABSTRACT

Listeria monocytogenes is an important food-borne pathogen with high hospitalization and case fatality rates. To cause disease, *L. monocytogenes* must gain access to a specific food matrix and in many cases be able to grow before the food is consumed. The fitness of this pathogen differs between individual foods and depends on its ability to adapt to various environmental stressors that are highly specific in each food matrix. Deli meats are an important cause of infections with *L. monocytogenes*. Here, we screened a transposon mutagenesis library in *L. monocytogenes* LL195 with 2640 individual mutants on a Lyoner-type deli meat to understand the fitness effect of individual genes. After the determination of 10 candidate mutants with confirmed phenotypes on Lyoner, in-frame deletion mutants of these genes were created by allelic replacement. The fitness effects of these in-frame mutants were then confirmed by growth experiments under cold stress and on Lyoner separately, and in a combined condition of both. Mutants with deletions in the cytosine-specific DNA modification methyltransferase *sau3AIM* and the penicillin-binding protein *pbp-B* were impaired in their growth at cold temperatures in rich medium as well as on Lyoner, suggesting a temperature-dependent phenotype. In contrast, the *purB* deletion mutant exhibited reduced fitness that was specific to growth on Lyoner. Our results indicate an important role for the *sau3AIM* and *pbp-B* genes in cold stress adaptation, while *purB*, which is a central component of purine biosynthesis, may play a more specialized role on the fitness of *L. monocytogenes* during growth on Lyoner.

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, which remains one of the most severe food-borne illnesses in the European Union (The European Union One Health 2021 Zoonoses Report, 2022) and worldwide. The latest available report by the European Food Safety Agency (EFSA) documents 23 foodborne outbreaks and 2183 cases with a case-fatality rate of 9% in the EU in 2021 (The European Union One Health 2021 Zoonoses Report, 2022). Ready-to-eat (RTE) foods like smoked fish, soft cheeses, and deli meats, are overrepresented among foods implicated in listeriosis cases and outbreaks (Lopez-Valladares et al., 2018). The primary reason these products represent a high risk is that there is either no killing step during their production or, in case of deli meats, events of re-contamination during processes like slicing. Sliced RTE deli meats have frequently been implicated in *L. monocytogenes* outbreaks and show a global prevalence of listeriosis of 2.9 % (with considerable heterogeneity) as determined by a systematic review and meta-analysis (Churchill et al., 2019). Several quantitative microbial risk analysis models estimate that most cases in the EU and the USA originate from RTE deli meats (Sampedro et al., 2022). The heating step in the production of most deli meats makes intact cooked sausages safe for consumption. The high number of listeriosis cases associated with deli meats results from post-processing contamination of the finished product during slicing and packaging (Sheen & Hwang, 2008).

Once *L. monocytogenes* is present on a given food, the intrinsic factors such as nutrient content, pH, water activity (a_w), presence of antimicrobial compounds and extrinsic factors such as oxygen availability, storage and transportation conditions determine whether and at what rate the pathogen is able to grow. As a saprophyte, *L. monocytogenes* is ubiquitously present in the natural environment and has a unique ability to adapt and survive under challenging conditions, including food-related stress conditions (Bucur et al., 2018). They can grow in a wide pH range (4.7–9.2), at temperatures from −0.5 to 45 °C, in high salt concentrations, and in the presence of preserving additives like

* Corresponding author.
E-mail address: philipp.beindorf@ls.vetmed.uni-muenchen.de (P.-M. Beindorf).

https://doi.org/10.1016/j.foodcont.2024.110393
Received 4 December 2023; Received in revised form 13 February 2024; Accepted 16 February 2024
Available online 29 February 2024

bacteriocins. As facultative anaerobes, they may also grow under anaerobic conditions (Chaturongakul et al., 2008).

Challenge tests on different food matrices show that phenotypic characteristics of *L. monocytogenes* vary on different food matrices (Spanu et al., 2014). Several studies have been conducted to establish genes and molecular mechanisms that underlie its adaptation to different niches to date: the alternative sigma factor σ^B acts as a global transcriptional regulator under many different stress conditions such as acidic, osmotic, or oxidative conditions, cold stress and nutrient limitations (Guerreiro et al., 2020). The response of *L. monocytogenes* to specific environmental stressors typically results in global changes in gene and protein expression profiles, and specific regulatory responses to pH, temperature, osmotic, oxidative, or abiotic stresses are well documented (reviewed in Bucur et al., 2018; Guerreiro et al., 2020). However, these studies were typically carried out in laboratory culture media. According to the limited studies done in food matrices, gene expression profiles of different *L. monocytogenes* strains varied significantly when they were grown in various foods (Olesen et al., 2010; Rantsiou et al., 2012). There are also variations in the expression level of the stress response, virulence, and adhesion genes between laboratory media and food matrix (Rantsiou, Mataragas, et al., 2012). One drawback of transcriptome studies is that a significant change in gene expression does not always significantly affect fitness (Feder & Walser, 2005). Therefore, screening random mutants for fitness effects of individual genes on the food matrix complements and expands these data.

Most of the available data on the resilience of *L. monocytogenes* in the food environment has been established using well-characterized laboratory reference strains such as EGD-e and 10403S. However, these strains do not represent clinically important clonal complexes, nor do they cover the clinically relevant lineage II or serotype 4b, and passaging may have led to the loss of crucial pathophysiological characteristics (Bécavin et al., 2014). Therefore, results should be interpreted not only considering the experimental conditions but also in the light of the genetic background of the bacterial strains used (Fux et al., 2005). In summary, there is a significant knowledge gap in our understanding of the interactions between clinically relevant strains of *L. monocytogenes* and the food matrices they are most associated with.

However, a better understanding of the molecular mechanisms behind the resilience of *L. monocytogenes* on different food matrices is crucial for the development of tailored and effective control methods. Therefore, the aim of this study was to elucidate the fitness contribution of individual genes of *L. monocytogenes* and to investigate their growth dynamics on a high-risk food matrix. We chose sliced Lyoner sausage as a model matrix because (i) Lyoner-type sausages are very popular in Europe, (ii) as deli-meats they are often associated with listeriosis cases, and (iii) their similarities with South-African "polony" that was responsible for the world's largest listeriosis outbreak in 2017–2018 (Smith et al., 2019). In this study, to overcome the disadvantages of using reference strains, we chose to work with an outbreak, serotype 4b strain of *L. monocytogenes* (Weinmaier et al., 2013).

2. Materials and methods

2.1. Bacterial strains, and growth conditions

The *L. monocytogenes* strain LL195 was used for the following experiments. This strain was isolated during an outbreak in Switzerland between 1983 and 1987, which was associated with Vacherin Mont-d'Or cheese (Weinmaier et al., 2013). *L. monocytogenes* LL195 was grown in Brain Heart Infusion (BHI, Merck KGaA, Germany) broth or on BHI agar (BHI + 1,5 % Agar Technical LP0012B, Oxoid Deutschland GmbH, Germany) unless otherwise stated. *Escherichia coli* Top10 strain was used as a host for the cloning experiments and grown in Luria Bertani (LB, Carl Roth GmbH, Germany) Broth or on LB agar (LB, Carl Roth GmbH + 1,5 % Agar Technical, Oxoid Deutschland GmbH, Germany) plates.

2.2. Cloning of the transposon library

To clone the Tn-library in LL195, we used pJZ037 and the protocol described by Zemansky et al. (Zemansky et al., 2009). pJZ037 contains a 1423 bp transposon that integrates into "TA" recognition sites via a mariner C9 transposase. In short, the plasmid pJZ037 was prepared using the Qiagen QIAprep Spin Miniprep kit (Qiagen, Cat. 27,104). To obtain electrocompetent *L. monocytogenes* LL195, cells were grown in 50 ml BHI (Biorad, Hercules, USA) with 0.5 M sucrose (Fluka Honeywell, Charlotte, USA) at 37 °C with shaking at 200 rpm until they reached an OD_{600} of 0.2. After addition of 10 μg/ml penicillin (Sigma-Aldrich, St. Louis, USA) and further incubation for another 2 h at 37 °C with shaking, the culture was cooled on ice, centrifuged at 5000×*g* at 4 °C and washed twice with 1 M HEPES (Sigma-Aldrich, St. Louis, USA) with 0.5 M sucrose. The resulting pellet was resuspended in 0.4 ml 1 M HEPES with 0.5 M sucrose. Aliquots of 100 μl of these electrocompetent cells were used to electroporate 1 μg pJZ037 in 0.1 mm cuvettes and 1.8 kV for 5 ms, followed by the addition of 1 ml BHI and incubation for 1 h at 30 °C without shaking. Dilutions of the resulting cultures were plated on BHI plates containing 7.5 μg/ml chloramphenicol (Sigma-Aldrich, St. Louis, USA) and incubated at 30 °C (permissive for plasmid replication) for 72 h. Colonies were then plated onto BHI agar plates containing 1 μg/ml erythromycin (Sigma-Aldrich, St. Louis, USA) and incubated at 43 °C (the non-permissive temperature for plasmid replication) for 48 h to cure the plasmid. From these plates, colonies that were resistant to erythromycin but sensitive to chloramphenicol were determined by replica-plating, picked and subcultured into 96-well plates. An additional passage in BHI with 1 μg/ml erythromycin at 43 °C, 24 h and control to confirm no growth in BHI with 7.5 μg/ml chloramphenicol at 30 °C, 24 h were performed in this 96-well format. The *L. monocytogenes* LL195 genome size is 2,904,662 bp (Weinmaier et al., 2013), and we aimed to produce a transposon insertion at least every 1200 bp on average. Therefore, mutant collection was continued until a collection of at least 2420 mutans was achieved. The final transposon library contains a collection of 2640 individual mutants, corresponding to a Tn insertion roughly every 1100 bp and was stored at −80 °C in 96-well plates in BHI containing 1 μg/ml erythromycin and 15 % glycerol (Sigma-Aldrich, St. Louis, USA).

2.3. Screening of the transposon library on a German deli meat

For the pre-screening, the frozen library was inoculated into 96-well plates (U bottom, Greiner BioOne, Germany), each containing 200 μl of BHI broth, using a 96 pin-replicator made of stainless steel. The plates were then incubated overnight at 37 °C with continuous shaking at 250 rpm in a microplate shaker (88-861-024 Fisherbrand™, Thermo Fisher, USA). Each plate contained three replicate wells with the wild type (WT) strain as a control. These pre-cultures were subcultured 1:100 into fresh BHI in 96 well plates and incubated until the WT strain reached early log phase corresponding to OD_{600} of 0,3–0,4 and 10^8 CFU/ml (Biophotometer 6131, Eppendorf SE, Germany) at 37 °C with 250 rpm shaking. This culture was used to inoculate miniaturized matrix pieces in 96-well plates. For the matrix, we chose slices of boiled chicken sausage called Lyoner (REWE BIO Hähnchen, REWE Markt GmbH, Germany). Using a custom-made stainless-steel punch (square base with 96 bars with diameter of 0.5 cm), pieces weighing an average of 45.3 mg were extracted and placed into individual wells of a 96-well plate. To ensure uniform positioning at the bottom of the wells, the plates were centrifuged (Eppendorf SE, Germany, Centrifuge 5804 R) at 500×*g* for 1 min. A total of 10^5 cells were inoculated on these slices of Lyoner and incubated at 8 °C for 48 h. Growth at the t_0 time point of the inoculum was compared with the growth on the food matrices after incubation at 8 °C for 48 h by using the running drop method on oxford agar (OX, Merck KGaA, Germany) (Küchbacher, Cossart, & Pizarro-Cerda, 2021). For this, 10 μl of the liquid cultures were carefully dispensed at the top of Oxford agar plates and tilted to allow the drops to run the length of the

plate in parallel tracks. In this manner, six samples can be plated on one plate. The plates were then incubated at 37 °C. Following the incubation period, the colonies were counted to determine the colony-forming units (CFUs). The propagation of the mutant library was compared with the propagation of the WT strain, *L. monocytogenes* LL195. This method was used to identify candidate mutants whose growth on Lyoner differed from the WT. The phenotype of these candidates was confirmed in a larger volume and compared with that of the WT. For this, candidate mutants were grown on BHI agar plates overnight at 37 °C. A few colonies were transferred into 5 ml BHI broth in tubes (Test Tube Soda glass, 100 × 16,00 × 0,8 - 1 mm, round bottom, VWR International GmbH, Germany) and were incubated overnight (MaxQ 6000, Thermo Fisher, USA) at 37 °C with continuous shaking at 200 rpm. They were then subcultured 1:100 into 5 ml fresh BHI broth and incubated until the WT strain reached early exponential phase corresponding to OD_{600} of 0, 3–0,4 corresponding to roughly 10^8 CFU/ml at 37 °C with 250 rpm shaking. Two hundred microliters of the main culture were inoculated onto a slice of Lyoner (10 g, diameter of each slice was 9,3 cm), placed in sterile petri dishes (633,180, Greiner BioOne, Germany), and incubated at 8 °C for 48 h. Quantification was performed on oxford agar (Merck KGaA) plates that were incubated at 37 °C for 24 h. These larger volume screenings were repeated three times.

2.4. Identification of transposon insertion sites in candidate mutants

In candidate mutants with a confirmed phenotype on Lyoner, candidate genes were identified by determining the exact location of the Tn in the genome using a slightly modified, nested PCR protocol as described in (Zemansky et al., 2009). Primers used to identify the exact location of transposon are listed in Supplementary Table 1. A colony touchdown PCR was performed using the Tn1 and Arb1 primers. The PCR mixture contained 0.5 U of Taq DNA Polymerase (GoTaq® DNA Polymerase, Promega Corporation, USA), 1*X* GoTaq DNA Polymerase Buffer, 200 μM of each dNTP (Meridian Bioscience Inc., USA) and 0.2 μM of each primer in a final volume of 50 μl. The Touch-down PCR program was designed as follows: pre-denaturation at 95 °C for 5 min, followed by 16 cycles of denaturation at 98 °C for 30 s, primer annealing starting at 48 °C with a 1 °C decrease per cycle for 30 s, and elongation at 72 °C for 2 min. This was followed by an additional 16 cycles of denaturation at 98 °C for 10 s, primer annealing at 61 °C for 30 s, and elongation at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The products were cleaned up with the QIAquick® PCR Purification Kit (cat. No. 28104, QIAGEN GmbH, Germany) following the manufacturer's protocol. 1 μl of these PCR products was employed as templates for a second PCR which is performed to increase specificity. The second PCR mixture was prepared in the same manner as the first PCR but this time with the primers Tn3 and Arb2. The second Touch-down PCR program was designed as follows: pre-denaturation at 98 °C for 3 min, followed by 20 cycles of denaturation at 98 °C for 10 s, primer annealing starting at 60 °C with a 0,5 °C decrease per cycle for 30 s, and elongation at 72 °C for 2 min. This was followed by an additional 20 cycles of denaturation at 98 °C for 10 s, primer annealing at 50 °C for 30 s, and elongation at 72 °C for 2 min, and a final extension at 72 °C for 5 min. Subsequently, all samples were sequenced (Eurofins Genomics Europe Shared Services GmbH, Germany). The resulting sequences were aligned to the LL195 genome to determine the transposon insertion sites (Geneious Prime, Biomatters Inc., USA). Among those confirmed candidate genes, we selected candidates for the creation of in-frame, non-polar deletion mutants according to the following criteria: genes that were identified with several independent Tn insertions, genes that belong to a pathway that was identified as relevant several times, and, if applicable, genes in processes that might offer the potential for food safety interventions.

2.5. Creation of in-frame deletion mutants

To exclude polar effects and multiple transposon insertions, in-frame, non-polar mutants of the candidate genes were generated in a LL195 background. To generate in-frame deletion mutants by allelic replacement, pKSV7 (Smith & Youngman, 1992) and pMAD (Arnaud et al., 2004) suicidal plasmids were used. The genomic DNA of *L. monocytogenes* LL195 was isolated by using the ISOLATE II Genomic DNA Kit (Bioline GmbH, Germany). The quantity and the quality of the genomic DNA was measured by nanophotometer (NP80-Mobile, SN M81074, IMPLEN GmbH, Germany). Mutants were created using splicing by overlap extension (SOE). Two separate PCRs were performed to amplify the upstream and downstream regions of each candidate gene using primers shown in Supplementary Table 2. The PCR mixture contained 1× of Q5 High-Fidelity 2× Master Mix (New Englands Biolabs GmbH, Germany), and 0.5 μM of each primer in a final volume of 50 μl. The PCR program was designed as follows: pre-denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 55 °C for 30 s, and elongation at 72 °C for 20 s with a final extension at 72 °C for 2 min.

PCR products were purified with the QIAquick PCR Purification Kit according to manufacturer's instructions. Subsequently, overlap extension PCR was performed using the purified fragments as templates, resulting in the fusion of these fragments. The PCR mixture contained 1× of Q5 High-Fidelity 2× Master Mix (New Englands Biolabs GmbH, Germany), and 0.5 μM of each primer (Primer A and Primer D) in a final volume of 50 μl. The PCR program was designed as follows: pre-denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 59 °C for 30 s, and elongation at 72 °C for 40 s and a final extension at 72 °C for 2 min.

The final PCR products were ligated into pMAD (candidates: hypothetical protein 2, *Sau3AIM*), at the *Bam*HI-HF and *Nco*I-HF restriction sites (New England Biolabs GmbH, Germany) or into pKSV7 (candidates: *pbp-B*, *purB*, *DNA binding protein*, hypothetical protein 1, *Yham*), at the *Sac*I-HF and *Sal*I-HF restriction sites (New England Biolabs GmbH, Germany). The ligated products were introduced into *E. coli* Top10 electrocompetent cells by electroporation. Transformed cells were plated on LB with ampicillin (100 μg/ml) agar and positive colonies were screened by PCR for the presence of the insert and sequenced. Plasmids with the correct insert were isolated from *E. coli* using the Qiagen QIAprep® MiniPrep Kit. These plasmids were electroporated into *L. monocytogenes* LL195 and transformants were selected on BHI + chloramphenicol (10 μg/ml, Carl Roth GmbH, Germany) for pKSV7 and casein-peptone soymeal-peptone broth (TSB, Merck KGaA, Germany) + erythromycin (5 μg/ml, Merck KGaA, Germany) + X-gal (50 μg/L) for pMAD.

For pKSV7: A few colonies from the transformant plates were selected and propagated in BHI + chloramphenicol (10 μg/ml) medium for 5–6 generations at 42 °C with shaking at 70 rpm for plasmid integration. Colonies in which the plasmid had chromosomally integrated in *L. monocytogenes* were chosen from those that grew on BHI + chloramphenicol (10 μg/ml) agar plates. These selected colonies were further propagated in BHI without antibiotics at 30 °C with shaking at 70 rpm for at least 14 generations to eliminate the plasmid. Several colonies were screened to detect the loss of chloramphenicol resistance through replica plating on both BHI + chloramphenicol (10 μg/ml) and BHI plates. Among those displaying chloramphenicol sensitivity, identification of individuals harboring a mutant allele within the chromosome was achieved using PCR with A and D primers and subsequently confirmed by sequencing.

For pMAD: A few colonies from the transformant plates were chosen, pooled, and cultured in TSB + erythromycin (5 μg/ml) at 37 °C with shaking at 120 rpm for 5 h. Subsequently, the temperature was shifted to 42 °C, and incubation continued overnight to force the integration of the plasmid into the LL195 chromosome. Blue colonies, in which the plasmid had chromosomally integrated in *L. monocytogenes*, were chosen

from those that grew on TSB + erythromycin (5 μg/ml) and X-gal (50 μg/L) agar plates. These selected colonies were further propagated in TSB without antibiotics at 37 °C with shaking at 120 rpm for at least 14 generations to eliminate the plasmid. Several white colonies were screened to detect the loss of erythromycin resistance through replica plating on both TSB + erythromycin (5 μg/ml) and TSB plates. Among those displaying erythromycin sensitivity, identification of individuals harboring a mutant allele within the chromosome was achieved using PCR with A and D primers and subsequently confirmed by sequencing. All in-frame deletion mutants were stored at −80 °C in 35% glycerol.

2.6. Confirmation of the phenotype using in frame deletion mutants

Seven out of ten in-frame deletion mutants were successfully created: *DNA binding protein*, hypothetical protein 1, *Yham*, hypothetical protein 2, *Sau3AIM*, *pbp-B*, and *purB*. These were then used to confirm the phenotype on 10 g slices of Lyoner, using the same protocol as described for large volume confirmation screening of the transposon mutants above. However, this time, the main cultures were prepared in culture tubes containing 5 ml of BHI medium. To determine the effect of temperature, these experiments were carried out at 37, 25 and 8 °C in BHI and on Lyoner, separately and repeated three times.

2.7. Scanning electron microscopic (SEM) imaging

The in-frame deletion mutants *Sau3AIM*, *pbp-B*, and *purB* strains exhibited significant differences compared to the WT, and we followed up on the hypothesis that the mutations may cause morphological changes with imaging. The strains were cultured in 5 ml BHI overnight at 37 °C with shaking. These pre-cultures were diluted 1:100 into fresh BHI and incubated at 37 °C with shaking until the OD_{600} reached 0,3–0,4 which is the exponential phase of the culture. These cultures were inoculated into fresh BHI and incubated at 12 °C for 48 h. One milliliter of each 37 °C and 12 °C cultures were pelleted and washed with PBS twice and then fixed by resuspending the cells in equal amounts of 2% paraformaldehyde (Alfa Aesar GmbH & Co. KG, Germany) and incubating at room temperature for 2 h. The samples were stored at 4 °C until imaging. One drop of liquid samples was coated with a gold/palladium layer (approx. 2 nm) in a sputter coater and examined under high vacuum conditions in a field emission SEM (Philipps XL 30SFEG, Netherlands) at 5 KV using the in-lens secondary electron detector. These samples were also visualized by Gram staining under the light microscope.

2.8. Data analysis

All analyses were performed in RStudio version 2023.09.1 + 494 (RStudio Team, 2015) using the packages lme4 version 1.1–34 (Bates et al., 2015), sjPlot version 2.8.15 (Lüdecke, 2023), dplyr version 1.1.2 (Wickham, 2011) and ggplot2 version 3.4.2 (Wickham, 2016). To assess the effect of individual in-frame deletion mutants on the fitness of the strain a linear mixed effect model was calculated with lme4. It models log10 fold changes (defined as the log10 CFU/g or ml difference before and after the relevant stress condition) as a function of the mutant strain, temperature (8 °C vs 25 °C) and medium (Lyoner vs. BHI). An artificial variable created from the combination of the biological replicate, mutant and temperature was used as a random effect in the model to control for differences between experimental replicates. To verify that all in-frame deletion mutants had the same growth rate in BHI, an ANOVA was performed on the time it took the individual strains to reach an OD_{600} of 0.4.

3. Results

3.1. Phenotype of the L. monocytogenes LL195 transposon insertion library on Lyoner

In this study, we screened almost 2640 transposon insertion mutants on a miniature Lyoner model. This initial small volume screening revealed that while the WT strain was able to grow on Lyoner at 8 °C (1,88 log10 CFU/g ±0,23), the growth of 106 mutants was reduced by at least 0,5 log10 CFU/g or they showed no growth on Lyoner compared to the WT strain, suggesting that the genes affected by the transposon insertion might be important for growth on Lyoner. Additionally, 16 mutants exhibited better growth on Lyoner compared to the WT strain, suggesting that the genes affected in these mutants might hinder growth on Lyoner.

To efficiently handle the large number of mutants in the library, we initially conducted a small volume screening. This small volume screening allowed us to quickly obtain an initial overview of the mutants' growth patterns and identify potential candidates that exhibited notable differences in growth on Lyoner compared to the WT strain. This pre-screening process was essential in prioritizing the most promising mutants for subsequent analysis in a larger volume. As a next step, we performed a second, larger volume screening on Lyoner with a total of 122 candidate mutants in triplicate.

In the subsequent larger volume screening, no significant difference in growth was found among the 16 mutants that had initially demonstrated enhanced growth compared to the WT strain on Lyoner during the small volume screening. However, out of 106 candidate mutant strains that initially exhibited reduced, or no growth compared to the WT strain, 13 mutants were confirmed to have a negative effect on the growth of the respective strains on Lyoner (Table 1). The fact that genes, *sau3AIM* and *pbp-B*, and the promoter region of the *DNA binding protein* were hit by several transposon insertions confirmed the feasibility of the method.

3.2. Identification of the candidate genes

Transposon insertion sites of the candidate genes were identified using a slightly modified nested PCR protocol as described by (Zemansky et al., 2009). The localization of the transposon insertion sites, and their genetic context is shown in Fig. 1.

3.3. Growth profiles of the in-frame deletion mutants

To confirm the observed phenotypic changes in the mutants, we created in-frame deletion mutants by SOE PCR and screened them on Lyoner. Among the initial thirteen confirmed candidates, there were several that were located within the same genes, resulting in ten candidate genes (Fig. 1). Out of these, a total of 7 in-frame deletion mutants (*DNA binding protein*, hypothetical protein 1, *Yham*, hypothetical protein 2, *Sau3AIM*, *pbp-B*, and *purB),* were successfully created. Despite several attempts to create in-frame deletion mutants for *manY*, *manZ* and *prsa2* using pKSV-7 and pMAD, these efforts remained unsuccessful.

All seven in-frame mutants had the same growth rate as the WT in BHI at 37 °C and took an average of 3.32 h (sd = 0.2 h) to grow to an OD_{600} of 0.4 (*p*-value for the comparison of growth time to OD_{600} of 0.4 by mutant: 0.20).

While none of the seven in-frame mutants showed a growth defect at 37 °C in BHI, the *sau3AIM* ($p < 0.01$), *pbp-B* ($p < 0.01$) and *purB* ($p = 0.04$) mutants were impaired when grown under the tested stress conditions (Fig. 2).

The growth defect of the *sau3AIM* and *pbp-B* mutants was temperature dependent, since the *sau3AIM* and *pbp-B* mutants showed reduced fitness in BHI at 8 °C compared to the WT. This growth defect was also matrix dependent, since these mutants showed reduced fitness on the

Table 1
Genes that affected *L. monocytogenes* LL195 fitness on Lyoner.

Mutant Name	Locus Tag	Interval	Disrupted Gene	Protein Name	Growth Difference Compared to WT (log reduction)	Function
P28C2 P28C3 P28C10	BN389_03290	346,524 … 347,923	*sau3AIM*	Cytosine Specific DNA Modification Methlytransferase	No Survival	Methylates C on GATC sequence and protect the host DNA from cleavage
P21G11 P30C4	BN389_04850	493,081 … 495,132	*pbp-B*	Penicillin Binding Protein	No Survival	Has a role in the cell wall assembly, cell division and cell wall reshaping
P29H8	BN389_01150 BN389_01160	115,272 … 116,078, 116,100 … 117,011	*manY-manZ*	Phosphoenolpyruvate-dependent sugar phosphotransferase system	0,82 ± 0,2	A major carbohydrate active transport system, involves in mannose transport
P1B3	BN389_7980	796,753 … 797,505	Hypothetical Protein 1	–	0,76 ± 0,07	Similar to transcriptional regulators of the GnTr family in *L. monocytogenes*
P24A5	BN389_01040	99,035 … 104,914	Hypothetical Protein 2	–	0,72 ± 0,16	Similar to HisZ, a regulatory subunit of the ATP phosphoribosyltransferase
P18F2 P16F10	BN389_27,880	2,850,688 … 2,851,515	DNA Binding protein	– –	1,29 ± 0,40	Similar to XRE family transcriptional regulator
P16E1	BN389_22,520	2,301,560 … 2,302,441	*prsA2*	Post translocation chaperone	2,18 ± 0,61	Promotes the activity and stability of 2 virulence factors in *L. monocytogenes*
P27B9	BN389_18,000	1,826,027 … 1,824,717	*purB*	Adenylosuccinate lyase	1,11 ± 0,19	Takes part in the purine biosynthesis pathway, affect the colonization ability of *L. monocytogenes* in gastrointestinal tract
P16E1	BN389_22,530	2,303,471 … 2,302,482	*yham*	–	2,18 ± 0,61	3′ to 5′ exonuclease activity

Lyoner matrix at both temperatures compared to the WT. (Fig. 3).

On the other hand, the *purB* mutant had a fitness effect that was only apparent while growing on Lyoner matrix as shown by its significantly lower mean log fold change on Lyoner compared to the WT at both 8 °C and 25 °C. This mutation did not affect fitness in a temperature-dependent way, since its growth was the same as the WT in BHI at 37 °C and 8 °C.

3.4. Morphology of the mutant strains compared to the wild type

Given the growth differences of the *pbp-B, sau3AIM* and *purB* mutant under some or all tested stress conditions, we wondered whether these mutations might cause morphological changes in the cells. This hypothesis was based on the fact that PBP-B is involved in cell wall synthesis, and PurB is part of the purine synthesis pathway that has a broad involvement in cellular processes including the expression of cell wall components.

A Gram-stain revealed that while *pbp-B* mutant and WT cells grown at 12 °C and 37 °C looked normal, *purB* mutants had increased frequency of elongated cells when they were grown at 37 °C, and in *sau3AIM* mutants elongated cells were observed at both temperatures.

We therefore proceeded to analyze the cells grown at 37 °C by SEM (Fig. 4). These images show a high frequency of elongated cells of *purB* mutants that seem to have formed rudimentary division septa without fully separating the two daughter cells. While some incomplete divisions were also visible in the *sau3AIM* mutant cells, there was a high frequency of very long cells absent of any visible signs of attempts to divide. In contrast, the *pbp-B* mutant did not show any morphological differences compared to the WT.

4. Discussion

4.1. To ensure reliable results, independent confirmation of small volume screening of a transposon library is necessary

Out of 122 candidate mutants from the initial screen in the miniature "Lyoner model", 13 mutants were confirmed to have a phenotype when repeated on a larger scale (corresponding to a confirmation rate of 0.15). In comparable studies using high-throughput screening of individual transposon mutant libraries in *L. monocytogenes* followed by confirmation in larger volumes, comparable confirmation rates of 0.12 (range: 0.03–0.19) (Alonso et al., 2014; Hingston et al., 2015; Mains et al., 2021; Narayanan et al., 2022) were observed. Potential reasons for the relatively low confirmation rates possibly lay in the infeasibility of replicates in large screens and small volumes causing more variation, or a combination of the two. This highlights the need for confirmation of results from transposon insertion libraries in larger volumes and with in-frame deletion mutants. Potential reasons for the fact that we were unable to create three (*manY, ManZ and prsa2*) out of the 10 target mutants (Fig. 1) include the possibility that the transposon inserted in a way that left some residual function intact, whereas a in-frame deletion mutant would abolish all activity. It is also possible that the transposon insertion left some *cis*- or *trans*-acting regulatory sequences intact that we may have attempted to delete in the in-frame mutant. Strain-specific effects may be possible, since a *prsa2* mutant has been successfully created by others in *L. monocytogenes* 10403S (Alonzo & Freitag, 2010). In other organisms, a *manXYZ* mutant was viable in *Klebsiella pneumoniae* (Bieler et al., 2006).

4.2. The sau3AIM and pbp-B in-frame mutant showed a temperature-dependent phenotype

While the *sau3AIM* and *pbp-B* deletion mutants grew at the same rate to OD_{600} as the WT at 37 °C, the mutations affected fitness under all tested stress conditions compared to the WT. Taking these results into consideration, we concluded that these mutations cause a general growth defect under stress conditions. *L. monocytogenes* encodes five high-molecular-weight penicillin binding proteins (HMW PBPs): PBP-A1, PBP-A2, PBP-B1, PBP-B2, PBP-B3, that are all present in the LL195 genome (where they are annotated as *pbpF, pbpB, ponA, pbpC* and *pbpC*) (Weinmaier et al., 2013). The one identified here was annotated as penicillin-binding-protein-B (*pbp-B*) in LL195 and corresponds to *pbc-3/lmo0441* in EGDe and LMRG00133 in 10403S. In agreement with other authors (Rismondo et al., 2015), the in-frame deletion mutant for *pbp-B* did not have a discernible phenotype when grown in BHI at 37 °C. A deletion of the *Bacillus subtilis* homologue of *pbp-B*, *pbpC,* did not produce a phenotype either (Murray et al., 1996). However, to our knowledge none of these authors tested their mutants under cold stress. In this study, the *pbp-B* mutant showed at least a 2.5 $\log_{10}$ CFU/g reduction when incubated under any condition we tested under cold temperatures. This effect seems to be only present at cold temperatures, since Rismondo et al. (Rismondo et al., 2015) did not observe reduced

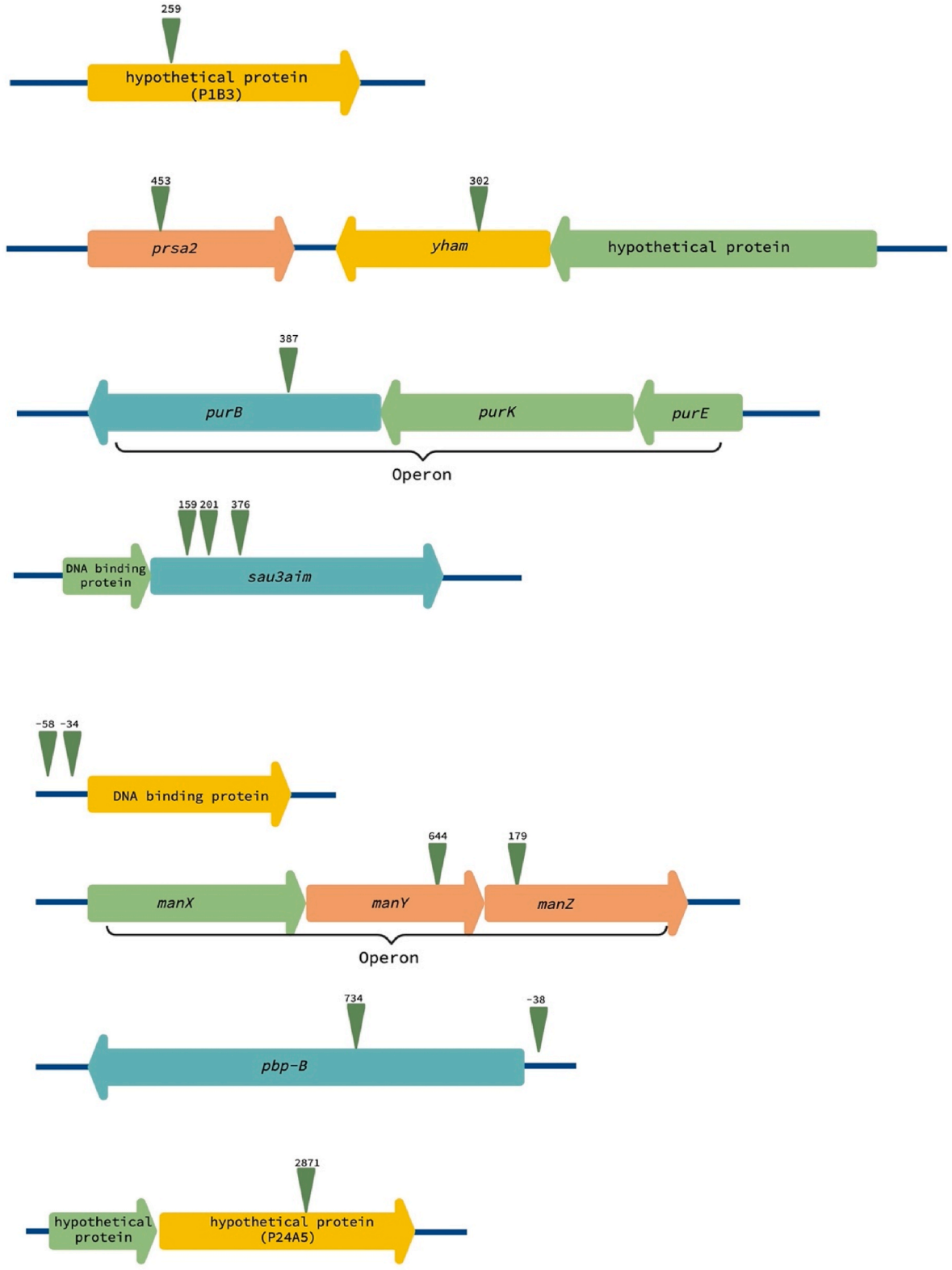

Fig. 1. Localization of transposon insertion sites. Upper green arrows represent sites of transposon insertions, and numbers above these arrows correspond to mapped transposon locations.

growth of a *pbp-3* mutant in EGD-e at 42 °C compared to 37 °C, and in accordance with this study the *pbp-B* mutant grown at 37 °C also showed normal morphology in SEM images (Fig. 4). A possible reason for this is that PBP-B might play a role in the reorganization of the cell wall during cold stress, and it might be interesting to see whether the observed cold phenotype aggravates in a cold-shock protein mutant background. Consistent with this theory suggesting a role in cell wall organization but not in cell division, PPB-3 localized to the cell wall, but not to the division septum (Guinane et al., 2006; Rismondo et al., 2015). Since PPB-3 has no transglycosylase domain (Rismondo et al., 2015), its role is solely

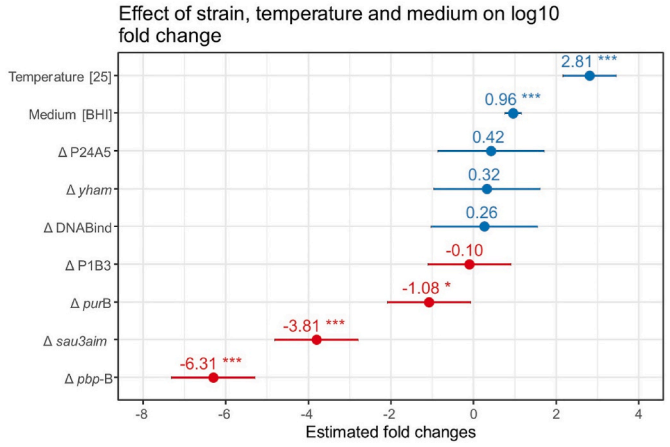

Fig. 2. Linear mixed effect model estimates for $\log_{10}$ fold changes of the in-frame mutants after growth for 48 h on Lyoner at 8 °C and 25 °C, as well as in BHI at 8 °C. Data points for mutants denoted by an asterisk were significantly different from the WT grown under the same condition. Data points for medium and temperature denoted with an asterisk were significantly different from the respective other condition, e.g. growth on Lyoner or growth at 8 °C.

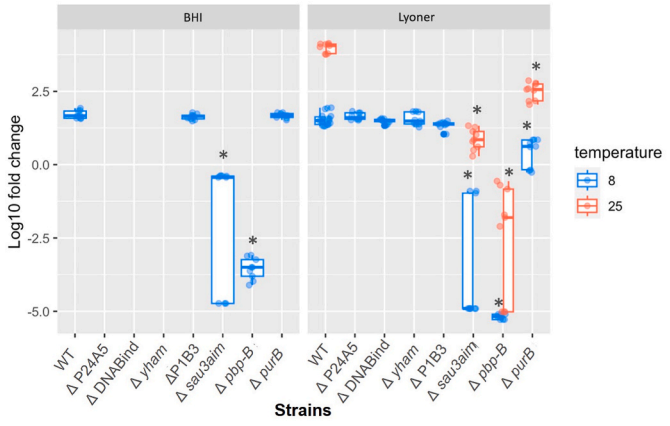

Fig. 3. Log fold change of the in-frame mutants after growth for 48 h on Lyoner at 8 °C and 25 °C, as well as in BHI at 8 °C. Data points denoted by an asterisk were significantly different from the WT grown under the same condition.

in the transpeptidation of peptidoglycan. Possibly, under standard laboratory conditions there might be functional redundancies between the different HMW PBPs, as suggested and demonstrated by other authors (Guinane et al., 2006; Rismondo et al., 2015) and supported by the normal morphology of the cells in SEM images. In this context, only PBP B2 has been demonstrated to be essential (Guinane et al., 2006; Rismondo et al., 2015). This redundancy may not be effective enough to balance the effect of a *pbp-B* mutation under the stress conditions tested here. Future studies on the expression of the different pbp's under cold conditions may clarify this question.

The *sau3AIM* gene encodes the methylase of a restriction-modification system that was first described in *Staphylococcus carnosus* (Seeber et al., 1990). Sau3AIM methylates the cysteine in the recognition site GATC which in turn prevents cleavage by the enzyme *Sau*3AI. In particular, *L. monocytogenes* strains with serotype 4b showed a high prevalence of the *sau3AIM* restriction-modification system in their genome (Yildirim et al., 2004), fitting with its presence in LL195 that is also a serotype 4b strain (Weinmaier et al., 2013).

One possible explanation for the reduced fitness of the *sau3AIM* mutant at low temperatures may be that the activity of the cognate restriction enzyme *Sau*3AI is temperature dependent. We might speculate that the *L. monocytogenes Sau*3AI is not active at higher temperatures and therefore cells might be able to tolerate the missing methylation by Sau3AIM due to the lack of restriction activity. This is, however, contradicted by the fact that commercially available *Sau*3AI restriction enzymes have a temperature optimum at 37 °C. Whether the *L. monocytogenes* Sau3AIM enzyme is an exception would have to be investigated.

Temperature-dependent R-M systems have been described in *L. monocytogenes* (Jae-Won & Sophia, 2009) as well as in other organisms, e.g., *Lactococcus lactis* (O'Driscoll et al., 2004) and *Streptococcus lactis* (Sanders & Klaenhammer, 1984). In *L. monocytogenes*, a temperature-dependent phage resistance that is mediated by a temperature-dependent R-M system (LmoH7) with the recognition site GTATCC (Kim et al., 2012) has been shown in ECII strains (Jae-Won & Sophia, 2009). In these strains, the optimal expression of the R-M system was at 19 °C and decreased with increasing temperatures.

Whether there is a link between the temperature-dependent phenotype of the *sau3AIM* and *pbp-B* mutant with established temperature-dependent regulatory systems in *L. monocytogenes* remains to be investigated. For instance, virulence gene expression in *L. monocytogenes* is regulated via a temperature-sensitive riboswitch in the transcriptional regulator PrfA that mediates virulence gene translation at host body temperatures of 37 °C, while this is suppressed at temperatures below 30 °C (Loh et al., 2009). It would be interesting to see whether the temperature-dependent effect of the *sau3AIM* and *pbp-B* mutants is affected in a PrfA* background where PrfA is constitutively active (Vega et al., 2004) or in a *prfA* null mutant. However, in the latest screen of the PrfA regulon at 37 °C, neither *sau3AIM* nor *pbp-B* were identified as PrfA dependent (Henderson et al., 2020).

4.3. PurB is important for growth on Lyoner

In contrast, the negative fitness effect of a *purB* deletion was only evident when the strains were grown on Lyoner, but not in BHI at either 8 or 37 °C. We therefore concluded that the product of *purB* is positively affecting growth on Lyoner, while it is not needed for efficient growth in BHI at optimal as well as cold temperatures. In *L. monocytogenes*, *purB* expression is transcriptionally co-regulated by the alternative sigma factors SigB and SigL at 3 °C in BHI but not at 37 °C (Mattila et al., 2020).

PurB is part of the purine biosynthesis pathway in *L. monocytogenes* (Ogata et al., 1999). Purines are essential for DNA replication, RNA transcription, and protein translation. Either of these mechanisms might be responsible for the elongated phenotype of *purB* mutant cells (Fig. 4) that we observed when they were grown at 37 °C. Bacteria can either synthesize purines *de novo* by a metabolically costly pathway, or through salvaging pre-formed nucleobases from the environment. PurB catalyzes two reactions within the *de novo* purine synthesis pathway (KEGG RN: R01083, RN:R04559 (Kanehisa & Sato, 2020), both of which yield fumarate as a side product. The *de novo* purine synthesis pathway is relevant for bacterial fitness in environments that do not offer abundant nucleobases, such as the intracellular environment during infection. Accordingly, attenuated virulence of purine biosynthesis mutants has been demonstrated in *L. monocytogenes* (Faith et al., 2012) as well as in *Salmonella* Typhimurium (McFarland & Stocker, 1987), *Brucella abortus* (Alcantara et al., 2004), *Brucella melitensis* (Crawford et al., 1996), *E. coli* (Shaffer et al., 2017) and *Staphylococcus aureus* (Connolly et al., 2017). *L. monocytogenes* auxotrophs for purine biosynthesis have also shown severe fitness effects when grown in minimal medium supplemented with single carbon sources (Narayanan et al., 2022) and in porcine bile (Dowd et al., 2011).

Since both Lyoner and BHI as growth substrates are based on meat that should offer an abundance of exogenous nucleobases, it is unlikely that *L. monocytogenes* would meet a shortage of nucleobases that results in the need for *de novo* purine biosynthesis under the observed growth conditions. Also, a polar effect of the *purB* mutation on other genes within the same transcriptional unit can be excluded since the phenotype was confirmed in a in-frame, non-polar *purB* mutant. It is possible

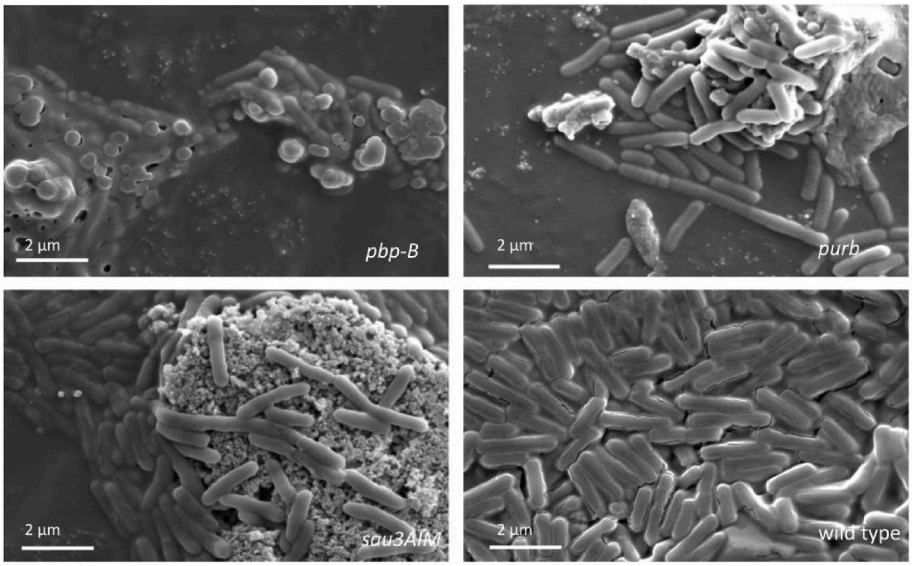

Fig. 4. SEM images of the *pbp-B, purB, sau3AIM* mutants and the WT, grown at 37°

that the observed fitness effect of the *purB* mutant during growth on Lyoner is due to an inability to efficiently use the exogenous purines present in meat, the lack of fumarate that is produced by the reactions catalyzed by PurB or due to other, yet unknown pleiotropic effects of PurB. We therefore hypothesize that some difference between the growth condition in Lyoner and BHI triggers a fitness effect due to the absence of *purB* in the mutant.

There are obvious differences in the composition and nutrient supply provided by a complex food matrix like Lyoner sausage and BHI as a laboratory medium. Lyoner sausage is specified as a cured (1.6–1.9 % nitrite curing salts) cooked sausage containing tendon-free meat, a fat content of around 20%, and a pH of 6–6.2, according to the German specifications for meat products (Landwirtschaft, 2022). Carbon sources are limited in meat, which is addressed by supplementing BHI medium with glucose as a carbon source.

Therefore, Lyoner in comparison to BHI has a lower pH (pH Lyoner: around 6, BHI: 7.4), higher fat and salt content (Lyoner: NaCl approximately 0.3 M NaCl, BHI: 0.08 M) and offers a lower abundance of carbon sources. In addition, various spices added to Lyoner sausage may exert stress on the microbial populations in Lyoner sausage (Liu et al., 2017). It is possible that either of these differences may cause the observed growth defect of the *purB* mutant.

These differences may also affect the pathways responsible for purine import into the cell. In the absence of *de novo* purine synthesis in a *purB* mutant, *L. monocytogenes* would have to rely solely on the import of nucleobases via the purine salvage pathway. Several genes have previously been shown to participate in this pathway: *lmo 1845* (Krawczyk-Balska et al., 2021), *lmo 1884* (Krajewski et al., 2017), *lmo 1885* (Knudsen et al., 2016), *lmo0573* (Ellinger et al., 2023), *pyrR* (Knudsen et al., 2016), *lmo2254* (Ledala et al., 2010), *lmo0456* (Fischer et al., 2022) and *lmo1839/pyrP* (Fischer et al., 2022). One might speculate that some of the specific conditions during growth in Lyoner may render this pathway ineffective and therefore be responsible for the observed phenotype. Some limited information is available on the regulatory mechanisms of the above genes. *lmo 1845* (Wurtzel et al., 2012), *lmo1885* and *lmo0573* (Ellinger et al., 2023) depend on riboswitches for their expression. *Lmo1885* was differentially expressed after exposure to sublethal concentrations of tetracycline and co-trimoxazole (Knudsen et al., 2016). *Lmo0573* was downregulated under anaerobic conditions (Ferrari et al., 2017) and upregulated in BHI compared to during host infection (Eimerman, 2011). Lmo2254 was downregulated in biofilms (Assisi et al., 2021), in response to iron limitation (Ledala et al., 2010), and during acid exposure (Li, 2020). This last regulatory mechanism might hint towards the purine import pathway being less effective at pH 6.4 than pH 7.4.

In summary, our study shows a role of the methylase Sau3AIM and the penicillin binding protein Pbp-3 for the growth of *L. monocytogenes* under food-associated stress conditions and of the adenylsuccinate lyase *purB* specifically for efficient growth of *L. monocytogenes* LL195 in Lyoner, a food matrix that is highly relevant as a vehicle for human infections with *L. monocytogenes*. This effect may in the future be exploited for highly targeted interventions to increase food safety. For instance, small molecule libraries of substances that are generally recognized as safe (GRAS) by the FDA may be screened for their effect on *purB* expression and provide an inhibitor that could serve as a food additive to reduce the fitness of *L. monocytogenes* in Lyoner and similar products like Polony. Currently, the use of designer temperate phage is being discussed as a potential strategy to mitigate the antibiotic resistance crisis in human therapy (Jaroszewicz et al., 2022). Once integrated into the host genome, these designer prophages are engineered to be unable to excise from the host genome to repress phage-mediated spread of antibiotic resistance, while they express repressors of virulence gene expression and therefore decrease virulence of the host bacteria. These same mechanisms may also be put to future use to reduce the fitness of pathogens in food and to complement the use of strictly lytic phage, thus broadening the available tools. However, these novel strategies are currently only applicable in experimental setups due to a lack of data on the long-term interaction of designer prophages with their hosts as well as regulatory hurdles.

CRediT authorship contribution statement

Philipp-Michael Beindorf: Writing – original draft, Methodology, Investigation, Data curation. **Jule Anna Horlbog:** Writing – review & editing, Methodology. **Irene Esteban Cuesta:** Writing – review & editing, Formal analysis, Conceptualization. **Claudia Guldimann:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Irmak Şah:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

5. Acknowledgements

We thank Dan Portnoy for sharing pJZ037 to create the transposon library and Barbara Fritz for her valuable help with the experiments. We thank Gebze Technical University (GTU) for SEM Analysis. We thank Eugen Gorich and Henri Funk from LMU Munich, Institute of Statistics for their valuable contribution on statistical analysis of the data.

This work was supported by the DFG (Deutsche Forschungsgemeinschaft) (Project number: 494875429) and the Adalbert RAPS Stiftung.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110393.

References

Alcantara, R. B., Read, R. D. A., Valderas, M. W., Brown, T. D., & Roop, R. M. (2004). Intact purine biosynthesis pathways are required for wild-type virulence of *Brucella abortus* 2308 in the BALB/c mouse model. *Infection and Immunity, 72*(8). https://doi.org/10.1128/IAI.72.8.4911-4917.2004

Alonso, A. N., Perry, K. J., Regeimbal, J. M., Regan, P. M., & Higgins, D. E. (2014). Identification of *Listeria monocytogenes* determinants required for biofilm formation. *PLoS One, 9*(12). https://doi.org/10.1371/journal.pone.0113696

Alonzo, F., & Freitag, N. E. (2010). *Listeria monocytogenes* PrsA2 is required for virulence factor secretion and bacterial viability within the host cell cytosol. *Infection and Immunity, 78*(11). https://doi.org/10.1128/IAI.00532-10

Arnaud, M., Chastanet, A., & Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Applied and Environmental Microbiology, 70*(11). https://doi.org/10.1128/AEM.70.11.6887-6891.2004

Assisi, C., Forauer, E., Oliver, H. F., & Etter, A. J. (2021). Genomic and transcriptomic analysis of biofilm formation in persistent and transient *Listeria monocytogenes* isolates from the retail deli environment does not yield insight into persistence mechanisms. *Foodborne Pathogens and Disease, 18*(3). https://doi.org/10.1089/fpd.2020.2817

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bécavin, C., Bouchier, C., Lechat, P., Archambaud, C., Creno, S., Gouin, E., Wu, Z., Kühbacher, A., Brisse, S., Graciela Pucciarelli, M., García-del Portillo, F., Hain, T., Portnoy, D. A., Chakraborty, T., Lecuit, M., Pizarro-Cerdá, J., Moszer, I., Bierne, H., & Cossart, P. (2014). Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e highlights genomic differences underlying variations in pathogenicity. *mBio, 5*(2). https://doi.org/10.1128/mBio.00969-14

Bieler, S., Silva, F., Soto, C., & Belin, D. (2006). Bactericidal activity of both secreted and nonsecreted microcin E492 requires the mannose permease. *Journal of Bacteriology, 188*(20). https://doi.org/10.1128/JB.00688-06

Bucur, F. I., Grigore-Gurgu, L., Crauwels, P., Riedel, C. U., & Nicolau, A. I. (2018). Resistance of *Listeria monocytogenes* to stress conditions encountered in food and food processing environments. *Frontiers in Microbiology, 9*(NOV). https://doi.org/10.3389/fmicb.2018.02700

Chaturongakul, S., Raengpradub, S., Wiedmann, M., & Boor, K. J. (2008). Modulation of stress and virulence in *Listeria monocytogenes*. *Trends in Microbiology, 16*(8). https://doi.org/10.1016/j.tim.2008.05.006

Churchill, K. J., Sargeant, J. M., Farber, J. M., & O'connor, A. M. (2019). Prevalence of *Listeria monocytogenes* in select ready-to-eat foods—deli meat, soft cheese, and packaged salad: A systematic review and meta-analysis. *Journal of Food Protection, 82*(2). https://doi.org/10.4315/0362-028X.JFP-18-158

Connolly, J., Boldock, E., Prince, L. R., Renshaw, S. A., Whyte, M. K., & Foster, S. J. (2017). Identification of *Staphylococcus aureus* factors required for pathogenicity and growth in human blood. *Infection and Immunity, 85*(11). https://doi.org/10.1128/IAI.00337-17

Crawford, R. M., Van De Verg, L., Yuan, L., Hadfield, T. L., Warren, R. L., Drazek, E. S., Houng, H. S. H., Hammack, C., Sasala, K., Polsinelli, T., Thompson, J., & Hoover, D. L. (1996). Deletion of purE attenuates *Brucella melitensis* infection in mice. *Infection and Immunity, 64*(6). https://doi.org/10.1128/iai.64.6.2188-2192.1996

Dowd, G. C., Joyce, S. A., Hill, C., & Gahan, C. G. M. (2011). Investigation of the mechanisms by which *Listeria monocytogenes* grows in porcine gallbladder bile. *Infection and Immunity, 79*(1). https://doi.org/10.1128/IAI.00330-10

Eimerman, P. R. (2011). *Characterization of Listeria monocytogenes growth and colonization of the murine gallbladder*. Stanford University.

Ellinger, E., Chauvier, A., Romero, R. A., Liu, Y., Ray, S., & Walter, N. G. (2023). Riboswitches as therapeutic targets: Promise of a new era of antibiotics. *Expert Opinion on Therapeutic Targets, 27*(Issue 6). https://doi.org/10.1080/14728222.2023.2230363

The European union one health 2021 Zoonoses report. *EFSA Journal, 20*(12), (2022). https://doi.org/10.2903/j.efsa.2022.7666

Faith, N. G., Kim, J. W., Azizoglu, R., Kathariou, S., & Czuprynski, C. (2012). Purine biosynthesis mutants (purA and purB) of serotype 4b *Listeria monocytogenes* are severely attenuated for systemic infection in intragastrically inoculated A/J mice. *Foodborne Pathogens and Disease, 9*(5). https://doi.org/10.1089/fpd.2011.1013

Feder, M. E., & Walser, J. C. (2005). The biological limitations of transcriptomics in elucidating stress and stress responses. *Journal of Evolutionary Biology, 18*(4). https://doi.org/10.1111/j.1420-9101.2005.00921.x

Ferrari, E., Walter, M. C., Huptas, C., Scherer, S., & Müller-Herbst, S. (2017). Complete circular genome sequence and temperature independent adaptation to anaerobiosis of *Listeria weihenstephanensis* DSM 24698. *Frontiers in Microbiology, 8*(SEP). https://doi.org/10.3389/fmicb.2017.01672

Fischer, M. A., Engelgeh, T., Rothe, P., Fuchs, S., Thürmer, A., & Halbedel, S. (2022). *Listeria monocytogenes* genes supporting growth under standard laboratory cultivation conditions and during macrophage infection. *Genome Research, 32*(9). https://doi.org/10.1101/gr.276747.122

Fux, C. A., Shirtliff, M., Stoodley, P., & Costerton, J. W. (2005). Can laboratory reference strains mirror 'real-world' pathogenesis? *Trends in Microbiology, 13*(2), 58–63. https://doi.org/10.1016/j.tim.2004.11.001

Guerreiro, D. N., Arcari, T., & O'Byrne, C. P. (2020). The σb-mediated general stress response of *Listeria monocytogenes*: Life and death decision making in a pathogen. *Frontiers in Microbiology, 11*. https://doi.org/10.3389/fmicb.2020.01505

Guinane, C. M., Cotter, P. D., Ross, R. P., & Hill, C. (2006). Contribution of penicillin-binding protein homologs to antibiotic resistance, cell morphology, and virulence of *Listeria monocytogenes* EGDe. *Antimicrobial Agents and Chemotherapy, 50*(8). https://doi.org/10.1128/AAC.00167-06

Henderson, L. O., Gaballa, A., Orsi, R. H., Boor, K. J., Wiedmann, M., & Guariglia-Oropeza, V. (2020). Transcriptional profiling of the *L. monocytogenes* PrfA regulon identifies six novel putative PrfA-regulated genes. *FEMS Microbiology Letters, 367*(22). https://doi.org/10.1093/femsle/fnaa193

Hingston, P. A., Piercey, M. J., & Hansen, L. T. (2015). Genes associated with desiccation and osmotic stress in *Listeria monocytogenes* as revealed by insertional mutagenesis. *Applied and Environmental Microbiology, 81*(16). https://doi.org/10.1128/AEM.01134-15

Jae-Won, K., & Sophia, K. (2009). Temperature-dependent phage resistance of *Listeria monocytogenes* epidemic clone II. *Applied and Environmental Microbiology, 75*(8), 2433–2438. https://doi.org/10.1128/AEM.02480-08

Jaroszewicz, W., Morcinek-Orłowska, J., Pierzynowska, K., Gaffke, L., & Węgrzyn, G. (2022). Phage display and other peptide display technologies. *FEMS Microbiology Reviews, 46*(2). https://doi.org/10.1093/femsre/fuab052

Kanehisa, M., & Sato, Y. (2020). KEGG Mapper for inferring cellular functions from protein sequences. *Protein Science, 29*(1). https://doi.org/10.1002/pro.3711

Kim, J. W., Dutta, V., Elhanafi, D., Lee, S., Osborne, J. A., & Kathariou, S. (2012). A novel restriction-modification system is responsible for temperature-dependent phage resistance in *Listeria monocytogenes* ECII. *Applied and Environmental Microbiology, 78*(6). https://doi.org/10.1128/AEM.07086-11

Knudsen, G. M., Fromberg, A., Ng, Y., & Gram, L. (2016). Sublethal concentrations of antibiotics cause shift to anaerobic metabolism in *Listeria monocytogenes* and induce phenotypes linked to antibiotic tolerance. *Frontiers in Microbiology, 7*(JUL). https://doi.org/10.3389/fmicb.2016.01091

Krajewski, S. S., Isoz, I., & Johansson, J. (2017). Antibacterial and antivirulence effect of 6-N-hydroxylaminopurine in *Listeria monocytogenes*. *Nucleic Acids Research, 45*(4). https://doi.org/10.1093/nar/gkw1308

Krawczyk-Balska, A., Ładziak, M., Burmistrz, M., Ścibek, K., & Kallipolitis, B. H. (2021). RNA-mediated control in *Listeria monocytogenes*: Insights into regulatory mechanisms and roles in metabolism and virulence. *Frontiers in Microbiology, 12*. https://doi.org/10.3389/fmicb.2021.622829

Küchbacher, A., Cossart, P., & Pizarro-Cerda, J. (2021). Internalization assays for *Listeria monocytogenes*. In E. M. Fox, H. Bierne, & B. Stessl (Eds.), *Methids in molecular biology: Vol. 2220*. *Listeria monocytogenes*. New York, NY: Humana. https://doi.org/10.1007/978-1-0716-0982-8_15.

Landwirtschaft, B.f. E.u. (2022). *Leitsätze für Fleisch und Fleischerzeugnisse*. https://Www.Bmel.de/SharedDocs/Downloads/DE/_Ernaehrung/Lebensmittel-Kennzeichnung/LeitsaetzeFleisch.Html.

Ledala, N., Sengupta, M., Muthaiyan, A., Wilkinson, B. J., & Jayaswal, R. K. (2010). Transcriptomic response of *Listeria monocytogenes* to iron limitation and Fur mutation. *Applied and Environmental Microbiology, 76*(2). https://doi.org/10.1128/AEM.01389-09

Li, M. (2020). *Exploring the connection between acid exposure and virulence in Listeria monocytogenes*. Utah State University.

Liu, Q., Meng, X., Li, Y., Zhao, C. N., Tang, G. Y., & Li, H. B. (2017). Antibacterial and antifungal activities of spices. *International Journal of Molecular Sciences, 18*(Issue 6). https://doi.org/10.3390/ijms18061283

Loh, E., Dussurget, O., Gripenland, J., Vaitkevicius, K., Tiensuu, T., Mandin, P., Repoila, F., Buchrieser, C., Cossart, P., & Johansson, J. (2009). A trans-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. *Cell, 139*(4). https://doi.org/10.1016/j.cell.2009.08.046

Lopez-Valladares, G., Danielsson-Tham, M. L., & Tham, W. (2018). Implicated food products for listeriosis and changes in serovars of *Listeria monocytogenes* affecting humans in recent decades. *Foodborne Pathogens and Disease, 15*(7). https://doi.org/10.1089/fpd.2017.2419

Lüdecke, D. (2023). *sjPlot: Data visualization for statistics in social science.* R Package Version 2.8.14.

Mains, D. R., Eallonardo, S. J., & Freitag, N. E. (2021). Identification of *Listeria monocytogenes* genes contributing to oxidative stress resistance under conditions relevant to host infection. *Infection and Immunity, 89*(4). https://doi.org/10.1128/IAI.00700-20

Mattila, M., Somervuo, P., Korkeala, H., Stephan, R., & Tasara, T. (2020). Transcriptomic and phenotypic analyses of the sigma b-dependent characteristics and the synergism between sigma b and sigma l in *Listeria monocytogenes* EGD-e. *Microorganisms, 8*(11). https://doi.org/10.3390/microorganisms8111644

McFarland, W. C., & Stocker, B. A. D. (1987). Effect of different purine auxotrophic mutations on mouse-virulence of a Vi-positive strain of Salmonella dublin and of two strains of Salmonella typhimurium. *Microbial Pathogenesis, 3*(2). https://doi.org/10.1016/0882-4010(87)90071-4

Murray, T., Popham, D. L., & Setlow, P. (1996). Identification and characterization of pbpC, the gene encoding *Bacillus subtilis* penicillin-binding protein 3. *Journal of Bacteriology, 178*(20). https://doi.org/10.1128/jb.178.20.6001-6005.1996

Narayanan, L., Ozdemir, O., Alugubelly, N., Ramachandran, R., Banes, M., Lawrence, M., & Abdelhamed, H. (2022). Identification of genetic elements required for *Listeria monocytogenes* growth under limited nutrient conditions and virulence by a screening of transposon insertion library. *Frontiers in Microbiology, 13*. https://doi.org/10.3389/fmicb.2022.1007657

O'Driscoll, J., Glynn, F., Cahalane, O., O'Connell-Motherway, M., Fitzgerald, G. F., & Van Sinderen, D. (2004). Lactococcal plasmid pNP40 encodes a novel, temperature-sensitive restriction-modification system. *Applied and Environmental Microbiology, 70*(9). https://doi.org/10.1128/AEM.70.9.5546-5556.2004

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., & Kanehisa, M. (1999). Kegg: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research, 27*(1). https://doi.org/10.1093/nar/27.1.29

Olesen, I., Thorsen, L., & Jespersen, L. (2010). Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. *International Journal of Food Microbiology, 141*(SUPPL). https://doi.org/10.1016/j.ijfoodmicro.2010.01.042

Rantsiou, K., Greppi, A., Garosi, M., Acquadro, A., Mataragas, M., & Cocolin, L. (2012). Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes* in meat juices as determined by microarray. *International Journal of Food Microbiology, 152*(3). https://doi.org/10.1016/j.ijfoodmicro.2011.08.009

Rantsiou, K., Mataragas, M., Alessandria, V., & Cocolin, L. (2012). Expression of virulence genes of *Listeria monocytogenes* in food. *Journal of Food Safety, 32*(2). https://doi.org/10.1111/j.1745-4565.2011.00363.x

Rismondo, J., Möller, L., Aldridge, C., Gray, J., Vollmer, W., & Halbedel, S. (2015). Discrete and overlapping functions of peptidoglycan synthases in growth, cell division and virulence of *Listeria monocytogenes*. *Molecular Microbiology, 95*(2). https://doi.org/10.1111/mmi.12873

RStudio Team. (2015). *RStudio: Integrated development for R. RStudio, Inc*, 2015. Boston, MA.

Sampedro, F., Pérez-Rodríguez, F., Servadio, J. L., Gummalla, S., & Hedberg, C. W. (2022). Quantitative risk assessment model to investigate the public health impact of varying *Listeria monocytogenes* allowable levels in different food commodities: A retrospective analysis. *International Journal of Food Microbiology, 383*. https://doi.org/10.1016/j.ijfoodmicro.2022.109932

Sanders, M. E., & Klaenhammer, T. R. (1984). Phage resistance in a phage-insensitive strain of *Streptococcus lactis*: Temperature-dependent phage development and host-controlled phage replication. *Applied and Environmental Microbiology, 47*(5). https://doi.org/10.1128/aem.47.5.979-985.1984

Seeber, S., Kessler, C., & Götz, F. (1990). Cloning, expression and characterization of the Sau3AI restriction and modification genes in *Staphylococcus carnosus* TM300. *Gene, 94*(1). https://doi.org/10.1016/0378-1119(90)90465-4

Shaffer, C. L., Zhang, E. W., Dudley, A. G., Dixon, B. R. E. A., Guckes, K. R., Breland, E. J., Floyd, K. A., Casella, D. P., Algood, H. M. S., Clayton, D. B., & Hadjifrangiskou, M. (2017). Purine biosynthesis metabolically constrains intracellular survival of uropathogenic *Escherichia coli*. *Infection and Immunity, 85*(1). https://doi.org/10.1128/IAI.00471-16

Sheen, S., & Hwang, C. A. (2008). Modeling transfer of *Listeria monocytogenes* from slicer to deli meat during mechanical slicing. *Foodborne Pathogens and Disease, 5*(2). https://doi.org/10.1089/fpd.2007.0049

Smith, A. M., Tau, N. P., Smouse, S. L., Allam, M., Ismail, A., Ramalwa, N. R., Disenyeng, B., Ngomane, M., & Thomas, J. (2019). Outbreak of *Listeria monocytogenes* in South Africa, 2017-2018: Laboratory activities and experiences associated with whole-genome sequencing analysis of isolates. *Foodborne Pathogens and Disease, 16*(7). https://doi.org/10.1089/fpd.2018.2586

Smith, K., & Youngman, P. (1992). Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis* spollM gene. *Biochimie, 74*(7–8). https://doi.org/10.1016/0300-9084(92)90143-3

Spanu, C., Scarano, C., Ibba, M., Pala, C., Spanu, V., & De Santis, E. P. L. (2014). Microbiological challenge testing for *Listeria monocytogenes* in ready-to-eat food: A practical approach. *Italian Journal of Food Safety, 3*(4). https://doi.org/10.4081/ijfs.2014.4518

Vega, Y., Rauch, M., Banfield, M. J., Ermolaeva, S., Scortti, M., Goebel, W., & Vázquez-Boland, J. A. (2004). New *Listeria monocytogenes* prfA* mutants, transcriptional properties of PrfA* proteins and structure-function of the virulence regulator PrfA. *Molecular Microbiology, 52*(6). https://doi.org/10.1111/j.1365-2958.2004.04052.x

Weinmaier, T., Riesing, M., Rattei, T., Bille, J., Arguedas-Villa, C., Stephan, R., & Tasara, T. (2013). Complete genome sequence of *Listeria monocytogenes* LL195, a serotype 4b strain from the 1983-1987 listeriosis epidemic in Switzerland. *Genome Announcements, 1*(1). https://doi.org/10.1128/genomeA.00152-12

Wickham, H. (2011). The split-apply-combine strategy for data analysis. *Journal of Statistical Software, 40*(1). https://doi.org/10.18637/jss.v040.i01

Wickham, H. (2016). ggplot2: Elegant graphics for data analysis. In , *Vol. 35. Media*. New York: Springer-Verlag. Issue July).

Wurtzel, O., Sesto, N., Mellin, J. R., Karunker, I., Edelheit, S., Bécavin, C., Archambaud, C., Cossart, P., & Sorek, R. (2012). Comparative transcriptomics of pathogenic and non-pathogenic Listeria species. *Molecular Systems Biology, 8*(1). https://doi.org/10.1038/msb.2012.11

Yildirim, S., Lin, W., Hitchins, A. D., Jaykus, L. A., Altermann, E., Klaenhammer, T. R., & Kathariou, S. (2004). Erratum: Epidemic clone I-specific genetic markers in strains of *Listeria monocytogenes* serotype 4b from foods (Applied and Environmental Microbiology (2004) 70:7 (4158-4164)) *Applied and Environmental Microbiology, 70*(12). https://doi.org/10.1128/AEM.70.12.7581.2004.

Zemansky, J., Kline, B. C., Woodward, J. J., Leber, J. H., Marquis, H., & Portnoy, D. A. (2009). Development of a mariner-based transposon and identification of *Listeria monocytogenes* determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype. *Journal of Bacteriology, 191*(12). https://doi.org/10.1128/JB.00016-09