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RESEARCH ARTICLE

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Characterisation of the growth behaviour of *Listeria monocytogenes* in *Listeria* synthetic media

Lisa Maria Schulz | Alicia Konrath | Jeanine Rismondo

Department of General Microbiology, Institute of Microbiology and Genetics, GZMB, Georg-August University Göttingen, Göttingen, Germany

Correspondence
Jeanine Rismondo, Department of General Microbiology, Institute of Microbiology and Genetics, GZMB, Georg-August University Göttingen, Grisebachstr. 8, 37077 Göttingen, Germany.
Email: jrismon@gwdg.de

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Abstract

The foodborne pathogen *Listeria monocytogenes* can grow in a wide range of environmental conditions. For the study of the physiology of this organism, several chemically defined media have been developed over the past decades. Here, we examined the ability of *L. monocytogenes* wildtype strains EGD-e and 10403S to grow under salt and pH stress in *Listeria* synthetic medium (LSM). Furthermore, we determined that a wide range of carbon sources could support the growth of both wildtype strains in LSM. However, for hexose phosphate sugars such as glucose-1-phosphate, both *L. monocytogenes* strains need to be pre-grown under conditions, where the major virulence regulator PrfA is active. In addition, growth of both *L. monocytogenes* strains was observed when LSM was supplemented with the amino acid sugar *N*-acetylmannosamine (Man*N*Ac). We were able to show that some of the proteins encoded in the operon *lmo2795-nanE*, such as the Man*N*Ac-6-phosphate epimerase NanE, are required for growth in the presence of Man*N*Ac. The first gene of the operon, *lmo2795*, encodes a transcriptional regulator of the RpiR family. Using electrophoretic mobility shift assays and quantitative real-time PCR analysis, we were able to show that Lmo2795 binds to the promoter region of the operon *lmo2795-nanE* and activates its expression.

INTRODUCTION

The foodborne pathogen *Listeria monocytogenes* is a Gram-positive, facultative anaerobic, rod-shaped bacterium, which is ubiquitously found in nature. It can survive as a saprophyte on decaying plant material, in soil or wastewater. *L. monocytogenes* and other *Listeria* species can also enter food processing facilities and food chains, for instance, due to contaminated water or raw materials (Lourenco et al., 2022). Several recent outbreaks of *L. monocytogenes* were associated with contaminated fruits and vegetables, such as melons, apples or mushrooms or contaminated meat products (Lachmann et al., 2021; Matle et al., 2020; Townsend et al., 2021). The intracellular pathogen *L. monocytogenes* is the causative agent of the disease listeriosis. In healthy individuals, the intake of *L. monocytogenes* via the consumption of contaminated food products mostly leads to mild symptoms, such as fever and self-limiting gastroenteritis. However, for immunocompromised individuals, the elderly, newborns and pregnant women, the infection with *L. monocytogenes* can cause more severe symptoms, such as muscle aches, vomiting, diarrhoea, encephalitis or meningitis. For pregnant women, it can also lead to premature or still birth (Radoshevich & Cossart, 2018). This is achieved by its ability to cross all human barriers, namely the intestinal, placental and blood–brain barriers (Lecuit, 2005).

The survival of *L. monocytogenes* in food processing facilities and within the host is enabled due to its ability to grow under diverse stress conditions (reviewed in (Gaballa et al., 2019; Osek et al., 2022; Wiktorczyk-Kapischke et al., 2021)). Previous studies reported that *L. monocytogenes* can grow at

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temperatures between 1 and 45°C, in a pH range from 4.0 to 9.5 or in the presence of up to 10% salt (Arizcun et al., 1998; Patchett et al., 1992; Vasseur et al., 2001). *L. monocytogenes* is also able to use a variety of carbon sources ranging from simple monosaccharides (glucose and fructose) to more complex sugars (maltodextrin) and polyols (glycerol, D-arabitol and D-xylitol) (Deutscher et al., 2014; Gopal et al., 2010; Kentache et al., 2016). The ability to take up such a wide range of carbon sources is facilitated by a diverse set of transporters, which are encoded in the genome of *L. monocytogenes*. Most of the carbon sources are imported into the cell via the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS). The genome of *L. monocytogenes* wildtype strain EGD-e contains 86 PTS genes, which encode for 29 complete and 12 incomplete PTS complexes (Glaser et al., 2001; Stoll & Goebel, 2010). These PTS transporters are involved in the transport of diverse carbon sources such as glucose, fructose, D-arabitol and D-xylitol (Deutscher et al., 2014; Kentache et al., 2016). Some of these PTS transporters also have overlapping substrate specificities. For instance, the two PTS systems ManLMN (MptACD) and MpoABCD, encoded by *lmo0096-0098* and *lmo0784-0781*, respectively, are both involved in the transport of glucose and mannose (Aké et al., 2011; Stoll & Goebel, 2010). Inactivation of all PTS systems, e.g. by deleting the PTS enzyme I encoding gene *ptsI*, did not abolish fermentation of some sugars such as glucose, fructose or mannose, suggesting that *L. monocytogenes* possesses also other sugar transporters (Deutscher et al., 2014). Indeed, *L. monocytogenes* encodes three GlcU-like non-PTS permeases (Lmo0169, Lmo0176 and Lmo0424), which could be involved in the import of these sugars (Aké et al., 2011; Deutscher et al., 2014). The import of L-rhamnose is accomplished by the major facilitator superfamily protein Lmo2850, which uses a proton symport mechanism (Deutscher et al., 2014; Fieseler et al., 2012; Zeng et al., 2021). Within the host, *L. monocytogenes* uses mainly glucose-1-phosphate, glucose-6-phosphate and glycerol as carbon sources (Grubmüller et al., 2014; Ripio et al., 1997). The transporter Hpt, also named UhpT, takes up hexose phosphates from the host cell cytosol (Chico-Calero et al., 2002), while the glycerol uptake facilitator GlpF facilitates the import of glycerol (Joseph et al., 2008). The expression of *hpt* thereby depends on PrfA, the main virulence regulator of *L. monocytogenes* (Chico-Calero et al., 2002; Ripio et al., 1997). *L. monocytogenes* possesses eight predicted carbohydrate ATP-binding cassette (ABC) transporters. One of these transporters, which is encoded by *lmo2123-2125*, is required for the import of maltose and maltodextrin (Gopal et al., 2010). The remaining seven ABC transporters have not yet been studied.

For many studies on the physiology and stress tolerance of *L. monocytogenes*, bacteria were cultured in a complex medium such as brain heart infusion (BHI) or tryptic soy broth (TSB). In their natural habitat or within their host, *L. monocytogenes* likely encounters more limiting growth conditions. Within the last decades, several minimal media have been developed which can be used to grow *L. monocytogenes* (Jarvis et al., 2016; Phan-Thanh & Gormon, 1997; Premaratne et al., 1991; Tsai & Hodgson, 2003; Welshimer, 1963; Whiteley et al., 2017). In this study, we aimed to characterise the growth of the two widely used *L. monocytogenes* wildtype strains EGD-e and 10403S, which belong to serotype 1/2a, in the recently developed *Listeria* synthetic medium (LSM) (Whiteley et al., 2017). *L. monocytogenes* strains belonging to serotype 1/2a are often recovered from food, environmental and clinical samples (Orsi et al., 2011; Ward et al., 2008) and thus encounter diverse environmental conditions. We thus determined the growth behaviour of EGD-e and 10403S in LSM under stress conditions, which they encounter in food or the environment or within the host during infection, namely high salt and low pH stress. To our knowledge, the growth of *L. monocytogenes* in LSM was so far only assessed with glucose as a sole carbon source. We therefore determined, which other carbon sources can facilitate the growth of *L. monocytogenes* EGD-e and 10403S in LSM. While 95% of the open reading frames are conserved between EGD-e and 10403S, the genomes differ by over 30,000 single nucleotide polymorphisms (Bécavin et al., 2014; Glaser et al., 2001). It is, thus, not surprising that we could observe differences in the growth behaviour between these two strains.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium and *Listeria monocytogenes* strains in BHI medium or LSM at 37°C unless otherwise stated. Where required, antibiotics and supplements were added to the medium at the following concentrations: for *E. coli* cultures, ampicillin (Amp) at 100 μg mL^{-1} and kanamycin (Kan) at 50 μg mL^{-1}, and for *L. monocytogenes* cultures, chloramphenicol (Cam) at 7.5 μg mL^{-1} and Kan at 50 μg mL^{-1}.

LSM was prepared in accordance with the recipe from Whiteley et al. with minor modifications (Whiteley et al., 2017). L-leucine, L-methionine and L-valine were used instead of the corresponding DL-enantiomers. L-arginine·HCl, riboflavin, $Na_2HPO_4 \cdot 7\ H_2O$, $CoCl_2 \cdot 7\ H_2O$ and L-cysteine·2·HCl were substituted for L-arginine, riboflavin 5′ monophosphate sodium salt

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TABLE 1 Bacterial strains used in this study.

Unique ID	Strain name and resistance	Source
Escherichia coli strains		
ANG1264	DH5α pKSV7; AmpR	(Smith & Youngman, 1992)
EJR149	XL10-Gold pWH844; AmpR	(Schirmer et al., 1997)
EJR88	XL1-Blue pKSV7-Δ*lmo2795*; AmpR	This study
EJR121	XL1-Blue pKSV7-Δ*lmo2800*; AmpR	This study
EJR122	XL10-Gold pKSV7-Δ*lmo2799*; AmpR	This study
EJR123	XL10-Gold pKSV7-Δ*lmo2798*; AmpR	This study
EJR131	DH5α pWH844-*lmo2795*; AmpR	This study
EJR164	XL1-Blue pKSV7-Δ*lmo2797*; AmpR	This study
EJR210	XL10-Gold pKSV7-Δ*nanE*; AmpR	This study
EJR236	XL10-Gold pKSV7-Δ*lmo2796*; AmpR	This study
Listeria monocytogenes strains		
ANG873	EGD-e	(Glaser et al., 2001)
ANG1263	10403S; StrepR	(Bishop & Hinrichs, 1987)
BUG2214	EGD-e Δ*prfA*	(Mandin et al., 2007)
LJR123	EGD-e Δ*lmo2795*	This study
LJR132	EGD-e Δ*lmo2798*	This study
LJR135	EGD-e Δ*lmo2799*	This study
LJR204	EGD-e Δ*lmo2797*	This study
LJR205	EGD-e Δ*lmo2799* Δ*lmo2797*	This study
LJR206	EGD-e Δ*lmo2800*	This study
LJR207	EGD-e Δ*nanE*	This study

hydrate, $Na_2HPO_4 \cdot 12\ H_2O$, $CoCl_2 \cdot 6\ H_2O$ and L-cysteine·HCl·H_2O, respectively (see Table 2 for detailed LSM composition and preparation). The pH of LSM was adjusted with HCl, where indicated. Sodium chloride was added to LSM for characterisation of the growth of *L. monocytogenes* strains under salt stress at the following concentrations: 1% (0.17 M), 2% (0.34 M), 3% (0.51 M), 4% (0.68 M), 5% (0.86 M) and 6% (1.03 M). To test the ability of *L. monocytogenes* to metabolise different carbon sources, 1% glucose, which is usually contained in LSM, was replaced by 1% of the following carbon sources: glycerol, glucosamine (Glc*N*), *N*-acetylglucosamine (Glc*N*Ac), *N*-acetylmannosamine (Man*N*Ac), glucose-1-phosphate (Glc-1-P), glucose-6-phosphate (Glc-6-P), mannose, maltose, mannitol, sucrose, rhamnose, trehalose, succinate, cellobiose, galactose and *N*-acetylneuraminic acid (Neu5Ac).

A previous study showed that the expression of *prfA* is induced in the presence of low levels of branched-chain amino acids (Lobel et al., 2015). For the growth of *L. monocytogenes* under PrfA-activating conditions, the concentration of branched-chain amino acids was 10-fold reduced in freshly prepared LSM yielding 80 μM L-isoleucine, 80 μM L-leucine and 90 μM L-valine.

Growth assays

Overnight cultures of the indicated *L. monocytogenes* strains were diluted in fresh BHI medium to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.3 was reached. Cells of 2 mL culture were collected per condition, washed twice with 1 mL of the corresponding LSM and re-suspended in 1 mL LSM. The cultures were adjusted to an OD_{600} of 0.2 and 100 μL of the cell suspension were transferred into wells of a 96-well plate containing 100 μL of the corresponding LSM. The plate was incubated at 37°C with orbital shaking in the Bio-Tek Epoch 2 microplate reader and the OD_{600} was measured every 15 min for at least 36 h. Averages and standard deviations of at least three independent growth assays were plotted.

To test whether the growth of *L. monocytogenes* was possible in LSM Glc-1-P and Glc-6-P after pre-growth of the indicated strains under PrfA-activating conditions, overnight cultures of the indicated *L. monocytogenes* strains were diluted in LSM glucose with low concentrations of BCAA to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.3 was reached. Cells were collected, washed and used to inoculate a 96-well plate containing the corresponding LSM with low concentrations of BCAA as described above.

Strain and plasmid construction

All primers used in this study are listed in Table 3. For markerless deletion of *lmo2795*, 1-kb fragments up- and downstream of *lmo2795* were amplified by PCR using primer pairs LMS346/LMS347 and LMS348/LMS349, respectively. The resulting PCR fragments were fused by PCR using primers LMS346/LMS349. The deletion fragment was subsequently digested with *Xba*I and *Sac*I and ligated into plasmid pKSV7 that had

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TABLE 2 Composition of the *Listeria* synthetic medium (LSM).

Stock name	Stock dilution factor	Stock volume (mL)	Ingredient	Final concentration	MW (g/Mol)	Final concentration (g/L)	Total in stock (g)
MOPS pH 7.5[a]	10	1000	MOPS	0.1 M	209.3	20.93	209.3
Glucose	50	1000	Glucose	55.5 mM	180.2	10	500
Phosphate	100	250	KH_2PO_4	4.8 mM	136.1	0.656	16.3
			$Na_2HPO_4 \cdot 12\ H_2O$	11.5 mM	358.14	4.12	103
Magnesium	100	250	$MgSO_4 \cdot 7\ H_2O$	1.7 mM	246.5	0.409	10.48
Micro-nutrients[b]	100	250	Biotin	2.05 μM	244.3	0.0005	0.0125
			Riboflavin 5′ monophosphate sodium salt hydrate	1.33 μM	478.33	0.0006	0.0158
			Para-aminobenzoic acid	7.29 μM	137.1	0.001	0.025
			Lipoic acid	0.02 μM	206.3	0.000005	0.0001
			Nicotinamide	8.19 μM	122.12	0.001	0.025
			D-pantothenic acid hemicalcium salt	4.2 μM	238.27	0.001	0.025
			Pyridoxal·HCl	4.91 μM	203.62	0.001	0.025
			Thiamine·HCl	2.96 μM	337.27	0.001	0.025
Complete amino acids[c]	50	500	L-arginine	500 μM	174.2	0.1	2.18
			L-histidine·HCl·H_2O	500 μM	209.6	0.1	2.62
			L-isoleucine	800 μM	131.17	0.1	2.62
			L-leucine	800 μM	131.17	0.1	2.62
			L-methionine	700 μM	149.21	0.1	2.61
			L-phenylalanine	600 μM	165.19	0.1	2.48
			L-tryptophan	500 μM	204.23	0.1	2.55
			L-valine	900 μM	117.15	0.1	2.64
Adenine[d]	100	250	Adenine	18.5 μM	135.13	0.0025	0.0625
Trace metals	100	250	$FeCl_2 \cdot 4\ H_2O$	5 μM	198.8	0.00099	0.025
			$MnSO_4 \cdot H_2O$	50 μM	169	0.00845	0.212
			$ZnSO_4 \cdot 7\ H_2O$	1 μM	287.6	0.00029	0.007
			$CaCl_2 \cdot 2\ H_2O$	10 μM	147	0.00147	0.037
			$CuSO_4 \cdot 5\ H_2O$	0.1 μM	249.7	0.00002	0.0005
			$CoCl_2 \cdot 6\ H_2O$	0.1 μM	237.93	0.00003	0.0005
			H_3BO_3	0.1 μM	61.8	0.00001	0.00015
			$Na_2MoO_4 \cdot 2\ H_2O$	0.1 μM	242	0.00002	0.0005
			NaCl	8.555784 mM	58.44	0.5	12.5
			Sodium citrate tri-sodium salt	100 μM	294.1	0.2941	0.74
Added fresh[e]	10	100	L-cysteine·HCl·H_2O	634.44 μM	175.6	0.1	0.1
			L-glutamine	4.106 mM	146.14	0.6	0.6

Note: The recipe of LSM was adapted from Whiteley et al. (2017). All stock solutions were sterile filtrated prior to use and stored at 4°C, except for Phosphate, which is stored at room temperature. LSM is prepared by combining the stock solutions in accordance with their dilution factor and in the order they are listed in the table. The final LSM medium is again sterile filtrated.

[a]Adjust pH of MOPS stock solution to pH 7.5 with 10 N NaOH.

[b]Dissolve ingredients in boiling water followed by filter sterilisation.

[c]Dissolve amino acids in hot 1 N NaOH followed by filter sterilisation.

[d]Dissolve adenine in 20 mL 0.2 M HCl and fill up with H_2O to 250 mL.

[e]These ingredients are usually freshly added to LSM according to Whiteley et al. (2017); however, a stock solution could be prepared and is stable for at least 4 weeks.

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TABLE 3 Primers used in this study.

Number	Name	Sequence
AK004	pWG844-*lmo2795* fw	AAAGGATCCATGACGATTTTAAATGAAATCCAACAAAATTA
AK005	pWG844-*lmo2795* rev	TTTGTCGACTTAATGAAATTCATCGGTAACAGTATTGA
JR142	*lmo2797* up fw	CGCGGATCCGTTGCAGACCGAGAACAAAAG
JR143	*lmo2797* up rev	GTGAAATTATTATAACATGCCCGTACCCCC
JR144	*lmo2797* down fw	ACGGGCATGTTATAATAATTTCACTAAAGAGGTGAAG
JR145	*lmo2797* down rev	CCGGAATTCCTGCTGTATCGAACATCGGC
JR211	*lmo2800* up fw	CGCGGATCCGTTAGTGGTTTCCTGCCAAG
LMS169	qRT-PCR *gyrB* fw	GGTGGCTCATGGATAACGTCT
LMS170	qRT-PCR *gyrB* rev	GCGAACTTGCTTTCTTGAATCGT
LMS346	*lmo2795* down rev	AAAGAGCTCCGGTGAAATCTAGGTCCATTCC
LMS347	*lmo2795* down fw	CAACAAAATTACATCAATACTGTTACCGATGAATTTCATTAA
LMS348	*lmo2795* up rev	AACAGTATTGATGTAATTTTGTTGGATTTCATTTAAAATCGTCAT
LMS349	*lmo2795* up fw	AAATCTAGACGAAGCAAATTCAAGCCAAGCA
LMS369	qRT-PCR *nanE* fw	GTGGGGGTGATAAATACGTCCG
LMS375	qRT-PCR *rplD* fw	ATGCCTTGTAAGTTGCGTGC
LMS376	qRT-PCR *rplD* rev	GGTTTGACTTTCGATGCACCT
LMS377	qRT-PCR *nanE* rev	TTCAAGGTGGTGCTGTTGGT
LMS384	qRT-PCR *lmo2796* fw	AGCCCACTTCACATGCTGTA
LMS385	qRT-PCR *lmo2796* rev	CTTGGCGAGTTTTGGTTGGG
LMS395	*lmo2796* up fw	AAAGGATCCCCGTGAATTAAATGAGAAGAATCAAATGG
LMS396	*lmo2796* up rev	ATCTTTTTCGGCGTCGACACAAAGATAATTTTCCATTTCTTC
LMS397	*lmo2796* down fw	CTTTGTGTCGACGCCGAAAAAGATGACGATTTTAAATGA
LMS398	*lmo2796* down rev	TTTCTGCAGGTGAAAAAGGCATCTGGGTCC
LMS401	*nanE* up fw	AAAGGATCCAATAATGAGCTAAAATATACGGTATAAGCC
LMS402	*nanE* up rev	TTGAATTTTTTGGATTTTTTCCATAACAGAATTGCCCAC
LMS403	*nanE* down fw	ATGGAAAAAATCCAAAAAATTCAAGAAGAAAGAGGTAAGTGA
LMS404	*nanE* down rev	TTTCTGCAGCCAATCATATGTGTGGCGCTATTTT
LMS408	*lmo2800* up rev	TCCTTCAGCAATAATTACGCCAACTTTGATTTGCTTTG
LMS409	*lmo2800* down fw	GTTGGCGTAATTATTGCTGAAGGAAAAGAAGTATCACTATAA
LMS410	*lmo2800* down rev	TTTCTGCAGATGTGGTCGCCGGGTG
LMS411	*lmo2799* up fw	AAAGGATCCCAAAGCAAATCAAAGTTGGCGTAATT
LMS412	*lmo2799* up rev	GTCTGCAACTATCTTTTGTACATGTACCCGAATAGACAT
LMS413	*lmo2799* down fw	CATGTACAAAAGATAGTTGCAGACCGAGAACAAAAG
LMS414	*lmo2799* down rev	TTTCTGCAGCATCAGCCAATGTTGCAAAGGT
LMS417	*lmo2798* up fw	AAAAAGCTTCCCACTAACGGCTTTAATCATTGAG
LMS418	*lmo2798* up rev	CATTATTTCTGTGTCAAAGTCCATCACAATCGCTTT
LMS419	*lmo2798* down fw	ATGGACTTTGACACAGAAATAATGGCTGAATATAATAAATAAGGG
LMS420	*lmo2798* down rev	TTTCTGCAGAGCCCACTTCACATGCTGTAA
LMS428	EMSA unspecific fw	CTTTAATATCCTCTAAGCCAGCATTTTTG
LMS429	EMSA unspecific rev	CAAGATGCAGCGAAGAAAATGGAA
LMS430	EMSA *lmo2795* rev	CTAACCCACCTTTGATTTTTTCCATAAC
LMS431	EMSA *lmo2795* fw	CGGAACACCCAACAAATTACCAAT

been cut with the same enzymes. Plasmid pKSV7-Δ*lmo2795* was recovered in *E. coli* XL1-Blue yielding strain EJR88. For markerless deletion of *lmo2796*, *lmo2799*, *lmo2800* and *nanE*, 1-kb fragments up- and downstream of the corresponding gene were amplified by PCR using primer pairs LMS395/LMS396 and LMS397/LMS398 (*lmo2796*), LMS411/LMS412 and LMS413/LMS414 (*lmo2799*), JR211/LMS408 and LMS409/LMS410 (*lmo2800*), LMS401/LMS402 and LMS403/LMS404 (*nanE*). The resulting PCR fragments

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were fused by PCR using primers LMS395/LMS398 (*lmo2796*), LMS411/414 (*lmo2799*), JR211/LMS410 (*lmo2800*) and LMS401/LMS404 (*nanE*). The Δ*lmo2799*, Δ*lmo2800* and Δ*nanE* deletion fragments were digested with *BamH*I and *Pst*I and ligated into *BamH*I/*Pst*I cut pKSV7. Plasmids pKSV7-Δ*lmo2796*, pKSV7-Δ*lmo2799* and pKSV7-Δ*nanE* were recovered in *E. coli* XL10-Gold yielding strains EJR238, EJR122 and EJR210, respectively. pKSV7-Δ*lmo2800* was recovered in *E. coli* XL1-Blue yielding strain EJR121. For the construction of pKSV7-Δ*lmo2798* and pKSV7-Δ*lmo2797*, 1-kb fragments up- and downstream of the corresponding gene were amplified by PCR using primer pairs LMS417/LMS418 and LMS419/LMS420 (*lmo2798*) and JR142/JR143 and JR144/JR145 (*lmo2797*). The resulting PCR fragments were fused by PCR using primers LMS417/LMS420 (*lmo2798*) and JR142/JR145 (*lmo2797*), cut with *BamH*I and *EcoR*I and ligated into *BamH*I/*EcoR*I cut pKSV7. pKSV7-Δ*lmo2798* was transformed into *E. coli* XL10-Gold yielding strain EJR123. Plasmid pKSV7-Δ*lmo2797* was recovered in *E. coli* XL1-Blue yielding strain EJR164. Plasmids pKSV7-Δ*lmo2795*, pKSV7-Δ*lmo2796*, pKSV7-Δ*lmo2797*, pKSV7-Δ*lmo2798*, pKSV7-Δ*lmo2799*, pKSV7-Δ*lmo2800* and pKSV7-Δ*nanE* were transformed into *L. monocytogenes* strain EGD-e and genes *lmo2795*, *lmo2797*, *lmo2798*, *lmo2799*, *lmo2800* and *nanE* deleted by the allelic exchange as previously described (Camilli et al., 1993) yielding strains EGD-e Δ*lmo2795* (LJR123), Δ*lmo2796* (LJR264), EGD-e Δ*lmo2797* (LJR204), EGD-e Δ*lmo2798* (LJR132), EGD-e Δ*lmo2799* (LJR135), EGD-e Δ*lmo2800* (LJR206) and EGD-e Δ*nanE* (LJR207). To construct strain EGD-e Δ*lmo2799* Δ*lmo2797* (LJR205), pKSV7-Δ*lmo2797* was transformed into *L. monocytogenes* strain LJR204 and *lmo2797* deleted by allelic exchange.

For the purification of Lmo2795 with an N-terminal His-tag, *lmo2795* was amplified using primer pair AK004/AK005. The resulting PCR product was digested with BamHI and SalI and ligated into pWH844. Plasmid pWH844-*lmo2795* was recovered in *E. coli* DH5α yielding strain EJR131.

RNA extraction

RNA of the indicated *L. monocytogenes* strains was isolated following a previously published method with minor modifications (Hauf et al., 2019). Briefly, a single colony was used to inoculate 10 mL LSM medium with 1% glucose as the sole carbon source and cultures were grown overnight at 37°C. The next day, cultures were used to inoculate 30 mL of fresh LSM medium with 1% glucose to an OD_{600} of 0.1. When the cultures reached an OD_{600} of 0.5 ± 0.05, 25 mL of the cell suspensions were harvested by centrifugation for 15 min at 4000 rpm and 4°C and the pellet was snap frozen in liquid nitrogen and stored at −80°C. To isolate the RNA, pellets were re-suspended in 1 mL killing buffer (20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 20 mM NaN_3), transferred to 1.5 mL microtubes and centrifuged at 13,000 rpm for 60 sec. Cells were then re-suspended in 1 mL lysis buffer I (25% sucrose; 20 mM Tris–HCl pH 8, 0.25 mM EDTA) and 2 μL lysozyme (100 mg mL^{-1}) and incubated for 5 min on ice, followed by centrifugation at 5000 rpm and 4°C for 5 min. Pellets were then re-suspended in 300 μL lysis buffer II (3 mM EDTA; 200 mM NaCl) and added to pre-heated (95°C) lysis Buffer III (3 mM EDTA; 200 mM NaCl; 1% SDS). Samples were incubated for exactly 5 min at 95°C and 600 μL phenol/chloroform/isoamyl alcohol (25:24:1) (PCI) was added. After shaking the samples at 700 rpm for 5 min, the two phases were separated by centrifugation at 13,000 rpm for 5 min and the upper aqueous phase was transferred to a new 1.5 mL microtube containing 600 μL PCI. The extraction was repeated with 600 μL chloroform/isoamyl alcohol (25:1). Finally, the upper phase was transferred into an RNAse-free tube and RNA was precipitated by the addition of 0.1 x volume 3 M sodium acetate (pH 5.2) and 1.5 volumes 96% ethanol and incubated at 20°C overnight. To precipitate the RNA, samples were centrifuged at 13,000 rpm for 15 min, washed in 70% ethanol and dried under the fume hood. Finally, samples were re-suspended in 25 μL RNAse-free H_2O.

To avoid DNA contamination, 5 μg isolated RNA were digested with 5 μL DNase I (1 U $μl^{-1}$, Thermo Scientific) for 40 min at 37°C. The reaction was stopped by the addition of 2.5 μL 25 mM EDTA and incubation at 65°C for 10 min. To verify that the isolated RNA is free of DNA, a check PCR was performed using primers LMS169 and LMS170. Genomic DNA from *L. monocytogenes* EGD-e was used as a control.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out using the One-Step reverse transcription PCR kit, the Bio-Rad iCycler and the Bio-Rad iQ5 software (Bio-Rad, Munich, Germany). Three biological replicates were performed. Primer pairs LMS375/LMS376 and LMS169/170 were used to determine the transcript amounts of *rplD* and *gyrB*, respectively, which were used as internal controls. Transcript amounts for *lmo2796* and *nanE* were monitored using primer pairs LMS384/LMS385 and LMS369/LMS377, respectively. The average of the cycle threshold (C_T) values of *rplD* and *gyrB* were used to normalise the C_T values obtained for *lmo2796* and *nanE*. For each strain, the fold changes in *lmo2796* and *nanE* expression were calculated using the $\Delta\Delta C_T$ method.

Purification of His-Lmo2795

For the overexpression of His-Lmo2795, plasmid pWH844 was used, which contains the *lacI* gene,

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enabling the strictly IPTG-dependent overproduction of His-tagged proteins (Schirmer et al., 1997). pWH844-*lmo2795* was transformed into *E. coli* BL21 and grown in 2x LB. When cultures reached an OD_{600} of 0.6–0.8, expression was induced by the addition of IPTG at a final concentration of 1 mM, and the cells were grown for 2 h at 37°C. Cells were collected by centrifugation, washed once with 1x ZAP buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl) and the cell pellet stored at −20°C. The next day, cells were re-suspended in 1x ZAP (50 mM Tris–HCl, pH 7.5, 200 mM NaCl) and lysed by three passages (18,000 lb/in2) through an HTU DIGI-F press (G. Heinemann, Germany). The resulting crude extract was centrifuged at 46,400x g for 60 min followed by protein purification using a Ni^{2+} nitrilotriacetic acid column (IBA, Göttingen, Germany). His-Lmo2795 was eluted using imidazole and elution fractions analysed using SDS-PAGE. Selected elution fractions were subsequently subjected to dialysis against 1x ZAP buffer at 4°C overnight. The protein concentration was determined according to the method of Bradford (Bradford, 1976) using the Bio-Rad protein assay dye reagent concentrate. Bovine serum albumin was used as standard. The protein samples were stored at −80°C until further use.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described with some modifications (Dhiman et al., 2014). Briefly, the upstream region of the *lmo2795-nanE* operon containing a putative promoter site was amplified using primer pair LMS430/LMS431. A 204 bp region within the operon, which served as an unspecific control, was amplified using primers LMS428/LMS429. Binding reactions were performed at 25°C for 20 min in 20 μL binding buffer containing 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10% glycerol, 5 mM EDTA, 20 mM DTT and varying amounts of His-Lmo2795 (3.5–14 μg), as well as 250 pmol of DNA. To ease the loading of the samples into the wells, 1 μL DNA loading dye was added to each sample. A 60-min pre-run was carried out at 70 V prior to sample loading. The samples were separated on an 8% polyacrylamide native gel in 0.5x TBE buffer (50 mM Tris–HCl, pH 10, 50 mM boric acid, 1 mM Na_2EDTA) at 4°C for 2.5 h at 50 V. The gel was stained in 50 mL 0.5x TBE buffer containing 5 μL HDGreen™ Plus DNA dye (INTAS, Göttingen, Germany) for 3 min, followed by a 5 min washing step in 0.5x TBE buffer, three 20 sec washing steps in H_2O and an additional 30 min washing step in H_2O. DNA bands were visualised using a GelDoc™ XR+ (Bio-Rad, Munich, Germany).

RESULTS AND DISCUSSION

Growth of *L. monocytogenes* wildtype strains under salt and pH stress

For most physiological studies, which aimed at the characterisation of salt and pH resistance of *L. monocytogenes*, bacteria were grown in complex media such as BHI or TSB. In nature as well as within the host, *L. monocytogenes* likely encounters limited access to nutrients, such as carbon and nitrogen sources or vitamins, which likely affects their ability to adapt to environmental stress conditions. We, therefore, assessed the ability of the two widely used *L. monocytogenes* wildtype strains EGD-e and 10403S to grow under high salt and low pH stress in the chemically defined medium LSM. Growth of EGD-e was barely affected in the presence of 1% NaCl. In the presence of 2, 3 and 4% NaCl, the growth of EGD-e was delayed as compared to LSM. At a concentration of 5% salt, EGD-e was still able to grow but only reached an optical density of around 0.3 after 36 h. The *L. monocytogenes* wildtype strain 10403S showed a lower salt resistance as compared to EGD-e. Growth of 10403S was already impaired in LSM containing 3% salt. In the presence of 4% and 5% salt, 10403S could only reach an optical density of around 0.2 after 36 h (Figure 1A). In contrast, *L. monocytogenes* is able to grow in the presence of up to 10% salt, when a complex medium is used (Patchett et al., 1992; Vasseur et al., 2001). To assess the pH resistance, we compared the growth of *L. monocytogenes* strains grown in standard LSM, which has a pH of 8.7, and LSM adjusted to a pH of 8, 7, 6 and 5.5. Growth of the *L. monocytogenes* wildtype strain EGD-e was similar in LSM with a pH of 7 as compared to standard LSM, while the growth of 10403S was slightly reduced. Both strains had a severe growth defect in LSM with a pH of 6 and were unable to grow in LSM with a pH of 5.5 (Figure 1B). Overall, we observed that the *L. monocytogenes* wildtype strain 10403S was less tolerant to salt and pH stress than EGD-e (Figure 1). Interestingly, 10403S grew better in the BHI medium, which was adjusted to a low pH, than EGD-e (Cheng et al., 2015). Resistance of *L. monocytogenes* to pH stress is mainly conferred by the activity of the glutamate decarboxylase activity (GAD) system, a process, which depends on the availability of extracellular glutamate (Cotter et al., 2001; Feehily et al., 2014). The two *L. monocytogenes* wildtype strains EGD-e and 10403S seem to use two distinct GAD systems, which confer a different degree of acid resistance. The GAD system used by 10403S was shown to give a higher acid tolerance (Cheng et al., 2015; Feehily et al., 2014). However, complex medium such as BHI likely contains higher glutamate levels than LSM, which can be used by the GAD system. Thus, the GAD system of 10403S

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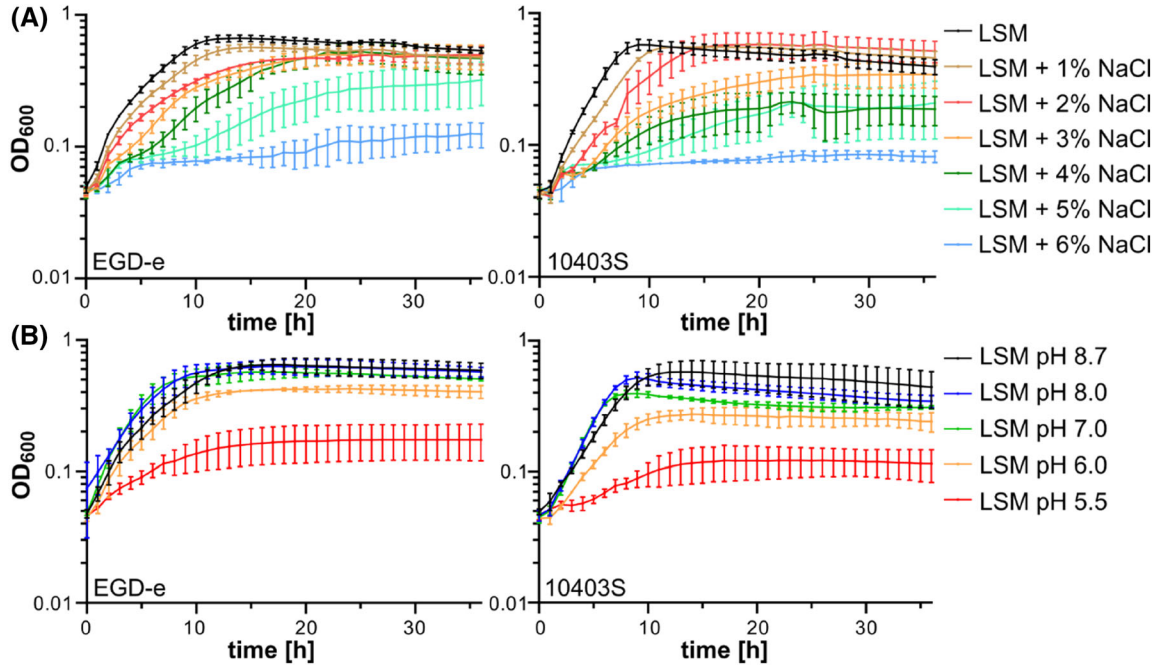

FIGURE 1 Salt and pH resistance of *L. monocytogenes* wildtype strains. (A) *L. monocytogenes* strains EGD-e and 10403S were grown in LSM containing 1% glucose and increasing concentrations of NaCl as described in the methods section. (B) *L. monocytogenes* strains EGD-e and 10403S were grown in LSM containing 1% glucose (pH 8.7). The pH of LSM was adjusted to the indicated pH values using HCl. The average values and standard deviations of three independent experiments were plotted.

might be less efficient when the cells are grown in LSM and are therefore less resistant to acid stress.

Growth of *L. monocytogenes* in LSM with different carbon sources

Over the last decades, several chemically defined media have been developed that support growth of *L. monocytogenes* (Jarvis et al., 2016; Phan-Thanh & Gormon, 1997; Pine et al., 1989; Premaratne et al., 1991; Tsai & Hodgson, 2003; Whiteley et al., 2017). Most of these media contain glucose as the sole carbon source. Other carbon sources such as fructose and mannose were shown to support the growth of *L. monocytogenes* in Hsiang-Ning Tsai medium (HTM) or modified Welshimer's Broth (MWB) (Premaratne et al., 1991; Tsai & Hodgson, 2003). Glucose could also be replaced by the amino sugars Glc*N*Ac and *N*-acetylmuramic acid in MWB (Premaratne et al., 1991). Interestingly, lactose and rhamnose could support the growth of *L. monocytogenes* in ACES-buffered chemically defined (ABCD) medium but not in HTM and/or MWB (Table 4) (Pine et al., 1989; Premaratne et al., 1991; Tsai & Hodgson, 2003). Similarly, glycerol was shown to support the growth of *L. monocytogenes* in HTM medium, while only weak growth could be observed for MWB with glycerol as the sole carbon source (Table 4) (Premaratne et al., 1991; Tsai & Hodgson, 2003). To our knowledge, no information is available on the carbon sources that support the growth of *L. monocytogenes* in LSM. We, therefore, performed growth experiments with *L. monocytogenes* wildtype strains EGD-e and 10403S in LSM containing 1% of selected carbon sources. This analysis revealed that growth of both strains was similar in LSM containing Glc*N*Ac, Glc*N*, mannose, cellobiose or trehalose as the sole carbon source compared to glucose (Figure 2, Table 4). Both strains were also able to use glycerol as carbon sources, while only weak growth was obtained for maltose, rhamnose and succinate (Figure 2, Table 4). No growth was observed for both strains, when glucose was replaced with galactose, sucrose, glucose-1-phosphate (Glc-1-P), glucose-6-phosphate (Glc-6-P) or mannitol (Figure 3A, Table 4). The hexose phosphate transporter Hpt, which is required for the import of Glc-1-P and Glc-6-P, is expressed in a PrfA-dependent manner. The expression and activity of PrfA is controlled on transcriptional, translational and post-translational level (reviewed in (Gaballa et al., 2019; Xayarath & Freitag, 2012)). One factor involved in the control of *prfA* expression is the global regulator CodY, which senses the presence of branched-chain amino acids (BCAAs). In the presence of high levels of BCAAs, CodY acts as a *prfA* repressor, while expression of *prfA* is induced when BCAA levels drop, which occurs upon invasion of host cells (Lobel et al., 2015). We, therefore, tested whether reduction of the BCAA concentration in LSM could lead to the growth of

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TABLE 4 Carbon sources supporting the growth of *L. monocytogenes* in chemically defined media.

	(Pine et al., 1989)		(Tsai & Hodgson, 2003)		(Premaratne et al., 1991)		This study	
	ABCD medium		HTM		MWB		LSM	
Carbon source[a]	Conc.	Growth	Conc.	Growth	Conc.	Growth	Conc.	Growth
Glucose	0.25%	Yes	1%	Yes	1%	Yes	1%	Yes
Fructose			1%	Yes	1%	Yes		
Galactose	0.25%	No	1%	No	1%	No	1%	No
Mannose			1%	Yes	1%	Yes	1%	Yes
Mannitol			1%	No	1%	No	1%	No
Arabinose			1%	No	1%	No		
Ribose			1%	No	1%	No		
Xylose	0.25%	No	1%	No	1%	No		
Lactose	0.25%	Yes	1%	No	1%	No		
Rhamnose	0.25%	Yes	1%	No			1%	Weak
Maltose			1%	No	1%	Weak	1%	Weak
Sucrose			1%	No	1%	No	1%	No
Cellobiose					1%	Yes	1%	Yes
Trehalose					1%	Yes	1%	Yes
Glc-1-P					1%	No	1%	Yes[b]
Glc-6-P					1%	No	1%	Yes[b]
Glc*N*					1%	Yes	1%	Yes
Glc*N*Ac					1%	Yes	1%	Yes
Mur*N*Ac					1%	Yes		
Man*N*Ac							1%	Yes
Neu5Ac							1%	No
Glycerol			1%	Yes	1%	Weak	1%	Yes
Succinate					1%	No	1%	Weak

Abbreviations: ABCD medium, *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered, chemically defined medium; Conc., concentration of carbon source; Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate; Glc*N*, glucosamine; GlcNAc, *N*-acetylglucosamine; HTM, Hsiang-Ning Tsai medium; LSM, *Listeria* synthetic medium; Man*N*Ac, *N*-acetylmannosamine; Mur*N*Ac, *N*-acetylmuramic acid; MWB, Modified Welshimer's broth; Neu5Ac, *N*-acetylneuraminic acid.
[a]The following carbon sources do not support growth: melibiose, raffinose, sorbose, sorbitol, galactose-1-phosphate, galactose-6-phosphate, gactate, citrate, isocitrate, α-ketoglutarate, fumarate, malate, pyruvate, acetate, chitin (Premaratne et al., 1991).
[b]Pre-growth in LSM glucose with low concentrations of BCAA, a PrfA-activating condition, is required.

L. monocytogenes wildtype strains EGD-e and 10403S on Glc-1-P and Glc-6-P as the sole carbon source. While both strains grew fine in LSM with low concentrations of BCAA and glucose as the sole carbon source, no growth was observed in LSM with Glc-1-P or Glc-6-P (Figure 3A). For growth analysis, *L. monocytogenes* strains were pre-grown in a BHI complex medium, in which PrfA is inactive (Renzoni et al., 1997). We, thus, wondered whether the strains have simply not sufficient time to activate PrfA and by this also do not induce the expression of *hpt*, encoding the Glc-1-P and Glc-6-P importer, when they are transferred into LSM containing the hexose phosphate sugars. To test this, we pre-grew *L. monocytogenes* strains EGD-e and 10403S in LSM glucose with low levels of BCAA and subsequently transferred them either into the same medium as a control, or into LSM with low levels of BCAA and containing Glc-1-P or Glc-6-P as sole carbon source. A strain lacking the virulence regulator PrfA was used as a control. Indeed, EGD-e and 10403S were now able to metabolise Glc-1-P and Glc-6-P, while the *prfA* mutant was unable to grow (Figure 3B). In contrast, the absence of PrfA results in a growth advantage in LSM with glucose as the sole carbon source, suggesting that the expression and/or activity of PrfA is a burden for *L. monocytogenes*, which has previously been shown (Friedman et al., 2017; Vasanthakrishnan et al., 2015). Therefore, characterisation of growth and the general physiology of *L. monocytogenes* in the presence of Glc-1-P and Glc-6-P as sole carbon source, requires a pre-growth under PrfA-activating conditions, for instance, in a chemically defined medium or BHI medium containing activated charcoal, Chelex or Amberlite XAD (Chico-Calero et al., 2002; Gaballa et al., 2021; Portman et al., 2017).

The amino sugar Man*N*Ac, which has not been tested for any of the other chemically defined media,

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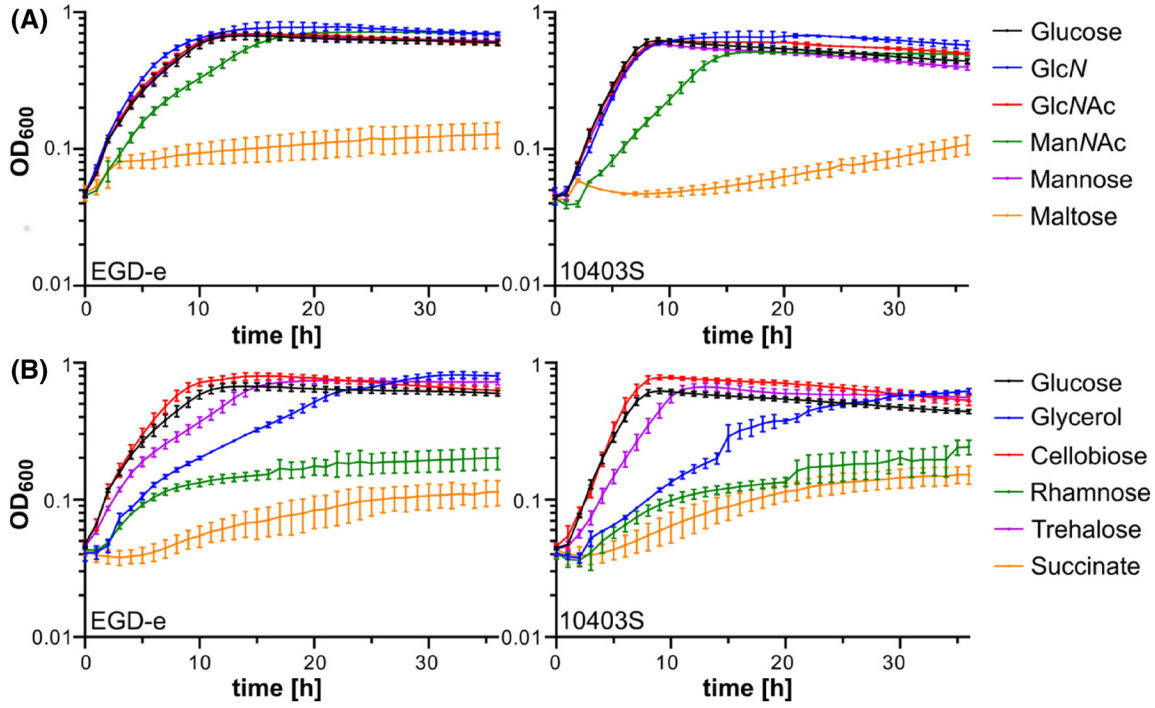

FIGURE 2 Growth in LSM media with different carbon sources. (A, B) *L. monocytogenes* strains EGD-e and 10403S were grown in *Listeria* synthetic medium (LSM) containing 1% of the indicated carbon sources as described in the methods section. The average values and standard deviations of three independent experiments were plotted. Glc*N*Ac, *N*-acetylglucosamine; Man*N*Ac, *N*-acetylmannosamine; Glc*N*, glucosamine.

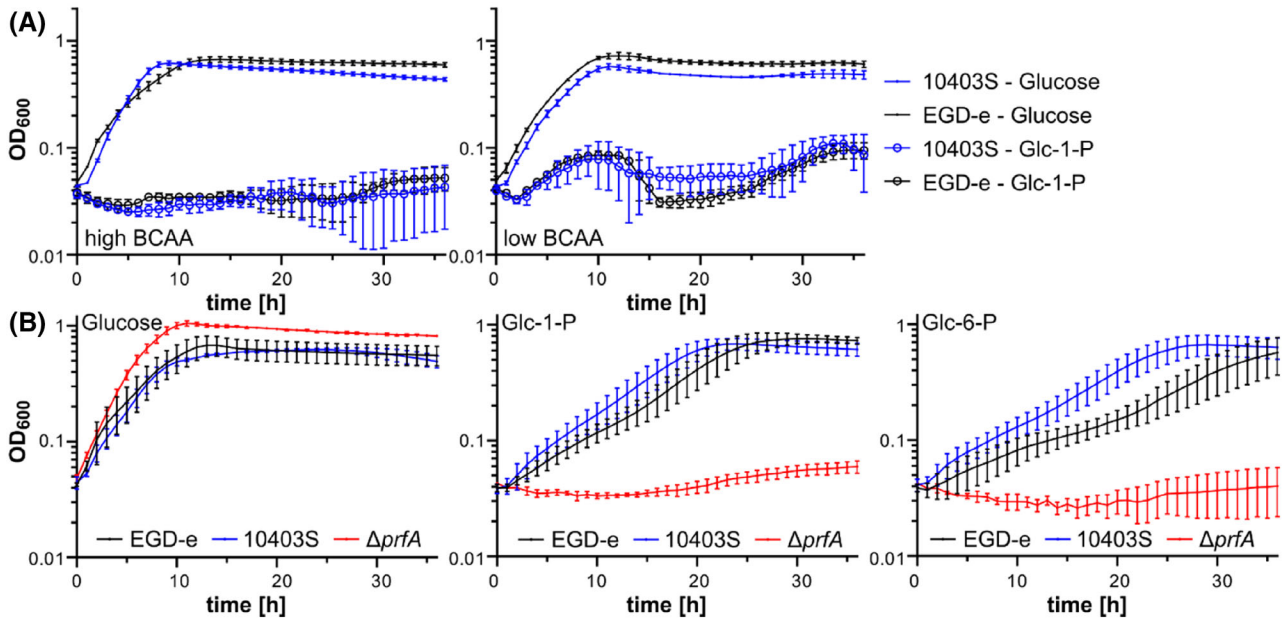

FIGURE 3 Pre-growth of *L. monocytogenes* in LSM glucose enables growth on glucose-1-phosphate and glucose-6-phosphate. (A) *L. monocytogenes* strains EGD-e and 10403S were grown in *Listeria* synthetic medium (LSM) containing 1% of the indicated carbon sources as described in the methods section. LSM was either prepared with the standard complete amino acid mix (high BCAA) or the complete amino acid mix with 10-fold less branched-chain amino acids (low BCAA). The average values and standard deviations of three independent experiments were plotted. (B) *L. monocytogenes* strains EGD-e, 10403S and Δ*prfA* were pre-grown in *Listeria* synthetic medium (LSM) containing 1% glucose and subsequently transferred to LSM containing the indicated carbon sources as described in the methods section. The average values and standard deviations of four independent experiments were plotted. Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate.

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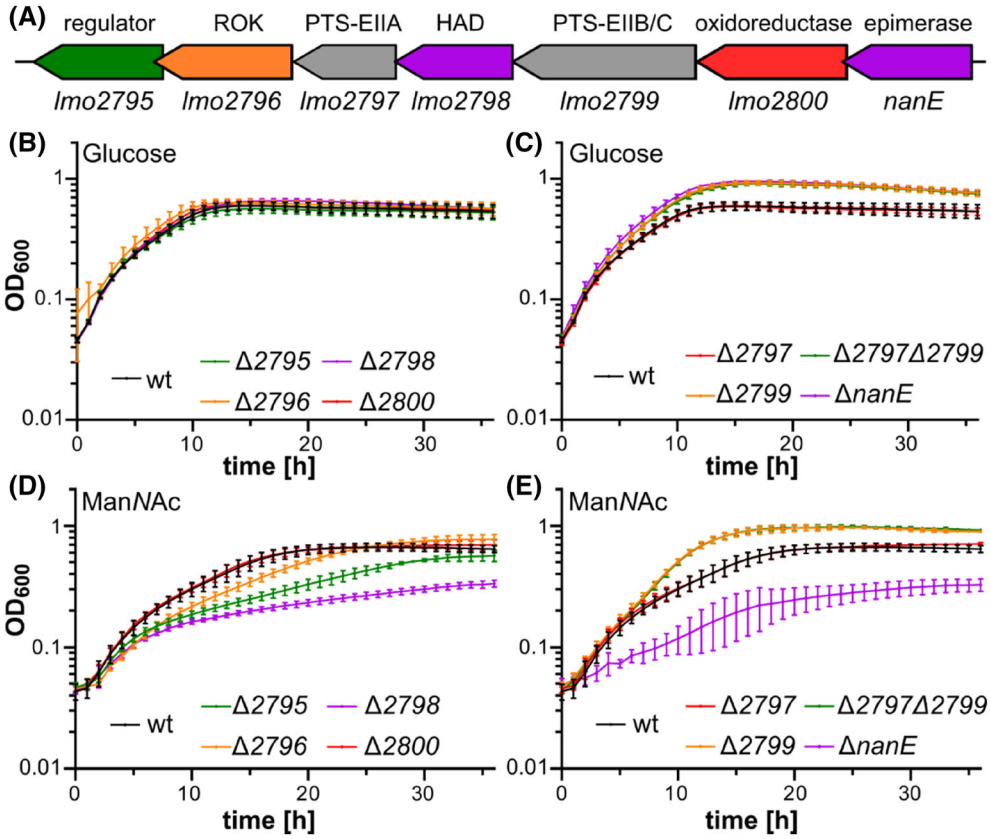

FIGURE 4 The *lmo2795-nanE* operon is required for Man*N*Ac utilisation. (A) Gene arrangement of the *lmo2795-nanE* operon of *L. monocytogenes*. Predicted protein functions are indicated above the genes. ROK, member of the repressor, ORF, kinase family; PTS-EIIA and PTS-EIIB/C, components of a phosphotransferase system; HAD, member of the haloacid dehydrogenase-like hydrolase superfamily. (B–E) Growth curves. The indicated *L. monocytogenes* strains were grown in LSM containing (B, C) 1% glucose, (D, E) 1% Man*N*Ac as the sole carbon source. The average values and standard deviations of three independent experiments were plotted.

was able to support the growth of *L. monocytogenes* strains EGD-e and 10403S (Figure 2A, Table 4). Man*N*Ac is the precursor of the sialic acid *N*-acetylneuraminic acid (Neu5Ac), which can serve as a carbon source for *Staphylococcus aureus* inside the host (Angata & Varki, 2002; Olson et al., 2013; Vimr et al., 2004). However, Neu5Ac could not support the growth of *L. monocytogenes*, suggesting that this organism does not contain the necessary catabolic pathway (Table 4).

The *lmo2795-nanE* operon is required for ManNAc catabolism

In *E. coli*, Man*N*Ac is imported into the cell by the PTS transporter ManXYZ yielding Man*N*Ac-6-phosphate. Man*N*Ac-6-phosphate is subsequently converted into Glc*N*Ac-6-phosphate by the Man*N*Ac-6-phosphate epimerase NanE. The genome of *L. monocytogenes* strain EGD-e contains one homologue of NanE encoded by *lmo2801* (37% identity and 58% similarity). Based on the presence of the NanE homologue, it was already previously proposed that the *lmo2795-nanE* operon might be involved in the transport of Man*N*Ac (Deutscher et al., 2014). In addition to NanE, the *lmo2795-nanE* operon encodes a RpiR-type regulator, a member of the repressor, ORF, kinase (ROK) family, the EIIA and EIIBC components of a PTS system, a putative HAD hydrolase and a putative oxidoreductase (Figure 4A). To test whether these proteins are required for the catabolism of Man*N*Ac, single deletion mutants and a mutant lacking both PTS components, Lmo2797 and Lmo2799, were constructed in the EGD-e wildtype background and their growth was analysed. All deletion mutants were able to grow in LSM containing glucose; however, growth was enhanced for *L. monocytogenes* strains Δ*lmo2799* and Δ*lmo2797* Δ*lmo2799*, lacking one or two of the PTS components and the *nanE* deletion mutant (Figure 4B, C). In contrast, growth on Man*N*Ac as the sole carbon source was reduced for the *lmo2795* and *lmo2798* deletion strains lacking the RpiR transcriptional regulator and the putative HAD hydrolase, respectively, and was nearly abolished for the strain lacking NanE (Figure 4D,E). Surprisingly, deletion strains Δ*lmo2799* and Δ*lmo2797* Δ*lmo2799*

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showed better growth in LSM with Man*N*Ac, similar to what was observed for the glucose-containing LSM (Figure 4C–E), indicating that this PTS system is either not involved in the import of Man*N*Ac or that Man*N*Ac can also be imported by other PTS systems of *L. monocytogenes*. The absence of individual PTS systems can also lead to the overexpression of another PTS system (Stoll & Goebel, 2010), which could explain why strains lacking the EIIB/C component Lmo2799 grew better than the wildtype in LSM with glucose or Man*N*Ac.

Expression of the *lmo2795-nanE* operon is controlled by Lmo2795

RpiR-type transcriptional regulators are often involved in the control of sugar metabolic pathways in Gram-positive and Gram-negative bacteria (Aleksandrzak-Piekarczyk et al., 2019; Jaeger & Mayer, 2008; Li et al., 2017; Sørensen & Hove-Jensen, 1996; Yamamoto et al., 2001). We, thus, wondered whether Lmo2795 regulates the expression of the *lmo2795-nanE* operon. First, we conducted EMSA experiments to assess whether Lmo2795 binds to the promoter region of the operon. For this purpose, increasing concentrations of purified His-Lmo2795 (Figure 5A) were incubated with the DNA fragment containing the putative promoter of the *lmo2795-nanE* operon. The migration of the DNA fragment was retarded in the presence of His-Lmo2795 as compared to the DNA sample that did not contain the protein, suggesting that a protein-DNA complex was formed. In addition, a so-called supershift could also be observed, whose intensity increases with increasing protein concentrations. In contrast, no retardation of the unspecific DNA control was observed in the presence of His-Lmo2795 (Figure 5B). To test whether Lmo2795 activates or represses the expression of the *lmo2795-nanE*, we quantified the expression of *lmo2796* and *nanE* via quantitative real-time PCR. To allow for a better comparison of the gene expression, we decided to isolate RNA of the *L. monocytogenes* wildtype strain EGD-e and the *lmo2795* mutant that were grown in LSM with glucose as the sole carbon source due to the observed growth deficit of the *lmo2795* mutant in Man*N*Ac-containing LSM. This analysis revealed that the expression of both genes, *lmo2796* and *nanE*, was reduced in the *lmo2795* mutant as compared to the wildtype strain (Figure 5C), suggesting that Lmo2795 acts as a transcriptional activator under the tested condition. Based on the observation that a strain lacking NanE had a growth deficit in LSM with Man*N*Ac as the sole carbon source, we assume that Lmo2795 also activates the expression of the *lmo2795-nanE* under this condition as we would expect at least wildtype-like growth of the *nanE* mutant if Lmo2795 acts as a repressor. Further analysis is required to support this hypothesis.

CONCLUSION

The human pathogen *L. monocytogenes* is able to withstand and adapt to a variety of environmental stress

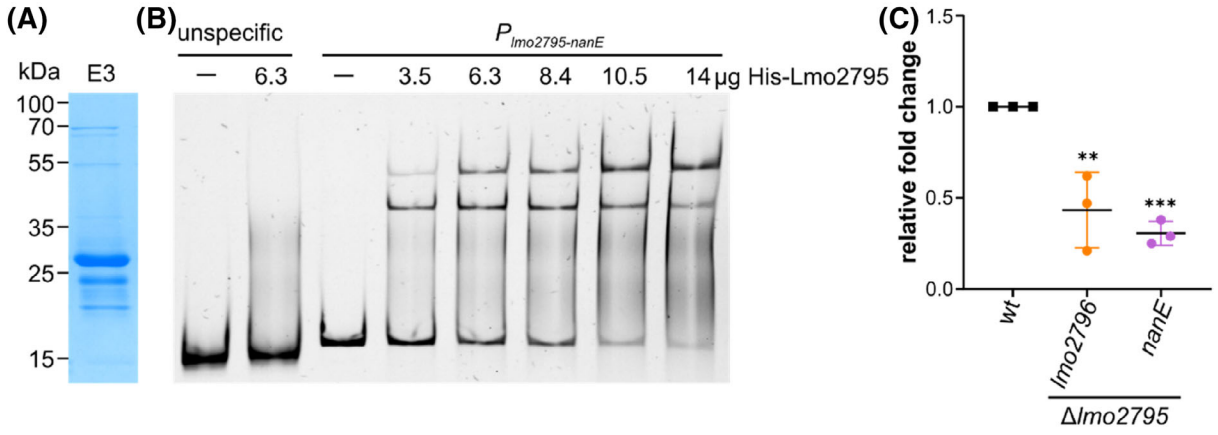

FIGURE 5 Lmo2795 regulates the expression of the *lmo2795-nanE* operon. (A) SDS-PAGE of elution fraction 3 containing purified His-Lmo2795. The expected molecular weight of His-Lmo2795 is 31.5 kDa. (B) EMSA assay. Increasing concentrations of His-Lmo2795 were incubated with the DNA fragment containing the predicted promoter region of the *lmo2795-nanE* operon. The DNA-protein complexes were separated on an 8% polyacrylamide gel, which was subsequently stained using HDGreen™. A DNA fragment containing a short region from within the operon was used as the unspecific control. Reactions without protein were used as negative controls (−). (C) Analysis of *lmo2796* and *nanE* expression by qRT-PCR. RNA was isolated from *L. monocytogenes* strains EGD-e (wt) and Δ*lmo2795* grown in LSM containing 1% glucose as the sole carbon source as described in the methods section. Expression of *lmo2796* and *nanE* was normalised to the expression of *gyrB* and *rplD* and fold changes were calculated using the $\Delta\Delta C_T$ method. Averages and standard deviations of three independent RNA extractions were plotted. For statistical analysis, a one-way ANOVA coupled with Dunnett's multiple comparison test was performed (**$p \leq 0.01$; ***$p \leq 0.001$).

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conditions. Most studies on the physiology and stress tolerance of this organism were performed in complex media without any nutrient limitation; however, this condition is rarely found in their natural habitat or within the host during infection. Our study focused on the characterisation of the growth of two widely used *L. monocytogenes* wildtype strains in the newly developed LSM. We were able to show that both strains can utilise a variety of carbon sources when supplied as the sole carbon source. To our knowledge, this is the first time that the catabolism of Man*N*Ac has been investigated for *L. monocytogenes*. We were able to show that the growth of this pathogen depends on the presence of NanE, which is encoded in the *lmo2795-nanE* operon. The expression of the *lmo2795-nanE* operon is regulated by the RpiR-type regulator Lmo2795, which acts as a transcriptional activator under the tested conditions.

Our detailed characterisation of the growth of *L. monocytogenes* in LSM in the presence of diverse stress conditions or carbon sources can serve as a starting point for future studies focusing on the adaptation of this important human pathogen under changing environmental conditions.

AUTHOR CONTRIBUTIONS

Lisa Maria Schulz: Conceptualization (supporting); formal analysis (lead); investigation (supporting); methodology (lead); supervision (supporting); validation (lead); writing – review and editing (equal). **Alicia Konrath:** Formal analysis (supporting); investigation (supporting); writing – review and editing (equal). **Jeanine Rismondo:** Conceptualization (lead); funding acquisition (lead); investigation (lead); supervision (lead); writing – original draft (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

ORCID

Jeanine Rismondo https://orcid.org/0000-0001-5337-3829

REFERENCES

Aké, F.M.D., Joyet, P., Deutscher, J. & Milohanic, E. (2011) Mutational analysis of glucose transport regulation and glucose-mediated virulence gene repression in *Listeria monocytogenes*. *Mol Microbiol*, 81, 274–293.

Aleksandrzak-Piekarczyk, T., Szatraj, K. & Kosiorek, K. (2019) GlaR (YugA)—a novel RpiR-family transcription activator of the Leloir pathway of galactose utilization in *Lactococcus lactis* IL1403. *Microbiology*, 8, e00714.

Angata, T. & Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. *Chem Rev*, 102, 439–469.

Arizcun, C., Vasseur, C. & Labadie, J.C. (1998) Effect of several decontamination procedures on *Listeria monocytogenes* growing in biofilms. *J Food Prot*, 61, 731–734.

Bécavin, C., Bouchier, C., Lechat, P., Archambaud, C., Creno, S., Gouin, E. et al. (2014) Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. *mBio*, 5, e00969–e00914.

Bishop, D.K. & Hinrichs, D.J. (1987) Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. *J Immunol*, 139, 2005–2009.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytic Biochem*, 72, 248–254.

Camilli, A., Tilney, L.G. & Portnoy, D.A. (1993) Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Molecular Microbiol*, 8, 143–157.

Cheng, C., Yang, Y., Dong, Z., Wang, X., Fang, C., Yang, M. et al. (2015) *Listeria monocytogenes* varies among strains to maintain intracellular pH homeostasis under stresses by different acids as analyzed by a high-throughput microplate-based fluorometry. *Front Microbiol*, 6, 15.

Chico-Calero, I., Suárez, M., González-Zorn, B., Scortti, M., Slaghuis, J., Goebel, W. et al. (2002) Hpt, a bacterial homolog of the microsomal glucose-6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. *Proc Natl Acad Sci USA*, 99, 431–436.

Cotter, P.D., Gahan, C.G. & Hill, C. (2001) A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Molecul Microbiol*, 40, 465–475.

Deutscher, J., Aké, F., Zebre, A., Cao, T., Kentache, T., Pham, Q. et al. (2014) *Carbohydrate utilization by Listeria monocytogenes and its influence on virulence gene expression*. Listeria monocytogenes: Food sources, prevalence and management strategies: Nova science publishers, pp. 49–76.

Dhiman, A., Bhatnagar, S., Kulshreshtha, P. & Bhatnagar, R. (2014) Functional characterization of WalRK: a two-component signal transduction system from *Bacillus anthracis*. *FEBS Open Bio*, 4, 65–76.

Feehily, C., Finnerty, A., Casey, P.G., Hill, C., Gahan, C.G.M., O'Byrne, C.P. et al. (2014) Divergent evolution of the activity and regulation of the glutamate decarboxylase systems in *Listeria monocytogenes* EGD-e and 10403S: roles in virulence and acid tolerance. *PLoS One*, 9, e112649.

Fieseler, L., Schmitter, S., Teiserskas, J. & Loessner, M.J. (2012) Rhamnose-inducible gene expression in *Listeria monocytogenes*. *PLoS One*, 7, e43444.

Friedman, S., Linsky, M., Lobel, L., Rabinovich, L., Sigal, N. & Herskovits, A.A. (2017) Metabolic genetic screens reveal multidimensional regulation of virulence gene expression in *Listeria*

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monocytogenes and an aminopeptidase that is critical for PrfA protein activation. *Infect Immun*, 85, e00027–e00017.

Gaballa, A., Guariglia-Oropeza, V., Wiedmann, M. & Boor, K.J. (2019) Cross talk between SigB and PrfA in *Listeria monocytogenes* facilitates transitions between extra- and intracellular environments. *Microbiol Mol Biol Rev*, 83, e00034–e00019.

Gaballa, A., Sunil, S., Doll, E., Murphy, S.I., Bechtel, T., Guariglia-Oropeza, V. et al. (2021) Characterization of the roles of activated charcoal and Chelex in the induction of PrfA regulon expression in complex medium. *PLoS One*, 16, e0250989.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F. et al. (2001) Comparative genomics of *Listeria* species. *Science*, 294, 849–852.

Gopal, S., Berg, D., Hagen, N., Schriefer, E.-M., Stoll, R., Goebel, W. et al. (2010) Maltose and maltodextrin utilization by *Listeria monocytogenes* depend on an inducible ABC transporter which is repressed by glucose. *PLoS One*, 5, e10349.

Grubmüller, S., Schauer, K., Goebel, W., Fuchs, T.M. & Eisenreich, W. (2014) Analysis of carbon substrates used by *Listeria monocytogenes* during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism. *Front Cell Infect Microbiol*, 4, 156.

Hauf, S., Herrmann, J., Miethke, M., Gibhardt, J., Commichau, F.M., Müller, R. et al. (2019) Aurantimycin resistance genes contribute to survival of *Listeria monocytogenes* during life in the environment. *Mol Microbiol*, 111, 1009–1024.

Jaeger, T. & Mayer, C. (2008) The transcriptional factors MurR and catabolite activator protein regulate *N*-acetylmuramic acid catabolism in *Escherichia coli*. *J Bacteriol*, 190, 6598–6608.

Jarvis, N.A., O'Bryan, C.A., Ricke, S.C., Johnson, M.G. & Crandall, P.G. (2016) A review of minimal and defined media for growth of *Listeria monocytogenes*. *Food Control*, 66, 256–269.

Joseph, B., Mertins, S., Stoll, R., Schär, J., Umesha, K.R., Luo, Q. et al. (2008) Glycerol metabolism and PrfA activity in *Listeria monocytogenes*. *J Bacteriol*, 190, 5412–5430.

Kentache, T., Milohanic, E., Cao, T.N., Mokhtari, A., Aké, F.M., Ma Pham, Q.M. et al. (2016) Transport and catabolism of pentitols by *Listeria monocytogenes*. *J Mol Microbiol Biotechnol*, 26, 369–380.

Lachmann, R., Halbedel, S., Adler, M., Becker, N., Allerberger, F., Holzer, A. et al. (2021) Nationwide outbreak of invasive listeriosis associated with consumption of meat products in health care facilities, Germany, 2014–2019. *Clin Microbiol Infect*, 27, 1035.e1.

Lecuit, M. (2005) Understanding how *Listeria monocytogenes* targets and crosses host barriers. *Clin Microbiol Infect*, 11, 430–436.

Li, J., Evans, D.R., Freedman, J.C. & McClane, B.A. (2017) NanR regulates *nanI* Sialidase expression by *Clostridium perfringens* F4969, a human enteropathogenic strain. *Infect Immun*, 85, e00241–e00217.

Lobel, L., Sigal, N., Borovok, I., Belitsky, B.R., Sonenshein, A.L. & Herskovits, A.A. (2015) The metabolic regulator CodY links *Listeria monocytogenes* metabolism to virulence by directly activating the virulence regulatory gene prfA. *Mol Microbiol*, 95, 624–644.

Lourenco, A., Linke, K., Wagner, M. & Stessl, B. (2022) The saprophytic lifestyle of *Listeria monocytogenes* and entry into the food-processing environment. *Front Microbiol*, 13, 789801.

Mandin, P., Repoila, F., Vergassola, M., Geissmann, T. & Cossart, P. (2007) Identification of new noncoding RNAs in *Listeria monocytogenes* and prediction of mRNA targets. *Nucleic Acids Res*, 35, 962–974.

Matle, I., Mbatha, K.R. & Madoroba, E. (2020) A review of *Listeria monocytogenes* from meat and meat products: epidemiology, virulence factors, antimicrobial resistance and diagnosis. *Onderstepoort J Vet Res*, 87, e1–e20.

Olson, M.E., King, J.M., Yahr, T.L. & Horswill, A.R. (2013) Sialic acid catabolism in *Staphylococcus aureus*. *J Bacteriol*, 195, 1779–1788.

Orsi, R.H., den Bakker, H.C. & Wiedmann, M. (2011) *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. *Int J Med Microbiol*, 301, 79–96.

Osek, J., Lachtara, B. & Wieczorek, K. (2022) *Listeria monocytogenes*—how this pathogen survives in food-production environments? *Front Microbiol*, 13, 866462.

Patchett, R.A., Kelly, A.F. & Kroll, R.G. (1992) Effect of sodium chloride on the intracellular solute pools of *Listeria monocytogenes*. *Appl Environ Microbiol*, 58, 3959–3963.

Phan-Thanh, L. & Gormon, T. (1997) A chemically defined minimal medium for the optimal culture of *Listeria*. *Int J Food Microbiol*, 35, 91–95.

Pine, L., Malcolm, G.B., Brooks, J.B. & Daneshvar, M.I. (1989) Physiological studies on the growth and utilization of sugars by *Listeria* species. *Can J Microbiol*, 35, 245–254.

Portman, J.L., Dubensky, S.B., Peterson, B.N., Whiteley, A.T. & Portnoy, D.A. (2017) Activation of the *Listeria monocytogenes* virulence program by a reducing environment. *mBio*, 8, e01595–e01517.

Premaratne, R.J., Lin, W.J. & Johnson, E.A. (1991) Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl Environ Microbiol*, 57, 3046–3048.

Radoshevich, L. & Cossart, P. (2018) *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nat Rev Microbiol*, 16, 32–46.

Renzoni, A., Klarsfeld, A., Dramsi, S. & Cossart, P. (1997) Evidence that PrfA, the pleiotropic activator of virulence genes in *Listeria monocytogenes*, can be present but inactive. *Infect Immun*, 65, 1515–1518.

Ripio, M.T., Brehm, K., Lara, M., Suárez, M. & Vázquez-Boland, J.A. (1997) Glucose-1-phosphate utilization by *Listeria monocytogenes* is PrfA dependent and coordinately expressed with virulence factors. *J Bacteriol*, 179, 7174–7180.

Schirmer, F., Ehrt, S. & Hillen, W. (1997) Expression, inducer spectrum, domain structure, and function of MopR, the regulator of phenol degradation in *Acinetobacter calcoaceticus* NCIB8250. *J Bacteriol*, 179, 1329–1336.

Smith, K. & Youngman, P. (1992) Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis spoIIM* gene. *Biochimie*, 74, 705–711.

Sørensen, K.I. & Hove-Jensen, B. (1996) Ribose catabolism of *Escherichia coli*: characterization of the *rpiB* gene encoding ribose phosphate isomerase B and of the *rpiR* gene, which is involved in regulation of *rpiB* expression. *J Bacteriol*, 178, 1003–1011.

Stoll, R. & Goebel, W. (2010) The major PEP-phosphotransferase systems (PTSs) for glucose, mannose and cellobiose of *Listeria monocytogenes*, and their significance for extra- and intracellular growth. *Microbiology*, 156, 1069–1083.

Townsend, A., Strawn, L.K., Chapman, B.J. & Dunn, L.L. (2021) A systematic review of *Listeria* species and *Listeria monocytogenes* prevalence, persistence, and diversity throughout the fresh produce supply chain. *Foods*, 10, 1427.

Tsai, H.-N. & Hodgson, D.A. (2003) Development of a synthetic minimal medium for *Listeria monocytogenes*. *Appl Environ Microbiol*, 69, 6943–6945.

Vasanthakrishnan, R.B., de Las Heras, A., Scortti, M., Deshayes, C., Colegrave, N. & Vázquez-Boland, J.A. (2015) PrfA regulation offsets the cost of *Listeria* virulence outside the host. *Environ Microbiol*, 17, 4566–4579.

Vasseur, C., Rigaud, N., Hébraud, M. & Labadie, J. (2001) Combined effects of NaCl, NaOH, and biocides (monolaurin or lauric acid) on inactivation of *Listeria monocytogenes* and *Pseudomonas spp*. *J Food Prot*, 64, 1442–1445.

Vimr, E.R., Kalivoda, K.A., Deszo, E.L. & Steenbergen, S.M. (2004) Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev*, 68, 132–153.

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A. & Bielawski, J.P. (2008) Multilocus genotyping assays for single nucleotide

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polymorphism-based subtyping of *Listeria monocytogenes* isolates. *Appl Environ Microbiol*, 74, 7629–7642.

Welshimer, H.J. (1963) Vitamin requirements of *Listeria monocytogenes*. *J Bacteriol*, 85, 1156–1159.

Whiteley, A.T., Garelis, N.E., Peterson, B.N., Choi, P.H., Tong, L., Woodward, J.J. et al. (2017) c-di-AMP modulates *Listeria monocytogenes* central metabolism to regulate growth, antibiotic resistance and osmoregulation. *Mol Microbiol*, 104, 212–233.

Wiktorczyk-Kapischke, N., Skowron, K., Grudlewska-Buda, K., Wałecka-Zacharska, E., Korkus, J. & Gospodarek-Komkowska, E. (2021) Adaptive response of *Listeria monocytogenes* to the stress factors in the food processing environment. *Front Microbiol*, 12, 710085.

Xayarath, B. & Freitag, N.E. (2012) Optimizing the balance between host and environmental survival skills: lessons learned from *Listeria monocytogenes*. *Future Microbiol*, 7, 839–852.

Yamamoto, H., Serizawa, M., Thompson, J. & Sekiguchi, J. (2001) Regulation of the glv operon in *Bacillus subtilis*: YfiA (GlvR) is a positive regulator of the operon that is repressed through CcpA and *cre*. *J Bacteriol*, 183, 5110–5121.

Zeng, Z., Li, S., Boeren, S., Smid, E.J., Notebaart, R.A. & Abee, T. (2021) Anaerobic growth of *Listeria monocytogenes* on rhamnose is stimulated by vitamin B(12) and bacterial microcompartment-dependent 1,2-propanediol utilization. *mSphere*, 6, e0043421.

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