Listeria monocytogenes growth limits and stress resistance mechanisms

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Te weten wat men weet
en te weten wat men niet weet,
dat is kennis

Confucius

Content

Abstract

The food-borne pathogen *Listeria monocytogenes* is a Gram-positive facultative anaerobic rod, which is the causative agent of listeriosis. Due to the severity of the disease and the fact that its incidence is increasing in numerous European countries, *L. monocytogenes* is of great public health concern. This bacterium shows relatively high resistance to environmental insults compared with many other non-spore-forming food-borne pathogens. It is able to grow at low pH, at high salt concentrations, and low temperatures. The possibility that this pathogen (cross-) contaminates food products is a major concern for the food industry. Therefore, it is important to investigate the diversity in growth potential and stress resistance (mechanisms) of *L. monocytogenes* strains at both the serotype as well as the population level, during exposure to commonly used preservation conditions. Several approaches are described in this thesis, including comparison of stress resistance and growth limits of a large collection of natural isolates, screening a mutant library for stress sensitive mutants, and transcription profiling of stress responses. The function of various stress response genes and mechanisms including the so-called SOS response are described in detail.

The SOS response in *L. monocytogenes* appears to be important for stress resistance and for the induction of mutations, resulting in *L. monocytogenes* variants. The SOS regulon of *L. monocytogenes* was shown to consist of 29 genes encoding proteins with functions in error-prone DNA synthesis and DNA repair. It also includes the bile extrusion system BilE, which is known to affect virulence properties of *L. monocytogenes* by conferring resistance to bile. The SOS response may therefore contribute to the generation and persistence of *L. monocytogenes* variants in food processing environments and in the human host.

This thesis provides novel insights in the natural diversity of *L. monocytogenes* strains, and it has been revealed that specific groups of strains (serotypes) can be more persistent in specific niches such as food processing environments and retail establishments. Functional assessment of specific transcriptional regulators revealed an overlap in target genes and added to further understanding of the activation and function of the complex stress response network in this human pathogen.

1. General introduction and outline of the thesis

Stijn van der Veen, Tjakko Abee, Marjon H. J. Wells-Bennik

Abstract

The food-borne pathogen *Listeria monocytogenes* is a Gram-positive facultative anaerobic rod, which is the causative agent of listeriosis. Due to the severity of the disease, the high mortality rate, and the fact that its incidence is increasing in numerous European countries, *L. monocytogenes* is of great concern to public health. This bacterium shows relatively high resistance to environmental insults compared with many other non-spore-forming food-borne pathogens. It is able to grow at a wide pH range (from pH4.5 to pH 9), at high salt concentrations (up to 13%), and at a wide temperature range (-0.4 to 46 ºC) and it is able to survive more extreme acid conditions, salt concentrations, and temperatures for extended periods of time. The ability of *L. monocytogenes* to proliferate under adverse conditions and survive environmental insults results from various mechanisms that allow for rapid responses and adaptation to changing environments. Due to consumer's demands for less heavily preserved foods and more convenience foods, processing conditions in the food industry are becoming milder. *L. monocytogenes* is able to adapt to such milder conditions, making it of major concern to the food industry. To adequately inactivate *L. monocytogenes* and/or to prevent growth during storage, more knowledge is required about the stress response mechanisms of this bacterium that determine resistance to stress and about (genetic) diversity between different strains and serotypes. This chapter gives a short history on the food-borne pathogen *L. monocytogenes*, shows an overview of the current knowledge on the most important stress responses, and provides an outline of this thesis.

History

The first reports of "listiric" infections go back as far as 1891 when the presence of Gram-positive rods was described in tissue sections of deceased patients. In 1926 E.G.D. Murray et al. (110) was the first to describe this bacterium as a non-spore-forming Gram-positive rod that infects monocytes of the blood, resulting in diseases in rabbits and guinea pigs. This strain was therefore named *Bacterium monocytogenes*. Notably, a derivative of this strain (EGD-e, after Murray's initials) later became the first *L. monocytogenes* strain to be completely sequenced (58).The first confirmed reported case of *L. monocytogenes* in human was made in 1929 by Nyfeldt (62). In 1940 its present name was given by Pirie (62). After a period in which only sporadic cases of listeriosis were reported, mostly involving workers that had been in contact with diseased animals, interest in *L. monocytogenes* grew rapidly in the 1980s after reports of several food-borne outbreaks. In one of these outbreaks pasteurised milk was identified as the vehicle, raising the question whether *L.*

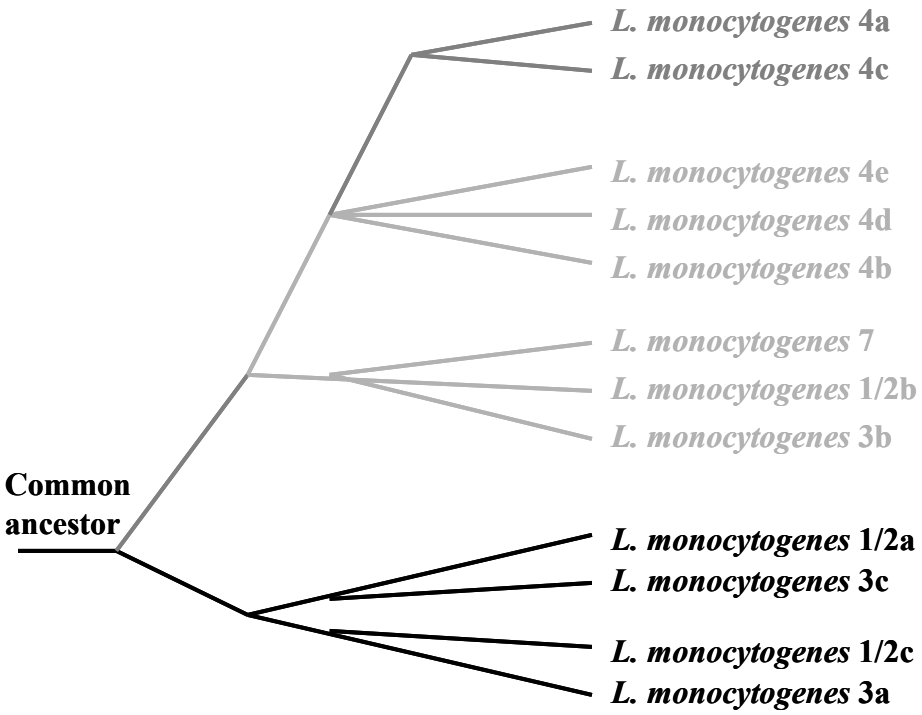

Fig. 1. Evolutionary scheme of the *L. monocytogenes* lineages and serovars based on the antigenic properties and the presence and absence of genes (derived from (43)).

monocytogenes was capable of surviving a pasteurisation step (53). The strain ScottA, which was isolated during this outbreak, is still the most researched strain in thermal inactivation studies. Due to the work on pathogenicity following these outbreaks, *L. monocytogenes* is now regarded as one of the invasive model strains in pathogen research (25).

Species

L. monocytogenes is part of the *Listeria* genus together with five other species: the pathogenic species *L. ivanovii* and the non-pathogenic species *L. seeligeri*, *L. welshimeri*, *L. innocua*, and *L. grayi* (128). The species *L. monocytogenes* contains 13 different serotypes, which are divided over three lineages according to the presence of specific marker genes (Fig. 1): lineage I contains the serotypes 1/2a, 1/2c, 3a, and 3c, lineage II contains the serotypes 1/2b, 3b, 7, 4b, 4d, and 4e, lineage III contains the serotypes 4a and 4c (43). The serotype 4ab strains were not designated to a lineage in this study. Of the 13 identified serotypes, serotypes 1/2a, 1/2b, 1/2c, and 4b account for over 95% of the isolates from foods and patients (42). The majority of the food isolates are of serotype 1/2a and 1/2b (72, 161), which might be related to the growth capabilities of these strains at low temperature (7 ºC) in combination with other stresses (Chapter 2). The majority of the clinical isolates are of serotype 4b and all major outbreaks of food-borne listeriosis are caused by strains from this serotype (12).

Food-borne pathogen

L. monocytogenes is the causative agent of listeriosis, which is often manifested as meningitis, encephalitis, sepsis, intrauterine infections, spontaneous abortion, and gastroenteritis (147). The affected population consist mainly of elderly, pregnant women (and their unborn children), infants, and immuno-compromized persons (75). The number of listeriosis cases is relatively low compared with other food-borne pathogens, but the mortality rate is extremely high (Table 1). The incidence of listeriosis is increasing in many countries of the European Union (33). In The Netherlands the incidence in 2006 was 3.9 per million and the mortality rate was 28% (40). Estimations in the transmission of listeriosis showed that 99 % of the cases are caused by contaminated food products (105). *L. monocytogenes* is ubiquitously present in the rural environments and contaminates raw materials used in the food-industry. The ability of this bacterium to tolerate high concentrations of salt (up to 13%), a wide pH range (pH4.5 to pH 9), and the capability to multiply at refrigeration conditions pose a serious risk to food safety (Chapter 2 and (79)).

Table 1. Outbreaks of Listeriosis with known food vehicles.

Year	Food Vehicle	Country (State)	Cases	Deaths (%)	Serotype	Reference
1978-1979	Vegetables (raw)	Australia	12	0 (0)	Unknown	(117)
1979	Raw vegetables or cheese	USA (MA)	20	3 (15.0)	4b	(71)
1980	Raw seafood (finfish and mollusks)	New Zealand	22	6 (27.3)	1b	(95)
1981	Miscellaneous Dairy Products	England	11	5 (45.5)	1/2a	(125)
1981	Vegetables (raw)	Canada	41	17 (41.5)	4b	(127)
1983	Pasteurized fluid milk	USA (MA)	32	14 (43.8)	4b	(53)
1983-1987	Vacherin Mont d'Or cheese	Switzerland	122	31 (25.4)	4b	(13)
1985	Mexican-style cheese (raw milk)	USA (CA)	142	48 (33.8)	4b	(97)
1986	Unpasteurized milk, organic vegetables	Austria	28	5 (17.9)	Unknown	(3)
1986-1987	Ice cream, salami, brie cheese	USA (PA)	36	16 (44.4)	4b,1/2b, 1/2a	(131)
1986-1987	Raw eggs	USA (CA)	2	Unknown	4b	(130)
1987	Butter	USA (CA)	11	Unknown	Unknown	(125)
1987-1989	Pâté and meat spreads	England	355	94 (26.5)	4b	(102)
1989-1990	Semi-soft Cheese (blue)	Denmark	23	0 (0)	4b	(77)
1990	Pâté and meat spreads	Australia	11	6 (54.5)	1/2a	(125)
1991	Smoked mussels	Tasmania, Australia	4	0 (0)	1/2a	(107)
1992	Smoked mussels	New Zealand	4	0 (0)	1/2	(9)
1992	Pork tongue in jelly	France	280	63 (22.5)	4b	(76)
1993	Rillettes	France	38	11 (28.9)	4b	(61)
1994-1995	Smoked Seafood (finfish and mollusks)	Sweden	9	2 (22.2)	4b	(45)
1995	Soft Ripened Cheese, >50% moisture	France	33	4 (20.0)	4b	(60, 76)

Table 1. Continued.

Year	Food Vehicle	Country (State)	Cases	Deaths (%)	Serotype	Reference
Not specified	Frozen vegetables	USA (TX)	7	Unknown	4b	(136)
1997	Pon l'Eveque cheese	France	14	0 (0)	4b	(125)
1998-1999	Butter	Finland	25	6 (24.0)	3a	(99)
1998-1999	Hot dogs, deli meats	USA (22 states)	101	21 (20.8)	4b	(103)
1999	Pâté	USA (CT, MD, NY)	11	unknown	1/2a	(14)
1999-2000	Pigs tongue in aspic	France	26	7 (26.9)	Unknown	(41)
2000	Deli turkey meat	USA (10 states)	29	7 (24.1)	unknown	(15)
2000-2001	Homemade Mexican-style cheese (raw milk)	USA (NC)	12	5 (41.7)	unknown	(16)
2002	Deli turkey meat	USA (9 states)	54	8	4b	(59)
2003	Sandwiches	UK	5	0 (0)	1/2	(32)
2005	Tomme cheese	Switzerland	10	3 (30.0)	1/2a	(8)
2006-2008	soft cheese, salads and fish	Czech Republic	75	12 (16.7)	1/2b	(148)
Not specified	Frankfurter meat	USA (24 states)	108	14 (13.0)	4b	(104)
2007-2008	Pasteurized milk	USA (Massachusetts)	?	4 (?)	Unknown	
2008	Deli meat	Canada	?	19 (?)	Unknown	

Food-borne outbreaks of *L. monocytogenes* have been associated with many different categories of food products (Table 1), including dairy products, seafood, vegetables and meat products (49). In particular, food products that do not require further cooking before consumption or those with an extended shelf life at refrigeration conditions are a serious risk for *L. monocytogenes* contamination and outgrowth (89). Because the infective dose of *L. monocytogenes* for humans is not known, a zero-tolerance policy was in effect for these types of products. However, in January 2006, regulation 2073/2005 (48) has been implemented in the European Union, providing new microbiological criteria for ready-to-eat (RTE) food products, providing they are not intended for infants and special medical purposes. In RTE food products that do not support growth of *L. monocytogenes*, a maximum concentration of 100 cfu/g is allowed. The criteria for these products are: pH ≤

4.4, or $a_w \leq 0.92$ (approximately 13 % NaCl), or pH ≤ 5.0 and $a_w \leq 0.94$ (+/- 10 % NaCl). Due to consumer's demands for less heavily preserved foods and more RTE and convenience foods, processing conditions in the food industry are becoming milder. To maintain bacteriocidal conditions in food processing, combinations of mild processing steps (hurdles) are being used (94). These hurdles can include combinations of heating, cold-shock, acidification, brining, and high hydrostatic pressure (HHP) treatments. Exposure to sub-lethal stresses might result in adaptation of *L. monocytogenes* to similar subsequent stresses or cross-protection to other stresses (1, 70). To design optimal mild processing conditions that result in adequate inactivation of pathogens like *L. monocytogenes*, insight in the diversity of this bacterium in relation to stress resistance is required. Furthermore, more knowledge on the stress response and adaptation mechanisms of this organism and the diversity of these mechanisms in *L. monocytogenes* populations needs to be obtained. This knowledge can be used to predict the optimal sequence of stress exposures to prevent cross-protection between different stresses, and to decide what level of exposure to certain inhibitory compounds or treatments is required for effective inactivation.

Diversity

Currently, the genomes of two *L. monocytogenes* strains have been completely sequenced and annotated. These strains are EGD-e, which is a serotype 1/2a strain (58), and F2365, which is of serotype 4b (116). In addition, the genome sequences of two other strains, which are of serotype 1/2a (F6854) and 4b (H7858), have nearly been completed (116). Recently, 18 *L. monocytogenes* genomes have been sequenced to near completion by the Broad Institute (www.broad.mit.edu/seq/msc/) and annotation for some of the genomes is in progress. These genomes are of strains belonging to serotype 1/2a, 1/2b, 1/2c, 4a, and 4b and include two of the most widely studies strains worldwide (10403S and LO28). All of the sequenced genomes are circular with approximately 89% coding sequences (65). The fully sequenced genomes showed high synteny in genetic organization and gene content (65, 116). Inversions or shifting of large genetic elements were not observed, probably due to the low occurrence of IS elements (11). However, genetic differences between strains and serotypes were observed, indicating that despite the low genetic variation phenotypic traits can still be different. A direct comparison of four sequenced strains showed 51, 97, 69, and 61 strain-specific genes for F2362, F6854, H7858, and EGD-e, respectively. Most differences between strains consisted of phage insertions (116). Furthermore, this study revealed that 83 genes were restricted to serotype 1/2a strains and 51 genes to serotype 4b strains. In some cases, genetic differences between strains and serotypes showed a relation to resistance to heat (160), acid (28), or bile salts (7, 142), to pathogenic potential (154), or to motility (63). A direct comparison between the pathogenic species *L. monocytogenes* and

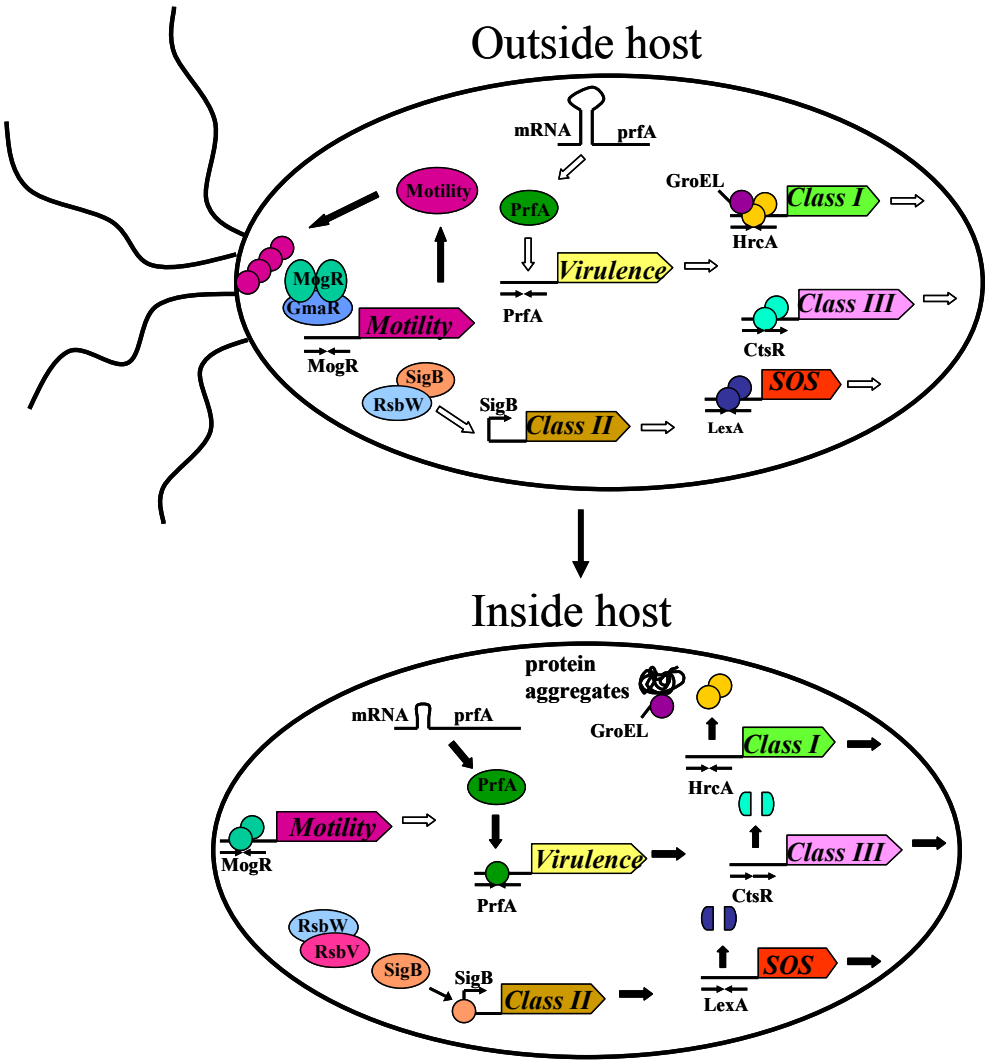

Fig. 2. Activation of specific virulence and stress mechanisms by the environment of host organisms. Outside the host, the motility genes are expressed (black arrows) resulting in the production of flagella, while the virulence genes and stress response genes are generally not activated (white arrows). Inside the host, the virulence genes and stress response genes are activated (black arrows), while the motility genes are repressed (white arrow). For details on the activation and repression mechanisms of the specific responses, see text.

the non-pathogenic species *L. innocua* revealed that the most important difference between these species was the insertion of a pathogenicity island (LIPI-1) (147) in the *L. monocytogenes* genome between the genes *prs* and *ldh* (58, 67). This island encodes proteins that are responsible for the ability of *L. monocytogenes* to survive and multiply in host cells and induce cell-to-cell spread.

Stress response and adaptation

Bacteria have evolved various strategies and networks to survive and adapt to changing conditions encountered in the environment, e.g. during food processing and inside host organisms. Strategies used by *L. monocytogenes* to survive adverse conditions are transcriptional regulation and activation of stress genes, inducing genetic diversity that result in stress resistant subpopulations, and formation of biofilms. Bacteria contain various signal transduction mechanisms to sense and respond to stress by modulation of gene expression. *L. monocytogenes* contains 15 putative two-component systems of which both the sensor histidine kinase and the cognate response regulator have been identified, and one orphan response regulator (DegU) (155, 156). Two component systems have been identified with roles in stress resistance (Chapter 5 and (10, 137)), biofilm formation (120), virulence (4), and motility (52, 156). Motility depends on the orphan response regulator DegU, which activates transcription of the motility genes in a temperature-dependent manner through activation of *gmaR* (64, 135). At 37 ºC and above, MogR represses the motility genes, while at 30 ºC and below MogR is antagonized by GmaR.

Furthermore, specific responses can be induced during exposure to stresses. Following the completion of the genome sequence of *L. monocytogenes*, several groups conducted whole-genome transcription profiling studies. These studies generated more insight in the specificity of these responses after stress exposure (Chapter 4 and (66, 73, 74, 119, 132)).

L. monocytogenes contains two specific heat-shock responses, namely, the HrcA regulated class I response, and the CtsR regulated class III response. These heat-shock responses are transcriptionally activated during exposure to elevated temperatures or other stresses like cold-shock, high salt concentrations, and acid-shock (Chapter 4 and (44, 98)). The heat-shock response genes encode molecular chaperones and proteases that protect cells from aggregation of damaged and misfolded proteins. Chaperones are able to reactivate protein aggregates (19, 92, 150) and proteases clean-up these protein aggregates when reactivation fails (112). Maintenance of protein quality is essential during exposure to stress conditions (96).

L. monocytogenes contains another important stress response mechanism, namely the class II stress response, which is controlled by the alternative sigma factor SigB. This

stress response mechanism is activated during exposure to many different stresses and in virulence (Chapter 4 and (5, 50, 153)). The class II stress response contains genes encoding decarboxylases for acid resistance (51, 152), osmolyte transporters for salt and low temperature resistance (140, 151), reductases for oxidative stress resistance (Chapter 4 and (66)), and chaperonins and proteases for heat- and HHP-resistance (Chapter 4 and (152)).

The SOS response of *L. monocytogenes* encodes alternative DNA polymerases and DNA repair proteins (Chapter 7) and is regulated by RecA and LexA. It is involved in DNA repair or restarting of stalled replication forks (29, 31). For many bacteria it was shown that the SOS response was activated during exposure to different stresses that result in DNA damage or replication fork stalling (24, 38, 39, 141). The SOS response is also involved in induction of genetic variation in many bacteria, including *L. monocytogenes* (Chapter 7 and (54, 144)), which could result in the generation of mutants with increased resistance to stress.

L. monocytogenes contains a specific temperature-dependent response that is important for colonizing the host environment. This virulence response is regulated by the PrfA activator (18, 93). The secondary structure of the untranslated region of *prfA* mRNA prevents translation at temperatures below 37 ºC (78), while at 37 ºC and above *prfA* mRNA is translated and the virulence response is activated. Besides the virulence response, several other (stress) responses are activated or repressed by the specific conditions of the host environment (Fig. 2). Previous studies showed a partial overlap between the PrfA regulated virulence response and the class II stress response (74, 106), indicating that this class of stress genes performs a role in colonization and growth inside host organisms. Furthermore, the class I and class III heat-shock response, the class II stress response, and the SOS response are activated during intracellular growth of murine macrophage cells (21). These results demonstrated that specific stress response mechanisms are important for growth and survival during exposure to stressful environments outside and inside host organisms.

Class I heat-shock response

The class I heat-shock genes encode molecular chaperones and include *dnaK*, *dnaJ*, *grpE*, *groES*, and *groEL*. Their products form the two chaperonin systems KJE (DnaK, DnaJ, and GrpE) and GroESL (37, 133). These genes are transcribed from two locations on the *L. monocytogenes* chromosome (Fig. 3). The class I heat-shock genes are regulated by the autoregulatory HrcA repressor, which binds to the CIRCE (Controlling Inverted Repeat of Chaperone Expression (162)) operator sequence (TTAGCACTC-N_9-GAGTGCTAA), located in the promoter region of this class of genes (129). In both *Bacillus subtilis* (108) and *Chlamydia trachomatis* (157) the binding affinity of HrcA to the CIRCE operator was dependent on the presence of the class I heat-shock protein GroEL. GroEL increases the

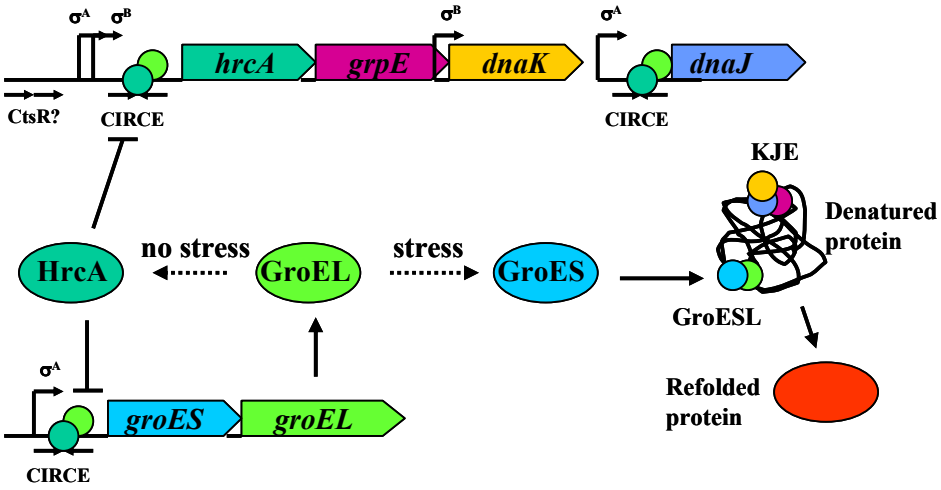

Fig. 3. Regulation and titration model for the activation of the class I heat-shock genes. Activation results in the induction of the KJE and GroESL chaperonin systems that refold denatured proteins. For details, see text.

binding affinity of HrcA to the CIRCE operator, resulting in repression of the class I heat-shock genes. Activation of the class I heat-shock genes could therefore be described by a GroEL titration model (Fig. 3) (115). Higher temperatures result in increased numbers of denatured proteins, which engages the GroESL chaperonin system in refolding of these proteins, thereby depleting the GroEL pool. Since the class I heat-shock response of *L. monocytogenes* is induced upon heat-shock (Chapter 4), the above described temperature-dependent titration model might be similar in *L. monocytogenes*. The class I chaperones have been shown to be involved in protection of *L. monocytogenes* to various stresses. Exposure to high salt concentrations resulted in induction of DnaK (44). Furthermore, a *dnaK* mutant of *L. monocytogenes* showed reduced growth at temperatures above 39 ºC and increased acid sensitivity compared with the wild-type strain (68). Expression of *groESL* was induced after growth at low temperatures (98) and after exposure to ethanol, acid, or bile salts compared with growth at 37 ºC (56). Both *dnaK* and *groESL* showed increased expression in mouse macrophages (56).

Recent evidence indicates that the class I heat-shock response is also partially regulated by the class II stress response regulator SigB (73). The *hrcA-grpE-dnaK* operon contains a SigB promoter overlapping the SigA promoter, and a second SigB promoter is located upstream of the *dnaK* coding region (Fig. 3). This indicates that *dnaK* could be induced by stress conditions that result in *sigB* activation while the *hrcA-dnaK* operon is still repressed by HrcA. For *Staphylococcus* it was shown that the *hrcA-dnaK* operon was

also under control of CtsR, showing regulatory interactions between the two classes of heat-shock genes (20). Interestingly, the *hrcA-dnaK* operon of *L. monocytogenes* contains a perfect CtsR binding site repeat on the lagging strand in front of the promoter 119 bp upstream of the ATG startcodon (unpublished results). Whether binding of CtsR to this repeat affects expression of the *hrcA-dnaK* operon remains to be established.

Class III heat-shock response

The class III heat-shock response consists of molecular chaperones and ATP-dependent Clp proteases, including ClpB, ClpC, ClpE, and two ClpP ATPases (112). Their functions and the genetic structures of their encoding genes are shown in Figure 4. The class III heat-shock genes are regulated by an autoregulatory dimeric CtsR (class three stress-gene repressor) repressor, which binds to a heptanucleotide repeat (A/GGTCAAA-NAN-A/GGTCAAA) in the promoter region of these genes (34-36, 90, 112). In previous

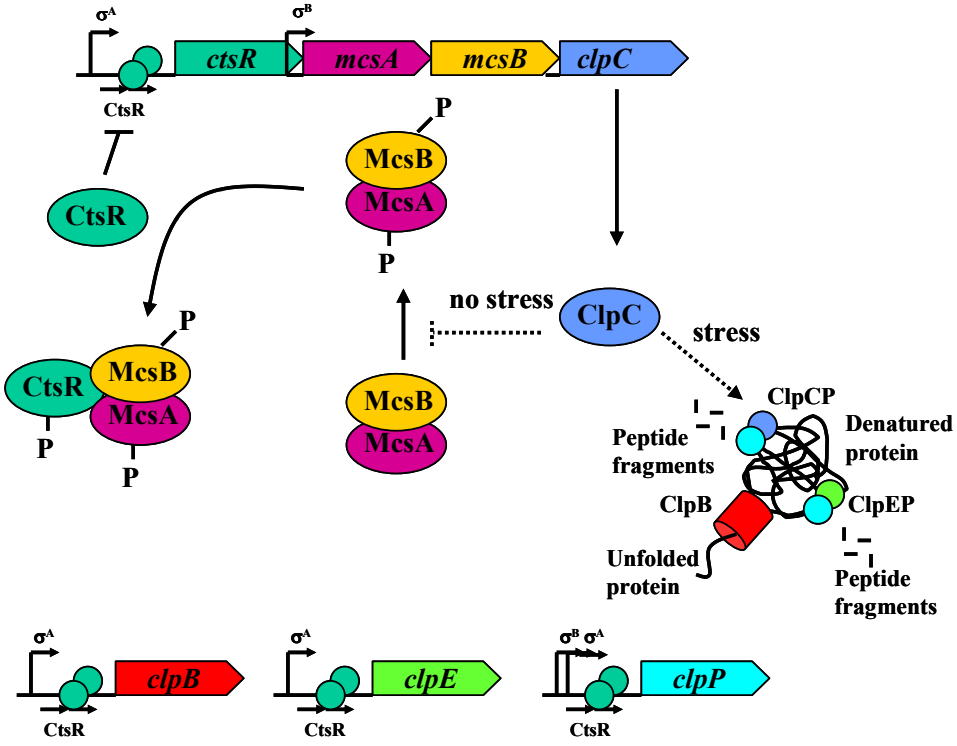

Fig. 4. Regulation and titration model for the activation of the class III heat-shock genes. Activation results in the induction of chaperonin systems or proteases that refold or degrade denatured proteins . For details, see text.

research the temperature-dependent regulatory mechanism has been described for *B. subtilis* (88). A similar mechanism is believed to be present in *L. monocytogenes* and involves all products of the *ctsR-mcsA-mcsB-clpC* operon and ClpP. CtsR of *L. monocytogenes* is thought to form a ternary complex with MscA and McsB, encoding a putative kinase, which prevents binding of CtsR with the operator sequence. McsA activates McsB, which in turn phosphorylates McsA, McsB and CtsR, and targets CtsR for degradation. Degradation of CtsR dependents on the activity of the ClpCP protease (91). Furthermore, the kinase activity of McsB is inhibited by ClpC. Similar to the class I heat-shock response, a titration model can be used to describe the activation of the class III heat-shock response, which depends on ClpC (Fig. 4). During exposure to higher temperatures elevated concentrations of unfolded or damaged proteins result in the relief of the kinase McsB from ClpC inhibition, thereby targeting CtsR for degradation and initiating the heat-shock response of the class III genes. Upon administering a heat shock, all *L. monocytogenes* class III heat-shock genes that are mentioned above show increased expression (Chapter 4). Noteworthy is that *L. monocytogenes* ScottA cultures contain a subpopulation of about six cells per 10^4 in which the class III heat-shock genes are constitutively induced due to mutations in *ctsR* that render this repressor inactive (81). This subpopulation of cells grows slower than the wild-type cells, but shows increased fitness upon exposure to stresses like heat, HHP, high salt concentrations, acid, and H_2O_2 (80, 82, 112). The *ctsR* mutations are likely reversible and are the result of strand slippage over a repeat encoding four consecutive glycines (82). The Clp ATPases are very important for both stress survival and virulence. The *clpC* mutant shows reduced capabilities to infect hepatocytes in the liver and epithelial cells of infected mice (114). Growth is restricted in macrophages and at stress conditions (42 ºC or 2% NaCl) in the *clpC* mutant (124). Furthermore, suggestions for cross-talk between ClpC and the central virulence regulator PrfA have been made (121) and it has been shown that ClpC is involved in the correct expression of the virulence factors InlA, InlB, and ActA (114). ClpE is required for prolonged survival at 42 ºC (113). Furthermore, ClpE is involved in virulence and cell division, since the *clpE-clpC* double mutant was avirulent in a mouse model and cell division was affected (113). ClpB is a chaperonin that is involved in reactivation of protein aggregates in collaboration with the KJE system (92). ClpB is tightly regulated by both the CtsR and MogR (motility gene repressor (63, 134, 135)) repressors. It plays an important role in *L. monocytogenes* heat- and HHP-resistance (Chapter 6) and in virulence, since the *clpB* mutant showed reduced virulence in a mouse infection model (19). While *clpB* is an important gene in the heat-shock response, it was also induced during growth at low temperatures (98). ClpP is important for stress resistance and virulence (57). A *clpP* mutant showed impaired virulence in a mouse infection model, which was partly the result of

reduced expression of the virulence factor listeriolysin O (LLO). Furthermore, this mutant showed reduced growth at 42 ºC or in the presence of 12% NaCl.

Previous studies showed a partial overlap between the class III heat-shock response and the class II stress response. Gene expression and protein studies on the *sigB* mutant showed that ClpP and ClpC are SigB activated (23, 152). Recently, a SigB promoter was identified in front of *clpP* and in the *ctsR-clpC* operon upstream of *mcsA* (Fig. 4) (74). This indicates that *clpC* could be induced during conditions in which SigB is activated while CtsR repression is still functional.

Class II stress response

The class II stress genes encode a group of proteins that play roles in the response to various stress conditions (5, 6, 50, 153). The class II stress genes are regulated by the autoregulatory alternative sigma factor SigB, which recognizes alternative –35 and –10 promoter sequences (GTTT-$N_{13–17}$-GGGWAT) (84). *SigB* is co-transcribed with seven regulatory genes from two operons, which are involved in post-translational regulation of SigB (Fig. 5) (22, 66, 69). The *rbsR-rbsS-rbsT-rbsU* operon is transcribed from a SigA promoter and the *rbsV-rbsW-sigB-rbsX* operon is transcribed from a SigB promoter. Under conditions in which SigB activity is not required, SigB is antagonized by the anti-SigB kinase RsbW. RsbW phosphorylates the anti-anti-SigB factor RsbV, thereby preventing its antagonizing activity on RsbW. Under conditions in which SigB is required, RsbV is dephosphorylated by the phosphatase RsbU, which in turn binds to RsbW to prevent SigB antagonizing activity. RsbU is allosterically activated by the kinase RsbT. RsbT phosphorylates RsbS, thereby preventing an antagonizing effect of RsbS. To activate the RsbT antagonizing activity of RsbS, RsbS is dephosphorylated by the phosphatase RsbX. Finally, the RsbS antagonizing activity on RsbT is also modulated by RsbR. The autoregulatory function of this system depends on the SigB activator activity and the RsbX repressing activity (22, 66, 69). RsbX, which is co-transcribed with SigB, acts as a feedback signal by dephosphorylation of RsbS which in turn antagonizes RsbT, and thereby prevents activation of RsbU. Finally, SigB is antagonized by RsbW. Furthermore, this SigB regulation module contains two energy (ATP) dependent phosphorylation steps, which are believed to function in energy dependent regulation of SigB (22, 66, 69). ATP depletion in for instance the stationary phase will result in accumulation of unphosphorylated RsbV, which antagonizes the SigB binding activity of RsbW, thereby activating the SigB stress response. On the other hand, ATP depletion will also result in accumulation of unphosphorylated RsbS, which will antagonize the RsbT activator. This will inhibit dephosphorylation of RsbV by RsbU and could potentially result in SigB inhibition by RsbW. In a recent whole genome expression profiling study, SigB regulated genes in *L. monocytogenes* were identified (66). In total, the expression of 216 genes were dependent

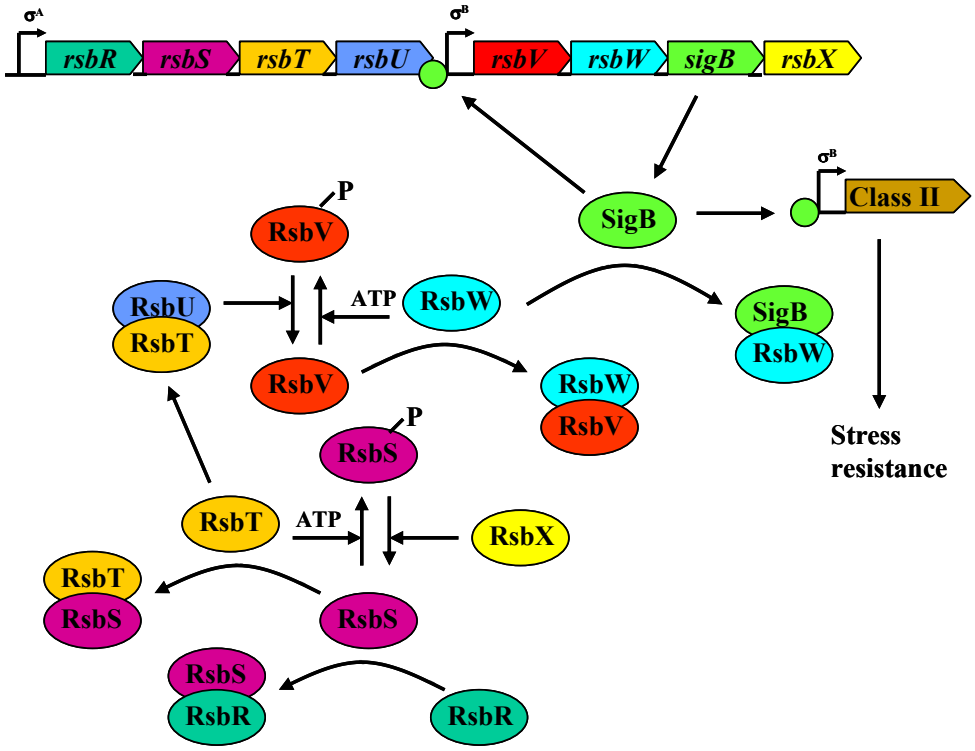

Fig. 5. Regulation model for the activation of the class II stress genes. After SigB inhibition by the RsbW antagonist is lost, SigB activates the class II stress genes by binding their SigB-dependent promoter, resulting in stress resistance. For details, see text.

on SigB, of which 105 genes in 76 transcription units were up-regulated by SigB and 111 genes in 94 transcription units were down-regulated. These genes comprise approximately 8% of the *L. monocytogenes* genome, showing the importance of the SigB response. Of the 76 positively controlled transcriptional units, 23 did not contain a consensus SigB promoter, while only 13 consensus SigB promoters were detected in the 94 down-regulated transcriptional units. SigB dependent genes include some of the class I and class III heat-shock genes (23, 73, 74, 152), and many other genes involved in other resistance mechanisms. SigB, for instance, regulates the expression of genes encoding for osmolyte transporters, including OpuC (55, 152), DtpT (66, 158), Gbu (17), and BetL (139). Osmolyte transporters are involved in protection against osmotic and cold stress by transporting osmo- and cryoprotective compounds such as glycine-betaine, carnitine, ornithine, and small peptides (140, 151). SigB is also involved in acid tolerance by controlling the expression of genes that encode the GAD decarboxylase system (51, 152).

This system consists of a glutamate/GABA antiporter for transportation and a glutamate decarboxylase, which consumes a intracellular proton by converting glutamate into GABA (26, 27). Other genes that are regulated by SigB include those encoding three universal stress proteins (Usp) of *L. monocytogenes*, the general stress protein Ctc, (Chapter 4 and (66)), and in addition, some reductases involved in oxidative stress (Chapter 4 and (66)). Previous research furthermore showed that SigB plays an important role in virulence (85, 111, 153). This role is two-fold as SigB can activate stress resistance mechanisms involved in bile resistance and also control the expression of virulence genes directly. The SigB controlled bile resistance systems are BilE (138) and Bsh (142), which are involved in bile exclusion and bile hydrolysis, respectively. The virulence genes that contain SigB dependent promoters encode the major virulence regulator PrfA, which is directly regulated by SigB in a growth phase dependent manner (85, 111), and internalins (87, 100, 101).

SOS response

The SOS response encodes DNA repair proteins and alternative DNA polymerases (29, 122). The SOS response is regulated by the autoregulatory LexA repressor and the RecA activator (Fig. 6) (46). The LexA repressor inhibits transcription of the SOS response genes by binding to an inverted repeat in the promoter region of these genes. The *L. monocytogenes* LexA consensus binding sequence is AATAAGAACATATGTTCGTTT

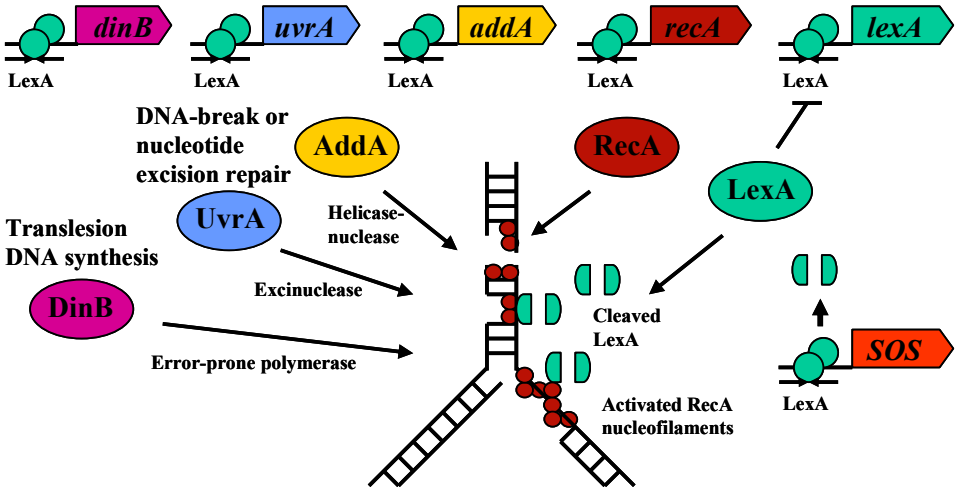

Fig. 6. Regulation and activity model for the SOS response. DNA damage or replication fork stalling results in activation of RecA by ssDNA. Activated RecA stimulates cleavage of the LexA repressor, resulting in induction of the SOS response genes. The SOS response encodes alternative DNA polymerases, excinucleases, and helicases that repair damaged DNA and stalled replication forks. For details, see text.

(Chapter 7). The SOS response is generally activated by processes that result in accumulation of single stranded DNA (ssDNA). RecA, which is present at basic levels, will bind to these ssDNA filaments, resulting in a nucleoprotein complex that activates RecA by inducing structural changes. These activated RecA-ssDNA complexes then stimulate autocleavage of the LexA repressor, resulting in induction of the SOS response (2, 126). This response is activated by stresses that induce accumulation of ssDNA after DNA damage, e.g. UV-exposure or mitomycin C-exposure (30, 86), or replication fork stalling, e.g. heat-shock or ciprofloxacin-exposure (Chapter 4 and (24)). Interestingly, low pH and high salt concentrations also resulted in structural changes in LexA and RecA, respectively, which could induce the SOS response (38, 39, 141). The *L. monocytogenes* SOS response consists of 29 putative genes, which are transcribed from 16 operons (Chapter 7). Two alternative DNA polymerases are part of the SOS response of *L. monocytogenes*, namely Pol IV (DinB) and Pol V (UmuDC) (Chapter 4). These alternative DNA polymerases were first discovered in *Escherichia coli*, and their presence has in the meantime been established in many other bacteria (118). DinB and UmuDC are error-prone lesion bypass polymerases without proofreading activity and are involved in introducing adaptive mutations (143-145, 149). In addition to the alternative DNA polymerases, the SOS response encodes several DNA repair systems. This includes the UvrAB system, which is an excinuclease system that recognizes DNA lesions and is responsible for repair of most of the DNA damage (47, 146), and the AddA helicase, which is involved DNA break processing (159). In *L. monocytogenes* this helicase is important for growth at elevated temperatures (Chapter 5). Remarkably, the SigA and SigB controlled bile exclusion system BilE appears to be part of the *L. monocytogenes* SOS response (Chapter 7). Given the role of BilE in virulence, the SOS response may play a role in the pathogenicity of *L. monocytogenes*. For some bacteria, it has been shown that activation of the SOS response results in inhibitions of cell division, thereby preventing transection of the genome after replication fork stalling (83, 109). This effect of interrupted Z-ring formation in the vicinity of the nucleoid is called “nucleoid occlusion” (123). In *L. monocytogenes*, YneA prevents septum formation by accumulating at the mid-cell. In this thesis, we demonstrate that this mechanism is important for heat-resistance (Chapter 7).

Outline of the thesis

Several genomics-based approaches were used to study the diversity in stress-resistance and survival strategies of *L. monocytogenes* during food preservation conditions. These results can be used by the food industry to develop new (mild) preservation techniques that are effective to inactivate *L. monocytogenes* or prevent growth of this

organism. The diversity between different strains and serotypes of *L. monocytogenes* with regard to stress resistance is addressed in Chapter 2 and 3. The stress responses of *L. monocytogenes* during exposure to elevated temperatures is described in Chapter 4 and 5. Lastly, specific characteristics of two important *L. monocytogenes* stress response mechanisms are described in Chapter 6 and 7, namely, the regulation of the class III heat-shock gene *clpB* by the MogR repressor, and the function and regulon of the SOS response in *L. monocytogenes*.

In Chapter 2 the growth limits of 138 *L. monocytogenes* strains from diverse origin were assessed using combinations of different temperatures, low pH conditions, high salt concentrations, and the addition of the preservative sodium lactate. The observed growth limits showed serotype- and niche-specific traits. The outcome of the growth limits experiments showed that the new European Union regulation 2073/2005 provide good criteria to prevent growth of *L. monocytogenes* in RTE food products and a potential new criterion was reported in this chapter. Furthermore, all strains were screened for the presence of biomarkers that might explain the observed difference in growth limits. Two potential biomarkers were identified for selected *L. monocytogenes* serotypes.

Chapter 3 reports on the diversity of *L. monocytogenes* strains with respect to heat-resistance. The heat inactivation kinetics of the most heat-resistant strains and a reference strain were determined in a continuous flow through heating system (micro-heater) that mimics an industrial heat-exchanger. In a pre-screen, the heat-resistance of 48 *L. monocytogenes* strains was assessed in a batch heating process, showing large variation in heat-resistance. Two of the most heat-resistant strains (1E and NV8) and a reference strain (ScottA) were tested under pasteurization conditions in the micro-heater. For the heat-resistant strain 1E it was calculated that approximately 10.7 $\log_{10}$ reduction is achieved during the minimal HTST pasteurization process of 15 s at 71.7 ºC in milk, while the calculated inactivation of the reference strain ScottA was approximately 78 $\log_{10}$ under the same conditions. These results show that HTST pasteurization is in principle sufficient to inactivate heat-resistant *L. monocytogenes* strains.

In Chapter 4, the heat-shock response of *L. monocytogenes* was investigated by comparing whole-genome expression profiles of cells before and after heat-shock. Genes belonging to the class I and class III heat-shock response, the class II stress response, and notably, the SOS response were induced upon heat-shock. Furthermore, cell elongation and inhibition of cell division was observed, which could be correlated with differential expression levels of a number of genes that encode products with a role in cell wall synthesis or cell division.

In Chapter 5, a mutant library was screened for mutants with reduced abilities to grow at elevated temperatures. The genetic origin of the vector insertion was identified in 28 different mutants and the morphology, growth characteristics, and heat-resistance of

these mutant strains with impaired growth at high temperatures were further investigated. Several temperature sensitive mutants showed insertions in genes belonging to specific stress responses or in genes with a function in translation or cell wall synthesis and turnover. Some of the mutants indeed showed altered cell morphologies, including elongated cells, cells with reduced length, or sickle-shaped cells. Also, most mutants showed increased sensitivity to heat-inactivation.

Chapter 6 focuses specifically on the link between the motility gene repressor, MogR, and the CtsR regulated class III heat-shock gene, *clpB*. In this chapter, *clpB* was identified as a new member of the MogR regulon. *ClpB* expression is regulated by both MogR and CtsR repressors in a temperature dependent manner, showing the importance of correct temperature dependent expression of this gene. Furthermore, the role of ClpB and MogR in stress resistance was highlighted in heat- and HHP-inactivation experiments. These results showed that MogR is a generic repressor, which is involved in both motility regulation and stress resistance.

Chapter 7 describes an analysis of the SOS response of *L. monocytogenes*. SOS response genes were identified by a combination of micro-array experiments and iterative motif searches. Furthermore, a role for the SOS response in mutagenesis and stress resistance was shown. The SOS response gene *yneA* was found to play a role in cell elongation or inhibition of cell division, resulting in increased heat-resistance.

In the final chapter, Chapter 8, the impact of diversity on stress resistance is discussed. The first part of this chapter focuses on the diversity of strains and serotypes with respect to stress resistance in relation to the presence of genetic biomarkers. In the second part, the role of mutagenesis mechanisms, such as the SOS-response, is discussed in relation to the generation of mutants with increased stress resistance and their impact on food safety.

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2. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits

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Abstract

Aims: The aim of this study was to associate the growth limits of *Listeria monocytogenes* during exposure to combined stresses with specific serotypes or origins of isolation, and identify potential genetic markers. Methods and Results: The growth of 138 strains was assessed at different temperatures using combinations of low pH, sodium lactate, and high salt concentrations in Brain Heart Infusion (BHI) broth. None of the strains was able to grow at pH ≤ 4.4, a_w ≤ 0.92, or pH ≤ 5.0 combined with a_w ≤ 0.94. In addition, none of the strains grew at pH ≤ 5.2 and NaLac ≥ 2 %. At 30 ºC, the serotype 4b strains showed the highest tolerance to low pH and high NaCl concentrations at both pH neutral (pH 7.4) and mild acidic conditions (pH 5.5). At 7 ºC, the serotype 1/2b strains showed the highest tolerance to high NaCl concentrations at both pH 7.4 and pH 5.5. Serotype 1/2b meat isolates showed the highest tolerance to low pH in the presence of 2 % sodium lactate at 7 ºC. ORF2110 and *gadD1T1* were identified as potential biomarkers for phenotypic differences. Conclusions: Differences in growth limits were identified between specific *L. monocytogenes* strains and serotypes, which could in some cases be associated with specific genetic markers. Significance and Impact of Study: Our data confirm the growth limits of *L. monocytogenes* as set out by the European Union for ready-to-eat foods, and provides an additional criterion. The association of *L. monocytogenes* serotypes with certain stress responses might explain the abundance of certain serotypes in retail foods while others are common in clinical cases.

Introduction

The pathogen *Listeria monocytogenes* has been associated with many food-related outbreaks world-wide. It is the causative agent of listeriosis, which is estimated to be the cause of 28% of all deaths from diseases due to known food-borne pathogens in the United States (32). Furthermore, the reported incidence of listeriosis cases is increasing in many European countries (11, 17, 23). The ability of *L. monocytogenes* to withstand relatively high concentrations of (organic) acids and salts, and its ability to grow at refrigerated as well as mild-heating temperatures leads to rising concerns for the perseverance of this pathogen in the food-chain. Food manufacturers, particularly those producing ready-to-eat foods, have had to re-evaluate their processes in order to maintain safe food products (FDA, http://www.foodsafety.gov/~dms/lmr2-toc.html).

To date, 13 different serotypes of *L. monocytogenes* have been identified, of which serotypes 1/2a, 1/2b, 1/2c and 4b account for over 95% of the strains isolated from food and patients (12). All major outbreaks of food-borne listeriosis were caused by serotype 4b strains (5), while the majority of the strains isolated from foods are of serotype 1/2 (15). Whole genome analyses showed high synteny between different *L. monocytogenes* strains at both sequence and protein level (34). However, strain-specific and serotype-specific differences have been identified. Previous research showed approximately 8% difference between a partially sequenced epidemic 4b strain and a completely sequenced non-epidemic 1/2a strain (13). Furthermore, in another study, four completely sequenced strains showed between 50 and 100 unique genes (34). The diversity that has been observed between *L. monocytogenes* strains may be the result of horizontal gene transfer, which is probably required for adaptation to specific niches (19, 35). Some genetic differences between *L. monocytogenes* strains showed influence on their pathogenic potential or stress response (7, 46, 51).

For *L. monocytogenes*, large variation in stress tolerance has been observed under different conditions of salinity, acidity, and temperature in both culture broth and in different kind of food products. Previous studies were focused on modeling the growth limits of the organism at a combination of temperature, pH, NaCl concentration, and lactic acid for two strains (39), or a cocktail of five strains (42). In other studies the effect of combination treatments with lactic acid and hot water on a five-strain cocktail of *L. monocytogenes* (25), or the growth and cell wall properties under acid and saline conditions of two strains of the pathogen were investigated (3). In these cases no link was made between the serotype or origin of isolation and the stress tolerance. A limited number of studies are available in which serotype or isolation differences were investigated. (29) investigated the inactivation kinetics of three virulent and three avirulent strains at various pH and salt concentrations. They showed that avirulent strains displayed a slightly higher

tolerance to alkali than virulent strains. Another study focused on the difference between clinical and seafood isolates in acid and osmotic environments (43); four clinical isolates associated with seafood and four seafood isolates were compared, but no clear difference was identified. In the above investigations only limited numbers of strains were used. In some studies, where more extensive numbers of strains were used, differences in stress tolerance were apparent. (28) investigated 25 strains of various serotypes and origins for their thermal and acid resistance. They showed that the group of serotype 4b strains had lower heat resistance than the other serotypes, and the outbreak-related strains of serotype 4b had lower acid death rates than the other serotype 4b strains. In another study, the serotypes 4b and 1/2a were compared for sensitivity to bacteriocins and for heat inactivation after cold storage, using 81 strains (6). The group of serotype 4b strains tended to be more heat resistant than the group of 1/2a strains. So far, the growth limits of a large collection of *L. monocytogenes* strains have not been determined.

According to a report from the ILSI Research Foundation (22) one of the most important strategies to reduce the incidence of food-borne listeriosis is to prevent growth of *L. monocytogenes* to high numbers in contaminated foods. The current study was designed to determine the limits at which different *L. monocytogenes* strains fail to grow, using isolates from different origins and with different serotypes. Information about the so-called growth/no growth interface is very useful for the food industry in order to design optimized processes and product types that ensure the highest level of food safety. In the current study, 138 *L. monocytogenes* strains of diverse origins representing the most important serotypes were characterized for their growth limits using several industrially relevant stresses. Furthermore, a number of genes have been identified that are lineage or serotype specific and play a particular role in growth or survival during stress exposure (9, 50). To investigate the involvement of these genes in particular stress responses during growth at combinations of stresses, their presence was verified in these 138 strains.

Materials and Methods

L. monocytogenes strains

A total of 138 strains with diverse origins and genetic backgrounds were used in this study (Table S1, online manuscript). The collection contained human (epidemic and sporadic cases; n=42), meat (n=38), dairy (n=17), and food processing environment (n=23) isolates and had an abundant number of the serotypes 1/2a (n=32), 1/2b (n=17), and 4b (n=36). Strains were stored at -80 ºC in Brain Heart Infusion (BHI, Difco) broth containing 15% (v/v) sterile glycerol (BDH).

Determination of the growth limits

The total collection of strains was screened for detectable growth in BHI broth at a combination of high salt concentrations, low pH, and different temperatures. Specifically, the growth/ no growth interface was determined at high temperatures (43.5 ºC to 47 ºC; increments of 0.5 °C). In addition, growth was assessed at both 30 ºC and 7 ºC in combination with the stresses: 1) low pH (pH 4.0 to pH 5.4 at 30 °C and pH 4.2 to pH 5.8 at 7 °C); 2) low pH with supplementation of 2 % (w/v) sodium lactate (Fluka) (pH 4.5 to pH 6.2 at both 30 °C and 7 °C); 3) high NaCl concentrations (Merck) at pH 7.4 (1.5 $mol \cdot l^{-1}$ to 2.2 $mol \cdot l^{-1}$ at 30 °C and 1.3 $mol \cdot l^{-1}$ to 2.2 $mol \cdot l^{-1}$ at 7 °C); 4) high NaCl concentrations at pH5.5 (1.3 $mol \cdot l^{-1}$ to 2.0 $mol \cdot l^{-1}$ NaCl at 30 °C and 1.0 $mol \cdot l^{-1}$ to 2.0 $mol \cdot l^{-1}$ at 7 °C); 5) low pH with supplementation of 1.0 $mol \cdot l^{-1}$ NaCl (pH 4.3 to pH 5.8 at 30 °C and pH 4.5 to pH 5.9 at 7 °C). The pH increments were 0.2 to 0.3 and the NaCl concentration increments were 0.1 $mol \cdot l^{-1}$. The pH was set with 10 % HCl using a pH meter (713 pH Meter, Metrohm). Acidified conditions were buffered with 0.02 M potassium phosphate buffer (KH_2PO_4/K_2HPO_4, Merck). Each strain was tested in duplicate in 96-well flat bottom tissue culture plates (Greiner). The wells were inoculated to a level of approximately 10^6 $CFU \cdot ml^{-1}$ in a volume of 250 µl, using an inoculum from 18-h cultures grown at 30 ºC in BHI broth. The plates were incubated at high temperatures (43.5 ºC to 47 ºC) in the C24KC incubator (New Brunswick) for two days, at 30 ºC in the BD 400 incubator (Binder) for three weeks and at 7 ºC in a temperature controlled cooling chamber for three months. The absorbance was regularly measured using the EL808 IU-PC (Bio-Tek) at 630 nm and compared with two wells serving as negative controls.

Data analysis

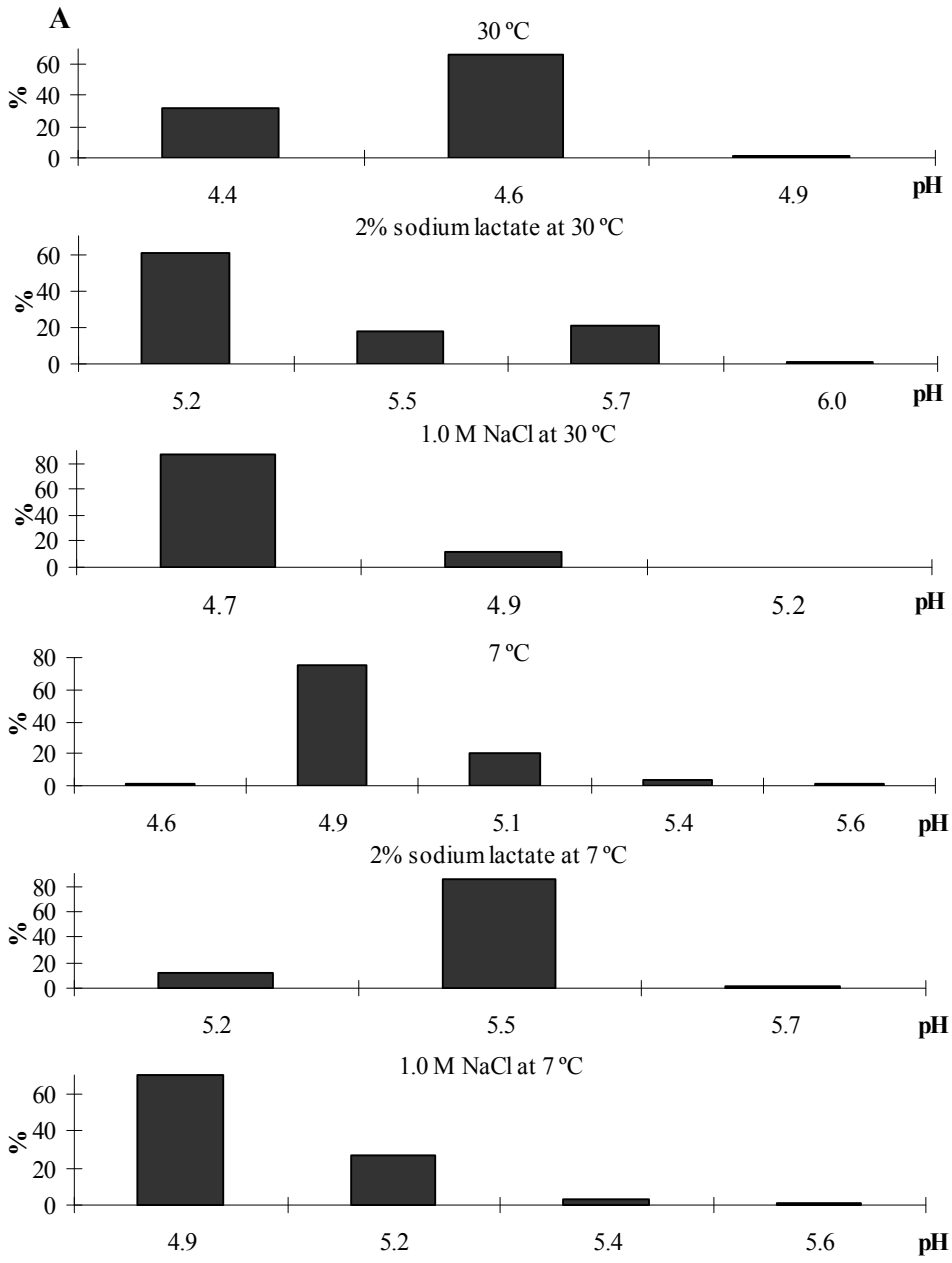
All strains were analyzed in duplicate for each treatment and temperature condition. The reported values for the growth/no growth interface are the conditions at which no growth was detected. Cluster analysis on the complete dataset for all stresses was performed by a euclidic distance analysis using the ward method on the results, scaled between zero and one, zero being the lowest reported value and one the highest. Statistical significant differences between subgroups of the collection (serotype or origin of isolation) were identified by a two-group pair-wise fixed reallocation randomization test (10000 randomizations; $p<0.05$) (21), which is freely available at (http://www.bioss.ac.uk/smart).

Gene specific PCR analysis

To link potential differences in stress tolerance to the genetic make-up of strains, a gene specific PCR analysis was performed. These genes are known to have a variable presence in *L. monocytogenes* and are associated with specific phenotypes. We selected candidate genes, including *LMOf2365_1900*, encoding a putative serine protease, *lmo0423*,

encoding sigma factor SigC, *pli0067*, encoding a putative UV-damage repair protein, and *lmo0447* and *lmo0448*, encoding a decarboxylase system. The putative serine protease LMOf2365_1900, was previously identified as an unknown extra-cellular protein, which was specific for the serotypes 4b, 4d, and 4e, and was named ORF2110 (12). This gene was selected because of its putative function that may resemble the serine protease HtrA, which has been shown to be important for growth at low pH, high temperatures, high osmolarity, and virulence (38, 47, 48). The ECF sigma factor SigC (lmo0423), is part of a lineage specific heat shock system (50), and is important for survival at elevated temperatures. Previous research showed diverse presence of a probe from a shotgun library in 50 *L. monocytogenes* strains, and was identified as part of a UV-damage repair protein (7). We identified this probe as being part of the *L. innocua* gene pli0067, which is located on the approximately 82 kb *Listeria*-specific plasmid (18). This plasmid is also present in some *L. monocytogenes* strains (34). Gene *pli0067* shows sequence similarity with the gene encoding the alternative DNA polymerase UmuD. This gene is induced during heat-shock due to activation of the SOS-response possibly after replication fork stalling (41). Therefore, the presence of gene *pli0067* might give a benefit for growth at higher temperatures, because replication fork stalling at these temperatures might be reduced due to the presence of an extra alternative DNA polymerase. As candidate marker genes for growth at low pH we identified the glutamate decarboxylase system encoded by the genes *gadD1* (lmo0447) and *gadT1* (lmo0448). These genes were previously identified to be important for growth at low pH (9). Although the system appears to be serotype 1/2 specific, it is not present in all serotype 1/2 strains (7, 9).

Strains were grown for 18 h at 30 ºC in BHI broth and pellets of 0.5 ml culture were obtained by centrifugation (2 min, 5000 x g, 20 ºC; 5417R Eppendorf). The pellets were suspended in 0.5 ml MilliQ and boiled for 10 min. The suspensions were centrifuged and 1 µl of the supernatant was used as DNA template for the PCR-reactions. The PCR was performed in 35 cycles of 15 s at 94 ºC, 30 s at 50 ºC, and 1 min at 72 ºC using the 2 x Taq PCR mix (Invitrogen) and 0.3 µM primers (Table S2, online manuscript). The presence of genes *lmo1134* (positive control) (30), *ORF2110*, *lmo0423*, *lmo0447*, *lmo0448*, and *pli0067* in the complete wild-type collection was verified using primers lmo1134-fwd and lmo1134-rev, ORF2110-fwd and ORF2110-rev, lmo0423-fwd and lmo0423-rev, lmo0447-fwd and lmo0447-rev, lmo0448-fwd and lmo0448-rev, and pli0067-fwd and pli0067-rev, respectively.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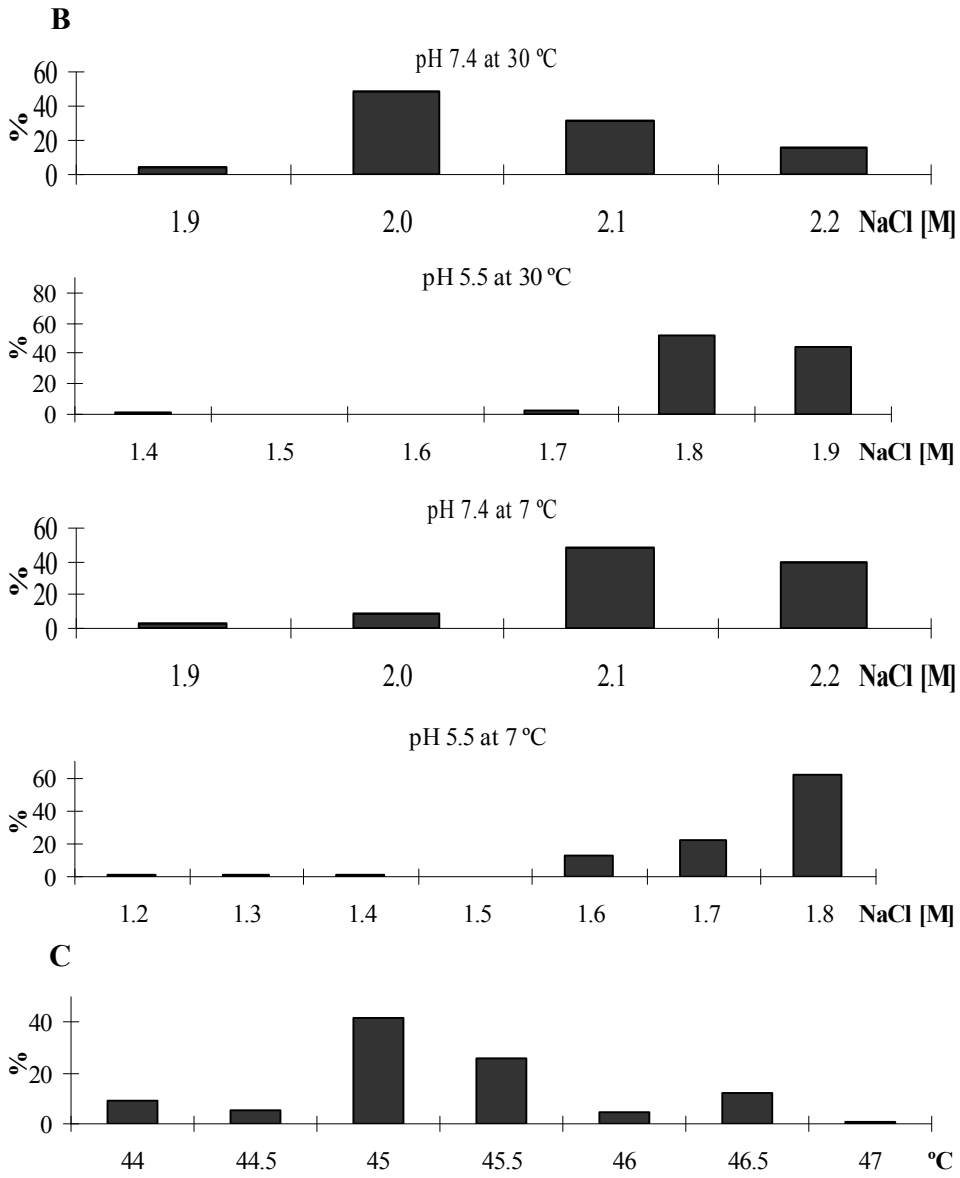

Fig. 1. The growth limits (x-axis) of *L. monocytogenes* strains (y-axis) at different conditions. A) The growth limits at low pH at 30 °C, and the shift of the growth limits at low pH in combinations with 2 % sodium lactate, with 1.0 M NaCl, and with growth at 7 °C. B) The growth limits at high NaCl concentrations at pH 7.4 at 30 °C, and the shift of the growth limits at high NaCl concentrations in combination with growth at pH 5.5 and 7 °C. C) The minimum inhibitory temperatures.

RESULTS

Growth limits

Each strain was tested under all conditions in duplicate. The variation between the duplicate measurements was low and did not influence the final results of the growth limits. The average coefficient of variation (CV: standard deviation divided by mean value) for the absorbance measurements of the different stresses were: 1) 0.084 for high temperatures; 2) 0.023 for low pH at 30 ºC; 3) 0.030 for low pH at 7 ºC; 4) 0.025 for low pH with supplementation of 2% sodium lactate at 30 ºC; 5) 0.017 for low pH with supplementation of 2% sodium lactate at 7 ºC; 6) 0.061 for high NaCl concentrations at pH 7.4 at 30 ºC; 7) 0.036 for high NaCl concentrations at pH 7.4 at 7 ºC; 8) 0.028 for high NaCl concentrations at pH 5.5 at 30 ºC; 9) 0.029 for high NaCl concentrations at pH 5.5 at 7 ºC; 10) 0.022 for low pH with supplementation of 1.0 $mol \cdot l^{-1}$ NaCl at 30 ºC; 11) 0.025 for low pH with supplementation of 1.0 $mol \cdot l^{-1}$ NaCl at 7 ºC. The results of the growth limits for the complete collection for all stresses are presented in Figure 1 and described further below.

Growth limits at low pH

At 30 °C, almost all strains failed to show growth at either pH 4.4 (32%) or pH 4.6 (67%), while only a few strains failed to grow at higher pH (Fig. 1A). In combination with the preservative NaCl (1 $mol \cdot l^{-1}$), 87% of the strains showed growth inhibition at pH 4.7. The addition of 2 % sodium lactate showed a larger effect than the addition of 1 $mol \cdot l^{-1}$ NaCl, since 61% of the strains failed to show growth at pH 5.2 or lower. At low temperature (7 °C), the growth limit shifted up to pH 4.9 for 75% of the strains. The addition of 1 $mol \cdot l^{-1}$ of NaCl at 7 °C did not show a large effect on the growth limits at low pH, since 70 % of the strains showed this limit still at pH 4.9. Also lowering the temperature to 7 °C in the presence of 2 % sodium lactate did not result in an additional shift of the growth limits at low pH. The majority of the strains showed growth inhibition at pH 5.5 or lower (86%), but for a small group of strains growth inhibition was still shown at pH 5.2 or lower (12%) under these conditions.

Growth limits at high NaCl concentrations

At high saline conditions, the majority of the strains failed to grow between 2.0 and 2.2 $mol \cdot l^{-1}$ NaCl at pH 7.4 at 30 °C (Fig 1B). By lowering the pH to pH 5.5 most of the strains did not show growth at 1.8 $mol \cdot l^{-1}$ NaCl (52 %) or 1.9 $mol \cdot l^{-1}$ NaCl (44 %). Lowering the temperature to 7 °C showed a negative effect on the growth limits at high saline conditions at pH 7.4 ($P=0.000$), since more strains showed growth inhibition at slightly higher NaCl concentrations, at 2.1 to 2.2 $mol \cdot l^{-1}$ NaCl at this temperature. At 7 °C

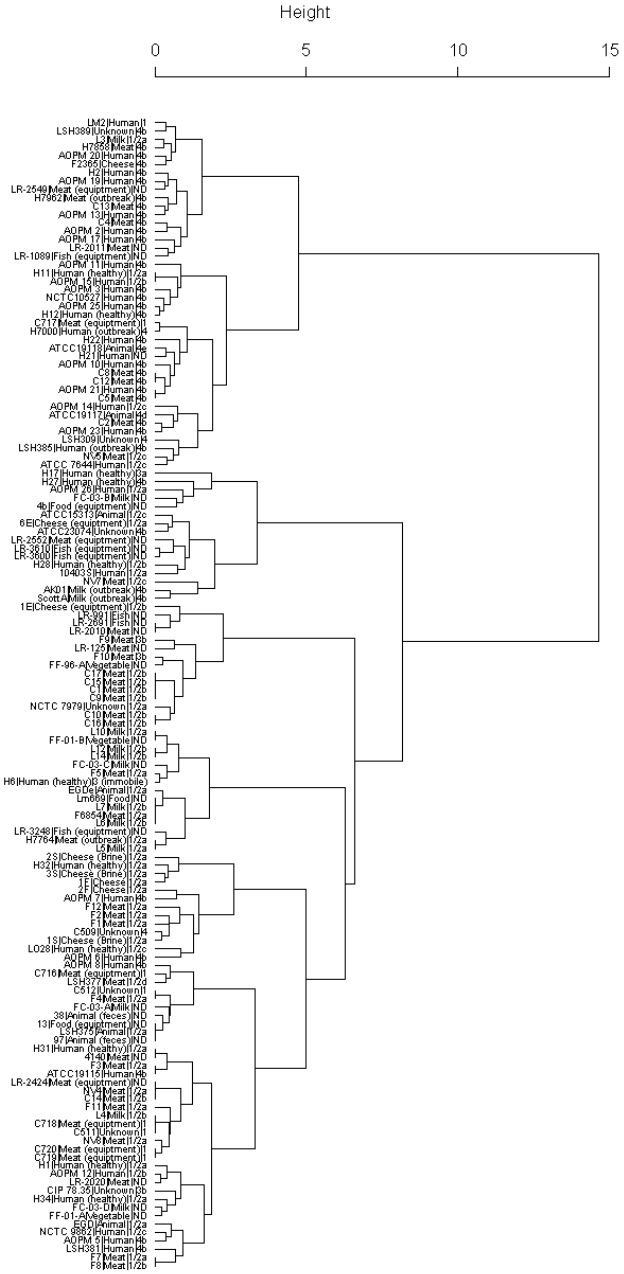

Fig. 2. Cluster dendogram of the growth limits of the 138 *L. monocytogenes* strains. The strains were clustered after an euclidic distance analysis using the ward method. If known, the serotypes and origin of isolation are indicated.

and pH 5.5, the growth limits at high NaCl concentrations remained 1.8 mol·l^{-1} for the majority of the strains, but no strain was able to grow at 1.9 mol·l^{-1} NaCl anymore.

Growth limits at high temperatures

All strains showed the growth limits between 44 °C and 47 °C (Fig. 1C). Most strains failed to grow at 45.0 °C (42%) and 45.5 °C (26%). Seventeen other strains showed growth inhibition at 46.5 °C. One serotype 4b strain isolated from a human in an Italian clinic, AOPM7 (8), was able to grow up to 47 °C.

Cluster analysis

The growth limits for the individual strains are reported in Table S3 (online manuscript). The complete collection of strains was clustered according to the scaled results of the growth limits. The results of this cluster analysis are reflected in a dendrogram (Fig. 2). The dendrogram shows two main branches dividing the majority of the serotype 4b strains from the other strains. Branch 1 contains 42 strains of which 26 are of serotype 4b (62 %), two of serotype 1/2a, one of serotype 1/2b, and three of serotype 1/2c. Branch 2 contains 96 strains, among which 10 are of serotype 4b, 30 of serotype 1/2a, 16 of serotype 1/2b, and 4 of serotype 1/2c. The main identifier in the division of these two branches is the growth limits at low pH at 30 °C. All strains from branch 1 showed the growth limits at pH 4.4, while only two strains from branch 2 showed the limits at this pH.

Serotype differences

At 30 °C, the group of serotype 4b strains showed higher acid tolerance than the serotype 1/2a strains (P=0.000) and 1/2b strains (P=0.000) (Fig. 3A). In total, 75% of the serotype 4b strains showed growth inhibition at pH 4.4. In contrast, the group of serotype 4b strains showed lower acid tolerance than the group of serotype 1/2a strains at 7 °C (P=0.018) (data not shown). No significant difference was observed between the group of serotype 4b strains and the group of serotype 1/2b strains (P=0.125). More serotypes differences were observed for acid tolerance at 7 °C in combination with the preservatives sodium chloride and sodium lactate. In the presence of 1 mol·l^{-1} NaCl the group of serotype 4b strains showed lower acid tolerance than the group of serotype 1/2a strains (P=0.042) and 1/2b strains (P=0.006) (data not shown). After addition of 2 % sodium lactate, the group of serotype 1/2b strains showed higher acid tolerance than the group of serotype 1/2a strains (P=0.001) and 4b strains (P=0.000) (Fig 3B).

At 30 °C, the group of serotype 4b strains showed higher tolerance to high saline conditions at pH 7.4 than the group of serotype 1/2a strains (P=0.000) and 1/2b strains (P=0.000), and only strains from serotype 4b showed the growth limits at 2.2 mol·l^{-1} NaCl (Fig. 3C). Also, the group of serotype 4b strains showed higher tolerance to high saline

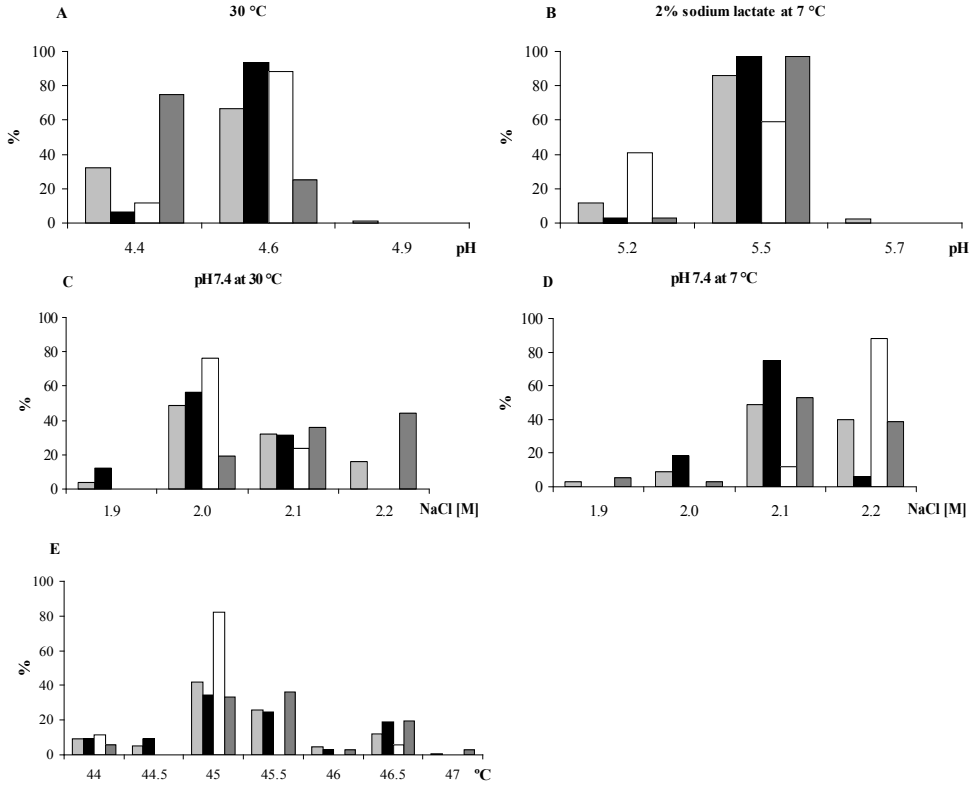

Fig. 3. Growth limits for the complete collection of *L. monocytogenes* strains and for the serotypes 1/2a, 1/2b, and 4b in Brain Hearth Infusion (BHI) broth. A) The percentage of strains of the complete collection (light grey) or strains belonging to the serotypes 1/2a (black), 1/2b (white), and 4b (dark grey) that showed the growth limits at the reported pH in BHI broth adjusted with HCl at 30 ºC. B) The percentage of strains of the complete collection (light grey) or strains belonging to the serotypes 1/2a (black), 1/2b (white), and 4b (dark grey) that showed the growth limits at the reported pH in BHI broth adjusted with HCl at 7 ºC in the presence of 2 % sodium lactate. C) The percentage of strains of the complete collection (light grey) or strains belonging to the serotypes 1/2a (black), 1/2b (white), and 4b (dark grey) that showed the growth limits at the reported NaCl concentration in BHI broth at pH 7.4 at 30 ºC. D) The percentage of strains of the complete collection (light grey) or strains belonging to the serotypes 1/2a (black), 1/2b (white), and 4b (dark grey) that showed the growth limits at the reported NaCl concentration in BHI broth at pH 7.4 at 7 ºC. E) The percentage of strains of the complete collection (light grey) or strains belonging to the serotypes 1/2a (black), 1/2b (white), and 4b (dark grey) that showed the growth limits at the reported temperature in BHI broth.

conditions at mildly acidic conditions (pH 5.5) than the group of serotype 1/2a strains (P=0.004) and the group of serotype 1/2b strains (P=0.017) (data not shown). At 7 °C, the group of serotype 1/2b strains showed higher tolerance for high saline conditions at pH 7.4 than the group of serotype 1/2a strains (P=0.000) and the group of serotype 4b strains (P=0.003) (Fig. 3D). Also at pH 5.5, the group of serotype 1/2b strains showed higher tolerance for high saline conditions than the group of serotype 1/2a strains (P=0.013) and the group of serotype 4b strains (P=0.002) (data not shown).

The group of serotype 4b strains were able to grow at a higher temperature than the group of 1/2b strains (P=0.004) (Fig. 3E). No significant difference was observed between the groups of serotype 4b and 1/2a strains (P=0.235) or between the groups of serotype 1/2a and 1/2b strains (P=0.105).

Differences between strains isolated from different origins

At 30 °C, the group of human isolates showed higher acid tolerance than the group of dairy isolates (P=0.001), the group of meat isolates (P=0.005), and the group of food processing environment isolates (P=0.001) (Fig 4A). In the presence of 1 mol·l^{-1} NaCl, the group of meat isolates showed higher acid tolerance than the group of human isolates (P=0.013), the group of dairy isolates (P=0.003), and the group of food processing environment isolates (P=0.000) (data not shown). In the presence of 2 % sodium lactate, the group of dairy isolates showed lower acid tolerance than the group of human isolates (P=0.000), the group of meat isolates (P=0.001), and the group of food processing environment isolates (P=0.027) (data not shown). At 7 °C in the presence of 2 % sodium lactate, the group of meat isolates showed higher acid tolerance than the group of human isolates (P=0.005), and the group of dairy isolates (P=0.049) (Fig. 4B). The differences between the group of meat isolates and the group of food processing environment isolates were not significant (P=0.117).

At 30 °C, the group of human isolates showed higher tolerance to high saline conditions at pH 7.4 than the group of meat isolates (P=0.003) and the group of food processing environment isolates (P=0.020) (Fig. 4C). The differences between the group of human isolates and the group of dairy isolates were not significant (P=0.287). In contrast, the group of human isolates showed lower tolerance to high saline conditions than the group of dairy isolates (P=0.026) and the group of food processing environment isolates (P=0.033) at pH 7.4 at 7 °C (data not shown). The differences between the group of human isolates and the group of meat isolates were not significant (P=0.064).

The group of meat isolates showed the growth limits at a lower temperature than the group of dairy isolates (P=0.015) and the group of human isolates (P=0.002) (data not shown). The differences between the group of meat isolates and the group of the food processing environment isolates were not significant (P=0.112).

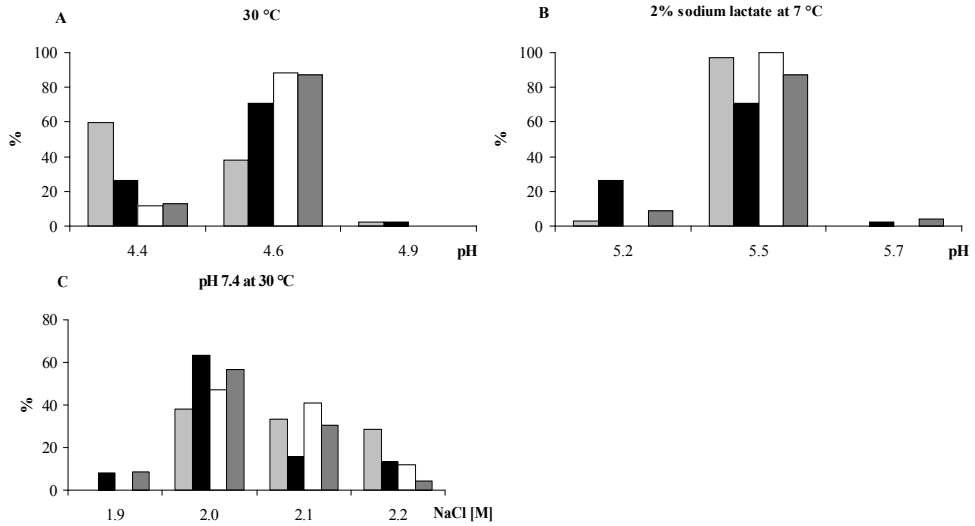

Fig. 4. Growth limits of *L. monocytogenes* strains isolated from human cases, meat, dairy, and the food processing environment in Brain Hearth Infusion (BHI) broth. A) The percentage of strains isolated from human cases (light grey), meat (black), dairy (white), and the food processing environment (dark grey) that showed the growth limits at the reported pH in BHI broth adjusted with HCl at 30 ºC. B) The percentage of strains isolated from human cases (light grey), meat (black), dairy (white), and the food processing environment (dark grey) that showed the growth limits at the reported pH in BHI broth adjusted with HCl at 7 ºC in the presence of 2 % sodium lactate. C) The percentage of strains isolated from human cases (light grey), meat (black), dairy (white), and the food processing environment (dark grey) that showed the growth limits at the reported NaCl concentration in BHI broth at pH 7.4 at 30 ºC.

Can phenotypic differences be linked to specific gene presence?

Certain genes may play a role in growth and survival during stress exposure and these genes may not be present in all strains or serotypes, in particular acid/ salt tolerance (*gadD1T1*, and *ORF2110*), and heat tolerance (*sigC* and *pli0067*).

Only strains of serotype 4b, 4d, and 4e showed a positive PCR-product using the *ORF2110* specific primers. The group of serotype 4b strains showed the growth limits at higher temperatures (Fig. 3E) and lower pH and higher salt concentrations at 30 °C (Fig. 3A and 3C) than the group of serotype 1/2a strains and the group of serotype 1/2b strains. Therefore, the enhanced performance of serotype 4b strains under these stress conditions may be associated with the presence of *ORF2110*.

The PCR analysis showed a positive product for all serotype 1/2a, 1/2c, 3a, and 3c strains using *sigC* specific primers, verifying the lineage specificity. Since no significant difference was observed for the maximum growth temperature between the group of

serotype 1/2a strains and the group of 1/2b and 4b strains, this system might not have an explicit role in growth at elevated temperatures.

A positive PCR product was observed in 56 strains (41%) using the specific primers for *pli0067*. Of these strains, 22 were of serotype 1/2a, 11 of serotype 1/2b, two of serotype 1/2c, and three of serotype 4b. However, strains containing this gene did not show higher maximum growth temperatures, and an explicit role of gene *pli0067* in growth at elevated temperatures could therefore not be established.

Positive PCR products for the *gadD1T1* specific primers were shown in nine serotype 1/2a strains, 13 serotype 1/2b strains, and six serotype 1/2c strains. The group of serotype 1/2 strains containing this decarboxylase system showed the growth limits in the presence of 1 mol·l^{-1} NaCl at 7 ºC at lower pH than the group of serotype 1/2 strains lacking this cluster (P=0.000) or the group of serotype 4b strains (P=0.000), while the group of serotype 1/2 strains that lack this system did not show a significant difference with the group of serotype 4b strains (P=0.091) (Fig. 5).

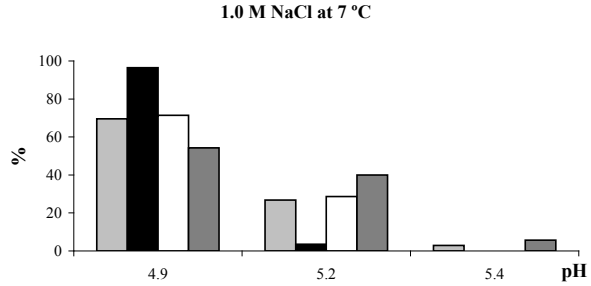

Fig. 5. Growth limits at low pH at 7 ºC in the presence of 1.0 M NaCl. The percentage of strains of the complete collection (light grey) or strains belonging to serotype 1/2 with (black) or without (white) the decarboxylase system *gadD1T1*, and serotype 4b (dark grey) that showed the growth limits at the reported pH in Brain Hearth Infusion (BHI) broth adjusted with HCl.

Discussion

One of the most important strategies to reduce the incidence of food-borne listeriosis is prevention of growth of *L. monocytogenes* to high numbers in contaminated foods (22). In this study, the growth limits of *L. monocytogenes* for combinations of low pH without and with sodium lactate, and high salt concentrations at 30 ºC and 7 ºC were investigated to identify differences between serotypes and isolates from specific niches. Notably, the group of serotype 4b strains were more tolerant to low pH in the absence of sodium lactate and to high saline conditions at 30 ºC than the group of serotype 1/2a and 1/2b strains, which might be attributed to the presence of ORF2110 in the serotype 4b strains. At 7 ºC, the

group of serotype 1/2b strains were more tolerant to high saline conditions at both neutral and mild acidic pH than the group of serotype 1/2a and 4b strains. The highest tolerance to low pH in the presence of 2 % sodium lactate at 7 ºC was observed for serotype 1/2b strains isolated from meat products. The assessment of various lineage- or serotype-specific genes with a known or anticipated role during stress exposure revealed a specific glutamate decarboxylase system (*gadD1* and *gadT1*) to be present in the group of serotype 1/2 strains with the highest tolerance to low pH in the presence of 1 $mol \cdot l^{-1}$ NaCl at 7 ºC. The group of serotype 1/2 strains that did not contain this system and the group of serotype 4b strains displayed both lower tolerance to low pH under these conditions. These findings are important for food preservation since strains that have adapted to selective pressures used to control them might be encountered in the food processing environment.

Commonly used strategies in food preservation are (combinations of) lowering the pH and the addition of preservatives like sodium chloride and sodium lactate. In particular, the shelf life of many ready-to-eat meat and deli products depends on these preservation strategies (2, 24). Previous reports showed that *L. monocytogenes* can grow at pH levels as low as pH 4.3 to pH 4.8 (4, 26, 42), at concentrations as high as 10 to 14 % NaCl (14, 31), and at various combinations of low pH and high NaCl concentrations (39) depending on the strain, the medium, the temperature, the physiological state of cells, and the inoculum size. Throughout this study an inoculum size of approximately 10^6 $CFU \cdot ml^{-1}$ was used, which is much higher than the contamination levels that occur in food products. Some studies showed that higher inoculum sizes resulted in growth under more severe stress conditions due to a higher probability to initiate growth under these conditions (27, 37). Recently, it was shown that *Bacillus cereus* cultures exposed to inhibitory salt concentrations (5% NaCl) contain a population of growing and non-growing cells (10). Under severe stress conditions that allow marginal growth, an initial small population that is able to grow might not reach the detection limits when a low inoculation size is used. Taking these phenomena into account, it is not inconceivable that the growth limits observed in this study overestimate growth capabilities of *L. monocytogenes* in practice. In this study, none of the 138 strains investigated showed the growth limits at lower pH or higher NaCl concentrations than observed in previous studies, indicating that the growth limits for these stresses are conserved within the *L. monocytogenes* species. However, the pH of many food products is higher than these growth limits (FDA, www.cfsan.fda.gov/~comm/lacf-phs.html). Also the combinations of low pH and high NaCl concentrations or 2 % sodium lactate, which is a commonly used concentration in ready-to-eat meat and deli products (16), do allow for growth of *L. monocytogenes* in many of these products (24), thereby posing a potential hazard. In January 2006, new microbiological criteria for *L. monocytogenes* have been implemented in the European Union through regulation 2073/2005 (40). Ready-to-eat foods that do not support growth of *L. monocytogenes* no

longer require zero-tolerance, but allow a maximum concentration of 100 CFU·g^{-1} during their shelf-life. The criteria for products that do not support growth of *L. monocytogenes* are pH ≤ 4.4, a_w ≤ 0.92 (+/- 13 % NaCl), or pH ≤ 5.0 and a_w ≤ 0.94 (+/- 10 % NaCl). None of the *L. monocytogenes* strains in our collection showed growth limits at lower pH or higher NaCl concentrations at both 30 and 7 ºC, indicating that these parameters are a good standard. Furthermore, our results show that a category of products using sodium lactate as a preservative could be added to the list of products that do not support growth of *L. monocytogenes*. The criteria for these products should be: pH ≤ 5.2 and NaLac ≥ 2 %.

Determination of the growth limits of strains belonging to different serotypes or strains isolated from specific niches and subsequent cluster analysis revealed significant differences to exist between groups of strains. Most of the serotype 4b strains branched separately from the serotype 1/2a and 1/2b strains, even though serotype 4b and 1/2b are of the same lineage (13). As a group, the serotype 4b strains showed the growth limits at lower pH, higher salt concentrations, and at a lower pH in the presence of 1 mol·l^{-1} NaCl at 30 ºC than the group of serotype 1/2a and 1/2b strains. In addition, the group of serotype 4b strains displayed higher maximum growth temperatures than the group of serotype 1/2b strains. Serotype 4b strains are abundantly found in clinical isolates (5). Their relatively high tolerance to acid and salt might contribute to their incidence in human infection, as these are also important traits for virulence (16, 33, 49).

In contrast, the group of serotype 4b strains was less tolerant to acid in the presence of 1 mol·l^{-1} NaCl at 7 ºC than the group of serotype 1/2a and 1/2b strains. The latter serotypes have predominantly been isolated from ready-to-eat food products (20, 52). Preservation conditions applied to these type of products likely are more favourable to the serotype 1/2 strains. Only at 7 °C, the group of serotype 1/2b strains grew at higher NaCl concentrations than the group of serotype 1/2a and 4b strains both at neutral pH and at pH 5.5. This particular serotype is most frequently isolated from retail foods (52) and hard and semi-hard cheeses (36). The capacity of serotype 1/2b strains to grow at the highest NaCl concentrations at 7 °C at neutral and mild acidic conditions may contribute to their presence in retail foods and on hard and semi-hard cheeses. The majority of the serotype 1/2b strains showed growth limits at 7 °C at NaCl concentrations similar to those of the serotype 4b strains at 30 °C. Low temperatures and high NaCl concentrations can induce overlapping salt and cold stress protection mechanisms (1, 44, 45). Clearly, some *L. monocytogenes* strains and serotypes are better able to adapt to these conditions than others, and growth of adapted strains to high numbers could occur after prolonged storage for this reason. The group of serotype 1/2b strains furthermore displayed the growth limits at a lower pH in the presence of 2 % sodium lactate than the group of serotype 1/2a and 4b strains at 7 °C. Within the group of serotype 1/2b strains, the strains isolated from smoked meat products were able to grow at the lowest pH. Since sodium lactate is a commonly used preservative

for meat products, this may indicate that certain serotype 1/2b strains found in specific niches may have adapted to their environment and/or that the environment has selected for these variants. These results show that selection of strains for growth experiments under different stress conditions is very important. Serotype 1/2 strains are generally able to grow at lower pH and higher salt concentrations at 7 ºC than the serotype 4b strains. In particular, serotype 1/2b strains are able to grow at high salt concentrations and low pH in the presence of sodium lactate at this temperature. Therefore, we recommend that ready-to-eat food manufacturers claiming their products do not support growth of *L. monocytogenes* show data of challenge tests performed with strains of this serotype.

The observed differences in growth limits between the serotype 1/2 strains and serotype 4b strains may be due to the presence of genes with a role in stress resistance specific for certain serotypes. Genetic screening of strains in our collection identified a putative serine protease (LMOf2365_1900 or ORF2110) that was found only in strains of serotype 4b, 4d and 4e. Its function may resemble the function of the serine protease HtrA, which is important for growth at low pH, high osmolarity, and high temperatures (38, 47, 48). It is conceivable that the protein encoded by ORF2110 not only contributes to higher acid and salt tolerance at 30 ºC of the 4b strains compared with the 1/2a and 1/2b strains, but also to higher thermo-tolerance of the 4b strains compared with the 1/2b strains. However, it should be noted that it is not possible to distinguish ORF2110 specific traits from general differences between serotype 4b and 1/2 strains, since none of the 1/2 strains contains this ORF.

In previous research a glutamate decarboxylase system (*gadD1* and *gadT1*) was identified which enhanced the ability of *L. monocytogenes* to grow at low pH (9). Strains that were lacking these genes generally grew more slowly at pH 5.1 at 37 ºC. This system was detected in only 50 % of our serotype 1/2 strains. At low pH at 30 ºC, the growth limits of the group of serotype 1/2 strains containing this system were similar to the group of serotype 1/2 strains lacking this system. However, in the presence of 1 $mol \cdot l^{-1}$ NaCl at 7 ºC, serotype 1/2 strains containing this system were more tolerant to low pH than serotype 1/2 strains lacking this system, or serotype 4b strains in which this system was absent. From these observations we can conclude that this decarboxylase system plays a role in resistance to these combined stresses. Therefore, this decarboxylase system might be a suitable biomarker for *L. monocytogenes* strains in food products in which preservation depends on combinations of low pH, high NaCl concentrations and low temperatures.

In short, this study identified the growth/no growth interface for 138 *L. monocytogenes* strains at combinations of low pH, high salt concentrations, temperatures and addition of 2% sodium lactate. Our results indicate that the preservation conditions of many food products may permit growth of all *L. monocytogenes* strains in our collection or specific serotypes or strains isolated from specific niches. The group of serotype 4b strains,

which are most commonly isolated in cases of clinical listeriosis, showed the highest acid and salt tolerance at 30 °C. This could possibly be explained by the presence of the putative biomarker ORF2110 in serotype 4b strains. Also, we identified a group of serotype 1/2b strains isolated from smoked meat products that showed the highest tolerance for low pH in the presence of 2 % sodium lactate at 7 °C. Sodium lactate is a commonly used preservative in the meat industry, and these results showed that some *L. monocytogenes* serotypes have likely adapted to specific environmental stresses. Furthermore, this study demonstrated that a specific decarboxylase system was present in a group of strains with higher tolerance to low pH in the presence of 1 $mol \cdot l^{-1}$ NaCl at 7 °C. The genes encoding this system might be suitable biomarkers for the presence of specific *L. monocytogenes* strains in food products that depend on the combination of these stresses for preservation. In general, we observed the highest tolerance to stress conditions at non-chilled temperatures for the group of serotype 4b strains, while the group of serotype 1/2 strains showed the highest tolerance to stress conditons at 7 °C. This might be associated with the abundance of these particular serotypes in clinical isolates and food isolates, respectively. The current findings increase our understanding of how specific *L. monocytogenes* strains and serotypes have evolved to become resident strains and/or selected for in specific niches like the food processing environment or retail establishments.

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3. Diversity assessment of heat resistance of *Listeria monocytogenes* strains in a continuous flow heating system

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Submitted for publication

Abstract

Listeria monocytogenes is a foodborne pathogen that has the ability to survive relatively high temperatures compared with other non-sporulating foodborne pathogens. Therefore, *L. monocytogenes* might pose a risk in food products that depend on thermal preservation steps. The heat-resistance of a large collection of strains was assessed in batch heating experiments. Subsequently, the heat inactivation kinetic parameters of two of the most heat-resistant strains (1E and NV8) and those of reference strain ScottA were determined in a continuous flow-through system that mimics industrial conditions. The three strains were cultures in whole milk and BHI at 30 ºC and 7 ºC. Strains 1E and NV8 were significantly more heat-resistant than strain ScottA after growth in BHI at 30 ºC and after growth in milk at 7 ºC. At 72 ºC, which is the minimal required temperature for a 15 s HTST pasteurization process, the D-values of the strains 1E, NV8, and ScottA in milk were 1.2 s, 1.0 s, and 0.17 s, respectively, resulting in 12.1, 14.2, and 87.5 $\log_{10}$ reduction for a 15 s HTST process at this temperature. These results demonstrate that industrial pasteurization conditions suffice to inactivate the most heat-resistant *L. monocytogenes* strains tested in this study. However, it is critical that the temperature during pasteurization is safeguarded, because a temperature drop of 2 ºC might result in survival of heat-resistant strains of this bacterium.

Introduction

Control of pathogenic and spoilage bacteria is needed to ensure food safety and to extend the shelf-life of foods. A common control measure that is used in industrial food manufacturing is heat treatment. Typical thermal inactivation conditions that are applied consist of pasteurization (i.e. 72 ºC, 15 seconds) and sterilization (i.e. 121 ºC, 3 minutes) (23). The effectiveness of a certain thermal treatment to reduce the number of pathogenic or spoilage bacteria in foods depends on the general heat-susceptibility of the organism, the physiological state of the organism, the composition of the food, and the heat-susceptibility of specific strains of the organism (6). In general, a pasteurisation process is sufficient to inactivate vegetative cells of the majority of species, while sterilization is required for spore inactivation.

The foodborne pathogen *Listeria monocytogenes* has a higher tolerance to heat than various other non-sporulating food-borne pathogens, such as *Escherichia coli* O157:H7, *Salmonella* ssp, and *Campylobacter* ssp (11, 15). If *L. monocytogenes* is not fully inactivated and conditions in a food allow for growth, the organism could reach high numbers and subsequently cause a foodborne disease (24, 26). Therefore, the legal requirement in the European Union for ready-to-eat foods that support growth of *L. monocytogenes* is absence in 25 g or a maximum of 100 cfu/g at the moment of consumption (9). Following an outbreak of listeriosis in 1983, in which pasteurized milk was identified as the source (13), various researchers evaluated the ability of *L. monocytogenes* to survive the pasteurization process (Reviewed by Farber and Peterkin (11)). In general, sufficient inactivation of *L. monocytogenes* was found after high-temperature short-time (HTST) pasteurization for 15 sec at 72 ºC. Thermal inactivation kinetics of *L. monocytogenes* has been established using laboratory strains or reference strains like ScottA (isolate from Massachusetts milk outbreak in 1983, as described by Fleming et al. (13). However, diversity in the thermal resistance of *L. monocytogenes* strains can occur, as has been shown by Edelson-Mammel et al. (8). Furthermore, thermo-resistant subpopulations in cultures of *L. monocytogenes* have been described: Karatzas et al (16, 17) showed that a sub-population of strain ScottA, containing a mutation in *ctsR* (class III heat-shock regulator), was more thermo-resistant than the wildtype strain.

Heating of liquid foods at an industrial scale is regularly performed using heat-exchangers with counter-current flow (22). The traditional way to estimate the expected reduction of specific organisms in a specific process (heating time and heating temperature combination) is based on the D/z-concept. The D-value represents the time to obtain 1 $\log_{10}$ reduction at a certain temperature while the z-value represents the temperature increase required to reduce the D-value with a factor 10. The D/z-values of *L. monocytogenes* as determined in many research publications for batch processes can not easily be translated to

continuous processes like HTST pasteurization, because effects of shear forces and physical stress are not taken into account (20). In heat-exchangers, it is possible to vary the heating temperatures, but in general the heating times can not be adjusted due to fixed holders. Instead of calculating D/z-values based on batch heating at varying exposure times, the inactivation kinetics in continuous flow systems with varying exposure temperatures are described by the first order exponential arrhenius relation with the energy of activation (E_a) and the inactivation constant (k_0). These constants can subsequently be used to derive the D/z-values (19).

To establish the variation in heat resistance for different *L. monocytogenes* strains, we screened a large number of *L. monocytogenes* strains for their heat resistances. The heat inactivation kinetic parameters of the strains with the highest heat resistance were subsequently determined in a continuous flow-through system, mimicking industrial food-processing conditions.

Materials and Methods

L. monocytogenes strains and growth conditions

Strains were stored in Brain Hearth Infusion (BHI) broth (Difco) containing 15% sterile glycerol (BDH) at -80 ºC. Single colonies of the strains were inoculated and grown overnight in BHI broth with shaking (200 rpm; New Brunswick type C24KC) at 30 ºC.

Screening of *L. monocytogenes* strains

In a preliminary screening, the heat resistance of 48 *L. monocytogenes* strains was assessed at 55 ºC in BHI broth. Cultures were grown overnight at 30 ºC and 200 rpm (New Brunswick C24KC) and subsequently inoculated (1% v/v) in 10 ml fresh BHI broth. The incubation was continued at 30 ºC and 200 rpm until an absorbance (OD_{600}) of approx. 0.2-0.3 was reached. These cultures were diluted (1:10) in fresh pre-warmed broth of 55 ºC and placed in a waterbath (GFL type 1083) that was set at this temperature. Samples were collected after 0, 60 and 180 minutes, and serially diluted in a peptone physiological salt (PPS) solution (0.85 % NaCl, 0.1 % peptone; Tritium Microbiology). Viable counts were determined on BHI agar plates after incubation at 30 ºC for 3-5 days.

Microbial inactivation experiment

Cells of cultures that were grown overnight in BHI broth (30 ºC and 200 rpm) were harvested by centrifugation at 4300 rpm for 10 min at room temperature (Heraeus type megafuse 1.0R) and resuspended in an equivalent volume of BHI broth or full fat milk (Long life UHT: fat 3.5 % w/w, proteins 3.3 % w/w, carbohydrates 4.8 % w/w, Calcium 0.12 % w/w; Milbona). These cell suspensions in BHI broth and milk were used to

inoculate 500 ml BHI broth (30 ºC or 7 ºC) and full fat milk (7 ºC), respectively, in 500 ml Scott flasks (1% v/v). These flasks were incubated statically at 30 ºC for 24 h (Binder type BD400) or at 7 ºC for 7 days (temperature controlled chamber). The 500 ml cultures obtained by growth in BHI broth (30°C and 7 °C) or full fat milk (7°C) were suspended in 4.5 litre fresh BHI broth (30 ºC or 7 ºC) or full fat milk (7 ºC), which were directly used for the heat-inactivation experiments. The heat-treatments were carried out using an in-house continuous flow micro-heater device. This device consists of a heating section where cell suspensions are heated to the desired temperature in a heat-exchanger, a holder section that is submerged in an oil bath at the selected temperature, and a cooling section where cell suspensions are cooled in a heat-exchanger. The residence times in the heating and cooling sections were 1.7 s. For each combination of strain, medium, and growth temperature, three different heating times of 3, 6 and 10 s were applied, by using three different holders with a flow of 5 l/h. The exposure temperature in the holder section was set using incremental steps of 2 ºC, covering a temperature range that resulted in no inactivation, measurable inactivation and complete inactivation of the culture during the exposure times of 3, 6 or 10 s. After heat treatment, samples were collected and serially diluted in a PPS solution. Viable counts were determined by plating on BHI agar plates and incubation at 30 ºC for 3-5 days.

Data analysis

The thermal inactivation data were analyzed by the first order exponential arrhenius relation:

$$\frac{N_t}{N_0} = e^{-k_0 t} \quad (1)$$

were t is the treatment time, N_t and N_0 are the populations of living cells at time t and time zero, and k_0 is the inactivation constant. The k_0 is determined by linear regression analysis of the time versus $\ln(N_t/N_0)$ plot. However, in heat-exchangers, not the time, but the temperature is the parameter that can be varied. Therefore, equation 1 was modified, resulting in the following equation:

$$\ln(k) = \ln(k_0) - \frac{E_a}{R \cdot (T + 273{,}15K)} \quad (2)$$

were k is the logarithmic inactivation rate, E_a is the energy of activation constant, R is the gas constant (8,314 J/mol/K), and T is the heating temperature (ºC). The kinetic constants

E_a and k_0 can be derived by linear regression analysis of ln(k) versus 1/RT. E_a is the reciprocal of the slope and ln(k_0) is the intercept. Part of the residence time in the heating and cooling sections of the micro-heater may contribute to the heat-inactivation of the cells. These contributions have been taken into account in our calculations by transformation of these sections to an extra residence time in the holder. These calculations are based on the F0-concept, which is used for lethality indication (extensively described by De Jong (5)). The cumulative normal distribution function in Excel (Microsoft) was used to identify significant differences ($p<0.05$) of E_a and ln(k_0) between the different strain, medium, and temperature combinations.

The constants E_a and ln(k_0) can be used directly to derive the D/z-values. The D-value (s) at temperature T (D_T) can be calculated by using the k-value at temperature T (k_T) from equation 2 in the following equation:

$$D_T = \frac{\ln(10)}{k_T} \quad (3)$$

The z-value (ºC) at temperature T (z_T) can be calculated using the following equation:

$$z_T = \frac{\ln(10) \cdot R \cdot (T + 273{,}15K)^2}{E_a - \ln(10) \cdot R \cdot (T + 273{,}15K)} \quad (4)$$

The D- and z-values and their standard error were calculated by simulation using the risk analysis software @RISK (Palisade corporation), based on the predicted E_a, ln(k_0), and error estimates.

Results

Preliminary screening for heat resistant *L. monocytogenes* strains

A preliminary screening was performed to determine the heat-resistance of 48 *L. monocytogenes* strains of diverse serotypes and origins of isolation (Fig. 1A). The strains with the highest heat resistance were strains 1E (isolated from a cheese plant) and NV8 (isolated from a bovine carcass) (21). The reductions in viable numbers of the reference strain ScottA was 2 $\log_{10}$ higher after 3 hours incubation in BHI broth at 55 ºC (Fig 1B). The most sensitive strains were not recovered after 3 hours exposure to this temperature and one strain showed already 2.5 $\log_{10}$ reduction after exposure for one hour. The two heat

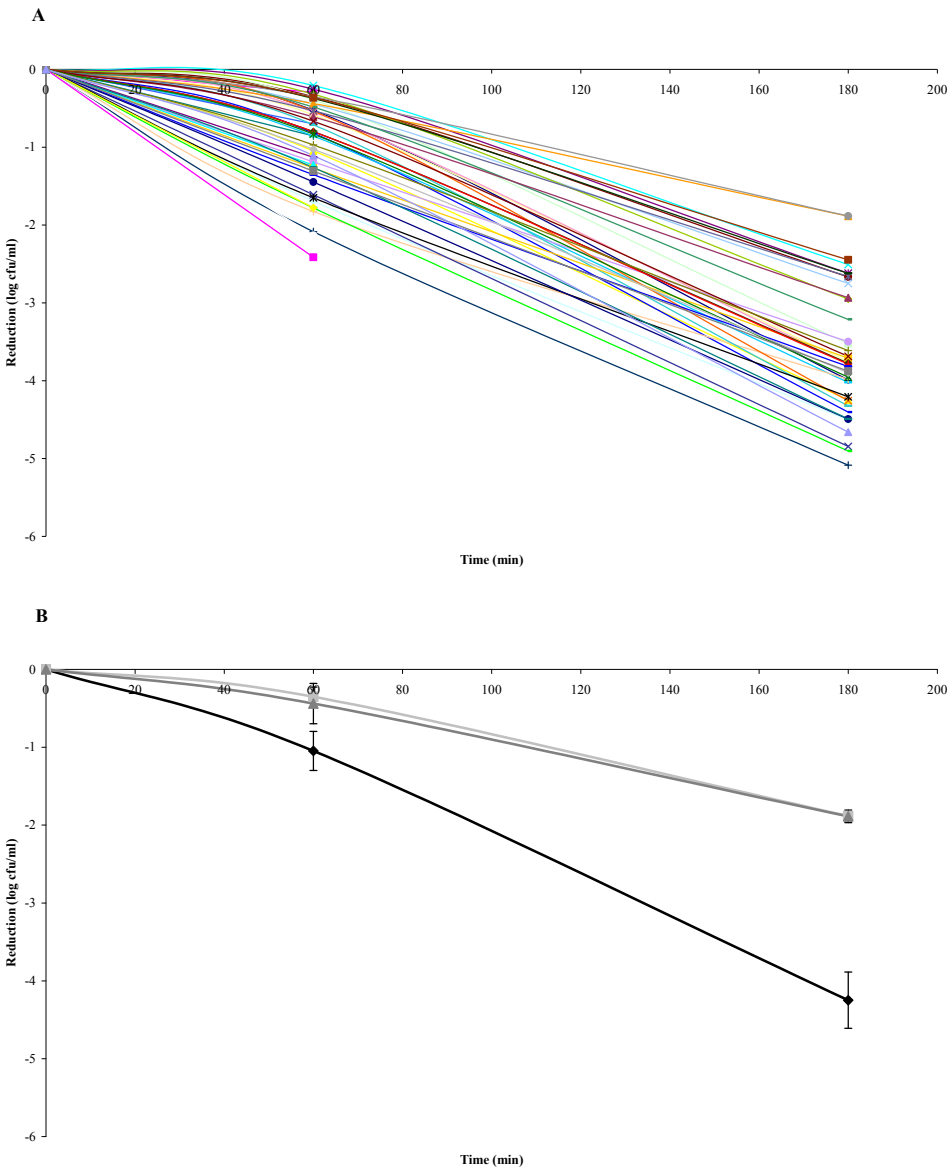

Fig. 1. Heat inactivation of *L. monocytogenes* strains in BHI broth after exposure to 55 ºC for 3 hours. A) Reduction in viable counts of 48 *L. monocytogenes* strains in a single experiment. B) Average reduction in viable counts of the strains ScottA, 1E, and NV8 in a duplicate experiment.

resistant strains 1E and NV8, and the reference strain ScottA were used in further experiments to characterise their heat-resistances in the micro-heater.

Heat inactivation kinetic parameters

In general, first order inactivation kinetics can be applied to heat inactivation data of *L. monocytogenes* obtained in liquid media with an a_w close to 1 and a temperature between 60 ºC and 80 ºC (12). For each strain, the inactivation data obtained using the corrected heating times for 3, 6, or 10 s were used to calculate the E_a and ln(k_0) in BHI broth or full fat milk at 30 ºC and/or 7 ºC by linear regression of ln(k) versus 1/RT (Fig. 2). The correlation coefficient (R^2), which is a measure of how well inactivation data fit the prediction, ranged from 0.81 to 0.98, indicating a good fit. The results of the parameters E_a and ln(k_0) for the heat-inactivation experiments are reported in Table 1. Statistical analysis was performed to determine whether these parameters differed significantly between the different strains, media, and temperatures. The significant differences are presented in Table 2. For strain-medium combinations that are absent from this table, no significant differences were observed. From equations 1 and 2 it can be depicted that higher E_a and lower ln(k_0) values result in a higher fraction of surviving cells after a heat treatment and consequently more resistant bacteria. When comparing different strains under the same inactivation conditions, the E_a and ln(k_0) values of strains ScottA and 1E in BHI broth at 30

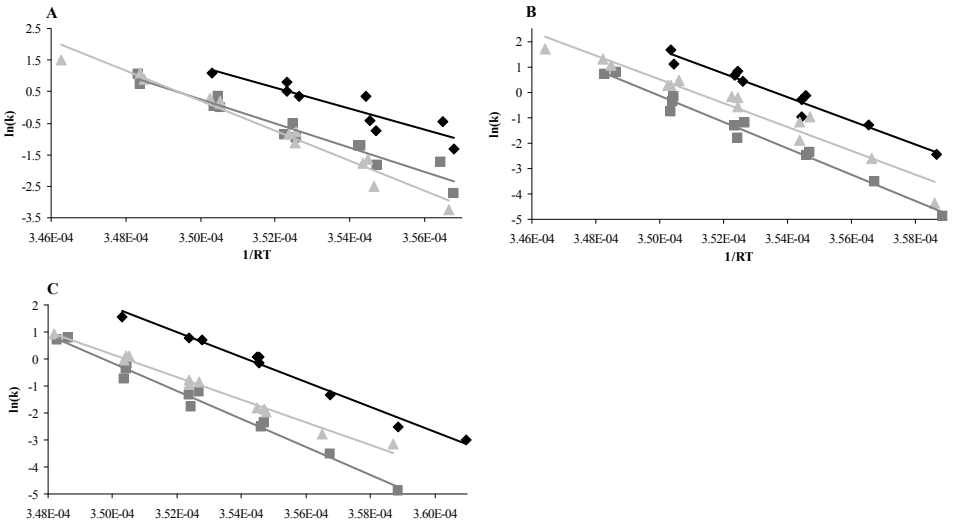

Fig. 2. Values of calculated ln(k) plotted against 1/RT, based on the inactivation of strains ScottA (black), 1E (dark grey), and NV8 (light grey) grown in: A) BHI at 30 ºC, B) BHI at 7 ºC, and C) Milk at 7 ºC. The parameters E_a and ln(k_0) were calculated by regression analysis of the fitted line. The parameters are presented in Table 1.

ºC were significantly lower than the values of strain NV8 under these conditions. Furthermore, strains ScottA and NV8 showed significantly lower E_a values than strain 1E in milk at 7 ºC. In addition, strain NV8 showed a significantly lower ln(k_0) value than strains ScottA and 1E in milk at 7 ºC.

When comparing the heat inactivation kinetic parameters of a specific strain under different growth conditions, significant differences were found for the following cases. For strain ScottA, the E_a and ln(k_0) values in BHI at 30 ºC were significantly lower than those in BHI at 7 ºC and milk at 7 ºC. No significant differences for the E_a and ln(k_0) values of ScottA were observed between BHI and milk at 7 ºC. For strain 1E, significantly lower E_a and ln(k_0) values were observed following heat inactivation of a culture grown in BHI at 30 ºC compared with growth in milk at 7 ºC. Significantly lower values were also observed after growth in BHI at 7 ºC compared with milk at 7 ºC. Lastly, for strain NV8, the E_a and ln(k_0) values were significantly higher for cultures grown in BHI at 30 ºC compared with cultures grown in milk at 7 ºC.

Table 1. Kinetic parameters of heat-inactivation for the 3 *L. monocytogenes* strains in the 3 heating media using first order linear regression.

Strain	**Medium**	E_a		**Ln(k_0)**		**R^2**
		Value	**SE**	**value**	**SE**	
ScottA	BHI 30	334570	61379	118	22	0,81
	BHI 7	469368	38048	166	13	0,95
	Milk 7	461393	23266	163	8	0,98
1E	BHI 30	386034	27651	135	10	0,95
	BHI 7	403731	50445	142	18	0,85
	Milk 7	518734	22717	181	8	0,98
NV8	BHI 30	477006	27247	167	10	0,97
	BHI 7	467244	35269	164	12	0,94
	Milk 7	416456	16497	146	6	0,98

D/z-values

The parameters E_a and ln(k_0) from Table 1 were used to calculate the D/z-values at 68, 70, and 72 ºC for the 3 strains grown in BHI at 30 ºC or 7 ºC or in milk at 7 ºC (Table 3). For all three medium - growth temperature combinations, the D-values of the heat-resistant strains 1E and NV8 are higher than the D-values of strain ScottA. The z-values ranged from 4.23 to 7.14. Furthermore, Table 3 shows the $\log_{10}$ reduction for a 15 s HTST pasteurization process at the reported temperatures. At 72 ºC, all strains show 12.1 or higher $\log_{10}$ reduction values for a 15 s heat inactivation process. This is sufficient for eradication of any potential *L. monocytogenes* contamination. However, if the pasteurisation temperature were to drop to 70 ºC due to for instance equipment failure,

contamination with exceptionally high initials numbers of strain 1E (> 700 cfu/ml) might pose a risk factor in milk. A further temperature drop of 2°C to 68 ºC could result in survival of both heat-resistant strains 1E and NV8 following 15 s heat-treatment in BHI and milk, since under these conditions, the expected inactivation is between 1.5 and 2.6 $\log_{10}$ units.

Table 2. Significant different E_a and $\ln(k_0)$ values ($p<0.05$) between different strains and media are indicated (x).

Strain	**Broth**	**ScottA**				**1E**		**NV8**			
		BHI 7		**Milk 7**		**Milk 7**		**BHI 30**		**Milk 7**	
		E_a	$\ln(k_0)$	E_a	$\ln(k_0)$	E_a	$\ln(k_0)$	E_a	$\ln(k_0)$	E_a	$\ln(k_0)$
ScottA	BHI 30	x	x	x	x			x	x		
	Milk 7					x					x
1E	BHI 30					x	x	x	x		
	BHI 7					x	x				
	Milk 7									x	x
NV8	BHI 30									x	x

Discussion

Most of the research on heat-resistance of *L. monocytogenes* has been performed with laboratory strains in batch processes. Data obtained using such experimental setup cannot directly be extrapolated to heating processes in continuous flow heat-exchangers due to effects of additional stresses (20). Furthermore, only limited information is available on strain variation with regard to heat resistance and on the heat resistance of industrial isolates. The present study was performed to investigate the potential of heat-resistant *L. monocytogenes* strains to survive in HTST pasteurisation processes in a counter-current continuous flow heat-exchanger.

Heat-resistant strains were selected following preliminary screening of a random selection of 48 strains with diverse serotypes and origins of isolation using a batch heating process. Hereby, two heat-resistant strains (1E and NV8) were identified which showed 2 $\log_{10}$ cfu/ml higher survival than the scottA reference strain after 3 hours exposure at 55 ºC in BHI broth. As strain ScottA was isolated from the listeriosis outbreak in 1983 in which pasteurized milk was the vehicle, the heat inactivation of this particular strain has been best characterised. In a previous study on heat-resistance of *L. monocytogenes* in dairy products, it was shown that strain ScottA displays relatively high heat resistance (2). Remarkably, strain ScottA showed an average heat-resistance in our initial screening experiments, although this experiments was performed using different conditions. A previous study on

Table 3. D/z-values for the 3 strains in the 3 heating media at 72, 70, and 68 ºC and the resulting $\log_{10}$ reduction after 15 s exposure to these temperatures.

Temp. (ºC)	Strain	BHI 30					BHI 7					milk 7				
		D_T (s)	se	z_T (ºC)	se	$\log_{10}$	D_T (s)	se	z_T (ºC)	se	$\log_{10}$	D_T (s)	se	z_T (ºC)	se	$\log_{10}$
	ScottA	0.40	0.01	7.14	0.24	37.1	0.22	0.00	4.81	0.32	68.6	0.17	0.00	4.83	0.28	87.5
72	1E	1.03	0.03	5.71	0.35	14.6	0.89	0.03	4.87	0.61	16.8	1.24	0.02	4.33	0.19	12.1
	NV8	0.95	0.02	4.66	0.25	15.9	0.85	0.04	4.60	0.38	17.5	1.05	0.02	5.42	0.20	14.2
	ScottA	0.80	0.02	6.79	0.31	18.8	0.57	0.01	4.69	0.27	26.5	0.44	0.01	4.82	0.20	34.3
70	1E	2.24	0.04	5.65	0.36	6.7	2.03	0.09	4.81	0.62	7.4	3.54	0.10	4.28	0.19	4.2
	NV8	2.49	0.06	4.61	0.24	6.0	1.92	0.05	4.55	0.36	7.8	2.46	0.02	5.36	0.20	6.1
	ScottA	1.58	0.04	6.55	0.31	9.5	1.49	0.03	4.62	0.27	10.1	1.13	0.02	4.75	0.20	13.3
68	1E	4.96	0.18	5.59	0.35	3.0	4.65	0.25	4.77	0.60	3.2	10.29	0.16	4.23	0.18	1.5
	NV8	6.64	0.13	4.56	0.24	2.3	5.00	0.12	4.50	0.36	3.0	5.78	0.07	5.29	0.19	2.6

thermal inactivation of 13 strains in BHI broth at pH 3.0 and a_w 0.987 or at pH 7.0 and a_w 0.970 showed that relative resistance of strains were different for these conditions (8). In our experiments, the selected heat-resistant strains 1E and NV8 were significantly more heat-resistant than strain ScottA following growth in BHI at 30 ºC and in milk at 7 ºC. For strain 1E, the heat resistance was higher in milk than in BHI. However, for the strains ScottA and NV8, no significant differences in the heat inactivation parameters for cultures grown in BHI or milk at 7 ºC were observed. Previous studies have shown a protective effect of milk fat (2.5-5.0%) (3) or low a_w (7% NaCl) (18, 23) on heat-resistance. The a_w of full fat milk is 0.97 with a fat content of 3.5%. A protective effect of milk on the heat-inactivation of *l. monocytogenes* compared with inactivation in BHI (a_w=0.995) was only observed for one out of three strains, therefore we can not conclude from our data that the milk matrix protects *L. monocytogenes* from heat inactivation.

Following a HTST pasteurization process of 15 s at 71.7 ºC ($D_{71.7}$=1.45), our study showed approximately 10.3 $\log_{10}$ reduction for the most heat-resistant strain that was cultured in milk at 7 ºC (1E). Similar results were obtained by Bunning et al (1). Their study showed that inactivation of strain ScottA in raw milk in a slug-flow heat-exchanger resulted in a D-value of 1.3 s at 71.7 ºC and consequently 11.5 $\log_{10}$ reduction for the 15 s HTST pasteurisation process at this temperature. Remarkably, our results for strain ScottA showed 76 $\log_{10}$ reduction in milk using this HTST pasteurisation process ($D_{71.7}$=0.20). Our study shows that a minimal HTST pasteurization process (15 s at 72 ºC) is in principle sufficient to inactivate the most heat-resistant *L. monocytogenes* strains. However, relatively small deviations in the temperature settings might result in survival of heat-resistant strains of this bacterium. A study on the contamination level of *L. monocytogenes* in raw milk from Swedish bulk tanks showed that only in rare cases levels as high as 10^2 cfu/ml could be expected, while contamination levels <10 cfu/ml are more common (25). The legal requirement for *L. monocytogenes* in milk in the European Union is absence in 25 g (9). To completely eradicate *L. monocytogenes* from 25 g of raw milk with contamination levels as high as 10^2 cfu/ml, approximately 3.4 $\log_{10}$ reduction is required. Using the E_a and $\ln(k_0)$ values obtained with strain 1E in milk, the minimal temperature of the 15 s pasteurisation process should be 69.6 ºC. These results are in contrast to some previous studies in which *L. monocytogenes* was capable of surviving minimal HTST pasteurization processes. Doyle et al. (7) showed that *L. monocytogenes* was able to survive the HTST process in industrial pasteurizers in milk at temperatures of 71.7 ºC to 73.9 ºC for 16.4 s. Similar results were obtained in a pilot scale pasteurizer when milk was pasteurized at 72 ºC for 15 s using an inoculum of 6.5 $\log_{10}$ cfu/ml (14). Finally, Farber et al (10) showed that *L. monocytogenes* could be recovered from a HTST pasteurizer after 16.2 sec exposure to 72 ºC if the cells were grown at relatively high temperatures (39 ºC and above), but they

concluded that under normal processing conditions and storage of milk, minimal pasteurisation (15 s at 71.7 ºC) provided a good safety margin.

It should be noted that *L. monocytogenes* cells surviving HTST pasteurisation are probably injured and unable to recover or multiply during the period of cold storage (4). Surviving bacteria in a commercial process would therefore likely not grow to high numbers during storage. Also, milk is generally treated with a subpasteurization treatment (thermization) at 57 to 68 ºC for 15 to 30 s. This treatment results in initial damage of *L. monocytogenes* cells, thereby ensuring complete eradication of this bacterium during the pasteurization process. Furthermore, minimal pasteurization conditions (15 s at 72 ºC) are seldomly used in a commercial process. The settings of commercial pasteurizers tend to be around 74-76 ºC to ensure sufficient inactivation of microbes. For the most heat-resistant strain that was cultured in milk, a heat treatment at 75 ºC for 15 s (NV8) would result in a 50 $\log_{10}$ reduction. In conclusion, this study showed that if commercial HTST pasteurization processes are performed without any failures, heat-resistant *L. monocytogenes* strains are inactivated. However, it is critical to safeguard the temperature during the pasteurisation process, because a drop in the temperature to 70 ºC could result in survival of heat-resistant *L. monocytogenes* strains.

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4. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat-shock, cell division, cell wall synthesis, and the SOS response

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Abstract

The food-borne pathogen *Listeria monocytogenes* has the ability to survive extreme environmental conditions due to an extensive interactive network of stress responses. It is able to grow and survive at relatively high temperatures in comparison with other non-sporulating food-borne pathogens. To investigate the heat-shock response of *L. monocytogenes*, whole-genome expression profiles of cells that were grown at 37°C and exposed to 48°C were determined using DNA micro-arrays. The transcription levels were measured over a 40-min period after exposure of the culture to 48 °C and compared with the unexposed culture at 37 °C. After 3 min, 25 % of all genes were differentially expressed, while after 40 min only 2 % of all genes showed differential expression, indicating a transient nature of the heat-shock response. The global transcriptional response was validated by analysing the expression of a set of 13 genes by quantitative real-time PCR. Genes previously identified as part of the class I and class III heat-shock response and the class II stress response showed induction at one or more of the time-points investigated. This is the first study to report that several heat-shock induced genes are part of the SOS response in *L. monocytogenes*. Furthermore, numerous differentially expressed genes that play a role in the cell division machinery and cell wall synthesis were down-regulated. This expression pattern is in line with the observation that heat-shock resulted in cell elongation and prevention of cell division.

Introduction

The food-borne pathogen *Listeria monocytogenes* is a Gram-positive facultative anaerobic rod and the causative agent of listeriosis. Due to the severity of the disease and the fact that its incidence is increasing in numerous European countries, *L. monocytogenes* is of great public health concern (9, 17, 23). This bacterium shows relatively high resistance to environmental insults compared with many other non-spore-forming food-borne pathogens. It is able to grow at a wide pH range (from pH 5 to pH 9), at high salt concentrations (up to 12 %), and at a wide temperature range (-0.4 to 44 ºC) (19, 20). The ability of *L. monocytogenes* to proliferate under adverse conditions and survive environmental insults is mediated by various mechanisms that allow for rapid responses and adaptation to changing environments. Due to consumer's demands for less heavily preserved foods and more convenience foods, processing conditions in the food industry are becoming milder. *L. monocytogenes* is able to adapt to such milder conditions, making it of major concern for the food industry.

Variation in temperature is a stress that is commonly encountered in nature and during the processing of foods. DNA micro-arrays provide an excellent tool to study the expression profiles of a complete genome during exposure to heat-stress. Previous studies involving transcriptional analysis of the heat-shock response in various bacteria showed induction of several protection mechanisms, including general protection mechanisms and specific heat-shock responses. The heat-shock response is a common phenomenon among bacteria that enables them to survive a wide variety of stresses, in particular heat stress. Most heat-stress-induced genes encode molecular chaperones or proteases that can either protect other proteins/enzymes against misfolding and damage or mediate degradation when this fails. Maintenance of protein quality is important for normal growth of cells, and is essential under stress conditions. In *L. monocytogenes*, two specific heat-shock response mechanisms and a general stress-response mechanism can be distinguished, namely, the class I and class III heat-shock response, and the class II stress response (3, 25, 40) . Class I heat-shock genes are controlled by the HrcA repressor, which binds to the CIRCE operator sequence (TTAGCACTC-N_9-GAGTGCTAA) preceding this class of genes. Class I heat shock genes include *dnaK*, *dnaJ*, *groES*, and *groEL*, encoding chaperones. Class III heat-shock genes encode chaperones and ATP-dependent Clp proteases, which degrade damaged or misfolded proteins. This class is regulated by the CtsR repressor (class three stress gene repressor), which binds specifically to a heptanucleotide repeat in the promoter region (A/GGTCAAA NAN A/GGTCAAA). The class II stress genes encode general stress proteins, of which the expression is regulated by the alternative sigma factor SigB. This sigma factor recognizes alternative -35 and -10 sequences (GTTT-N_{13-17}-GGGWAT) in the promoter region of the class II stress genes (22).

The complete heat-shock regulon of *L. monocytogenes* in response to a temperature up-shift has not been investigated before, even though heating is an important preservation strategy for the food industry during minimal processing. The aim of this study was to determine the global transcriptional response of *L. monocytogenes* to heat stress. Our data show that exposure to elevated temperatures triggers the classical heat-shock genes, and in addition, a transient effect on expression of genes involved in the cell replication machinery was observed. Another novel finding is that heat-shock triggers the SOS response in *L. monocytogenes*.

Materials and Methods

Strains and sample conditions

L. monocytogenes EGD-e (11) was grown in Brain Hearth Infusion Broth (Difco) with shaking (200 rpm, New Brunswick C24KC) using 10 ml culture medium in 100 ml conical flasks. An exponentially growing culture was used to inoculate 100 ml of fresh pre-warmed BHI broth in a 500 ml flask. This culture was incubated at 37 °C with agitation at 200 rpm until an optical density of 1.0 at 600 nm (OD_{600}) was reached. At this point (designated time-zero), a 5 ml aliquot was removed for RNA extraction and 10 ml aliquots were transferred to pre-warmed 100 ml flasks at 48 °C. The cultures were incubated in a shaking water bath at 48°C (GFL Type 1083, 60 % shaking speed) and samples for RNA extraction and microscopy analysis were taken after 3, 10, 20, and 40 min.

RNA isolation, labelling, hybridization, imaging, and micro-array analysis

Samples of 0.5 ml were rapidly removed and diluted in 1.0 ml of RNAprotect (Qiagen). After incubation for 5 min at room temperature and centrifugation at 5000 x g for 5 min pellets were stored at –80 °C. Micro-array experiments (including micro-array generation, total RNA extraction, labelling, hybridization, imaging and micro-array analysis) were performed as described in great detail in our previous manuscript (Chatterjee *et al.*, 2006). The program SAM (Significance Analysis of Microarrays) was used to analyse the data. The cut-off for significantly differentially expressed genes was set with a q-value (false discovery rate) of ≤ 1 % and a fold-change ≥ 2. In three independent experiments, the whole-genome expression profiles of cells that had undergone heat-shock for 3, 10, 20 and 40 min were compared with those of cells at time-zero in a dye-swap hybridization experiment. The micro-array platform and micro-array data are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession numbers A-MEXP-752 and E-MEXP-1118, respectively.

Microscopy and image analysis

Samples of 1 ml were removed from cultures (see Strains and sample conditions) and centrifuged at 5000 x g for 2 min. Cells were dissolved in nigrosin solution (Sigma-Aldrich) and dried on a glass slide. Images of the cells were taken at 100 x magnification under a Dialux 20 microscope (Leica). The ImageJ program (http://rsb.info.nih.gov/ij/download.html) was used to analyse the images. The images were loaded in 8-bit type and the threshold was adjusted to black & white. The number of pixels per cell was counted and distribution graphs were constructed in Excel (Microsoft) by analyses of 7 images from two cell preparations for each time-point.

Quantitative PCR (Q-PCR)

Superscript III Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA using 1 µg of DNase-treated total RNA. The RNA samples were controlled for DNA contamination by omitting this cDNA synthesis step. Q-PCR reactions were performed using 10 µl 2 x Sybr Green PCR Master Mix (Applied Biosystems), 200 nM primers, and 1 µl cDNA sample in a 20 µl final volume. For each primer set a standard curve was generated using both genomic DNA and cDNA, and negative control samples using pure water were included. Reactions were run on the 7500 real-time PCR System (Applied Biosystems) with an initial step of 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. To verify single product formation a dissociation cycle was added. Forward and reverse primers (Table S1, online manuscript) were designed with an amplicon length of about 100 bp and a Netprimer rating above 80 (http://www.premierbiosoft.com/netprimer).

Results

Global gene expression analysis

Whole-genome expression profiles of cells at four time points (3, 10, 20, 40 min) following the temperature shift from 37 °C to 48 °C were compared with those from cells harvested prior to the upshift (time zero). In total, 714 genes showed ≥ 2-fold differential expression during at least one of the four time points compared with the time zero samples with a q-value ≤ 1 % (significant values in Table S2, online manuscript). Of these 714 genes, 427 showed increased expression and 287 showed decreased expression upon heat-shock, constituting 15 % and 10 % of the total number of 2857 genes, respectively. The maximum level of gene induction observed was 50.3-fold (lmo1883), while the maximum level of gene repression was 31.6-fold (lmo0048). Most of the differentially expressed genes showed a transient pattern. The highest number of differentially expressed genes, which accounts for 24 % of all genes, was observed 3 min after the temperature up-shift.

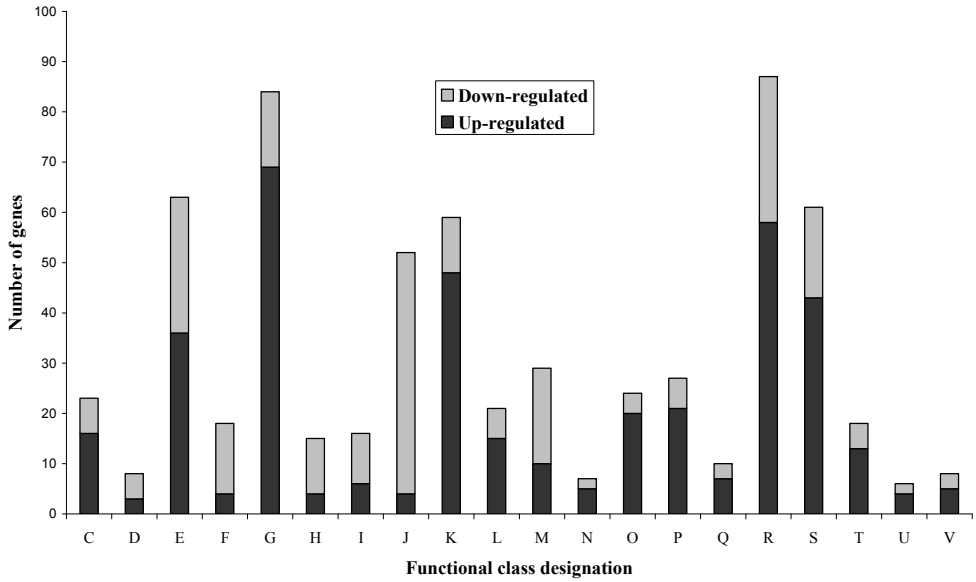

Fig. 1. Differentially expressed genes (fold-change ≥ 2, q-value ≤ 1%) grouped by functional classification according to the NCBI database (www.ncbi.nlm.nih.gov/COG/). Columns: C, energy production and conversion; D, cell cycle control, mitosis and meiosis; E, amino-acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation; K, transcription; L, replication, recombination and repair; M, cell wall/membrane biogenesis; N, cell motility; O, posttranslational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking and secretion; V, defence mechanisms.

After 40 min, only 2 % of all genes were differentially expressed. The differentially expressed genes were grouped into functional classes (Fig. 1). The classes containing the highest numbers of differentially expressed genes were carbohydrate transport and metabolism, transcription, translation, and amino-acid transport and metabolism, with 84, 59, 52, and 63 genes, respectively. Most genes involved in carbohydrate transport and metabolism (82 %) and transcription (81 %) showed up-regulation and most genes involved in translation (92 %) showed down-regulation. Numerous genes belonging to the class of amino-acid transport and metabolism showed differential expression, both up- and down-regulation (36 and 27 genes, respectively).

Stress response

L. monocytogenes encodes different classes of (heat) stress-response genes and general mechanisms to survive a wide variety of environmental insults. During exposure to elevated temperatures these classes of genes show differential expression of constant or transient nature. The number of differentially expressed genes belonging to different classes that are significantly up-regulated and down-regulated at the measured time-points is shown in Table 1. A detailed list of the actual differentially expressed genes belonging to these different classes is presented in Table 2 and further described below.

Table 1. Number of up- and down-regulated genes of *L. monocytogenes* at designated time-points after exposure to 48 ºC in comparison with time-zero expression at 37 ºC, belonging to different classes of (heat) stress response and general mechanisms that are important for surviving a wide variety of environmental insults.

	3 min		**10 min**		**20 min**		**40 min**	
Response	**up**	**down**	**up**	**down**	**up**	**down**	**up**	**down**
Group I heat-shock	6	0	2	0	6	0	5	0
Group III heat-shock	9	0	4	0	6	0	4	0
Group II stress	54	0	33	0	21	0	5	0
SOS response & DNA repair	7	0	4	0	7	0	8	0
Cell division	0	12	0	2	0	1	0	0
Autolysis & cell wall hydrolases	0	8	0	3	0	3	0	1
Cell wall synthesis	7	7	3	4	3	4	2	4
Cell wall associated	6	2	1	1	3	0	0	0
Virulence associated	3	4	1	3	2	4	2	4

Stress response genes

The specific heat-shock regulons consisting of class I (molecular chaperones) and class III (ATP-dependent proteases) heat-shock genes were induced during the heat-shock experiments. Genes belonging to the class I heat-shock response (*dnaJ*, *dnaK*, *grpE*, *hrcA* [lmo1472 to lmo1475] and *groEL*, *groES* [lmo2068 and lmo2069]) showed between 2- and 4-fold higher expression levels. In contrast, the class III heat-shock genes (*ctsR* [lmo0229], lmo0230, lmo0231, *clpC* [lmo0232], *clpE* [lmo0997], lmo1138, *clpY* [lmo1279], *clpB* [lmo2206] and *clpP* [lmo2468]) showed a transient differential expression pattern. Another transiently expressed gene (*htrA* or lmo0292), encoding a serine protease, showed approximately 5-fold higher expression levels (Table S2, supplemental material). This

Table 2. Differentially expressed genes of *L. monocytogenes* EGD-e at designated time-points after exposure to 48 ºC in comparison with time-zero expression at 37 ºC*.

Gene	Fold change at time (min)				Name	Product description
	3	10	20	40		
Class I heat-shock genes						
lmo1472	2.67	1.64	2.15	2.50	*dnaJ*	Heat-shock protein DnaJ
lmo1473	3.14	1.94	2.52	2.41	*dnaK*	Heat-shock protein DnaK
lmo1474	2.97	1.48	2.06	2.10	*grpE*	Heat-shock protein GrpE
lmo1475	3.04	1.81	2.42	2.30	*hrcA*	Transcription repressor of class I heat-shock genes HrcA
lmo2068	2.96	2.97	2.96	2.71	*groEL*	Heat-shock protein GroEL
lmo2069	3.06	2.66	3.26	2.94	*groES*	Heat-shock protein GroES
Class III heat-shock genes						
lmo0229	2.95	1.02	1.13	1.22		Transcription repressor of class III heat-shock genes CtsR
lmo0230	4.98	1.13	1.57	1.56		Similar to *B. subtilis* YacH protein
lmo0231	8.12	1.81	2.27	1.95		Similar to arginine kinase
lmo0232	6.86	1.74	2.23	2.14	*clpC*	Endopeptidase Clp ATP-binding chain C
lmo0997	29.0	3.42	4.37	3.32	*clpE*	ATP-dependent protease
lmo1138	7.10	2.75	3.13	2.49		Similar to ATP-dependent Clp protease
lmo1279	1.97	1.28	1.18	-1.1	*clpY; hslU*	Similar to ATP-dependent Clp protease
lmo2206	16.1	8.20	6.42	4.00	*clpB*	Similar to endopeptidase Clp ATP-binding chain B
lmo2468	6.38	4.94	4.76	3.34	*clpP*	ATP-dependent Clp protease
Class II stress genes (SigB regulated)						
lmo0200	6.63	4.29	5.06	4.35	*prfA*	Listeriolysin positive regulatory protein
lmo0211	2.32	1.22	1.50	1.36	*ctc*	Similar to *B. subtilis* general stress protein
lmo0405	5.69	1.54	1.16	-1.0		Similar to phosphate transport protein
lmo0593	2.04	1.50	-1.0	-1.3		Similar to transport proteins
lmo0669	5.30	1.17	1.66	1.30		Similar to oxidoreductase
lmo0670	6.49	1.17	1.74	1.23		Hypothetical protein
lmo0781	**5.30**	1.49	1.30	-1.2		Similar to mannose-specific PTS component IID

Table 2. Continued.

Gene	Fold change at time (min)				Name	Product description
	3	10	20	40		
lmo0782	**4.83**	1.56	1.33	-1.2		Similar to mannose-specific PTS component IIC
lmo0783	**6.08**	1.83	1.47	-1.2		Similar to mannose-specific PTS component IIB
lmo0794	**11.6**	**2.03**	1.77	1.01		Similar to *B. subtilis* YwnB protein
lmo0880	**8.15**	1.43	1.37	-1.1		Similar to wall associated protein precursor (LPXTG motif)
lmo0911	**9.90**	**2.10**	**2.36**	1.20		Hypothetical protein
lmo0956	**7.93**	**3.30**	**4.29**	**2.80**		Similar to N-acetylglucosamine-6-phosphate deacetylase
lmo0957	**7.19**	**2.52**	**3.33**	2.41	*nagB*	Similar to glucosamine-6-phosphate isomerase
lmo0958	**5.39**	**2.56**	**3.27**	**2.47**		Similar to transcription regulator
lmo0994	**6.59**	1.59	1.62	-1.1		Hypothetical protein
lmo1421	**3.03**	1.19	1.15	-1.0	*bilEA*	Bile exclusion system
lmo1425	**5.01**	**2.52**	1.56	-1.3	*opuCD*	Similar to betaine-carnitine-choline ABC transporter
lmo1426	**5.09**	**2.03**	1.58	-1.3	*opuCC*	Similar to glycine betaine-carnitine-choline ABC transporter
lmo1427	**5.54**	1.49	1.35	-1.3	*opuCB*	Similar to glycine betaine-carnitine-choline ABC transporter
lmo1428	**4.86**	1.44	1.28	-1.4	*opuCA*	Similar to glycine betaine-carnitine-choline ABC transporter
lmo1433	**4.10**	1.27	1.42	-1.2		Similar to glutathione reductase
lmo1538	**4.93**	1.09	1.12	-1.2	*glpK*	Similar to glycerol kinase
lmo1539	**7.02**	1.70	1.41	-1.0		Similar to glycerol uptake facilitator
lmo1580	**2.85**	-1.0	-1.1	-1.3		Similar to unknown protein
lmo1601	**2.79**	**2.63**	**2.03**	-1.5		Similar to general stress protein
lmo1602	**3.03**	1.94	1.46	-1.4		Similar to unknown proteins
lmo1694	**8.96**	**3.48**	2.27	1.18		Similar to CDP-abequose synthase
lmo1883	**50.3**	**2.48**	**3.22**	2.18		Similar to chitinases
lmo2067	**8.22**	**3.22**	**2.88**	1.84		Similar to conjugated bile acid hydrolase
lmo2085	**10.8**	**2.38**	**2.02**	1.43		Putative peptidoglycan bound protein (LPXTG motif)

Table 2. Continued.

Gene	Fold change at time (min)				Name	Product description
	3	10	20	40		
lmo2157	**10.5**	**3.76**	**5.04**	1.12	*sepA*	SepA
lmo2205	**11.9**	**7.47**	**5.55**	**3.05**	*gpmA*	Similar to phosphoglyceromutase 1
lmo2230	**11.0**	**5.12**	**4.59**	**2.44**		Similar to arsenate reductase
lmo2269	**2.66**	**2.14**	**2.36**	2.17		Hypothetical protein
lmo2386	**10.3**	**2.02**	1.92	1.42		Similar to *B. subtilis* YuiD protein
lmo2391	**15.5**	**2.35**	1.72	-1.1		Similar to *B.subtilis* conserved hypothetical protein YhfK
lmo2398	**3.93**	1.99	1.48	1.01	*ltrC*	Low temperature requirement C protein
lmo2434	**19.4**	**2.79**	**2.24**	1.51		Highly similar to glutamate decarboxylases
lmo2463	**12.8**	**2.89**	1.82	1.19		Similar to transport protein
lmo2484	**11.9**	**2.62**	**2.58**	1.34		Similar to *B. subtilis* YvlD protein
lmo2485	**9.04**	**2.35**	**2.18**	1.36		Similar to *B. subtilis* yvlC protein
lmo2511	**4.39**	1.71	1.27	-1.2		Similar to *B. subtilis* conserved hypothetical protein YvyD
lmo2570	**11.7**	**3.04**	**2.40**	1.41		Hypothetical protein
lmo2571	**12.6**	**3.13**	**2.37**	1.37		Similar to nicotin amidase
lmo2572	**15.2**	**2.86**	**2.07**	1.40		Similar to Chain A, dihydrofolate reductase
lmo2573	**15.9**	**3.11**	**2.53**	1.23		Similar to zinc-binding dehydrogenase
lmo2602	**7.66**	**2.16**	1.69	1.14		Conserved hypothetical protein
lmo2673	**12.8**	**3.36**	**2.73**	1.61		Conserved hypothetical protein
lmo2695	**13.8**	**3.91**	2.27	-1.1		Similar to dihydroxyacetone kinase
lmo2696	**13.6**	**3.06**	1.90	-1.1		Similar to hypothetical dihydroxyacetone kinase
lmo2697	**11.8**	**3.00**	2.00	-1.2		Hypothetical protein
lmo2748	**11.9**	**2.34**	1.68	1.07		Similar to *B. subtilis* stress protein YdaG
SOS response and DNA repair						
lmo0233	**3.32**	1.04	1.19	1.29	*radA; sms*	Similar to DNA repair protein Sms
lmo0496	**6.51**	**3.58**	**5.04**	**3.36**	*ynzC*	Similar to YnzC of *B. subtilis*
lmo1303	**2.48**	1.27	**3.14**	**5.15**		Similar to *B. subtilis* YneA protein
lmo1368	**2.00**	1.14	1.11	1.10	*recN*	DNA repair and genetic recombination

Table 2. Continued.

Gene	Fold change at time (min)				Name	Product description
	3	10	20	40		
lmo1398	1.40	**2.29**	**4.47**	**5.23**	*recA*	Recombination protein RecA
lmo1975	**3.99**	**3.15**	**4.53**	**5.52**	*dinB*	DNA polymerase IV
lmo2488	**2.29**	2.15	**2.55**	**4.54**	*uvrA*	Excinuclease ABC (subunit A)
lmo2489	**2.65**	**2.26**	**3.60**	**5.24**	*uvrB*	Excinuclease ABC (subunit B)
lmo2675	1.82	1.38	**2.00**	**2.90**	*umuD*	DNA polymerase V
lmo2676	1.60	1.30	1.41	**2.60**	*umuC*	DNA polymerase V
Cell division						
lmo2687	**3.40**	1.14	1.24	1.50		Similar to cell division protein FtsW
lmo2688	**3.56**	1.12	1.27	1.59		Similar to cell division protein FtsW
lmo1071	**-2.1**	-1.2	-1.2	-1.0		Similar to cell-division protein RodA and FtsW
lmo1544	**-2.5**	-1.4	-1.6	-1.7	*minD*	Similar to septum placement protein MinD
lmo1545	**-3.3**	-1.8	-2.0	-2.0	*minC*	Similar to septum placement protein MinC
lmo1546	**-3.5**	-1.5	-1.3	-1.6	*mreD*	Similar to cell-shape-determining protein MreD
lmo1547	**-3.0**	-1.6	-1.7	-1.8	*mreC*	Similar to cell-shape-determining protein MreC
lmo1548	**-2.9**	-1.5	-1.8	-1.6	*mreB*	Similar to cell-shape-determining protein MreB
lmo2020	**-4.3**	-1.6	-1.9	-1.9	*divIVA*	Similar to cell-division initiation protein
lmo2039	**-2.4**	-1.2	-1.4	-1.4	*pbpB*	Similar to penicillin-binding protein 2B, cell division protein FtsI
lmo2506	**-10.3**	**-2.7**	-2.3	-1.3	*ftsX*	Highly similar to cell-division protein FtsX
lmo2507	**-11.4**	**-2.7**	**-2.3**	-1.4	*ftsE*	Highly similar to the cell-division ATP-binding protein FtsE
Autolysins and cell wall hydrolases						
lmo0582	**-3.2**	-2.1	**-3.0**	**-3.2**	*iap*	P60 extracellular protein, invasion associated protein Iap
lmo1076	**-2.7**	-1.5	-1.7	-1.5	*auto*	Similar to N-acetylmuramoyl-L-alanine amidase (autolysin)
lmo1216	**-5.5**	**-2.0**	**-2.1**	-1.5		Similar to N-acetylmuramoyl-L-alanine amidase (autolysin)

Table 2. Continued.

Gene	Fold change at time (min)				Name	Product description
	3	10	20	40		
lmo1855	**-2.4**	-1.5	-1.6	-1.6		Similar to similar to D-alanyl-D-alanine carboxypeptidases
lmo2505	**-8.8**	**-3.0**	-2.6	-1.3	*spl*	Peptidoglycan lytic protein P45
lmo2558	**-2.2**	-1.5	-1.7	-1.5	*ami*	Amidase (autolysin)
lmo2691	**-8.0**	**-2.8**	**-3.2**	-2.3	*murA*	Similar to N-acetylmuramidase (autolysin)
lmo2754	**-2.4**	-1.7	-2.0	-1.9		Similar to D-alanyl-D-alanine carboxypeptidase
Cell wall synthesis						
lmo0877	**2.39**	1.13	1.04	-1.05		Similar to glucosamine-6-phosphate isomerase
lmo0956	**7.93**	**3.30**	**4.29**	**2.80**		Similar to N-acetylglucosamine-6-phosphate deacetylase
lmo0957	**7.19**	**2.52**	**3.33**	2.41		Similar to glucosamine-6-phosphate isomerase
lmo0958	**5.39**	**2.56**	**3.27**	**2.47**		Similar to transcription regulator
lmo1998	**3.36**	1.07	-1.02	1.03		Weakly similar to glucosamine-fructose-6-phosphate aminotransferase
lmo1999	**14.4**	1.22	1.39	1.31		Weakly similar to glucosamine-fructose-6-phosphate aminotransferase
lmo2108	**9.88**	1.49	1.86	1.76		Similar to N-acetylglucosamine-6-phosphate deacetylase
lmo0971	**-6.8**	**-2.9**	**-5.0**	**-5.0**	*dltD*	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
lmo0972	**-6.1**	**-2.2**	**-3.3**	**-3.6**	*dltC*	D-alanyl carrier protein
lmo0973	**-6.5**	**-2.0**	**-2.8**	**-2.6**	*dltB*	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
lmo0974	**-10.1**	**-2.9**	**-4.4**	**-3.8**	*dltA*	D-alanine-activating enzyme (dae), D-alanine-D-alanyl carrier protein ligase (dcl)
lmo1420	**-2.2**	-1.2	-1.2	-1.3	*murB*	Similar to UDP-N-acetylglucosaminyl-3-enolpyruvate reductase
lmo2537	**-4.6**	-2.3	-2.6	-2.0		Similar to UDP-N-acetylglucosamine 2-epimerase
lmo1075	**-2.15**	-1.37	-1.45	-1.44		Similar to teichoic acid translocation ATP-binding protein TagH (ABC transporter)

Table 2. Continued.

Gene	Fold change at time (min)				Name	Product description
	3	10	20	40		
Cell wall associated						
lmo0576	**2.20**	1.11	1.28	1.20		Hypothetical cell wall associated protein
lmo0610	**6.17**	1.33	1.12	-1.13		Similar to internalin proteins, (LPXTG motif)
lmo0880	**8.15**	1.43	1.37	-1.13		Similar to wall associated protein precursor (LPXTG motif)
lmo1413	1.63	1.33	**2.07**	1.91		Putative peptidoglycan bound protein (LPXTG motif)
lmo1666	**2.13**	1.34	1.07	-1.16		Peptidoglycan bound protein (LPXTG motif)
lmo2027	**2.31**	1.44	**2.05**	1.74		Putative cell surface protein, similar to internalin proteins
lmo2085	**10.8**	**2.38**	**2.02**	1.43		Putative peptidoglycan bound protein (LPXTG motif)
lmo2504	**-4.4**	**-2.1**	-1.5	-1.5		Similar to cell wall binding proteins
lmo2522	**-5.91**	-1.42	-1.30	1.21		Similar to hypothetical cell wall binding protein from *B. subtilis*
PrfA regulated genes						
lmo0200	**6.63**	**4.29**	**5.06**	**4.35**	*prfA*	Listeriolysin positive regulatory protein
lmo0201	1.74	1.25	**2.13**	**3.86**	*plcA; pic*	Phosphatidylinositol-specific phospholipase C
lmo0433	**3.88**	1.07	1.09	-1.1	*inlA*	Internalin A
lmo0434	**2.30**	1.82	1.61	1.43	*inlB*	Internalin B
agr operon						
lmo0048	**-31.6**	**-3.2**	**-6.3**	**-6.7**		Similar to AgrB
lmo0049	**-19.4**	**-3.8**	**-5.8**	**-8.1**		Similar to two-component protein AgrD
lmo0050	**-11.8**	**-2.4**	**-3.7**	**-3.8**		Similar to two-component sensor histidine kinase AgrC
lmo0051	**-8.9**	-1.9	**-3.3**	**-3.5**		Similar to 2-component response regulator protein AgrA

* The significant values of the genes (q-value ≤ 1 % and fold change ≥ 2) are given in bold.

protease does not belong to the class III heat-shock genes but to a general group of stress genes. Helmann *et al.* (14) showed 5-fold induction for two *htrA* paralogues in *B. subtilis* as well, and designated this general class of heat-shock genes class U.

The class II stress response represents a general stress response mechanism that is regulated by the alternative sigma factor SigB. The gene encoding SigB (lmo0895) did not show ≥ 2-fold differential expression on the micro-arrays during the heat-shock. Verification of *sigB* expression using q-PCR showed 1.5-fold up-regulation after 3 and 10 min (Fig. 2). In total, 51 genes previously identified as being SigB regulated (22) showed increased expression at one or more of the selected time-points. Among these up-regulated genes were *ctc* (lmo0211), *lmo1601* and *ydaG* (lmo2748). These genes encode proteins that show sequence similarity with general stress proteins. Increased expression was also observed for the SigB-regulated *opuC* operon (lmo1425 to lmo1428). This operon encodes a betaine/carnitine/choline ABC transporter and has previously been shown to be responsible for the accumulation of osmolytes in response to salt, acid and cold stress (41, 44). Notably, these osmolytes have been shown also to provide protection during heat exposure of *B. subtilis* (16) and *Escherichia coli* (4), though no increase in their intracellular concentration was observed.

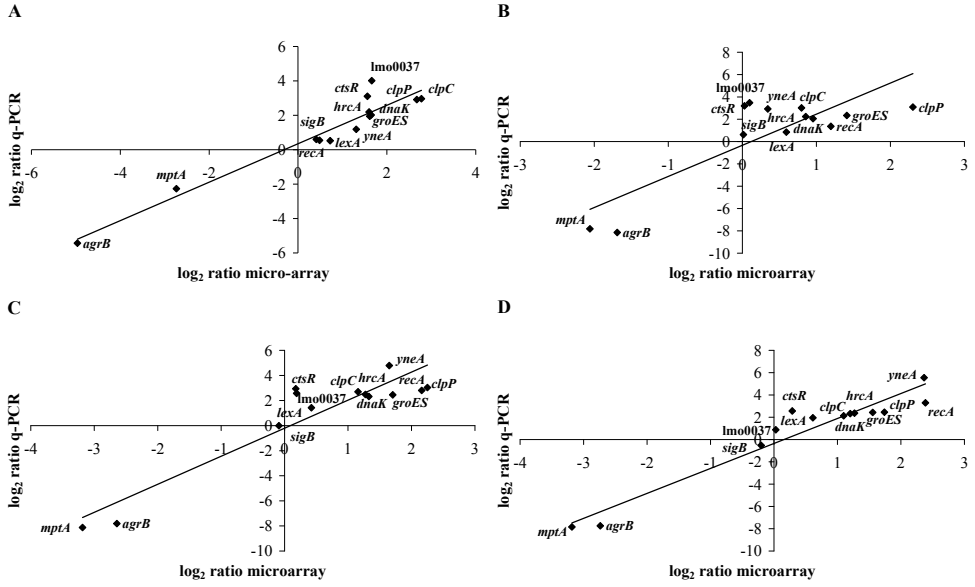

Fig. 2. Validation of micro-array data with quantitative real-time PCR analysis. A, Comparison of differentially expressed genes after 3 min. B, Comparison of differentially expressed genes after 10 min. C, Comparison of differentially expressed genes after 20 min. D, Comparison of differentially expressed genes after 40 min.

SOS response and DNA repair

The SOS response is a mechanism involved in the repair of DNA damage and restart of stalled replication forks (30) or in the introduction of adaptive point mutations (32, 33). Numerous genes that are part of the SOS response and the DNA repair machinery showed increased expression after heat exposure. The gene *recA* (lmo1398), encoding the major activator of the SOS response RecA, showed gradual increase in expression over time, from 1.4-fold to 5.2-fold induction between 3 and 40 min. Differential expression of *lexA* (lmo1302), the repressor of the SOS response, was not observed using micro-arrays, but q-PCR showed a change from 1.9-fold repression at 3 min to 2.0-fold induction at 40 min (Fig. 2). Transcription of *dinB* (lmo1975), and *umuDC* (lmo2675 and lmo2676), coding for DNA polymerases IV and V, respectively, was also up-regulated. These gene products constitute important alternative polymerases in the SOS response. The genes encoding DNA repair proteins *radA* (lmo0233), *recN* (lmo1368), *uvrA* (lmo2488), and *uvrB* (lmo2489), showed increased expression as well. Two other genes belonging to the SOS response were induced, namely *ynzC* (lmo0496) and *yneA* (lmo1303). The latter is transcribed divergently from *lexA* and the product has been shown to be responsible for cell elongation and suppression of cell division (21). Thus, the expression of *yneA* is consistent with the observation that exposure of *L. monocytogenes* to elevated temperatures resulted in elongated cells (Fig. 3). After heat exposure of cells growing at 37 ºC to 48 ºC the OD_{600} continued to increase (Fig. 3A) whereas the concentration of cells (cfu/ml) was found to remain constant (Fig. 3B). Cell size measurements of microscopic images showed increased cell size in time after heat exposure compared with cells that were grown continuously at 37 ºC (Fig. 3C to 3E).

Cell division

Cell division is a complicated mechanism involving different pathways that are intertwined. The expression of two genes, lmo2687 and lmo2688, encoding proteins that show sequence similarity to the cell division protein FtsW, was induced after 3 min exposure to 48 ºC. Ten other genes involved in cell division showed reduced expression mainly in the initial phase of the heat-shock. Repression of the genes lmo1071, *minDE* (lmo1544 and lmo1545), *mreDCB* (lmo1546 to lmo1548), *divIVA* (lmo2020), and *pbpB* (lmo2039), encoding proteins similar to RodA and FtsW, cell division inhibitors (septum placement), cell shape determining proteins, cell division initiation protein (septum placement), and cell division protein FtsI (penicillin-binding protein 3) respectively, was observed only after 3 min heat-shock. Transcription of the genes *ftsX* (lmo2506) and *ftsE* (lmo2507), whose products are highly similar to cell division protein FtsX and cell division

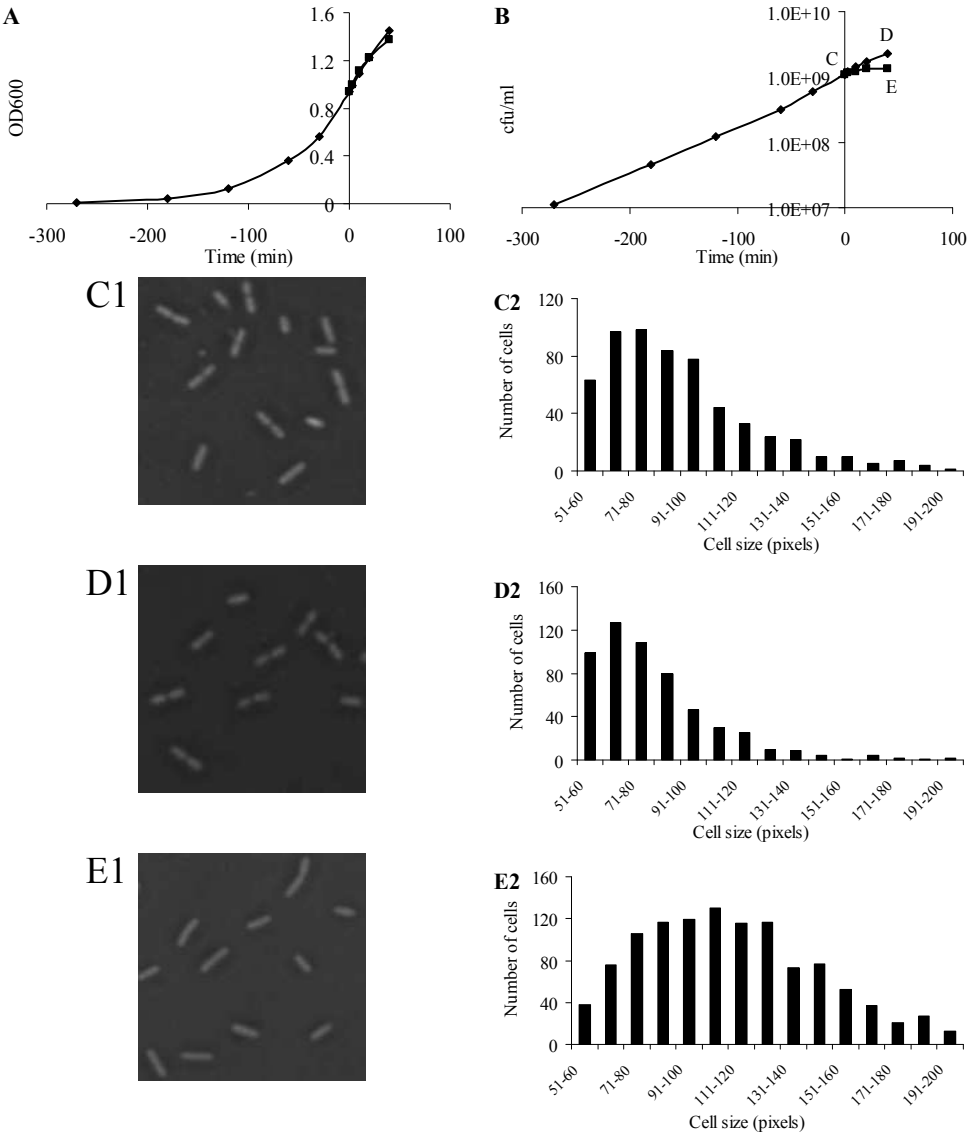

Fig. 3. Increasing cell size of *L. monocytogenes* cells exposed to 48 ºC in comparison with cells continuously grown at 37 ºC. The optical density (A) and viability count (B) were measured of a *L. monocytogenes* culture grown at 37 ºC (Diamonds). At time-zero (point C) the culture was transferred to 48 ºC (squares) or continuously grown at 37 ºC (diamonds). The cultures were incubated for 40 min and samples were taken from the culture at 37 ºC (point D) and from the culture at 48 ºC (point E). C1, D1, and E1; Microscopic images of the cells collected from points C, D, and E. C2, D2, and E2; Analyses of the microscopic images of cells from point C, D, and E using ImageJ. The graphs show the distribution of cell sizes in number of pixels per cell.

ATP-binding protein FtsE, was transiently down-regulated for 10 and 20 min after heat exposure, respectively.

Cell wall-associated genes

The bacterial cell wall is a complex structure and plays an important role as the first protection against environmental stresses. Cell wall hydrolases (including autolysins) play important roles in numerous cellular processes including cell division and cell wall turnover. In line with the results described above, all genes encoding cell wall hydrolases, namely *aut* (lmo1076), lmo1216, lmo1855, *spl* (lmo2505), *ami* (lmo2558), *murA* (lmo2691), and lmo2754, showed a transient repressed expression pattern, except for *iap* (lmo0582), which showed a constant 3-fold repression. The intermediate glucosamine-6-phosphate plays a central role in the regulation of cell wall biosynthesis versus glycolysis (24). Genes controlling the regulation between these two pathways showed induction after heat exposure. These genes are lmo0877 and lmo0957, of which the encoded products show sequence similarity to glucosamine-6-phosphate isomerases, lmo0957 and lmo2108, which encode putative N-acetylglucosamine-6-phosphate deacetylases, and lmo1998 and lmo1999, which encode products that show low sequence similarity to glucosamine-fructose-6-phosphate aminotransferases. The essential gene in the biosynthetic pathway, *murB* (lmo1420), was down-regulation after 3 min of heat-shock. Another operon that plays a role in cell wall synthesis and showed decreased expression upon heat-shock was the *dlt* operon (lmo0971 to lmo0974), encoding proteins important in catalysing the incorporation of D-alanine residues into lipoteichoic acids and wall teichoic acids. Furthermore, the gene lmo1075, encoding a protein with 64% sequence similarity to *B. subtilis* teichoic acid tra nslocation ATP-binding protein TagH, was also down-regulated after 3 min of heat-shock. Nine genes encoding putative cell wall-associated proteins were differentially expressed during heat exposure. Five of these genes encode products containing an LPXTG peptidoglycan-binding motif, namely lmo0610, lmo0880, lmo1413, lmo1666, and lmo2085.

Virulence-associated genes

A constant induction was observed for *prfA* (lmo0200) encoding the major regulator of the virulence genes. This gene is both SigB and SigA regulated and its product induces genes containing a PrfA box in their promoter sequence. Accordingly, numerous PrfA-regulated virulence genes (34) showed up-regulation after heat exposure, including *plcA* (lmo0201), encoding a phospholipase, *inlA* and *inlB* (lmo0433 and lmo0434), encoding *Listeria*-specific internalins, lmo2067, encoding a conjugated bile acid hydrolase, and lmo0596, encoding an unknown protein. However, Rauch *et al.* (37) showed that PrfA had no direct influence on the transcription of lmo2067 and lmo0596. Significant down-

regulation was found for the *agr* locus (lmo0048 to lmo0051). This locus encodes a two-component system that has been shown to play a role in bacterial virulence (2).

Validation of micro-array gene expression

The micro-array data was validated by quantitative real-time PCR analysis using a set of 13 genes which cover a range of expression values in the micro-array data. The relative gene expression levels obtained by the quantitative real-time PCR were normalized to that of 3 genes (*tpi*, *rpoB*, *16S rRNA*) that did not fluctuate during temperature changes. Relative quantitative values were obtained using the comparative threshold cycle method ($\Delta\Delta C_T$) in which the C_T value corresponds to the cycle at which the fluorescent signal crosses the threshold line. The relative expression of the genes was determined in quadruplicate for three independent temperature up-shift experiments. The resulting ratios were $\log_2$ transformed and plotted against the $\log_2$ values from the micro-array analysis. Figure 2 shows a strong correlation with $r>0.8$ for all time-points, which is considered the threshold for strong correlation. Generally, the micro-array analysis underestimated the differential expression values by an average of 5-fold. Similar observations have been reported previously when using orf arrays in microbial transcriptome analysis (10, 14, 43).

Discussion

Variation in temperature is frequently encountered in nature and bacteria have evolved to cope with such fluctuations by employing various mechanisms. Micro-arrays were used to investigate the whole-genome expression profiles of *L. monocytogenes* in response to a temperature up-shift from 37 ºC to 48 ºC over a 40 min period. Our data show differential expression for genes involved in different cellular processes, including SOS response, cell division, and specific (heat) stress responses. For many differentially expressed genes a transient expression pattern was observed between 3 and 40 min. The highest number of differentially expressed genes was observed 3 min after temperature upshift. Whole-genome expression profiles in response to heat shock have been reported for *Bacillus subtilis*, *Shewanella oneidensis*, and *Campylobacter jejuni* (10, 14, 43). These studies also showed transient expression patterns, albeit that peak numbers of differentially expressed genes were observed later in time upon heat exposure. The ability of *L. monocytogenes* to rapidly alter the transcript levels allows for a quick response to sudden environmental changes.

In *L. monocytogenes*, genes belonging to the class I and class III heat-shock regulons showed increased expression during heat exposure. Interestingly, the class I heat-shock genes showed a constant induction in expression levels over the 40 min period, whereas the

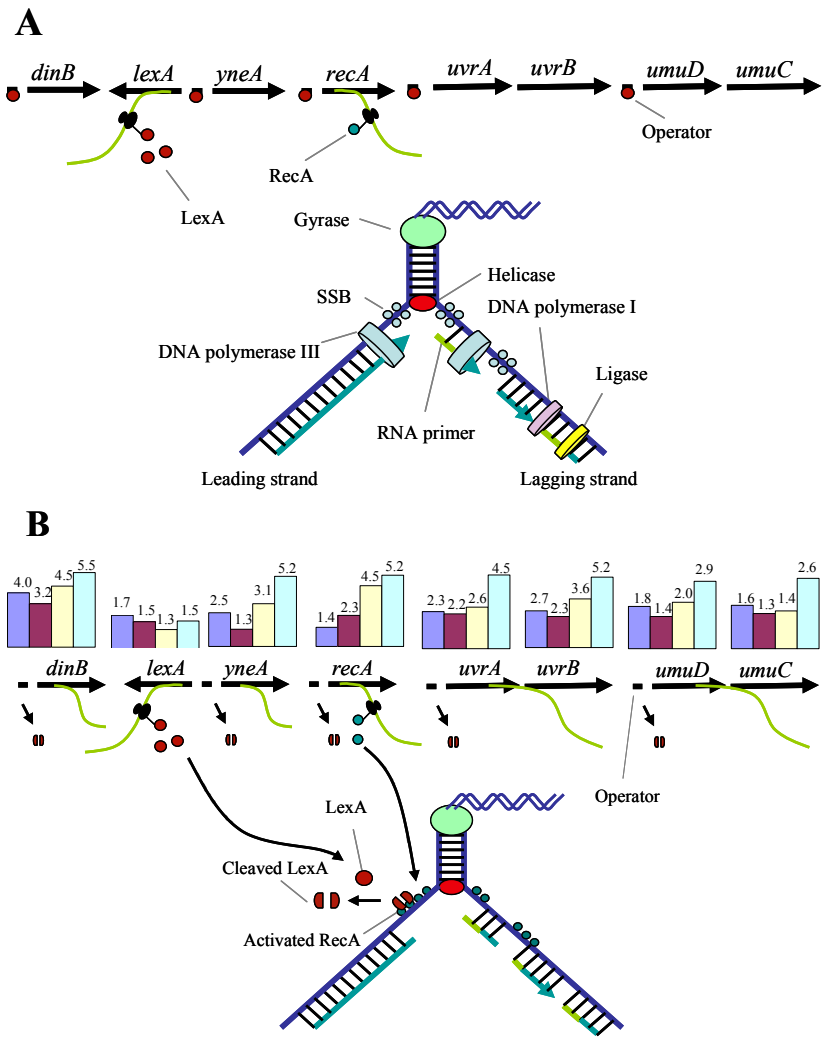

Fig. 4. Activation of the SOS response of *L. monocytogenes* during exposure to elevated temperatures. A. In the cells with an active replication fork (before exposure), LexA represses the transcription of *lexA*, *recA*, and the other genes of the SOS response (*dinB*, *yneA*, *uvrAB* and *umuDC*) by binding to the operator in the promoter region. B. When the replication fork is stalling after exposure to elevated temperatures, RecA is activated by the resulting ssDNA. Activated RecA promotes cleavage of LexA, and consequently the SOS response genes are induced. The genes of the SOS response are illustrated schematically and are not to scale. The induction of the genes after heat-shock is represented by the graphs above each gene (3 min, blue; 10 min, purple; 20 min, yellow; 40 min, green).

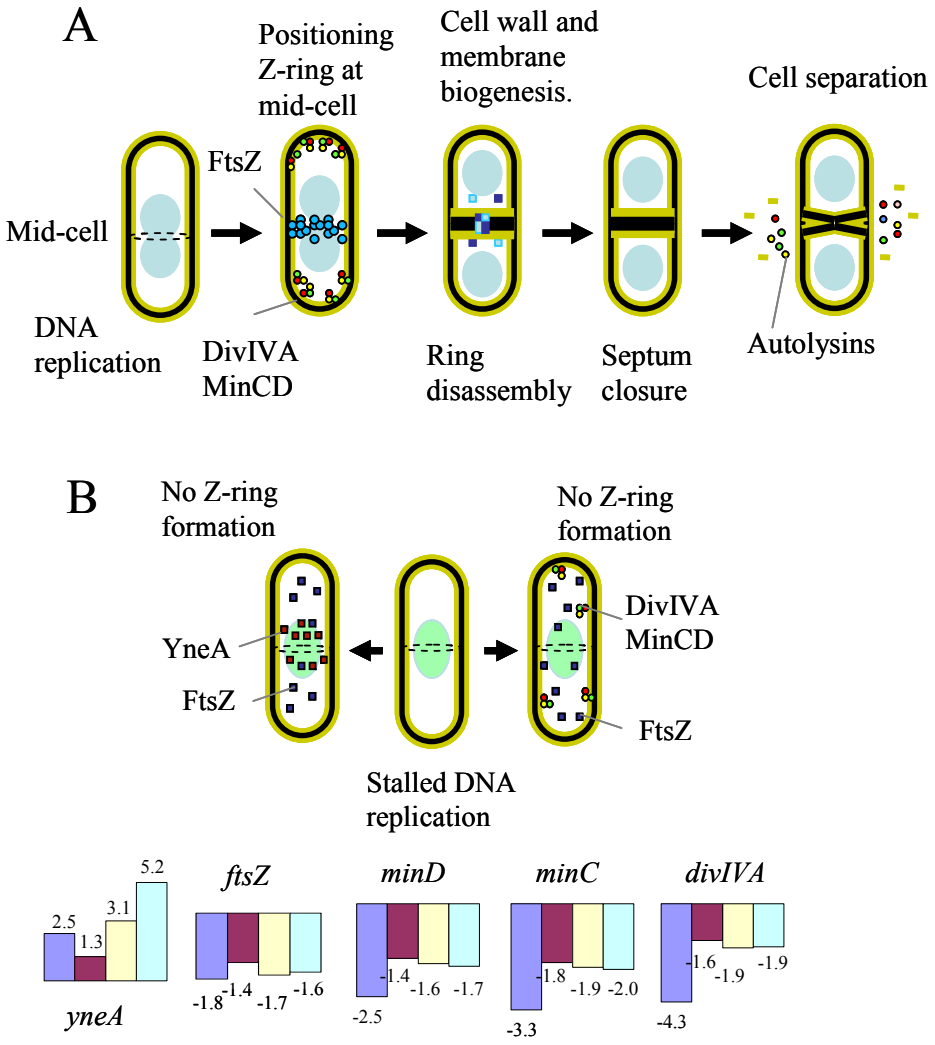

Fig. 5. Nucleoid occlusion. A. Normal replicating cells. B. Stalling DNA replication results in perturbation of cell division. Two possible explanations: 1, An activated SOS response results in increased numbers of YneA at the nucleoid in the mid-cell. YneA reduces the concentration of FtsZ, preventing septum formation. 2, Reduction in numbers of MinCD and DivIVA prevents the concentration of FtsZ at the mid-cell by allowing FtsZ at the poles. Differential expression of the genes after heat-shock is represented by the graphs (3 min, blue; 10 min, purple; 20 min, yellow; 40 min, green).

class III heat-shock genes showed a transient expression pattern. This is likely related to the different roles the two classes fulfil during heat-shock. The Clp-ases are specifically required at the early stages of the heat-shock to remove the initially damaged and misfolded proteins. Following adaptation to heat, Clp-ase levels are reduced while higher levels of chaperones are present to prevent accumulation of damaged and misfolded proteins. Q-PCR showed 1.5 fold induction of the gene encoding SigB after 3 and 10 min. Considering the role for SigB as a regulator, this slight increase in expression appears to be enough to induce genes containing a SigB promoter as evidenced by the transient nature of the class II stress response. Whole genome expression profiles upon heat shock have been reported for a number of bacteria, namely, *B. subtilis*, *C. jejuni*, and *S. oneidensis* (7, 10, 14, 43). When present in these bacteria, genes belonging to the class I and class III heat-shock response, and SigB regulated class II stress response were induced. Given the genetic resemblance and similar heat-shock conditions applied, a direct comparison of up-regulated genes of *L. monocytogenes* and *B. subtilis* (14) was possible. A total of 25 orthologues of the 130 induced class II stress genes in *B. subtilis* showed induction in *L. monocytogenes* as well. Of these 25 genes, 8 genes were previously indentified in *L. monocytogenes* to be SigB regulated (22). In *B. subtilis*, 76 other genes, designated class U, showed increased expression upon heat-shock. In *L. monocytogenes*, 9 orthologues of these genes showed induction, among which was *htrA*. Previous research showed that HtrA was important for survival during exposure to environmental and cellular stresses (42, 45).

In *L. monocytogenes*, several genes that play a role in the SOS response and DNA repair machinery showed increased expression after heat exposure, including *recA*, which is the major activator of the SOS response. RecA is involved in various processing steps of DNA replication proteins and repair proteins, and plays a role in the restart of stalled replication forks (8), homologous recombination, repair of double-stranded DNA breaks (29), and introducing adaptive point mutations (31). Furthermore, it stimulates cleavage of the LexA repressor of the SOS response, activating expression of the SOS response genes (Fig. 4). The LexA repressor is auto-regulatory, and binds to the operator sequence in the promoter region of the SOS response genes (CGAACATATGTTCG in *B. subtilis* (1)). Several lexA-regulated genes showed increased expression in *L. monocytogenes,* including genes encoding the two alternative DNA polymerases *umuDC* and *dinB*. DNA polymerases Pol IV (DinB) and Pol V ($UmuD_2$'C) have previously been shown to respond to inhibition of replication fork progression in a damage-independent manner (12, 13). Elevated temperatures may cause the *L. monocytogenes* DNA replication fork to stall, leading to exposure and possible degradation of the arrested replication fork without rescue by Pol IV and Pol V. This theory is consistent with the observation that heat-shock resulted in cell elongation and prevention of cell division. This effect is called "nucleoid occlusion", and is generally interpreted as prevention of Z-ring formation in the vicinity of the nucleoid,

thereby preventing cell division without complete replication and segregation of the nucleoid (38). The expression profiles showed two possible mechanisms that might lead to prevention of Z-ring formation after heat-shock (Fig. 5). The first mechanism would be the result of increased expression of *yneA* (as part of the SOS response), which is responsible for suppression of cell division in *B. subtilis* by reducing FtsZ proteins at the cell division site, thereby preventing FtsZ ring formation (21). The second mechanism is the result of a decreased expression of the genes *minDE*, and *divIVA*, which are involved in spatial correct septum placement. The MinCD complex negatively regulates formation of the cytokinetic Z ring to midcell by preventing its formation near the poles (46). Another strategy of *L. monocytogenes* to suppress cell division might be to decrease expression of the genes *fteX* and *ftsE*. These genes encode proteins with sequence similarity to ABC transporters and are localized at the division site. In *E. coli* they are directly involved in cell division and are important for septal ring formation (39). Mutants for *ftsXE* constitutively showed induction of the SOS response in *E. coli* (36).

A possible strategy of the *L. monocytogenes* cells to prevent continuous elongation after suppression of cell division is to reduce the expression of genes encoding proteins involved in cell wall biosynthesis and turnover. The down-regulated genes *rodA* and *mreDCB* encode proteins involved in cell wall synthesis and determination of cell shape. The MreDCB complex forms actin-like cables beneath the cell surface and requires RodA for control of cell shape (26). Research in *B. subtilis* showed that cells depleted of RodA were impaired in their cell division (15). Reduced expression levels were also observed for the genes encoding autolysins, *murB*, which is an essential gene in the biosynthetic pathway of peptidoglycan, and the *dlt* operon, which encodes products that catalyse incorporation of D-alanine residues into teichoic acids. While most bacteria showed slower growth rates after mutations in the *dlt* operon, *Streptococcus gordonii* showed abnormal septation and defective cell separation (35).

PrfA and various other virulence genes showed induction upon heat exposure, which has not been reported before. PrfA is the major virulence regulator in *L. monocytogenes* (5, 28). Previous studies showed maximal induction of the virulence genes at 37 ºC (18, 27). However, expression levels at temperatures above 37 ºC were not measured in these studies. Destabilizing mutations in the secondary structure of the untranslated region of *prfA* mRNA resulted in increased levels of PrfA at 37 ºC (18), indicating that this structure is not completely unfolded at this temperature. Increasing the temperature above 37 ºC probably results in a more relaxed secondary structure allowing more PrfA translation.

A comparative analysis of the transcription profiles of *L. monocytogenes* during heat-shock and in the intracellular environment (6) showed a high number of genes that were differentially expressed during both exposures. Of the 714 differentially expressed genes during heat-shock, 236 genes showed differential expression in the intracellular

environment as well. Among genes that were commonly regulated during both exposures, 172 genes showed increased expression and 64 genes showed decreased expression. Exposure of *L. monocytogenes* to the intracellular environment triggered numerous (heat) stress responses that were shown to be differentially expressed during heat-shock in this study. All genes belonging to the class I and class III heat-shock genes and 24 of the SigB-regulated class II stress response genes showed induction in the intracellular environment. Remarkably, similar expression profiles for the SOS response genes, the cell division genes, and the cell wall related genes were observed. This comparative analysis showed that numerous responses induced during heat exposure contribute to the pathogenicity of *L. monocytogenes* by allowing survival during the stressful conditions encountered intracellularly and during passage through the stomach and the intestinal tract.

This study shows the importance of the SOS response as a common protection mechanism during heat-shock and its possible role in suppression of cell division to prevent transection of the genome as a result of incomplete chromosomal segregation. Whether replication fork stalling is the actual cause leading to both the SOS response and suppression of cell division remains to be elucidated. This opens up interesting possibilities for future research. Our transcriptome analysis has revealed several aspects of the heat-shock response of *L. monocytogenes* that may include targets for inactivation. The role of specific factors such as the SOS response in *L. monocytogenes* stress survival and virulence will be assessed in future studies.

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5. Genome-wide screen for *Listeria monocytogenes* genes important for growth at high temperatures

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Abstract

Listeria monocytogenes is a Gram-positive food-borne pathogen that is able grow over a wide temperature range. While the class I and class III heat-shock genes are known to play an important role in heat-shock, information on genes that are essential for growth at high temperatures is scarce. To determine which genes are important for growth at high temperatures (42.5-43 ºC), we performed a random insertion screen in *L. monocytogenes*, rendering 28 temperature sensitive mutants. These mutants showed insertions in genes that play a role in transcription regulation, cell wall-biosynthesis, cell division, translation, transport, sensing, and specific stress responses like the SOS response and the class III heat-shock response. Some of these mutants showed altered morphological characteristics such as cell elongation, reduced cell length, or sickle-shapes. Furthermore, the majority of the mutants showed increased heat-inactivation after exposure to 55 ºC compared with the wild-type strain. The role of the specific genes in relation to growth at high temperatures is discussed.

Introduction

The Gram-positive food-borne pathogen *Listeria monocytogenes* is the causative agent of listeriosis. In many European countries the incidence of listeriosis cases is increasing (8, 18), and in the United States it has been estimated that 28% of all deaths due to known food-borne pathogens are caused by *L. monocytogenes* (25). This bacterium has the ability to survive a range of stresses related to food processing and is able to grow over a wide temperature range, spanning from -0.3 ºC to 46 ºC (16, 35, 36). The persistence of this pathogen in the food-chain is leading to rising concerns (15).

The response of *L. monocytogenes* to temperature stress has so far been assessed for growth at low temperatures (10 ºC and below) (33) and exposure to heat-shock (32, 34). Only limited information is available on the capacity of this organism to grow at high temperatures (44 ºC and above) (35) and it is not known which genes are important for growth at high temperatures. To identify such genes and to understand the molecular mechanisms involved in growth at high temperatures, a random insertion mutant library was created and screened for mutants with growth deficiency at 42.5 ºC on Brain Hearth Infusion (BHI; Difco) agar plates, which is the highest temperature that is not growth limiting for the *L. monocytogenes* EGD-e wild-type strain (13) on BHI agar. These mutants were subsequently investigated for heat-resistance, and altered morphology or growth characteristics in BHI broth.

Materials and Methods

The mutagenesis library was constructed in strain EGD-e using the temperature-sensitive plasmid pGh9:ISS1 (22). *L. monocytogenes* EGD-e was transformed with plasmid pGH9:ISS1 using the protocol described by Park and Stewart (29). Transposition of the plasmid into the bacterial genome was performed using the method described by Maguin et al. (22). The growth of approximately 6500 mutants was assessed at both 42.5 ºC and 37 ºC on BHI agar plates. Mutants that showed a deficiency in colony formation at 42.5 ºC compared to 37 ºC were selected. A semi-quantitative sensitivity analysis on these temperature sensitive mutants was performed in triplicate using a dilution assay as described by Frees et al. (12). In this assay, 10 ul drops of a 10-fold serial dilution of cultures grown overnight at 37 °C were spotted on BHI agar plates and incubated at 42.5 ºC and 37 ºC for 48 h. The vector insertion site for these mutants was determined by inverse PCR on HindIII digested circularized DNA using primers ISS1-fwd (5'-CATTGATATATCCTCGCTGTC-3') and ISS1-rev (5'-CTTAATGGGAATATTAGCTTAAG-3'). The growth of the mutants in BHI broth was followed in two independent experiments using two replicates in 96-well flat bottom tissue

culture plates (Greiner) using the EL808 IU-PC (Biotek) at 630 nm. The morphology of the mutants, suspended in nigrosin solution (Sigma) and dried on glass slides, was investigated at 100 x magnification using a Dialux 20 microscope (Leica). To determine the heat resistance of the different mutant strains and the wild-type strain, 100 µl of exponential phase cultures (OD_{600} 0.3-0.8), grown at 37 ºC in BHI broth at 200 rpm, were pelleted (2 min, 5000 x g) and suspended in 100 µl PBS in 1.5 ml Eppendorf tubes. The tubes were placed in a water bath at 55 ºC. Inactivation was determined after 20 min heat exposure. Samples were taken and appropriate dilutions were plated on BHI agar. The plates were incubated at 30 ºC for 3-5 days and colonies were enumerated. The experiment was performed in triplicate.

Table 1. Genetic location of the vector insertion for the temperature sensitive mutants and the resulting growth reduction at 42.5 ºC. Entries in the growth reduction include fold changes.

Vector integration site			**Description of product**	**Growth reduction[b]**
Gene	**Length[a]**	**Site[a]**		
lmo0197	309	-33[c]	Stage V sporulation protein G (SpoVG)	10^2
lmo0220	2076	1782	ATP-dependent metalloprotease FtsH	10^5
lmo0590	1767	704	Similar to a fusion of two types of conserved hypothetical proteins	10^2
lmo0674	921	216	Motility gene repressor (MogR)	10^4
lmo0776	867	563	Similar to transcription regulator (repressor)	10^1
lmo0971	1275	746	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid	10^3
lmo0986	912	62	Similar to antibiotic ABC transporter, ATP-binding protein	10^4
lmo1010	879	22	Similar to transcription regulator (LysR family)	10^3
lmo1327	345	7	Highly similar to ribosome-binding factor A (RbfA)	10^1
lmo1333	480	439	Similar to B. subtilis YqzC protein	10^1
lmo1355	558	302	Highly similar to elongation factor P (EFP)	10^5
lmo1420	897	890	UDP-N-acetylmuramate dehydrogenase (MurB)	10^3
lmo1487	576	90	Similar to unknown proteins	10^4
lmo1495	624	38	hypothetical protein	10^2
lmo1619	870	157	D-amino acid aminotransferase (DaaA)	10^4
lmo1672	1410	146	Similar to O-succinylbenzoic acid-CoA ligase (MenE)	10^1
lmo1741	1041	325	Similar to two-component sensor histidine kinase (VirS)	10^2
lmo1746	1980	1565	Similar to ABC transporter (permease)	10^1

Table 1. Continued.

Vector integration site			Description of product	Growth reduction[b]
Gene	**Length[a]**	**Site[a]**		
lmo2045	387	106	Hypothetical protein	10^2
lmo2206	2601	814	Similar to endopeptidase Clp ATP-binding chain B (ClpB)	10^1
lmo2267	3708	3311	Similar to ATP-dependent deoxyribonuclease (AddA)	10^4
lmo2302	540	277	Hypothetical protein	10^5
lmo2473	969	173	Conserved hypothetical protein	10^3
lmo2520	1125	842	Similar to *B. subtilis* O-succinylbenzoate-CoA synthase (MenC)	10^1
lmo2598	747	639	Pseudouridylate synthase (TruA)	10^1
lmo2694	1380	338	Similar to lysine decarboxylase	10^2
lmo2754	1338	63	Similar to D-alanyl-D-alanine carboxypeptidase (PBP5)	10^1
lmo2810	1890	1780	Highly similar glucose inhibited division protein A (GidA)	10^3

[a] Base pairs (bp)

[b] Growth reduction at 42.5 ºC compared to growth at 37 ºC, as determined by the dilution essay

[c] Vector integrated in promoter region 33 base pairs upstream of the start codon

Results and Discussion

Screening of the mutant library rendered 28 temperature sensitive mutants of which colony formation was reduced on BHI agar at 42.5 ºC compared with 37 ºC. Southern hybridization analyses using a probe for the erythromycin resistance gene of vector pGH9:ISS1 confirmed that the vector was integrated at a single location (results not shown). Genetic analysis revealed that the temperature sensitive mutants contain insertions in genes encoding transcriptional regulators (3 mutants) and in genes involved in cell wall biosynthesis (5 mutants), cell division (1 mutant), translation (4 mutants), transport (2 mutants), sensing (1 mutant), and specific stress responses (3 mutants). Table 1 shows the genetic location of the vector insertion for the different mutants and the resulting growth deficiency at 42.5 ºC as determined by the dilution essay.

Growth at 37 ºC in BHI broth showed comparable growth rates or maximum cell density reached for the wild-type and insertion mutant strains (results not shown). At 43 ºC, for the majority of the mutants differences were observed for the maximum growth rate or the maximum cell density reached in BHI broth compared with the wild-type strain (Fig. 1).

Five mutants showed a reduced maximum growth rate, while for two mutants the maximum growth rate was increased (Fig. 1A). In total, 20 mutants showed reduced maximum cell densities and, surprisingly, five mutants showed increased cell densities (Fig. 1B). Several mutants showed morphological characteristics that were different from the wild-type strain at high temperatures, such as cell elongation (Fig. 2). These altered morphologies were also observed at 37 ºC, albeit in a milder form in some cases.

To investigate a potential role for the identified genes in heat-resistance, the mutants were tested for differences in heat-inactivation. Several mutants showed decreased or increased resistance to heat-inactivation at 55 ºC compared with the wild-type strain (Fig. 3). The specific genes belonging to different functional groups will be discussed below in relation to their putative role in growth at high temperatures.

Three mutants with reduced growth at high temperatures showed insertions in genes encoding for transcription regulators (*mogR* [lmo0674], *lmo0776*, and *lmo1010*). It is unknown which regulons are controlled by lmo0776 and lmo1010. MogR represses the temperature dependent expression of the motility genes (31). The sensitivity for growth at temperatures above the optimum temperature might be related to de-repression of the motility genes in the *mogR* mutant and overproduction of the motility-associated proteins at 37 ºC and above. Since *mogR* expression is transiently induced during heat-shock in *L. monocytogenes* (34), another possible explanation is that MogR regulates genes other than the motility genes with an important function at high temperatures. Interestingly, the *mogR* mutant showed increased resistance to heat-inactivation compared to the wild-type strain (Fig. 3).

Five other high temperature-sensitive mutants contained insertions in genes encoding for proteins with a role in the cell wall synthesis, namely *spoVG* [lmo0197], *dltD* [lmo0971], *murB* [lmo1420], *daaA* [lmo1619], and *lmo2754*. Except for *dltD*, all of these mutants showed increased sensitivity to heat-inactivation compared to the wild-type strain (Fig. 3). SpoVG is involved in capsular polysaccharide (CP) production and capsule formation in *Staphylococcus aureus* through regulation of transcription of *capA*, encoding CapA, which is an important protein for CP production. Deletion of *spoVG* resulted in decreased *capA* expression and abolished capsule formation (26). A previous study in which a random mutant library in *L. monocytogenes* was screened for bile sensitive mutants resulted in a *capA* (lmo0516) mutant and the authors hypothesized that this gene could be involved in cell envelope biogenesis (3). In *L. monocytogenes*, an insertion in *spoVG* might also result in decreased expression of *capA*, thereby effecting cell envelope biogenesis.

DltD is important for incorporation of D-alanine esters into the cell wall teichoic acids. Proper cross-linking of teichoc acids in the cell wall is thus important for growth of *L. monocytogenes* at high temperatures on BHI agar (Table 1) and in BHI broth (Fig. 1).

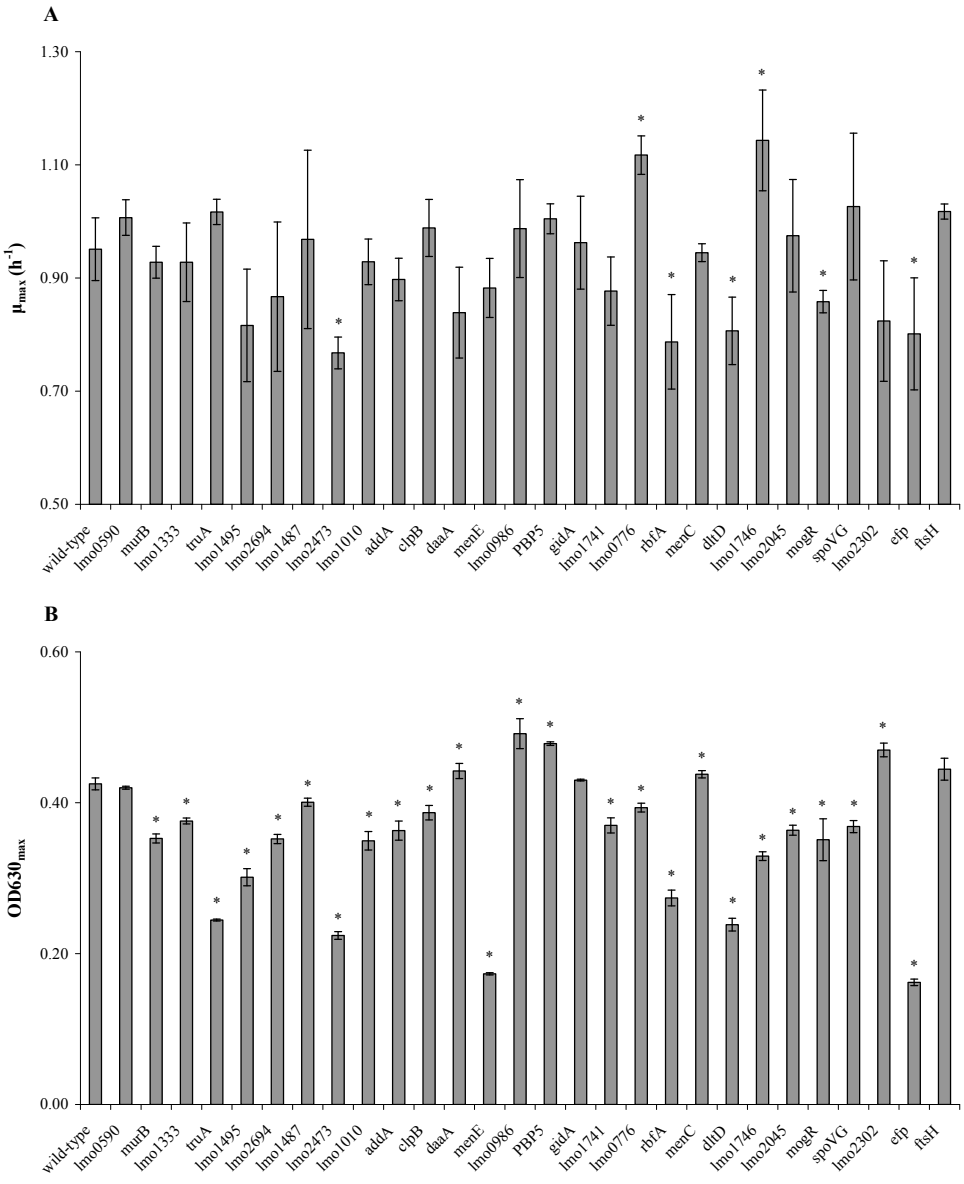

Fig. 1. Growth characteristics of the wild-type strain and its respective insertion mutants in BHI broth as determined by OD_{630} at 43 ºC. A) Maximum growth rate. B) Maximum cell density. Significant different values (t-test) compared with the wild-type strain are indicated by an asterisk.

The *dlt*-operon has also been shown to be important for stress resistance in other bacteria. A *dltD* mutant of *Lactococcus lactis* for instance shows UV-sensitivity (9), and a *dlt*-mutant of *Lactobacillus reuteri* shows impaired growth under acidic conditions (37). Furthermore, Abachin et al. (1) previously showed impaired virulence of a *dltA* mutant of *L. monocytogenes* in a mouse infection model, indicating that a properly formed teichoic acid network is also important for correct display of potential virulence traits allowing efficient colonization of the hosts.

Another gene required for growth at high temperatures is *murB*. MurB is involved in the biosynthesis of the peptidoglycan of the cell wall and is actually down-regulated during heat-shock in *L. monocytogenes* (34). Mutations in *murB* also resulted in temperature sensitivity of *S. aureus*; peptidoglycan synthesis was reduced and cell walls were thin at 43 ºC (24). The insertion mutant of *L. monocytogenes* also showed reduced growth at 43 ºC in BHI broth (Fig. 1) and a high number of elongated cells in cultures (Fig. 2), indicating that it might have a function during cell separation as well.

Inactivation of the gene *daaA* resulted in impaired growth at 42.5 ºC as well. DaaA is a putative D-amino acid aminotransferase, and in *Bacillus spaericus*, this enzyme plays a role in synthesis of cell wall precursors (11).

Lastly, Penicillin-Binding Protein V (PBP5), encoded by gene *lmo2754*, was found to be important for growth at high temperatures. As previously shown by Korsak et al. (19), *L. monocytogenes* Δ*lmo2754* mutants produce thickened cell walls with more cross-linking,

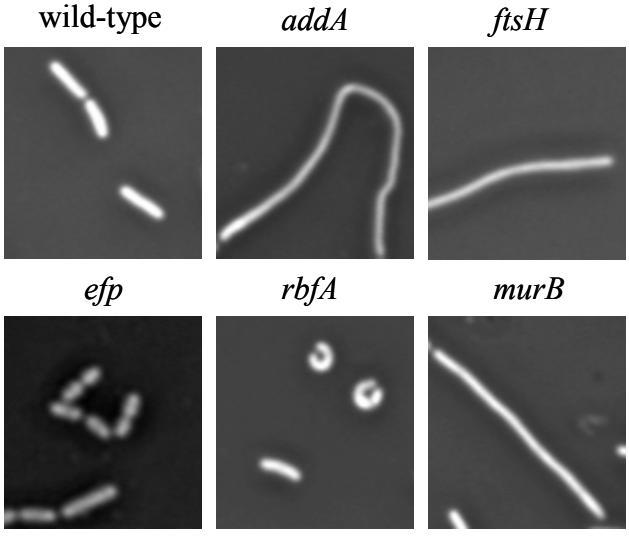

Fig. 2. Microscopic images of cells of the wild-type strain and its respective insertion mutants during exponential growth at 42.5 ºC.

and slightly slower growth at 37 ºC. PBP5 removes the terminal D-alanine residue from the murein penta-peptide side chains and thereby plays an important role in cell wall turnover during cell division (30).

Taken together, our results show that a properly formed cell wall is very important for bacteria to grow at high temperatures. Mutations in genes affecting cell wall formation, cross-linking of the peptidoglycan layer, and synthesis of teichoic acids resulted in abolished growth under these conditions, whereas growth at 37 ºC is not affected.

The ability to grow at 42.5 ºC on BHI agar was impaired profoundly in the mutant with an insertion in the gene encoding metalloprotease FtsH (lmo0220; Table 1), which has a function in cell division. Furthermore, the *ftsH* mutant showed increased sensitivity to heat-inactivation compared to the wild-type strain (Fig. 3). In *B. subtilis,* FtsH accumulates at the septum during cell division (38). *FtsH* expression is transiently induced after a temperature upshift in *B. subtilis* (6) and in *Caulobacter crescentus* (10), but overexpression did not result in increased thermotolerance in *C. crescentus*. In *B. subtilis*, Δ*ftsH* mutations resulted in filamentous growth due to over-expression and production of Penicillin-Binding Protein IV (PBP4) (44). In *L. monocytogenes* PBP4 catalyzes glycan polymerization and cross-linking (43). We found that the *L. monocytogenes ftsH* insertion

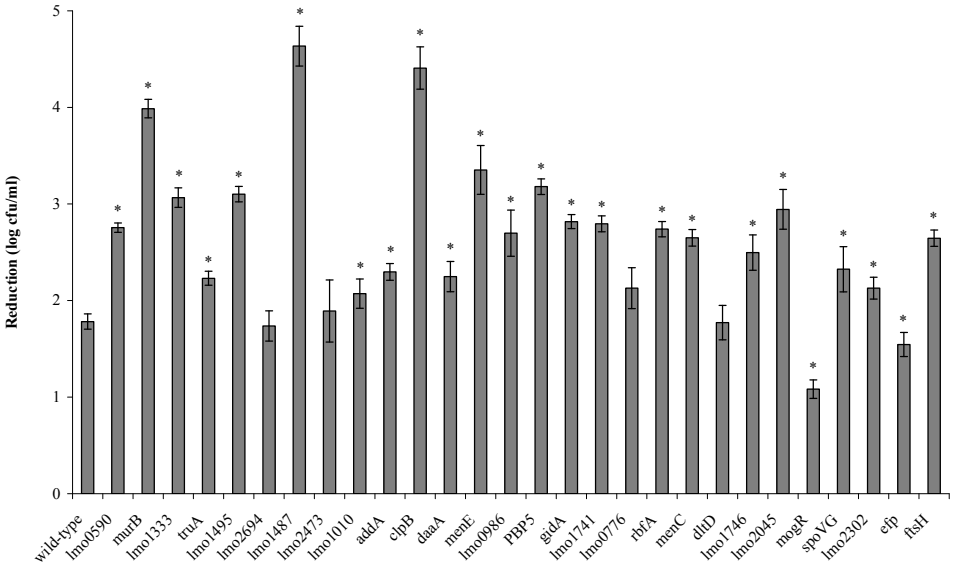

Fig. 3. Heat-inactivation of exponential phase cells of the wild-type and mutant strains after growth at 37 ºC. The graph shows the reduction in viable counts after 20 min exposure to 55 ºC. Significant different values (t-test) compared with the wild-type strain are indicated by an asterisk.

mutant indeed showed filamentation (Fig. 2). This shows that a properly functioning cell division system is very important at high temperatures. Where such defects are seemingly compensated at optimal temperatures, this ability is lost at elevated temperatures. Interestingly, the *ftsH* insertion mutant did not show reduced growth in BHI broth at 43 ºC compared to the wild-type strain (Fig. 1).

Four high temperature sensitive mutants showed insertions in genes encoding products involved in translation (*rbfA* [lmo1327], *efp* [lmo1355], *truA* [lmo2598], and *gidA* [lmo2810]). RbfA processes the part of 16S rRNA involved in mRNA decoding and tRNA binding (5), and it was demonstrated that this gene is essential for *Escherichia coli* to adapt to low temperatures (40). Our study shows that RbfA is also important for growth of *L. monocytogenes* at elevated temperatures on BHI agar (Table 1) and in BHI broth (Fig. 1). Furthermore, the *rbfA* insertion mutant showed a sickle shaped cell (Fig. 2), indicating that cell wall synthesis is influenced in this mutant. This altered cell shape might explain the observed increased sensitivity to heat-inactivation of this mutant compared to the wild-type strain (Fig. 3). Another ribosome related protein, EFP, influences the peptidyltransferase activity of 70S ribosomes. EFP genes are universally conserved in bacteria (20). In *E. coli*, EFP is essential for viability (2), while in *B. subtilis*, *efp* mutants only affected sporulation, but not growth (28). In our study, growth of the *efp* insertion mutant was not affected in BHI broth at 37 ºC, while at 43 ºC reduced growth was clearly shown (Fig. 1). Apparently, in *L. monocytogenes* EFP is not essential for viability, but growth at high temperatures is affected in *efp* mutants. It is noteworthy that microscopic images showed reduced cell length in this mutant (Fig. 2).

The proteins TruA and GidA are involved in modification of the uridines of tRNA (14, 42). A *gidA* mutant of *L. lactis* was shown to be UV-sensitive (9), while a *gidA* mutant of *Lactococcus garvieae* was unable to survive in a fish host (27). Our results showed that the *gidA* mutant was more sensitive to heat-inactivation compared to the wild-type strain (Fig. 3). These results show that *L. monocytogenes* is able to compensate for mutations affecting the translation machinery at 37 ºC, but fails to do so at above optimum temperatures. The ability to modify the ribosome and the uridines of tRNA seems important for proper functioning of the translation machinery at higher temperatures.

Two high temperature-sensitive mutants showed insertions in putative ABC-transporters (*lmo0986* and *lmo1746*). The expression of *lmo0986* was transiently down-regulated during heat-shock (34). The insertion in *lmo1746* probably resulted in polar effects on *virS* (lmo1741), which is located in the same operon. We also found a mutant with an insertion in *virS*, which rendered a high temperature sensitive phenotype. VirS is a histidine kinase and forms a two-component system with VirR (lmo1745) (39). Both the *dlt*-operon and the class I heat-shock response are down-regulated in a Δ*virS* mutant (23), which might explain the temperature sensitivity of these mutants.

Three mutants showed insertions in genes with a known stress related function. One of these temperature-sensitive mutants showed the insertion in the CtsR-regulated class III heat-shock gene *clpB* (lmo2206). This mutant showed 3 $\log_{10}$ higher reduction in cfu's after 20 min exposure to 55 ºC compared with the wild-type strain (Fig. 3). *ClpB* expression is induced during heat-shock in *L. monocytogenes* EGDe (34) and its product functions as a molecular chaperone, thereby protecting proteins during exposure to various stresses (21). For *L. monocytogenes* it was shown that ClpB is important for virulence as well (4).

Another temperature-sensitive mutant contained an insertion in a gene encoding a putative AddA type helicase (lmo2267), which is generally involved in processing of DNA breaks. Whole genome transcription profiling studies showed that this gene is transiently up-regulated after heat-shock (34). It contains a putative LexA binding site, suggesting it is part of the SOS-response (results not shown). Deletion of *addA* has been shown to result in sensitivity to DNA damaging agents and reduced viability in numerous bacteria (41). The *addA* insertion mutant showed increased numbers of elongated cells (Fig. 2), which might be the result of reduced capabilities for DNA repair during replication.

Notably, a gene encoding a putative lysine decarboxylase (lmo2694) was found to be important for high temperature growth. This gene was also transiently induced during heat-shock in *L. monocytogenes* (34). However, this mutant did not show increased sensitivity to heat-inactivation at 55 ºC compared with the wild-type strain (Fig. 3). Decarboxylase systems are usually involved in low pH and oxidative stress response (17). Various researchers have speculated that exposure to stress, including heat-stress, is associated with concomitant oxidative stress at a molecular level due to metabolic disorders (7). Decarboxylase systems are involved in superoxide radical scavenging, thereby preventing DNA damage (17).

Taken together, these results show that growth at high temperatures requires adequate mechanisms to counteract effects of protein instability and DNA damage. In *L. monocytogenes*, these systems include e.g. the heat-shock response, the SOS-response, and specific stress responses.

In short, we identified a number of genes of *L. monocytogenes* that are important for growth at high temperatures. Some belong to specific stress responses (e.g. heat-shock-response or SOS-response). These responses are induced during heat-shock as well, indicating a potential overlap in the heat-shock-response and growth at high temperature. Another group of genes important for growth at high temperatures includes genes encoding factors involved in cell-wall synthesis, indicating the importance of a properly synthesized cell-wall under such conditions. One other remarkable outcome of this study is the importance of the translation machinery under high temperature conditions. Mutations in genes involved in ribosome processing or tRNA modification resulted in reduced growth at high temperatures, which indicates that ribosomes and tRNA need to be processed for

proper functioning at high temperatures. We anticipate that further molecular characterization of the here described mutants by advanced functional genomics studies will provide insight in how *L. monocytogenes* adapts to high temperatures that can be encountered during food processing.

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6. The *Listeria monocytogenes* motility regulator MogR controls expression of *clpB* and affects stress resistance

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Abstract

Listeria monocytogenes is a food-borne pathogen that can survive adverse environmental conditions and colonize its hosts due to an interactive network of responses. It contains a large number of regulators, including the recently identified motility gene repressor MogR, which controls the expression of the flagellar motility genes in a temperature-dependent manner. In this study we demonstrated that the class III heat-shock gene *clpB* also belongs to the MogR regulon. ClpB is required for proper refolding of damaged proteins. *ClpB* gene expression was increased in the absence of MogR repression in a Δ*mogR* strain, resulting in increased resistance of *L. monocytogenes* to heat and high hydrostatic pressure (HHP). A pivotal role for *clpB* in heat stress and HHP resistance was further substantiated by our findings that a Δ*clpB* mutant and a Δ*mogR*-Δ*clpB* mutant showed decreased resistance to heat and to HHP compared with the wild-type strain. The specific role that ClpB plays in the resistance to these stresses was demonstrated by overexpression of *clpB*. The MogR-controlled concomitant expression of flagellar motility genes and *clpB* may indicate a role for this chaperone in the maintenance of flagella protein quality.

Introduction

Listeria monocytogenes is a Gram-positive food-borne pathogen, which is the causative agent of listeriosis. This bacterium can survive and even grow under adverse conditions due to a complex network of different stress responses. In the last decade, *L. monocytogenes* been studied extensively and is now regarded as one of the model Gram-positive organisms for virulence (29, 37), motility (31, 33), and stress response (26, 32, 35, 36). The different pathways involved in stress survival, virulence, and motility are complex and their potential interactions have not all been unraveled. Many important central regulators have so far been identified in *L. monocytogenes*, including CtsR (23) and HrcA (10) for heat-shock, SigB (39) for general stress response, PrfA (21) for virulence, and MogR (9) and DegU (40) for motility. Moreover, various interactions between these central regulators and their regulons have been identified (11, 12, 15, 18, 36, 39), whereby overlapping functions are possible.

L. monocytogenes can grow and survive in various niches, ranging from cold wet environments to warm-blooded hosts. Upon uptake by the host, expression of virulence and stress factors is often induced by the temperature-shift. Temperature-dependent expression of specific genes and regulators is therefore important for the pathogenicity, motility, and stress response of *L. monocytogenes*. So far, four temperature-sensitive central regulators have been identified in *L. monocytogenes*, namely, PrfA, MogR, CtsR, and HrcA. PrfA is the major virulence regulator. Translation of *prfA* mRNA to PrfA protein depends on the conformation of a thermo-sensor in the untranslated mRNA preceding the *prfA* gene; PrfA protein is only synthesized at temperatures of 37 ºC and above, followed by activation of transcription of virulence genes (13). The activity of the central motility regulator MogR is also temperature dependent: at 37 ºC and above MogR represses motility genes, while at lower temperatures MogR repression is antagonized by the GmaR protein, resulting in flagella synthesis (31). Studies on *L. monocytogenes* motility and virulence have shown that flagellum based motility is important for the initial phase of host invasion but that flagella do not play a role in adhesion to the host cells (25). Since flagella are potential targets for the host immune response, their expression is repressed during infection. Lastly, the activities of the heat-shock response regulators HrcA and CtsR are temperature dependent. HrcA and CtsR are repressors that regulate the class I and class III heat-shock response, respectively. These responses are activated upon intracellular accumulation of protein aggregates and damaged proteins during exposure to stressful conditions such as high temperatures or the host environment, to maintain the quality and functionality of the protein pool (4, 6, 17, 24).

Recent findings suggest that the MogR regulator might have a role that is more generic than just the regulation of motility genes. The MogR protein binds directly to a

minimum of two TTTT-N5-AAAA recognition sites typically separated by 7 to 9, or alternatively, by 17 to 19 base pairs. While these recognition sites are present upstream of motility genes of *L. monocytogenes*, various other genes have also been predicted to contain a MogR binding site in their promoter region (30). However, these genes have not been investigated in relation to MogR thus far.

In this study, we identified two MogR binding sites in the promoter region of the heat-shock gene *clpB,* which belongs to the CtsR regulon, and demonstrated that MogR co-regulates *clpB*. Furthermore, induction of ClpB in the absence of MogR repression contributed to survival upon exposure to heat and high hydrostatic pressure (HHP) of *L. monocytogenes*. Given the role of ClpB in protein quality control, regulation of ClpB by MogR likely plays an important role in quality maintenance of protein tertiary structure and proper folding during flagella synthesis.

Materials and Methods

Strains, media, and plasmids

L monocytogenes EGD-e (8) and mutants thereof (Table 1) were used throughout the study. Single colonies of the strains were inoculated and grown in Brain Heart Infusion (BHI) broth (Difco) with shaking (200 rpm; New Brunswick type C24KC). When appropriate, antibiotics were added to the medium (10 $\mu g \cdot ml^{-1}$ erythromycin [Sigma] or 2 $\mu g \cdot ml^{-1}$ chloramphenicol [Sigma]). Recombinant DNA techniques were performed following standard protocols (27). The Δ*clpB* deletion mutant was constructed by using the suicide plasmid pAULa-Δ*clpB*, which contains the flanking regions of the *clpB* gene, resulting in a 2577 bp internal deletion. This plasmid was constructed using the temperature sensitive suicide plasmid pAULa (1) and the primers clpB-A, clpB-B, clpB-C, and clpB-D (Table 2) following the protocol described by Wouters et al. (41). The Δ*mogR* deletion mutant was constructed with the temperature sensitive suicide plasmid pSvS26 (Table 1) and the primers mogR-A, mogR-B, mogR-C, and mogR-D (Table 2) following the protocol described by Lambert et al. (19), resulting in an 897 bp internal deletion. Plasmid pSvS26 was constructed by cloning the XhoI-ScaI digested origin of replication fragment from vector pGH9:ISS1 (22) in the NheI-ScaI digested plasmid pNZ5319 (19). The Δ*clpB*-Δ*mogR* double deletion mutant was constructed by using the suicide plasmid pAULa-Δ*clpB* (Table 1) in the Δ*mogR* strain following the protocol from Wouters et al. (41). To complement *clpB* in Δ*clpB* strains and to over-express *clpB* in various backgrounds, *clpB* was introduced in the inducible plasmid pSPAC (7) by cloning a PCR fragment using the primers clpB-E and clpB-F (Table 2) in the XbaI-PstI digested plasmid pSPAC, which contains an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter, resulting in plasmid pSPAC-*clpB* (Table 1).

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant genotype or characteristics	Reference
L. monocytogenes		
EGD-e	Wild-type serotype 1/2a strain	(8)
Δ*mogR*	EGD-e Δ*mogR*	This study
Δ*clpB*	EGD-e Δ*clpB*	This study
Δ*mogR*-Δ*clpB*	EGD-e Δ*mogR* Δ*clpB*	This study
EGD-e pSPAC	EGD-e strain containing plasmid pSPAC	This study
Δ*clpB* pSPAC	EGD-e Δ*clpB* strain containing plasmid pSPAC	This study
Δ*clpB* pSPAC-*clpB*	EGD-e Δ*clpB* strain containing plasmid pSPAC-*clpB*	This study
Δ*mogR*-Δ*clpB* pSPAC	EGD-e Δ*mogR* Δ*clpB* strain containing plasmid pSPAC	This study
Δ*mogR*-Δ*clpB* pSPAC-*clpB*	EGD-e Δ*mogR* Δ*clpB* strain containing plasmid pSPAC-*clpB*	This study
Plasmids		
pAULa	Em^r; Cloning plasmid for gene replacements in Gram-positive bacteria	(1)
pAULa-Δ*clpB*	Em^r; pAULa derivative containing homologous regions up- and downstream of EGD-e *clpB*	This study
pSvS26	Cm^r Em^r; pNZ5319 derivative containing the temperature sensitive origin of replication of plasmid pGHost9:ISS1	This study
pSvS28	Cm^r Em^r; pSvS26 derivative containing homologous regions up- and downstream of EGD-e *mogR*	This study
pGHost9:ISS1	Em^r; Plasmid for random mutagenesis of Gram-positive bacteria	(22)
pNZ5319	Cm^r Em^r; Cloning plasmid for gene replacements in Gram-positive bacteria	(19)
pSPAC	Cm^r; Shuttle plasmid containing IPTG-inducible P_{spac} promoter	(7)
pSPAC-*clpB*	Cm^r; pSPAC derivative containing *clpB*	This study

Quantitative-PCR (Q-PCR)

To determine the *clpB*-expression levels, strains were grown overnight and inoculated (0.5%) in 10 ml BHI broth in 100 ml conical flasks. In three independent experiments, cultures were grown at 30 ºC, 37 ºC, and 43 ºC until OD_{600} 0.5 and samples of 0.5 ml were rapidly removed and diluted in 1.0 ml RNAprotect (Qiagen). After 5 min incubation at room temperature and 5 min centrifugation at 5000 x g (Eppendorf type 5417R) pellets were stored at -80 ºC. RNA extraction was performed as described previously (4). First strand cDNA synthesis and q-PCR reactions were performed as

described previously by Van der Veen et al. (36) using primers clpB-G and clpB-H (Table 2). Relative expression levels were corrected using the housekeeping genes tpi (tpi-A and tpi-B), rpoB (rpoB-A and rpoB-B), and 16S rRNA (16SrRNA-A and 16SrRNA-B) (Table 2 for primers).

Table 2. PCR primers used in this study

Primer	Sequence (5'-3')a
clpB-A	GTGGGGATCCCTTGCGCTGAATTTATACCT
clpB-B	GTGGGCGGCCGCTTGTAAATCCATTCATTCGTC
clpB-C	GTGGGCGGCCGCGTCACAGAATAACCTAAAAAAGG
clpB-D	GTGGGTCGACTAATGGCAGAATTCGTTCTT
clpB-E	GTGGACTAGTCTTTTATAAGGAGGACGAATGA
clpB-F	GTGGCTGCAGGAAGTGTCAAACCTTTTTTAGG
clpB-G	CGAAGGATTAGCGCAACGTA
clpB-H	GCTCCAGCAATAAGGGAACC
mogR-A	AACCCTCGAGACTTGGTTGGGCGGCAAATT
mogR-B	TGATTTAGGCATACAATCAC
mogR-C	AAACAAATGTAATTTAGAAG
mogR-D	AACCAGATCTTATTTCTCGCAATCATCAAC
tpi-A	AACACGGCATGACACCAATC
tpi-A	CACGGATTTGACCACGTACC
rpoB-A	CGTCGTCTTCGTTCTGTTGG
rpoB-A	GTTCACGAACCACACGTTCC
16SrRNA-A	GATGCATAGCCGACCTGAGA
16SrRNA-B	TGCTCCGTCAGACTTTCGTC

[a] Nucleotides introduced to create restriction sites are underlined

Heat-inactivation

To determine the heat resistance of the different mutant strains and with the wild-type strain, cultures were grown overnight and inoculated (0.5%) in 10 ml BHI broth in 100 ml conical flasks. In three independent experiments, cultures were grown at 30 ºC and 37 ºC until OD_{600} 0.5 and during the last two hours of growth 0.5 mM IPTG (Sigma) was added when appropriate. The cultures were centrifuged for 10 min

at 4300 rpm at room temperature (Heraeus type megafuse 1.0R) and the pellets were resuspended in 1 ml 1 x phosphate buffered saline (PBS; Sigma). The cells were added to 9 ml pre-warmed PBS (55 ºC) in 100 ml conical flasks and placed in a shaking water bath (60% shaking speed; GFL type 1083) at 55 ºC. Inactivation was determined after 18 min heat exposure. Samples were taken and appropriate dilutions were plated on BHI agar. The plates were incubated at 30 ºC for 3-5 days and colonies were counted.

HHP-inactivation

The HHP-inactivation experiments were started by inoculating (0.5%) of 25 ml BHI broth in 100 ml conical flasks using cultures that were grown overnight. Cultures were grown in three independent experiments at 30 ºC and 37 ºC to an OD_{600} 0.5. During the last two hours of growth 0.5 mM IPTG was added when appropriate. The cultures were harvested by centrifugation at 2600 x g for 5 min at room temperature (Eppendorf type 5810R). The pellets were washed twice with N-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma) buffer (pH 7.0) and resuspended in 1.5 ml ACES buffer. Sterile plastic stomacher bags (Seward) of 1.5 x 6 cm were filled with 0.7 ml cell suspension and vacuum sealed. The bags were placed in glycol in the HHP unit (Resato) and exposed to 350 MPa at 20 ºC. The pressure was built up at a rate of 400 MPa/min during which the temperature of the sample transiently increased with 15 ºC. Four min after the start of the pressure built up the temperature was back at 20 ºC. Inactivation of *L. monocytogenes* was determined after 20 min HHP exposure at the set conditions. Samples were serial diluted and plated on BHI agar. The plates were incubated at 30 ºC for 5 days and colonies were counted.

Results

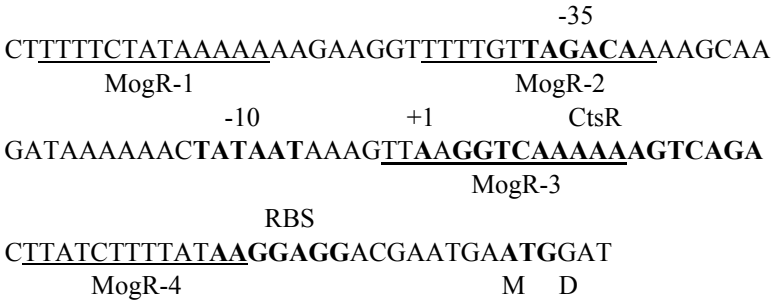

Fig. 1. The *clpB* promoter region contains four putative MogR recognition sites. The MogR recognition sites are indicated and underlined. The –35 and -10 promoter sequences, the +1 transcriptional start site, the CtsR binding site, the potential RBS sequence, and the translational start site are indicated and given in bold (adapted from Chastanet et al. (2)). The first two amino acids of ClpB are indicated below the nucleotide sequence.

MogR binding sites in the *clpB* upstream region

Analysis of the promoter region of *clpB* revealed the presence of four TTTT-N_5-AAAA MogR recognition sites resulting in two putative MogR binding sites (Fig. 1). The first two recognition sites (MogR-1 and MogR-2) were separated by 7 to 9 base pairs overlapping the –35 sequence of the promoter. A single mismatch was observed in the

second recognition site. The two other recognition sites (MogR-3 and MogR-4) were separated by 9 bp, and had overlap with the CtsR binding site and the transcriptional start site of *clpB* (2). Each of the latter two MogR recognition sites contained two mismatches with the consensus sequence.

Impact of temperature and MogR on *clpB* expression

To determine whether MogR regulates *clpB* expression, a Δ*mogR* strain was constructed (Table 1) and the expression levels of *clpB* were determined in this mutant and in the isogenic wild-type strain by Q-PCR after growth at 30 ºC, 37 ºC, and 43 ºC (Fig. 2). The *clpB* expression levels were lower in the wild-type strain than in the Δ*mogR* strain at all temperatures, indicating a repressor function of MogR on *clpB*. Both the wild-type and Δ*mogR* mutant strains showed induced expression of *clpB* at 37 °C and 43 °C, which results from a loss of CtsR repression at these temperatures (6).

MogR influences heat- and HHP-survival through *clpB*-repression

To determine whether MogR represses *clpB* expression and thereby affects survival upon heat and HHP exposure, we performed inactivation experiments of the wild-type strain, the Δ*mogR* strain, the Δ*clpB* strain, and the Δ*mogR*-Δ*clpB* double mutant strain after

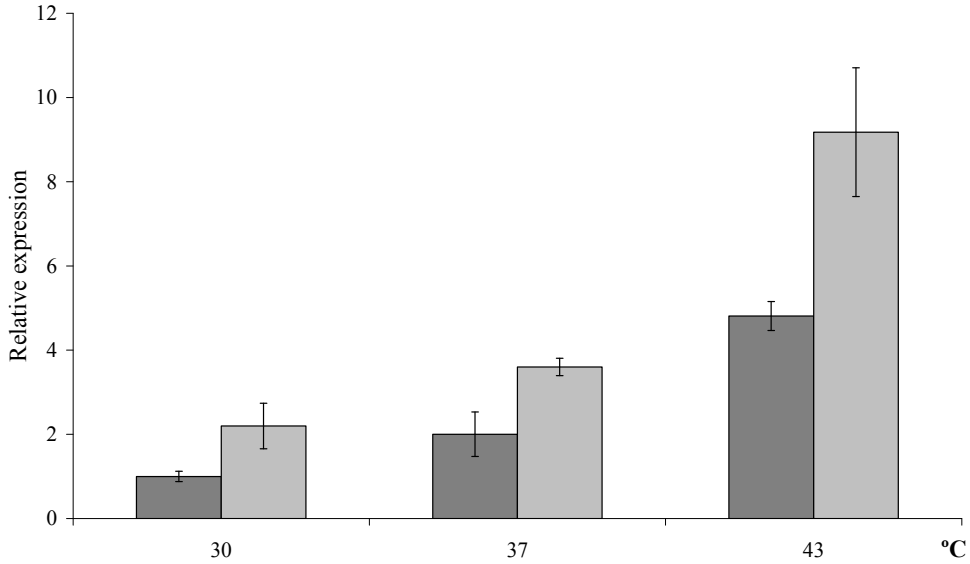

Fig. 2. Impact of temperature and MogR on the *clpB* expression levels. The graph presents the relative expression levels of *clpB* during exponential growth at 30 ºC, 37 ºC, and 43 ºC in BHI broth for the wild-type (dark grey) and Δ*mogR* (light grey) strains. The *clpB* expression of the wild-type strain grown at 30 ºC was set at one.

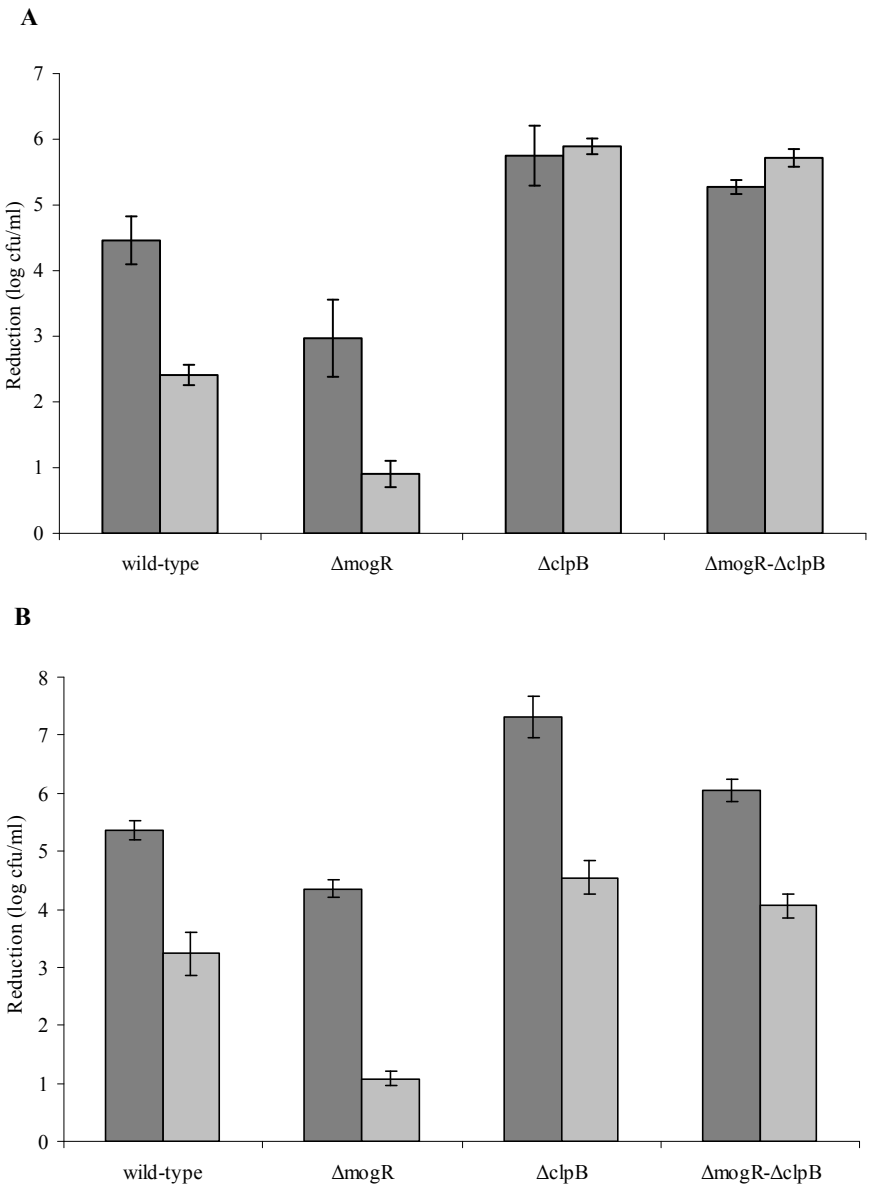

Fig. 3. MogR influences heat- and HHP-survival through *clpB*-repression. The graphs present heat and HHP inactivation of the wild-type and mutant strains after exponential growth at 30 ºC (dark grey) and 37 ºC (light grey) in BHI broth. A) Reduction in viable count after 18 min exposure to 55 ºC. B) Reduction in viable counts after 20 min exposure to 350 MPa at 20 ºC.

growth at 30 ºC and 37 ºC (Fig. 3). The Δ*mogR* mutant strain showed higher resistance to heat-inactivation (Fig. 3A) and HHP-inactivation (Fig. 3B) than the wild-type strain after growth at both 30 ºC and 37 ºC. Furthermore, both of these strains showed increased resistance to heat and HHP after growth at 37 ºC compared with growth at 30ºC. These results are consistent with the observed *clpB* expression levels, supporting a pivotal role for ClpB in heat- and HHP-resistance. This role was further substantiated by the results of the Δ*clpB* and the Δ*mogR*-Δ*clpB* mutant strains. Both of the latter mutants showed lower heat- and HHP-resistance than the wild-type strain after growth at 30 ºC and 37 ºC.

Complementation and overexpression of *clpB*

To verify whether the observed differences between the wild-type and mutant strains with regard to heat- and HHP-inactivation could be attributed to ClpB, the Δ*clpB* and the Δ*mogR*-Δ*clpB* strains were complemented for *clpB* using the IPTG-inducible plasmid pSPAC-*clpB* (Table 1). Experiments were performed after growth at 30 ºC and 37 ºC using the *clpB* complemented strains and wild-type strains transformed with the empty plasmid pSPAC (Fig. 4). The inactivation of the mutant strains was compared with inactivation of the wild-type with empty plasmid. The Δ*clpB* and Δ*mogR*-Δ*clpB* strains complemented with the plasmid pSPAC-*clpB* showed higher resistance to heat- and HHP-inactivation than the wild-type strain. The *clpB* expression levels of the complemented strains after growth at both 30 ºC and 37 ºC were verified by Q-PCR analyses (Fig. 5). The complemented mutant strains showed higher *clpB* expression levels than the wild-type strain, which is consistent with the results from the heat- and HHP-inactivation experiments.

Discussion

In this study, we demonstrated that MogR regulates the stress resistance gene *clpB*, which establishes ClpB as a new MogR regulon member. MogR mediated repression of *clpB* expression resulted in reduced heat- and HHP-resistance of *L. monocytogenes*, demonstrating a significant impact of this Clp protein on the survival capacity of this human pathogen.

The promoter region of *clpB* contains two putative MogR binding sites and four MogR recognition sites overlapping the promoter, the transcriptional start site, and the CtsR binding site. Tight repression of *clpB* expression by CtsR has previously been demonstrated (2), but the presence of MogR binding sites also suggests repression of *clpB* expression by MogR. The presence of two mismatches with the TTTT-N_5-AAAA consensus sequence in two of the MogR recognition sites (MogR-3 and MogR-4) does not rule out MogR binding, since Shen and Higgins (30) showed that it requires four mismatches within one of the recognition sites to prevent binding of MogR. We demonstrated that *clpB* expression is

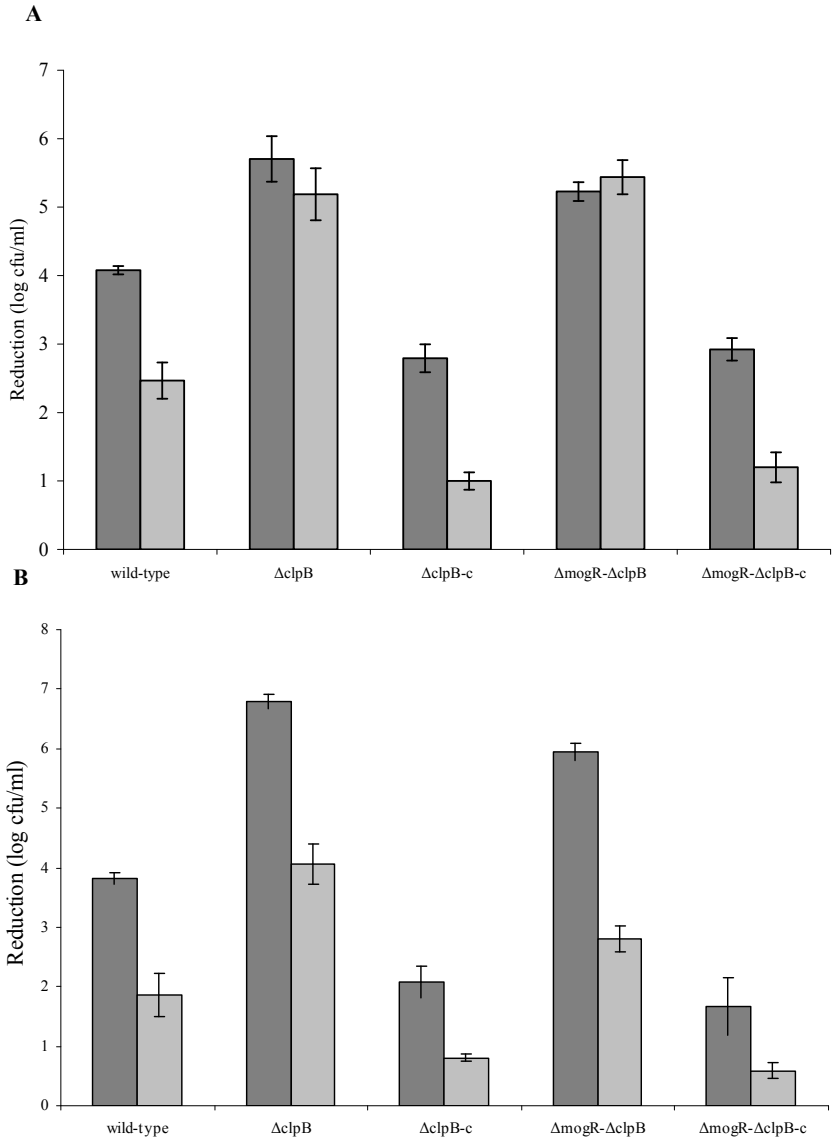

Fig. 4. Overexpression of *clpB* results in increased heat- and HHP-resistance. The graphs present heat and HHP inactivation of the wild-type and mutant strains transformed with the empty plasmid pSPAC or the complementation plasmid pSPAC-*clpB* (-c) after exponential growth at 30 ºC (dark grey) and 37 ºC (light grey) in BHI broth with 0.5 mM IPTG induction for 2 hours. A) Reduction in viable count after 18 min exposure to 55 ºC. B) Reduction in viable counts after 20 min exposure to 350 MPa at 20 ºC.

indeed regulated by MogR by showing that higher *clpB* expression levels were present in a Δ*mogR* mutant than in the wild-type strain after growth at different temperatures.

ClpB expression at 30 ºC was higher in the Δ*mogR* mutant than in the wild-type. At this temperature, MogR repression is antagonized by the protein GmaR in the wild-type strain, resulting in a functional knockout strain for MogR repression activity. However, the differences might be partially explained by the background repression activity of MogR at this temperature in the wild-type strain.

The two repressors CtsR and MogR regulate *clpB* expression. At low temperatures (30 ºC and below), *clpB* expression is repressed by CtsR, while at high temperatures (37 ºC and above), *clpB* expression is repressed by MogR. This indicates that tight regulation of *clpB* expression is important for the *L. monocytogenes*. ClpB is a molecular chaperone that plays a role in unfolding and reactivating aggregated proteins in both prokaryotes and eukaryotes (42).

Simultaneous regulation of the motility genes and *clpB* by MogR indicates a role for the chaperone ClpB in the production and quality maintenance of flagellar proteins. Notably, flagella are very complex structures that require coordinate expression and production of large numbers of proteins for its synthesis and function (for review see (5)). A well functioning protein maintenance system seems therefore important.

ClpB seems to play an important role in stress resistance. Previous research showed that a natural *ctsR* mutant in *L. monocytogenes* strain ScottA was more resistant to heat-inactivation (14) and HHP-inactivation (15) due to overexpression of the *clp*-genes. For *Tetragenococcus halophilus* it has been demonstrated that ClpB forms homohexameric ring structures under regular conditions and dissociates into monomers and dimers upon a stress response (34). A recent study in *Escherichia coli* revealed that ClpB is involved in the reactivation of protein aggregates which accumulate under thermal stress, suggesting a role for ClpB in maintenance of intracellular protein quality (38). The heat- and HHP-inactivation experiments performed in this study using the Δ*mogR*, Δ*clpB*, and Δ*mogR*-Δ*clpB* mutants, indeed showed that resistance to these stresses was repressed by MogR through repression of *clpB* expression. Complementation and overexpression of *clpB* in the Δ*clpB*, and Δ*mogR*-Δ*clpB* mutants resulted in increased stress resistance, showing that ClpB is important for both heat- and HHP-resistance.

Heat-resistance is dependent on the presence of ClpB, while HHP-resistance can also be induced by other factors like the KJE chaperonin system (*dnaK*, *dnaJ*, and *grpE*) or other class I or class III heat-shock genes (15, 23, 36) in absence of ClpB. In general, the CtsR regulated class III heat-shock genes (*clpB*), and the HrcA regulated class I heat-shock genes *dnaK*, *dnaJ*, and *grpE* are expressed at higher levels at 37 ºC than at 30 ºC (3, 6, 10). The KJE system forms a bichaperone system with ClpB, which solubilizes and reactivates protein aggregates (20, 28, 38). Large protein aggregates are reduced to medium sized

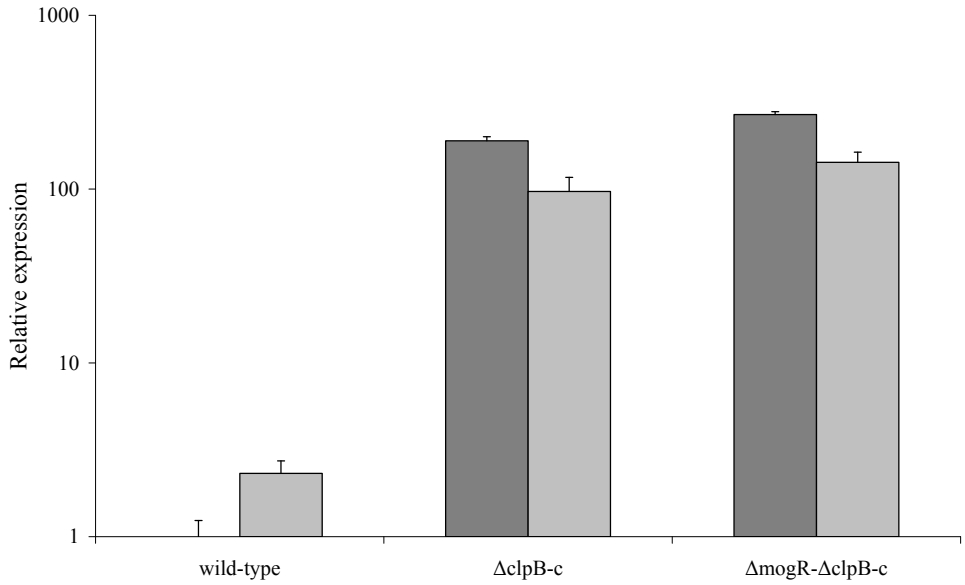

Fig. 5. Expression analysis in the *clpB* overexpressing mutant. The graph presents the relative *clpB* expression levels of the wild-type strain transformed with the empty plasmid pSPAC and mutant strains transformed with the complementation plasmid pSPAC-*clpB* (-c) after exponential growth at 30 ºC (dark grey) and 37 ºC (light grey) in BHI broth with 0.5 mM IPTG induction for 2 hours. The *clpB* expression of the wild-type strain transformed with pSPAC and grown at 30 ºC was set at one.

aggregates by ClpB, which in turn are either directly refolded in their native state by the KJE system or first translocated into unfolded protein by joint activity of the ClpB protein and the KJE system before refolding by the KJE system. Since both heat- and HHP-stress result in accumulation of protein aggregates (16, 38), wild-type strains are more resistant to these stresses after growth at 37 ºC than after growth at 30 ºC. This phenotype was confirmed by our heat- and HHP-inactivation experiments. The wild-type and Δ*mogR* mutant strains showed higher resistance after growth at 37 ºC than after growth at 30 ºC. However, in those cases where higher HHP-resistance for the Δ*clpB* and Δ*mogR*-Δ*clpB* mutants was observed after growth at 37 ºC than after growth at 30 ºC, no increase in heat-resistance was found. Conceivably, HHP-exposure results mainly in the accumulation of medium sized aggregates whereas heat-exposure results in the accumulation of large protein aggregates, enforcing a role for ClpB in the latter stress condition.

This study established a clear link between motility regulation and stress survival by showing that the class III heat-shock gene *clpB* is regulated by the motility gene repressor MogR. Furthermore, we showed that ClpB is crucial for *L. monocytogenes* to survive heat

and HHP treatments and that repression of *clpB* by MogR influences the survival capacity of *L. monocytogenes* during exposure to these stresses. Lastly, in combination with previous results on its function in *L. monocytogenes* motility and virulence (30), MogR can now be classified a generic regulator.

Acknowledgements

We thank Saskia van Schalkwijk for construction of the Δ*mogR* mutant strain.

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7. The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis

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Submitted for publication

Abstract

The SOS response is a conserved pathway that is activated under certain stress conditions and is regulated by the repressor LexA and the activator RecA. The food-borne pathogen *Listeria monocytogenes* contains RecA and LexA homologs, but their roles in *Listeria* have not been established. In this study, we identified the SOS regulon in *L. monocytogenes* by comparing the transcription profiles of the wild-type strain and the Δ*recA* mutant strain after exposure to the DNA damaging agent mitomycinC. In agreement with studies in other bacteria, we identified an imperfect palindrome AATAAGAACATATGTTCGTTT as the SOS operator sequence. The SOS regulon of *L. monocytogenes* consists of 29 genes in 16 LexA regulated operons, encoding proteins with functions in translesion DNA synthesis and DNA repair. It also includes the bile extrusion system BilE, which is known to affect virulence properties of *L. monocytogenes* by conferring resistance to bile. We furthermore identified a role for the product of the LexA regulated gene *yneA* in cell elongation and inhibition of cell division. Notably, the Δ*yneA* strain was less heat resistant than the wild-type, linking SOS response genes to heat resistance. As anticipated, RecA of *L. monocytogenes* plays a role in mutagenesis. This was demonstrated by considerably lower rifampicin and streptomycin resistant fractions of Δ*recA* cultures compared with wild-type cultures. The inability to induce the SOS response furthermore led to reduced resistance to various stresses, including heat, H_2O_2, and acid in Δ*recA* cultures compared with the wild-type. Our results indicate that the SOS response of *L. monocytogenes* contributes to survival of a range of stresses that may influence its persistence in the environment and in the host.

Introduction

Listeriosis is a food-borne infection caused by *Listeria monocytogenes*. The disease has a high case mortality rate and is therefore of great concern to public health (37). *L. monocytogenes* can grow and survive during exposure to severe environmental stresses. It has the ability to grow at a wide pH range (pH 4.6 to pH 9), at high salt concentrations (up to 13 %), and over a wide temperature range (-0.4 to 46 ºC) (31, 55). Exposure of *L. monocytogenes* to stress can lead to stress adaptation, due to the transcriptional activation of stress response genes (19). Several classes of stress response genes have been described in *L. monocytogenes*, which are activated during exposure to specific or general stresses. In particular, the class I and class III heat-shock and the SigB response have been investigated thoroughly (13, 24, 33). Recently, it has been shown that the SOS response of *L. monocytogenes* is activated upon mild heat exposure (54).

Certain environmental insults lead to undesirable DNA damage that requires repair, while under other circumstances increased mutation rates are needed to maximize chances of survival. The SOS response is an inducible pathway involved in DNA repair, restart of stalled replication forks (8, 10, 35), and in induction of genetic variation in stressed and stationary phase cells (20, 36, 49, 53). It is regulated by LexA and RecA. LexA is an autoregulatory repressor which binds to the CGAACATATGTTCG consensus sequence in the promoter region of the SOS response genes as determined for *Bacillus subtilis* (1), thereby repressing transcription. A consensus LexA binding motif for *L. monocytogenes* has not been identified thus far. Generally, the SOS response is induced under circumstances in which single stranded DNA accumulates in the cell. This results in activation of RecA, which in turn stimulates cleavage of LexA, and ultimately in the induction of the SOS response (48).

For an increasing number of bacteria it has been shown that the SOS response is activated during stress exposure (6, 14, 15, 51) or during pathogenesis (30, 34). A comparative analysis between the SOS regulon of *B. subtilis* and *Escherichia coli* showed a surprisingly small overlap (eight genes), while the regulons in each of these species contain over 30 genes (34). The SOS regulon and its role in *L. monocytogenes* has not been established, but activation of the SOS response was previously observed during heat-shock (54). Induction of the SOS regulon was postulated to suppress cell division, thereby preventing transection of the genome after replication fork stalling (54). This effect of interruption of Z-ring formation in the vicinity of the nucleoid is called “nucleoid occlusion” (44). For *Bacillus subtilis*, activation of the SOS response gene *yneA* leads to accumulation of YneA at the midcell, thereby preventing septum formation, which results

in cell elongation (32). Whether YneA has a similar function in *L. monocytogenes* remains to be elucidated.

In this study, we established the regulon of the SOS response in *L. monocytogenes* by comparing whole genome expression profiles of a Δ*recA* strain and the isogenic wild-type strain before and after exposure to the DNA damaging agent mitomycin C (MMC). Furthermore, we demonstrated that RecA-controlled functions of *L. monocytogenes* are involved in mutagenesis and stress survival, and that the *L. monocytogenes* SOS response gene *yneA* is involved in cell elongation and heat-survival.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant genotype or characteristics	References
L. monocytogenes		
EGD-e	Wild-type serotype 1/2a strain	(21)
ΔrecA	EGD-e ΔrecA	This study
ΔyneA	EGD-e ΔyneA	This study
Plasmids		
pAULa	Emr ; Cloning plasmid for gene replacements in Gram-positive bacteria	(4)
pAULa-ΔrecA	Emr ; pAULa derivative containing homologous regions up- and downstream of EGD-e recA	This study
pAULa-ΔyneA	Emr ; pAULa derivative containing homologous regions up- and downstream of EGD-e yneA	This study

Materials and Methods

Strains, media, and plasmids

Strain *L monocytogenes* EGD-e (21) was the wild-type parent strain in this study. This strain and its mutants (Table 1) were stored in Brain Hearth Infusion (BHI) broth (Difco) containing 15% sterile glycerol (BDH) at -80 ºC. Single colonies were inoculated in BHI broth and grown at 37 ºC and 200 rpm (New Brunswick type C24KC). Antibiotics were added to the medium to maintain plasmids (10 μg·ml^{-1} erythromycin [Sigma] or 2 μg·ml^{-1} chloramphenicol [Sigma]). Standard protocols were performed for recombinant DNA techniques (46). The temperature sensitive suicide plasmid pAULa (4) was used for Construction of the Δ*recA* and Δ*yneA* following the protocol described previously (57). The primers containing the flanking regions (recA-A to D for Δ*recA* and yneA-A to D for Δ*yneA*) are listed in Table 2. This resulted in a 915 bp and 306 bp internal deletions for *recA* and *yneA*, respectively.

Table 2. PCR primers used in this study.

Primer	Sequence (5'-3')[a]
recA-A	GTGGGGATCCCTGCTGATTTAAACGATTTG
recA-B	GTGGGCGGCCGCACGATCATTCACATTGTTGC
recA-C	GTGGGCGGCCGCACACACAGATATTCGTGATGAG
recA-D	GTGGGTCGACCGGTTTTCTGATTCTTTGAC
yneA-A	GTGGGGATCCATCCAAGGGAAGTCAGTTCT
yneA-B	GTGGGCGGCCGCTTTTAAAGTCATTAATAATCCCTC
yneA-C	GTGGGCGGCCGCGCAAATCAGTAAGGTCGATTTAG
yneA-D	GTGGGTCGACTAAAAGCATTGAGCCGTGT

[a] Nucleotides introduced to create restriction sites are underlined.

Sample collection and RNA isolation

In three independent experiments, cultures of the wild-type and Δ*recA* strain were grown in 50 ml BHI broth (250 ml conical flasks, 37 ºC, 200 rpm,) until an absorbance (OD_{600}) of approximately 0.5 was obtained. At that moment 1 mM MMC (Sigma) was added to the cultures. Ten ml samples were taken before exposure to MMC and one hour afterwards, and dissolved in 20 ml RNAprotect (Qiagen). The mixtures were incubated for 5 min at room temperature, centrifuged for 10 min at 3720 x g (Heraeus type megafuse 1.0R), and the pellets were stored at -80 ºC. The cell pellets were washed in 400 µl SET buffer (50 mM NaCl [Sigma], 5 mM EDTA [Sigma], and 30 mM Tris-HCl [pH 7.0; Sigma]) containing 10 % sodium dodecyl sulfate (Sigma) and treated for 30 min at 37 ºC in a shaker (350 rpm; Eppendorf Thermomixer Comfort) with 200 µl 50 mM Tris-HCl (pH 6.5) containing 50 mg/ml lysozyme (Merck), 2 mg/ml Proteinase K (Ambion), 2.5 U/ml mutanolysin (Ambion), and 4 U/ml SUPERase (Ambion). Total RNA was extracted using the RNeasy mini kit (Qiagen) with an on column DNase treatment according to the manufacturer's protocol. The quality of the RNA was analyzed on a 2100 Bioanalyzer (Agilent Technologies) and quantified on a ND-1000 spectrophotometer (NanoDrop Technologies).

cDNA synthesis and labeling and micro-array hybridization, washing, scanning, and analyzing

Five µg of total RNA of each sample was used for cDNA synthesis and labeling with both cyanine 3 (Cy3) and cyanine 5 (Cy5) dyes. The CyScribe cDNA post-labeling kit (RPN5660; GE Healthcare) was used according to the manufacturer's protocol. Aliquots of 0.3 µg labeled cDNA were used for hybridization on custom-made *L. monocytogenes* EGD-e micro-arrays (Agilent Technologies). These arrays (8 x 15K format) contained i*n situ* synthesized 60-mer oligomers with a theoretical melting temperature of approximately 82 ºC (following nearest neighbor calculations (41) using 1 M Na^+ and 10^{-12} M oligo-

nucleotides). The *L. monocytogenes* genes were represented on the array by 1 probe for 36 genes, 2 probes for 94 genes, 3 probes for 2701 genes, or 6 probes for 1 gene and a total of 23 genes was not represented on the arrays because no unique probe could be selected. The labeled cDNA samples were hybridized on 16 arrays for 17 hours at 60 ºC following a loop design. The micro-arrays were washed, scanned, and analyzed according to the protocol described extensively by Saulnier et al. (47). The micro-array data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) using accesion number GSE12634.

Prediction of SOS-response genes

The promoter region of putative SOS-response genes (300 bp) was collected. These genes were selected based on the following criteria: 1) significant up-regulation (fold-change>1.5 and $p<0.05$) in the wild-type strain after MMC exposure, 2) no significant up-regulation (fold-change>1.5 and $p<0.05$) in the Δ*recA* mutant strain after MMC exposure, and 3) the MMC treatment resulted in significant higher up-regulation (fold-change>1.5 and $p<0.05$) in the wild-type strain compared with the Δ*recA* mutant strain. The promoter regions were analyzed for conserved motifs by the MEME program (2). The MEME search criteria were set at a minimal length of the motif of 8 nt and a maximal length of 40 nt. The consensus *L. monocytogenes* LexA binding motif in the putative SOS response genes was visualized using the weblogo tool (11).

Heat-inactivation of wild-type and Δ*yneA* strains

To determine the heat-resistance of the Δ*yneA* mutant and the wild-type strain, cultures that were grown overnight were inoculated in 10 ml BHI broth (0.5%) in 100 ml conical flasks and subsequently grown at 37 ºC until an absorbance (OD_{600}) of approx 1.0 was reached. During the last hour of growth 1 mM MMC was added. Control experiments were performed without addition of 1 mM MMC. Samples were taken for microscopic image analysis before and after exposures. The cultures were collected (10 min, 3720 x g, room temperature; Heraeus type megafuse 1.0R) and washed with 1 ml PBS. To assess the heat resistance of Δ*yneA* and wild-type cultures with or without pre-treatment with MMC, cells were added to 9 ml pre-heated BHI broth (55 ºC) in 100 ml conical flasks and placed in a shaking waterbath (60% shaking speed; GFL type 1083) at 55 ºC. Samples were taken before and 2 h after exposure to 55 ºC and serial diluted in 1 x PBS. Appropriate dilutions were plated on BHI agar, plates were incubated at 30 ºC for 3-5 days and colonies were enumerated. All experiments were performed in triplicate.

Microscopic image analysis of wild-type and Δ*yneA* strains

Cell sizes were determined using microscopic image analysis. Culture samples of 100 µl were collected at 5000 x g for 1 min (Eppendorf type 5417R). The pellets were

dissolved in nigrosin solution (Sigma) and 5 µl of a cell suspension was dried on glass slides. A Dialux 20 microscope (Leica) was used to make images of the cells at 100 x magnification. The images were analyzed in eight-bit type after adjusting the threshold to black and white using the ImageJ program (http://rbs.info.nih.gov/ij/download.html). Distribution graphs of cell sizes (pixels/cell) were constructed in Excel (Microsoft) from a minimum of 500 cells of three independent experiments.

Mutagenesis in wild-type and Δ*recA* strains

To investigate the role of the SOS response in introducing mutations, exponentially growing cultures of wild-type and Δ*recA* mutant cells were plated on 0.05 µg/ml rifampicin or 75 µg/ml streptomycin. Cultures of the wild-type strain and the Δ*recA* mutant strain were grown in 10 ml BHI broth in 100 ml conical flasks at 37 ºC and 200 rpm. When an absorbance (OD_{600}) of 0.5-0.7 was reached, the cells were collected (10 min, 4300 rpm, room temperature; Heraeus type megafuse 1.0R) and dissolved in 1 ml 1 x phosphate buffered saline (PBS; Sigma). The cell suspensions were serially diluted in 1 x PBS and appropriate dilutions were plated on BHI agar and BHI agar containing 75 µg/ml streptomycin (Sigma) or 0.05 µg/ml rifampicin (Sigma). The plates were incubated at 37 ºC for 3 days and colonies were enumerated. The complete experiment was performed in triplicate.

Stress resistance of wild-type and Δ*recA* strains

Cultures of the wild-type strain and Δ*recA* mutant strain were grown in 10 ml BHI broth at 37 ºC and 200 rpm in 100 ml conical flasks until an absorbance (OD_{600}) of approximately 0.3 was obtained. At this moment the cultures were exposed to different stresses. The heat resistance was tested by transferring the cultures to a shaking water bath

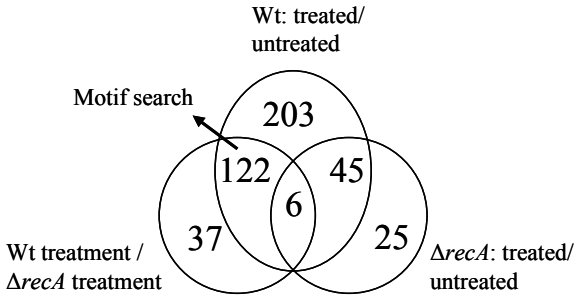

Fig. 1. Comparison of differentially expressed genes after MMC treatment between the wild-type and Δ*recA* mutant strains. The number of differentially expressed genes is indicated in the circles and the overlapping areas indicate that the same genes were differentially expressed. The arrow indicates the group of genes on which the LexA motif search was performed.

(60% shaking speed; GFL type 1083) set at 55 ºC, the oxidative stress resistance was tested by addition of 60 mM H_2O_2 (Merck), and the acid resistance was tested by dissolving the collected cultures (10 min, 3720 x g, room temperature; Heraeus type megafuse 1.0R) in 10 ml BHI (pH 3.4; adjusted with 10% HCl) in 100 ml conical flasks. Samples were taken before stress exposure and 1 hour after stress exposure and serially diluted in PBS. Dilutions were plated on BHI agar and colonies were enumerated after 3-5 days incubation at 30 ºC. Experiments were performed in triplicate.

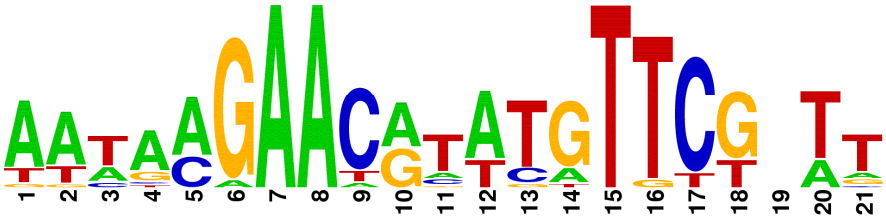

Fig. 2. Consensus sequence of the LexA binding motif of the putative SOS response genes (Table 3) in *L. monocytogenes*. visualized with WebLogo (11).

Results

Identification of SOS response genes

To identify genes belonging to the SOS response, transcriptional profiles of the wild-type and Δ*recA* strains were compared before and after exposure to MMC. A LexA motif search was carried out for 122 selected genes that showed significant up-regulation (fold-change >1.5 and $p<0.05$) in the wild-type strain after MMC exposure while no significant up-regulation was found in the Δ*recA* mutant strain. Furthermore, the MMC treatment resulted in significant higher up-regulation of these genes in the wild-type strain compared to the Δ*recA* mutant (Fig. 1). The upstream regions of these 122 genes were collected and compared for similar motifs. A consensus motif was identified in 16 promoter regions (E-value=$2.2e^{-13}$) (Fig. 2), representing 29 genes (Table 3). This included genes encoding the regulators of the SOS response RecA and LexA, the alternative DNA polymerases DinB and UmuDC, the excinuclease UvrBA, and the cell division inhibitor YneA. These SOS response genes were recently also found to be induced by heat stress (54). The newly identified SOS response genes encode (predicted) helicase systems (lmo0157-lmo0158, lmo1759-lmo1758, and lmo2268-lmo2264), alternative DNA polymerases (lmo1574 and lmo2828), and exo/excinuclease systems (lmo1640-lmo1638 and lmo2222-lmo2220). These results show that the majority of the SOS response genes of *L monocytogenes* encode DNA repair systems and alternative DNA polymerases that help during replication fork

Table 3. Genes belonging to the *L. monocytogenes* SOS response. The putative LexA binding sites and $\log_2$ expression ratios between the wild-type (wt) and Δ*recA* mutant after MMC exposure are given.

Gene[a]	Name	LexA binding site	Description product	MMC treatment		Δ*recA*/wt		
				wt	Δ*recA*	Untreated	MMC treated	MMC treatment
lmo0157		GTTGCGAACGTAGGTTCTGTG	Predicted ATP-dependent helicase	1.61	-0.13	0.99	-0.75	-1.74
lmo0158			Predicted hydrolase	1.18	0.21	0.85	-0.11	-0.96
lmo1302	*lexA*	AAAAAGAATGTATGTTCGCTT	Transcription repressor of SOS response	1.74	0.25	-1.58	-3.06	-1.48
lmo1303	*yneA*	AAAGCGAACATACATTCTTTT, TGTACGAACGGTTGTTCTATA	Similar to *B. subtilis* YneA protein	3.95	-0.06	-4.21	-8.22	-4.01
lmo1398	*recA*	AATACGAATAAATGTTCGCTT	Transcription activator of SOS response	2.58	0.50	-2.90	-4.98	-2.08
lmo1421	*bilEA*	ATATAGAACATACATTCGATT	Osmoprotectant transport system ATP-binding protein, bile resistance	1.13	0.30	-1.55	-2.38	-0.83
lmo1422	*bilEB*		Osmoprotectant transport system permease protein, bile resistance	1.21	0.17	-1.23	-2.27	-1.04
lmo1574	*dnaE*	AACACGAACACACTTTCTTTT	DNA polymerase III alpha subunit	1.37	0.03	-1.18	-2.53	-1.34
lmo1640		AAACAGAACATATGTTTTATC	Hypothetical protein	1.90	-0.52	-1.58	-4.00	-2.42
lmo1639			DNA-3-methyladenine glycosidase, base excision repair	2.24	-0.53	-1.54	-4.31	-2.77
lmo1638			Predicted peptidase	2.22	-0.24	-1.47	-3.94	-2.47
lmo1759	*pcrA*	AATAAGAACAAATGTTTGTAT	ATP-dependent DNA helicase	0.93	-0.20	-0.14	-1.27	-1.13
lmo1758	*ligA*		NAD-dependent DNA ligase	0.65	-0.20	-0.16	-1.01	-0.85
lmo1975	*dinB*	AATAAGAACGCTTGTTCGTTT	DNA polymerase IV	3.08	-0.34	1.88	-1.54	-3.41

Table 3. Continued.

Gene	Name	LexA binding site	Description product	MMC treatment		ΔrecA/wt		
				wt	ΔrecA	Untreated	MMC treated	MMC treatment
lmo2222		AATAAGAACGTATATTCGGTT	Predicted DNA repair exonuclease	1.95	-0.48	-0.26	-2.69	-2.42
lmo2221			Hypothetical protein	2.38	-0.21	-0.15	-2.75	-2.60
lmo2220			Predicted exonuclease	1.52	0.16	0.17	-1.19	-1.36
lmo2268	*addB*	AATAAAAACATATGTTCGGTG	Predicted ATP-dependent helicase	1.59	-0.15	0.47	-1.26	-1.73
lmo2267			Predicted ATP-dependent helicase	1.74	0.09	0.05	-1.61	-1.65
lmo2266			Predicted hydrolase	1.56	-0.06	0.11	-1.52	-1.62
lmo2265			Hypothetical protein	1.61	0.06	-0.04	-1.59	-1.55
lmo2264			Hypothetical protein	1.28	-0.39	0.26	-1.42	-1.67
lmo2271		TTCAAGAACGTTTGTTCGTAT	Bacteriophage A118 protein	3.40	-0.65	1.34	-2.72	-4.05
lmo2332	*int*	AAAAAGAACGTATGTGCGAAA	Site-specific DNA recombinase, integrase (Bacteriophage A118)	1.58	0.05	0.50	-1.03	-1.53
lmo2489	*uvrB*	AATGCGAAAATATGTTCGGTT	Excinuclease ABC (subunit B)	2.76	0.10	1.40	-1.26	-2.66
lmo2488	*uvrA*		Excinuclease ABC (subunit A)	2.63	0.10	1.08	-1.45	-2.53
lmo2675	*umuD*	AATAAGAACATTTGTTCGTAT	DNA polymerase V	2.93	-0.51	0.52	-2.92	-3.44
lmo2676	*umuC*		DNA polymerase V	2.14	-0.30	0.19	-2.26	-2.44
lmo2828		TTTAAGAACGTTTGTTCGTAT	Similar to UmuD polymerase V subunit from gram-negative bacteria	4.56	0.13	2.67	-1.76	-4.43

[a]The first gene of a putative operon is given in bold

stalling. Furthermore, two of the SOS response genes are part of a bile resistance system (lmo1421-lmo1422). Since bile exposure may result in DNA damage (42), activation of this system as part of the SOS response may provide additional protection of cellular DNA. Finally, the first and the last gene of the *comK* integrated bacteriophage A118 (lmo2271 and lmo2332) are LexA controlled.

YneA in cell elongation and heat-resistance

To investigate the role of the SOS response gene *yneA* in cell elongation, cell size distribution graphs were constructed from the wild-type strain and the Δ*yneA* strain before and after triggering the SOS response by MMC exposure (Fig. 3). Exposure to MMC resulted in a significant increase in cell size for the wild-type strain compared with the unexposed cells, while the Δ*yneA* mutant strain did not show an increase in cell size compared with unexposed cells. Similar results were obtained after triggering the SOS response by exposure to 48 °C for 40 min (as in (54)), although cell elongation was less pronounced (data not shown). These results show that YneA activity is associated with cell elongation after triggering of the SOS response. To investigate the potential role of YneA mediated cell elongation in heat-resistance, heat inactivation studies were performed before and after MMC exposure using the wild-type and Δ*yneA* mutant strains (Fig 4). A pre-exposure to MMC resulted in increased resistance to heat inactivation for the wild-type

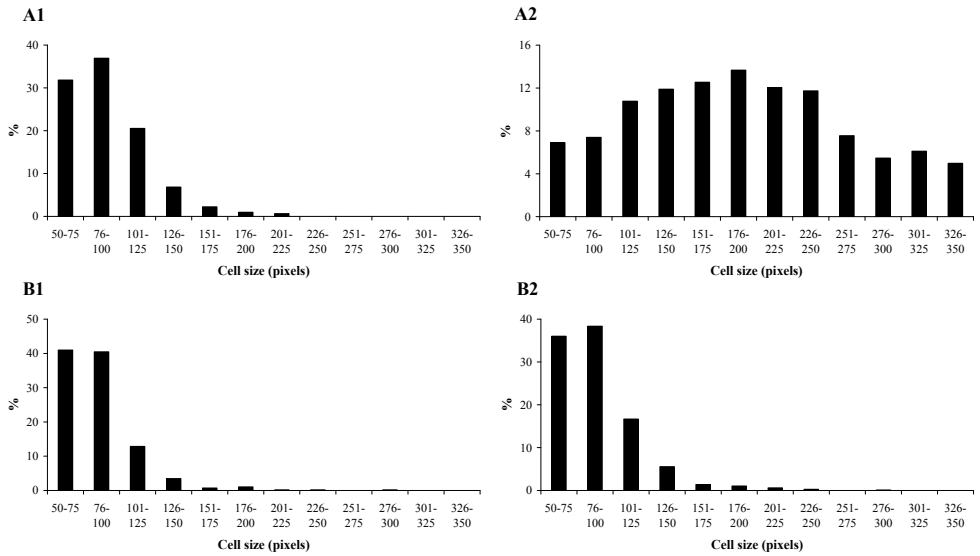

Fig. 3. Microscopic image analysis of the wild-type (A) and Δ*yneA* (B) strains before [1] and after [2] exposure for 1 hour to MMC. The graphs show the distribution of cell sizes in pixels per cell; for details see Materials and Methods.

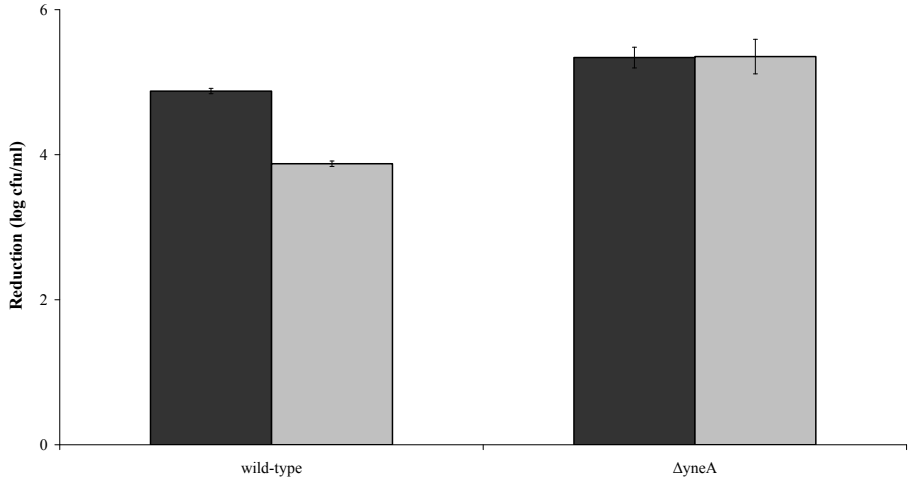

Fig. 4. Heat inactivation of the wild-type and Δ*yneA* strain in BHI broth at 55 ºC. The graph shows the reduction in viable counts of strains before (dark grey) and after (light grey) 2 hours heat exposure of cells pre-adapted for 1 hour with 1 mM MMC.

strain but not for the Δ*yneA* mutant. These results show that YneA-mediated cell division inhibition or cell elongation contributes to heat resistance.

RecA dependent mutagenesis

Exponentially growing cultures of wild-type and Δ*recA* mutant cells were plated on 0.05 µg/ml rifampicin or 75 µg/ml streptomycin. These concentrations of antibiotics were the minimal inhibitory concentration (MIC) for the wild-type strain (data not shown). The rifampicin resistant fraction of the wild-type cultures was $1.25 \cdot 10^{-7}$, which was 14 times higher than the resistant fraction of the Δ*recA* cultures (Fig. 5). Furthermore, the Δ*recA* cultures did not show a resistant fraction to streptomycin ($<10^{-9}$), while a resistant fraction for the wild-type strain of $1.33 \cdot 10^{-8}$ was observed. These results indicate that in the absence of RecA, mutation rates in the cell are lower due to the inability of LexA cleavage and derepression of the SOS response.

RecA dependent stress resistance

The role of *recA* in stress resistance was investigated by exposing the wild-type and Δ*recA* strains to heat (55 ºC), oxidative stress (60 mM H_2O_2), and acid (pH 3.4). The wild-type strain showed higher resistance to these stresses than the Δ*recA* strain (Fig. 6). In particular, high sensitivity of the Δ*recA* strain to heat and oxidative stress was observed under the conditions used. The Δ*recA* mutant strains showed approximately 3 log higher reductions in cell counts after 1 hour exposure to 55 ºC and 60 mM H_2O_2 than the wild-type strain.

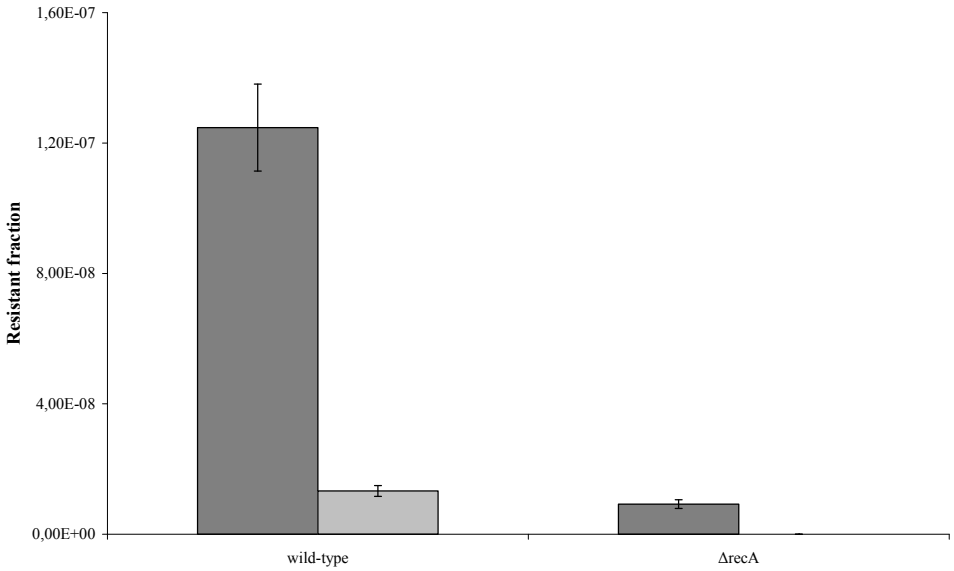

Fig. 5. RecA dependent mutagenesis. The graph shows the resistant fractions of exponentially growing wild-type and Δ*recA* mutant cultures after exposure to 0.05 µg/ml rifampicin (dark grey) and 75 µg/ml streptomycin (light grey).

Discussion

In this study, the SOS regulon of *L. monocytogenes* was characterized and its role in mutagenesis and stress resistance assessed. A consensus motif for LexA binding was identified upstream of the differentially expressed genes. Sixteen putative binding sites were found controlling the expression of 29 genes with roles in DNA repair and translesion DNA synthesis. The SOS response gene *yneA* was shown to be involved in cell elongation or inhibition of cell division, and contributed to heat-resistance. Furthermore, a role for RecA in the introduction of mutations and in the resistance to stress was established by antibiotic resistance essays and stress resistance tests.

The regulon of the SOS response in *L. monocytogenes* was determined by comparing the transcription profiles of wild-type and an SOS deficient Δ*recA* strain after exposure to a DNA damaging agent. This approach was previously used to identify the SOS regulons of *E. coli* (9), *Staphylococcus aureus* (6), and *Pseudomonas aeruginosa* (7). The complete SOS regulon has furthermore been determined for *Caulobacter crescentus* (12), *Pseudomonas fluorescens* (28), and *B. subtilis* (1). The various SOS regulons in these bacteria consists of 43 genes in *E. coli*, 33 genes in *B. subtilis*, 15 genes in *P. aeruginosa*, 37 genes in *C. crescentus*, 17 genes in *P. fluorescens*, and 16 genes in *S. aureus*, and here

we identified 29 genes in *L. monocytogenes*. Only 5 SOS genes are commonly present in the bacteria analysed thus far, namely *lexA*, *recA*, *uvrBA*, and *dinB*. In *L. monocytogenes* the other SOS response genes encode proteins involved in DNA repair (excinucleases, helicases, and recombinases) or translesion DNA synthesis (alternative DNA polymerases). A number of these proteins have been investigated in other bacteria as part of their specific SOS response (for a review see (18)). Noteworthy is that the *L. monocytogenes* SOS response contains a LexA regulated bile exclusion system (BilE). Like several other SOS response genes of *L. monocytogenes*, the genes encoding this system were induced during heat-shock (54). BilE has been shown to play a role in *L. monocytogenes* bile resistance and virulence; increased sensitivity to bile and reduced virulence was observed in absence of *bilE* in a murine model (50). This system is transcribed from both a SigA and a SigB promoter (50), indicating an overlap between the SOS response and the SigB regulated class II stress response (23, 33). For *Salmonella enterica* and *Escherichia coli* it was shown previously that exposure to bile resulted in DNA damage and in induction of several LexA controlled DNA damage repair systems or error-prone DNA polymerases (29, 42, 43). Conceivably, activation of the BilE system as part of the SOS response may contribute to the survival capacity of *L. monocytogenes* during passage of the human GI tract. This novel finding may point to a role of the SOS response in the pathogenicity of *L. monocytogenes*.

Inhibition of cell division is a common phenomenon that has been associated with activation of the SOS response. Cell division in bacteria is initiated by accumulation of FtsZ at the mid-cell, and is a complex process involving many proteins. For several bacteria the products of a number of SOS response genes were found to inhibit this process. Such genes include *sulA* for *Escherichia coli* (27), *yneA* for *Bacillus subtilis* (32), Rv2719c for *Mycobacterium tuberculosis* (5), and *divS* for *Corynebacterium glutamicum* (40). These studies reported the occurrence of cell elongation as a consequence of this process. In a previous study, we found that *yneA* was up-regulated during heat-shock and that YneA had a potential role in cell elongation and cell division (54). This role of YneA was confirmed in this study. Induction of the SOS response by MMC exposure resulted in cell elongation of wild type cells, while this was not observed in the Δ*yneA* strain. Notably, cells of the latter mutant appeared to be more sensitive to heat-inactivation than the wild-type strain. The parameters involved in sensitization of the Δ*yneA* mutant to heat remain to be elucidated. However, we anticipate that it might be related to prevention of transection of the genome during replication fork stalling after heat exposure. This process allows bacteria to rescue their genome by re-initiation of chromosomal replication and segregation due to RecA-dependent activation of specific SOS response genes.

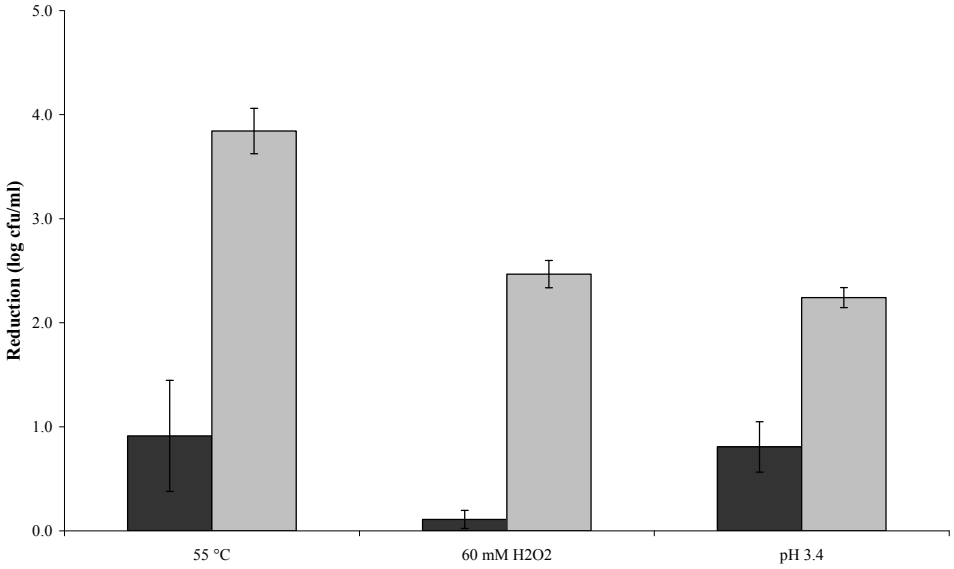

Fig. 6. RecA dependent stress resistance. The graph shows the reduction in cell counts of the wild-type (dark grey) and Δ*recA* (light grey) strain in BHI broth after 1 hour exposure at 55 ºC, 60 mM H_2O_2 or pH 3.4. Cell counts were made after 3 days incubation at 30 ºC.

One of the major functions of RecA is the activation of translesion DNA synthesis polymerases and DNA repair mechanisms (8, 25). Therefore, we investigated these specific functions of RecA in *L. monocytogenes*. RecA-dependent mutagenesis in *E. coli* is dependent on the derepression of genes encoding any of the alternative DNA polymerases Pol II (*polB*), Pol IV (*dinB*), or Pol V (*umuDC*) (22, 39). For *B. subtilis*, an additional Y-family polymerase DnaE was required (16, 52). The *L. monocytogenes* SOS response contains homologs of these genes, except for *polB*, suggesting that mechanisms involved in RecA-dependent mutagenesis are similar. Our results confirmed that RecA performs an important function in mutagenesis, as shown by the rifampicin and streptomycin resistant fractions of wild-type and Δ*recA* cultures. In the presence of RecA, rifampicin resistant mutants arose with a frequency of 10^{-7}, which was similar to the frequencies that were reported in previous studies for *L. monocytogenes* (3), *E. coli* (45), or *Streptococcus uberis* (56). The frequency of rifampicin resistant mutants in the Δ*recA* mutant was 14-fold lower than in the wild-type strain. Streptomycin resistant mutants were found with a frequency of 10^{-8} in the wild-type strain, while no resistant mutants were detected in the Δ*recA* mutant strain. Streptomycin resistant mutants were found at 10-fold lower frequencies than rifampicin resistant mutants. This lower frequency might be related to the occurrence of specific mutations in the *L. monocytogenes* genes *rpoB* and *rpsL*, which are required for resistance to the antibiotics rifampicin and streptomycin, respectively (26, 38).

A variety of stresses can iduce DNA damage (oxidative stress) or replication fork stalling (heat stress), indicating that RecA may play an important role in survival during stress exposure. Duwat et al. (17) showed that RecA of *L. lactis* is involved in survival of oxidative and heat stress. Furthermore, it was shown for *E. coli* that exposure to acidic pH could activate the SOS response (51), indicating a potential function of the SOS response in acid resistance. Our study revealed that RecA of *L. monocytogenes* is involved in stress resistance, because the wild-type strain showed higher survival after exposure to heat, oxidative, and acid stress than the Δ*recA* mutant strain. It was shown that YneA also contributes to heat-resistance, although its contribution is apparently not as prominent as that of other RecA-dependent factors (Fig. 4 and 6). The exact role of YneA in *L. monocytogenes* stress resistance remains to be elucidated.

In conclusion, the SOS regulon of *L. monocytogenes* was characterized and shown to contain genes encoding alternative DNA polymerases, DNA repair proteins, and a bile resistance system. Furthermore, our results showed that the SOS response of *L. monocytogenes* plays an important role in stress survival, mutagenesis and conceivably pathogenesis. These results indicate an important role for the SOS response in persistence of *L. monocytogenes* in the environment and in the host.

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8. General discussion and conclusions

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Introduction

Listeria monocytogenes is ubiquitously found in the environment and as a result raw materials used in the food industry could be contaminated with this bacterium. Furthermore, *L. monocytogenes* is able to form biofilms on surfaces of processing equipment, which could result in contamination of food products. Food manufacturers have to ensure that *L. monocytogenes* is absent in 25 g of food products that support growth, or that the food contains a maximum of 100 cfu/g at the moment of consumption. When the products do not support growth, a level of 100 cfu/g is tolerated (18). Due to consumer trends toward convenient fresh food products with a natural flavour and texture, a range of products rely on minimal preservation strategies, often referred to as "hurdle technology". *L. monocytogenes* might be able to survive some preservation steps used by the food industry to control food-borne pathogens, posing an increased risk for extended survival and/or growth of *L. monocytogenes* during storage. For this reason it is important to investigate the diversity in stress resistance and growth potential of the *L. monocytogenes* population during exposure to commonly used preservation conditions. It is known that mild stress conditions can trigger stress responses that might lead to increased survival during subsequent preservation steps. Therefore insight in the diversity and relevance of specific stress responses is required. In this thesis several approaches were used to study the diversity of the stress response of *L. monocytogenes* strains. This included a comparison of a large collection of wild-type strains for diversity in stress resistance, construction and screening of a mutant library for stress sensitive mutants, and transcription profiling of cells before and after stress exposure. The roles of various genes and regulation mechanisms in a number of stress responses were established. In particular, one mechanism referred to as the "SOS response" was investigated in more detail, because of its ability to generate genetic diversity. The role of the SOS response in *L. monocytogenes* stress resistance, generation of genetic diversity and virulence is discussed.

Diversity in stress resistance and growth limits

The complete genome sequences of *Listeria monocytogenes* strains with various serotypes have been determined. High synteny was observed at both the DNA and protein level (27, 50). Despite this high synteny, the stress resistance of different strains can vary significantly upon exposure to (combinations of) different stresses. Variation in stress resistance has been reported upon exposure to heat (Chapter 3), exposure to combinations of heat and lactic acid (35, 40), exposure to combinations of heat and bacteriocins (8), and exposure to combinations of acid and high salt concentrations (Chapter 2 and (4, 41)). The

differences in stress resistance of strains should be considered during selection of conditions and strains used in challenge testing. Strains from specific origins or with specific serotypes might be better able to grow and survive during exposure to certain stressful conditions. As shown in Chapter 3, experiments performed with the most resistant strains under certain stress condition will provide insight in worst case growth and survival conditions for *L. monocytogenes*.

It still remains a challenge to relate phenotypic differences in stress resistance between certain serotypes and strains to the presence of specific genes and their expression levels. Several attempts have been made to identify such genes or their products, as these could be used as indicators or "biomarkers" for the presence of certain *Listeria* strains with particular stress resistance phenotypes. Strains of lineage I (serotypes 1/2a, 3a, 1/2c, and 3c) contain a specific heat-shock system that increases their heat-resistance (60). Approximately 50% of the serotype 1/2 strains contain a specific decarboxylase system, which enhances the ability of growth at pH 5.1 (13) and lowers the growth limits at low pH in the presence of 1.0 M NaCl at 7 ºC (Chapter 2). This study also reveals the unique presence of a specific serine protease in all serotype 4b strains, which might enhance the ability of these strains to grow at low pH, high salt concentrations, and high temperatures. Further studies on this serine protease using genetic knock-out mutants indicated a role for this protease in growth at high temperatures. The temperature at which this mutant ceased to grow was 0.5 ºC lower than that of the wild-type strain (unpublished data). Another serotype specific gene is *bsh*, which encodes a bile salt hydrolase that favours gastrointestinal persistence (3, 55). This gene is specific for serotype 1/2 strains and is present at the same locus as the serotype 1/2 specific decarboxylase system (3).

L. monocytogenes likely harbours various other so far unidentified genetic traits that result in diversity in stress resistance. Such traits could for instance be introduced by plasmids or bacteriophages. Approximately 41% of the *L. monocytogenes* strains contain a plasmid (pLM80) of 82 kb (Chapter 2) and all strains sequenced up to date, appear to contain one to five prophage regions (7). Two serotype specific phages were isolated thus far from *L. monocytogenes* (29). Phage A118 and phage PSA show specificity for serotype 1/2a and serotype 4b, respectively, and have been completely sequenced (44, 61). Phages could influence stress resistance either directly by their encoded products or indirectly by changing the genetic structure after genomic insertion. The effect of phage insertions on stress resistance was exemplified by the finding that a mutant containing a vector insertion in the phage A118 region (lmo2302) was less able to grow at elevated temperatures and was more sensitive to heat-inactivation (Chapter 5). This might indicate that the presence of some of the phage A118 products enhance growth at high temperatures and induce heat-resistance. Targets for phage insertion in *L. monocytogenes* include the gene *comK* and several tRNA genes (27, 50). ComK is an important regulator in many bacteria, which

controls the expression of many genes involved in competence development, cell wall synthesis, and stress response (30). For *B. subtilis* it was shown that the SOS response was activated by ComK in a LexA independent manner (26). The role of ComK in *L. monocytogenes* remains to be elucidated. Bacteriophage A118 was furthermore found to harbour two genes that are regulated by the *L. monocytogenes* SOS response (Chapter 7). This indicates that these specific phage genes may be functional during the specific stress conditions that result in activation of the SOS response and provide additional protection or stimulate the increase of genetic diversity.

Another group of genes that show diverse presence between strains and serotypes are genes encoding cell wall modifying enzymes and cell wall components. The bacterial cell wall can play an important role as part of the primary defence during exposure to various stresses (Chapter 4 and (24, 32)). A high proportion of predicted proteins in *L. monocytogenes* are expressed as surface exposed proteins (7) and this bacterium contains the highest number of LPXTG surface proteins of all Gram-positive bacteria sequenced to date (5). Many of these surface proteins are internalins and the diversity of internalin distribution over the strains and serotypes is high (7, 27). Many of the internalin encoding genes show temperature dependent expression and both SigB and PrfA have been shown to regulate several of these (Chapter 4 and (46)), suggesting specific roles of the different internalins in specific conditions and/or environments such as the human host. Whether the diversity in internalins between strains and serotypes can be linked to functional differences in stress resistance and pathogenesis remains to be elucidated. However, a more detailed characterization of these genetic differences may allow for the use in strain or serotype identification or for selection of strains to be used in challenge tests.

Gene mutations and stress resistance

Over a hundred years ago Darwin postulated his evolution theory of selection of species (14). He suggested that variability was generated by stress and that the natural environment selects for beneficial mutations. However, this statement remains controversial, since it is difficult to determine whether stress generates variability or only allows for selection of mutants that arise independent of stress (53). The majority of mutations are harmful or non-favourable, and bacteria have evolved repair systems to cope with DNA damage (16). Bacteria are regularly exposed to adverse conditions that may induce DNA damage, such as high temperatures, high or low pH, UV light, and nutrient starvation. In response to such conditions, transcription patterns change to relieve unfavourable effects of these stresses. One of these responses is the SOS response, which encodes DNA repair mechanisms (45) and alternative lesion-bypass DNA polymerases that could introduce mutations due to their somewhat lower specificity (51). Increasing genetic

variation could be beneficial to obtain phenotypes that allow survival (20). This process of "mutagenesis", referred to as "adaptive mutagenesis", has been defined as a process that produces advantageous mutations during exposure to non-lethal stress, even though other, non-beneficial, mutations occur at the same time (9, 21).

Nowadays, more and more mechanisms are being identified that induce genetic changes under influence of stressful conditions, including starvation-induced or stationary phase mutagenesis (22, 37, 52, 54, 56), mutagenesis in aging colonies or resting organisms (6, 57, 58), and mutagenesis after exposure to antibiotics (10). Furthermore, adaptive mutagenesis or natural selection of resistant cells may occur in bacterial biofilms and some evidence exists that these biofilms are responsible for the persistence of adapted strains in the industry or in human infections (1, 12, 36). While the underlying mechanism of adaptation is not known exactly, biofilms are a source of natural diversity within bacterial populations (11).

All of the mechanisms leading to adaptive mutagenesis require one or more factors of the SOS response, in particular RecA or one of the alternative DNA polymerases (Pol II, Pol IV, or Pol V). Stresses that result in DNA damage or replication fork stalling (heat-shock, UV, high hydrostatic pressure, radiation) activate the SOS response, resulting in increased mutagenesis due to error-prone polymerases (Chapter 7 and (23, 34)). However, factors and mechanisms involved in the class II stress response and the class I and class III heat-shock response have been described to induce adaptive mutagenesis as well. For *Escherichia coli*, it was shown that the alternative sigma factor RpoS (SigB for Gram-positives), which is the regulator of the class II stress response (28, 31, 33), activates the gene encoding Pol IV (*dinB*) in the late-stationary phase (38). A closer look at the promoter region of *dinB* in *L. monocytogenes* revealed the presence of two potential SigB dependent promoters as well (unpublished data). Interaction between the SOS response and the heat-shock response has also been established in *L. monocytogenes* (Chapters 4 and 7). Previous studies in *E. coli* have shown that the heat-shock chaperone system GroE (*groESL*) interacts with the alternative DNA polymerases Pol IV (DinB) (39) and Pol V (UmuDC) (15, 43) and protects them from degradation. GroE deficient mutants only retained 10% of the wild-type Pol IV pool, resulting in reduced mutations (39). Furthermore, many SOS products showed instability and quick degradation by the heat-shock dependent Clp-proteases (47-49), resulting in active control of the SOS response by the heat-shock response. Mutations can also be induced by heat-shock proteins directly. Rearrangements of DNA repeats are dependent on the heat-shock chaperone DnaK (25). These results show that the class I and class III heat-shock responses and the class II stress response, which are induced during exposure to various stresses (Chapter 4 and (2, 17, 19, 42, 59)), could play an important role in adaptive mutagenesis. Induction of these responses or the SOS

response during mild food preservation could therefore potentially result in adapted strains which are more difficult to eradicate or have increased virulence potential.

Concluding remarks

This thesis describes several approaches to elucidate the function of stress resistance genes and mechanisms and their diversity in *L. monocytogenes* strains and serotypes, including a comparative analysis of wild-type strains and random and targeted deletion mutants. To obtain insight in the natural diversity of *L. monocytogenes* strains with respect to outgrowth under different stress conditions, a collection of 138 wild-type strains with diverse origins was tested under temperature stress, salt stress, lactate stress and pH stress. Importantly, serotype and niche-specific traits for the growth-limits were identified, indicating that certain strains or serotypes might have adapted to their environment. Furthermore, the European Union regulation for RTE food products that do not support growth of *L. monocytogenes* was adequate for all of the 138 tested strains, since none of these strains was able to grow at the conditions set in this regulation. Also a new criterion was identified. Based on our results a combination of sodium lactate and low pH, i.e., sodium lactate $\geq$ 2% and pH $\leq$ 5.2, efficiently prevented growth of all 138 *L. monocytogenes* isolates tested. However, more combinations of low pH and sodium lactate should be tested to elucidate the concentration of (undissociated) acid that prevents growth of *L. monocytogenes* before this criterion can be implemented. The complete collection was subsequently screened for the presence of genes in strains and serotypes with a link to stress resistance, which resulted in the identification of the two putative biomarkers ORF2110 and *gadD1T1*. Furthermore, a large collection of *L. monocytogenes* strains was screened for thermal inactivation at 55 ºC. More than 3 log-units difference in inactivation between these strains was found after 3 hours incubation in BHI broth. Thermal inactivation parameters were determined for two of these heat-resistant strains and a reference strain in BHI broth and in milk in the so-called "micro-heater", which mimics heat-inactivation in industrial heat-exchangers. The heat-resistant strains still showed significantly higher heat-resistance than the reference strain, but it should be noted that even the most heat-resistant *L. monocytogenes* strain was inactivated fully when applying normal and well performed pasteurization processes.

Screening of a random insertion mutant library of *L.* monocytogenes EGD-e for high temperature sensitive mutants resulted in the identification of 28 genes involved in growth at high temperatures. The relevance of two of the identified genes (*mogR* & *clpB*) was verified by constructing specific knock-out mutants. Experiments showed that *clpB*-expression (class III heat-shock) is repressed by MogR (Motility gene Repressor). In short, this work established MogR as the first motility regulator that controls a stress response

gene with a direct role in stress survival. These results established yet another overlap between different pathways of *L. monocytogenes* and showed the complex nature and coherence of these regulatory units.

Finally, to obtain insight in the stress response mechanisms that are important for heat-exposure whole genome expression profiles were analysed upon heat stress. Numerous differentially expressed genes were identified including the well-known class I and class III heat-shock genes, the SigB regulated class II stress genes, SOS-response genes, and numerous cell wall and cell division related genes. Additional experiments involving specific knock-out mutants of genes involved in the SOS-response (*recA* and *yneA*) were performed. These experiments identified a role for the SOS response in adaptive mutagenesis, stress survival, and virulence. These results are important because induction of the SOS response during food preservation might result in generation and subsequent selection of persistent *L. monocytogenes* variants in food processing environments.

In conclusion, this thesis describes the function and relevance of specific genes and stress response mechanisms for stress survival and provides insight in the diversity of stress resistance and growth limits of *L. monocytogenes*. These results increase our understanding of the activation and function of the complex stress response network in *L. monocytogenes* and how strains and serotypes adapt to conditions encountered during food preservation or in the human host.

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Samenvatting

De voedselpathogeen *Listeria monocytogenes* is een Grampositieve facultatief anaerobe bacterie die de ziekte listeriosis kan veroorzaken. De symptomen die met deze ziekte gepaard gaan zijn onder andere meningitis, encefalitis, sepsis en spontane abortus. In verscheidene Europese landen neemt de incidentie toe. *L. monocytogenes* komt overal in het milieu voor en is daarom in staat om ingredienten en procesapparatuur die gebruikt worden in de voedingsindustrie te besmetten. Omdat de vraag van consumenten naar minder geconserveerde en verse producten toeneemt worden de toegepaste conserveringscondities milder. *L. monocytogenes* kan zich aanpassen aan deze milde condities en daarna uitgroeien tijdens opslag van het voedsel. Uitbraken van listeriosis zijn geassocieerd met verschillende categorieën voedselproducten, zoals niet-gepasteuriseerde zuivelproducten, schaal- en schelpdieren, verse groentes en kant-en-klare vleesproducten. Met name voedselproducten die niet meer verder behandeld of gekookt dienen te worden voor consumptie of producten met een verlengde houdbaarheidsdatum die bereikt wordt door opslag onder gekoelde condities zijn geassocieerd met een verhoogd risico op *L. monocytogenes* besmetting en uitgroei. Verder kan deze bacterie relatief goed overleven en zelfs groeien onder conserveringsomstandigheden die afdoende zijn voor andere voedselpathogenen. Zo kan groei optreden bij lage pH, hoge zout concentraties en lage temperaturen.

L. monocytogenes stammen worden getypeerd op basis van aanwezigheid van bepaalde eiwitten in de celwand van de bacterie. Tot op heden zijn 13 verschillende serotypes van *L. monocytogenes* geïdentificeerd. De serotypen 1/2a, 1/2b en 4b zijn het meest gevonden, en meer dan 95% van de isolaten uit voedsel en patiënten behoort tot deze serotypes. Doorgaans zijn serotype 4b stammen het meest prominent aanwezig onder klinische isolaten terwijl de serotype 1/2a en 1/2b stammen meer worden aangetroffen in kant-en-klare voedselproducten. Deze verschillen zijn mogelijk gerelateerd aan genetische verschillen in stress tolerantie (mechanismen).

In dit proefschrift is meer inzicht verkregen in de functie van stress resistentie genen en stress mechanismen en in de diversiteit tussen *L. monocytogenes* stammen. De diversiteit in stress resistentie voor de *L. monocytogenes* populatie is onderzocht door de groeilimieten onder verschillende stress condities voor een grote collectie *L. monocytogenes* stammen te bepalen. Om inzicht te verkrijgen in de genen en mechanismen die een rol spelen in stress overleving is gebruik gemaakt van een specifieke stam (EGD-e) waarvan een genetische blauwdruk van het complete genoom bekend is. Van deze stam is een mutantenbank gecreëerd welke vervolgens is gescreend op stress gevoelige mutanten. Verder zijn transcriptieprofielen in deze stam vergeleken voor en na een stress blootstelling om vast te

stellen welke mechanismen specifiek worden geactiveerd tijdens blootstelling aan een bepaalde stress. Enkele specifieke mechanismen die belangrijk zijn voor stress overleving zijn vervolgens in meer detail bestudeerd.

Om inzicht te krijgen in het vermogen tot groei van *L. monocytogenes* stammen bij combinaties van verschillende zuurgraden en natriumlactaatconcenraties, hoge zoutconcentraties, en verschillende temperaturen is een collectie van 138 stammen getest. Deze collectie bevatte klinische isolaten en stammen geïsoleerd uit verschillende voedselbronnen waarvan de serotypes waren bepaald. Geen van de stammen was in staat om te groeien bij een pH $\leq$ 4,4, bij een $a_w \leq 0{,}92$ (ongeveer 13 % NaCl) of bij een combinatie van pH $\leq$ 5,0 en een $a_w \leq 0{,}94$ (+/- 10 % NaCl). Deze resultaten komen overeen met de criteria die door de Europese Unie zijn vastgesteld ten aanzien van *L. monocytogenes* voor kant-en-klare voedselproducten waarin deze bacterie niet kan groeien. Voor voedselproducten die voldoen aan deze eisen wordt 100 cfu/g product getolereerd, terwijl voor producten waarin *L. monocytogenes* wel kan groeien afwezigheid in 25 g wordt geëist. Verder is in dit proefschrift een nieuw criterium geïdentificeerd: bij een combinatie van minimaal 2% natriumlactaat en een pH $\leq$ 5,2 was geen van de 138 geteste stammen in staat om te groeien. Na clustering van de groeilimieten van stammen op basis van specifieke serotypes of op basis van de isolatiebron (bepaalde voedselproducten of de menselijke gastheer) zijn verschillen in groeilimieten geïdentificeerd. Deze verschillen kunnen belangrijk zijn voor het selecteren van de juiste stam voor het uitvoeren van stress experimenten. Verder is de hele collectie getest op de aanwezigheid van genen die een rol spelen bij stress resistentie. Zo zijn twee mogelijke “biomarkers” geïdentificeerd, namelijk een bepaald serotype 4b specifiek protease (ORF2110) en een glutamaat decarboxylase systeem (*gadD1T1*) dat een rol speelt bij het handhaven van een hoge intracelulair pH in zure omgevingen. Ten slotte is de hitteresistentie van een uitgebreide stammencollectie getest door blootstelling aan een temperatuur van 55 ºC. Na 3 uur blootstelling was meer dan 3 $\log_{10}$ units verschil in inactivatie tussen de verschillende stammen waarneembaar. De hitte-inactivatie van de twee meest hitte resistente stammen en een referentie stam zijn vervolgens nauwkeurig bepaald in een modelsysteem. Hieruit bleek dat niet alleen de referentie stam maar ook de meest hitte resistente stammen niet in staat zijn om een goed uitgevoerde pasteurisatiestap te overleven. Het is echter wel belangrijk om de temperatuur tijdens het pasteurizatieproces goed te bewaken, aangezien bij een daling van enkele graden (>4°C) er een mogelijkheid bestaat dat de meest hitte resistente stammen niet voldoende geinactiveerd worden en dus de behandeling overleven.

In dit proefschrift zijn 28 genen geïdentificeerd die belangrijk zijn voor groei bij verhoogde temperaturen door een mutantenbank van *L. monocytogenes* EGD-e te screenen op gevoeligheid voor groei bij verhoogde temperaturen. Deze genen coderen voor eiwitten met een functie in celwand synthese, in translatie en in stress resistentie. Sommige

mutanten laten een veranderde morfologie zien zoals verlengde cellen, kleinere cellen, of cellen met een sikkel vorm. De meeste mutanten zijn ook meer gevoelig voor hitte inactivatie. De relevantie van twee specifieke genen (*mogR* en *clpB*) is verder onderzocht door het maken van specifieke deletie mutanten. Deze experimenten lieten zien dat de expressie van het klasse III hitte schok gen *clpB* wordt onderdrukt door de mobiliteitsregulator MogR. Verder is aangetoond dat ClpB essentieel is voor de overleving van hitte en zeer belangrijk is voor de overleving van een blootstelling aan hoge druk.

Om inzicht te verkrijgen in de stress adaptatie mechanismen van *L. monocytogenes* na een hitte blootstelling zijn de transcriptieprofielen van cellen geanalyseerd voor en na een blootstelling. Verschillende genen en stress mechanismen vertoonden verhoogde expressie na de hitte blootstelling, waaronder de klasse I en klasse III hitte schok respons, de SigB gereguleerde klasse II stress respons, de SOS respons en veel genen die coderen voor eiwitten die deel uitmaken van de celwand of die een rol spelen bij celwandsynthese of tijdens celdeling. Verdere experimenten zijn uitgevoerd met specifieke deletie mutanten van genen uit de SOS response. De SOS response is een mechanisme dat over het algemeen wordt geactiveerd na DNA beschadiging of bij het optreden van problemen met de DNA replicatie vork. De SOS response bevat DNA herstel eiwitten en alternatieve DNA polymerases die beschadigd DNA kunnen herstellen en repliceren. De experimenten laten zien dat de SOS response een rol heeft in stress overleving, virulentie en het induceren van genetische diversiteit. Deze resultaten zijn belangrijk omdat de inductie van de SOS response tijdens blootstelling aan stress mogelijk resulteert in het genereren van stress resistente *L. monocytogenes* mutanten.

Dit proefschrift heeft nieuwe inzichten opgeleverd in de diversiteit in groeilimieten van *L. monocytogenes* stammen en de stress resistentie van deze bacterie. De verschillen in groeilimieten kunnen mogelijk verklaren waarom bepaalde serotypes meer worden aangetroffen onder klinische isolaten terwijl andere meer worden geïsoleerd uit specifieke voedselproducten. Daarnaast heeft dit werk meer inzicht opgeleverd in de functie en de complexiteit van het stress response netwerk van *L. monocytogenes*.

Nawoord

Na vier jaar hard werken is het dan eindelijk zover, mijn boekje is af! De tijd is echt voorbij gevlogen. Als ik terug kijk naar de afgelopen jaren is er echt ontzettend veel gebeurd, zowel in mijn privé leven als werkgerelateerd. Zo ben ik getrouwd met mijn allerliefste Jingjie, verhuisd naar een leuk appartementje in het centrum van Wageningen en heb zo ongeveer alle continenten bezocht tijdens de vele privé en werkgerelateerde reisjes, waarvan niet de minste mijn tweede bezoek aan China om onze bruiloft nog eens dunnetjes over te doen. Verder heb ik echt genoten van de vrijheid die ik kreeg om interessante resultaten binnen mijn onderzoek verder in detail uit te pluizen. Ik kan daarom ook iedereen die afstudeerd en wetenschappelijke interesse heeft aanraden om promotieonderzoek te doen.

Natuurlijk heb ik niet alles wat in dit boekje staat alleen voor elkaar gekregen. Ik wil beginnen met het bedanken van Marjon, mijn begeleider op het NIZO. Ik ben zeer blij dat jij de plek heb opgevuld die Jeroen achterliet na zijn vertrek naar de USA. De contacten met jou, in het begin bijna op dagelijkse basis aangezien jou bureau op ongeveer 3 meter van die van mij stond, heeft zeer stimulerend gewerkt in de ontwikkeling van mijn wetenschappelijke denken. Ook heb ik heel veel geleerd van jou over het schrijven van wetenschappelijke artikelen. Verder ben ik je zeer dankbaar voor je kritische blik op mijn schrijfwerk.

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List of Publications

van der Veen, S., T. Hain, J. A. Wouters, H. Hossain, W. M. de Vos, T. Abee, T. Chakraborty, and M. H. Wells-Bennik. 2007. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology **153:** 3593-3607.

van der Veen, S., R. Moezelaar, T. Abee, and M. H. J. Wells-Bennik. 2008 The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche specific traits. J Appl Microbiol **105:** 1246-1258.

van der Veen, S., T. Abee, W. M. de Vos, and M. H. J. Wells-Bennik. Genome-wide screen for *Listeria monocytogenes* genes important for growth at high temperatures. Submitted for publication.

van der Veen, S., A. Wagendorp, T. Abee, and M. H. J. Wells-Bennik. Diversity assessment of heat resistance of *Listeria monocytogenes* strains in a continuous flow heating system. Submitted for publication

van der Veen, S., I. K. H. Van Boeijen, W. M. de Vos, T. Abee, and M. H. J. Wells-Bennik. The *Listeria monocytogenes* motility regulator MogR controls expression of *clpB* and affects stress resistance. Submitted for publication.

van der Veen, S., S. van Schalkwijk, D. Molenaar, W. M. de Vos, T. Abee, and M. H. J. Wells-Bennik. The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. Submitted for publication.

Curriculum Vitae

Stijn van der Veen werd op 23 oktober 1978 geboren in het plaatsje Goirle, even ten zuiden van Tilburg. Hij heeft het VWO gedaan in Tilburg aan het Koning Willem II College. In 1997 is hij de studie bioprocestechnologie gaan volgen aan de Wageningen Universiteit met als specialisatie cellulair-moleculair. Hij heeft tijdens deze studie afstudeervakken gedaan aan de vakgroep Virologie en bij het Rikilt. Verder heeft hij een stage gedaan bij de University of Otago in Nieuw-Zeeland. In 2003 voltooide hij zijn studie waarna hij in 2004 is begonnen met zijn promotieonderzoek. Dit onderzoek is beschreven in dit proefschrift en werd uitgevoerd voor TI Food and Nutrition (voorheen WCFS) op het NIZO in samenwerking met de vakgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit. Het onderzoek werd begeleid op het NIZO door Dr. Ir. M. H. J. Wells-Bennik en aan de Wageningen Universiteit door Prof. Dr. T. Abee en Prof. Dr. W. M. de Vos. Aansluitend aan het promotieonderzoek is hij in 2008 begonnen als post-doc aan de vakgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit voor TI Food and Nutrition onder de leiding van Prof. Dr. T. Abee en Dr. R. Moezelaar.

VLAG graduate school activities

Discipline specific activities

Courses

Genetics and physiology of food-associated micro-organisms , VLAG, 2004
Systems biology: "Principles of ~omics data analysis", VLAG, 2005
Radiation expert 5B, Larenstein, Wageningen, 2004
GFP & Luc workshop, EPS, 2005
Working visit at University of Giessen, University of Giessen, Germany, 2005

Meetings

Isopol conference, Uppsala, Sweden, 2004
Food Microbiology conference, Bologna, Italy, 2006
IDF dairy conference, Quebec, Canada, 2008
GRC conference on Microbial stress response, Mount Holyoke, USA, 2008

General courses

VLAG PhD week, 2004
Techniques for Writing and Presenting a Scientific Paper, WGS, 2006

Optionals

Preparation PhD research proposal, 2004
PhD study tour, South-Africa, 2005
PhD study tour, California, USA, 2006
Microbiology / molecular biology discussion group, Nizo, 2004-2008
WCFS / TIFN days, 2004-2008
WCFS / TIFN C009 project meetings, 2004-2008

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