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EPIDEMIOLOGY AND STRESS RESPONSES OF *LISTERIA MONOCYTOGENES*

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ACADEMIC DISSERTATION

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Cover picture: *Listeria monocytogenes* EGD-e. Electron microscopic image by Esa Penttinen.

ABSTRACT

Listeria monocytogenes causes potentially fatal illness to susceptible people and is found in various foods. It typically enters processed foods via a contaminated processing environment, in which it may have persisted for years. To study the role of raw material as a potential source of contamination of food processing plants by *L. monocytogenes*, the prevalence and genetic diversity of this species in tonsils of pigs and in raw fish was examined. A total of 14% and 4% of tonsils of pigs and raw fish, respectively, harboured *L. monocytogenes*. From 38 pig tonsil isolates and 11 raw fish isolates, 24 and nine different types were recovered using pulsed-field-gel electrophoresis (PFGE) typing. The results indicate that a wide variety of *L. monocytogenes* strains enters pork slaughterhouses and fish processing plants in the raw materials, which are thus potential sources of direct or indirect contamination of processing plants by this pathogen.

Since identical PFGE types were recovered from both raw and processed fish, it is likely that raw fish are an initial source of the *L. monocytogenes* found in processed fish. Some strains entering a plant along with raw fish may contaminate and persist in the processing environment, causing recurrent contamination of the final products via contact surfaces. Alternatively, *L. monocytogenes* strains in raw fish may survive non-listericidal processes, resulting in contamination of the final product.

To identify novel factors contributing to survival of *L. monocytogenes* in food processing environment, the roles of specific genes in stress response were investigated, using *flhA* and *motA* that encode flagellar factors involved in cold stress tolerance, and *lmo0866, lmo1246, lmo1450*, and *lmo1722* encoding DEAD-box RNA helicases involved in cold, heat acid, alkali, osmotic, ethanol, and oxidative stress tolerance. Increased relative transcription levels of *flhA, motA, lmo0866, lmo1450,* and *lmo1722,* restricted growth of the single gene deletion mutant strains EGD-eΔ*flhA,* EGD-eΔ*motA,* Δ*lmo0866,* Δ*lmo1450,* and Δ*lmo1722* at 3°C, and increased minimum growth temperatures of Δ*lmo0866,* Δ*lmo1450,* and Δ*lmo1722* revealed that FlhA, MotA, Lmo0866, Lmo1450, and Lmo1722 had roles in growth of *L. monocytogenes* EGD-e under cold stress conditions. The restricted growth of Δ*lmo0866* in 3.5% ethanol, and its increased maximum growth temperature and growth rate at 42.5°C, indicated that Lmo0866 had roles also in ethanol and heat stress tolerance of strain EGD-e. The role of Lmo1450 in the growth of strain EGD-e under heat, alkali, and oxidative stress conditions was shown by the restricted growth rate of Δ*lmo1450* at 42.5°C, in pH 9.4, and in 5 mM H_2O_2. The slightly decreased growth rate and maximum optical density of Δ*lmo1246* at 3°C indicated that the role of Lmo1246 in cold stress tolerance was negligible. Under all the other conditions, the growth of Δ*lmo1246* and the wild-type EGD-e were identical,

suggesting that Lmo1246 had no role in growth of *L. monocytogenes* EGD-e under heat, pH osmotic, ethanol, or oxidative stress conditions.

The deletion of *flhA, motA, lmo0866, lmo1450,* and *lmo1722* impaired the motility of strain EGD-e, whereas the motility of Δ*lmo1246* did not differ from that of the wild type. This indicates that DEAD-box RNA helicases Lmo0866, Lmo1450, and Lmo1722 have roles in motility of strain EGD-e. Moreover, these results suggest that motility and cold stress tolerance of *L. monocytogenes* are linked, and that motile flagella may be needed for full cold stress tolerance of strain EGD-e.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications referenced in the text by their Roman numerals:

I Autio, T., Markkula, A., Hellström, S., Niskanen, T., Lundén, J., and Korkeala, H. (2004) Prevalence and genetic diversity of *Listeria monocytogenes* in the tonsils of pigs. *J Food Prot* **67**: 805–808.

II Markkula, A., Autio, T., Lundén, J., and Korkeala, H. (2005) Raw and processed fish show identical *Listeria monocytogenes* genotypes with pulsed-field gel electrophoresis. *J Food Prot* **68:** 1228–1231.

III Mattila, M., Lindström, M., Somervuo, P., Markkula, A., and Korkeala, H. (2011) Role of *flhA* and *motA* in growth of *Listeria monocytogenes* at low temperatures. *Int J Food Microbiol* **148:** 177–183.

IV Markkula, A., Mattila, M., Lindström, M., and Korkeala, H. (2012) Genes encoding putative DEAD-box RNA helicases in *Listeria monocytogenes* are needed for growth and motility at 3˚C. *Environ Microbiol* **14:** 2223–2232.

V Markkula, A., Lindström, M., Johansson, P., Björkroth, J., and Korkeala, H. (2012) Roles of four putative DEAD-box RNA helicase genes in growth of *Listeria monocytogenes* EGD-e under heat, pH, osmotic, ethanol, and oxidative stress conditions. *Appl Environ Microbiol* **78:** 6875–6882.

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ABBREVIATIONS

a_w	water activity
ADI	arginine deimidase
AFLP	amplified fragment length polymorphism
BHI	brain heart infusion
bp	base pair
cDNA	complementary DNA
GAD	glutamate decarboxylase
HK	histidine kinase of two-component signal transduction system
LB	Luria-Bertani
OD_{600}	optical density at 600 nm
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
(p)ppGpp	guanosine pentaphospate
ROS	reactive oxygen species
RR	response regulator of two-component signal transduction system
RT	reverse transcription
RTE	ready-to-eat
SOD	superoxide dismutase
TCS	two-component signal transduction system
TSA	tryptic soy agar

1 INTRODUCTION

The history of the zoonotic foodborne pathogen *Listeria monocytogenes* and listeriosis is traced to 1910, when a gram-positive bacterium was isolated from the necrotic liver of a rabbit in Sweden (Hülpfers, 1911). In 1918 the bacterium, albeit wrongly identified, was isolated from a human patient with meningitis (Dumont & Cotoni, 1921). The description of the bacterium, named as *Bacterium monocytogenes,* was published by Murray *et al.* in 1926. The name *Listeria monocytogenes* was established in 1940 (Pirie, 1940). Even though food as a significant source of listeriosis was suspected already in 1961 by Seeliger, the emergence of *L. monocytogenes* as an important foodborne pathogen became evident in 1980s, after several foodborne outbreaks proved to be caused by it (Schlech *et al.*, 1983; Fleming *et al.*, 1985; Linnan *et al.*, 1988).

In Europe, Australia, and the USA, approximately 0.3 listeriosis cases per 100 000 population are reported annually and the worldwide incidence seems to be increasing (Denny & McLauchlin, 2008; OzFoodNet Working Group, 2010; Dalton *et al.*, 2011; Silk *et al.*, 2012). In Finland, the incidence of listeriosis is clearly higher. Between 2008 and 2012, the incidence varied from 0.64 to 1.33 cases per 100 000 population, resulting in listeriosis in 34–71 citizens annually (Terveyden ja hyvinvoinnin laitos, THL, 2012). Even though listeriosis, mainly affecting people with another severe underlying disease, is rare compared to many other foodborne diseases, its severity and the economic losses resulting from massive recalls of contaminated foods make *L. monocytogenes* one of the most important foodborne pathogens in industrialized countries nowadays (Mead *et al.*, 1999; de Valk *et al.*, 2005; Vaillant *et al.*, 2005; Scallan *et al.*, 2011).

Foods may be contaminated with *L. monocytogenes* already before harvest, but the main source of contamination of processed foods has been reported to be endogenous strains that may have persisted at food-processing plants for years (Ericsson *et al.*, 1997; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Fonnesbech Vogel *et al.*, 2001; Keto-Timonen *et al.*, 2007). Detergents and disinfectants used for sanitizing the processing environment as well as processing, preservatives, additives, and storage of foods, may cause stress to the bacterium. Survival under these stresses improves its persistence in food processing environment and increases its occurrence in foods. Especially its ability to tolerate cold stress and thus grow in refrigerated conditions threatens the hygienic quality of foods stored for extended periods at low temperatures (Junttila *et al.*, 1988; Nolan *et al.*, 1992; Hudson *et al.*, 1994).

To efficiently control *L. monocytogenes* and decrease the number of listeriosis cases, the contamination routes in the food chain need to be identified. The aim of this study was to examine the role of raw material as a

source of *L. monocytogenes* in food-processing plants. Moreover, the role of flagellar factors FlhA and MotA, and DEAD-box RNA helicases Lmo0866, Lmo1246, Lmo1450, and Lmo1722 in the tolerance of *L. monocytogenes* EGD-e to various stresses the bacterium may meet in food chain was studied. Understanding mechanisms behind stress tolerance may provide new insight into the control of *L. monocytogenes* and listeriosis.

2 REVIEW OF THE LITERATURE

2.1 *Listeria monocytogenes* and listeriosis

The foodborne zoonotic pathogen, *L. monocytogenes*, is a mesophilic, facultatively anaerobic, gram-positive bacterium widespread in nature. It is able to grow in a wide temperature, pH, and water activity (a_w) range, whereas heating to 60˚C for 30 min kills it (Gray & Killinger, 1966; Nolan *et al.*, 1992; Hudson *et al.*, 1994; McLaughlin & Rees, 2009). As an opportunistic pathogen, *L. monocytogenes* causes a disease, listeriosis, mainly in susceptible people or animals with increased risk because of severe underlying disease or pregnancy.

2.1.1 *Listeria monocytogenes*

L. monocytogenes belongs to the family *Listeriaceae* in the class *Bacilli* of the phylum *Firmicutes,* together with *L. innocua, L. ivanovii, L. seeligeri, L. welchimeri, L. grayi* (McLaughlin & Rees, 2009)*,* and four novel species *L. rocourtiae* (Leclercq *et al.*, 2010), *L. marthii* (Graves *et al.*, 2010), *L. fleischmannii* (Bertsch *et al.*, 2013), and *L. weihenstephanensis* (Lang Halter *et al.,* 2013). All *Listeria* species are small, regular rods, 0.5 μm in diameter and 1–5 μm in length that do not form spores or capsula. They produce catalase but not oxidase. Except for *L. fleischmannii, Listeria* spp. are motile with peritrichous flagella under 30˚C (McLaughlin & Rees, 2009; Graves *et al.*, 2010; Leclercq *et al.*, 2010; Bertsch *et al.*, 2013; Halter *et al.,* 2013). *L. monocytogenes* is β-haemolytic and ferments rhamnose but not xylose (McLaughlin & Rees, 2009).

Based on cell surface antigens, *L. monocytogenes* strains are divided into 13 serotypes (McLaughlin & Rees, 2009). To further characterize it, genotyping methods, for example pulsed-field gel electrophoresis (PFGE) typing, ribotyping, and amplified fragment length polymorphism (AFLP) typing that are based on differences in DNA sequences can be used. PFGE, ribo and AFLP types are grouped into four genetic lineages with varying distribution and properties of *L. monocytogenes* (Nightingale, 2010) (Table 1).

L. monocytogenes has been found in decaying vegetation, cultivated and non-cultivated fields, forests, aquatic environments, food, feed, animal and human faeces, and food-processing environments (Weis & Seeliger, 1975; Low & Donachie, 1997; Giovannacci *et al.*, 1999; Chasseignaux *et al.*, 2001; Gudbjörnsdóttir *et al.*, 2004; Berzins *et al.*, 2010). Growth has been reported to occur in temperatures from -1.5˚C to approximately 45˚C, at pH of 4.3–9.6, and in a_w down to 0.90 (Gray & Killinger, 1966; Nolan *et al.*, 1992; Hudson *et al.*, 1994). It grows well in aerobic conditions, but restriction of

oxygen enhances its growth (Lungu *et al.*, 2009). Despite its tolerance to wide ranged temperature, pH, and a_w, *L. monocytogenes* is relatively fastidious in nutrient requirements and competing microbes inhibit its growth (Premaratne *et al.*, 1991; Al-Zeyara *et al.*, 2011).

Table 1. Distribution of clinical and environmental *Listeria monocytogenes* isolates between genetic lineages.

Genetic lineage	Serotype	Distribution
I	1/2a, 1/2c, 3a, 3c	Dominates among foods and food-associated environments. Dominates as a cause of sporadic listeriosis cases in several European countries and in Canada.
II	1/2b, 3b, 4b, 4d, 4e	Serotypes 1/2b and 4b are responsible for most listeriosis outbreaks worldwide. Dominates as a cause of sporadic listeriosis cases in the USA and Taiwan.
III	4a, 4b, 4c	Rare isolates. More common as causes of animal than human listeriosis.
IV	4a, 4b, 4c, 7	Rare isolates. More common as causes of animal than human listeriosis.

Derived from Doumith *et al.* (2004), Liu *et al.* (2006a), Clark *et al.* (2010), Nightingale (2010), Orsi *et al.* (2011), and Pontello *et al.* (2012).

2.1.2 Listeriosis

In susceptible people and animals *L. monocytogenes* can cause a life-threatening, invasive disease (Vázquez-Boland *et al.*, 2001; Silk *et al.*, 2012). The main predisposing factor is a decrease in cell-mediated immunity because of underlying disease or pregnancy, and the risk of listeriosis is increased also in neonates and the elderly (Wilesmith & Gitter, 1986; Unanue, 1997; Painter & Slutsker, 2007; Denny & McLauchlin, 2008; Dalton *et al.*, 2011; Silk *et al.*, 2012). About 20% of invasive listeriosis cases are fatal (Vázquez-Boland *et al.*, 2001; Silk *et al.*, 2012). In immunocompetent adults, the disease is rare and symptoms are typically mild.

Human listeriosis

In humans, 99% of listeriosis cases are foodborne (Mead *et al.*, 1999). Most cases are sporadic, leading to meningitis, encephalitis, sepsis, and abortion, and reported in people with another severe underlying disease (Denny & McLauchlin, 2008; Dalton *et al.*, 2011; Pouillot *et al.*, 2012; Silk *et al.*, 2012). Physiological reduction in cell-mediated immunity in pregnant women may result in listeriosis with influenza-like symptoms and miscarriages (Silver, 1998). In people with no predisposing factors, invasive listeriosis is rare, and the most typical symptom is mild gastroenteritis with fever, headache, nausea, diarrhoea, and abdominal pain (Miettinen *et al.*, 1999b; Ooi &

Lorber, 2005; Goulet *et al.*, 2012). Cutaneous and eye infections have rarely been reported, mainly in farmers and veterinarians in direct contact with afterbirths and infected foetuses (McLauchlin & Low, 1994; Regan *et al.*, 2005; Tay *et al.*, 2008). About 1% of asymptomatic humans occasionally excrete *L. monocytogenes* in their feces (Lamont & Postlethwaite, 1986; Grif *et al.*, 2001, 2003).

Vehicles of sporadic listeriosis cases usually remain unknown, at least partly because of the time lag between consumption of contaminated food and the appearance of symptoms, along with host-specific differences in sensitivity to listeriosis. In listeriosis outbreaks, the most commonly incriminated vehicles have been ready-to-eat (RTE) meat and dairy products (Lundén *et al.*, 2004; Swaminathan & Gerner-Smidt, 2007). Contaminated sandwiches were suspected to have caused several small outbreaks (Centers for Disease Control and Prevention, 2011; Gaul *et al.*, 2012; Little *et al.*, 2012; Yde *et al.*, 2012). One of the largest listeriosis outbreaks, affecting 147 persons in 2011, was caused by contaminated cantaloupe (Centers for Disease Control and Prevention, CDC, 2013).

The development of listeriosis seems to be dose-dependent (Miettinen *et al.*, 1999b; Maijala *et al.*, 2001; Ooi & Lorber, 2005). Prolonged consumption of food contaminated with as few as 14—2.2 x 10^3 colony forming units of *L. monocytogenes* per day may be sufficient to infect susceptible people (Maijala *et al.*, 2001). The clinical symptoms of invasive listeriosis typically begin 20—30 days after the ingestion, even though incubation period can be up to 72 days (Linnan *et al.*, 1988; Vázquez-Boland *et al.*, 2001). In non-susceptible people, food containing 1.9 x 10^5 colony forming units/g has been reported to cause gastroenteritis, although clearly higher infectious doses have also been reported (Dalton *et al.*, 1997; Miettinen *et al.*, 1999b; Vázquez-Boland *et al.*, 2001; Ooi & Lorber, 2005). Symptoms of gastroenteritis typically begin 24 hours after ingestion of the bacterium (Miettinen *et al.*, 1999b; Vázquez-Boland *et al.*, 2001; Ooi & Lorber, 2005).

In the stomach, the bacterium is exposed to low gastric pH, which reduces the number of viable cells (Schlech *et al.*, 1993; Vázquez-Boland *et al.*, 2001). The surviving cells pass into the intestine, passively cross the intestinal wall, proliferate mainly in Payers patches, and spread to neighbouring enterocytes basolaterally (Pron *et al.*, 1998; Vázquez-Boland *et al.*, 2001; Ooi & Lorber, 2005). The massive invasion of *L. monocytogenes* to epithelial cells is thought to cause the symptoms of gastroenteritis (Vázquez-Boland *et al.*, 2001; Ooi & Lorber, 2005). Following passage through the intestinal barrier, the bacterium enters the liver and, less extensively, the mesenteric lymph nodes and spleen through the lymph and blood (Marco *et al.*, 1992; Pron *et al.*, 1998; Vázquez-Boland *et al.*, 2001; Melton-Witt *et al.*, 2012). Kuppfer cells destroy most of the *L. monocytogenes* cells, and surviving cells start to proliferate and spread into hepatocytes (Cheers *et al.*, 1978; Ebe *et al.*, 1999; Gregory & Liu, 2000). In healthy humans, the immune system destroys *L. monocytogenes* in the liver (Cheers *et al.*, 1978; Mielke *et al.*, 1988; Gregory

& Liu, 2000). Disturbed cell-mediated immunity may enable the passage of the bacterium from the liver to the central nervous system and placenta, leading to the appearance of symptoms of invasive listeriosis (Cheers *et al.*, 1978; Mielke *et al.*, 1988; Gregory & Liu, 2000; Vázquez-Boland *et al.*, 2001).

Exceptionally, other *Listeria* spp. have been reported to cause human listeriosis (Perrin *et al.*, 2003; Rapose *et al.*, 2008; Guillet *et al.*, 2010). In these cases, symptoms have been similar to those of invasive listeriosis cases caused by *L. monocytogenes* (Rocourt *et al.*, 1986; Vázquez-Boland *et al.*, 2001; Perrin *et al.*, 2003; Rapose *et al.*, 2008; Guillet *et al.*, 2010).

Listeriosis in animals

Listeriosis has been detected in nearly all domestic animals (Gray & Killinger, 1966). Most listeriosis cases have been reported in sheep, among which *L. ivanovii* is also a significant cause of listeric infections, and also cows and goats, causing encephalitis, abortion, or septicemia (Beauregard & Malkin, 1971; Wilesmith & Gitter, 1986; Alexander *et al.*, 1992; Low & Donachie, 1997; Chand & Sadana, 1999; Wesley *et al.*, 2002). In sheep and cows, subclinical mastitis and gastroenteritis caused by *L. monocytogenes* have also been reported (Jensen *et al.*, 1996; Clark *et al.*, 2004; Rawool *et al.*, 2007).

In monogastric animals, listeriosis is rare, and large epidemics with generalized listeriosis and acute deaths have been reported only in farmed chinchillas (Finley & Long, 1977; Wilkerson *et al.*, 1997; Wesley, 2007). In swine, the primary manifestation of listeriosis is septicemia, whereas in horses, abortions and encephalitis are also typical (Wesley, 2007). Listeriosis of fowls is probably secondary to viral infections, and typically causes septicemia with accompanying cardiac lesions (Cummins *et al.*, 1988; Wesley, 2007).

Pathogenesis of *Listeria* spp. in fish differs from that in other animals. *L. monocytogenes* is less pathogenic to fish than to mice, wherease *L. innocua, L. ivanovii, L. seeligeri,* and *L. welchimeri,* which have little or no virulence in mammals, are virulent in fish (Menudier *et al.*, 1996).

In livestock, listeriosis is associated with indoor housing and consumption of bad quality feed, especially silage (Wilesmith & Gitter, 1986; Wiedmann *et al.*, 1996; Wesley, 2007). The sensitivity of pregnant animals to listeriosis has led to epidemics in which the only symptoms were abortions (Wilesmith & Gitter, 1986).

Symptomless faecal carriage of *L. monocytogenes* has been reported in primates, other mammals, and birds (Husu, 1990; Iida *et al.*, 1991; Miettinen & Wirtanen, 2006; Ho *et al.*, 2007; Lyautey *et al.*, 2007; Hellström *et al.*, 2008; Esteban *et al.*, 2009). The highest prevalence, up to 30%, has been reported in cattle followed by other ruminants, whereas companion animals seldom carry this species (Embil *et al.*, 1984; Husu, 1990; Iida *et al.*, 1991; Lyautey *et al.*, 2007; Esteban *et al.*, 2009).

2.2 *L. monocytogenes* in foods and in food-processing environments

This bacterium has been found in numerous raw and processed foods. The presence of low numbers of *L. monocytogenes* in fresh produce or in products that are cooked before consumption is considered as safe. Contamination of RTE foods that enable its growth during a long shelf life may pose a risk of listeriosis to consumers.

Contamination of processed foods is typically caused by strains that have persisted in the processing environment for extended periods (Lawrence & Gilmour, 1995; Rørvik *et al.*, 1995; Autio *et al.*, 1999; Giovannacci *et al.*, 1999; Miettinen *et al.*, 1999a). It is unclear how *L. monocytogenes* strains enter the plant and why some strains persist in food processing environments.

2.2.1 *L. monocytogenes* in foods

The prevalence of *L. monocytogenes* in carcasses and raw foods varies greatly (Table 2). The highest prevalences have been reported in poultry carcasses and poultry meat, whereas in most studies it is relatively seldom found in beef and pork carcasses, raw milk, and fresh produce. The most commonly contaminated RTE foods of animal origin have been reported to be fermented, air-dried, or cold-smoked meat and fish products that have not undergone heat treatment that reduces the occurrence of the bacterium (Table 3).

The prevalence of *L. monocytogenes* in foods increases during processing (Eklund *et al.*, 1995; Lawrence & Gilmour, 1995; Rørvik *et al.*, 1995; Autio *et al.*, 1999; Berzins *et al.*, 2010). Several studies have shown that the main strains contaminating the final products originate from the processing environments and are different from the strains present in raw material (Lawrence & Gilmour, 1995; Rørvik *et al.*, 1995; Autio *et al.*, 1999; Giovannacci *et al.*, 1999). Brining by injection has been associated with *L. monocytogenes* contamination of cold-smoked pork (Berzins *et al.*, 2007). Complex processing machines in contact with large product surfaces, including cutting, brining, and slicing machines, efficiently spread the contamination to the many products of a particular plant (Autio *et al.*, 1999; Berzins *et al.*, 2010). When the process does not include heat treatment, raw material may also serve as a direct source of contamination of the final products (Keto-Timonen *et al.*, 2007).

Table 2. Prevalence of *Listeria monocytogenes* in carcasses and raw foods in Europe.

Sample	No. of positive samples/total (%)	Country of origin	Reference
Carcasses			
Beef	10/406 (2)	Poland	Wieczorek *et al.*, 2012
	1/14 (7)	Italy	Peccio *et al.*, 2003
	0/200 (0)	Ireland	Madden *et al.*, 2001
	11/50(22)	Belgium	Korsak *et al.*, 1998
	0/20 (0)	The Netherlands	van den Elzen & Snijders, 1993
Pork	2/359 (1)	Finland	Hellström *et al.*, 2010
	6/50 (12)	Finland	Autio *et al.*, 2000
	1/49 (2)	Belgium	Korsak *et al.*, 1998
	0/960 (0)	Sweden, Norway	Nesbakken *et al.*, 1994
	4/90 (4)	The Netherlands	van den Elzen & Snijders, 1993
Poultry	38/100 (38)[a]	Greece	Sakaridis *et al.*, 2011
	75/150 (50)	Norway	Rørvik *et al.*, 2003
	15/100 (15)	Spain	Capita *et al.*, 2001
	152/635 (24)	Belgium, France	Uyttendaele *et al.*, 1997
	103/320 (32)	Denmark	Ojeniyi *et al.*, 1996
Raw meat			
Beef	3/190 (2)	Germany	Meyer *et al.*, 2011
	8/125 (6)	Bulgaria	Karakolev, 2009
	12/94 (13)	The Netherlands	van den Elzen & Snijders, 1993
Pork	6/179 (3)	Germany	Meyer *et al.*, 2011
	6/122 (5)	Bulgaria	Karakolev, 2009
	41/121 (34)	France	Thevenot *et al.*, 2005
	13/34 (38)	Greece	Samelis, 1999
	107/296 (36)	The Netherlands	van den Elzen & Snijders, 1993
Poultry	167/231 (72)	Estonia	Praakle-Amin *et al.*, 2006
	57/158 (36)	Spain	Vitas & Garcia-Jalon, 2004
	48/95 (51)	Norway	Rørvik *et al.*, 2003
	14/80 (18)	Ireland	Soultos *et al.*, 2003
	38/61 (62)	Finland	Miettinen *et al.*, 2001
	13/17 (76)	Greece	Samelis, 1999
	112/410 (27)	Belgium, France	Uyttendaele *et al.*, 1997
Raw fish	15/103 (15)[b]	Finland	Miettinen & Wirtanen, 2005
	15/33 (35)	UK	Dauphin *et al.*, 2001
	16/217 (7)	Norway, Faroe Islands	Fonnesbech Vogel *et al.*, 2001
	0/26 (0)	Italy	Pourshaban *et al.*, 2000
	1/60 (2)	Finland	Autio *et al.*, 1999
Raw milk			
Cow	10/183 (5)	Finland	Ruusunen *et al.*, 2013
	25/1459 (2)	France	Meyer-Broseta *et al.*, 2003
	3/294 (4)	Sweden	Waak *et al.*, 2002
	28/774 (1)	Spain	Gaya *et al.*, 1998
Fresh produce	0/314 (0)	Austria	Wagner *et al.*, 2007
	21/720 (2)	Ireland	Francis & O'Beirne, 2006
	88/2950 (3)	UK	Sagoo *et al.*, 2003
	2/3200 (0.06)	UK	Sagoo *et al.*, 2001
	82/350 (23)	Denmark	Nørrung *et al.*, 1999

[a]Each sample contained neck skins from three carcasses.

[b]Each sample contained five fish.

Table 3. Prevalence of *Listeria monocytogenes* in ready-to-eat meat, fish and dairy products in Europe.

Sample	No. of positive samples/total (%)	Country	Reference
Meat products			
Heat-treated	5/460 (1)	Sweden	Lambertz *et al.*, 2012
	0/65 (0)	Latvia	Berzins *et al.*, 2009
	3/166 (2)	Spain	Cabedo *et al.*, 2008
	13/139 (9)	Greece	Angelidis & Koutsoumanis, 2006
	15/255 (6)	Switzerland	Jemmi *et al.*, 2002
	658/7581 (9)	Denmark	Nørrung *et al.*, 1999
Fermented, air-dried	23/112 (21)	Italy	Di Pinto *et al.*, 2010
or cold-smoked[a]	37/117 (32)	Latvia	Berzins *et al.*, 2009
	120/312 (38)	Latvia and Lithuania	Berzins *et al.*, 2007
	4/49 (8)	Greece	Angelidis & Koutsoumanis, 2006
	25/274 (9)	Switzerland	Jemmi *et al.*, 2002
	134/685 (20)	Denmark	Nørrung *et al.*, 1999
Poultry products			
Heat-treated	0/5 (0)	Latvia	Berzins *et al.*, 2009
	7/103 (7)	Spain	Cabedo *et al.*, 2008
	0/25 (0)	Greece	Angelidis & Koutsoumanis, 2006
	4/55 (7)	Denmark	Ojeniyi *et al.*, 2000
	17/528 (3)	UK	Nichols *et al.*, 1998
Fish products			
Hot-smoked	2/113 (2)	Sweden	Lambertz *et al.*, 2012
	57/471 (12)	Switzerland	Jemmi *et al.*, 2002
	1/48 (2)	Finland	Johansson *et al.*, 1999
Cold-smoked	32/206 (16)	Sweden	Lambertz *et al.*, 2012
	104/1010 (10)	France	Beaufort *et al.*, 2007
	114/814 (14)	Switzerland	Jemmi *et al.*, 2002
	46/356 (13)	Finland	Hatakka *et al.*, 2002
	10/232 (4)	Finland	Hatakka *et al.*, 2001
	59/1000 (6)	Denmark	Fonnesbech Vogel *et al.*, 2001
	38/170 (22)	Spain	Dominguez *et al.*, 2001
Gravad	28/194 (14)	Sweden	Lambertz *et al.*, 2012
	5/82 (6)	Finland	Hatakka *et al.*, 2001
	16/32 (50)	Finland	Johansson *et al.*, 1999
	51/176 (29)	Denmark	Jørgensen & Huss, 1998
Dairy products			
Cheese	2/518 (0.4)	Sweden	Lambertz *et al.*, 2012
	1/73 (1)	Norway	Jakobsen *et al.*, 2011
	2/294 (0.7)	Italy	Di Pinto *et al.*, 2010
	47/2132 (2)	Italy	Prencipe *et al.*, 2010
	1/287 (0.3)	Spain	Cabedo *et al.*, 2008
Ice cream	6/1129 (0.5)	Finland	Miettinen *et al.*, 1999
	2/150 (2)	UK	Greenwood *et al.*, 1991
	0/82 (0)	Spain	Cabedo *et al.*, 2008

[a]Treatments do not contain heating to listericidal temperature.

2.2.2 *L. monocytogenes* in food-processing environments

In addition to raw and processed foods, equipment, contact and noncontact surfaces, and protecting clothing of employees in various food-processing plants have been proven to be contaminated with *L. monocytogenes* (Autio *et al.*, 1999; Giovannacci *et al.*, 1999; Miettinen *et al.*, 1999a; Chasseignaux *et al.*, 2001; Keto-Timonen *et al.*, 2007; Berzins *et al.*, 2010). The most heavily contaminated objects have been complex equipment in contact with large product surfaces, such as coolers, conveyers, and cutting, slicing, and brining machines that are often difficult to clean, so they can maintain the contamination in factories despite regular cleaning and disinfecting (Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Lundén *et al.,* 2003b; Keto-Timonen *et al.*, 2007; Berzins *et al.*, 2010).

L. monocytogenes may persist in food-processing plant for years (Rørvik *et al.*, 1995; Miettinen *et al.*, 1999a; Keto-Timonen *et al.*, 2007). Persistent contamination is typically caused by a few strains recurrently isolated from the same or changing sampling sites, in the presence of a background of several other sporadically occurring strains (Giovannacci *et al.*, 1999; Miettinen *et al.*, 1999a; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007; Berzins *et al.*, 2010). Persistent strains have been reported to tolerate particular disinfectants better than sporadic strains (Aase *et al.*, 2000; Lundén *et al.*, 2003a), possibly because the persistent strains attach more firmly to surfaces that contact food (Norwood & Gilmour, 1999; Lundén *et al.*, 2000; Latorre *et al.*, 2011). Attached cells tolerate mechanical and chemical cleaning and disinfecting better than free-living cells (Norwood & Gilmour, 2000; Mah & O'Toole, 2001; Renier *et al.*, 2011). The adherence of *L. monocytogenes* enables the formation of a biofilm, a surface-attached microbial community in which the resistance and persistence of the organism in food production lines is increased (O'Toole *et al.*, 2000; Renier *et al.*, 2011). The biofilm-forming ability of strains varies, with some strains being able to form three-dimensional mushroom-shaped biofilms, while others produce sparse aggregates or monolayers (Chae & Schraft, 2000; Kalmokoff *et al.*, 2001; Borucki *et al.*, 2003). Surface material, environmental condition, and the presence of other bacteria affect the survival of *L. monocytogenes* in a biofilm (Bremer *et al.*, 2001; Midelet & Carpentier, 2002; Nilsson *et al.*, 2011; Bae *et al.*, 2012b; Kostaki *et al.*, 2012).

Contamination may enter the processing plant from multiple sources, and the main source has been suspected to be raw material (Lawrence & Gilmour, 1995; Ojeniyi *et al.*, 1996; Giovannacci *et al.*, 1999; Katzav *et al.*, 2006; Berzins *et al.*, 2010; Hellström *et al.*, 2010). Several studies, however, have shown that raw material was free of *L. monocytogenes,* or that different *L. monocytogenes* strains were found in the raw materials and in the processing environment or processed foods (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Hoffman *et al.*, 2003). Other possible contamination sources could be personnel, pests, insects, and soil (Iida *et al.*, 1991; El-Shenawy, 1998; Sauders *et al.*, 2005; Schoder & Wagner, 2011; Pava-Ripoll *et al.*, 2012).

Persistent contamination has been attributed to the transfer of processing machinery between food processing plants (Lundén *et al.*, 2002).

2.3 Stress responses of *L. monocytogenes*

In foods and in food-processing environments, *L. monocytogenes* is exposed to various stresses that cause changes in cellular components and reactions, thus decreasing its viability. The severity of the stress determines whether the bacterium is killed or its growth is restricted, while the type of stress determines the mechanism by which these responses happen.

L. monocytogenes may escape unfavourable environmental conditions by "swimming" toward more favourable locations, or it may adjust to the adverse condition (Mitchell & Kogure, 2006; Marles-Wright & Lewis, 2007). During stress adaptation, changes take place to maintain structural integrity of the cell and to keep important systems functioning (Berry & Foegeding, 1997; Beales, 2004), including changes in morphology, protein synthesis and structure, nutrient uptake, and concentration of intracellular metabolites. Different stresses have different effects on *L. monocytogenes* and thus induce different responses, although there are some common alterations in cell structure and function (Duché *et al.*, 2002a; Gardan *et al.*, 2003a; Chan *et al.*, 2007; van der Veen *et al.*, 2007; Giotis *et al.*, 2008b; Cacace *et al.*, 2010). A complex regulatory network harmonizes the expression of the stress-associated genes according to the existing conditions (Kazmierczak *et al.*, 2006; Kamp & Higgins, 2009; Ollinger *et al.*, 2009; Toledo-Arana *et al.*, 2009; Chaturongakul *et al.*, 2011).

2.3.1 Stressors present in food chain

The modern food industry aims to decrease the use of preservatives and to increase shelf lives, so food safety is widely based on the cold chain. Even though temperatures lower than optimum decrease growth rates in all bacteria, growth inhibition of *L. monocytogenes* is not complete until temperatures below 0°C (Junttila *et al.*, 1988; Hudson *et al.*, 1994). Pasteurizing and cooking destroy this species, but it may survive heat stress caused by mild thermal processing (Doyle *et al.*, 2001).

Low pH restricts the growth of *L. monocytogenes* in fermented foods and it is subjected to acid stress also while passing through the stomach and in the phagosomes (de Chastellier & Berche, 1994). In the small intestine it is exposed to alkaline pancreatic secretions, and residues of alkaline detergents used in food-processing plants can cause sublethal stress.

Decrease in a_w due to either decreased water content (desiccation) or increased solute content (adding salt or sugar), causing dehydration and osmotic stress, has long been used to preserve foods. Naturally existing or added ethanol preserves foods and drinks, and it is used also as disinfectant

in food-processing plants together with other alcohols, quaternary ammonium compounds, peroxides, and halogens, that may cause stress to *L. monocytogenes* if only sub-lethal concentrations are reached in food-contact surfaces or if residues remain in the contaminated food (Ryan *et al.*, 2008). Peroxides such as H_2O_2, and other agents containing reactive oxygen species (ROS), cause oxidative stress similar to that used by phagosomes to kill engulfed pathogens. The mechanisms of action of stressors present in the food chain are represented in Table 4.

Table 4. Mechanisms of growth inhibition in bacteria by stressors present in the food chain.

Stressor	Mechanisms of growth inhibition	Reference
Low temperature	Hindered membrane-associated cellular functions due to decreased fluidity, slowing of cell metabolism, decelerated reaction kinetics, increased stability of DNA and RNA secondary structures, hampered function of ribosomes.	VanBogelen & Neidhardt, 1990; Yamanaka *et al.*, 1998; Hebraud & Potier, 1999; Bayles *et al.*, 2000; Tasara & Stephan, 2006
High temperature	Denaturation of proteins and enzymes, DNA damage, degradation of rRNA. Damage of cytoplasmic membrane increases leakage of cellular components.	Yamanaka *et al.*, 1998; Russell, 2003; Al-Qadiri *et al.*, 2008
Acid	Disturbed function of enzymes and cellular bioenergetics, damage of the cell membrane and DNA.	Zilberstein *et al.*, 1984; Cotter & Hill, 2003; Moorman *et al.*, 2008; Krulwich *et al.*, 2011
Alkali	Disturbed function of enzymes and cellular bioenergetics. Disruption of cell membrane and leakage of intracellular contents. Disturbed cell division.	Zilberstein *et al.*, 1984; Sampathkumar *et al.*, 2003; Giotis *et al.*, 2007; Krulwich *et al.*, 2011
Low a_w	Dehydration of the cell, loss of turgor pressure, inhibition of nutrient uptake and other essential cellular functions.	Kempf & Bremer, 1998; Beales, 2004
Ethanol	Increased permeability of cell membrane, denaturation of proteins, and altered folding and interactions of macromolecules in all cell compartments.	Ingram, 1990; Barker & Park, 2001; Huffer *et al.*, 2011
H_2O_2, O_2, and other agents with strong oxidizing capacity	Peroxidation of lipids results in decreased fluidity of cell membrane. Damage of DNA and formation of crosslinkages between DNA and other molecules, modification of protein structure and folding, and thus function. Formation of aldehydes that subsequently damage molecules.	Storz & Imlay, 1999; Cabiscol *et al.*, 2000; Mishra & Imlay, 2012

2.3.2 Global alterations in *L. monocytogenes* following stress exposure

Stress induces alterations in *L. monocytogenes*, of which some are direct effects of a stressor on the morphology and function of the cell, whereas the others result from an attempt by the bacterium to compensate for these changes. Under mild stress, the alterations are few, whereas severe stress causes numerous changes in various cellular components (Bayles *et al.*, 1996; Esvan *et al.*, 2000; Bereksi *et al.*, 2002; Duché *et al.*, 2002a, 2002b; Bae *et*

al., 2012a; Bergholz *et al.*, 2012). This chapter describes alterations in the phenotype, transcriptome, proteome, and metabolome of *L. monocytogenes* exposed to different stress conditions.

Whole genome microarray studies have revealed that of the approximately 2850 genes of *L. monocytogenes,* 411, 714, 425, and 355 genes are differentially transcribed during growth under cold, heat, osmotic, and alkali stress conditions, respectively, compared with control growth condition (Glaser *et al.*, 2001; Nelson *et al.*, 2004; Chan *et al.*, 2007; van der Veen *et al.*, 2007; Giotis *et al.*, 2008b; Bergholz *et al.*, 2012). Similarly, 380 proteins showed relative changes in expression levels under growth-enabling acid stress conditions (Bowman *et al.*, 2012). Despite increases in the relative transcription and protein levels of numerous factors, overall protein synthesis is severely restricted under stress, as shown by only 12–38 proteins synthesized under growth-inhibiting cold, heat, acid, alkali, osmotic, and ethanol stress conditions (Phan-Thanh & Gormon, 1995, 1997; Bayles *et al.*, 1996; Esvan *et al.*, 2000; Agoston *et al.*, 2009).

Under cold, acid, and osmotic stress conditions, the lag time of *L. monocytogenes* growth increases and growth rate and yield decreases (Walker *et al.*, 1990; Bereksi *et al.*, 2002; Beales, 2004; Shabala *et al.*, 2008; Bowman *et al.*, 2012). Under these conditions, the relative amounts of folding chaperones and proteins with roles in energy production are increased, probably because of increased energy demand (Duché *et al.*, 2002a; Cacace *et al.*, 2010; Bowman *et al.*, 2012). Moderate acid and osmotic stresses result in slightly decreased cell length, whereas under severe acid and osmotic stresses as well as under heat and alkali stresses, filamentous cell structures appear and cell size increases (Isom *et al.*, 1995; Bereksi *et al.*, 2002; Giotis *et al.*, 2007; van der Veen *et al.*, 2007). This may be due to down-regulation of genes associated with cellular growth and cell division under heat, alkali, and osmotic stresses (van der Veen *et al.*, 2007; Giotis *et al.*, 2008b; Bergholz *et al.*, 2012).

Attachment of *L. monocytogenes* to inert food-contact surfaces is increased under acid, osmotic, and ethanol stresses (Briandet *et al.*, 1999a, 1999b; Gravesen *et al.*, 2005). Under acid and osmotic stress conditions, increased attachment is due to changes in cell surface charge and the electron donor and acceptor properties of the cells (Briandet *et al.*, 1999a). The formation of biofilms has been reported to be more efficient under acidic or alkaline conditions than at neutral pH, and at relative humidity of 100% rather than 85% or less (Nilsson *et al.*, 2011; Bae *et al.*, 2012b). Higher adherence of *L. monocytogenes* between 20°C and 45°C than between 8°C and 15°C indicates a temperature effect (Smoot & Pierson, 1998; Briandet *et al.*, 1999b). Surprisingly, the flagella, with its role in the initial attachment to inert surfaces, is not expressed at temperatures above 30°C (Vatanyoopaisarn *et al.*, 2000; Lemon *et al.*, 2007; Todhanakasem & Young, 2008; McLaughlin & Rees, 2009).

Many stress-induced alterations result from hindered growth, but stresses also induce specific changes in *L. monocytogenes* (Phan-Thanh & Gormon, 1995, 1997; Bayles *et al.,* 1996; Esvan *et al.*, 2000; Chan *et al.*, 2007; Cacace *et al.*, 2010; Bowman *et al.*, 2012). Under cold stress, transcription of 30 genes was up-regulated, including those with roles in transport systems for compatible solutes and oligopeptides, modifications of cell membranes, and motility, and transcription of 10 genes was down-regulated during both stationary and logarithmic growth phases (Liu *et al.*, 2002; Chan *et al.*, 2007). Accordingly, the intracellular concentrations of carnitine and the other cryoprotective osmolytes glycinebetaine, glutamate and trehalose, together with about half of the rest of the small organic compounds identified, were elevated during cold-temperature growth (Singh *et al.*, 2011). Heat stress induces expression of class I, II, and III stress-response genes, several virulence genes, and the SOS response genes involved in the repair of DNA damage, restart of stalled replication forks, and in the induction of adaptive point mutations (McKenzie *et al.*, 2000; Maul & Sutton, 2005; van der Veen *et al.*, 2007). Acid stress depresses motility, chemotaxis, and cold-shock proteins and induces energy production elements such as F_0F_1-ATPase (Bowman *et al.,* 2012). Alkali stress seems to induce genes involved with phosphate uptake, phosphorylation or dephosphorylation, and DNA damage response (Giotis *et al.*, 2008b, 2010), and osmotic stress increases the transcription of genes with roles in ion transport and uptake of osmolytes (Bae *et al.*, 2012a; Bergholz *et al.*, 2012).

2.3.3 Factors with roles in stress tolerance of *L. monocytogenes*

The roles of many regulatory and non-regulatory factors in tolerance of *L. monocytogenes* to temperature, pH, osmotic, ethanol, and oxidative stresses have been confirmed by comparing the phenotypes of genetically modified and unmodified strains (Table 5). The number of stress tolerance factors is rapidly increasing.

Two-component systems

One way in which bacteria sense environmental stress is through two-component regulatory systems (TCS). These consist of an autophosphorylable sensory histidine kinase (HK), typically embedded in the cell membrane and an intracellular response regulator (RR) that when receiving a phosphoryl group from a cognate HK, either activates or represses the transcription of the target genes. *L. monocytogenes* has 16 TCSs one of which is an orphan RR (Glaser *et al.*, 2001). LisKR has a role in tolerance to temperature, acid, osmotic, ethanol, and oxidative stresses (Cotter *et al.*, 1999, 2002; Kallipolitis & Ingmer, 2001; Sleator & Hill, 2005; Stack *et al.*, 2005; Williams *et al.*, 2005a; Chan *et al.*, 2008). CesRK controls

the transcription of several cell-envelope related genes and affects growth under heat, osmotic, and ethanol stresses (Kallipolitis & Ingmer, 2001; Kallipolitis *et al.*, 2003; Williams *et al.*, 2005a; Gottschalk *et al.*, 2008). An orphan RR, DegU, involved in the temperature-dependent regulation of motility and chemotaxis, is needed for heat and ethanol stress tolerance of (Knudsen *et al.*, 2004; Williams *et al.*, 2005b). DegU and motility-associated RR AgrA, with a role in osmotic stress tolerance, are needed for the formation of the wild-type biofilm on abiotic surfaces (Rieu *et al.*, 2007; Gueriri *et al.*, 2008; Garmyn *et al.*, 2012). RR KdpE is associated with heat and osmotic stress tolerance (Kallipolitis & Ingmer, 2001; Brøndsted *et al.*, 2003). TCSs PhoRP, ResDE, VirRS, Lmo1060-1061, and Lmo1172-1173 have been linked solely to cold or ethanol stress tolerance (Williams *et al.*, 2005a; Chan *et al.*, 2008).

Alternative sigma factors

Sigma factors are dissociable subunits of RNA polymerase responsible for the recognition of a specific promoter region and transcription initiation at that promoter site. The *L. monocytogenes* EGD-e genome contains five sigma factors (Glaser *et al.*, 2001), of which the main one, RpoD (σ^D), is an essential housekeeping factor (Metzger *et al.*, 1994). Under changing environmental conditions, the alternative sigma factors σ^B, σ^C, σ^H, and σ^L associate with core RNA polymerase with varying intensities, enabling transcription of specific sets of genes, thus serving as important regulators of gene expression (Chaturongakul *et al.*, 2008).

Deletion of σ^B substantially decreases acid resistance and σ^B also has roles in temperature, alkali, osmotic, and oxidative stress tolerance of *L. monocytogenes* (Becker *et al.*, 2000; Ferreira *et al.*, 2001; Moorhead & Dykes, 2004; Wemekamp-Kamphuis *et al.*, 2004b; Abram *et al.*, 2008; Giotis *et al.*, 2008a; Chaturongakul *et al.*, 2011; Ait-Ouazzou *et al.*, 2012) More than 140 genes have a putative σ^B-dependent promoter (Raengpradub *et al.*, 2008). Under optimal or acidic growth conditions, transcription of a further set of more than 140 genes is affected by σ^B (Kazmierczak *et al.*, 2003; Hain *et al.*, 2008; Raengpradub *et al.*, 2008). The main glutamate decarboxylase system (GAD) genes, activated under acid stress to increase intracellular pH, and several compatible solute transport systems, are affected by the factor (Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003, 2006; Cetin *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004b; Raengpradub *et al.*, 2008). Flagellar biosynthesis genes are repressed by σ^B (Raengpradub *et al.*, 2008).

The contribution of σ^B to heat, acid, osmotic, and oxidative stress tolerance seems to be strain-dependent (Becker *et al.*, 2000; Ferreira *et al.*, 2001; Moorhead & Dykes, 2003; Wemekamp-Kamphuis *et al.*, 2004b; Ait-Ouazzou *et al.*, 2012), as the role of σ^B in the stress tolerance was greater in a serotype 1/2a strain than in serotype 4c strain (Moorhead & Dykes, 2003,

2004). Moreover, differences have been reported in the role of σ^B in the heat stress tolerance of three different serotype 1/2a strains (Ferreira *et al.*, 2001; Moorhead & Dykes, 2003; Hu *et al.*, 2007b; Ait-Ouazzou *et al.*, 2012).

Deletion of σ^L decreases tolerance to cold, acid, osmotic, and ethanol stresses. In an optimal or cold environment, σ^L directly or indirectly affects the expression of up to 708 genes, of which approximately 20 belong to the σ^L regulon (Arous *et al.*, 2004; Chaturongakul *et al.*, 2011; Mattila *et al.*, 2012). Several flagellar biosynthesis, motility, and chemotaxis genes are promoted by σ^L, and this factor also has a role in carbohydrate metabolism (Arous *et al.*, 2004; Chaturongakul *et al.*, 2011; Mattila *et al.*, 2012).

σ^H contributes to growth and survival under cold, acidic, and alkaline conditions and in minimal media (Rea *et al.*, 2004; Chan *et al.*, 2008; Chaturongakul *et al.,* 2011). It contributes to the transcription of at least 169 genes, of which about 60 belong to the σ^H regulon (Chaturongakul *et al.*, 2011). The genes of the phosphotransferase system operon are stimulated by σ^H, whereas transcription of several genes with roles in protein and nucleotide biosynthesis is repressed by the factor (Chaturongakul *et al.*, 2011).

Temperature stress-associated σ^C is present solely in lineage I *L. monocytogenes* strains dominating in various non-host environments (Zhang *et al.*, 2005; Chan *et al.*, 2008). Only 11 genes have been reported to belong to the σ^C regulon (Chaturongakul *et al.*, 2011). Under heat stress, the transcription of five genes was reported to be affected by σ^C, but none was affected in optimal conditions (Zhang *et al.*, 2005; Chaturongakul *et al.*, 2011). At least two of these five genes have roles in heat stress tolerance (Zhang *et al.*, 2005).

Some genes are controlled by more than one sigma factor (Chaturongakul *et al.*, 2011; Nielsen *et al.*, 2012). The greatest overlap was reported between σ^B and σ^H, with 92 genes including several associated with energy metabolism being promoted by these two factors (Chaturongakul *et al.*, 2011). The transcription of 21 genes is increased by both σ^B and σ^L and of the five genes controlled by σ^C, two are controlled also by σ^B (Chaturongakul *et al.*, 2011).

Other regulators

Stress responses of *L. monocytogenes* are under the control of complex, hierarchial network of TCSs, sigma factors and numerous other regulators (Kazmierczak *et al.*, 2006; Kamp & Higgins, 2009; Ollinger *et al.*, 2009; Toledo-Arana *et al.*, 2009; Chaturongakul *et al.*, 2011). Independent of the type of the stress, at least three of the seven regulators located in the *sigB* operon are needed for activation of σ^B (Chaturongakul & Boor, 2004; Shin *et al.*, 2010). σ^B controls the expression of Hfq, a small RNA-binding regulatory protein involved in the tolerance of osmotic and ethanol stresses and of ArgR, a regulator of the arginine deimidase (ADI) pathway needed for

survival under acidic environment (Christiansen *et al.*, 2004, 2006; Ryan *et al.*, 2009).

The transcriptional repressor CtsR negatively regulates class III stress-response genes and decreases resistance to heat, acid, osmotic, and oxidative stress (Nair *et al.*, 2000; Karatzas & Bennik, 2002; Karatzas *et al.*, 2003; Hu *et al.*, 2007b). It also has a role in regulation of motility (Karatzas & Bennik, 2002; Karatzas *et al.*, 2003; Grundling *et al.*, 2004; Shen & Higgins, 2006; Lemon *et al.*, 2007; Kamp & Higgins, 2011). HrcA negatively regulates class I stress-response genes and has roles in heat stress resistance and biofilm formation (Hu *et al.*, 2007a; van der Veen & Abee, 2010). Both CtsR and HrcA affect the transcription level of several genes, together with σ^B and the major virulence-gene regulator PrfA (Hu *et al.*, 2007b; Hu *et al.*, 2007a; Ollinger *et al.*, 2009; Toledo-Arana *et al.*, 2009; Chaturongakul *et al.*, 2011). Genes controlled by both σ^B and PrfA have roles in the acid and osmotic stress responses and survival inside the host (Ferreira *et al.*, 2001; Abram *et al.*, 2008; Toledo-Arana *et al.*, 2009; Bruno & Freitag, 2010).

Non-regulatory factors

As is the case for most of the regulatory factors, the roles of most stress-associated non-regulatory factors of *L. monocytogenes* have roles in multiple stresses. A serine protease HtrA, responsible for degradation of misfolded proteins and regulated by LisRK, is associated with at least four different stresses (Pallen & Wren, 1997; Stack *et al.*, 2005). All three cold-shock proteins (Csp), that belong to a highly conserved group of structurally related nucleic acid binding proteins, play roles not only under cold but also under osmotic and oxidative stress conditions (Ermolenko & Makhatadze, 2002; Schmid *et al.*, 2009; Loepfe *et al.*, 2010). Proteins of this family act as chaperones by assisting in folding and un-folding of nucleic acids, and potentially regulate both transcription and translation under various stresses (Phadtare, 2004; Horn *et al.*, 2007). Universal stress proteins (Usps), thought to protect cells from DNA damage and respiratory distress, have been reported to have roles in cold and oxidative stress tolerance (Kvint *et al.*, 2003; Seifart Gomes *et al.*, 2011).

Under low temperature, *L. monocytogenes* strives to sustain the fluidity of cell membranes by regulating its fatty acid composition (Annous *et al.*, 1997; Edgcomb *et al.*, 2000). Accordingly, the genes of the Bkd cluster, controlling the branched-chain fatty acid composition of the cell wall, were confirmed to have a role in cold stress tolerance (Zhu *et al.*, 2005). The role of PgpH and RelA in cold stress tolerance seems to be due to adjustment of cellular guanosine pentaphosphate [(p)ppGpp] levels during low-temperature growth (Liu *et al.*, 2006b). The (p)ppGpp is an important intracellular signal molecule that affects association of sigma factors to core RNA polymerases under various stress conditions (Dalebroux & Swanson, 2012).

The accumulation of cryoprotective osmolytes increases the tolerance of *L. monocytogenes* to cold, heat, and osmotic stress (Ko *et al.*, 1994; Kempf & Bremer, 1998; Angelidis & Smith, 2003a; Wemekamp-Kamphuis *et al.*, 2004a; Ells & Truelstrup Hansen, 2011). These substrates restore cellular turgor pressure, stabilize enzymatic functions, and affect the membrane lipid bilayer (Ko *et al.*, 1994; Russell *et al.*, 1995; Kempf & Bremer, 1998; Ells & Truelstrup Hansen, 2011). Several factors involved with intracellular accumulation of compatible solutes have roles in tolerance to cold or osmotic stress (Borezee *et al.*, 2000; Angelidis & Smith, 2003a, 2003b; Christiansen *et al.*, 2004; Chassaing & Auvray, 2007; Ells & Truelstrup Hansen, 2011; Burall *et al.*, 2012). OpuC, Gbu, and BetL are needed for importation of carnitine and betaine (Angelidis & Smith, 2003a, 2003b; Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003; Cetin *et al.*, 2004; Sleator & Hill, 2010). OppA associated with cold stress, is responsible for the importation of unknown, potentially cryoprotective peptides (Borezee *et al.*, 2000). TreA is needed for the breakdown of trehalose, thus decreasing tolerance to osmotic stress, and it has role in heat stress tolerance (Ells & Truelstrup Hansen, 2011).

Under acid stress, *L. monocytogenes* strives to maintain pH homeostasis. F_0F_1-ATPase serves as a channel for proton translocation across the cell membrane (Cotter *et al.*, 2000). Four out of five GAD pathway factors are associated with the internalization and conversion of glutamate to proton-consuming gamma-aminobutyrate (Cotter *et al.*, 2001; Karatzas *et al.*, 2012). Two GAD pathway genes and three adjacent genes are generally missing from serotype 4 *L. monocytogenes* strains that are under-represented in various non-host environments (Cotter *et al.*, 2005; Ryan *et al.*, 2010; Orsi *et al.*, 2011). ADI pathway factors involved in the conversion of arginine to ornithine, carbon dioxide and ammonia protect *L. monocytogenes* from acid stress by increasing intracellular pH (Ryan *et al.*, 2009).

Catalase, the superoxide dismutase (SOD) system, and ferritin synergistically protect bacteria from oxidative stress caused by ROS (Fisher *et al.*, 2000; Mongkolsuk & Helmann, 2002; Dussurget *et al.*, 2005; Archambaud *et al.*, 2006; Azizoglu & Kathariou, 2010b). SOD converts superoxide anions to H_2O_2 that is converted to water and oxygen by catalase (Dussurget *et al.*, 2005; Azizoglu & Kathariou, 2010b). Ferritin binds iron that reacts with oxygen intermediates to produce deleterious free radicals (Dussurget *et al.*, 2005). Ferritin also has roles in cold and heat tolerance and catalase also has a role in cold stress tolerance of *L. monocytogenes* (Dussurget *et al.*, 2005; Azizoglu & Kathariou, 2010b), and the roles of several factors with unknown functions in stress tolerance has been confirmed (Table 5).

2.3.4 Stress adaptation and cross adaptation of *L. monocytogenes*

Exposure to cold, heat, acid, alkali, osmotic, ethanol, and oxidative stress conditions increase tolerance of *L. monocytogenes* to the same factor,

resulting in either a more rapid transition from lag to growth phase, or a higher lethal level of the stress (Stephens *et al.*, 1994; Lou & Yousef, 1997; Aase *et al.*, 2000; Phan-Thanh *et al.*, 2000; To *et al.*, 2002; Lundén *et al.*, 2003a; Shabala *et al.*, 2008; Skandamis *et al.*, 2008; Mastronicolis *et al.*, 2011). The enhanced stress tolerance of these adapted cells is partly due to stress-induced expression of enzymes involved in the synthesis of metabolites needed under stress (Beales, 2004; Giotis *et al.*, 2008b). Constitutively expressed proteins that are active only under stress conditions have also been reported (Berry & Foegeding, 1997).

Adaptation to one stress factor often protects an organism against the lethal effects of another stressor. Cross adaptation of *L. monocytogenes* to osmotic, ethanol, and oxidative stresses has been reported to result from adaptation to acid stress (Lou & Yousef, 1997; Phan-Thanh *et al.*, 2000; Faleiro *et al.*, 2003). Heat, alkali, osmotic, or ethanol stress exposure also protects *L. monocytogenes* against other stress factors (Lou & Yousef, 1997; Taormina & Beuchat, 2001; Skandamis *et al.*, 2008). Even though resistance to oxidative stress increases following adaptation to several other stresses, adaptation to oxidative stress itself does not seem to cross adapt *L. monocytogenes* to other stresses (Lou & Yousef, 1997; Bergholz *et al.*, 2012).

2.3.5 Stress tolerance of *L. monocytogenes* in biofilms

L. monocytogenes tolerates various stresses better in mature biofilms than as planktonic cultures or in immature biofilms (Robbins *et al.*, 2005; Nilsson *et al.*, 2011). Higher concentrations of ozone, chlorine, and H_2O_2 are needed to destroy the bacterium in biofilms than as free-living cells (Robbins *et al.*, 2005; Yun *et al.*, 2012). Moreover, its tolerance to quaternary ammonium compounds is better in mature biofilms than in immature ones (Nilsson *et al.*, 2011), attributable to the decreased physiological activity of the bacteria inside the biofilm (Chae & Schraft, 2001). The extracellular matrix of the biofilm may also protect the bacterium from the lethal effects of the stressors (Hoyle *et al.*, 1990; Chae & Schraft, 2001).

Table 5. Factors/systems with phenotypically confirmed roles in tolerance of *Listeria monocytogenes* to temperature, pH, osmotic, ethanol and oxidative stress.

Factor/ system	Functional description	Role in stress[a]							References
		Cold	Heat	Acid	Alkali	Osmotic	Ethanol	Oxidative	
LisR	Response regulator	↑L	↑L			↑L	↓L	↑L	Kallipolitis & Ingmer, 2001; Williams *et al*., 2005a; Chan *et al*., 2008
LisK	Histidine kinase			↑L ↓S		↑L	↓L		Cotter *et al*., 1999; Sleator & Hill, 2005
CesR	Response regulator		↑L			↑L	↓L		Kallipolitis & Ingmer, 2001; Williams *et al*., 2005a
DegU	Response regulator		↑L				↑L		Williams *et al*., 2005a; Gueriri *et al*., 2008)
KpdE	Response regulator		↑L			↑L			Kallipolitis & Ingmer, 2001; Brøndsted *et al*., 2003
AgrA	Response regulator					↑L			Garmyn *et al*., 2012
PhoP	Response regulator						↑L		Williams *et al*., 2005a
ResD	Response regulator						↑L		Williams *et al*., 2005a
VirR	Response regulator						↑L		Williams *et al*., 2005a
Lmo1060	Response regulator	↑L							Chan *et al*., 2008
Lmo1172	Response regulator	↑L							Chan *et al*., 2008
δ^B	Alternative sigma factor B	↑L, S	(↑S)[b]	(↑S)[b]	↑S	↑↓L[c]		(↑S)[b]	Becker *et al*., 2000; Ferreira *et al*., 2001; Moorhead & Dykes, 2004; Wemekamp-Kamphuis *et al*., 2004b; Abram *et al*., 2008; Giotis *et al*., 2008a; Chaturongakul *et al*., 2011; Ait-Ouazzou *et al*., 2012
δ^L (RpoN)	Alternative sigma factor L	↑L		↑L ↓S[d]		↑L	↑L		Okada *et al*., 2006; Raimann *et al*., 2009; Chaturongakul *et al*., 2011; Mattila *et al*., 2012
δ^H	Alternative sigma factor H	↑L		↓S	↑L				Rea *et al*., 2004; Chan *et al*., 2008; Chaturongakul *et al*., 2011
δ^C	Alternative sigma factor C	↑L	↑S						Zhang *et al*., 2005; Chan *et al*., 2008
Hfq	Putative RNA binding protein					↑L	↑L		Christiansen *et al*., 2004

Table 5. *Continued*

Factor/ system	Functional description	Role in stress [a]							References
		Cold	Heat	Acid	Alkali	Osmotic	Ethanol	Oxidative	
ArgR	Putative activator of ADI genes			↑L, S					Ryan *et al*., 2009
CtsR	Repressor of class III heat shock genes		↓S						Nair *et al*., 2000; Karatzas *et al*., 2003
HrcA	Putative negative regulator of stress response		↑S						Hu *et al*., 2007a
PerR	Putative ferric uptake regulator						↑L	↑L, S	Rea *et al*., 2004, 2005
MarR	Putative transcriptional regulator				↑L		↑L		Rea *et al*., 2004
Fur	Putative ferric uptake regulator						↑L	↑L, S	Rea *et al*., 2004
HtrA	Serine protease		↑L	↑L		↑L		↑L	Pallen & Wren, 1997; Wonderling *et al*., 2004; Stack *et al*., 2005
Fri	Ferritin	↑L	↑L					↑L	Dussurget *et al*., 2005
Csps	Cold shock domain proteins	↑L				↑L		↑L	Ermolenko & Makhatadze, 2002; Schmid *et al*., 2009; Loepfe *et al*., 2010
Lmo0581	Putative methyl transferase	↓L			↑L		↑L		Rea *et al*., 2004; Burall *et al*., 2012
Usp	Universal stress proteins			↑L				↑L	Kvint *et al*., 2003; Seifart Gomes *et al*., 2011
Kat	Catalase	↑L						↑L, S	Fisher *et al*., 2000; Azizoglu & Kathariou, 2010b
OpuC, Gpu, BetL	Osmolyte transporters	↑L				↑L			Angelidis *et al*., 2002; Angelidis & Smith, 2003a, 2003b
Hfq	RNA chaperone					↑L	↑L		Christiansen *et al*., 2004
Iap	Invasion associated protein	↑L				↑L			Burall *et al*., 2012
Lmo1078	Putative UDP-glucose pyrophosphorylase	↑L				↑L			Chassaing & Auvray, 2007
TreA	Phosphotrehalase		↓S			↓S			Ells & Truelstrup Hansen, 2011

Table 5. *Continued*

Factor/ system	Functional description	Role in stress [a]							References
		Cold	Heat	Acid	Alkali	Osmotic	Ethanol	Oxidative	
Lmo0038	Ornithine and putrescine carbamoyltransferase		↑L	↑L					Chen *et al.*, 2009
OppA	Oligopeptide permease	↑L							Borezee *et al.*, 2000
PgpH	Putative integral membrane and (p)ppGpp hydrolase	↑L							Liu *et al.*, 2006b
Bkd	Branched chain α-keto acid dehydrogenase enzyme cluster	↑L							Zhu *et al.*, 2005
LMOf2365_1746	Putative DEAD-box RNA helicase	↑L							Azizoglu & Kathariou, 2010a
F_0F_1-ATPase	Proton efflux membrane ATPase			↑S					Cotter *et al.*, 2000
ADI	Arginine deiminase system			↑L, S					Ryan *et al.*, 2009
GAD	Glutamate decarboxylase system			↑S					Cotter *et al.*, 2001
RelA	(p)ppGpp-synthetase and -degradase					↑L			Okada *et al.*, 2002
Mrp	Ion transporter					↑L			Bergholz *et al.*, 2012
ProBA	Proline synthesis factor					↑L			Sleator *et al.*, 2001
Ctc	50S ribosomal protein L25					↑L			Gardan *et al.*, 2003b
SOD	Superoxide dismutase							↑L, S	Fisher *et al.*, 2000; Archambaud *et al.*, 2006
Lmo0668	Putative transporter				↑L	↑L			Gardan *et al.*, 2003a
Lmo0992	Unknown function				↑L	↑L			Gardan *et al.*, 2003a

Table 5. *Continued*

Factor/ system	Functional description	Role in stress [a]							References
		Cold	Heat	Acid	Alkali	Osmotic	Ethanol	Oxidative	
Lmo1431	Putative transporter				↑L	↑L			Gardan *et al*., 2003a
Lmo1432	Unknown function				↑L	↑L			Gardan *et al*., 2003a
Lmo1443	Unknown function				↑L	↑L			Gardan *et al*., 2003a
Lmo2232	Unknown function				↑L	↑L			Gardan *et al*., 2003a

Modified from Soni *et al.* (2011).

[a] ↑, inactivation of a factor/system decreases tolerance of *L. monocytogenes* to the stress; ↓, inactivation of a factor/system increases tolerance of *L. monocytogenes* to the stress; L, logarithmic growth phase; S, stationary phase of growth.

[b] The role of the factor has been confirmed in only part of the strains analysed.

[c] The presence of the factor increases stress tolerance in complex growth media and decreases tolerance in defined media.

[d] The role of the factor differs between the strains.

3 AIMS OF THE STUDY

The objectives of this work were to trace the initial sources of *L. monocytogenes* contamination in food processing plants and processed foods, and to identify genetic factors with roles in tolerance to stresses this pathogen may be exposed to in the food chain.

The specific aims were to examine:

1. the role of raw materials as potential sources of *L. monocytogenes* contamination of pork slaughterhouses and fish processing plants (I, II).
2. the role of raw material as a potential source of *L. monocytogenes* contamination of fish products (II).
3. the role of flagellar genes *flhA* and *motA* in the cold tolerance of *L. monocytogenes* (III).
4. the role of putative DEAD-box RNA helicase-encoding genes *lmo0866, lmo1246, lmo1450,* and *lmo1722* in the tolerance of *L. monocytogenes* to temperature, pH, osmotic, ethanol, and oxidative stresses (IV, V).
5. the correlation between cold tolerance and motility of *L. monocytogenes* (III, IV).

4 MATERIALS AND METHODS

4.1 *L. monocytogenes* isolates (II)

A total of 101 fish product isolates from 21 fish processing plants from 1996 to 2002, and belonging to the culture collection of Department of Food Hygiene and Environmental Health (former Department of Food and Environmental Hygiene) were included.

4.2 Bacterial strains and plasmids (III-V)

The sequenced *L. monocytogenes* strain EGD-e (Glaser *et al.*, 2001) was used for genetic modifications. Strains and plasmids used are presented in Table 6.

4.3 Sampling (I, II)

A total of 271 pig tonsils, 132 from fattening pigs and 139 from sows (I), from five different slaughterhouses in various parts of Finland, along with 257 raw fish samples (II), sampled at the slaughterhouse during slaughtering (45 samples) or at the processing factory before processing (212 samples), were collected between 1998 and 2001. Each fish sample contained slime, skin, gills, and fins from one to five fish heads. Samples were homogenized in half-Fraser broth (Oxoid, Basingstoke, UK) in a ratio of 1:10.

4.4 Determination of *L. monocytogenes* (I, II)

L. monocytogenes was isolated from pig tonsil and raw fish samples according to the guidelines of the International Organization for Standardization (Anonymous, 1996), using Oxford (Oxoid), Palcam (Oxoid), and *L. monocytogenes* blood agars (Johansson, 1998). Gram-staining, catalase reaction, and the API *Listeria* kit (bioMérieux, Inc, Marcy l'Etoile, France) were used for identification of *L. monocytogenes*.

4.5 Growth conditions (III-V)

In the control, *L. monocytogenes* was grown at 37˚C on blood or brain heart infusion (BHI) agar plates (BD, Franklin Lakes, NJ, USA), or in BHI broth (BD) supplemented with antibiotics (Sigma-Chemicals) when appropriate. *Escherichia coli* was grown on Luria-Bertani (LB) agar (BD) or in LB broth

(BD), supplemented with antibiotics (Sigma-Chemicals) when appropriate. To study cold and heat stress responses, the wild-type *L. monocytogenes* EGD-e and mutant strains were grown in BHI broth at 3˚C and at 42.5˚C, respectively. Acid and alkali stress responses were studied

Table 6. Bacterial strains and plasmids used in studies III-V.

Strain or plasmid	Genotype or characteristic [a]	Reference or source[b]
Listeria monocytogenes		
EGD-e	Parental serotype 1/2a strains	Glaser *et al.*, 2001
EGD-eΔ*flhA*	1274 bp out-of-frame deletion of EGD-e *flhA* gene	III
EGD-eΔ*motA*	472 bp out-of-frame deletion of EGD-e *motA* gene	III
Δ*lmo0866*	Deletion of EGD-e *lmo0866* cds with seven upstream and 170 downstream nucleotides	IV
Δ*lmo1246*	Deletion of EGD-e *lmo1246* cds with ten upstream and one downstream nucleotides	IV
Δ*lmo1450*	Deletion of EGD-e *lmo1450* cds with three downstream nucleotides	IV
Δ*lmo1722*	Deletion of EGD-e *lmo1722* cds	IV
Δ*lmo0866*c	Δ*lmo0866*, tRNA ^{Arg}::p*lmo0866*c, complemented strain	IV
Δ*lmo1450*c	Δ*lmo1450*, tRNA ^{Arg}::p*lmo1450*c, complemented strain	IV
Δ*lmo1722*c	Δ*lmo1722*, tRNA ^{Arg}::p*lmo1722*c, complemented strain	IV
EGD-epPL2	EGD-e, tRNA ^{Arg}::pPL2,	IV
Δ*lmo0866*pPL2	Δ*lmo0866*, tRNA ^{Arg}::pPL2	IV
Δ*lmo1450*pPL2	Δ*lmo1450*, tRNA ^{Arg}::pPL2	IV
Δ*lmo1722*pPL2	Δ*lmo1722*, tRNA ^{Arg}::pPL2	IV
Escherichia coli		
TOP10	Electrocompetent strain	Invitrogen
NEB5α	Electrocompetent strain	New England Biolabs
HB101	Conjugation donor containing helper plasmid pRK24	CRBIP
Plasmid		
pMAD		Arnaud *et al.*, 2004
pMAD- Δ*flhA*	pMAD containing homologous region up- and downstream of EGD-e *flhA*	III
pMAD- Δ*motA*	pMAD containing homologous region up- and downstream of EGD-e *motA*	III
pMAD- Δ*lmo0866*	pMAD containing homologous region up- and downstream of EGD-e *lmo0866*	IV
pMAD- Δ*lmo1246*	pMAD containing homologous region up- and downstream of EGD-e *lmo1246*	IV
pMAD- Δ*lmo1450*	pMAD containing homologous region up- and downstream of EGD-e *lmo1450*	IV
pMAD- Δ*lmo1722*	pMAD containing homologous region up- and downstream of EGD-e *lmo1722*	IV
pPL2	Site-specific integration vector	Lauer *et al.*, 2002
p*lmo0866*c	pPL2 containing ~500bp upstream nucleotides, cds and downstream terminator sequence of EGD-e *lmo0866*	IV
p*lmo1450*c	pPL2 containing ~200bp upstream nucleotides and cds of EGD-e *lmo450*	IV
p*lmo1722*c	pPL2 containing ~620bp upstream nucleotides, cds and downstream terminator sequence of EGD-e *lmo1722*	IV

[a] cds, coding sequence

[b] CRBIP, Biological Resource Centre of Institut Pasteur

at 37˚C in BHI broth adjusted to pH 5.6 and pH 9.4, respectively, by using 1 M HCl or NaOH. To study osmotic, ethanol, or oxidative stress responses at 37˚C, BHI broth was supplemented with 6% NaCl, 3.5% ethanol (vol/vol) (99.5%) or 5 mM H_2O_2 (30%). The minimum and maximum growth temperatures were examined in tryptic soy agar (TSA) (BD) containing 1.5 g and 2.5 g agar per liter, respectively.

4.6 Typing

4.6.1 Serotyping (II)

Serotyping of 142 *L. monocytogenes* isolates from raw and processed fish was done using commercial *Listeria* antisera (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions.

4.6.2 PFGE typing (I, II)

In situ DNA isolation and PFGE of *L. monocytogenes* isolates were done according to Autio *et al.* (2002) using proteinase K (Finnzymes, Helsinki, Finland) or Pronase (Roche Diagnostics GmbH, Mannheim, Germany). Restriction enzymes *Asc*I (New England Biolabs) (I, II) and *Apa*I (Boeringer Mannheim, Mannheim, Germany) (I) were used for DNA digestion.

*Asc*I and *Apa*I macrorestriction patterns were analyzed using BioNumerics software (Applied Maths, Kortrijk, Belgium). The Dice coefficient was calculated as a measure of the similarity of restriction patterns, based on band position. The position tolerance was optimal when set at 1.1% (I) or 1.0% (II) for the total length of restriction patterns with no increase. The clustering and construction of dendrograms were performed by the unweighted pair-group method with arithmetic averages (UPGMA).

4.7 Transcriptional analysis (III-V)

4.7.1 Total RNA isolation

To compare the transcription levels of *flhA, motA*, *lmo0866, lmo1246, lmo1450*, and *lmo1722* in different stress conditions with control values, three replicate cultures of the wild-type EGD-e were grown to mid-logarithmic growth phase in each treatment, and total RNA was extracted using the Qiagen RNeasy Midi-kit (Qiagen, Valencia, CA, USA) according to the manufacturers instructions. The cells were lysed in Tris-EDTA buffer using lysozyme 25 mg/ml (Sigma-Aldrich, St.Louis, MO, USA) and mutanolysin 250 U/ml (Sigma-Aldrich) for 30 min at 37˚C. DNase treatment

Table 7. Primers used in the study.

Application	Primer	Sequence in 5' to 3' direction [a, b]	Reference
Quantitative real-time PCR	*flhA* forward	GGAGCACAACCAAGCATTT	III
	flhA reverse	CGTTTCCCCGTTCATTTTT	III
	motA forward	CGTGCTTTGGACACCATTT	III
	motA reverse	TCTGCTTTTCTTCCCTTTTCC	III
	16S *rRNA* forward	GATGCATAGCCGACCTGAGA	III
	16S *rRNA* reverse	CTCCGTCAGACTTTGCTCCA	III
	gap forward	AAAGCTGGCGCTAAAAAAGTTG	Kim *et al.*, 2005
	gap reverse	TTCATGGTTTACATTGTAAACGATTG	Kim *et al.*, 2005
	lmo0866-5	AGCGGTTTACGGTGGTAGTG	IV
	lmo0866-6	GCCCATGTTTAGCATTTCGT	IV
	lmo1246-5	CGACAAGGTTGCCCATTTAG	IV
	lmo1246-6	GTTTTGGTAAGCGGCTAAGG	IV
	lmo1450-5b	AACGTTTTCGCCGTATGAAC	IV
	lmo1450-6b	GAACGCCTGGTCGTATCAAT	IV
	lmo1722-5	GACATTGGCTCCTCCGATTA	IV
	lmo1722-6b	ACGGAAGTAATCCGTTCGTG	IV
Generation of deletion mutants	Pre-*flhA*-forward	AATTGGATCCCGACACATATTTCCGTCGTG	III
	Pre-*flhA*-reverse	AATTGAATTCGCACGTTTTTCTTTCGCTTC	III
	Post-*flhA*-forward	AATTGAATTCGCGCTGCTTTTACAGGATTC	III
	Post-*flhA*-reverse	AATTACGCGTACTGTTTTGCTCACGCTCCT	III
	Pre-*motA*-forward	AATTGGATCCCGTTTTAGACGGCACGATTT	III
	Pre-*motA*-reverse	AATTGAATTCACAGCCGTAATTGTCCCAAG	III
	Post-*motA*-forward	AATTGAATTCTGGACACCATTTGCCAATAA	III
	Post-*motA*-reverse	AATTGAATCCTGGACACCATTTGCCAATAA	III
	lmo0866-1	GCGCGGATCCTGTTCGCGATAAAGATGCAG	IV
	lmo0866-2	*ATATGGTAACTATTTGTCT*ATGATTCACGGCATCAGAAA	IV
	lmo0866-3	*CTGATGCCGTGAATCAT*AGACAAATAGTTACCATATAGATAGA	IV
	lmo0866-4	GCCGTGAATTCTGCGATAATTGCCATCGTTA	IV
	lmo1246-1	GCGCGGATCCCTAAATCCACCGCTCCCATA	IV
	lmo1246-2	*TCCATTGTTTGGACCTTCTT*CAGTCCTCATTTCCTCATATCATC	IV
	lmo1246-3	*TGATATGAGGAAATGAGGAC*AAGAAGGTCCAAACAATGGAAA	IV
	lmo1246-4	GCCGACGCGTCGGAAGATTGGCCAGAAATA	IV
	lmo1450-1	GCGCGGATCCGAAGGGCATCAAAGCCAATA	IV
	lmo1450-2	*ATAGAACGGAGTGGAAATAT*GTGAGGAATGAATTATGCTAA	IV
	lmo1450-3	*TAGCATAATTCATTCCTCAC*ATATTTCCACTCCGTTCTATACC	IV
	lmo1450-4	GCCGACGCGTTGCTCATCGTTGTTTGGTTC	IV
	lmo1722-1	GCGCGGATCCAAGCGGTGGATTTCATCAAG	IV
	lmo1722-2	*ACTAATAAAGGAGTCGGGTT*GATGCGTCGAATGTTTTTCT	IV
	lmo1722-3b	*ATTCGACGCATC*AACCCGACTCCTTTATTAGTTCTTC	IV
	lmo1722-4b	CGCGACGCGTTTTGTTGGTTGGCTGATTTG	IV
Sequencing	lmo0866-7	CGGATACGATGATCAACAACG	IV
	lmo0866-8	CAGCTGTTGTTGCCCAAGTA	IV
	lmo0866-9	CGGTGAAACGGAAGAAATTG	IV
	lmo0866-10	GTAAGTCCACCCCAAAACGA	IV
	lmo1246-7	CGGGATTAACCAAGCAAAAA	IV
	lmo1246-8	TGGGAACACTGGAGCATGT	IV
	lmo1246-9	TTTTGGGGGCTTAGAGTTCA	IV
	lmo1246-10	GAATTCCCAACGATGTCACC	IV
	lmo1450-7	TGGCGATTTCCCATTTGTAT	IV
	lmo1450-8	AAACTTGGGCTAAACGAGCA	IV
	lmo1450-9	CGCGTACGTTCAATTTCAGA	IV
	lmo1450-10	CATCGATGACTCCGTAGCAA	IV
	lmo1722-7	TTCTGCAAGGGATGGTAAGG	IV
	lmo1722-8	ATTAGAATGGAACGCGCATC	IV
	lmo1722-9	TGAAGCAATTCTGCGACATC	IV
	lmo1722-10	TCCAGATGGGTTTTAATCTTTTG	IV
Complementation	lmo0866-11	CCGGGATCCGCTGGAAATCATTTTTGGATG	IV
	lmo0866-12	GGTCTAGATAGCACCACACTCCCGTATC	IV
	lmo1450-11	GGTCTAGAATTCCTCACTTATCGTTTTT	IV
	lmo1450-12	CGGGGATCCCATCGATGACTCCGTAGCAA	IV

Table 7. *Continued*

Application	Primer	Sequence in 5' to 3' direction [a, b]	Reference
	lmo1722-11	GGTCTAGATTAAATGGCATCCTCGCAAC	IV
	lmo1722-12	CGGGGATCCGGCGATGGTGTTTTCATAGC	IV
	NC16	GTCAAAACATACGCTCTTATC	Lauer *et al.*, 2002
	PL95	ACATAATCAGTCCAAAGTAGATGC	Lauer *et al.*, 2002

[a] Restriction sites for cloning are underlined.

[b] Overlapping 5'-end extensions of splicing-by-overlap extension PCR primers for construction insert from two PCR fragments are indicated in italics.

was done as an on-column treatment using the Qiagen RNase-Free DNase set (Qiagen). Additional DNase treatment was done for extracted RNA by utilizing the Ambion DNA-free kit (Ambion, Austin, TX, USA). The RNA yield was determined by using the Nanodrop ND-1000 (Nano Drop Technologies, Wilmington, DE, USA) and the integrity was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

4.7.2 Reverse transcription, real-time PCR, and quantification of gene expression

Reverse transcription (RT) was performed using the Finnzymes Dynamo cDNA synthesis kit (Finnzymes, Espoo, Finland) in two replicates for each RNA sample. As a control, an additional RT reaction was performed without reverse transcriptase enzyme (no-RT control). A sample of 800 ng (III) or 400 ng (IV-V) of total RNA from each sample was reverse-transcribed into complementary DNA (cDNA) according to the manufacturer's instructions, with predenaturation at 65˚C for 5 min, and extension at 40˚C for 40 min. cDNA samples were diluted 1:10 with an additional 1:100 dilution for rRNA analysis (III), or 1:5000 (IV-V). The real-time polymerase chain reactions (PCR) were performed using a Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia) with two replicates for each cDNA sample using SYBR Green chemistry (Dynamo Flash SYBR Green qPCR kit, Finnzymes) according to the manufacturer's instructions. The relative expression of *flhA, motA, lmo0866, lmo1246, lmo1450,* and *lmo1722* was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The 16S rRNA gene (III-V) and *gap* (III), the most stably expressed housekeeping genes of *L. monocytogenes* (Tasara & Stephan, 2007), were used as reference genes. The amplification efficiencies of the six target genes and reference genes using the primers listed in Table 7 ranged between 0.96 and 1.07.

4.8 Genetic modification of *L. monocytogenes*

4.8.1 Construction of deletion mutant strains (III, IV)

To construct flagellar gene deletion mutant strains EGD-eΔ*flhA* and EGD-eΔ*motA,* and DEAD-box RNA helicase gene deletion mutant strains Δ*lmo0866,* Δ*lmo1246,* Δ*lmo1450,* and Δ*lmo1722* without an associated antibiotic resistance gene in *L. monocytogenes* EGD-e, allelic replacement according to Arnaud *et al.* (2004) was used. Restriction-ligation (III) or splicing-by-overlap extension PCR protocol (IV) was used to construct target gene-deficient fragments for allelic replacement from two PCR products. Deletion mutants were confirmed by PCR with target gene-specific PCR primers and by sequencing at the Institute of Biotechnology, University of Helsinki, Finland. The primers used for construction of deletion mutants, PCR, and sequencing are presented in Table 7.

4.8.2 Complementation of deletion mutations (IV)

Functional complementation of the phenotypically affected Δ*lmo0866*, Δ*lmo1450,* and Δ*lmo1722* mutants was performed according to Lauer *et al.* (2002) by restoring the wild-type copy of each deleted gene into the respective deletion mutant strain, using the site-specific integration vector pPL2, received from Prof. Martin Loessner, Swiss Federal Institute of Technology, Zurich, Switzerland. The coding sequence and approximately 500-base pair (bp), 200-bp and 600-bp upstream regions, including the putative promoters of *lmo0866*, *lmo1450* and *lmo1722*, respectively, were amplified by PCR and ligated between the *Spe*I and *Bam*HI cloning sites of pPL2 (Table 6). The plasmids were transformed into the recipient *L. monocytogenes* strains by conjugation, according to Ma *et al.* (2011), except that selective Oxoid Chromogenic Listeria Agar (Oxoid) was used for selection of transconjugants. Strains carrying the pPL2 constructs were selected with chloramphenicol and confirmed by PCR using gene-specific primer pairs (Table 6). Integration of the pPL2 constructs into the chromosome of the recipient cells was confirmed by PCR with primers NC16 and PL95 (Lauer *et al.*, 2002).

4.9 Characterization of genetically modified *L. monocytogenes*

4.9.1 Electron microscopy (III)

To examine the presence of flagella in the wild-type EGD-e and EGD-eΔ*flhA* and EGD-eΔ*motA* mutant strains, cells grown in BHI broth to optical density

at 600 nm (OD_{600}) of approximately 0.4 were washed with physiological saline. A drop of bacterial suspension was placed on a carbon-coated grid, and the preparation was negatively stained in 3% uranyl acetate for 20 to 30 s. Grids were examined with a Jeol 1200 EX II transmission electron microscope (Jeol Ltd, Tokyo, Japan) at the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki.

4.9.2 Growth curve analyses (III-V)

Single colonies of EGD-e and each mutant strain grown on blood agar plates were inoculated into 10 ml of BHI broth at 37°C in three or five replicates. Cultures grown for 16-17 h (III) or 20 h (IV, V), were diluted 1:100 in test or control growth media. The strains were grown in the Bioscreen C Microbiology Reader (Growth Curves Ltd, Helsinki, Finland) under the aforementioned test conditions and in BHI broth at 37°C as a control. The OD_{600} levels of the cultures were monitored at 15-min intervals at 37°C and 42.5°C for 48 h, or at 1-h intervals at 3°C for 21 days. The OD_{600} data were fitted to growth curves to obtain the growth rates and maximum OD_{600} levels for the wild-type EGD-e and the deletion mutant strains, using the nonlinear least squares function in the statistical computing package R (Venables & Ripley, 2002) (III), or DMFit software version 2.1. (Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, UK) (Baranyi & Roberts, 1994) (IV, V).

4.9.3 Correspondence between viable cell numbers and OD_{600} readings (III, IV)

The correspondence between the viable cell numbers and OD_{600} of the wild-type EGD-e and deletion mutant strains at 3°C and at 37°C were examined by plating cultures grown in BHI broth at two time points, representing early logarithmic and late logarithmic growth phases (III), or at three time points, representing early logarithmic, late logarithmic, and early stationary growth phases (IV) of the wild-type EGD-e, to plate count agars (Difco Laboratories, Detroit, MI, USA).

4.9.4 Minimum and maximum growth temperatures (IV, V)

The mean minimum and maximum growth temperatures of the wild-type EGD-e and mutant strains were examined with three or five replicate cultures of each strain, according to Hinderink *et al.* (2009), using the Gradiplate W10 temperature gradient incubator (BCDE Group, Helsinki, Finland), with the following modifications. Cultures grown for 20 h in BHI broth and diluted 1:100 in the same medium were plated by the stamping technique onto TSA. To study minimum growth temperatures, the strains

were grown for 21 days aerobically in the Gradiplate incubator using temperature gradients from 1.0°C to 9.5°C and from 8.7°C to 16.8°C. To analyse maximum growth temperatures, the strains were grown for 48 h using a temperature gradient from 39.1°C to 45.6°C. The growth boundaries were observed with a stereomicroscope. The temperatures under and above which dense growth was abolished and separate colonies appeared were determined as the minimum and maximum growth temperatures, respectively.

4.9.5 Swarming motility (III, IV)

The swarming motility of the wild-type EGD-e, all six deletion mutant strains, and complementation strains on semisolid tryptic soy medium was examined in triplicate, according to Kathariou *et al.* (1995), using tryptic soy broth (BD) solidified with 0.25% agar (III, IV). The plates were incubated at 3°C for three (III) or eight weeks (IV), or at 25°C and 37°C for 24 h.

4.10 Statistical testing

Significance of differences in the prevalence of *L. monocytogenes* in tonsils of fattening pigs and sows, between two sampling sites of raw fish, and in the distribution of *L. monocytogenes* isolates recovered from raw and processed fish to serotypes was examined using the χ^2 chi-square test. The *t*-test was used to determine the significance of differences in the transcription levels of the *flhA, motA, lmo0866, lmo1246, lmo1450,* and *lmo1722* in test and control growth conditions, and in growth rates (III, V) and in maximum optical densities (III) of the wild-type EGD-e and the deletion mutant strains under test and control growth conditions.

5 RESULTS

5.1 Prevalence and diversity of *L. monocytogenes* in tonsils of pigs and in raw fish (I, II)

A total of 14% (38/271) of pig tonsils and 4% (11/257) of raw fish samples harbored *L. monocytogenes*. The prevalence in tonsils of fattening pigs (22%) was significantly higher than in sows (6%) ($P<0.001$), whereas there were no differences in prevalence between the two sampling sites of raw fish ($P>0.05$). From the 38 positive tonsil samples, 24 different *L. monocytogenes* genotypes were recovered with PFGE typing. The positive raw fish samples represented nine different PFGE types and three serotypes.

5.2 Comparison of *L. monocytogenes* isolated from raw and processed fish (II)

From 101 fish product isolates, 32 different PFGE types were recovered. Two fish product PFGE types, found from non-heat treated, sugar-salted "gravad" fish, were indistinguishable from raw fish PFGE types. Serotyping divided fish product isolates and raw fish isolates similarly into serotypes, except that serotype 3a was more prevalent in raw fish ($P<0.05$).

5.3 Relative expression of *flhA, motA, lmo0866, lmo1246, lmo1450,* and *lmo1722* during growth at various stress conditions (III-V)

During mid-logarithmic growth in BHI broth at 3˚C, the transcription levels of flagellar genes *flhA* and *motA* were 465-fold and 238-fold higher, respectively, than at 37˚C ($P<0.01$). The relative expression of DEAD-box RNA helicase-encoding genes *lmo0866, lmo1246, lmo1450,* and *lmo1722* was also increased during growth at 3˚C ($P<0.05$) (Fig. 1). In 6% NaCl, the relative expression of *lmo0866* and *lmo1246* was lower than under control growth condition ($P<0.05$), while the relative expression of *lmo1450* and *lmo1722* was not affected. In 3.5% ethanol, the relative expression of *lmo1246, lmo1450,* and *lmo1722* was increased ($P<0.05$), whereas the transcription level of *lmo0866* was unaffected. At 42.5˚C, pH 5.6, pH 9.4, and in 5 mM H_2O_2, the transcription levels of the DEAD-box RNA helicase-encoding genes did not differ from those in the control growth condition.

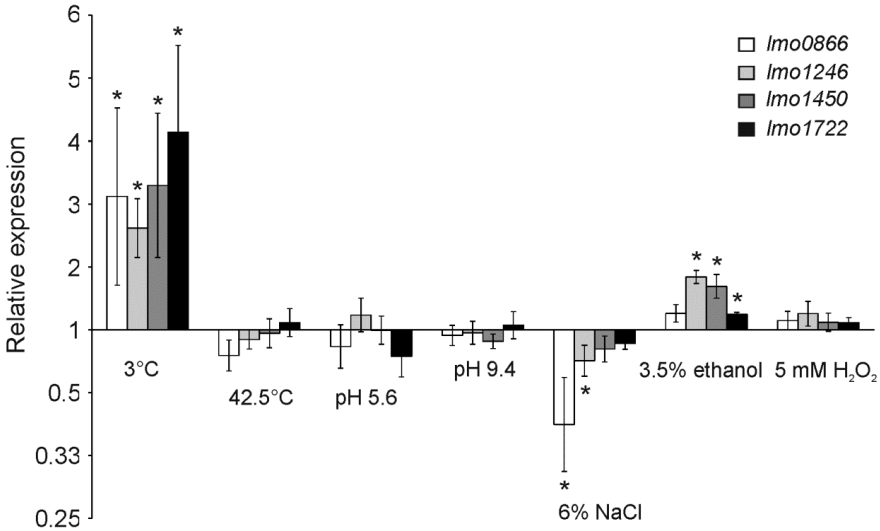

Fig. 1. Relative expression of the DEAD-box RNA helicase genes *lmo0866, lmo1246, lmo1450,* and *lmo1722* in *Listeria monocytogenes* EGD-e during mid-logarithmic growth at 3˚C, at 42.5˚C, at pH 5.6, at pH 9.4, in 6% NaCl, in 3.5% ethanol, and in 5 mM H_2O_2 in relation to midlogarithmic growth at 37˚C in BHI broth. Error bars represent the standard deviation of three independent replicates. Significant differences (paired *t*-test, $P<0.05$) in relative expression levels are indicated by an asterisk. Adopted from IV and V.

5.4 Characteristics of genetically modified *L. monocytogenes*

5.4.1 Morphology (III, IV)

After 24 h at 37˚C on blood agar, EGD-eΔ*flhA,* EGD-eΔ*motA,* Δ*lmo0866,* Δ*lmo1246,* Δ*lmo1722,* Δ*lmo0866*c, Δ*lmo1450*c, and Δ*lmo1722*c formed colonies similar to the wild-type strain EGD-e. Colonies of Δ*lmo1450* were more rounded and opaque, and the diameter was approximately one-third of that of the wild-type EGD-e colonies. All strains were β-haemolytic.

Electron microscopy showed that the wild-type EGD-e produced flagella whereas EGD-eΔ*flhA* and EGD-eΔ*motA* appeared nonflagellated at 3˚C. At 37˚C all three strains appeared nonflagellated.

5.4.2 Stress tolerance (III-V)

Deletion of *flhA* or *motA* decreased the growth rate of the wild-type strain EGD-e by 25% at 3˚C (Table 8A). At this temperature, the maximum optical densities of EGD-eΔ*flhA* and EGD-eΔ*motA* were 27% and 22% lower, respectively, than those of the wild-type EGD-e. At 37˚C, there were no significant differences in growth rates or in the maximum optical densities between these three genotypes.

The growth of Δ*lmo0866* was prevented at 3˚C (Fig. 2). In 3.5% ethanol, the mean maximum growth rate of Δ*lmo0866* was 75% less than that of the wild-type EGD-e (Table 8B). In control growth conditions and in 6% NaCl, the mean growth rate of Δ*lmo0866* was 17% and 14% lower, respectively, than that of the wild-type, whereas at 42.5˚C, the mean growth rate of Δ*lmo0866* was increased by 22%.

There was no detectable growth of Δ*lmo1450* at 42.5˚C and at pH 9.4 (Fig. 2). At 3˚C and in 5 mM H_2O_2, the growth of this mutant was restricted. At pH 5.6, in 6% NaCl, in 3.5% ethanol, and in the control, the mean growth rate of Δ*lmo1450* was 20%, 29%, 50%, and 41%, respectively, less than that of the wild-type EGD-e (Table 8B).

Deletion of *lmo1722* resulted in no detectable growth at 3˚C (Fig. 2, Table 8B). The mean growth rate of Δ*lmo1722* was increased by 22% and 25% at 42.5°C and in 3.5% ethanol, respectively, and decreased by 10% in control condition. The growth of Δ*lmo1246* was similar to that of the wild-type EGD-e under all the conditions tested. From cultures of the wild-type EGD-e and

Table 8A. Average maximum growth rates of *Listeria monocytogenes* EGD-e and the flagellar gene deletion mutants EGD-eΔ*flhA* and EGD-*e*Δ*motA* at 37˚C and 3˚C. Adopted from III.

	Growth rate ± SD (OD_{600} units/h)		
Growth condition	EGD-e	EGD-eΔ*flhA*	EGD-eΔmotA
37˚C, control	0.47±0.007	0.45±0.02	0.46±0.03
3˚C	0.04±0.002	0.03±0.001*	0.03±0.002*

*Significant difference (Student's *t*-test, $P<0.001$) compared with the corresponding value of the wild-type EGD-e.

Table 8B. Average maximum growth rates of *Listeria monocytogenes* EGD-e and the DEAD-box RNA helicase gene deletion mutants Δ*lmo0866*, Δ*lmo1246*, Δ*lmo1450* and Δ*lmo1722* under different growth conditions. Adopted from VI and V.

	Growth rate ± SD (OD_{600} units/h)				
Growth condition	EGD-e	Δ*lmo0866*	Δ*lmo1246*	Δ*lmo1450*	Δ*lmo1722*
37˚C, control	0.29±0.004	0.24±0.006*	0.28±0.007	0.17±0.004*	0.26±0.006*
3˚C	0.12±0.009	0.00±0.002*	0.09±0.003*	0.01±0.001*	0.00±0.000*
42.5˚C	0.09±0.004	0.11±0.003*	0.10±0.003	0.01±0.001*	0.11±0.003*
pH 5.6	0.05±0.002	0.05±0.005	0.05±0.003	0.04±0.005*	0.05±0.002
pH 9.4	0.07±0.014	0.05±0.006	0.06±0.006	0.02±0.023	0.05±0.006
6% NaCl	0.07±0.002	0.06±0.003*	0.07±0.001	0.05±0.004*	0.07±0.001
3.5% ethanol	0.04±0.002	0.01±0.004*	0.04±0.003	0.02±0.001*	0.05±0.001*
5 mM H_2O_2	0.21±0.041	0.17±0.015	0.21±0.015	0.02±0.019*	0.22±0.013

*Significant difference (Student's *t*-test, $P<0.001$) compared with the corresponding value of the wild-type EGD-e.

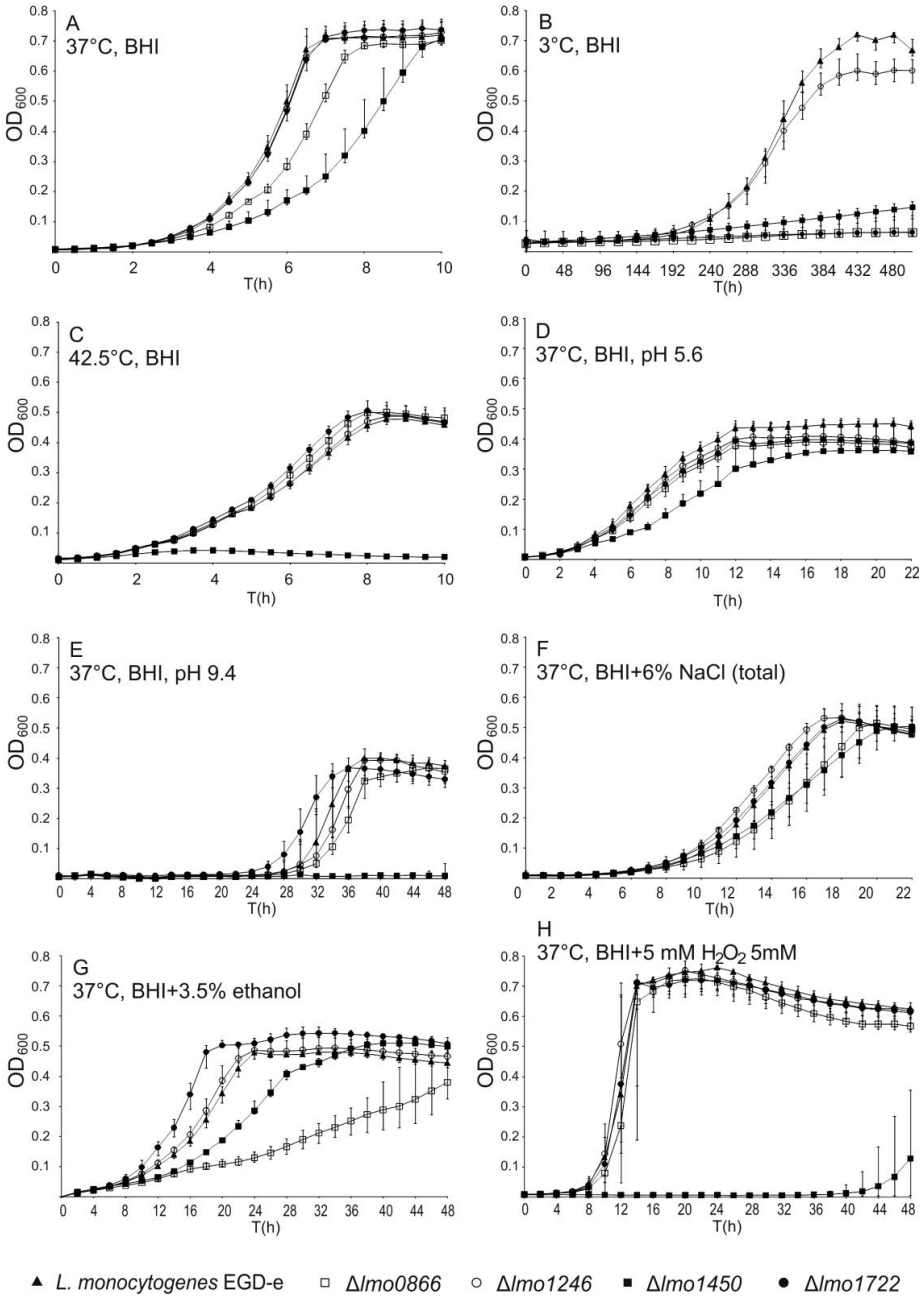

Fig. 2. Growth of *Listeria monocytogenes* EGD-e and the DEAD-box RNA helicase gene deletion mutants Δ*lmo0866*, Δ*lmo1246*, Δ*lmo1450*, and Δ*lmo1722* in BHI broth at 37°C (A), 3°C (B), 42.5°C (C), pH 5.6 (D), pH 9.4 (E), in 6% NaCl (F), 3.5% ethanol (G), and 5mM H_2O_2 (H). The OD_{600} was monitored at 15-min intervals for 48 h (A, C-H) or at 1-h intervals for three weeks (B). The data shown represent median OD_{600} values of four (G) and five (A–F, H) independent cultures. The error bars indicate the range of the replicate cultures. Adopted from IV and V.

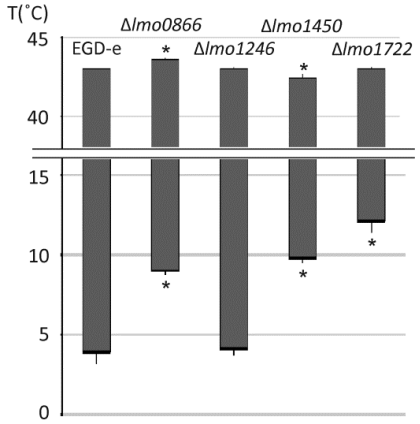

Fig. 3. Temperature range of growth of the wild-type *Listeria monocytogenes* EGD-e and the DEAD-box RNA helicase gene deletion mutant strains Δ*lmo0866,* Δ*lmo1246,* Δ*lmo1450,* and Δ*lmo1722.* Mean minimum and maximum growth temperatures were examined with five replicate cultures of each strain, by growing the strains in tryptic soy agar containing 1.5% agar for three weeks and tryptic soy broth solidified with 2.5% agar for 48 h, respectively, in a Gradiplate W10 temperature gradient incubator at temperature ranges from 1.0°C to 9.5°C, from 8.7°C to 16.8°C, and from 39.1°C to 45.6°C. Significant differences (paired *t*-test, $P<0.05$) in minimum and maximum growth temperatures compared to that of the wild-type EGD-e are indicated by an asterisk. Derived from IV and V.

all six deletion mutant strains with the same OD_{600} values, similar total cell numbers were determined by plating.

Growth curves of the Δ*lmo0866*c complementation strain at 3°C and in 3.5% ethanol, Δ*lmo1450*c complementation strain at 3°C, at 42.5°C, at pH 9.4, and in 5 mM H_2O_2, and Δ*lmo1722*c complementation strain at 3°C were similar to that of the EGD-epPL2 control strain.

Minimum growth temperatures of Δ*lmo0866,* Δ*lmo1450,* and Δ*lmo1722,* and maximum growth temperatures of Δ*lmo0866* and Δ*lmo1450* differed significantly from that of the wild-type EGD-e (Fig. 3). After 21 days in a temperature gradient incubator, the minimum growth temperatures of Δ*lmo0866,* Δ*lmo1450,* and Δ*lmo1722* were 5.1°C, 4.9°C and 8.8°C higher, respectively, than that of the wild-type EGD-e. Following 48 h incubation, maximum growth temperatures of Δ*lmo0866* and Δ*lmo1450* were 0.6°C higher and 0.6°C lower, respectively, than the maximum growth temperature of the wild-type EGD-e. The maximum growth temperatures of Δ*lmo0866*c and Δ*lmo1450*c complementation strains were restored to the wild-type value.

5.4.3 Motility (III, IV)

The wild-type EGD-e and Δ*lmo1246* were motile in semi-solid agar plates at 25°C and at 3°C. The diameter of the corona of growth around the inoculation point of Δ*lmo1722* on the semi-solid agar plate at 25°C was

approximately half of that of the wild-type EGD-e. The motility of Δ*lmo1722* could not be evaluated at 3°C because of growh inhibition. At 37°C, the wild-type EGD-e, Δ*lmo1246,* and Δ*lmo1722* were non-motile. EGD-eΔ*flhA,* EGD-eΔ*motA,* Δ*lmo0866,* and Δ*lmo1450* were non-motile under all the conditions tested. The motilities of Δ*lmo0866*c, Δ*lmo1450*c, and Δ*lmo1722*c complementation strains were restored to the wild-type level.

6 DISCUSSION

6.1 Tonsils of pigs and raw fish as a potential source of contamination of pork and fish processing plants by *L. monocytogenes* (I, II)

The figure of 14% of pigs carrying *L. monocytogenes* in their tonsils is in agreement with a previous Finnish study showing a carrier rate of 12% in tonsils of pigs slaughtered at low-capacity slaughterhouses (Autio *et al.*, 2000). In the present study, the prevalence was significantly higher in fattening pigs (22%) than in sows (6%). Even higher prevalences, 32% and 45%, were reported in fattening pig tonsils in Germany and former Yugoslavia, respectively (Buncic, 1991; Fredriksson-Ahomaa *et al.,* 2009). The 4% prevalence in raw fish falls within the 0-50% range of previous studies (Ben Embarek, 1994; Autio *et al.*, 1999; Nørrung *et al.*, 1999; Fonnesbech Vogel *et al.*, 2001; Norton *et al.*, 2001; Hoffman *et al.*, 2003). The occurrence in pigs is at least partly attributable to husbandry and feeding practices (Skovgaard & Nørrung, 1989; Buncic, 1991; Hellström *et al.*, 2010) and that in fish to water quality (Ben Embarek, 1994). The differences in prevalence between fattening pigs and sows may result from acquired resistance or immunity of sows to this pathogen, since they are older at slaughtering than fattening pigs.

The totals of 24 and nine different PFGE types in pig and fish samples, respectively, demonstrate the high genetic diversity of *L. monocytogenes* and suggests that a wide range of strains enters pig slaughterhouses and fish processing plants. Clearly, raw material serves as a potential source of *L. monocytogenes* contamination of pork and fish entering the food chain. When pig tonsils are removed, the pathogen can spread from the tonsils to the pluck set and carcass. During evisceration of fish, *L. monocytogenes* can spread from gills, the most heavily contaminated part (Miettinen & Wirtanen, 2005). Moreover, *L. monocytogenes* in these raw materials can contaminate equipment in the processing chain.

6.2 Raw fish as a potential source in contamination of fish products by *L. monocytogenes* (II)

In this study, identical *L. monocytogenes* PFGE and serotypes were found from raw and processed fish as also demonstrated by Fonnesbech Vogel *et al.* (2001) in Denmark and Di Ciccio *et al.* (2012) in Italy. The excistence of the same PFGE type both in raw and cold-smoked fish can be attributed to persistence of strains that entered the plant along with raw material and contaminated the final products via the processing environment, or to the

initial contaminant's survival of a non-listericidal process through to the final product. Nevertheless, the same PFGE type in the final product may also have originated from a source other than raw fish (Autio *et al.*, 2002).

Contamination of fish products has been reported to occur mainly during the processing stage and primarily by endemic *L. monocytogenes* strains that may have persisted in plants for years (Ericsson *et al.*, 1997; Autio *et al.*, 1999; Fonnesbech Vogel *et al.*, 2001). Our results support the hypothesis that certain *L. monocytogenes* strains entering the plants along with raw material may contaminate the processing environment and persist there, causing recurrent contamination of the final products via processing machines.

6.3 The role of *flhA* and *motA* in cold tolerance of *L. monocytogenes* (III)

In this study, the role of *flhA* and *motA*, encoding FlhA and MotA needed for temperature-dependent synthesis of flagella (Dons *et al.*, 1992; Kathariou *et al.*, 1995), in cold tolerance of *L. monocytogenes* was examined. The decreases in growth rates and maximum optical densities of the flagella-deficient and non-motile deletion mutant strains EGD-eΔ*flhA* and EGD-eΔ*motA* under cold stress conditions, compared to those of the wild-type EGD-e, confirm that FlhA and MotA have roles in cold-temperature growth of *L. monocytogenes*. This conclusion was supported by the clearly increased transcription levels of *flhA* and *motA* under cold stress conditions compared to control values. How *flhA* and *motA* support the growth of *L. monocytogenes* at low temperatures is unknown. It may be that motile flagella are needed for optimal cold stress response, but these genes might also have other, yet undescribed functions in cold tolerance of this species.

6.4 The role of *lmo0866, lmo1246, lmo1450,* and *lmo1722* in the stress tolerance of *L. monocytogenes* (IV, V)

DEAD-box proteins are conserved RNA helicases present in most living organisms and associated with various aspects of RNA metabolism (Silverman *et al.*, 2003; Cordin *et al.*, 2006; Fairman-Williams *et al.*, 2010; Linder & Jankowsky, 2011). The best known functions of DEAD-box proteins include separation of short duplex regions of RNA (helicase activity), and chaperone activity that facilitates native folding of structured RNAs (Jarmoskaite & Russell, 2011). Presumably due to the increased stability of RNA secondary structures at low temperatures, inactivity of DEAD-box proteins has been linked to cold sensitivity in some bacteria (Charollais *et al.*, 2004; Hunger *et al.*, 2006; Pandiani *et al.*, 2010; Palonen *et al.*, 2012). DEAD-box proteins have also been reported to contribute to heat, alkali, and oxidative stress tolerance in *Bacillus cereus* (Pandiani *et al.*, 2011). The

increased minimum growth temperature of the deletion mutant Δ*lmo0866*, its inhibited growth at 3˚C and restricted growth rate in 3.5% ethanol suggest that Lmo0866 has supportive roles in growth of *L. monocytogenes* EGD-e under both cold and ethanol stress conditions. The decreased maximum growth temperature and increased growth rate of Δ*lmo0866* at 42.5˚C indicate that Lmo0866 represses growth under heat stress. The findings were confirmed when complementation of the deletion restored the maximum growth temperature and growth of Δ*lmo0866* at 3˚C and 3.5% ethanol to the wild-type level. The slightly restricted growth of Δ*lmo0866* compared to the wild-type EGD-e at pH 9.4 and in 6% NaCl, but also in control growth condition, suggests no specific role for Lmo0866 under alkali and osmotic stresses.

The observed growth characteristics of Δ*lmo1450* at 3˚C, at 42.5˚C, at pH 9.4, and in 5 mM H_2O_2 indicate an important role for Lmo1450 under cold, heat, alkali, and oxidative stress condition, and this was confirmed when complementation of the *lmo1450* deletion restored the wild-type phenotype. The role of DEAD-box RNA helicases in heat and oxidative stress tolerance is confirmed by the roles of *cshA, cshB,* and *cshC,* orthologous with *lmo0866, lmo1450,* and *lmo1722,* respectively, in heat and oxidative stress tolerance in *B. cereus* (Pandiani *et al.,* 2011). The slightly restricted growth of Δ*lmo1450* compared to that of the wild-type EGD-e in the control condition, at pH 5.6, in 6% NaCl, and in 3.5% ethanol suggests that Lmo1450 has a more general role in growth of *L. monocytogenes*.

The inhibited growth of Δ*lmo1722* at 3˚C, and its restoration in Δ*lmo1722*c complementation strain, suggests that Lmo1722 is needed for growth under cold stress. This conclusion is supported by the restricted low-temperature growth of a mutant of *L. monocytogenes* F2356 carrying a transposon insertion in its *lmo1722* homologue (Azizoglu & Kathariou, 2010a). The effect of slightly increased growth rate of Δ*lmo1722* at 42.5˚C was not confirmed by altered maximum growth temperature or transcript level of *lmo1722* at 42.5˚C, so it may be concluded that the role of Lmo1722 under heat stress is not significant. A further test of the growth of Δ*lmo1722* under ethanol stress (data not shown) suggests that its slightly increased growth rate in 3.5% ethanol was not specific to ethanol stress. This study and the previous study by Azizoglu and Kathariou (2010a) show that Lmo1722 has an important role in growth of *L. monocytogenes* under cold stress and is not associated with heat, pH, osmotic, ethanol, or oxidative stress tolerance. This differentiates Lmo1722 from Lmo0866 and Lmo1450, both of which play roles in tolerance to several stresses.

The negligible role of *lmo1246* in low-temperature growth, and the identical growth of the wild type and Δ*lmo1246* under all the other conditions tested, indicate that Lmo1246 has no role in the tolerance of *L. monocytogenes* EGD-e to temperature, pH, osmotic, ethanol, or oxidative stress. Similarly, the DEAD-box RNA helicase CshD of *B. cereus*, containing the conserved carboxy-terminal domain DpbA present also in Lmo1246 and

not found in the other DEAD-box RNA helicases of *L. monocytogenes* or *B. cereus*, has no role in temperature, pH, or oxidative stress tolerance (Pandiani *et al.*, 2010, 2011). Further studies are needed to reveal if Lmo1246 is complementary to the paralogous DEAD-box RNA helicases or if it has a role in growth of *L. monocytogenes* under other stresses.

In cold conditions, the relative expression of DEAD-box RNA helicase genes of *L. monocytogenes* increased in a comparable manner to that of their counterparts in *B. cereus,* which showed higher transcript levels at 10˚C than at 37˚C (Pandiani *et al.*, 2010). Similarly, Chan *et al.* (2007) showed that the expression of the *lmo0866, lmo1450,* and *lmo1722* homologues of *L. monocytogenes* 10403S increased at low temperatures. Moreover, transcript levels of *lmo0866* and *lmo1246* decreased under osmotic stress and those of *lmo1246, lmo1450,* and *lmo1722* increased under ethanol stress, compared to the transcript levels of controls. Our findings are supported by the lack of significant changes in the expression of DEAD-box RNA helicase genes of alkali stressed strain 10403S (Giotis *et al.,* 2010) and the decreased transcription levels of *lmo0866* 3 min after heat shock without any subsequent change in the expression of DEAD-box RNA helicase genes (van der Veen *et al.,* 2007). Thus, tolerance of *L. monocytogenes* to cold stress, associated with DEAD-box RNA helicase genes, is likely regulated at the transcriptional level, whereas *lmo0866*- or *lmo1450*-associated tolerance to heat, alkali, ethanol, or oxidative stress is likely controlled through another mechanism. Under heat, alkali, ethanol, and oxidative stresses, the expression of *lmo0866* and *lmo1450* may be controlled at a translational or post-translational level, a well-known phenomenon in the regulation of RNA helicase activity in eucaryotes but yet unknown in bacteria (Owttrim, 2006).

6.5 Association between motility and cold tolerance of *L. monocytogenes* (III, IV)

No motility was detected in EGD-eΔ*flhA* and EGD-eΔ*motA* with reduced tolerance to cold stress, and cold-sensitive mutants Δ*lmo0866* and Δ*lmo1450*. In cold-sensitive Δ*lmo1722,* motility was restricted to about half of that of the wild-type EGD-e. Deletion of *lmo1246* did not affect motility and had a negligible effect on the cold tolerance of strain EGD-e. These results suggest an association between bacterial motility and the ability to grow at low temperatures. Palonen *et al.* (2011) showed reduced growth and motility of the c*heA* chemotaxis gene inactivation mutant strain of *Yersinia pseudotuberculosis* at low temperature, which supports this hypothesis. It seems that in addition to flagellin gene *flhA* and flagellar motor gene *motA*, DEAD-box RNA helicase-encoding genes *lmo0866, lmo1450,* and *lmo1722* are needed for synthesis or function of flagella, which is needed for optimal cold stress response of *L. monocytogenes*. Moreover, *lmo0866*, *lmo1450*, and

lmo1722 likely affect cold stress tolerance of *L. monocytogenes* through an additional, currently unknown mechanism.

7 CONCLUSIONS

1. Pigs and raw fish were shown to be potential sources of contamination of slaughterhouses and processing plants by wide variety of *L. monocytogenes* strains. The strains entering the plants may directly or indirectly contaminate the processing environment.

2. Similar PFGE types detected in raw and processed fish indicate that *L. monocytogenes* isolates originating from raw fish may directly or indirectly contaminate final fish products. *L. monocytogenes* originating from raw fish, and entering and persisting in the processing plant, may contaminate the final products via the processing environment. Alternatively, *L. monocytogenes* in raw fish may survive non-listericidal processes, resulting in contamination of final product.

3. The flagellar biosynthesis gene *flhA* and flagellar motor protein-encoding gene *motA* played roles in cold tolerance of *L. monocytogenes* EGD-e. This suggests that FlhA and MotA are needed for the optimal growth of *L. monocytogenes* under cold stress.

4. Putative DEAD-box RNA helicase-encoding genes *lmo0866, lmo1450*, and *lmo1722* played important roles in the tolerance of *L. monocytogenes* EGD-e to cold, alkali, ethanol, and oxidative stress. Gene *lmo0866* was needed for the wild-type growth under cold and ethanol stress conditions, and its deletion enhanced growth under heat stress conditions. Gene *lmo1450* seems to play a universal role in the growth of strain EGD-e, and it may have roles in cold, heat, alkali, and oxidative stress tolerance. Gene *lmo1722* played a role solely in cold tolerance. Whether *lmo1246* plays a role in growth under extremely cold conditions warrants further investigation. None of the putative DEAD-box RNA helicase genes had a role in acid or osmotic stress tolerance. These results suggest that DEAD-box RNA helicases Lmo0866, Lmo1450, and Lmo1722 have roles in growth of *L. monocytogenes* under cold, heat, alkali, ethanol, and oxidative stress conditions.

5. The motility of cold-sensitive, flagellar gene deletion mutants EGD-eΔ*flhA* and EGD-eΔ*motA,* and DEAD-box RNA helicase gene deletion mutants *Δlmo0866, Δlmo1450,* and *Δlmo1722* was restricted. This suggests that cold tolerance and motility of *L. monocytogenes* EGD-e are linked.

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