Screening of glutamate decarboxylase activity and bile salt resistance of human asymptomic carriage, clinical, food, and environmental isolates of Listeria monocytogenes

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Keywords: *Listeria monocytogenes*, bile salts, glutamate decarboxylase acid resistance system

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Abstract

Following consumption, stomach acidity is the first major barrier encountered by the food-borne pathogen *Listeria monocytogenes*. Analysis of low pH sensitivity and glutamate decarboxylase (GAD) acid resistance system of 14 isolates of *L. monocytogenes* carried asymptomatically by humans showed that levels of GAD activity was subjected to strain variation. Similar variations were observed for strains responsible of 18 listeriosis, whereas in comparison, 13 strains isolated from food and from food-processing plant environment showed lower GAD activity. Following survival of the stomach barrier, *L. monocytogenes* also has to resist to bile salts encountered in the small intestine. Analysis revealed that all strains tested were able to grow in presence of bile salts with concentrations as high as those encountered in the small intestine and had the previously identified Bile Salt Hydrolase (BSH) activity. Strain variation was observed but there was no relationship between the origin of the strains and the ability to degrade bile salts.

1. Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that has been recognized as a major food-borne pathogen since transmission of human listeriosis was first shown to be food borne (Schlech et al., 1983; Schlech, 1984). Based on present knowledge, any strain of *L. monocytogenes* detected in food should be considered as potentially pathogenic. However, assessment of their pathogenic process by *in vivo* and *in vitro* models suggest that *L. monocytogenes* virulence is heterogeneous (Del Corral et al., 1990; Brosch et al., 1993; Wiedmann et al., 1997; Van Langendonck et al., 1998; Norrung and Andersen, 2000; Barbour et al., 2001; Olier et al., 2003). Consequently, detection of markers that could predict their level of virulence in human are currently underway. Markers of virulence are generally studied using strains isolated from patients with listeriosis or from food or environment; in our laboratory isolates carried asymptomatically by humans have been studied in order to ascertain their virulence levels (Olier et al., 2003): of the 14 human carriage strains studied, 10 were virulence attenuated toward 14-day-old chick embryos. Five of these 10 isolates were impaired in their internalization process in Caco-2 cells because they produced a truncated form of Internalin, a well-known virulence factor. For the other 5 strains, internalization into host cells was similar to sporadic or epidemic-associated strains (Olier et al., 2003). Consequently, these human carriage strains must be considered as potentially pathogenic.

Ability to resist to low pH and bile, two stresses encountered in stomach and intestine, could be important factors for survival and asymptomatic carriage of a *L. monocytogenes* strain. In reponse to low pH in gastric fluids, intracellular homeostasis of *L. monocytogenes* is partially dependent on the ability of the F_0F_1-ATPase to pump excess protons from cytoplasm (Cotter et al., 2000). In addition, *L. monocytogenes* apparently utilizes a glutamate decarboxylase system (GAD) homologous to that used by *E. coli* (Cotter et al., 2001). When the cell is exposed to low pH, the GAD system converts one molecule of extracellular glutamate to one extracellular γ-aminobutyrate (GABA), while consuming an intracellular proton. The net effect of the GAD system is to reduce the proton concentration within the cell, thus alleviating acidification of the cytoplasm and alkalinize the environment since γ-aminobutyrate is less acidic than glutamate (Small and Waterman, 1998). Variations in levels of GAD activity between strains of *L. monocytogenes* correlates significantly with levels of tolerance to gastric fluids in the presence of glutamate and suggests that its expression is an absolute requirement for survival in the stomach environment and for a subsequent colonization of the gut (Cotter et al., 2001). Deliberate addition of free glutamate

in a large variety of food for flavor enhancement or for adjustment of acidity contributes significantly to the survival of *L. monocytogenes* strains with GAD activity, even with low concentrations (0.22mM) (Cotter et al., 2001). The well-characterized LO28 strain, which was recovered from the feces of healthy pregnant women, possesses the highest GAD activity among strains tested by Cotter et al. (2001). So, one of our objectives was to evaluate GAD activity of human carriage strains in comparison to the GAD activity of food, environmental and clinical isolates of *L. monocytogenes*.

Similarly, in comparison to clinical isolates, *L. monocytogenes* LO28 strain was capable of growing in the presence of bovine, porcine, and human bile salts with concentrations higher than those encountered *in vivo* (Begley et al., 2002). Moreover, *L. monocytogenes* have been isolated from gallblader, where bile salts are concentrated before they are released in the duodenum during digestion (Briones et al., 1992). Bile salts are amphipatic molecules, which can act as biological detergents by degrading lipid-containing bacterial membranes and have so antimicrobial properties. Several mechanisms to resist the detergent action of bile salts have been recently identified. Firstly, homologues of genes involved in maintenance of the cell enveloppe in species closely related to *L. monocytogenes* have been identified as putative genes involved in bile stress responses (Begley et al., 2002; Begley et al., 2003). Secondly, similarly to bacteria of numerous species isolated from the human gastrointestinal tract, Dussurget et al. (2002) found that the sequenced EGD-e *L. monocytogenes* strain was capable of hydrolysing the amide bond of conjugated bile salts, liberating free bile salts with markedly lower detergent properties in the emulsification of fat. Analysis of bile salt hydrolase (BSH) of EGD-e strain revealed that BSH was regulated by PrfA, the transcriptional activator of known virulence genes, and a PrfA DNA binding site was detected in the promoter of the *bsh* gene (Dussurget et al., 2002). Moreover, analysis of fecal carriage in guinea pig orally inoculated with EGD-e versus EGD-eΔ*bsh* showed that BSH activity was an adaptative advantage for intestinal persitence and colonization of this enteropathogen (Dussurget et al., 2002).

Despite the fact that *L. monocytogenes* is not considered as a normal inhabitant of the gastrointestinal tract, human beings and animals are known fecal carriers (Bojsen-Moller, 1972; Weber et al., 1995) and could have an important role in the transmission and spread of the bacteria. Therefore objectives of this study was to characterize further these human carriage strains by analysing their tolerance to low pH and bile, to evaluate their GAD and BSH activity in comparison to food, environmental and clinical isolates of *L. monocytogenes*.

2. Material and methods

2.1. L. monocytogenes *strains and media*

Fourteen human carriage strains, 4 animal carriage isolates, 18 human sporadic and epidemic clinical isolates and 14 food and environmental isolates were used (Table 1). Isolation methods, species identification (Rousset et al., 1994) and virulence potential of human isolates have been previously described (Olier et al., 2003). Strains Scott A (obtained from the Institut Pasteur Collection, Paris, France), strains LO28 and EGD-e (kindly provided by P. Cossart, Institut Pasteur, Paris, France) were added as reference strains for comparative analysis. Food and environmental strains came from the strain collection of "Laboratoire de Microbiologie UMR INRA/UB 1232" (Dijon, France) except strains NV4, NV5, NV7 and NV8, which have been provided by the "Laboratoire départemental de la Haute Vienne"(Limoges, France).

Stock cultures were maintained at – 80°C in cryobank (AES Laboratoires, Combourg, France). All strains were resuscitated before use by inoculation onto Brain Heart Infusion (BHI, Biomérieux, Marcy l'Etoile, France) agar plates supplemented with acriflavine (Sigma, St Louis, MO, USA) 10 mg l^{-1} and ceftazidime (GlaxoSmithkline, Marly-le Roi, France) 50 mg l^{-1}, followed by incubation at 37°C. Subculture and culture medium was BHI broth (Biomérieux).

2.2. Survival of L. monocytogenes strains in synthetic gastric fluid in vitro

Firstly, in order to detect GAD activity of human asymptomatic carriage strains of *L. monocytogenes*, a preliminary study was carried accordingly to the method of Cotter et al. (2001) with the well-studied LO28, Scott A and EGD-e strains considered as reference strains. Briefly, overnight cultures grown in BHI at 37°C were pelleted by centrifugation (Eppendorf, Hamburg, Germany), resuspended in equal volumes of synthetic gastric fluids (8.3 g Proteose Peptone (Difco, Becton Dickinson, Le Pont de Claix, France), 3.5 g D-glucose (Prolabo, Fontenay sous Bois, France), 2.05 g NaCl (Prolabo), 0.6 g KH_2PO4 (Prolabo), 0.4 g $CaCl_2$ (Prolabo), 0.37 g KCl (Prolabo), 0.1 g lysozyme (Prolabo), 50 mg porcine bile salts (Sigma), and 13.3 mg pepsin (Sigma) per liter of distilled water adjusted to pH 2.5 with 1N HCl) prewarmed at 37°C. To assesss protective effect of glutamate, 10 mM of monosodium glutamate (Sigma) were added to synthetic gastric fluid before pH adjustment. Sensitivity of the strains to synthetic gastric fluid with and without addition of monosodium glutamate was monitored by removing samples at 15-min intervals during 60 minutes. The CFU values for viable bacteria were determined by plating suitable dilutions of the samples onto BHI agar.

Table 1. Characteristics of *Listeria monocytogenes* strains used in this study

L.monocytogenes isolates	Origin	Serogroup
Human fecal isolates		
H1	Healthy pregnant carrier (Beaune, France, 1991)	1
H2	Healthy 3-year infant (Beaune, France, 1992)	4
H6	Healthy 30-year women (Beaune, France, 1991)	
H11	Healthy 35-year man (Beaune, France, 1992)	1
H17	Healthy 10-year infant (Beaune, France, 1992)	
H27	Healthy 2-year infant (Beaune, France, 1994)	4
H28	Healthy 7 year infant (Beaune, France, 1994)	1
H31	Healthy 55-year man (Beaune, France, 1995)	1
H32	Healthy 19-year women (Beaune, France, 1996)	1
H34	Healthy 8-year infant (Beaune, France, 1997)	1
H35	Healthy 11-year infant (Beaune, France, 1997)	1
H38	Healthy carrier (Beaune, France, date not communicated)	
LO28	Healthy pregnant carrier (Spain)	1/2c
Animal fecal isolates		
23	Rook feces (Besançon, France, 1995)	4
38	Rook feces (Besançon, France, 1995)	1
81	Rook feces (Besançon, France, 1995)	1
97	Rook feces (Besançon, France, 1995)	1
Human oral isolate		
H12	Healthy 39-year man (Beaune, France, 1991)	4
Sporadic patients isolates		
H3	Blood culture (Chalon/Saône, France, 1991)	1
H4	Blood culture (Beaune, France, 1992)	1
H5	Cephalorachid liquids (Chalon/Saône, France, 1991)	1
H8	Blood culture (Chalon/Saône, France, 1992)	1
H10	Peripheral swab (Chalon/Saône, France, 1991)	4
H13	Blood culture and cephalorachid liquids (Chalon/Saône, France, 1991)	4
H15	Blood culture (Rennes, France, 1992)	1
H16	Blood culture (Rennes, France, 1992)	1
H18	Blood culture (Strasbourg, France, 1992)	1/2b
H19	Blood culture (Strasbourg, France1992)	4b
H21	Blood culture (Strasbourg, France, 1992)	4
H22	Blood culture (Strasbourg, France, 1992)	4b
H23	Liver (Strasbourg, France, 1992)	1
H24	Placenta (Strasbourg, France, 1992)	4b
H25	Placenta (Strasbourg, France, 1992)	1/2a
Epidemic-associated strains		
Scott A	Massachusetts milk outbreak (USA, 1983)	4b
H36	Epoisses cheese outbreak (France, 1999)	4b
EGD-e	Derivative strain of animal outbreak (England, 1924)	1/2a
Food isolates		
NV4	Minced beef (Limoges, France)	1/2a
NV5	Minced beef (Limoges, France)	1/2c
NV7	Bovine carcass (Limoges, France)	1/2c
NV8	Bovine carcass (Limoges, France)	1/2a
1 F	Cheese (Dijon, France, 1990)	1/2a
2 F	Cheese (Dijon, France, 1990)	1/2a
7 F	Cheese (Dijon, France, 1990)	3b
Environmental isolates		
1 E	Machine of an industrial cheese-making plant (Dijon, France, 1990)	1/2b
3 E	Sink of an industrial cheese-making plant (Dijon, France, 1990)	1/2b
6 E	Wall of an industrial cheese-making plant (Dijon, France, 1990)	1/2a
1 S	Brine (Dijon, France, 1990)	1/2a
2 S	Brine (Dijon, France, 1990)	1/2a
3 S	Brine (Dijon, France, 1990)	1/2a
C9	Compost (Dijon, France, 2002)	nd

Secondly, in order to ease the monitoring of differences between human asymptomatic carriage strains, survival was carried out for 120 min with gastric fluids adjusted to pH 3.5. Results are means of triplicate independent experiments.

2.3. Glutamate decarboxylase activity assay

All 50 strains were tested in a rapid colorimetric assay using bromocresol green as pH indicator as described by Cotter et al. (2001). Volumes (1 ml) of overnight culture in BHI were centrifuged (Eppendorf), washed in 0,9 % NaCl solution and resuspended in 0.5 ml of test solution (1 g L-glutamic acid (Sigma), 0.3 ml Triton X-100 (Prolabo), 90 g NaCl and 0.05 g bromocresol green (Sigma) in 1 l of distilled water adjusted to pH 4). No change in yellow color after 4 h incubation at 37°C was considered as a negative result for GAD activity. Development of a green or blue colour was considered as low or high activity respectively. Three independent experiments were done for each strain tested.

2.4. Porcine Bile Salts tolerance

Overnight cultures (carriage, clinical, food and environmental isolates) grown in BHI were patched onto BHI agar plates supplemented with 1% (wt/vol) of porcine bile salts (Sigma). Plates were incubated for 48 h at 37°C, observed for growth and for the development of zones of bile salts degradation around colonies. Isolates were grouped into 4 activity classes based on the diameter of the degradation zone: no detectable activity; low activity (zone of up to 8 mm); medium activity (zone of up to 10 mm); high activity (zone of up to 12 mm) according to activity classes described by Franz et al. (2001). Initial size of the colonies was 4.5 mm.

2.5. Bile salt hydrolase activity

L. monocytogenes strains were screened for BSH activity by adapting the methods of Franz et al. (2001) and Dussurget et al. (2002). Colonies grown on BHI agar plates were patched onto BSH screening medium which consisted of Man Rogosa and Sharpe (MRS) agar medium (Difco) supplemented with 0.37 g l^{-1} $CaCl_2$ (Sigma) and 0.5% (wt/vol) glycodeoxycholic acid (Larodan AB, Malmö, Sweden). Plates were incubated anaerobically at 37°C for 5 days. BSH activity was present when deoxycholic acid precipitated in the agar medium below and around a colony.

2.6.Extraction of genomic DNA

Overnight cultures of each strain grown in BHI broth were harvested in 0.4 ml buffer (50 mM Tris-HCl [pH 8] [Euromedex, Mundolsheim, France], 25 mM EDTA [Euromedex], 2% [wt/vol] glucose, 200 µg ml^{-1} RNAse [Eurogentec, Angers, France], 3 mg ml^{-1} lysozyme [Euromedex]). The mixture was incubated for 60 min at 37°C. One hundred microliters of 10% (wt/vol) SDS solution (Euromedex) were added and the mixture was incubated for 10 min at 60°C. Before undergoing phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) extraction, 125 µl of 5M $NaClO_4$ (Sigma) were added. After centrifugation, DNA was precipitated by addition of isopropanol (Sigma); samples were then centrifuged (Eppendorf), and the pellets resuspended in 30 µl of ultrapure water (Fisher Scientific, Elancourt, France).

2.7. Analysis of sequence variation of the PrfA DNA binding site uptstream of the bsh *gene by PCR*

Presence of *bsh* gene was assessed in all strains by PCR amplification with primers GRO-LMO1 and GRO-BSH (Table 2). Comparison of the *bsh* promoter of the sequence of strains EGD-e (lmo2067, Glaser et al., 2001) and a representative strain of serotype 4b (Institute for Genomic Research website at http://www.tigr.org) revealed differences in the PrfA box upstream *bsh* (Fig 1.). The sequence of the serotype 4b strains was found to be 5'-TAAAAAATTTTTA-3' whereas the perfect palindromic sequence identified by Dussurget et al. (2002) for EGD-e strain was 5'-TTAAAAATTTTTAA-3'. The two different types of PrfA DNA binding site were screened by using GRO-LMO1 as universal forward primer and reverse primers PRFABOX-EGD or PRFABOX-4b (Table 2), which were able to amplify the PrfA DNA binding site of EGD-e or 4b sequenced strains, respectively. Amplification of *inlA* gene with primers seq01 and seq02 (Olier et al., 2002) was considered as positive control of amplification (Table 2).

PCR was performed in a reaction volume of 20 µl containing 40 ng of *L. monocytogenes* target DNA; 1x Taq polymerase buffer (QBiogene, Illkirch, France); 10 µM of each deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP; Qbiogene); 0.1 µM of each primer; and 0.4 U of AmpliTaq polymerase (Qbiogene). PCR was carried out in a i-Cycler thermocycler (BioRad Laboratories, Hercules, CA, USA) run for 30 cycles (94°C for 45 s, 57°C for 1 min, and 72°C for 1min 50 s with a final extension at 72°C for 7 min). After amplification, 10 µl of each PCR product was analysed by agarose gel (2%) electrophoresis in Tris-Acetate-EDTA buffer, stained with ethidium bromure and examined by UV transillumination using Gel Doc 2000 system (Bio-Rad).

Table 2. PCR primers used in this study.

Primer	Sequence (5' to 3')	PCR product size
GRO-LMO1	TGAACGCTTGAAACACGAAG	832 bp with GRO-BSH
GRO-BSH	CAGTAGCGCATTGACCAAGA	832 bp with GRO-LMO1
PRFABOX-EGD	CAA**T**ATGATTTGGCTC**CTT**[a]	383 bp with GRO-LMO1
PRFABOX-4b	CAA**G**ATGATTTGGCTC**TGA**[a]	383 bp with GRO-LMO1
Seq01	AATCTAGCACCACGTTCGGG	733 bp with Seq02
Seq02	TGTGACCTTCTTTTACGGGC	733 bp with Seq01

a. base changes between sequences of EGD-e and 4b strains (change in bold)

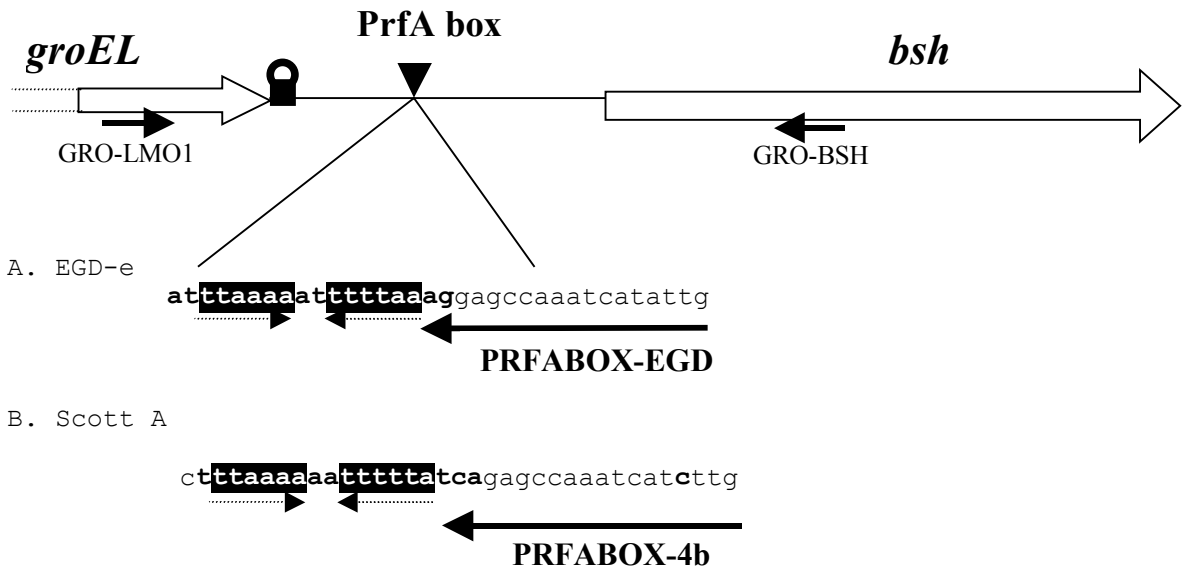

Fig. 1. Graphic representation of the genomic organisation of the *bsh* gene region. Hollow arrows indicate gene orientation, solid arrows indicate primers orientation and dotted arrows indicate palindromes of the putative PrfA box. Hairpins depict putative terminators. The black triangle shows the putative PrfA box. A. Promotor sequence regulated by PrfA activator of EGD-e strain and primer sequence (PRFABOX-EGD) used for amplification of this type of promotor. B. Promotor sequence regulated by PrfA activator of 4b strain and primer sequence (PRFABOX-4b) used for amplification of this type of promotor.

3. Results and discussion

3.1. Survival in synthetic gastric fluids in vitro

By using gastric fluids ajusted to pH 2.5 in the presence and the absence of 10 mM glutamate (Fig. 2), LO28 reference strain was inactivated within less that 50 min (kill of over 7 logs) without glutamate, whereas in the presence of glutamate (10 mM) less than 1 log decrease was observed within 50 min. This protection offered by glutamate reflects GAD activity (Cotter et al., 2001). Furthemore, we confirmed that GAD activity is subject to strain variation. Indeed, GAD activity of Scott A was moderate compared to LO28 pattern and acid resistance in presence of free glutamate did not occur for EGD-e strain. These levels of GAD activity are in accordance with these described by Cotter et al. (2001) but in our experiments, resistance towards synthetic gastric fluids in presence of glutamate was generally lower.

By increasing pH to 3.5 and time of exposition to 120 minutes, differences in survival kinetics between human asymptomatic carriage strains were improved (Fig. 3). Without addition of monosodium glutamate, EGD-e, LO28, H27 and H38 were the most gastric fluid-sensitive strains (kill of over 7 logs within 120 min), whereas H1, H17, H28, H32, H34 and H35 were the most gastric fluid-resistant strains (kill of approximatively 3.5 to 4.5 logs within 120 min). Survival of 4 out of the 14 human carriage strains was not improved in presence of glutamate (Fig. 3A) while H1, H2, H11, H12, H17, H28, H32, H34 and H38 exhibited GAD activity (Fig. 3B). The highest protective effect of glutamate was observed with strains LO28 and H34. Considering that free glutamate is largely encountered in food and sometimes deliberately added in food manufacture for flavor enhancement (usually between 0,1 and 0,8%), Cotter et al. (2001) suggested that expression of GAD by *L. monocytogenes* could be critical to overcome the human stomach barrier leading to infection of the small intestine. Considering that fecal carriage by human may reflect intestinal persistance, it can be postulated that for the majority of fecal carriage strains efficient GAD system was a favoring factor for reaching the intestine. However, presence of human fecal carriage strains as strains H6, H27, H31 and H35 with no apparent GAD activity suggest that high GAD activity is not compulsory for overcoming the stomach barrier and remind us that presence of pathogens in the human gastrointestinal tract is probably multifactorial. Similarly, these GAD negative strains were average gastric juice-resistant strains.

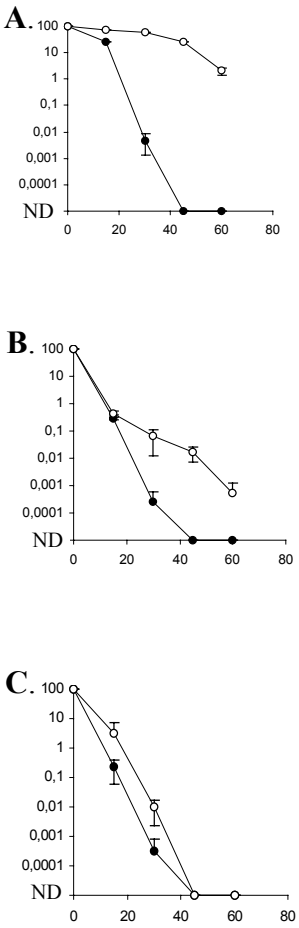

Fig. 2. Survival of reference *L. monocytogenes* strains in synthetic human gastric fluids (pH 2.5) in the presence (open symbols) and absence (closed symbols) of 10 mM monosodium glutamate. A. LO28 strain with high GAD activity. B. Scott A strain with moderate GAD activity. C. EGD-e strain with low GAD activity. Error bars represent standard deviations from the mean of triplicate experiments. Data points on x-axis correspond to survival not being detected (ND).

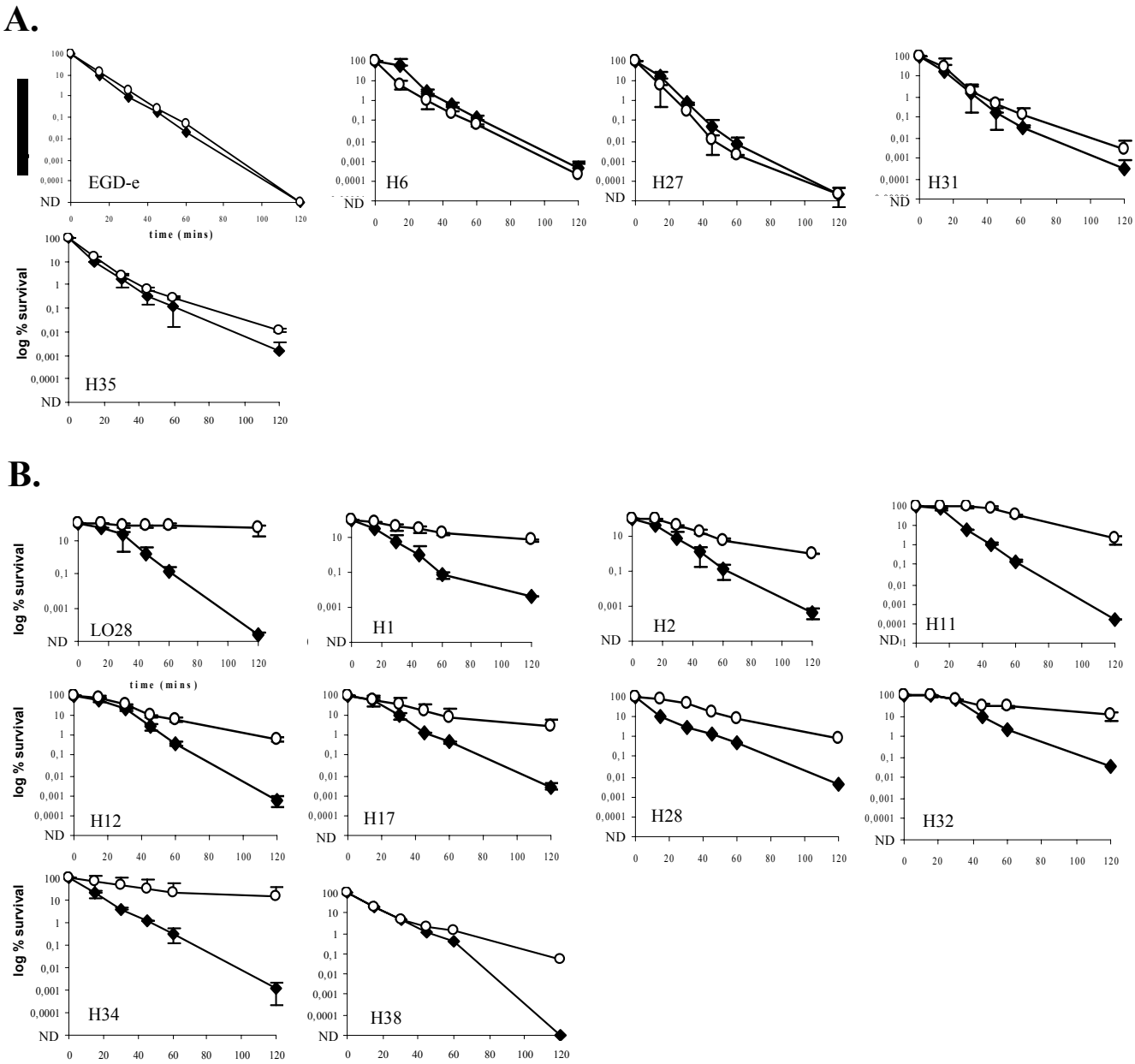

Fig. 3. Survival of human carriage *L.monocytogenes* strains in synthetic human gastric fluids (pH 3.5) in the presence (open symbols) or absence (closed symbols) of 10 mM monosodium glutamate. A. Strains similar to EGD-e with low GAD activity. B. Strains with GAD activity (similar to LO28). Error bars represent standard deviations from the mean of triplicate experiments. Data points on x-axis correspond to survival not being detected (ND)

3.2. Assessment of the glutamate decarboxylase activity of human carriage isolates in comparison to clinical, food and environmental isolates of L. monocytogenes

pH increase associated with GAD activity was determined by color change of the bromocresol green indicator (Table 3). This qualitative assay confirmed results observed in gastric fluids with and without glutamate and 3 levels of GAD activity were found. Human carriage strains for which presence of glutamate had little or no effect on their acid-resistance in gastric fluids were grouped here in classes with no (H6, H27 and H31) or low GAD activity (H35). All human carriage strains that had enhance survival in synthetic gastic fluids in presence of glutamate also had GAD activity. Among these GAD positive strains, H28 and H32 exhibited the lowest GAD activities. Interestingly, the three strains showing low GAD activity belonged to the most gastric juice-resistant strains (without glutamate) at pH 3.5. The 4 animal carriage strains that had already encountered low pH of the bird's stomach, showed high GAD activity.

Using initial pH 4 in this qualitative assay, reference epidemic strain Scott A exhibited high GAD activity, whereas EGD-e, as previously observed at pH 2,5 and 3,5 in gastric fluids, was not able to raise the pH of its environment. High GAD activity was observed for most of the clinical strains (11 strains). However, 2 out of 18 strains had low activity and 5 out of 18 strains had no detectable activity. Epidemic episode with strains such as EGD-e (the derivative strain of animal outbreak) and H36 (isolated during Epoisses cheese human outbreak) in spite of the absence of GAD activity could be explained by consumption of foodstuff highly contaminated. In fact, mice infection has been observed following intragastric inoculation of EGD-e with doses of approximately 10^9 bacteria (Barbour et al., 1996; Marco et al., 1997; Barbour et al., 2001; Lecuit et al., 2001; Czuprynski and Faith, 2002) and the DL50 value was 10^{11} bacteria after oral infection of guinea-pigs (Lecuit et al., 2001). Furthermore, infection with these strains that did not have detectable GAD activity, could be favored in patients with abnormally high gastric pH. Indeed, Czuprynski et al. (2002) showed that neutralization of gastric pH could enhance infectivity in mice. From these observations, it appears that strains carried asymptomatically by humans and animal had similar ability to use monosodium glutamate as acid resistance system in comparison to clinical isolates.

Table 3. Glutamate decarboxylase activity of human carriage isolates, clinical isolates, food and environmental isolates of *L. monocytogenes*.

Origin of isolates	Activity class		
	No detectable activity (n=16)	Low activity (n=9)	High activity (n=25)
Human carriage isolates (n=14)	H6 H27 H31	H28 H32 H35	**LO28** H1 H2 H11 H12 H17 H34 H38
Animal carriage isolates (n=4)			23 38 81 97
Sporadic and outbreak isolates (n=18)	**EGD-e** H18 H23 H25 H36	H4 H10	**Scott A** H3 H5 H8 H13 H15 H16 H19 H21 H22 H24
Food isolates (n=7)	NV4 NV5 NV7 NV8	1F 7F	2F
Environmental isolates (n=7)	1E 3E 6E 1S	2S 3S	C9

Conversely, among the 14 food and environmental isolates, 8 strains had no detectable GAD activity and 4 had low activity. Interestingly, strains with no or low GAD activities were isolated from food and food-processing plant, whereas only one strain from food origin (2F) showed high GAD activity. Proportion of food associated strains with high GAD activity (1 out 13) is low in comparison to strains isolated from human (19 out 31). This observation suggests that GAD acidity resistance system was also an adaptative advantage for growth within foods and particularly acidic foods, to overcome the stomach barrier and pass to the intestine. However, isolation of *L. monocytogenes* strains with no GAD activity from healthy human carrier suggest that some strains with no GAD activity could pass from environmental niches such as foodstuff and food plant environment to the human gut reservoir.

3.3. Bile salts tolerance

Bile salts resistance was determined by plating bacteria onto BHI agar plates supplemented with 1% porcine bile salts (Table 4). All strains tested grew even at high porcine bile salts concentration (2%). Colonies were surrounded by light zones of bile salts degradation. The degradation zone was strain dependent. No degradation was observed with strains H13, NV7 and 6E. *L. monocytogenes* Scott A produced the largest zone (12 mm), whereas the other epidemic reference strain EGD-e exhibited zone with diameter considered as low (5.5 mm). Similarly, growth of strain EGD-e was lower than Scott A in BHI broth supplemented with 1% bile salts. Compared to growth in BHI broth, final cell number in presence of bile salts was 1.18-log-unit lower for EGD-e strain, while the decrease was only 0.34-log-unit for strain Scott A. Growth was still observed in BHI broth supplemented with 5% bile salts even with strains with low zones of bile salts degradation (data not shown). Interestingly, while we observed an inherent ability to tolerate high concentrations of bile salts, King et al. (2003) recently observed that addition of only 0,3 % bile salts appeared to have a deleterious effect on the exponential and stationary cells of both Scott A strain and three other isolates of *L. monocytogenes* isolated from food and environment. These discrepancies between these results could be explained by experimental differences concerning growth conditions such as broth, atmosphere and pH used. For example, as observed by Begley et al. (2002), growth in the presence of some specific bile acids is strongly pH dependent; we observed that in BHI broth supplemented with 1% bile salts, growth was observed at pH 7.4 but at pH 6.5, the pH used in the bile broth system used by King et al. (2003), strain Scott A did not grow in our study.

Reference human carriage strain LO28 produced zones with intermediate size (9 mm). These levels reflected well bile tolerance observed by Begley et al. (2002) and suggested that size of the bile salts degradation zones reflected bile salts tolerance. Nineteen strains belonged to LO28 group of strain, 17 strains behaved similarly to *L. monocytogenes* EGD-e and 9 strains behaved similarly to *L. monocytogenes* Scott A. However no relationship was observed between level of activity and origin of isolates.

All strains isolated from feces of healthy humans in this study were able to growth in presence of bile salts concentrations relevant to concentrations encountered *in vivo* in human intestinal lumen (0.2 to 2%) but this was also true with all the other strains. Similarly, human and animal isolates (asymptomatic carriage and clinical) did not exhibit any particular bile salts degradation abilities in comparison to these observed for food and environmental isolates.

Table 4. Degradation of porcine bile salts on BHI agar plates containing 1% porcine bile salts by carriage, clinical and environmental isolates of *L. monocytogenes*. Classes are based on diameter of bile salts degradation zones.

Origin of isolates	Activity class			
	No detectable activity (n=2)	Low activity (n=17)	Medium activity (n=20)	High activity (n=10)
Human carriage isolates (n=14)		H1 H31 H34 H38	**LO28** H11 H17 H27 H28 H32	H2 H6 H12 H35
Animal carriage isolates (n=4)		38 81 97		23
Clinical isolates (n=18)	H13	**EGD-e** H3 H4 H5 H8 H15 H16	H10 H18 H19 H22 H23 H25 H36	**Scott A** H21 H24
Food isolates (n=7)	NV7	NV4 NV8	NV5 1F 2F 7F	
Environmental isolates (n=7)	6E	3S	1S 2S C9	1E 3E

3.4. Bile salt hydrolase activity

Considering that BSH activity is currently screened for commensal bacteria of the gastrointestinal tract (De Boever and Verstraete, 1999; Kociubinski et al., 1999; De Boever et al., 2000; Franz et al., 2001; Knarreborg et al., 2002), it was interesting to assess BSH activities of strains carried asymptomatically by humans in comparison to BSH activities of clinical, food and environmental isolates. Unfortunately, growth of *L. monocytogenes* on MRS agar was subjected to strain variation and did not allow quantitative comparison of BSH activity between strains. Then, pH drop due to cell metabolism resulted in growth inhibition of the most pH sensitive strains in this medium thus preventing observation of any precipitate. Similarly use of agar medium (MRS or BHI) previously adjusted to pH 6.8 did not support growth in presence of glycodeoxycholic acid as previously observed by Begley et al. (2002). Nevertheless, all 50 strains tested were positive for BSH activity.

3.5. Analysis of sequence variation of the PrfA DNA binding site uptstream bsh *gene by PCR*

Comparative analysis of *bsh* gene of the sequenced strains EGD-e (Glaser et al., 2001) and Scott A (Institute for Genomic Research) showed that BSH proteins had 99% identity. However differences in putative PrfA DNA binding site upstream *bsh* was observed. Primers were designed in order to compare these variations (Fig 1.) and allowed classification of strains on the basis of their PCR products with primer sequence similar to EGD-e (Fig 4A, B and C) or similar to Scott A (Fig 4D). Among the 14 isolates carried asymptomatically by humans, 8 strains were closely related to EGD-e and 6 similar to Scott A strain. Among the 16 other clinical strains, 8 were closely related to EGD-e and 8 were closely related to Scott A strain. Among the 18 food and environmental strains, 14 were closely related to EGD-e and 4 were closely related to Scott A strain. Based on the strains with known serotype, primer PRFABOX-4b allowed amplification of the target in serotype 1/2 b and 4b strains, and primer PRFABOX-EGD allowed amplification of the target in serotype 1/2a, 1/2c and 3b strains. There was no relation between origin of strains and type of putative PrfA boxes.

Several authors have already shown that variation in the PrfA box could determine the level of expression of some virulence genes such as *hly* and *mpl* (Sheehan et al., 1995; Williams et al., 2000). However, expression of some other genes of the PrfA regulon such as *inlA*, *actA* and *plcB* were only partially dependent on PrfA and could depend on additional factors. Taken into account that semi-quantitative analysis of the BSH activity was not possible in this study, comparison between sequences within putative PrfA binding sites and BSH activity for each strain was not possible. Interestingly, a recent transcriptome analysis revealed that upregulation of *bsh* gene by PrfA was twice higher for a serotype 4b strain (P14) and was 10,3 higher for EGD-e in BHI broth (Milohanic et al., 2003). In our experiments, strains that produced no or small zones in the presence of 1% bile salts on BHI-agar plates had putative PrfA binding site similar to those of EGD-e and strains that produced high zone of bile salts degradation had putative PrfA binding site similar to these of Scott A, suggesting effectively that promotor of the *bsh* gene of strains such as Scott A could allow higher activation than these of strains such as EGD-e. Nevertheless, the link between bile salt degradation and the level of BSH activity may not be straight forward because BSH is only the first identified factor involved in bile salts degradation and additional unknown activities could be involved. Relationship between ability in bile salts degradation and polymorphism of the promotor of *bsh* gene could correspond to general polymorphism between the genome of the two types of strains and so correspond to the relative polymorphism for the other putative genes involved in bile salts degradation.

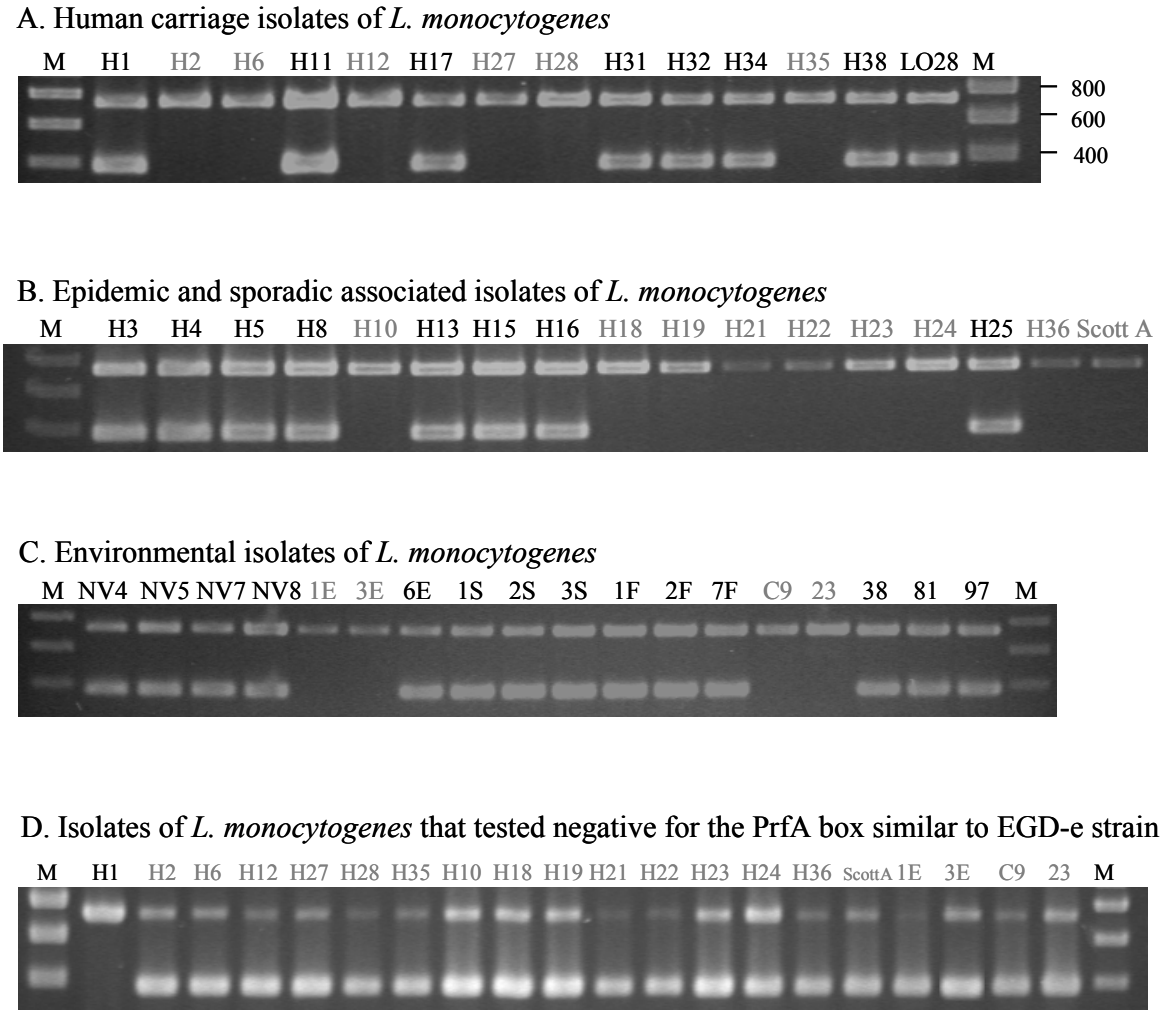

Fig. 4. Agarose gel electrophoresis showing the distribution of PrfA box types (EGD-e type or 4b type; 383 bp) among *L.monocytogenes* strains from different sources and the amplification of their *inlA* gene (control; 733 bp). Gels A, B and C contained amplification products obtained from the primers GRO-LMO1, PRFABOX-EGD, Seq01 and Seq02. Gel D contained amplification products obtained from the primers GROLMO1, PRFABOX-4b, Seq01 and Seq02. M, molecular size marker.

The present study provides important data on the variations in response towards acid and bile salts stress among *L. monocytogenes* wild strains from different origins. Bile salts resistance is not specific to isolates carried asymptomatically by humans, since all *L. monocytogenes* strains tested were able to grow in presence of 5% bile salts. Although the levels of response observed did not correlate with their origin (asymptomatic carriers, patients with listeriosis, food and environment), the higher levels of GAD activity were observed mainly for strains isolated from human or rook and suggested that these strains are acid resistant in presence of glutamate. These observations are consistant with those of Dykes and Moorhead (2000), who hypothetised that gut passage is likely to select for acid resistant strains from food origin. Some of these strains with high GAD activity had the higher bile salts degradation ability too (Scott A, H2, H12, H21, H24, 23). Interestingly, ability to tolerate low pH and bile salts, are properties that, in addition to adhesion to intestinal surfaces, relates to commensal and probiotic microorganisms (Jacobsen et al., 1999; Kimoto et al., 1999).

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