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Stress Survival Islet 1 (SSI-1) Survey in *Listeria monocytogenes* Reveals an Insert Common to *Listeria innocua* in Sequence Type 121 *L. monocytogenes* Strains▽†

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***Listeria monocytogenes* strains ($n = 117$) were screened for the presence of stress survival islet 1 (SSI-1). SSI-1$^+$ strains (32.5%) belonged mainly to serotypes 1/2c, 3b, and 3c. All sequence type 121 (ST-121) strains included ($n = 7$) possessed homologues to *Listeria innocua* genes *lin0464* and *lin0465* instead of SSI-1.**

The genus *Listeria* comprises eight species: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, and the newly described *L. rocourtiae* and *L. marthii* (7, 11, 18). From a human perspective, the food-borne pathogen *L. monocytogenes* is the most important species of this genus. Recently, a stress survival islet (stress survival islet 1 [SSI-1]) has been identified in *L. monocytogenes* (29). Deletion mutants showed impaired growth at low pH and high salt concentrations. The islet was present in about 50% of tested *L. monocytogenes* strains, mainly those not belonging to serogroup 4. According to the sequence of *L. monocytogenes* EGD-e (serotype 1/2a), the islet contains five genes, *lmo0444*, *lmo0445*, *lmo0446* (*pva*), *lmo0447* (*gadD1*), and *lmo0448* (*gadT1*), positioned between two genes highly conserved across different *Listeria* spp. An open reading frame (ORF) transcribed in the opposite direction is present at this location in *L. monocytogenes* strains without the islet (e.g., strain F2365, serotype 4b), and in *L. innocua* CLIP 11262, two genes transcribed in opposite directions (*lin0464* and *lin0465*) are present at the same location (29). *L. welshimeri* SLCC 5334 does not contain any genes at this position. In *L. seeligeri* SLCC 3954, this region was similar to that in *L. welshimeri* SLCC 5334, with no open reading frame and with small differences in the lengths of the intergenic region, i.e., 281 bp (*L. welshimeri* FN557490) and 166 bp (*L. seeligeri* AM263198). There was 63% identity between a 46-bp stretch at the 5′ end in *L. welshimeri* and that in *L. seeligeri*, both of which were followed by an *L. welshimeri*-specific portion of 112 bp and a 123-bp stretch at the 3′ end with 73.2% identity.

In the present study, a collection of 117 *L. monocytogenes* strains, including isolates from cheese dairies, meat and meat products, fish, and veterinary and human infections, was screened by PCR for the presence of SSI-1 (Table 1; also see Table S1 in the supplemental material) (29). Serotypes were determined by classical serotyping and by a multiplex PCR method for grouping strains into four major serotype-related groups (9, 31). The predicted 9.7-kbp (SSI-1$^+$) and 1.1-kbp (SSI-1$^-$) fragments were observed in 32.5% and 61.5% of strains, respectively (Fig. 1; Table 2). SSI-1 was present in the majority of 1/2c, 3b, and 3c strains tested but in only one strain each of serotypes 4a and 4b. A single serotype 4c strain was included in the survey, and it contained SSI-1. The majority of serotype 4b, 1/2a, and 4a strains, as well as all strains with ambiguous serotype 4d or 4e, lacked SSI-1. Earlier findings suggest that SSI-1 is more prevalent in non-serogroup 4 strains, which was confirmed by our results: 50% of non-serogroup 4 strains but only 7.5% of serogroup 4 strains contained SSI-1 ($\chi^2 = 20.33$, $P < 0.0005$) (29). However, seven strains yielded PCR fragments of about 2.2 kbp (Table 2), all of them serotype 1/2a and representing 25% of tested strains belonging to this serotype (Fig. 1). The sequence of this 2.2-kbp fragment was determined for one strain (CDL67) by primer walking (Macrogen Inc., Seoul, South Korea) using primers lmo0443 Fwd and lmo0449 Rev (Table 1). A BLAST search revealed 95% identity with *L. innocua* CLIP 11262 (GenBank accession no. AL596165). The sequence contained homologues to *lin0464* (98% identity, 15 substitutions, five of them nonsynonymous leading to five amino acid substitutions) and *lin0465* (94% identity, 35 substitutions, 16 of them nonsynonymous leading to 14 amino acid substitutions). Based on sequence information of *L. innocua* CLIP 11262, primers lmo0443 Fwd and lmo0449 Rev were expected to amplify a 2,166-bp fragment. Indeed, a survey of nine *L. innocua* strains from culture collections (DSM 20649, NCTC 10528 and 12210, and CIP 78.44, 79.45, 80.11, 80.12, 106065, and 107775) and 27 *L. innocua* strains obtained from dairy plants yielded a 2.2-kbp

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TABLE 1. Oligonucleotides used in this study

Primer or probe	Sequence (5′–3′)	Target(s)	Reference
lmo0443 Fwd	GGC ACA ATG AGC GAA TTG	SSI-1	29
lmo0449 Rev	GTC CTT CTG GAA CAT TGC	SSI-1	29
gadD1 F	GGT ATT GTG GGT ATT CTG G	*gadD1*	29
gadD1 R	CTG ACC GAT AAT CTG ACT C	*gadD1*	29
LI1	CTC CAT AAA GGT GAC CCT	16S rRNA gene	1
U1	CAG CAG CCG CGG TAA TAC	16S rRNA gene	1
LM1	CCT AAG ACG CCA ATC GAA	*hly*	1
LM2	AAG CAC TTG CAA CTG CTC	*hly*	1
Siwi2	TAA CTG AGG TAG CGA GCG AA	*iap*	2
Ino2	ACT AGC ACT CCA GTT GTT AAA C	*iap*	2
MonoA	CAA ACT GCT AAC ACA GCT ACT	*iap*	2
Mugra	CCA GCA GTT TCT AAA CCT GCT	*iap*	2
Lis1B	TTA TAC GCG ACC GAA GCC AAC	*iap*	2
inlAF1	TTA CAT CAG TCC CCT AGC AGG T	*inlA*	32
inlAR1	TCC AAT AGT GAC AGG TTG GCT A	*inlA*	32
inlB-forward	CTC GCA CCG CTG TAA AGC T	*inlB*	14
inlB-reverse	TTA TTT CTG TGC CCT TAA ATT A	*inlB*	14
LIP1	GAT ACA GAA ACA TCG GTT GGC	*prfA*	28
LIP2	GTG TAA TCT TGA TGC CAT CAG G	*prfA*	28
LIP probe 2	FAM-CAG GAT TAA AAG TTG ACC GCA-MGB[a]	*prfA*	28
Lin0198F	ATG AAC AAA TTA GTT AGT CAA AGT AAT G	*lin0198*	15
Lin0198R	TAT CGA TGT CTT GAG GTC ACA CAA AGT TC	*lin0198*	15
LinNCR1F	GGA TTT GGT AAA TTA TAC AAA GGT TTT AAG	*lin0454* and *lin0455*	15
LinNCR1R	TGC TTC TTG GCA TTT TAG TAA TCT TTC	Intergenic region	15
inlAseqF1	CAC CAT TGG AAA AGG AAC GA	*inlA*	This study
seq02	TGT GAC CTT CTT TTA CGG GC	*inlA*	28a

[a] FAM, 6-carboxyfluorescein; MGB, minor groove binder.

fragment in all cases (Fig. 1). Thus, the seven *L. monocytogenes* strains yielding PCR fragments of about 2.2 kbp contained a region common in *L. innocua* at that genomic site. PCR analysis targeting the 16S rRNA gene, *hly*, *iap*, *prfA*, *inlA*, and *inlB* yielded results typical for *L. monocytogenes* (1, 2, 14, 28, 32), whereas PCR results for the *L. innocua*-specific targets *lin0198* and the noncoding intergenic region between *lin0454* and *lin0455* were negative (15). Pulsed-field gel electrophoresis (PFGE) profiles (ApaI and AscI) of these *L. monocytogenes* strains were similar to each other (Fig. 2) (13). Multilocus sequence typing (MLST) was performed as outlined on the Institute Pasteur *Listeria monocytogenes* MLST Database website (www.pasteur.fr/mlst) and revealed a profile consistent with sequence type 121 (ST-121): *abcZ*-7, *bglA*-6, *cat*-8, *dapE*-8, *dat*-6, *ldh*-37, and *lhkA*-1 (26).

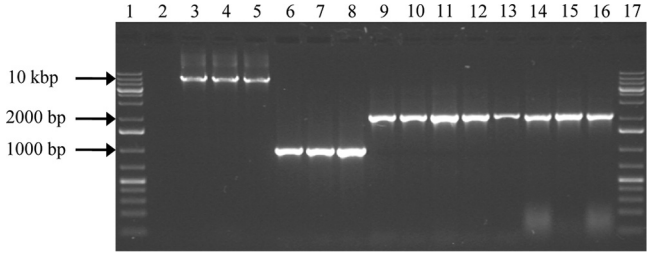

FIG. 1. PCR analysis of the *lmo0443* and *lmo0449* intergenic region of *L. monocytogenes*. Lanes 1 and 17, 1-kb Plus DNA ladder (Fermentas International Inc., Burlington, Ontario, Canada); lane 2, no-template control; lanes 3 to 13, *L. monocytogenes* strains (lane 3, CDL73; lane 4, CDL74; lane 5, CDL75; lane 6, CDL201; lane 7, CDL211; lane 8, CDL218; lane 9, CDL64; lane 10, CDL67; lane 11, L56/65; lane 12, L58/55; lane 13, L37/35); lanes 14 to 16, *L. innocua* strains (lane 14, CDL25; lane 15, CDL26; lane 16, CDL192).

The ST-121 strains analyzed in this study were isolated in Austria and Belgium from different ecological niches, including food and human cases, over several years (Table 3). In two instances, two strains each represent clones which were consistently isolated from the same dairy plant over a course of at least 3 years. Isolation of *L. monocytogenes* ST-121 strains has also been reported in France, Italy, and Spain (25, 26, 30) (Table 3). Two French strains, CLIP 73068 and CLIP 71322, were available for testing and yielded the 2.2-kbp amplicon with primers lmo0443 Fwd and lmo0449 Rev. Both strains had PFGE profiles similar to those of the other

TABLE 2. Amplicons observed when screening *L. monocytogenes* strains of different serotypes for the presence of SSI-1

Serotype	No. (%) of isolates with:		
	9.7-kbp fragment	2.2-kbp fragment	1.1-kbp fragment
1/2a	6 (21.4)	7 (25.0)	18 (64.3)
1/2b	8 (50.0)	0 (0.0)	8 (50.0)
1/2c	11 (78.6)	0 (0.0)	3 (21.4)
3a	2 (50.0)	0 (0.0)	2 (50.0)
3b	3 (60.0)	0 (0.0)	2 (40.0)
3c	4 (80.0)	0 (0.0)	1 (20.0)
4a	1 (20.0)	0 (0.0)	4 (80.0)
4b	1 (3.6)	0 (0.0)	27 (96.4)
4c	1 (100.0)	0 (0.0)	0 (0.0)
4d/e	0 (0.0)	0 (0.0)	5 (100.0)
4e	0 (0.0)	0 (0.0)	1 (100.0)
7	1 (50.0)	0 (0.0)	1 (50.0)
All	38 (32.5)	7 (6.0)	72 (61.5)

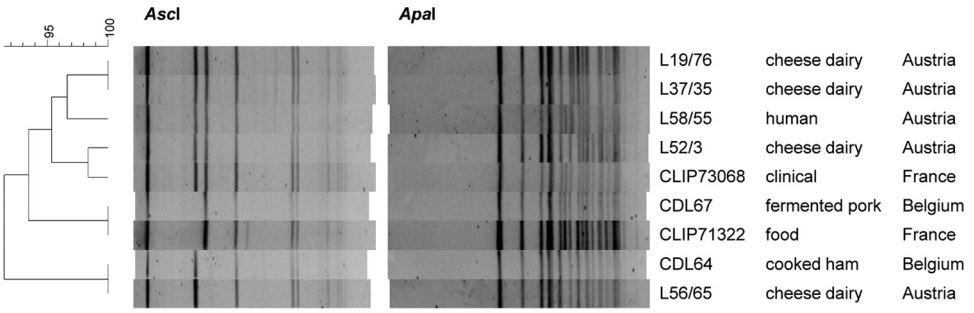

FIG. 2. PFGE analysis of *L. monocytogenes* strains harboring *lin0464* and *lin0465* homologues. Cluster analysis was performed with Fingerprinting II software (Bio-Rad, Hercules, CA) using the following settings: 0.2% optimization, 0.5% position tolerance, and the Dice similarity coefficient. For dendrogram construction, the UPGMA (unweighted-pair group method using average linkages) algorithm was used. Strain designations, sources, and countries of origin are given on the right, and percentages of similarity are indicated on the left.

ST-121 strains, with those of CLIP 71322 being identical to those of CDL67 (Fig. 2).

A transition from C to T at nucleotide position 1474 of the *inlA* gene has been reported in two French ST-121 strains. This transition resulted in a premature stop codon (PMSC) and a truncated form of InlA, which is secreted rather than anchored to the cell wall (22, 26). Sequencing of the respective region in our strains yielded an identical transition (Table 1). Several mutations associated with PMSCs have been described for this gene (26). Although *inlA* PMSCs yield virulence-attenuated strains, such strains have been isolated from human cases. Immunosuppression has been suggested to be a risk factor present in these cases (21, 22). One of our ST-121 strains is also a human isolate. ST-121 represents an *L. monocytogenes* clone which is spatially, temporally, and ecologically widespread.

A BLAST search of ST-121 homologues to *lin0464* and *lin0465* revealed homologues in other *Firmicutes* as well (Fig. 3). The DNA-based identity ranged from 65% to 79%. Protein-based identities were similar to the DNA-based data (data not shown). In *Acetivibrio cellulolyticus*, *Anaerostipes caccae*, *Eubacterium limosum*, *Clostridium asparagiforme*, and an *Enterococcus faecalis* plasmid, homologues to both genes were present. The arrangement of these genes was identical to that of *lin0464* and *lin0465*: two successive ORFs with opposing directions of transcription. *lin0464* belongs to the GntR family of transcriptional regulators, with a winged helix-turn-helix DNA-binding domain. *lin0465* is characterized by a type 1 glutamine amidotransferase-like domain found in a subgroup of proteins similar to the ATP-independent intracellular protease PfpI from *Pyrococcus furiosus* (12). The conservation of the arrangement of both genes in different species suggests that they are a functional unit and that *lin0464* or the homologues thereof might act as transcriptional regulators for the intracellular protease encoded by *lin0465* or the *lin0465* homologue. The substrate of the *Pyrococcus furiosus* protease has not been identified so far. In *Pseudomonas aeruginosa*, a similar protease provides general stress protection (27).

TABLE 3. *L. monocytogenes* ST-121 strains with *lin0464* and *lin0465* homologues identified in this study and ST-121 strains reported by others

Strain	Country of origin	Yr of isolation	Source	Reference
CDL64	Belgium	2001–2006	Cooked ham	This study
CDL67	Belgium	2001–2006	Salami	This study
L52/3	Austria	1998	Cheese dairy	This study
L56/65	Austria	2000	Cheese dairy	This study
L58/55	Austria	2001	Clinical isolate	This study
L19/76	Austria	1997	Cheese dairy	This study
L37/35	Austria	1999	Cheese dairy	This study
CLIP 71322	France	1996	Food	26
CLIP 73068	France	1996	Clinical isolate	26
829	Spain	2000	Pork	30
21-P	Italy	1993–2004	Meat product	25
174	Italy	1993–2004	Ready-to-eat product	25
350	Italy	1993–2004	Meat product	25
170	Italy	1993–2004	Meat product	25
204	Italy	1993–2004	Meat product	25
90-P	Italy	1993–2004	Cured fish	25
87	Italy	1993–2004	Soil	25
206	Italy	1993–2004	Meat product	25
159	Italy	1993–2004	Meat product	25
115	Italy	1993–2004	Mammalian stool	25

The presence of homologues to *lin0464* and *lin0465* in the conjugative *E. faecalis* plasmid pBEE99 hints at the mobility of these genes. In this plasmid, these genes are flanked upstream by a transcriptional regulator also belonging to the GntR family and a transposase and downstream by a site-specific recombinase and a transposase. These genes are located in a 5,000-bp region containing genes with 54 to 60% identity to genes found in species other than *E. faecalis*, whereas the rest of the plasmid contains almost exclusively genes associated with *E. faecalis* (6). The sequenced ST-121 strains possess a short inverted repeat 66 bp upstream of the *lin0464* homologue (5′-AAGAT TTT-3′) and 73 bp downstream of the *lin0465* homologue (5′-AAAATCTT-3′), which might indicate mobility of that genomic region. However, this inverted repeat is not present in the *L. innocua* CLIP 11262 genome.

In *Clostridium hathewayi* and a *Clostridiales* and an *Erysipelotrichaceae* bacterium, a *lin0464* homologue was present but was followed by an isochorismatase protein instead of a homologue to *lin0465*. On the other hand, *L. seeligeri* SLCC 3954 contains only a *lin0465* homologue. The 5′ end of the homologue region is part of *lse_0574*, and the rest is part of *lse_0575*. The homologue to *lmo_0443* in *L. seeligeri* is *lse_0377*. Thus, the *lin0465* homologue region is integrated in this *L. seeligeri*

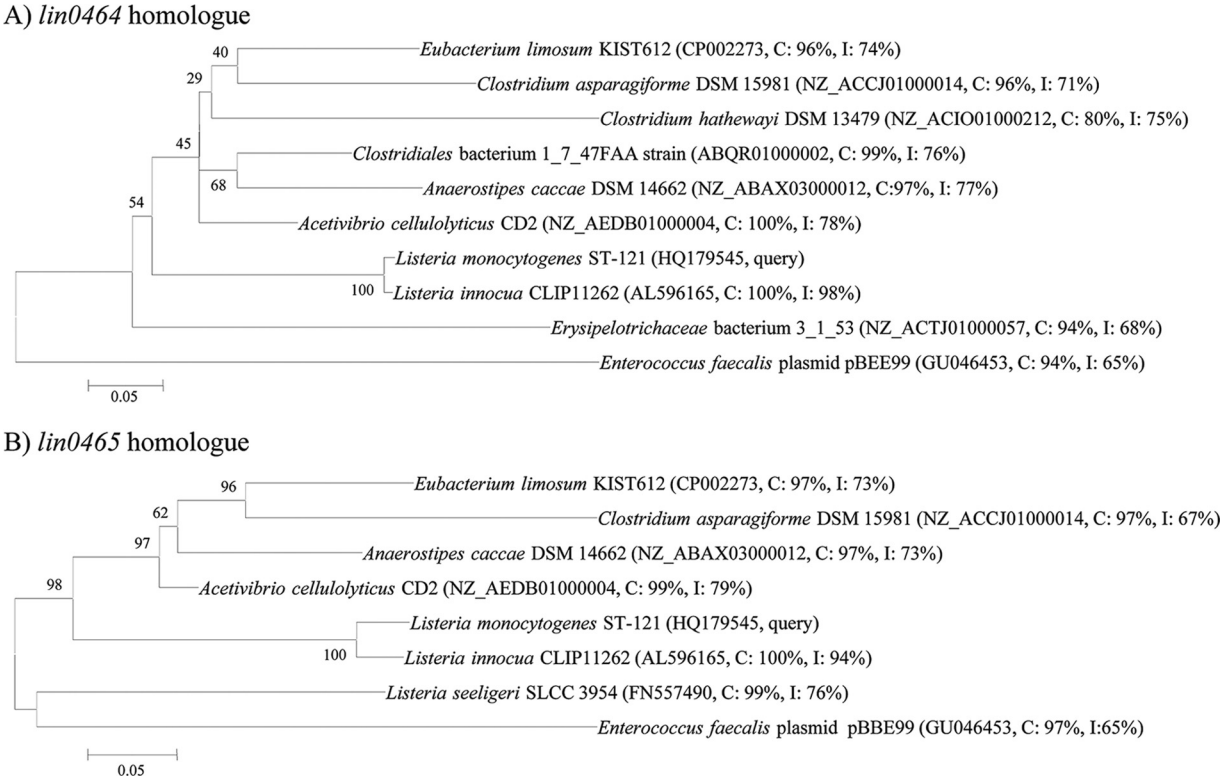

FIG. 3. Maximum-parsimony tree of the BLAST hits against the *lin0464* homologue (top) and the *lin0465* homologue (bottom) of *L. monocytogenes* ST-12. The tree was generated by using MEGA (v. 4.0) software (16). The scale bar represents a distance of 5%. Accession numbers are from GenBank. Bootstrap values of 500 trees are indicated as percent confidence values for the branching. GenBank accession numbers, query coverages (C), and identities (I) are given in parentheses.

strain in a genomic region different from that in *L. innocua* or *L. monocytogenes* ST-121 strains.

Cross-reactivity of a PCR system targeting *lin0464*, which was initially thought to be specific for *L. innocua*, has been reported for four *L. monocytogenes* serotype 4c strains belonging to phylogenetic lineage IIIA (19, 20). Thus, it seems that the presence of a region commonly found in *L. innocua* at that genomic site in *L. monocytogenes* is not restricted to ST-121 strains.

With the exception of *L. marthii*, as the closest relative to *L. monocytogenes*, *L. monocytogenes* and *L. innocua* are more closely related to each other than to the other *Listeria* species and coexist in various ecological niches (4, 11). Comparative genomics and DNA array data suggest that *L. innocua* evolved by successive gene loss from an ancestor of *L. monocytogenes* serogroup 4 strains (10). Recently, *L. monocytogenes* 4a strains that harbor *L. innocua*-specific *lin0372* and *lin1073* but not *lin0464* and share some gene deletions with *L. innocua* have been identified (5). On the other hand, *L. innocua* strains containing *L. monocytogenes*-specific genes have been identified as well. These genes include *Listeria* pathogenicity island 1 (LIPI-1) genes, *inlA*, partial *inlB*, and *gtcA*, which is involved in teichoic acid glycosylation and characteristic of *L. monocytogenes* serogroup 4 strains (17, 32). In addition, a 10-gene MLST scheme provided evidence for interspecific recombination between these two species (7). Serotype 4c belongs to lineage IIIA, whereas serotype 1/2a belongs to lineage II. Recombination rates are higher in lineage II strains, and a large number of imports from lineage IIIA to lineage II was observed (8, 23, 24). In addition, SSI-1 is located within a 616-kbp region in the first third of the genome, which may be a hot spot for horizontal gene transfer (3). Taking into account the high degree of identity to *lin0464* and *lin0465*, the homologous genes in *L. monocytogenes* ST-121 strains might have been acquired rather recently from *L. innocua*. It is tempting to speculate that this might have happened not directly but by recombination with *lin0464*-positive *L. monocytogenes* serotype 4c strains belonging to lineage IIIA. An alternative scenario might be independent acquisition from an external source or a common ancestor harboring the insert now common in *L. innocua*, albeit these do not seem to be very likely. Comparison of the *lin0464* and *lin0465* homologues and the flanking regions of *lin0464*-positive *L. monocytogenes* 4c strains to the respective sequences in *L. innocua* and *L. monocytogenes* ST-121 strains might aid in reconstructing the evolutionary events leading to the present situation. The lower degree of identity, the different position in the genome, and the lack of a *lin0464* homologue suggest that the *lin0465* homologue in *L. seeligeri* SLCC 3954 might have been acquired in the more distant past or from a different source than the *lin0465* homologue in *L. innocua* and *L. monocytogenes* ST-121 strains.

In conclusion, *Listeria* strains harboring *lin0464* and *lin0465* or their homologues, e.g., *L. innocua*, *L. monocytogenes* serovar 4c, and *L. monocytogenes* serovar 1/2a ST-121 strains,

rarely cause diseases and are associated with the environment. It is likely that *lin0464* and *lin0465* and their homologues contribute to the fitness of these bacteria in the environment. These genes may also have contributed to the broad spatiotemporal distribution of the ST-121 clone. Thus, further investigations should focus on the function of *lin0464* and *lin0465* in *L. innocua* and their homologues in *L. monocytogenes*.

Nucleotide sequence accession number. The sequence of the 2.2-kbp fragment determined for *L. monocytogenes* CDL67 was deposited in GenBank under accession number HQ179545.

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