Heat Resistance Mediated by pLM58
Plasmid-Borne ClpL in Listeria monocytogenes

Anna Pöntinen, Mariella Aalto-Araneda, Miia Lindström, Hannu Korkeala
Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

ABSTRACT
Listeria monocytogenes is one of the most heat-resistant non-spore-forming food-borne pathogens and poses a notable risk to food safety, particularly when mild heat treatments are used in food processing and preparation. While general heat stress properties and response mechanisms of L. monocytogenes have been described, accessory mechanisms providing particular L. monocytogenes strains with the advantage of enhanced heat resistance are unknown. Here, we report plasmid-mediated heat resistance of L. monocytogenes for the first time. This resistance is mediated by the ATP-dependent protease ClpL. We tested the survival of two wild-type L. monocytogenes strains—both of serotype 1/2c, sequence type ST9, and high sequence identity—at high temperatures and compared their genome composition in order to identify genetic mechanisms involved in their heat survival phenotype. L. monocytogenes AT3E was more heat resistant (0.0 CFU/ml log10 reduction) than strain AL4E (1.4 CFU/ml log10 reduction) after heating at 55°C for 40 min. A prominent difference in the genome compositions of the two strains was a 58-kb plasmid (pLM58) harbored by the heat-resistant AT3E strain, suggesting plasmid-mediated heat resistance. Indeed, plasmid curing resulted in significantly decreased heat resistance (1.1 CFU/ml log10 reduction) at 55°C. pLM58 harbored a 2,115-bp open reading frame annotated as an ATP-dependent protease (ClpL)-encoding clpL gene. Introducing the clpL gene into a natively heat-sensitive L. monocytogenes strain significantly increased the heat resistance of the recipient strain (0.4 CFU/ml log10 reduction) at 55°C. Plasmid-borne ClpL is thus a potential predictor of elevated heat resistance in L. monocytogenes.

IMPORTANCE
Listeria monocytogenes is a dangerous food pathogen causing the severe illness listeriosis that has a high mortality rate in immunocompromised individuals. Although destroyed by pasteurization, L. monocytogenes is among the most heat-resistant non-spore-forming bacteria. This poses a risk to food safety, as listeriosis is commonly associated with ready-to-eat foods that are consumed without thorough heating. However, L. monocytogenes strains differ in their ability to survive high temperatures, and comprehensive understanding of the genetic mechanisms underlying these differences is still limited. Whole-genome-sequence analysis and phenotypic characterization allowed us to identify a novel plasmid, designated pLM58, and a plasmid-borne ATP-dependent protease (ClpL), which mediated heat resistance in L. monocytogenes. As the first report on plasmid-mediated heat resistance in L. monocytogenes, our study sheds light on the accessory genetic mechanisms rendering certain L. monocytogenes strains particularly capable of surviving high temperatures—with plasmid-borne ClpL being a potential predictor of elevated heat resistance.

KEYWORDS
ClpL, Listeria, heat stress, heat tolerance, plasmid-mediated resistance, protease, stress response

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Address correspondence to Anna Pöntinen, anna.pontinen@helsinki.fi.
\textit{Listeria monocytogenes} is a Gram-positive, non-spore-forming food-borne pathogen and the causative agent of listeriosis, a severe human illness with mortality reaching 35\% (1–4). \textit{L. monocytogenes} may persist in food-processing premises for several years (5, 6), which makes it a challenging contaminant in food production. In addition, it is able to cope with many stress conditions used for controlling bacterial contamination, including high temperatures (7, 8). \textit{L. monocytogenes} can grow at 45\°C and is more heat resistant than most other non-spore-forming food-borne pathogens (1, 9). Furthermore, a previous heat treatment may enhance the tolerance and adaptation of \textit{L. monocytogenes} to subsequent heat stress as well as other stressors encountered in food production, such as NaCl and ethanol (7, 10). However, distinct differences exist between \textit{L. monocytogenes} strains in their ability to survive high temperatures (10, 11). For example, Lundén et al. reported a 3-log-unit difference in the heat resistance (log$_{10}$ reduction) of \textit{L. monocytogenes} strains (11). While the general heat stress properties and adaptation responses of \textit{L. monocytogenes} have been reported (8, 12), investigations are required to reveal the accessory genetic mechanisms that provide certain strains with enhanced heat resistance.

Among mobile genetic elements, plasmids are self-replicating entities that are often costly, yet they may contribute to diversified adaptation and resistance of the host strain (13, 14). Plasmids are relatively prevalent among \textit{L. monocytogenes} strains: approximately one-third of \textit{L. monocytogenes} strains harbor plasmids (15–17), and they are particularly found in environmental and food-related strains (15). Thus, their potential to contribute to the environmental fitness of the host cannot be overlooked. Indeed, the involvement of listerial plasmids in resistance of \textit{L. monocytogenes} to antibiotics (18, 19), benzalkonium (20–22), and heavy metals (15, 23) has been reported. The role of plasmids is yet to be elucidated, however, in the adaptation of \textit{L. monocytogenes} strains into niches of food production environments, where high temperature is a key stressor for bacteria to surmount.

Due to the severe risk on food safety posed by markedly heat-resistant \textit{L. monocytogenes} strains, it is pivotal to better understand the variation in their ability to survive heat treatments. Here, we sought to elucidate the genetic mechanisms conferring heat resistance in \textit{L. monocytogenes} by comparing the genome composition and heat survival phenotypes. We show that heat resistance is mediated by the plasmid-borne ATP-dependent protease ClpL. To the best of our knowledge, this is the first report on plasmid-mediated heat resistance in \textit{L. monocytogenes}.

**RESULTS**

**Resistance and growth of \textit{L. monocytogenes} strains vary at high temperatures.**

We first tested the heat resistance at 55\°C, growth at 42\°C, and maximum growth temperature of \textit{L. monocytogenes} AL4E and AT3E (Table 1) in order to elucidate the differences between their thermostolerance and growth at high temperature. With 0.0 CFU/ml log$_{10}$ reduction, \textit{L. monocytogenes} AT3E proved to be more heat resistant than AL4E (1.4 CFU/ml log$_{10}$ reduction; \(P < 0.01\)) at 55\°C (Fig. 1). At 42\°C, the differences between their growth were negligible (Fig. 2). Strain AL4E exhibited 0.5\°C higher maximum growth temperature than the heat-resistant AT3E strain did (\(P < 0.01\)).

**\textit{L. monocytogenes} AL4E and AT3E share high chromosomal sequence identity.**

In order to identify genetic differences explaining the variation in heat resistance phenotypes between the strains, we compared the genome composition of the newly sequenced AL4E and AT3E strains. Both strains were of serotype 1/2c and multilocus sequence type (MLST) ST9 and had a GC content of 38\% (Table 2). They shared high chromosomal identity (Fig. 3). Genome comparison of the strains in SEED Viewer 2.0 (24) revealed 49 chromosomal genes unique to strain AT3E and 21 chromosomal genes unique to strain AL4E; most of these genes were hypothetical or phage related. PHASTER (25) predicted two intact phages, of which the 42.7-kb phage insert (designated $\delta$tRNA-Arg) adjacent to the arginine tRNA gene was related to \textit{Listeria} phage LP-101 and present in both strains. The 33.5-kb phage insert (designated $\delta$MT) down-
stream of a methyltransferase gene was related to *Listeria* phage A006 and absent in strain AL4E (Fig. 3).

**L. monocytogenes AT3E harbors a novel 58-kb plasmid.** Upon genome analysis, we discovered that the heat-resistant strain AT3E harbors a novel plasmid, which was designated pLM58. It is 58.5 kb in size and contains 70 predicted open reading frames (ORFs) and 19 predicted operons and has a GC content of 36.6% (Fig. 4 and Tables 2 and 3). In sequence comparison with *L. monocytogenes* plasmids deposited in GenBank at NCBI, pLM58 manifested a mosaic structure characteristic of listerial plasmids.

Annotation of pLM58 revealed an ORF that putatively encodes an ATP-dependent Clp protease ATP-binding subunit (ClpL) and is unrelated to any predicted operons (Fig. 4 and Table 3). An identical clpL sequence was harbored by 55% (12/22) of the different *L. monocytogenes* plasmids in GenBank. No *clpL* sequence was present in the remainder of the plasmids deposited in NCBI. Furthermore, *clpL* of pLM58 shared high nucleotide sequence identity (98%; E value of 0.0) with *clpL2* of *Lactobacillus rhamnosus* and moderate amino acid identity with plasmid-borne *clpK2* of *Escherichia coli* (46%; E value of 9E−175).

**FIG 1** Susceptibility of *L. monocytogenes* strains to heat stress at 55°C after 40 min. Plasmid curing in the heat-resistant strain AT3E led to significantly impaired survival at 55°C. Introducing *clpL* into the heat-sensitive 10403S strain resulted in significantly increased heat resistance, while control vector pPL2 without *clpL* did not have the same effect. These findings suggest that pLM58 is involved in heat resistance of *L. monocytogenes* and that this resistance is mediated by the plasmid-borne ATP-dependent protease ClpL. The log₁₀ reduction values are the means ± standard deviations (error bars) for three replicate cultures. Statistical significance was determined using the independent-samples two-tailed t test and indicated as follows: *, P < 0.01; **, P < 0.001; NS, not significant.

### TABLE 1 Bacterial strains and plasmids used in this study

| Strain or plasmid | Description or relevant phenotype or characteristic | Reference or source |
|-------------------|---------------------------------------------------|---------------------|
| **L. monocytogenes** | | |
| AL4E              | Wild-type strain; serotype 1/2c                    | 11                  |
| AT3E              | Wild-type strain; serotype 1/2c                    | 11                  |
| AT3Ep             | AT3E strain; plasmid-cured strain                 | This study          |
| 10403S            | Wild-type strain; serotype 1/2a; streptomycin resistant | 27                  |
| 10403SpcplL       | 10403S with tRNA<sup>Arg</sup>:clpL               | This study          |
| 10403SpPL2        | 10403S with tRNA<sup>Arg</sup>:pPL2               | This study          |
| **E. coli**       | | |
| NEBSα             | Chemically competent strain                        | New England Biolabs |
| HB101             | Conjugation donor containing helper plasmid pRK24 | CRBIP               |
| **Plasmids**      | | |
| pLM58             | Plasmid in the AT3E strain                         | This study          |
| p<sub>clpL</sub>  | pPL2 containing 423 bp of upstream nucleotides and coding sequence of ATP-dependent protease *clpL* | This study |
| pPL2              | Site-specific integration vector                   | 61                  |

<sup>a</sup>CRBIP, Biological Resource Centre of the Institut Pasteur.
pLM58 did not carry any antibiotic resistance genes but harbored genes similar to \textit{cadAC} that mediate cadmium resistance in \textit{Staphylococcus aureus} (23) (Table 3). The \textit{cadAC} genes were associated with a transposon Tn5422-related sequence (Fig. 4) and colocalized in an operon encoding predicted multicopper oxidase and a protein with unknown function (Table 3). The replication initiation protein of pLM58 shared high sequence identity (99%; E value of 0.0) with plasmids that have been allocated into group 1 of listerial plasmids by replicon-based distinction (26). Finally, no intact phages were found in pLM58.

**Plasmid pLM58 contributes to heat resistance of \textit{L. monocytogenes} AT3E.** In order to confirm whether heat resistance could be mediated by pLM58, the heat-resistant strain AT3E was cured of plasmid and subjected to heat resistance and growth assays. Plasmid curing resulted in the removal of plasmid from 2% (1/56) of \textit{L. monocytogenes} AT3E colonies. The cured derivative strain AT3Epc showed significantly impaired heat resistance compared to the AT3E parent, with a log$_{10}$ reduction of 1.1 CFU/ml (\( P < 0.001 \)) at 55°C (Fig. 1). The maximum growth temperatures and the kinetic growth parameters of the cured strain AT3Epc and its parent strain AT3E did not differ (\( P > 0.05 \)).

**Plasmid-borne ATP-dependent protease gene \textit{clpL} increases heat resistance in a natively heat-sensitive strain.** The \textit{clpL} gene was introduced into heat-sensitive \textit{L. monocytogenes} 10403S (Table 1) (27) in order to ascertain whether the plasmid-mediated heat-resistant phenotype was attributable to the ATP-dependent protease ClpL harbored by pLM58. Conjugation of \textit{clpL} in the pPL2 backbone increased the heat resistance of the recipient strain 10403S, which was observed by a significant decrease in log$_{10}$ reduction from 1.2 CFU/ml to 0.4 CFU/ml (\( P < 0.01 \)) at 55°C (Fig. 1). Conjugation of the control plasmid pPL2 lacking \textit{clpL} did not increase heat resistance of strain 10403S but did lead to a slight decrease in cell concentration (log$_{10}$ reduction 1.5 CFU/

**TABLE 2** General features of \textit{L. monocytogenes} strains and plasmid sequenced in this study

| Strain or plasmid | MLST type$^a$ | Assembly size (bp) | GC content (%) | No. of predicted CDS$^b$ | No. of RNAs | Avg length (bp) of CDS | Origin | Country | Yr of isolation |
|-------------------|--------------|-------------------|---------------|--------------------------|-------------|------------------------|--------|---------|--------------|
| AL4E              | ST9          | 3,027,995         | 38.0          | 3,002                    | 85          | 885                    | Equipment, conveyor | Finland | 1998         |
| AT3E              | ST9          | 3,057,808         | 38.0          | 3,049                    | 85          | 881                    | Product, sausage    | Finland | 1995         |
| pLM58             | NA           | 58,523            | 36.6          | 70                       | 0           | 714                    | AT3E strain         | Finland | 2017         |

$^a$NA, not applicable.

$^b$CDS, coding sequences.

FIG 2 Growth of \textit{L. monocytogenes} strains AL4E, AT3E, and 10403S, derivative cured strain AT3Epc, and conjugation strains 10403SpclpL and 10403SpPL2 displayed no significant differences at 42°C. All strains were grown in BHI broth for 10 h, and the OD$_{600}$ was measured every hour. Time (in hours) is shown on the x axis. The data represent the mean OD$_{600}$ values ± standard deviations (error bars) for three replicate cultures. The correspondence between the OD$_{600}$ values and viable-cell numbers was verified by plate counts at mid-logarithmic and stationary growth phase.
ml), although the difference was not statistically significant ($P > 0.05$). No significant differences were observed in the maximum growth temperature or kinetic growth parameters at 42°C between strain 10403S and conjugation strain 10403Spclpl or 10403SpPL2 ($P > 0.05$).

**pLM58 is putatively nonconjugative.** In order to confirm whether pLM58 is self-transmissible, standard plate mating was performed between strains AT3E and 10403S. The plasmid-borne cadAC genes confer cadmium resistance (17, 23) and were also found in pLM58 (Table 3). Thus, cadmium resistance facilitates the selection of recipient cells that have not received pLM58. The innate streptomycin resistance of strain 10403S facilitates the selection of possible transconjugants from the donor strain (28). The AT3E donor strain and 10403S recipient strain grew on positive-control plates containing 130 μg/ml CdSO₄ or 200 μg/ml streptomycin, respectively. However, no colonies were detected on selective plates containing both 130 μg/ml CdSO₄ and 200 μg/ml streptomycin. Colonies on selective plates containing less cadmium sulfate (65 μg/ml) tested positive by PCR for strain 10403S and negative for plasmid pLM58. Indeed, pLM58 also lacked the known type IV secretion system genes needed for the conjugation process of self-transmissible plasmids (29–31) (Table 3).

![Graphical representation of L. monocytogenes AL4E and AT3E strains sharing high chromosomal sequence identity](image-url)
DISCUSSION

For the purpose of elucidating the genetic mechanisms that render certain *L. monocytogenes* strains particularly resistant to heat, the genome sequences of a heat-resistant and heat-sensitive wild-type strain were compared. The two chromosomal sequences were highly similar. Yet, genome sequence analysis revealed a 58-kb plasmid exclusively harbored by the resistant AT3E strain, which suggested that the observed phenotypic difference in heat resistance between the two strains may be mediated by a plasmid. Indeed, plasmid curing resulted in significant reduction of cell concentration at 55°C, while the parent AT3E strain survived for the measured 40-min period. To the best of our knowledge, this is the first description of plasmid-mediated heat resistance in *L. monocytogenes*.

In comparison to previously reported listerial plasmids, heat resistance-mediating pLM58 is medium sized with 58 kb and 70 predicted ORFs (Tables 2 and 3). Listerial plasmids are mosaics of highly homologous fragments (32–34). Indeed, pLM58 is a novel plasmid harboring fragments both unique and highly similar to closely related plasmids. By replicon-based distinction of listerial plasmids, pLM58 was allocated into group 1 that manifests relatively small plasmid genomes (26).

Although plasmids have been shown to contribute to the resistance of *L. monocytogenes* strains to heat, the genetic mechanisms involved are still largely unknown. This study provides insights into the role of plasmids in heat resistance and opens up avenues for further research to understand the underlying mechanisms.

**FIG 4** Genetic map of *L. monocytogenes* plasmid pLM58 built using SnapGene Viewer 3.3.4. Initiation of the replication protein-encoding *ori* gene, ATP-dependent protease-encoding *clpL* gene, cadmium resistance genes *cadA* and *cadC*, transposon Tn5422, and unique restriction sites is indicated.
### TABLE 3

Putative open reading frames and their functions in pLM58

| ORF no. | Start position (bp) | Stop position (bp) | Length (bp) | Strand | GC content (%) | Predicted operon<sup>b</sup> | Function (RAST annotation)<sup>c</sup> |
|---------|---------------------|--------------------|-------------|--------|---------------|----------------|----------------------------------|
| 1       | 1635                | 1,635              | 1,635       | +      | 42.81         | Replication initiation protein | |
| 2       | 1900                | 165                | 1,635       | +      | 38.79         | Hypothetical protein          | Op1     |
| 3       | 2665                | 756                | 1,635       | +      | 41.53         | Hypothetical protein          | Op1     |
| 4       | 2699                | 603                | 1,635       | −      | 35.49         | Mobile element protein        | Op2     |
| 5       | 3378                | 756                | 1,635       | −      | 37.04         | Mobile element protein        | Op2     |
| 6       | 4144                | 1,227              | 1,635       | +      | 36.51         | Mobile element protein        | Op2     |
| 7       | 6304                | 591                | 1,635       | +      | 29.78         | Mobile element protein        | Op3     |
| 8       | 7224                | 918                | 1,635       | +      | 33.88         | FIG00775381; hypothetical protein | Op3     |
| 9       | 7861                | 240                | 1,635       | +      | 37.50         | PI0011 protein               | Op4     |
| 10      | 8313                | 441                | 1,635       | +      | 34.01         | PI0010 protein               | Op4     |
| 11      | 9001                | 1,341              | 1,635       | +      | 36.99         | PI0007 protein               | Op4     |
| 12      | 10028               | 372                | 1,635       | +      | 38.44         | PI0006 protein               | Op4     |
| 13      | 10812               | 765                | 1,635       | +      | 41.05         | PI0005 protein               | Op4     |
| 14      | 11456               | 331                | 1,635       | +      | 36.72         | PI0004 protein               | Op4     |
| 15      | 11493               | 336                | 1,635       | −      | 35.52         | Mobile element protein        | Op5     |
| 16      | 12400               | 399                | 1,635       | +      | 34.83         | Mobile element protein        | Op5     |
| 17      | 13906               | 1,503              | 1,635       | +      | 38.92         | Mobile element protein        | Op5     |
| 18      | 14443               | 453                | 1,635       | +      | 36.42         | Mobile element protein        | Op5     |
| 19      | 14472               | 681                | 1,635       | +      | 40.38         | Mobile element protein        | Op6     |
| 20      | 15183               | 42                | 1,635       | +      | 37.56         | Transposase and inactivated derivative-like protein | Op6     |
| 21      | 17140               | 1,635              | 1,635       | +      | 40.44         | NADH peroxidase (EC 1.11.1.1) | |
| 22      | 17386               | 870                | 1,635       | −      | 41.49         | l-Proline glycine betaine ABC transport system permease protein ProW (TC 3.A.1.12.1) | |
| 23      | 18615               | 240                | 1,635       | +      | 32.92         | Hypothetical protein          | Op7     |
| 24      | 18935               | 321                | 1,635       | +      | 29.91         | Mobile element protein        | Op7     |
| 25      | 19890               | 726                | 1,635       | +      | 39.94         | FIG00774644; hypothetical protein | Op8     |
| 26      | 21282               | 930                | 1,635       | +      | 44.95         | Lead-, cadmium-, zinc-, and mercury-transportsing ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4) | |
| 27      | 22235               | 966                | 1,635       | +      | 45.55         | Lead-, cadmium-, zinc-, and mercury-transportsing ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4) | |
| 28      | 22292               | 681                | 1,635       | −      | 38.18         | Mobile element protein        | |
| 29      | 23411               | 375                | 1,635       | −      | 33.60         | Hypothetical protein          | |
| 30      | 24006               | 126                | 1,635       | −      | 31.75         | Hypothetical protein          | |
| 31      | 24447               | 141                | 1,635       | −      | 33.83         | Hypothetical protein          | |
| 32      | 24829               | 269                | 1,635       | +      | 44.17         | Mobile element protein        | |
| 33      | 25010               | 189                | 1,635       | +      | 33.33         | Mobile element protein        | |
| 34      | 26156               | 423                | 1,635       | +      | 44.92         | Mobile element protein        | |
| 35      | 26893               | 654                | 1,635       | +      | 39.60         | Mobile element protein        | |
| 36      | 27654               | 765                | 1,635       | +      | 40.13         | Mobile element protein        | |
| 37      | 28036               | 198                | 1,635       | +      | 41.92         | Hypothetical protein          | |
| 38      | 28478               | 117                | 1,635       | −      | 30.77         | Hypothetical protein          | |
| 39      | 28578               | 120                | 1,635       | −      | 28.33         | Hypothetical protein          | |
| 40      | 29143               | 573                | 1,635       | +      | 37.87         | Nonspecific DNA-binding protein Dps/iron-binding ferritin-like antioxidant protein/ferroxidase (EC 1.16.3.1) | |
| 41      | 29273               | 162                | 1,635       | +      | 30.25         | FIG00630535; hypothetical protein | |
| 42      | 29992               | 705                | 1,635       | +      | 43.55         | Transcriptional regulator; Crp/Fnr family | Op11    |
| 43      | 30309               | 225                | 1,635       | +      | 42.67         | Prolipoprotein diacylglycerol transferase (EC 2.4.99.-) | Op11    |
| 44      | 30689               | 234                | 1,635       | +      | 36.32         | Copper chaperone              | |
| 45      | 32791               | 1,905              | 1,635       | +      | 41.99         | Copper-translocating P-type ATPase (EC 3.6.3.4) | |
| 46      | 33174               | 1,857              | 1,635       | −      | 29.35         | Bipolar DNA helicase HerA     | Op12    |
| 47      | 35023               | 1,194              | 1,635       | −      | 28.14         | FIG036446; hypothetical protein | Op12    |
| 48      | 36485               | 579                | 1,635       | −      | 39.90         | Resolvase/integrase bin       | |

(Continued on next page)
togenes to stressors such as antibiotics (18, 19), disinfectant (20–22), and heavy metals (15, 23), little evidence is available on thermal resistance attributable to listerial plasmids. Hingston et al. discovered by genetic characterization that the presence of plasmids is associated with cold sensitivity of L. monocytogenes (16). Studying Listeria innocua strains, Margolles and de los Reyes-Gavilán found no difference in thermal inactivation by pasteurization between the Li16 strain and its cured derivative Li16c (35). Therefore, they suggested that pLI59 harbored by strain Li16 does not encode genes related to heat resistance (35). Heat stress-related genes have been annotated in L. monocytogenes plasmids (36), but phenotypic evidence on their importance in growth or survival at high temperatures has been lacking thus far.

pLM58 harbored a 2,115-bp ORF annotated as clpL putatively encoding ATP-dependent protease ClpL that we considered a potential mediator of heat resistance in L. monocytogenes. Indeed, introducing the putative promoter and the coding sequence of clpL into the natively heat-sensitive L. monocytogenes 10403S innately lacking clpL, resulted in significantly increased survival at 55°C. Conjugation of the control vector pPL2 without clpL did not have the same effect, which indicates that the vector itself does not confer resistance to heat treatment. These findings suggest that plasmid-borne clpL plays a role in elevated heat resistance of L. monocytogenes. The presence of the same ORF in many other listerial plasmids suggests that heat resistance mediated by clpL may be widespread among L. monocytogenes strains harboring plasmids. Clp ATPases function both as ATP-dependent proteases degrading damaged and misfolded proteins and as chaperones involved in protein folding (37). The chromosomally encoded ClpC, ClpP, and ClpE class III heat shock proteins are involved in virulence and stress tolerance of L. monocytogenes (38, 39). However, our study is the first to describe heat resistance attributable to a plasmid-borne Clp in L. monocytogenes.

The clpL gene of pLM58 was nearly identical to the plasmid-borne clpL of L. rhamnosus. As the clpL homolog in L. rhamnosus is surrounded by transposase genes and

| ORF no. | Start position | Stop position | Length (bp) | Stranda | GC content (%) | Predicted operonb | Function (RAST annotationc) |
|---------|----------------|---------------|-------------|---------|---------------|-------------------|----------------------------|
| 49      | 37324          | 39369         | 2,046       | +       | 34.55         | Op13              | Lead-, cadmium-, zinc-, and mercury-transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); copper-translocating P-type ATPase (EC 3.6.3.4) |
| 50      | 39384          | 40544         | 1,161       | +       | 29.72         | Op13              | Multicopper oxidase |
| 51      | 44347          | 40532         | 2,916       | −       | 36.83         | Op14              | Mobile element protein |
| 52      | 44025          | 43471         | 555         | −       | 38.02         | Op14              | DNA invertase |
| 53      | 44305          | 44664         | 360         | +       | 35.28         | Op15              | Cadmium efflux system accessory protein |
| 54      | 44664          | 46799         | 2,136       | +       | 39.98         | Op15              | Cadmium-transporting ATPase (EC 3.6.3.3) |
| 55      | 46867          | 47271         | 405         | +       | 37.53         | Op15              | Multicopper oxidase |
| 56      | 47291          | 47836         | 546         | +       | 33.15         | Op15              | DUF1541 domain-containing protein |
| 57      | 47988          | 48110         | 123         | +       | 21.95         | Hypothetical protein |
| 58      | 48221          | 48820         | 600         | +       | 38.33         | Op16              | Site-specific recombinase; resolvase family |
| 59      | 48810          | 49037         | 228         | +       | 35.96         | Op16              | Hypothetical protein |
| 60      | 49435          | 49157         | 279         | −       | 36.92         | Hypothetical protein |
| 61      | 49398          | 49634         | 237         | +       | 34.60         | Hypothetical protein |
| 62      | 50062          | 52176         | 2,115       | +       | 40.00         | Hypothetical protein |
| 63      | 52443          | 53441         | 999         | +       | 35.04         | Op17              | Mobile element protein |
| 64      | 53457          | 53792         | 336         | +       | 36.01         | Op17              | Mobile element protein |
| 65      | 53946          | 54701         | 756         | +       | 32.54         | FIG00775080; hypothetical protein |
| 66      | 54906          | 55250         | 345         | +       | 41.16         | FIG00775387; hypothetical protein |
| 67      | 55238          | 56533         | 1,296       | +       | 41.82         | Op18              | ImpB/MucB/SamB family protein |
| 68      | 56832          | 56536         | 297         | −       | 29.63         | Op19              | Hypothetical protein |
| 69      | 57784          | 56810         | 975         | −       | 30.46         | Op19              | Replication-associated protein |
| 70      | 58142          | 58300         | 159         | +       | 28.93         | Hypothetical protein |

The location of the open reading frame on the plus (+) or minus (−) strand is indicated.

**Table 3 (Continued)**

Operon prediction was performed using FGENESB (54).

Annotations were performed using RAST 2.0 (52).
was mobilizable (40), it has been proposed that the ClpL protease is acquired via horizontal gene transfer (33). Canchaya et al. also suggest that ClpL of *L. monocytogenes* may originate from lactic acid bacteria (33). Interestingly, *clpL* of pLM58 is surrounded by genes related to site-specific recombinases, phages, and other mobile genetic elements (Table 3), further evidence for the putative horizontal transfer of *clpL*. *clpL* expressed from an *L. rhamnosus* plasmid was upregulated during heat shock (40). It is thus possible that plasmid-borne *clpL* plays a universal role in heat resistance of Gram-positive bacteria.

While ClpL is exclusively associated with Gram-positive bacteria (41), we found that *clpL* of pLM58 is moderately similar to plasmid-borne *clpK* of the Gram-negative *E. coli*. Intriguingly, plasmid-mediated heat resistance has been reported in an *E. coli* dairy isolate (42) and in a nosocomial *Klebsiella pneumoniae* strain (41). In both studies, thermostolerance was shown to correlate with the presence of a plasmid-borne ATPase-encoding *clpK* gene (41, 42).

Plate mating between the AT3E donor strain and the 10403S recipient strain yielded no transconjugants, which suggests that pLM58 is not self-transmissible. This is in line with the fact that pLM58 did not harbor any known type IV secretion system genes needed for the conjugation process of self-transmissible plasmids (29–31). It remains to be verified whether pLM58 is mobilizable. However, listerial plasmids manifest mosaic patterns (33), and we found *clpL* among the ones reported in NCBI. Therefore, the heat resistance-mediating *clpL* gene could also be found in a conjugative plasmid. Therefore, the conjugative ability of listerial plasmids harboring *clpL* should be further investigated.

In addition to the presence of pLM58, the heat-resistant AT3E strain harbored an intact phage insert, related to *Listeria* phage A006 and absent in the heat-sensitive AL4E strain. Many phages encode virulence factors contributing to bacterial pathogenesis as well as stress resistance genes specifically related to survival of bacteria in host cells (43). However, further investigations are needed to elucidate their potential in conferring bacterial heat resistance.

Although proven heat sensitive at 55°C, strain AL4E had a slightly higher maximum growth temperature than the heat-resistant AT3E strain did. In addition, the differences between their growth at 42°C were negligible. Furthermore, plasmid curing in the heat-resistant AT3E strain or introducing the *clpL* gene into the heat-sensitive 10403S strain had no effect on maximum growth temperature or kinetic growth parameters at 42°C. This suggests that the mechanisms underlying resistance to thermal kill are different from those permitting growth at the higher end of the growth temperature range. Bojer et al. demonstrated that the maximum growth temperature of *K. pneumoniae* was unaffected by a mutation in *clpK* that was shown to mediate heat resistance in the bacterium (41). Studying growth and survival under acid stress, Metselaar et al. demonstrated that increased acid resistance in *L. monocytogenes* was, in fact, correlated with decreased maximum growth rate (44). Heterogeneity under different stress conditions may be of advantage to *L. monocytogenes*, since it may benefit cell survival (44).

In addition to increased environmental fitness of bacteria, plasmid-borne stress resistance genes are of concern due to potential cotransfer with virulence and antibiotic resistance genes often harbored by plasmids (36, 41, 45). Thus, they may enhance the ability of plasmid-harboring pathogens to survive in different niches, which creates opportunities to infect new hosts.

This study is, to the best of our knowledge, the first description of a plasmid that plays a role in heat resistance of *L. monocytogenes*. We state that plasmid-borne ATP-dependent protease ClpL contributes to the survival of *L. monocytogenes* at high temperature. Plasmid-borne ClpL is a potential predictor of elevated heat resistance in *L. monocytogenes* and other Gram-positive bacteria. Our findings bring light to accessory genetic mechanisms that cause large variation in the ability of *L. monocytogenes* strains to survive heat treatments.
MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains and plasmids used are presented in Table 1. The strains were routinely grown at 37°C on blood agar, on tryptic soy agar (TSA) or in tryptic soy broth (TSB), on brain heart infusion (BHI) agar or in BHI broth, on Luria-Bertani (LB) agar or in LB broth (Oxoid, Cheshire, England), or on ALOA (Harlequin Listeria Chromogenic) agar (Labema, Helsinki, Finland). Appropriate antibiotics (Sigma-Aldrich, St. Louis, MO) and cadmium sulfate (3CdSO4 · 8H2O) (Merck, Darmstadt, Germany) were added when needed. L. monocytogenes AL4E and AT3E were serotyped according to the supplier’s instructions using the Listeria Antisera Set (Denka Seiken, Tokyo, Japan) including O- and H-factor antisera and by using the Pasteur typing tool (http://bigdb.pasteur.fr/perl/bigdb/bigdb.pl?db=pubmlst_listeria_seqdef_public) (accessed 13 July 2017).

Heat resistance assay. The wild-type strains 10403S, AL4E, and AT3E, cured derivative strain AT3Epclp and conjugation strains 10403SpPl2 and 10403Spclp were challenged in a heat survival test conducted by the method of Lundén et al. (11) with minor modifications. Briefly, three colonies of each strain were individually inoculated into TSB and grown overnight at 37°C, followed by dilution (1:100) of each culture into 10 ml of fresh TSB and subsequent overnight incubation at 37°C to reach a cell concentration of 10⁸ to 10⁹ CFU/ml. The cultures were then diluted (1:100) into 5 ml of fresh TSB and placed in a 55°C water bath for 40 min. Serial dilutions from room temperature before heat stress (0⁰) and after 40 min at 55°C were plated on PCA agar (Oxoid) to determine the decrease in cell concentration (log₁₀ reduction). Colony counting on PCA plates was performed after 2 days of incubation at 37°C. Strains showing a log₁₀ reduction of less than 1.0 log₁₀ were considered heat resistant.

Growth curve analyses. Three colonies of each L. monocytogenes strain were individually inoculated into 10 ml of BHI broth and incubated overnight at 37°C. The cultures were diluted (1:100) in fresh BHI broth and grown at 42°C for 10 h under continuous shaking, and the optical density at 600 nm (OD₆₀₀) was measured at 1-h intervals. Growth parameters, including the maximum growth rate, lag phase, maximum optical density, and area under the curve were obtained for each strain using the grofit package (47) with default settings in R 3.2.2 (R Core Team, Vienna, Austria). The parameters were determined using the logistic model, which fitted the data best based on the Akaike information criterion (47). Correspondence between the OD₆₀₀ values and viable-cell numbers of the strains was confirmed by plate counts at mid-logarithmic and stationary growth phases.

Differences in maximum growth temperatures. Differences in maximum growth temperatures of L. monocytogenes strains were examined using Gradiplate W10 incubator (BCDE Group, Helsinki, Finland) by the method of Hinderink et al. (48) with slight modifications. Briefly, three overnight cultures of each strain were individually diluted (1:100) in peptone water and plated on TSA (containing 25 g/liter agar) by the stamping technique in duplicate. The strains were grown for 24 h at a temperature gradient of 39.2°C to 45.7°C. Growth boundaries were determined by using a stereomicroscope (Olympus SZ61; Nikon, Tokyo, Japan).

Genome sequencing and comparative genomic analysis. Genomic DNA from L. monocytogenes AL4E and AT3E was extracted using guanidium thiocyanate by the method of Pitcher et al. (49). Whole-genome sequencing was performed by the Institute of Biotechnology (Helsinki, Finland) using single-molecule real-time (SMRT) sequencing in the PacBio RS platform with coverages of 199× (AL4E), 252× (AT3E), and 422× (pLM58). The genomes were de novo assembled using the RS_HGAP_Assembly3 protocol (Pacific Biosciences of California, Inc., Menlo Park, CA). Multilocus sequence types (MLSTs) of strains AL4E and AT3E were derived using the Pasteur MLST typing tool (50, 51). The genomes were annotated using RAST 2.0 (52), and genome comparison was performed in SEED Viewer 2.0 (24). Prophage prediction was performed using PHASTER (25, 53), and sequence-based operon prediction of pLM58 was performed using FGENE58 (54). Visual comparison of AL4E and AT3E chromosomes was generated using BRIG (BLAST ring image generator) (55), and pLM58 was visualized using SnapGene Viewer 3.3.4 (GSL Biotech LLC, Chicago, IL). Using BLASTN 2.2.26 (56), coding sequences of the replication initiation protein and the ATP-dependent protease ClpL harbored by pLM58 were compared to each L. monocytogenes plasmid sequence deposited in GenBank at NCBI (https://www.ncbi.nlm.nih.gov/genome/plasmids/1597; accessed 13 July 2017).

Plasmid curing. Plasmid curing in the heat-resistant AT3E strain was performed by the method of Margolles and de los Reyes-Gavilán (35). Briefly, strain AT3E was grown in TSB overnight at 37°C and inoculated (1:100) into TSB supplemented with a subinhibitory concentration of novobiocin (0.2 μg/ml) as a nonmutagenic curing agent (57, 58). The cultures were incubated at 40°C for 24 h, followed by nine identical subsequent inoculations (1:100) and incubations. The final cultures were plated on TSA plates containing novobiocin (0.2 μg/ml). The absence of the plasmid was verified from purified colonies by PCR using primers oriV-F (F stands for forward) and oriV-R (R stands for reverse) specific for the replication initiation protein-encoding gene of pLM58 and primers clpL-F and clpL-R specific for the ATP-dependent protease ClpL-encoding clpL gene harbored by pLM58 (Table 4). All the primers were designed using Primer3 (v. 4.0.0) (59, 60).

Introducing clpL into heat-sensitive L. monocytogenes. The coding sequence of clpL and a 423-bp upstream region, including the putative promoter, were conjugated into the heat-sensitive L. monocytogenes 10403S. This was done in order to verify the role of clpL in enhancing the heat resistance of L. monocytogenes. Conjugation was performed using the PSA prophage site-specific phage integration vector pPL2 kindly provided by Martin Loessner, Swiss Federal Institute of Technology, Zurich, Switzerland) by the methods of Lauer et al. and Ma et al. (61, 62). Briefly, the coding sequence and the putative promoter of clpL were amplified by PCR using primers BamHI clpL FN and BamHI clpL RN (Table 4). The insert was ligated into the BamHI restriction site of linearized pPL2 treated with recircularization-preventing Antarctic phosphatase (New England Biolabs, Ipswich, MA) and propagated in E. coli NEBα
This study (strain AT3E), and
11
This study
msphere.asm.org
61
CP023752
strain AL4E),
61
under accession numbers
insert was confirmed using
pLM58) within BioProject
Differences in log10 reductions at 55°C, growth parameters at 42°C, and maximum growth temperatures
streptomycin.

Table 4
Primers used in this study

| Primer | Sequence (5′ – 3′)a | Reference |
|--------|---------------------|-----------|
| orV-F  | GAACAAGCGATCGGTATGC | This study |
| orV-R  | TCGTGCTAGGACTGTGCTG | This study |
| clpL-F  | ACAGGCTCTGATGCTTATTC | This study |
| clpL-R  | ACCGGATATTTGAATTCGCG | This study |
| BamHI clpL FN | NNNNGATATGTTCAAGTAGTCTG | This study |
| BamHI clpL RN | NNNNGATATCCTCATCAAGAATCTCCTCTC | This study |
| NC16  | GTCAAAACATAGCGCTTATC | 61 |
| PL95  | ACATAATCAGTCCAAAGTAGATGC | 61 |
| ESAT-6 F  | GCATACAGTGGGAAAGGACTG | This study |
| ESAT-6 R  | ATCCATCGTGTATTTCCTGT | This study |
| secA-F  | ACTACTGCCAAAACATCGAAGC | This study |
| secA-R  | AAGAGGCACCTGGATCCCTC | This study |

aThe bases in the sequences are shown as follows: N, any of the bases, i.e., adenine (A), cytosine (C), guanine (G), or thymine (T). Restriction sites in the sequences are underlined.

(New England Biolabs). The pclpL and control plasmid pPL2 without an insert were separately trans-
formed into the conjugation donor E. coli HB101 and conjugated into the 10403S recipient strain by filter
matting. Transconjugants were selected on ALOA agar supplemented with 7.5 μg/ml chloramphenicol.
Integration of the plasmids was confirmed using primers NC16 and PL95 (61), and the presence of the
insert was confirmed using clpL gene-specific primers (Table 4).

Horizontal transfer experiments. To examine whether pLM58 is self-transmissible between two
wild-type L. monocytogenes strains, standard plate mating was performed with AT3E as the donor strain
and L. monocytogenes 10403S (kindly provided by Martin Wiedmann, Cornell University, Ithaca, NY) as the
recipient strain. Briefly, the donor strain was grown in BHI, and the recipient strain was grown in BHI
supplemented with 200 μg/ml streptomycin. After overnight growth at 37°C, 10403S cells were washed
twice with fresh BHI, and both strain cultures were diluted (1:100) into fresh BHI and grown to logarithmic
growth phase (OD600 of 0.5). Equal volumes (100 μl) of the donor and recipient were spotted on top of
each other on BHI agar and incubated at room temperature for 1 h followed by 24-h incubation at 37°C.
The cells were washed from the plate with BHI broth, and possible transconjugants were screened after
3 days of incubation at 37°C on BHI plates containing 200 μg/ml CdSO4. The experiment was done in two simultaneous repeats, and the visible colonies were screened by PCR using primers specific for pLM58 orV, ESAT-6 in the AT3E genome, and secA in the
10403S genome (Table 4). To serve as both positive and negative controls, the donor AT3E and recipient
10403S strains were individually plated on BHI agar containing 130 μg/ml CdSO4 or 200 μg/ml streptomycin.

Statistical analysis. Statistical analysis was conducted in IBM SPSS statistics 24 (IBM, Armonk, NY).
Differences in log10 reductions at 55°C, growth parameters at 42°C, and maximum growth temperatures
between the strains were tested using independent-samples two-tailed t tests.

Accession number(s). The nucleotide sequences have been deposited in the GenBank database
under accession numbers CP023754 (strain AL4E), CP023752 (strain AT3E), and CP023753 (plasmid
pLM58) within BioProject PRJNA412588.

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