A *Listeria monocytogenes* RNA-helicase essential for growth and ribosomal maturation at low temperatures, uses its C-terminus for appropriate interaction with the ribosome

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Abstract

Listeria monocytogenes, a Gram-positive food-borne human pathogen, is able to grow at temperatures close to 0°C and is thus of great concern for the food-industry. In this work, we have investigated the physiological role of one DExD-box RNA-helicase in Listeria monocytogenes. The RNA-helicase Lmo1722 was required for optimal growth at low temperatures, whereas it was dispensable at 37°C. A Δlmo1722 strain was less motile due to a down-regulation of the major subunit of the flagellum, FlaA, caused by decreased flaA expression. By ribosomal fractionation experiments, it was observed that Lmo1722 was mainly associated with the 50S subunit of the ribosome. Absence of Lmo1722 decreased the fraction of 50S ribosomal subunits and mature 70S ribosomes and affected the processing of the 23S precursor rRNA. The ribosomal profile could be restored to wild-type levels in a Δlmo1722 strain expressing Lmo1722. Interestingly, the C-terminal part of Lmo1722 was redundant for low temperature growth, motility, 23S rRNA processing and appropriate ribosomal maturation. However, Lmo1722 lacking the C-terminus showed a reduced affinity for the 50S and 70S fractions, suggesting that the C-terminus is important for proper guidance of Lmo1722 to the 50S subunit. Taken together, our results show that the Listeria RNA helicase Lmo1722 is essential for growth at low temperatures, motility, ribosomal RNA processing and is important for ribosomal maturation being associated mainly with the 50S subunit of the ribosome.
Introduction

With reducing temperature, most chemical reactions are slowed down. For bacteria to survive at low temperatures, it needs to maintain macromolecules active. Single stranded RNA molecules form secondary structures that by physical properties are more stable at low temperatures compared to high temperatures. This raises the question how psychrotolerant bacteria can survive and thrive at low temperatures. DExD-box RNA-helicases (subfamily of DEAD-box RNA-helicases) are enzymes that have been suggested to unwind occluding secondary RNA-structures and are believed to be particularly important during growth at low temperatures (16). For instance, some RNA-helicases have been shown to participate in the maturation and re-folding of the 23S rRNA (DbpA, YxiN) or/and assembly of the 50S ribosomal subunit (SrmB, CsdA) (14, 15, 21, 30, 36). Several RNA-helicases act together with additional partners, constituting an RNA degradosome (13, 35, 38, 52). Yet other helicases can be associated with different targets and partners (16, 29, 30). Hitherto, most studies of RNA-helicases in bacteria have been performed in Escherichia coli and Bacillus subtilis. However, neither of these bacteria can multiply at temperatures close to 0°C.

Listeria monocytogenes is a pathogenic Gram-positive bacteria causing listeriosis, manifested by gastrointestinal infection which can spread into blood, central nervous system and, in case of pregnant women, fetus (17). Although being a soil-bacterium, it can occasionally contaminate the food-chain and can thus be found in non-pasteurized and ready-to-eat food (48). Listeria is a major concern for the food-industry due to its ability to grow at very low temperatures, even below 0°C (32). Several factors have been identified that allow Listeria to grow at low temperatures (12). Among these are compatible solutes transporters encoded by opu and gbu operons, oligopeptide permease oppA, ferritin Fri, pgpH phosphohydrolase involved in ppGpp turnover, and others (7, 9, 20, 23, 28, 41, 42, 45, 46). L. monocytogenes harbors 4 putative DExD-box RNA-helicases and in a previous study, the genes encoding
three RNA helicases (lmo0866, lmo1450 and lmo1722) were identified to be induced at low temperature (11). A transposon insertion in a region directly upstream of a putative RNA helicase gene (homologous to lmo1722) in the *L. monocytogenes* strain F2365, induced a cold sensitive growth phenotype and reduced swarming motility on soft agar plates (4). However, the authors were unable to complement these phenotypes, suggesting additional mutations or downstream effects of the transposon mutant.

To our knowledge, only a few studies have investigated the role of RNA helicases in organisms able to grow at very low temperatures. Most studies have focused on the role of these RNA-helicases *in vitro* and not in their natural context *in vivo* (8, 10, 40, 49). One exception being the *B. cereus*, where the physiological function of five RNA-helicase genes was examined with regard to their role in cold growth and response to various stresses (50, 51). In this study, we have investigated the function of an RNA-helicase in a cold-growing bacterium, *Listeria monocytogenes*. Our data show that the RNA-helicase Lmo1722 is required for growth at low temperatures, for motility, for accurate 23S rRNA processing and for proper ribosome maturation. Intriguingly, a strain expressing a C-terminal deleted form of Lmo1722 displayed wild-type ribosomal maturation, 23S rRNA processing and complete growth restoration in cold, despite being dispersed in the cytoplasm (delocalized from ribosomal subunits).
Materials and methods

Strains and plasmid construction

E. coli and L. monocytogenes strains are listed in Table 1. Plasmids used in the study are listed in Table 2 and oligonucleotides in Supplementary Table 1. E. coli were grown in LB and L. monocytogenes in BHI, unless otherwise noted. Where needed, antibiotics were included in the growth media at these final concentrations: carbenicillin 100 µg/ml; kanamycin 50 µg/ml; nalidixic acid 50 µg/ml; colistin sulfate 10 µg/ml; chloramphenicol – 25 µg/ml for E. coli and 7 µg/ml for L. monocytogenes. Cloning was performed using standard techniques (53).

Flanking regions of lmo1722 were amplified with primer pairs: lmo1722-A and lmo1722-B for the upstream, and lmo1722-C and lmo1722-D for the downstream region. The PCR products were ligated and digested with SalI and NcoI endonucleases and cloned in tandem into SalI and NcoI sites of the pMAD vector (1). The deletion of lmo1722 was then performed as described previously (1). Complementation of lmo1722 deletion was done by cloning of the helicase gene into E. coli – L. monocytogenes shuttle vector pMK4P harboring a constitutively active promoter (2). Oligonucleotide primers 1722 Dp+/p- and 1722 Up- were used for PCR amplification. The plasmid with lmo1722 insert was transferred to L. monocytogenes by electroporation. For overexpression in E. coli the gene lmo1722 was amplified with Phusion DNA polymerase (Finnzymes) from EGDe chromosomal DNA template by PCR using lmo1722-F_64_KpnI and lmo1722-RH_66_XbaI primers. The appropriately cut DNA fragments purified from agarose gel were ligated with pBAD18 vector and E. coli DH5α was transformed with the ligation mixture. Selected clones were confirmed by sequencing the cloned region. Similarly, primer pair lmo1722-F_64_BamHI and lmo1722-R_62_SalI was used to generate plasmid pKVA791 (also pKVA742 and pKVA789, which contain random mutations). Primer pair lmo1722-F_64_BamHI and lmo1722-R_noC_64_SalI
was used to generate plasmid pKVA746. The resulting constructs were transferred to *L. monocytogenes* by conjugation with *E. coli* S17-1 strain carrying these plasmids (18). Transconjugants were selected by plating on BHI plates containing kanamycin, colistin sulfate and nalidixic acid.

**RNA isolation**

Bacterial cultures grown to a defined growth phase were mixed with 0.2 volumes of 5 % phenol in 95 % ethanol (61) and bacteria harvested by centrifugation. Bacterial pellets were frozen in liquid nitrogen and stored at -80 °C. RNA from *L. monocytogenes* was isolated using a modification of guanidinium thiocyanate-phenol-chloroform extraction (56).

**Northern blotting**

For northern blotting, 20 μg of total RNA was separated on a formaldehyde agarose gel prior to bloting as described (56). The Hybond-N membrane (GE Healthcare) was subsequently hybridized with $^{32}$P α-dATP-labeled DNA fragments amplified with corresponding primers using Megaprime DNA labeling system (GE Healthcare). Northern blots were developed, and band intensities were measured in the STORM 860 machine (Molecular Dynamics). PCR primer pairs used to generate DNA probes for detection of *flaA*, *degU* and tmRNA are listed in Supplementary Table 1.

**Primer extension**

A method of primer extension using a fluorescently labeled primer was described previously (43). Primer extension reactions were performed using RevertAid Premium reverse transcriptase (Thermo Scientific) according to manufacturer’s protocol. Each reaction contained 1 μg of total RNA and 2.4 pmol carboxyfluorescein (6-FAM) labeled primer 23S-
FAM 5’-catatcggtgttagtcccg-3’. The primer was allowed to anneal to the template RNA by slowly cooling down the reaction solution from 80 °C to 30 °C during 1 hour. The rest of reaction components were added to a final volume of 20 μl and primer extension proceeded at 50 °C for 1 hour. Reaction products were ethanol precipitated and resolved on 3130xl Genetic Analyzer using a GeneScan 500LIZ Size Standard (Applied Biosystems). Peaks of fluorescent products (corresponding to transcripts of different lengths) were analyzed by GeneMapper 4.0 software (Applied Biosystems) (Supplementary Figure 5). From this, the most prominent peak areas corresponding to mature 23S rRNA and immature 23S rRNA harboring a 160 nt 5’-precursor sequence were quantified and the ratio of immature/mature signal was plotted.

**Listeria chromosomal DNA isolation**

Chromosomal DNA was extracted after lysis with mutanolysin (19). Briefly, bacteria were grown in BHI medium at 37 °C in shaking culture. *Listeria* from 10 ml of overnight culture were harvested by centrifugation, suspended in 1 ml SET buffer (30 mM Tris-Cl pH 8, 50 mM NaCl and 5 mM EDTA), washed with 0.5 ml acetone and incubated for 1 h at 37 °C in 50 mM Tris-Cl pH 6.5, 200 U/ml mutanolysin (Sigma-Aldrich) and 0.01 mg/ml RNase A. A 10× Proteinase K incubation buffer was added to a final concentration of 50 mM Tris-Cl pH 7.5 and 10 mM CaCl₂, and treatment with 12000 U of Proteinase K (Fermentas) was continued for 30 min at 37 °C. After phenol/chloroform extraction chromosomal DNA was fished out from 75 % ethanol and suspended in TE buffer (10 mM Tris-Cl pH8, 1 mM EDTA).

**Lmo1722 purification and antibody production**

*E. coli* LMG194 strain carrying pKVA429 (pBAD18:lmo1722H₆) plasmid was grown at 37 °C in 3 l of LB medium supplemented with 100 μg/ml carbenicillin to an optical density OD₆₀₀ of 0.5. Lmo1722-H₆ expression was induced with 0.02 % arabinose and allowed to
proceed at 30 °C for 6 hours. The bacteria were harvested by centrifugation at 6000 ×g for 30 minutes at 4 °C. Bacteria were washed by suspending in 1/10th culture volume solution W (50 mM Tris-Cl pH 8, 200 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine) with 5 mM EDTA and 0.1 mg/ml lysozyme. After centrifugation the pellet was again suspended in the same volume of solution W and bacteria were disrupted by sonication. Insoluble material was removed by centrifugation at 30000 ×g for 1 hour. Polyethyleneimine pH 8 was added drop-wise into stirred supernatant to a final concentration of 0.2 %, allowed to precipitate at 0 °C for 30 min and pellet removed after centrifugation at 30000 ×g for 15 min (22, 59). Ammonium sulfate was added to 95 % saturation and allowed to stand overnight in ice. Protein precipitate was collected by centrifugation at 30000 ×g for 2 h, washed in solution W saturated with ammonium sulfate, centrifuged again, suspended in solution A (20 mM Tris-Cl pH 8, 500 mM NaCl, 50 mM imidazole, 10 % glycerol, 5 mM 2-mercaptoethanol, 1 mM PMSF) and loaded onto 1 ml HisTrap column (GE Healthcare). Lmo1722-H6 was then eluted with a linear imidazole gradient to a 300 mM concentration. The protein was further purified by size exclusion on a Superdex 75pg (GE Healthcare) column using a buffering solution S (50 mM Tris-Cl pH 8, 500 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF). For rabbit immunization 2 mg of purified protein was separated on 10 % SDS-PAGE, stained with Coomassie R250, cut out from the gel and used for injection into rabbit (Agrisera AB, Vännäs, Sweden).

**Ribosome profiling**

The ribosomal profiling was essentially performed as previously described (25), with a modification of the lysis method adapted for *Listeria*. *Listeria monocytogenes* strains were grown in BHI shaking culture at 16 °C 160 rpm to an OD₆₀₀ of 0.5. Bacterial growth was stopped with 100 µg/ml chloramphenicol, cells were
harvested by centrifugation of 100 ml culture at 10000 ×g, washed with half culture volume ice-cold solution RW (10 mM Tris-Cl pH 7.5, 60 mM KCl, 10 mM MgCl₂, 6 mM 2-mercaptoethanol, 1 mM PMSF, 100 μg/ml chloramphenicol). To the bacterial pellet, 0.5 ml of solution RM (10 mM Tris-Cl pH 6.5, 60 mM KCl, 10 mM MgCl₂, 6 mM 2-mercaptoethanol, 1 mM PMSF, 100 μg/ml chloramphenicol, 20 % sucrose, 2000 U/ml mutanolysin) was added. After incubation for 40 min at 37 °C samples were centrifuged for 10 min at 10000 ×g, supernatant discarded. Bacteria were lysed by keeping for 15 min in 0.5 ml ice-cold solution RL (10 mM Tris-Cl pH 7.5, 60 mM KCl, 10 mM MgCl₂, 6 mM 2-mercaptoethanol, 1 mM PMSF, 100 μg/ml chloramphenicol, 0.2 % Triton X100 Reduced (Sigma-Aldrich), 200 U/ml DNase I, RNase free (Roche)). After addition of 0.16 % sodium deoxycholate samples were centrifuged at 20000 ×g, 4 °C, for 1 h. Supernatant was either directly loaded for fractionation in sucrose gradient or frozen in liquid nitrogen to be processed later. The sucrose gradients were prepared using the Gradient Master apparatus (Biocomp, Fredericton, NB, Canada). The amount of lysates, corresponding to 7 units of A₂₆₀, was loaded on top of centrifugation tubes with formed 10-40 % sucrose gradient in solution R (10 mM Tris-Cl pH 7.5, 60 mM KCl, 10 mM MgCl₂). Samples were centrifuged for 3.5 h in SW41Ti rotor (Beckman) at 35000 rpm, 4 °C. The ribosomal profiles were generated by UV absorbance A₂₅₄ measurements of the gradients using an ISCO sucrose gradient fractionator equipped with an UA-6 absorbance detector (Teledyne ISCO, Lincoln, Nebraska, USA). When necessary 4-drop volume fractions were collected, the absorbance of each measured using Nanodrop (Thermo Scientific) and used further for SDS-PAGE and Western blot analysis. Proteins for SDS-PAGE were concentrated by 6 % trichloracetic acid and sodium deoxycholate precipitation (5).

SDS-PAGE, Western blotting
Listeria total protein samples for electrophoresis were prepared by mutanolysin lysis (19), and analysed by SDS-PAGE (37) and/or Western blotting on PVDF membrane (57). Briefly, the different cultures were grown in BHI before being disrupted. Protein samples were separated on a 12% Polyacrylamide gel electrophoresis and either stained with Commassie Brilliant Blue or transferred onto a PVDF membrane using a tank transfer apparatus (Bio-Rad). Development of the membrane essentially followed the protocol of the ECL+ Western blotting kit (Amersham) using anti-Lmo1722 as primary antibodies and HRP-conjugated anti-rabbit as secondary antibodies (Bio-Rad). Measurement of luminescence signal was carried out in LAS4000 machine (Fuji). Quantification of luminescence signal was done using Quantity One 4.6.3 1-D Analysis software (Bio-Rad).
Deletion of the DExD-box RNA-helicase Lmo1722 abolishes *L. monocytogenes* growth at temperatures below 10°C. To investigate whether DExD-box RNA helicases in *Listeria monocytogenes* are important for the overall bacterial physiology, the gene encoding one of them, Lmo1722, was deleted (Figure 1). There were several reasons for choosing Lmo1722. It shows an induced expression on the transcriptional level at low temperatures (11). Also, its *Bacillus subtilis* orthologue, YfmL, is an RNA helicase not well characterized and is probably not part of the RNA degradosome, given that it was not able to interact with RNA degradosome components or other helicases in a bacterial two hybrid system assay (38). The *lmo1722* orthologue *cshC* in *B. cereus* was among three RNA helicase genes out of five, which were required for cold growth and involved in adaptation to several stress conditions (50, 51). Lmo1722 has a core region similar to other DExD-box RNA helicases but also harbors a unique lysine-rich 72 amino-acid long C-terminus, not present in the *B. subtilis* orthologue YfmL (Supplementary Figure 1) (38, 44). Absence of Lmo1722 did not affect growth of *L. monocytogenes* at 37°C (Supplementary Figure 2, Table 3). However, the growth of the Δlmo1722 strain was severely impaired compared to the wild-type strain at lower temperatures (Figure 1B, Table 3). For instance, the growth rate was almost 3-fold slower for the Δlmo1722 strain compared with the wild-type at 16°C and the Δlmo1722 strain was unable to grow at 0°C. To rule out possible polar effects, the *lmo1722* gene was introduced into plasmid pMK4P harboring a constitutive promoter (Pprot) (2). When the resulting plasmid plmo1722 was introduced into the Δlmo1722 strain, growth was restored to wild-type levels at 26°C (Table 3). It also allowed the Δlmo1722 strain to grow at temperatures below 10°C (data not shown).
Absence of Lmo1722 decreases *L. monocytogenes* motility by downregulating *flaA* expression. *L. monocytogenes* is only motile at temperatures around and below 30°C (39). When plating the WT + pMK4P, the Δlmo1722 + pMK4P and the Δlmo1722 + plo1722 strains on low-agar motility plates at 26°C, it was observed that the Δlmo1722 + pMK4P showed reduced motility in comparison with the other strains (Figure 2A). To analyze the reason for the motility deficiency, whole cell protein preparations from wild-type and the Δlmo1722 strains grown at 37 and 16°C were separated on 1D SDS-PAGE. This revealed a protein band more prominent in the wild-type compared to the Δlmo1722 strain at 16°C (Figure 2B). By trypsin digestion/mass-spectrometry analysis, the protein was identified as FlaA, the major subunit of *Listeria* flagelin. To test if the regulation of the FlaA expression was exerted on transcriptional or post-transcriptional level, a northern blot was performed with RNA from different strains. As compared to the wild-type, the *flaA* mRNA level was decreased in the Δlmo1722 + pMK4P strain but could be re-established in the Δlmo1722 + plo1722 strain (Figure 2C). These results are compatible with Lmo1722 affecting expression of motility genes at the transcriptional level. The regulation of *Listeria* motility is complex (26, 33, 34). Both the gene encoding the repressor of motility (*mogR*) and the gene encoding the positive regulator of motility (*gmaR*) were downregulated in the Δlmo1722 strain as compared to the wild-type strain (data not shown), indicating that Lmo1722 functions upstream of these regulators. One regulator stimulating motility and regulating *gmaR* expression is DegU. Expression of *degU* was decreased in the Δlmo1722 + pMK4P strain, but could be restored to wild-type levels in the Δlmo1722 + plo1722 strain (Figure 2C). This indicates that Lmo1722 control motility by regulating *degU* expression directly, or by affecting expression of regulators upstream of *degU*.
Absence of Lmo1722 decreases the number of mature 70S ribosomes at low temperatures. Several studies in other bacterial species have shown that DEAD-box RNA-helicases are important for ribosomal maturation (14, 15, 21, 36). To test whether Lmo1722 was important for the formation of mature 70S ribosomes in \textit{L. monocytogenes}, ribosomal fractions of the WT + pMK4P; the \textit{Δ}lmo1722 + pMK4P and the \textit{Δ}lmo1722 + p\textit{lmo1722} strains grown at 16°C, were separated on sucrose gradients. The results showed that the \textit{Δ}lmo1722 + pMK4P strain formed less mature 70S ribosomes and instead contained more free 30S and 50S subunits, whereas the \textit{Δ}lmo1722 + p\textit{lmo1722} strain did only marginally increase the amount of mature 70S ribosomes (data not shown). This surprising result prompted us to measure the Lmo1722 levels in different strains. By western blot, we observed that the level of Lmo1722 protein was reduced in the \textit{Δ}lmo1722 + p\textit{lmo1722} strain as compared to the WT + pMK4P strain (Supplementary Figure 3). Why the complemented strain displayed low levels of Lmo1722 protein is unclear, but it could be due to transcript or plasmid instability.

To analyze complementation phenotypes at wild-type Lmo1722 levels, \textit{lmo1722} alleles were placed under the control of an IPTG-inducible promoter in the chromosomally integrative pIMK3 vector (47). The resulting constructs were streaked on agar-plates without or with 1 mM of IPTG and incubated at 16 or 37°C (Figure 3A, data not shown). Our results suggest that the \textit{Δ}lmo1722 + pIMK3:\textit{lmo1722}WT strain grew as fast as the WT + pIMK3 strain on plates lacking IPTG (Figure 3A) and the generation time was similar between these strain in bacterial cultures lacking IPTG (Table 3). During the cloning-process, we obtained a base-deletion in the promoter region of the construct (pIMK3:mut\textit{lmo1722}WT), decreasing Lmo1722WT levels approximately 2-fold without IPTG-induction compared to wild-type levels (Supplementary Figure 3). However, the \textit{Δ}lmo1722 + pIMK3:mut\textit{lmo1722}WT strain still grew as fast as the WT + pIMK3 strain at 16°C (Figure 3A, Table 3). A construct harbouring
a deletion of 19 base-pairs creating a premature stop-codon within the central core-region was also obtained during cloning. Unlike the other constructs, this strain (Δlmo1722 + pIMK3:mutlmo1722Frameshift) was unable to complement growth at 16°C even in the presence of IPTG, suggesting that the Lmo1722 protein but not the lmo1722 DNA or RNA is sufficient for growth complementation (Figure 3A and data not shown). In line with the above results were strains expressing Lmo1722 (WT + pIMK3; Δlmo1722 + pIMK3:lmo1722WT and Δlmo1722 + pIMK3:mutlmo1722WT) able to grow at 0 and 5°C (Supplementary Figure 4). This was in contrast to the Δlmo1722 + pIMK3 strain which was unable to grow at these lower temperatures. Some of the above strains were examined for their motility on low-agar plates. Not surprisingly, expression of Lmo1722 re-established motility to wild-type levels at 26°C (Figure 3B).

Ribosomal maturation in *L. monocytogenes* does not require the C-terminus of Lmo1722. Since the Δlmo1722 strain harboring the pMK4P:lmo1722 construct did not restore the ribosomal maturation to the wild-type profile, we were interested to investigate whether the pIMK3:lmo1722WT construct could re-establish the ribosomal profile. First, the expression level of Lmo1722 protein in various strains was determined. Our results showed that the expression of Lmo1722 followed the wild-type levels even in the absence of IPTG (Figure 4A, Supplementary Figure 3). We therefore performed the following experiments without IPTG induction. In contrast to the Δlmo1722 + pIMK3 strain, a Δlmo1722 strain harboring pIMK3:lmo1722WT could restore ribosome maturation to a profile observed in the wild-type strain (Figure 4B). A wild-type like ribosomal maturation profile was also observed for the Δlmo1722 + pIMK3:mutlmo1722WT strain, although this strain only express half of wild-type Lmo1722 levels (data not shown). The Lmo1722 orthologue in *Bacillus subtilis*, YfmL, lacks...
a major part of the C-terminal extension (Figure S1). To test if the C-terminus of Lmo1722 was required for growth at low temperatures, motility and ribosomal maturation, a C-terminal deleted form of Lmo1722 (Lmo1722_{ΔCT}) was created. Expression of Lmo1722_{ΔCT} re-established growth, motility as well as ribosome maturation equally well as full-length Lmo1722 (Figure 3 and 4), indicating that the C-terminal part of Lmo1722 is redundant for the above activities.

Absence of Lmo1722 alters 23S rRNA precursor processing A commonly observed phenotype of bacterial strains showing an immature ribosomal profile is the inability to properly process the 23S rRNA precursor transcript. *E. coli* RNA helicases SrmB and CsdA affect the 23S rRNA maturation, as reviewed in (54). To analyze if a Δlmo1722 strain displayed an altered 23S rRNA processing, a primer extension analysis was performed. Our results suggest that Lmo1722 indeed is important for proper 23S rRNA precursor processing, since a strain lacking Lmo1722 showed a large fraction of a non-processed precursor (Figure 4C, Supplementary Figure 5). This immature 23S rRNA harbors a 160 nucleotides long 5´- precursor not present in the mature 23S rRNA. The phenotype was restored in the Δlmo1722 + pIMK3:*lmo1722_{WT} strain (Figure 4C). Interestingly, a C-terminally truncated Lmo1722 could fully restore 23S rRNA processing to a wild-type appearance (Figure 4C).

Lmo1722 is associated with free 50S subunits and 70S ribosomes. To further investigate the role of Lmo1722 during ribosomal maturation, the association of Lmo1722 with the ribosomal subunits was examined. Sucrose gradient fractionated extracts from the Lmo1722 or Lmo1722_{ACT} expressing strains were used for western blotting. The results suggested that Lmo1722 was associated with the 50S subunit but also with mature 70S ribosomes. Almost
no full-length Lmo1722 could be detected in cytoplasmic or at 30S fractions (Figure 5). In contrast, Lmo1722\(_{\text{ΔCT}}\) was observed in all isolated fractions (also cytoplasmic and 30S fractions) indicating that the C-terminal part guides Lmo1722 to the 50S subunit and 70S ribosomes (Figure 5).

Discussion

In this work, we show that the *Listeria monocytogenes* DExD-box RNA helicase Lmo1722, is essential for growth at low temperatures, a condition *L. monocytogenes* encounter in nature but also when present in refrigerated food. In contrast, Lmo1722 is dispensable for growth at 37°C (Figure 1, Table 3). DExD-box RNA helicases in bacteria have mostly been studied in *Escherichia coli* and *Bacillus subtilis* (16, 30). However, neither of these strains are able to grow at extremely low temperatures, like *L. monocytogenes*. Considering that expression of *lmo1722* is induced during cold growth (11), it is not surprising that presence of Lmo1722 would be needed for growth at low temperatures (Figure 1 and 3, Supplementary Figure 4 and Table 3). In support of the above results, we observe an increase in Lmo1722 protein levels at 16°C compared to 37°C (Supplementary Figure 3). Absence of Lmo1722 decreases *L. monocytogenes* motility, due to a downregulated expression of *flaA*, encoding the main subunit of the flagellin, FlaA (Figure 2). The exact mechanism by how Lmo1722 controls flagellin expression remains to be elucidated, but it most probably lies somewhere upstream of the most prominent regulators (MogR, GmaR and DegU).

Absence of Lmo1722 decreases the amount of polysomes, mature 70S ribosomes and 50S subunits but increases the level of 30S subunits. Similar ribosomal profiles have been detected in other bacteria lacking certain RNA-helicases (31) However, we did not observe any ribosomal subunit intermediates (i.e. 40S and 45S) for the Δ*lmo1722* mutant strain, as found
in *E. coli* helicase mutant strains. If this is due to differences in the ribosomal maturation pattern between Gram-positive and Gram-negative strains remains to be investigated. A Δ*lmo1722* mutant strain supplemented with wild-type levels of Lmo1722 re-established the ribosomal profile to wild-type appearance (Figure 4B).

A Lmo1722 protein lacking its C-terminal part was not affected in growth or motility, and the ribosomal profile and ribosomal rRNA processing in that strain overlapped with the profile of a wild-type strain. In this respect the Lmo1722 helicase is comparable to several other well-characterized bacterial helicases. *E. coli* SrmB helicase having its 60 amino acids of lysine-rich C-terminus replaced with an affinity tag or deleted was able to completely restore the ribosomal profile of a Δ*srmB* deletion mutant (58). Yet another *E. coli* helicase CsdA involved in ribosomal maturation could not fully complement the bacterial growth at 15 °C when the C-terminus was truncated (3, 60). Here, the complementation with physiological levels of Lmo1722Δ_CT expression completely restored the analyzed phenotypes. However, in contrast to a wild-type strain where full-length Lmo1722 protein was detected almost exclusively with 50S subunits and mature ribosomes, a C-terminal deleted Lmo1722 could be found also in cytoplasmic and 30S subunit fractions (Figure 4C). This was not due to an overexpression of Lmo1722Δ_CT compared to the wild-type expression of Lmo1722 (Figure 4A).

The results suggest that the function of the C-terminal part of Lmo1722 involves appropriate guidance of Lmo1722 to the 50S subunit of the ribosome. The presence of Lmo1722 at polysomes indicates that it might be important for active translation (Figure 4C). Such a mechanism has been suggested previously for DEAD-box RNA helicases to be most important at the initiation step of translation (16, 29).
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References


Table 1. Bacteria strains

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<td>pMK4P</td>
<td><em>E. coli</em> – <em>L. monocytogenes</em> shuttle vector with constitutive Pprot promoter</td>
<td>(2)</td>
</tr>
<tr>
<td>plmo1722</td>
<td>pMK4P with <em>lmo1722</em> under constitutive promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD18</td>
<td>Arabinose inducible <em>E. coli</em> cloning vector, Cb&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(27)</td>
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<tr>
<td>pKVA429</td>
<td><em>lmo1722</em> with C-terminal 6× His tag in pBAD18</td>
<td>This study</td>
</tr>
<tr>
<td>pIMK3</td>
<td>IPTG inducible <em>Listeria</em> cloning vector, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>P. Casey (47)</td>
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<tr>
<td>pKVA791</td>
<td><em>lmo1722</em> in pIMK3</td>
<td>This study</td>
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<tr>
<td>pKVA746</td>
<td><em>lmo1722</em>, with a C-terminal deletion, clone in pIMK3</td>
<td>This study</td>
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<tr>
<td>pKVA742</td>
<td><em>lmo1722</em> in pIMK3, 1 bp ΔA deletion in the cloned region 5 nucleotides downstream of BamHI recognition site</td>
<td>This study</td>
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<td>pKVA789</td>
<td><em>lmo1722</em> in pIMK3, 19 bp deletion ΔG&lt;sub&gt;634&lt;/sub&gt;-G&lt;sub&gt;652&lt;/sub&gt; of <em>lmo1722</em> gene resulting in a frameshift after Val&lt;sub&gt;211&lt;/sub&gt; of Lmo1722</td>
<td>This study</td>
</tr>
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Table 3. Growth rates (minutes of doubling) at different temperatures of indicated strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>16°C</th>
<th>26°C</th>
<th>37°C</th>
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<tbody>
<tr>
<td>EGDe</td>
<td>134</td>
<td>67</td>
<td>35</td>
</tr>
<tr>
<td>Δlmo1722</td>
<td>345</td>
<td>80</td>
<td>37</td>
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<tr>
<td>EGDe + pMK4P</td>
<td>231</td>
<td>110</td>
<td>56</td>
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<tr>
<td>Δlmo1722 + pMK4P</td>
<td>393</td>
<td>147</td>
<td>61</td>
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<tr>
<td>Δlmo1722 + p/lmo1722</td>
<td>276</td>
<td>112</td>
<td>56</td>
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<tr>
<td>EGDe + pIMK3</td>
<td>176</td>
<td>69</td>
<td>45</td>
</tr>
<tr>
<td>Δlmo1722 + pIMK3</td>
<td>592</td>
<td>98</td>
<td>47</td>
</tr>
<tr>
<td>Δlmo1722 + pIMK3:mutlmo1722_{WT}</td>
<td>177</td>
<td>73</td>
<td>46</td>
</tr>
<tr>
<td>Δlmo1722 + pIMK3:lmo1722_{WT}</td>
<td>160</td>
<td>71</td>
<td>45</td>
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<tr>
<td>Δlmo1722 + pIMK3:lmo1722_{ACT}</td>
<td>167</td>
<td>70</td>
<td>45</td>
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</table>
**Figure legends**

**Figure 1.** The *lmo1722* gene locus. Arrows indicate transcriptional directions and lollipops indicate terminators. The DNA between the vertical hatched lines was deleted in the Δ*lmo1722* strain.

**Figure 2.** Motility and gene expression in different *L. monocytogenes* strains. **A.** Motility on BHI soft agar plates of indicated strains at 26°C. Motility was scored after 48 hours and measured as the diameter of the colonies originating from the center. Error-bars indicate standard-deviation (*** show a statistically significant difference (p<0.001, Student T-test) between the WT + pMK4P and the Δ*lmo1722* + pMK4P strains). **B.** SDS-PAGE of total proteins from *L. monocytogenes* and its isogenic *lmo1722* deletion strain grown at 37 or 16°C. **C.** Northern blot analysis of flagellin *flaA* and transcriptional regulators *degU* and *degV* expression of bacteria grown at 26°C.

**Figure 3.** Analysis of low temperature growth and motility of indicated strains. **A.** Growth of *L. monocytogenes* carrying different *lmo1722* allele constructs on solid BHI-plates lacking IPTG at 16°C or 37°C. **B.** Motility on BHI soft agar plates of indicated strains. Plates are shown after 48 hour incubation at 26°C. Expression of tmRNA is shown as a loading control.

**Figure 4.** Absence of Lmo1722 alters ribosomal RNA processing and ribosomal maturation. **A.** Western blot analysis of Lmo1722 expression levels in indicated strains grown at 16°C.
Arrows indicate Lmo1722 and Lmo1722_{ΔCT}, respectively. The background signal in the Δlmo1722 + pIMK3 strain probably reflects Lmo1450 expression, an RNA helicase 32% identical to Lmo1722 and with a similar calculated molecular mass of 50 kDa. **B.** Ribosomal profile analysis. Samples from indicated strains grown at 16°C, were prepared and separated by sucrose gradient. Gradients were normalized by the addition of equivalent A_{260}-units as described in the Materials and Methods. Numbered arrows indicate samples used in Figure 5. The different ribosomal subunits are indicated above the peaks for the wild-type + pIMK3 profile. **C.** Graph showing relative processing of immature versus mature 23S rRNA transcript in the indicated strains grown at 16°C (see text and Materials and Methods for details).

**Figure 5.** Localization of Lmo1722 in different fractions of ribosomal profiles from bacteria grown at 16°C. Equal volumes of fractions with either highest or lowest A_{260} value (indicated in Figure 4B), were pooled together, proteins concentrated by TCA precipitation and analyzed by Western blotting. Arrows indicate Lmo1722 and Lmo1722_{ΔCT}, respectively.
Figure 2

A. Motility (diameter/mm)

B. 

37°C 16°C

Std WT Δlmo1722 WT Δlmo1722

Δlmo1722

C. 

WT + pMK4P Δlmo1722 + pMK4P Δlmo1722 + pimo1722

flaA

degU-degV

degU

tmRNA
Figure 3

A

1. WT + pIMK3
2. Δlmo1722 + pIMK3
3. Δlmo1722 + pIMK3:mut/lmo1722WT
4. Δlmo1722 + pIMK3:lmo1722WT
5. Δlmo1722 + pIMK3:lmo1722frameshift
6. Δlmo1722 + pIMK3:lmo1722ΔCT

B

1. WT + pIMK3
2. Δlmo1722 + pIMK3
3. Δlmo1722 + pIMK3:lmo1722WT
4. Δlmo1722 + pIMK3:lmo1722ΔCT
Figure 4

A

WT + pIMK3
Δlmo1722 + pIMK3
Δlmo1722 + pIMK3::lmo1722 WT
Δlmo1722 + pIMK3::lmo1722act

Lmo1722
Lmo1722act

B

WT + pIMK3
Δlmo1722 + pIMK3
Δlmo1722 + pIMK3::lmo1722 WT
Δlmo1722 + pIMK3::lmo1722 act

C

Relative abundance (Immature/mature 23S rRNA)

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relative Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG21/pIMK3</td>
<td>0.00</td>
</tr>
<tr>
<td>Δlmo1722/pIMK3</td>
<td>0.00</td>
</tr>
<tr>
<td>Δlmo1722::lmo1722 WT/pIMK3</td>
<td>0.80 ± 0.09</td>
</tr>
<tr>
<td>Δlmo1722::lmo1722 act/pIMK3</td>
<td>0.80 ± 0.09</td>
</tr>
</tbody>
</table>
Figure 5

WT + pMK3 lysate
Chlorplastic
50S
70S
Polyome
1 2 3 4 5 6 7 8 9

Lmo1722
ΔCT

Lmo1722
Lmo1722_{ΔCT}