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DExD-box RNA-helicases in *Listeria monocytogenes* are important for growth, ribosomal maturation, rRNA processing and virulence factor expression

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RNA-helicases are proteins required for the unwinding of occluding secondary RNA structures, especially at low temperatures. In this work, we have deleted all 4 DExD-box RNA helicases in various combinations in the Gram-positive pathogen *Listeria monocytogenes*. Our results show that 3 out of 4 RNA-helicases were important for growth at low temperatures, whereas the effect was less prominent at 37°C. Over-expression of one RNA-helicase, Lmo1450, was able to overcome the reduced growth of the quadruple mutant strain at temperatures above 26°C, but not at lower temperatures. The maturation of ribosomes was affected in different degrees in the various strains at 20°C, whereas the effect was marginal at 37°C. This was accompanied by an increased level of immature 23S rRNA precursors in some of the RNA-helicase mutants at low temperatures. Although the expression of the PrfA regulated virulence factors ActA and LLO decreased in the quadruple mutant strain, this strain showed a slightly increased infection ability. Interestingly, even though the level of the virulence factor LLO was decreased in the quadruple mutant strain as compared with the wild-type strain, the *hly*-transcript (encoding LLO) was increased. Hence, our results could suggest a role for the RNA-helicases during translation. In this work, we show that DExD-box RNA-helicases are involved in bacterial virulence gene-expression and infection of eukaryotic cells.

Introduction

RNA-helicases are proteins involved in several steps of the maturation of RNA-molecules. Most RNA-helicases are either part of the DExH-family of processive RNA-helicases or the DExD-family of non-processive RNA-helicases where DExH or DExD refer to the consensus amino-acid sequence of the ATP-binding catalytic center. In eukaryotes, RNA-helicases have been assigned roles in RNA-splicing and editing, translation and mRNA degradation.[1] Also, RNA-helicases are important for appropriate ribosomal maturation and spliceosomal formation.[1] In bacteria, most attention has been drawn to the DExD-family of RNA-helicases. Generally, this family of RNA-helicases are involved in ribosomal maturation and mRNA decay.[2-4] The function of RNA-helicases of the DExH-family is less clear. In *Escherichia coli*, DExD-box RNA-helicases have been shown to be an important component of RNA degradosomes, protein complexes essential for RNA degradation and maturation.[5,6] RNA-helicases in Gram-positive bacteria are often associated with RNA degradosomes.[3,7,8] Interestingly, *Bacillus subtilis* and *Listeria monocytogenes* both harbor 4 DExD-box RNA-helicase genes whereas *Staphylococcus aureus* only has one (**Fig. 1**). The enzymatic core region of each DExD-box RNA-helicase is relatively conserved, resembling RecA, whereas the C-terminal part varies extensively.[9]

So far, only a few bacterial RNA-helicases have been implicated in virulence. HrpA, a DEAH-box RNA-helicase of *Escherichia coli* has been shown to be required for proper fimbrial processing.[10] More recently, the DEAH-box RNA-helicase HrpA of *Borrelia burgdorferi* was shown to be essential for mouse infectivity and tick transmission.[11,12] To our knowledge, only one DExD-box RNA-helicase, CshA of *Staphylococcus aureus*, has been shown to be involved in virulence factor expression.[13,14] It was shown that absence of CshA increased the stability of *agr* mRNA and hence hemolysis, whereas *S. aureus* biofilm formation was inhibited.

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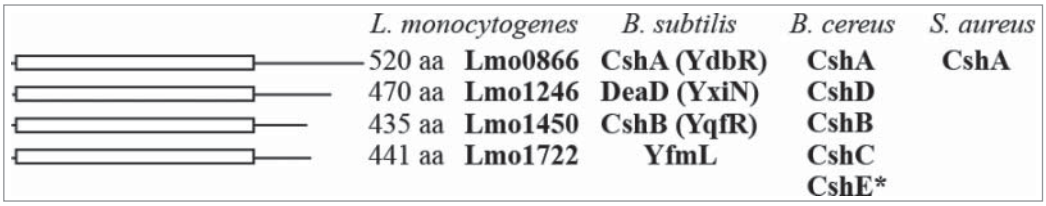

Figure 1. Schematic representation of DExD-box RNA-helicases in some Gram-positive species. The length of *L. monocytogenes* DExD-box RNA-helicases are shown together with the schematix drawing of the core-region (box) and the C-terminal part. *B. subtilis, B. cereus* and *S. aureus* RNA-helicase homologs are shown to the right. *CshE of *B. cereus* is most similar to Lmo0866 (e^{-128}, BLAST homology score).

Listeria monocytogenes is a Gram-positive, intracellular food-borne pathogen, able to cause severe infections in immunocompromised individuals.[15,16] Infection of human cells follow a strict temporal and spatial pattern, requiring the concerted action of several virulence factors.[16] Among these are different adhesins (internalins) required for bacterial attachment to eukaryotic cells; a haemolysin (Listeriolysin O) essential for escape from the phagosome and an actin-polymerization factor (ActA) needed for cell to cell spread.[16] Expression of these virulence factors are controlled by the transcriptional regulator PrfA.[17-19] We have previously shown that one DExD-box RNA-helicase, Lmo1722, is important for ribosome maturation and binds the 50S ribosomal subunit through its C-terminal domain.[20] Also, the *Listeria* RNA-helicases appear to be most important if the bacterium faces different stress-conditions, such as low temperature.[21,22]

In this work, we have characterized the function of all DExD-box RNA-helicases in the Gram-positive pathogen *Listeria monocytogenes* by combining different knock-out mutants. Our results indicate a hierarchy among the RNA-helicases, where Lmo1450 appears most important considering growth, motility and ribosome maturation at low temperatures (from 16 to 26°C). At permissive temperature (37°C), the RNA-helicases are less important for growth, 23S rRNA precursor processing, ribosomal maturation, but are still required for appropriate expression of virulence factors and infectivity. We also observe a putative function of RNA-helicases during translation.

Results

A *L. monocytogenes* strain lacking all DExD-box RNA helicases is viable

During the course of this work, it was shown that 3 out of 4 RNA-helicases in *Listeria monocytogenes* were important for maximal growth and motility.[23] We observed similar phenotypes in our single knockout mutants, with the largest effect observed at low temperatures (data not shown, **Table 1**, **Figs. S1–S3**). The individual knock-out mutants could be complemented by expression *in trans*, eliminating the possibility of polar effects in accordance with previous work ([23] and data not shown). In order to determine the overall function of RNA-helicases, strains lacking 2, 3 or all 4 RNA-helicases were constructed. These strains were examined for their growth abilities at different temperatures in liquid media (**Table 1**, **Figs. S1–S3**). From the results, it was clear that the RNA-helicases Lmo1722 and Lmo1246 play a minute, if any, role during growth at 37°C, since the growth-rate of the Δ*lmo1246*, Δ*lmo1722* double mutant strain was similar to the wild-type, whereas a strain lacking only Lmo1722 showed a reduced growth rate at low temperatures as shown previously (**Table 1**, **Figs. S1–S3** [20]). Absence of either Lmo0866 or Lmo1450 prolonged the generation time at all temperatures, although the reduced growth rates of the various RNA-helicase strains were not followed by an increased bacterial death (**Table 1**, **Figs. S1–S3**). Interestingly, absence of Lmo1450 in any combination (single, double, triple or quadruple mutant) lead to an almost identical growth rate of the bacterial strains at 37°C, indicating that the reduced growth-rate at that temperature was to a large degree due to the lack of Lmo1450 (**Table 1**, **Fig. S3**). When over-expressing the different RNA-helicases in the quadruple mutant at different temperatures, we observed a gradual

Table 1. Generation times (g ± SD) and viability (dilution test on agar plates) of various *L. monocytogenes* RNA helicase mutants at 16°C, 25°C and 37°C respectively

	16°C		25°C		37°C	
	g (min) ± SD	dilution test 10^0 10^{-4} 10^{-5} 10^{-6}	g (min) ± SD	dilution test 10^0 10^{-4} 10^{-5} 10^{-6}	g (min) ± SD	dilution test 10^0 10^{-4} 10^{-5} 10^{-6}
wt	181 ± 46		78 ± 10		46 ± 6	
Δ1722	355 ± 67		86 ± 15		46 ± 6	
Δ1246	184 ± 39		83 ± 8		44 ± 2	
Δ1246 Δ1722	500 ± 104		95 ± 8		44 ± 3	
Δ0866	463 ± 30		96 ± 6		55 ± 6	
Δ0866 Δ1722	688 ± 19		114 ± 10		56 ± 5	
Δ1450	553 ± 121		136 ± 13		68 ± 5	
Δ1450 Δ1722	497 ± 100		143 ± 10		67 ± 2	
Δ1450 Δ1246	553 ± 77		137 ± 5		66 ± 4	
Δ1450 Δ0866 Δ1722	500 ± 121		150 ± 19		68 ± 5	
Δ1450 Δ1246 Δ0866	306 ± 9		143 ± 11		70 ± 6	
Δ1450 Δ1246 Δ0866 Δ1722	585 ± 87		229 ± 5		73 ± 3	

improvement of growth in the following order of importance: Lmo1450 > Lmo0866 > Lmo1722 > Lmo1246 (**Fig. 2**). Although not reflecting a physiological condition, the results indicate that high levels of Lmo1450 can overtake the role of the other RNA-helicases and re-establish growth of the quadruple mutant at temperatures higher than 26°C. At 16°C, overexpression of Lmo1450 was only partially able to overcome the growth impairment of the quadruple mutant strain, indicating that the other RNA-helicases have specific functions at lower temperatures that excess Lmo1450 is unable to suppress.

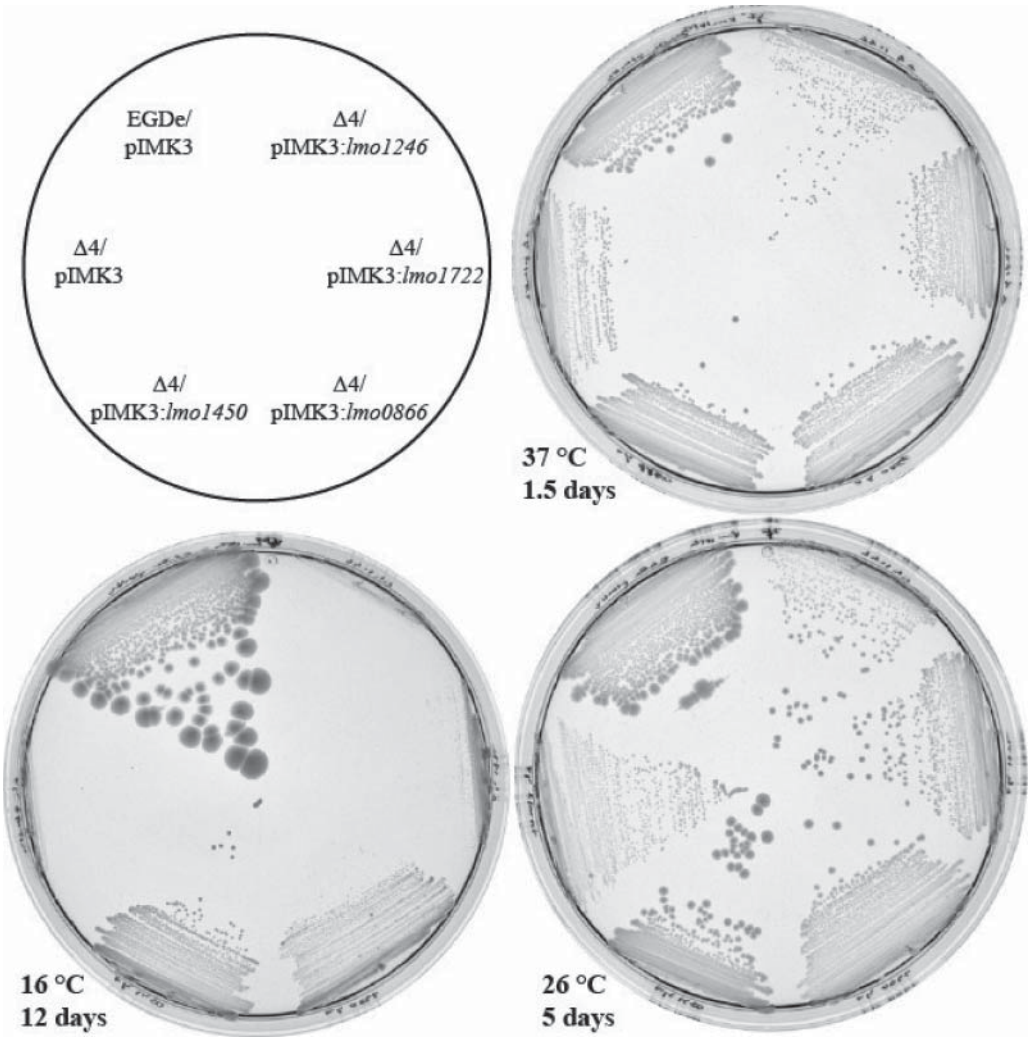

Figure 2. Growth of the quadruple RNA helicases mutant strain complemented with each helicase gene. Wild-type (EGDe) carrying the vector control pIMK3, or quadruple mutant (Δ4) strains carrying the RNA helicase genes of *lmo1246*, *lmo1722*, *lmo0866*, *lmo1450*, or pIMK3, respectively, were grown on BHI agar-plates at indicated temperatures and for designated times in presence of 1 mM IPTG.

Absence of RNA-helicases affect ribosomal maturation

In many cases, absence of RNA-helicases affects ribosomal maturation, especially at low temperatures.[24,25] We have previously observed that a *L. monocytogenes* strain lacking the RNA-helicases Lmo1722 had a reduced fraction of 50S ribosomal subunits.[20] This was in line with a C-terminal dependent association of Lmo1722 with the 50S subunit.[20] Here, we were interested to examine ribosomal maturation in strains lacking different combinations of RNA-helicases. Absence of Lmo1246 did not affect the overall ribosomal maturation at 20°C, whereas absence of Lmo1722, Lmo0866 or Lmo1450 showed a clear effect (**Fig. 3A**). The lack of Lmo1722 or Lmo0866 decreased the amount of 50S subunits and 70S ribosomes. Absence of Lmo1450 on the other hand lead to an increased level of 50S subunits and decreased 70S levels. The quadruple mutant strain essentially followed the ribosomal maturation pattern of the Δ*lmo1450* strain, furthermore emphasizing the important physiological role of Lmo1450. We were unable to detect any differences in the appearance of intermediate ribosomal subunits in the different strains (**Fig. 3A**). This was in contrast to *E. coli* strains lacking the RNA-helicases SrmB and CsdA which displayed an increased level of 40S subunits.[26,27] The absence of RNA-helicases gave a much less pronounced effect in ribosomal maturation at 37°C, although a similar trend compared with 20°C was observed (**Fig. 3B**). Importantly, the amount of mature 70S subunits was similar in all strains at 37°C, suggesting that initial loading of 70S ribosomes on mRNA was not affected. It is therefore of interest to note that the amount of polysomes (transcript harboring more than one translating ribosome) was almost absent in the Δ*lmo0866* strain, the Δ*lmo1450* strain and the quadruple mutant strain as compared with the wild-type strain at 37°C (**Fig. 3B**).

The DExD-box RNA-helicases are important for 23S rRNA precursor processing

In *L. monocytogenes*, the immature 23S rRNA harbor a 160 nucleotide long 5′precursor. Previously, it was shown that 23S rRNA processing required Lmo1722.[20] Surprisingly, direct

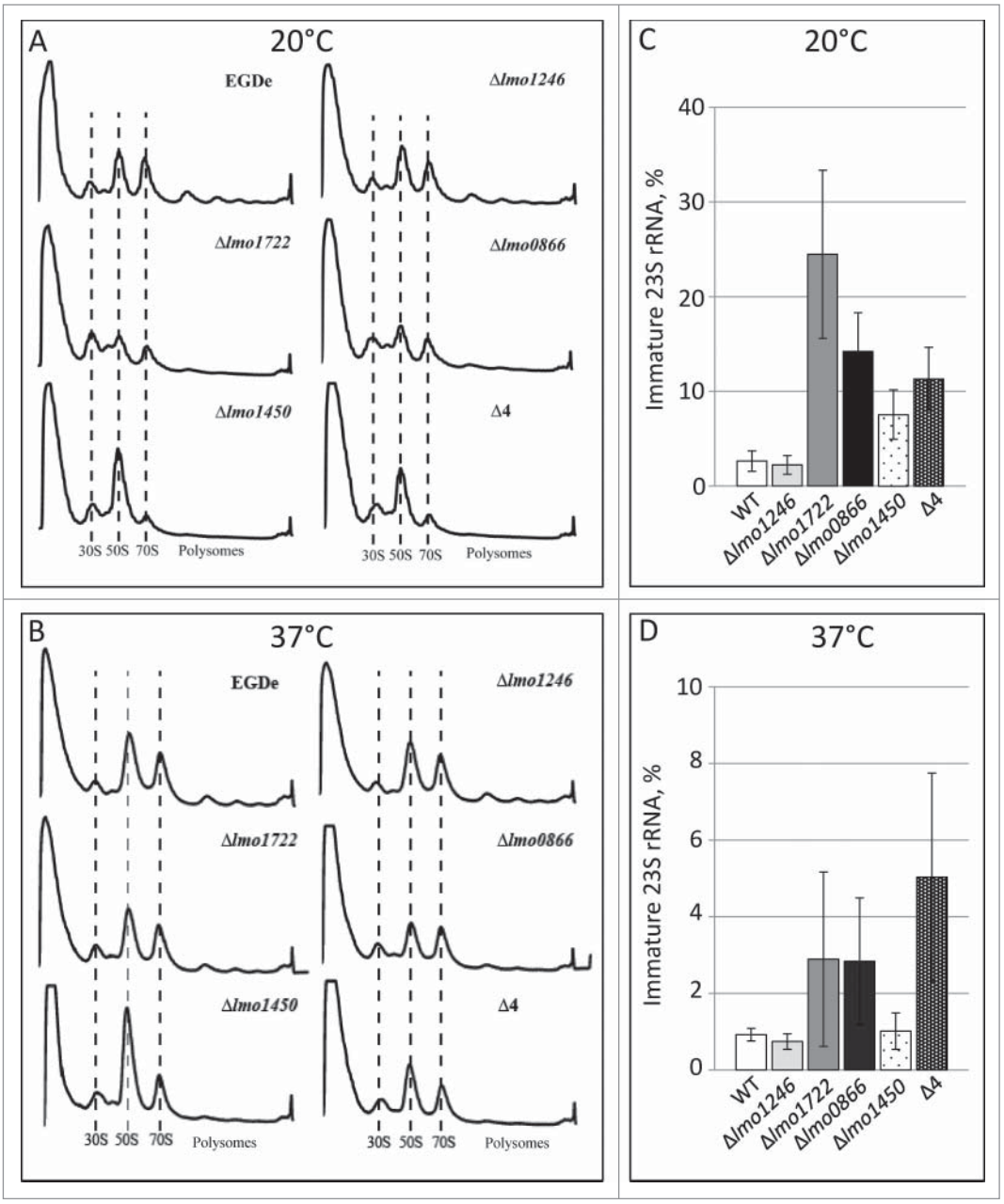

Figure 3. Ribosomal maturation and 23S rRNA processing at different temperatures. Ribosomal profiles of indicated strains were generated by the measurement of light absorbance at A_{254} of lysates fractionated in sucrose gradients at 20°C (**A**) or at 37°C (**B**). 30S, 50S, 70S and polysome profiles are shown below. Indicated strains were grown at 20°C (**C**) or 37°C (**D**) before RNA was isolated and the amount of non-processed compared with processed 23S rRNA precursor transcript (%) was determined.

contact between the 50S subunit and Lmo1722 through its C-terminal part was redundant for 23S rRNA processing activity. We were interested to examine if impaired ribosomal maturation observed in the different RNA-helicase mutants was accompanied by a deficiency of 23S rRNA precursor processing. Through a primer extension analysis, it was observed that the strains displaying a reduced ribosomal maturation also showed an increased level of immature 23S rRNA precursor. At 20°C, the largest effect was observed in the Δ*lmo1722* mutant strain (~25% immature 23S rRNA), although all other mutants, except the Δ*lmo1246* strain, also showed a reduced ability to process the 23S rRNA precursor (**Fig. 3C**). At 37°C, a similar pattern to 20°C was observed, while at this temperature, the quadruple mutant strain exhibited the most prominent increase of immature 23S rRNA precursors (**Fig. 3D**).

RNA-helicases affect virulence gene expression

Recently, it was shown that the DExD-box RNA helicase CshA was involved in *S. aureus* hemolysis and biofilm formation through modulation of the *agr*-mRNA stability.[13,14] We were therefore interested to examine the putative role of the Listerial DExD/box RNA-helicases in virulence and chose to investigate the levels of 2 major virulence factors, LLO and ActA. Expression of the actin-polymerization responsible factor ActA was decreased in all mutant strains, except the Δ*lmo1246* strain, compared with the wild-type strain (**Fig. 4A**). Also, the level of the phagosome lysing haemolysin LLO was measured. Absence of Lmo1722 and Lmo0866 decreased LLO expression by approximately 50% when relating to wild-type levels (**Fig. 4A**). Essentially no difference in LLO expression could be observed in the Δ*lmo1450* strain compared with the wild-type. This suggests that the reduced LLO-levels in the other RNA-helicase mutants were not caused by a decreased growth rate since the Δ*lmo1450* strain displayed the largest growth impairment at 37°C (**Table 1**, **Figs. S1-S3**). The LLO level was slightly reduced in the quadruple mutant strain compared with the wild-type strain. Surprisingly, the *hly* mRNA levels (encoding LLO) were increased in the quadruple mutant strain (**Fig. 4B**).

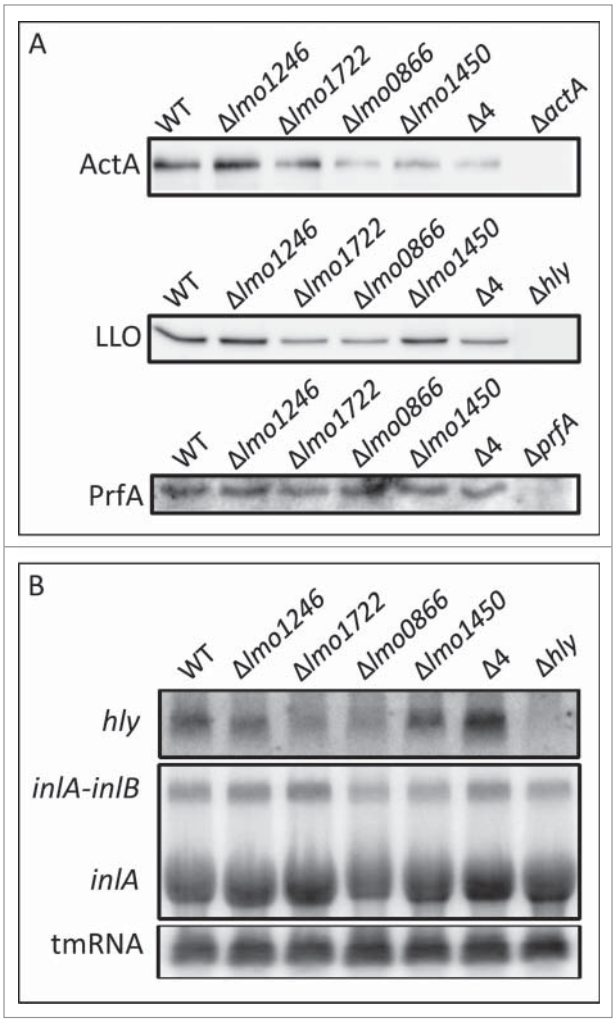

Figure 4. Expression levels of virulence-related genes in RNA helicase mutant strains. (**A**) Indicated strains were grown and levels of specified proteins were analyzed using Western blotting: ActA was extracted by boiling bacterial cells in 1×Laemmli sample loading buffer, LLO was analyzed in culture supernatants after trichloracetic acid precipitation, whereas PrfA was detected in whole bacterial lysates. (**B**) Indicated strains were grown and total RNA was isolated before separation on an agarose gel and RNA gel blot. Radioactively labeled DNA-probes specifically recognizing *hly, inlAB* and tmRNA transcripts were used for detection.

Thus, the increased *hly* transcript levels and decreased LLO-levels in the quadruple mutant strain could indicate a requirement for RNA-helicases during LLO translation. Since CshA in *S. aureus* was shown to destabilize the *agr*-transcript,[13,14] we were interested to examine if the *hly* transcript stability was affected in the quadruple mutant strain. No detectable differences in *hly*-transcript stability between the wild-type and the quadruple mutant strain could however be observed, suggesting that the increased level of *hly*-transcript observed in a quadruple mutant strain was due to elevated transcription and not higher stability (**Fig. 5**). Almost all virulence factors in *L. monocytogenes* are controlled by one single regulator, PrfA, a transcriptional activator of the Crp/Fnr family.[17,18,28] Since the levels of the virulence factors LLO and ActA were reduced in the quadruple mutant strain, it could be hypothesized that the PrfA-levels were affected. However, the *prfA* transcript amount and stability did not differ between the wild-type and the quadruple mutant strain nor did the PrfA protein levels (**Figs. 4A and 5**).

RNA-helicases are involved in bacterial uptake into eukaryotic cells

Do the altered levels of LLO and ActA observed in the different RNA-helicase mutant strains affect the infection capability? To address this, a cell-infection experiment was performed where the different strains were allowed to infect cultured Caco2-cells. To avoid indirect intracellular growth-related effects between the different RNA-helicase mutants, we only examined the uptake of the bacteria (early time-point – 1 hour post-infection) and not intracellular replication. Surprisingly, the Δ*lmo1450* and the quadruple mutant strains showed a significantly increased uptake compared with the wild-type strain, whereas absence of Lmo0866 and Lmo1722 did not show any differences (**Fig. S4**). Unexpectedly, absence of Lmo1246 displayed a significantly reduced uptake capacity as compared with the wild-type (**Fig. S4**). It could be hypothesized that the increased uptake of the Δ*lmo1450* strain and the quadruple mutant strain could be due to an increased activity of the stress-sigma factor σ^B which in turn activates expression of the *L. monocytogenes* cell-infection dependent adhesins InlA and InlB.[29] However, no difference in *inlAB* transcript levels could be observed between wild-type and the RNA-helicase mutant strains (**Fig. 4B**).

Discussion

RNA-helicases are proteins essential for the function of various RNA-species. In this work, we highlight roles for *L. monocytogenes* DExD-box RNA-helicases during growth, ribosomal maturation and virulence. Previous work has shown individual roles of *L. monocytogenes* RNA-helicases during growth and at different stress-responses.[20,21,23] Here, we show that a *L. monocytogenes* strain lacking all RNA-helicases is still viable at all temperatures tested and only show approximately a 50% longer generation time compared with the wild-type at 37°C (**Table 1**, **Figs. S1-S3**). Over-expression of Lmo1450, but none of the other RNA-helicases, was shown to be sufficient for restored growth of the quadruple mutant strain at temperatures above 26°C (**Fig. 2**). However, higher amounts of Lmo1450 could not re-establish growth of the quadruple mutant strain at 16°C, indicating specific roles of the other RNA-helicases at low temperatures. RNA-helicases in other Gram-positive bacteria have also been shown to be important for growth at and/or adaptation to low temperatures [13,30-34] but also at other stress-conditions.[31] As in *Listeria*, absence of all RNA-helicases in *B. subtilis* (Δ4 quadruple mutant strain), drastically prolonged the generation time at low (16°C) temperatures.[33] Another Gram-positive psychrotolerant foodborne pathogen, closely related to *L. monocytogenes* is *Bacillus cereus*. *B. cereus* harbors 5 DExD-box RNA-helicases of

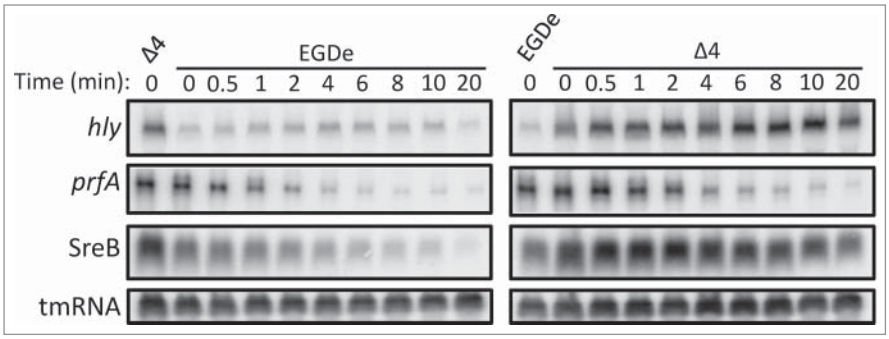

Figure 5. Transcript stability of selected genes in the WT and quadruple RNA helicase mutant *L. monocytogenes* strains. The stability of *hly*, *prfA,* SreB and tmRNA transcripts, respectively, was analyzed by Northern blotting of RNA isolated from wild-type (EGDe) or quadruple mutant (Δ4) strains harvested at indicated time points (in minutes) after rifampicin addition. Radioactively labeled DNA-probes specifically recognizing *hly, prfA,* SreB and tmRNA transcripts were used for detection.

which 3 (CshA, CshB and CshC, corresponding to Lmo0866, Lmo1450 and Lmo1722, respectively) have been shown to be important for stress-adaptation and growth at low temperatures (**Fig. 1**, [30-32]). CshD and CshE of *B. cereus* (corresponding to Lmo1246 and the core region of Lmo0866, respectively), appear to be redundant at these stress-conditions.[30,31] Whether or not the RNA-helicases of *B. cereus* contribute to virulence gene expression and pathogenesis remain to be analyzed.

The functional differences of the RNA-helicases were reflected by their involvement in the maturation of ribosomal subunits, especially evident at low temperatures (**Fig. 3**). This is a common trait in bacterial strains lacking RNA-helicases.[25] As also observed in *B. subtilis* RNA-helicase mutants, we were unable to detect any increase in intermediate subunits (between 30S and 50S) when analyzing the ribosomal maturation of the quadruple mutant.[33] Such intermediary ribosomal subunits (40S) have been identified in *E. coli* RNA-helicase mutants.[2] This could suggest differences in the assembly of ribosomes in Gram-positive and Gram-negative bacteria as has been proposed earlier.[33] A ribosomal maturation difference was also observed at 37°C, where absence of Lmo0866 or Lmo1450 had opposite effects on 50S subunit levels (**Fig. 3**). We observe a decreased processing of the 23S rRNA precursor, particularly in a strain lacking Lmo1722, in agreement with previous findings.[20] In *E. coli*, absence of SrmB and CsdA (DeaD) has been shown to affect processing of the 23S rRNA precursor.[26,27] However, the mechanism by which the RNA-helicases function in 23S rRNA processing might be different since in *E. coli* the 23S rRNA precursor is only 15 nucleotides longer than the mature form, compared with 160 nucleotides longer precursor in *Listeria*.

The Δ*lmo0866*, the Δ*lmo1450* and the quadruple mutant strains all showed reduced levels of polysomes in their ribosomal fractions at both 20 and 37°C (**Fig. 3**). This has previously been shown in a strain lacking Lmo1722 at 16°C.[20] Absence of the *E. coli* RNA-helicases SrmB and CsdA (DeaD) also decrease the number of polysomes, but only at low temperatures (30 and 20°C, respectively).[26,27] The reduced level of polysomes in *Listeria* could possibly explain to the reduced translation of *hly* mRNA that we observed at 37°C (i.e. the level of *hly* mRNA was higher, but the amount of LLO protein was reduced in the quadruple mutant as compared with the wild-type - **Fig. 4**). Whether this reflects a decreased translation capacity, less functional 70S ribosomes or increased mRNA turnover in strains lacking DExD-box RNA-helicases remains to be investigated. Talking against the latter 2 alternatives were the following findings: *1*. The level of 70S monosomes (single ribosomes loaded on the transcript) was almost identical in the wild-type and the quadruple mutant strain (**Fig. 3B**). *2*. The high mRNA/low protein expression pattern observed for *hly*/LLO in the quadruple mutant was not general for all gene-products (as it should be if the 70S ribosomes in this strain would be less functional), since the ratio of PrfA protein: *prfA* transcript was similar between the wild-type and the quadruple mutant strain (**Figs. 4 and 5**). *3*. The *hly* mRNA stability was similar in the wild-type and quadruple mutant strains (**Fig. 5**). Recently, a role for the *E. coli* RNA-helicase CsdA (DeaD) was observed during translation.[35] In that study, the author could show that the RNA-helicase stimulated translation of UvrY, most likely by making the *uvrY*-transcript accessible for ribosomes.[35] It remains to be investigated whether the listerial RNA-helicases also make target mRNAs more available for ribosomes or if they act by other mechanism(s). It has been suggested that CsdA (DeaD) stimulate *rpoS* translation at low temperatures in a mechanism involving the RNA-chaperone Hfq and the small RNA DsrA.[36] In this paper, we also observe a connection between RNA-helicases and a small regulatory RNA: the *trans*-acting riboswitch SreB, which inhibits *prfA*-translation by binding to its 5′-UTR, shows a higher level in the quadruple mutant strain, most probably by an increased stability in that as compared with the wild-type strain (**Fig. 5**.[37])

Recently, CshA, a DExD-box family RNA-helicase was shown to negatively regulate hemolytic activity and positively control biofilm formation in *Staphylococcus aureus*.[13,14] To our knowledge, no bacterial DExD-box RNA-helicase has previously been shown to be important during bacterial infection of eukaryotic cells. In this work, we show that several Listerial RNA-helicases are required for maximal expression of the virulence factors LLO and ActA, whereas expression of the virulence regulator PrfA was essentially unaffected (**Fig. 4**). This would suggest that the RNA-helicases act at the level of PrfA post-translational activation, possibly by affecting the nature of the yet unknown co-factor that is supposed to increase PrfA activity.[18,19] Surprisingly, the reduction in virulence factor expression in these strains was not accompanied by decreased uptake into eukaryotic cells (**Fig. S4**). Instead, the uptake of these mutant strains into cultured cells became higher as compared with the wild-type strain. The reason for this apparent contradiction is unknown, but it was not due to

Table 2. Bacterial strains used in this study.

Strain	Relevant genotype/phenotype	Reference
Escherichia coli DH5α	Cloning host	[51]
Escherichia coli S17-1	*E. coli* strain used for conjugative plasmid transfer to *L. monocytogenes*	[52]
Listeria monocytogenes EGDe	Wild-type *Listeria monocytogenes*	[53]
Δ*hly*	EGDe deleted of *hly*	[32]
Δ*actA*	EGDe deleted of *actA*	[32]
Δ*prfA*	EGDe deleted of prfA	[22]
Δ*lmo1722*	EGDe deleted of *lmo1722*	[20]
Δ*lmo1246*	EGDe deleted of *lmo1246*	This study
Δ*lmo0866*	EGDe deleted of *lmo0866*	This study
Δ*lmo1450*	EGDe deleted of *lmo1450*	This study
Δ*lmo1246* Δ*lmo1722*	EGDe deleted of *lmo1246* and *lmo1722*	This study
Δ*lmo0866* Δ*lmo1722*	EGDe deleted of *lmo0866* and *lmo1722*	This study
Δ*lmo1450* Δ*lmo1722*	EGDe deleted of *lmo1450* and *lmo1722*	This study
Δ*lmo1450* Δ*lmo1246*	EGDe deleted of *lmo1450* and *lmo1246*	This study
Δ*lmo1450* Δ*lmo0866* Δ*lmo1722*	EGDe deleted of *lmo1450*, *lmo0866* and *lmo1722*	This study
Δ*lmo1450* Δ*lmo1246* Δ*lmo0866*	EGDe deleted of *lmo1450*, *lmo1246* and *lmo0866*	This study
Δ4, quadruple mutant	EGDe deleted of *lmo1722*, *lmo1246*, *lmo0866* and *lmo1450*	This study
Δ4/pIMK3:*lmo0866*	Quadruple mutant strain overexpressing *lmo0866*	This study
Δ4/pIMK3:*lmo1246*	Quadruple mutant strain overexpressing *lmo1246*	This study
Δ4/pIMK3:*lmo1450*	Quadruple mutant strain overexpressing *lmo1450*	This study
Δ4/pIMK3:*lmo1722*	Quadruple mutant strain overexpressing *lmo1722*	This study
Δ4/pIMK3	Quadruple mutant strain carrying pIMK3 vector control	This study
EGDe/pIMK3	Wild-type strain carrying pIMK3 vector control	This study

an increased level of the mRNA encoding the InlA and InlB adhesins (**Fig. 4B**).

Materials and Methods

Strains and plasmid construction

E. coli and *L. monocytogenes* strains are listed in **Table 2**. *E. coli* were grown in LB and *L. monocytogenes* in BHI at indicated temperatures, 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. Cloning was performed using standard techniques.[38]

Flanking regions of *lmo1246*, *lmo0866* and *lmo1450* were amplified with primers listed in Supplementary **Table S1**. The PCR products were cloned in tandem into the pMAD vector, creating plasmids pMAD1246, pMAD0866 and pMAD1450 respectively (Supplementary **Table S2**). The constructs were sequenced to ensure wild-type sequences of clones. Gene deletions were then performed as described previously.[39] Multiple deletion mutants were constructed in a sequential manner. Complementation of helicases deletions was done by cloning of the helicase genes (using lmo0866 F and R; lmo1246 F and R; lmo1450 F and R oligonucleotides, respectively) into an IPTG-inducible pIMK3 vector,[40] creating pIMK3:*lmo0866*; pIMK3:*lmo1246* and pIMK3:*lmo1450*, respectively. The pIMK3:*lmo1722* was created previously.[20] The resulting constructs were transferred to *L. monocytogenes* by conjugation with *E. coli* S17-1 strain carrying these plasmids.[41] 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 [42] 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.[43]

Northern blotting

For northern blotting, 20 μg of total RNA was separated on a formaldehyde agarose gel prior to blotting as described.[43] The Hybond-N membrane (GE Healthcare) was subsequently hybridized with ^{32}P α-dATP-labeled DNA fragments amplified with corresponding primers using Prime-a-Gene DNA labeling system (Promega). Northern blots were developed, and band intensities were measured in the STORM machine (Molecular Dynamics). PCR primer pairs used to generate DNA probes for detection of *hly*, *prfA*, *inlA* and tmRNA are listed in Supplementary **Table S1**.

Polysome profile

The polysome profiling was essentially performed as previously described,[44] with a modification of the lysis method. *Listeria monocytogenes* strains were grown in BHI shaking culture at 37 or 20°C 160 rpm to an OD_{600} of 0.5. Bacterial growth was stopped with 100 μg/ml chloramphenicol, cells were harvested by centrifugation of 500 ml culture at 10000 ×g at 4°C, washed

with half culture volume ice-cold solution RW (10 mM Tris-Cl pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 mM PMSF, 100 μg/ml chloramphenicol) and frozen in liquid nitrogen. Bacterial cells were disrupted using a Freezer/Mill 6870 (Spex Sampleprep, Stanmore, UK). The grindates were suspended in solution RL (10 mM Tris-Cl pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 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 8 units of $A_{260,}$ 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 $MgCl_2$). Samples were centrifuged for 3.5 h in SW41Ti rotor (Beckman) at 35000 rpm, 4°C. The ribosomal profiles were generated by UV absorbance A_{254} measurements of the gradients using an ISCO sucrose gradient fractionator equipped with an UA-6 absorbance detector (Teledyne ISCO, Lincoln, Nebraska, USA).

Primer extension

A method of primer extension using a fluorescently labeled primer was described previously.[45] 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).[20] 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.

SDS-PAGE, Western blotting

Listeria total protein samples for electrophoresis were prepared by mutanolysin lysis,[20,46] and analyzed by SDS-PAGE [47] and/or Western blotting on PVDF membrane.[48] Different cultures were grown in BHI to an OD_{600}=1. Culture supernatant proteins were concentrated by 6 % trichloracetic acid and sodium deoxycholate precipitation,[49] ActA was extracted by boiling the bacteria in 1× Laemmli sample buffer [47] for 10 minutes. Protein samples were separated by a 10% 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 followed the protocol of the ECL Prime Western blotting kit (GE Healthcare) using primary antibodies against PrfA, Listeriolysin O or ActA, and HRP-conjugated anti-rabbit secondary antibodies (Bio-Rad). Measurement of luminescence signal was carried out in LAS4000 machine (Fuji).

Cell culture infection assay

Internalization efficiency of *L. monocytogenes* strains was tested using a Caco-2 gentamicin protection assay.[50] Caco-2 cells were grown in DMEM medium supplemented with 10 % fetal calf serum and non-essential amino acids at 37°C in presence of 5 % CO_2. Caco-2 were seeded 3×10^4 cells/well on BD BioCoat collagen coated 24-well plates (Corning, Tewksbury, MA, USA). Cells were grown for 24 hours until semi-confluency. Bacterial strains were harvested at OD_{600}=0.5, washed in room temperature with PBS and suspended in complete cell culture medium immediately before the infection. Bacterial strains were added 1.5 × cfu/well and allowed to penetrate for 1 h. After washing twice with warm DMEM medium, Caco-2 cells were incubated for another 1 h with 150 μg/ml gentamicin to kill extracellular bacteria and washed again. Internalized bacteria were released by lysing cells for 30 minutes with ice-cold water. PBS was added to the wells from a 10× stock solution and lysates transferred to Eppendorf tubes. Each tube was vortexed before plating appropriate volumes of bacterial suspension to determine the colony count on tryptic soy agar (TSA) plates.

Viable count dilution test

Single colonies of the EGDe and each mutant strain were inoculated into 10 ml of BHI overnight at 37°C. Cultures grown overnight were diluted to a start OD of 0.02 in fresh BHI. The strains were grown in water bath at 37°C, 25°C and 16°C until an OD600 of 0.5 when the viable cell numbers of the different strains was examined by drop test with indicated dilutions from original culture.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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