An unstructured 5′-coding region of the prfA mRNA is required for efficient translation

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ABSTRACT

Expression of virulence factors in the human bacterial pathogen Listeria monocytogenes is almost exclusively regulated by the transcriptional activator PrfA. The translation of prfA is controlled by a thermostensor located in the 5′-untranslated RNA (UTR), and is high at 37°C and low at temperatures <30°C. In order to develop a thermoregulated translational expression system, the 5′-UTR and different lengths of the prfA-coding sequences were placed in front of lacZ. When expressed in Escherichia coli, the β-galactosidase expression was directly correlated to the length of the prfA-coding mRNA lying in front of lacZ. A similar effect was detected with gfp as a reporter gene in both L. monocytogenes and E. coli, emphasizing the requirement of the prfA-coding RNA for maximal expression. In vitro transcription/translation and mutational analysis suggests a role for the first 20 codons of the native prfA-mRNA for maximal expression. By toe-print and RNA-probing analysis, a flexible hairpin-loop located immediately downstream of the start-codon was shown to be important for ribosomal binding. The present work determines the importance of an unstructured part of the 5′-coding region of the prfA-mRNA for efficient translation.

INTRODUCTION

The human pathogen Listeria monocytogenes causes perinatal infections, meningo-encephalitis, meningitis, septicemia and gastroenteritis. Listeria monocytogenes has turned out to be a very important model for the study of host–pathogen interactions and bacterial adaptation to mammalian hosts (1,2). Analysis of L. monocytogenes infections have provided considerable knowledge into how bacteria invade cells, escape the phagosome, move intracellularly and disseminate into deeper tissues. A majority of the proteins involved in the different infection steps are encoded on a 9-kb pathogenicity island, and the expression of these factors is dependent on the transcriptional activator PrfA. Expression of the virulence genes is maximal at 37°C, whereas it is very low at 30°C (3). At low temperatures, the 5′-UTR of prfA adopts a structure obstructing the binding of the ribosome to the ribosome-binding site. An increase in temperature induces a conformational change in the RNA structure, allowing binding of the ribosome and initiation of translation. Also, a riboswitch whose transcription terminates when binding S-adenosylmethionine, was recently identified as a regulator of PrfA expression, acting by an RNA:RNA antisense mechanism (4). The terminated riboswitch (SreA) binds to the 5′-end of the prfA thermostensor and represses prfA translation at least in part by destabilizing the prfA transcript. In contrast to the repressive effect of the thermostensor, the 5′-UTRs lying in front of inlA, hly and actA are each required for maximal expression of their gene-products (5–7). These proteins are essential for adhesion to cell, lysis of phagosome and actin-based motility, respectively. It has been speculated that the 5′-UTR in these cases are important to stabilize the transcript (7). However, no mechanism has yet been shown to explain the function of these 5′-UTRs.

In this article, we examined the role of the prfA-coding region for expression. We show that the first 20 codons of the prfA mRNA is required to be maintained in a flexible
manner, to allow ribosome binding and translation initiation.

MATERIALS AND METHODS

Oligonucleotides, strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Tables 1 and 2. The oligonucleotides used in this study are listed in Table 3. *Listeria monocytogenes* strains were grown in BHI broth (Fluka), and *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB-agar. For RNA isolation, *L. monocytogenes* and *E. coli* overnight cultures were diluted 100-fold and grown to the indicated optical density (0.4) in the presence of antibiotics at the following concentrations: carbenicillin, 100 μg ml⁻¹; chloramphenicol, 7 μg ml⁻¹; and kanamycin, 50 μg ml⁻¹. All strains were grown at 37°C or 30°C with aeration.

Plasmid constructions

DNA fragments covering the prfA operon region and different length of PrfA coding sequence (1aa, 4aa, 9aa, 20aa) were PCR amplified using the Pfu enzyme and plasmid plis35 as template (PCR primers are listed in Table 3). The DNA fragments were digested with ScaI and cloned into ScaI digested pJEM12 (9) to generate plasmid pBSN73, pBSN74, pBSN75, pBSN76. To construct PrfA-GFP fusions, pBSN73 and pBSN76 were digested with ScaI and KpnI and inserted into HindIII (blunt-end treated) and KpnI cut pEFGP-N2 (Clontech) to generate plasmid pBSN77 and pBSN78. A PrfA-GFP fragment was excised from pBSN77 and pBSN78 using *XhoI/NotI*, filled in and inserted into *SmaI* digested pMK4 to generate plasmid pBSN79 and pBSN80, respectively. Construction of pBSN83 (prfA20mut-lacZ): Primer pairs pJEM15-1 and prfA20mutD as well as LacZ-D and prfA20mut-U were used in a PCR-reaction with pBSN76 as template, creating fragments A and B. These fragments were purified and used together in a new PCR-mix together with primers pJEM15-1 and LacZ-D, creating fragment C. This fragment was purified, inserted into pJET1.2 and sequenced. The resulting construct was digested with *BamHI* and ScaI and the short fragment was purified and inserted into ScaI/BamHI digested pBSN76 (lacking the short fragment). All constructs were sequenced for accuracy.

RNA isolation

Total cellular RNA was isolated from *L. monocytogenes* and *E. coli* by dissolving pelleted culture (20 ml, A₆₀₀ = 0.4) in resuspension solution (10% glucose, 12.5 mM Tris [pH 7.6] and 5 mM EDTA) and fresh EDTA (0.5 M). Samples were immediately transferred to bead beater tubes with roughly 0.4 g glass beads and 500 μl of acid phenol (pH 4.5). The bacteria were disrupted using a mini bead beater (Biospec products) for 75 s. After centrifugation (5 min, 20 800g), RNA was recovered by addition of 1 ml of Trizol and 100 μl of chloroform/isoamylalcohol (24:1) followed by centrifugation. Samples were there-after subjected to two additional chloroform/IAA extractions. The aqueous phase was precipitated by adding isopropanol (0.7×) and incubated at −20°C for 20 min. For collection of the pellet, the RNA samples were centrifuged for 25 min. The pellet was dissolves in 200 μl of RNase-free water.

For removal of the remaining DNA, samples were treated with 20 U of DNasel (Ambion) for 45 min at 37°C. The reaction was terminated by addition of phenol/chloroform/IAA (25:24:1 [pH 6.6]). Centrifuged samples were chloroform/IAA extracted and ethanol precipitated. The pellet was resuspended in 200 μl RNase-free water, RNA concentration was measured on a Nanodrop (Nanodrop ND-1000 Spectrophotometer), and the RNA integrity was determined on a 1.2%
agarose gel. Only RNA samples showing distinct non-processed precursors to ribosomal RNA were used in the following experiments.

**Northern blot**

For northern blotting, 20μg of total RNA was separated on a formaldehyde agarose gel prior to blotting as described (12). The Hybond-N membrane was subsequently hybridized with 32P-ATP α-labelled DNA fragments amplified with corresponding primers. Northern blots were developed using a STORM machine (Molecular Dynamics). Primers used are listed in Table 3. To amplify a DNA fragment for detection of prfA, hns and tmRNA, we used GFP-U and GFP-D, hns-RT1 and hns-RT2 and tmRNA-U and tmRNA-D, respectively.

**RNA stability**

Indicated *E. coli* strains were grown at 37°C in a shaking water bath, until A600 = 0.4. Initiation of transcription was stopped by the addition of rifampicin to 250μg ml⁻¹, and samples were collected at indicated time points for RNA isolation.

**SDS-PAGE and western blotting**

The different cultures were grown in BH1 (*L. monocytogenes*) or LB medium (*E. coli*) to an optical density of OD₆₀₀ = 0.4. Bacteria were centrifuged and resuspended in buffer A (200mM KCl, 50mM Tris–HCl [pH 8.0], 1mM EDTA and 10% glycerol). The suspension was disrupted using a bead-beater for 1.5 min at maximum speed. After 2 min on ice, the suspension was centrifuged at 15,000 rpm for 5 min, and the supernatant (cytoplasmic fraction) was removed. Protein samples were separated on a 12% polyacrylamide gel electrophoresis before being transferred onto a PVDF membrane using a semidy membrane blotting apparatus. Development of the membrane essentially followed the protocol of the ECL+ western blotting kit (Amersham), using anti-β-galactosidase (GenWay), anti-β-lactamase (GenWay), anti-GFP (BD-living colours), or anti-GroEL (4) as primary antibodies and HRP-conjugated anti-rabbit or anti-mouse as secondary antibodies (Bio-Rad), respectively. Measurement of protein expression was carried out using a STORM machine (Molecular Dynamics).

**In vivo protein stability experiment**

To determine the intracellular stability of PrfA-LacZ, we used a technique described by (13). Protein stability was monitored after the protein synthesis had been inhibited by the addition of spectinomycin (100μg ml⁻¹) to bacterial cultures grown to OD₆₀₀ = 0.4 in LB medium supplemented with kanamycin, 50μg ml⁻¹ at 37°C. Samples to be analysed by western blotting were removed at indicated times.

**In vitro transcription/translation**

One microgram of pT7prfA₁-gfp and pT7prfA₂₀-gfp plasmids (T7 driven prfA₁-gfp and prfA₂₀-gfp, amplified using primers prfA-pT7 and GFP-D and GFP-D were inserted into pGEM-T) were in vitro transcribed in an S30 T7 high yield in vitro Transcription/Translation Kit (Promega) according to the manufacturer’s instructions. In brief, the mixtures were incubated at 25°C for 5 min before transfer to 37°C for an additional 5 min. Samples were acetone-precipitated, re-suspended in sample buffer, and separated on a 12% polyacrylamide gel before being transferred onto a PVDF membrane using a wet blotting apparatus (Biorad). Development of the membrane essentially followed the protocol of the ECL+ western blotting kit (Amersham), using anti-GFP (BD-living colours) and anti-β-lactamase (GenWay) as primary antibodies and HRP-conjugated anti-mouse and anti-rabbit as secondary antibodies (Bio-Rad).
Fluorescent imaging on agar plate

Bacterial strains were streaked onto a LB-plate containing carbenicillin (100 μg/ml) and were grown overnight. Fluorescence imaging was performed with an IVIS Spectrum imaging system (Xenogen). A GFP filter (excitation wavelength 445–490 nm and emission 515–575 nm) was used for acquiring fluorescence imaging. Identical illumination settings, such as exposure time (1 s) and field of views (15 × 15 cm), were used for acquiring all images. Fluorescence emission was normalized to photons per second per centimeter squared per steradian (p.s⁻¹.cm⁻².sr⁻¹). Images were acquired and analysed using Living Image 3.0 software (Xenogen).

β-galactosidase assay

For the β-galactosidase assay, samples were taken at OD₆₀₀ = 0.5. The β-galactosidase reactions were assayed essentially as described by (14), with the exception that we used chloroform and 0.002% SDS to disrupt the bacteria. All data represent the average from assays performed in duplicate in three independent experiments, and the means±standard deviations are shown in the plotted graphs.

Toe-print assay

Templates for in vitro transcription of prfA₂₀⁻lacZ and prfA₂₀mut⁻lacZ were constructed by PCR using the primers (prfA-pT7 and LacZ-D-Toe) listed in Table 3. The templates contain a 5'⁰-terminating lacZ promoter. In vitro transcription was performed using the RibomAX™ Large Scale RNA production sytems-SP6 and T7 kit as described by the manufacturer (Promega). In vitro transcribed RNA was ethanol precipitated, resuspended in formamide loading dye and separated on an 8% denaturing polyacrylamide gel. The RNA was visualized by UV shadowing, excised from the gel and transferred to RNAguard and eluted overnight at 4°C using NucAway spin columns (Ambion). The synthesized RNA was gel-precipitated and dissolved in water. An amount of 10 pmol of purified RNA was dephosphorylated with FastAP alkaline phosphatase (Fermentas) and ²³P-labelled using a T4 polynucleotide kinase (Fermentas) as described by manufacturer. Following chloroform extraction the unincorporated label was removed by size exclusion using ProbeQuant spin columns (GE). The labelled RNA was gel-purified as described above. For structural probing ~0.1 pmol of labelled RNA and 1 µg total yeast RNA (Ambion) were used per reaction. Before structural probing the RNA was denatured by incubating at 95°C for 1 min and cooling on ice for 5 min. Following denaturation the RNA was diluted in 1× Structure Buffer (Ambion) and renatured at 37°C for 20 min. An amount of 2 µl of appropriately diluted RNase T1 (Ambion) were added into 10 µl aliquots containing ~0.1 pmol labelled RNA and 1 µg total yeast RNA and continued to incubate at 37°C for 5 min. To generate RNase T1 sequencing ladder 2 µl of RNA (~0.2 pmol labelled RNA and 2 µg total yeast RNA) were mixed with 9 µl of 1× Sequencing Buffer (Ambion), and incubated with 1 µl of 0.4 U µl⁻¹ RNase T1 at 50°C for 5 min. Alkaline hydrolysis ladder was prepared by mixing 2 µl of RNA (~0.2 pmol labelled RNA and 2 µg total yeast RNA) with 10 µl 1× Alkaline Hydrolysis Buffer (Ambion) and incubation at 95°C for 15 min. Reactions were stopped by addition of 12 µl Gel Loading Buffer II (Ambion) and immediate freezing the tubes in dry ice. Prior to electrophoresis RNA samples for structure probing were incubated for 1 min at 95°C and kept on ice. The RNA was separated on a denaturing 8 M urea, 6% AA/bisAA (19:1) gel in TBE buffer.

T1 ribonuclease structure mapping

The prfA UTR region was amplified by PCR from pBSN76 and pBSN83 plasmids with primers prfA-PT7 and LacZ-New-D(toe) using Phusion DNA polymerase (Finnzymes). An amount of 4 µg of gel-purified products were used as template for in vitro transcription with a RiboMAX RNA Production System T7 (Promega) in a total volume of 100 µl according to manufacturer's instructions. After DNase treatment the reaction products were chloroform extracted, ethanol precipitated, resuspended in DEPC treated water and purified by size exclusion using NucAway spin columns (Ambion). The synthesized RNA was separated by electrophoresis on a denaturing 8 M urea, 6% AA/bisAA (29:1) gel in a TBE buffer. The bands were detected by UV shadowing, excised, and eluted overnight at 4°C into 500 µl of 500 mM ammonium acetate, 1 mM EDTA pH 6.5 in presence of 100 µl acid phenol/chloroform (Ambion). Eluted RNA was chloroform extracted, ethanol precipitated and dissolved in water. An amount of 10 pmol of purified RNA were dephosphorylated with FastAP alkaline phosphatase (Fermentas) and ³²P-labelled using a T4 polynucleotide kinase (Fermentas) as described by manufacturer. Following chloroform extraction the unincorporated label was removed by size exclusion using ProbeQuant spin columns (GE). The labelled RNA was gel-purified as described above. For structural probing ~0.1 pmol of labelled RNA and 1 µg total yeast RNA (Ambion) were used per reaction. Before structural probing the RNA was denatured by incubating at 95°C for 1 min and cooling on ice for 5 min. Following denaturation the RNA was diluted in 1× Structure Buffer (Ambion) and renatured at 37°C for 20 min. An amount of 2 µl of appropriately diluted RNase T1 (Ambion) were added into 10 µl aliquots containing ~0.1 pmol labelled RNA and 1 µg total yeast RNA and continued to incubate at 37°C for 5 min. To generate RNase T1 sequencing ladder 2 µl of RNA (~0.2 pmol labelled RNA and 2 µg total yeast RNA) were mixed with 9 µl of 1× Sequencing Buffer (Ambion), and incubated with 1 µl of 0.4 U µl⁻¹ RNase T1 at 50°C for 5 min. Alkaline hydrolysis ladder was prepared by mixing 2 µl of RNA (~0.2 pmol labelled RNA and 2 µg total yeast RNA) with 10 µl 1× Alkaline Hydrolysis Buffer (Ambion) and incubation at 95°C for 15 min. Reactions were stopped by addition of 12 µl Gel Loading Buffer II (Ambion) and immediate freezing the tubes in dry ice. Prior to electrophoresis RNA samples for structure probing were incubated for 1 min at 95°C and kept on ice. The RNA was separated on a denaturing 8 M urea, 6% AA/bisAA (19:1) gel in TBE buffer.

In silico RNA folding

RNA sequences of different constructs/mRNAs were analysed using the RNAfold web server of the Vienna
RNA package (http://rna.tbi.univie.ac.at/egi-bin/RNAfold.cgi). For each sequence, the minimum free energy in kcal mol$^{-1}$ was predicted (15).

**RESULTS**

The expression of PrfA is directly correlated to the amount of prfA-coding sequence

In order to develop a thermo-inducible translation system for Mycobacterial species, the prfA-thermosensor from *L. monocytogenes* was chosen as a scaffold due to its temperature-sensing properties (3). It has previously been shown that only six codons (18 bases) of the prfA-coding mRNA was sufficient for proper thermosensing, when fused in front of *gfp* (3). To investigate if a difference in the length of the prfA-coding region affected thermosensing, DNA encoding 1 codon (3 bases), 4 codons (12 bases), 9 codons (27 bases) or 20 codons (60 bases), respectively, was inserted in front of *lacZ* and the constructs were introduced into *Escherichia coli* (Supplementary Figure S1 and ‘Materials and Methods’ section). These constructs all harboured the identical native prfA promoters and were inserted in the identical *Scel* cloning site in the Multiple Cloning site (MCS) of the vector generating translation fusions (i.e. all constructs contained the same length of the MCS). To test whether the prfA-*lacZ* fusions still were thermoregulated, PrfA-gfp expression was measured at 30°C and 37°C. Except for the one codon construct, thermosensing was still retained in the different prfA-*lacZ* fusions, with 2- to 4-fold higher expression at 37°C compared to 30°C (Supplementary Figure S2). More strikingly though, was the correlation between the β-galactosidase expression and the length of the prfA-coding sequence (Figure 1A). The β-galactosidase expression was ~15-fold higher when 20 codons of *prfA* were inserted in front of *lacZ*, (creating prfA$_{20}$-lacZ), compared to one codon (prfA$_{1}$-lacZ). The constructs carrying either four (prfA$_{4}$-lacZ) or nine codons (prfA$_{9}$-lacZ) had a β-galactosidase expression lying in between prfA$_{20}$-lacZ and prfA$_{1}$-lacZ (Figure 1A). Importantly, the β-galactosidase activity was directly correlated with protein expression as determined by western blotting (Figure 1B). An equal amount of plasmids could be extracted from each strain grown to mid-log phase, demonstrating that the difference in β-galactosidase expression among the constructs were not due to variations in plasmid maintenance and stability (data not shown).

The increased prfA-expression is not due to the reporter mRNA

It could be hypothesized that the *lacZ*-gene would, by some mechanism, cause the differences in β-galactosidase expression observed. To test this, the prfA-UTR with either 1 or 20 codons was inserted in front of *gfp* in the identical site of the vector before introduction into *E. coli*. We reasoned that if the difference in expression between the prfA$_{1}$ and the prfA$_{20}$ was still detected with gfp as a reporter mRNA, it would furthermore demonstrate the importance of the 20 first codons for efficient PrfA expression and rule out effects caused by the reporter genes. A large difference in fluorescence was detected between strains expressing the PrfA$_{1}$-GFP or the PrfA$_{20}$-GFP on bacterial agar-plates (Figure 2A). By western blotting, we determined that the difference in the level of PrfA$_{1}$-GFP and PrfA$_{20}$-GFP was similar to the difference detected between the short and the long prfA-*lacZ* constructs (compare Figures 1B and 2B). Altogether, these results suggest that the 20 first codons of the prfA-coding mRNA are important for the expression of PrfA, in a mechanism independent of the reporter mRNAs (*lacZ* or *gfp*).

The prfA-coding sequence does not affect the stability of the prfA-*gfp* transcripts

One possible explanation of the above results would be that prfA-*gfp* mRNAs carrying 20 codons was more expressed or more stable than prfA-*gfp* mRNAs with only one codon. To test this, we isolated RNA from cultures (OD$_{600}$ = 0.4) of the strains carrying 1 or 20 codons of prfA in front of *gfp* (prfA$_{1}$-gfp and prfA$_{20}$-gfp, respectively). Differences in the length of prfA did not affect the steady-state levels of the prfA-*gfp* transcripts (data not shown). These data, however, did not rule out the possibility that the different lengths of prfA might affect stability of the transcripts and hence PrfA expression. We therefore performed a transcript stability experiment where rifampicin was added to cultures carrying 1 or 20 codons of prfA upstream of *gfp*. Samples were taken at 0, 5, 10 and 15 min after addition of rifampicin. Northern blot results revealed that the prfA$_{1}$-gfp and the prfA$_{20}$-gfp transcripts were equally stable with a half-life of ~8 min (Figure 3). As a control, the decay of the *hns*
transcript was followed, showing a half-life of ~3–5 min (Figure 3). The results show that the variation observed in PrfA expression of the different constructs is not due to an altered transcription or transcript stability.

The first 20 codons of the prfA-mRNA are important for PrfA expression in *L. monocytogenes*

To test whether the 20 first codons of the prfA-mRNA would be required for efficient PrfA-expression in its natural strain background and rule out *E. coli* specific artefacts, the prfA1-gfp and the prfA20-gfp constructs were introduced into *L. monocytogenes* and the protein expression measured by western blotting. As seen in Figure 4, the amount of PrfA20-GFP was higher than the amount of PrfA1-GFP when expressed in *L. monocytogenes*, similar to the difference detected in *E. coli* (compare Figures 2B and 4).

Similar protein stabilities are detected in the PrfA1 and PrfA20 codon constructs

In order to examine if the varied PrfA expression in the different constructs was due to a difference in protein stability, strains harbouring prfA1-lacZ or prfA20-lacZ, were grown to mid-log phase before translation was inhibited by the addition of spectinomycin. After 48 h of spectinomycin treatment, no proteolytic degradation could be observed for either PrfA1-LacZ or PrfA20-LacZ (Supplementary Figure S3). This suggests that an altered protein stability cannot explain the reduced amount of PrfA1-LacZ, at least during the time-period of our β-galactosidase experiments.

Thus far, our results indicate that the first 20 codons of the prfA-mRNA are necessary for efficient PrfA expression. The mechanism controlling PrfA expression is not governed by altered expression/stability of the prfA messenger, nor does it affect the stability of the PrfA protein. Moreover, the mechanism is functioning in both *E. coli* and *L. monocytogenes*.

The 20 first codons of prfA are required for efficient translation *in vitro*

To investigate whether the 20 first codons of prfA are important for translation, an *in vitro* transcription/translation assay was used (4). An equal amount of the prfA1-gfp and the prfA20-gfp plasmid constructs were transcribed and translated in a continuous manner. From the reactions, *in vitro* synthesized protein was extracted and the levels measured by western blotting. The level of

![Figure 2](image-url)

**Figure 2.** (A) Fluorescence measurement of PrfA-GFP fusions. *Escherichia coli* strains carrying the indicated plasmids were streaked on LB-agar plate and grown at 37°C for 24 h. Fluorescence was measured using an IVIS-Spectrum imaging system. Colour scale represents level of fluorescence intensity ranging from high (yellow) to low (dark-red). (B) Western blot analysis of PrfA-GFP expression. *E. coli* strains carrying the indicated plasmids were grown to an OD600 = 0.4 at 37°C. Total protein was isolated and subjected to western blot analysis. Membrane was probed with antibodies recognizing GFP or GroEL (loading control).

![Figure 3](image-url)

**Figure 3.** Northern blot analysis examining transcript stability. *Escherichia coli* strains carrying the indicated plasmids were grown until an OD600 = 0.4 when rifampicin was added to block further transcription. Samples were isolated at indicated time-points prior to RNA isolation. RNA samples (20 µg) were separated on agarose/formaldehyde gel and subjected to a northern blot analysis. The membrane was hybridized with gfp, hns and tmRNA (loading control) probes, respectively.
PrfA20-GFP was higher than the level of PrfA1-GFP construct in a range similar to prfA20-gfp and prfA1-gfp in E. coli and L. monocytogenes (Compare Figures 5 with Figures 2B and 4). As a control, expression of β-lactamase (encoded on the same plasmid as prfA-gfp) was analysed from the same extracts. Expression of β-lactamase did not alter between the samples, showing that the reduced expression of PrfA1-GFP compared with PrfA20-GFP was not due to a general expression-defect of the plasmid.

A mutation stabilizing the prfA20-lacZ secondary RNA-structure dramatically decreases PrfA expression

Previous reports have shown a correlation between the stability of the mRNA secondary structure and translation. This has been indicated for regions just downstream of the startcodon (within the coding RNA) (16). Also, strong mRNA secondary structures can inhibit an initial interaction between the ribosome and the mRNA at ribosome standby sites located on the mRNA (17,18). Therefore, the mRNA secondary structure stability for the entire 5’-UTR + 60 extra bases downstream of A in AUG were predicted for the different constructs (Supplementary Figure S4). The results indicated that the thermosensor remained relatively intact in all constructs, which is in agreement with the β-galactosidase expression results at different temperatures (Supplementary Figure S2). Instead, the predicted RNA-structure differed downstream of the startcodon, with the shorter constructs (1 or 4 codons) being more stable than the longer constructs [9 or 20 codons (Supplementary Figure 4)]. If the in silico predictions of RNA secondary structure stabilities were correct, an increased stability of the prfA RNA secondary structure should decrease PrfA expression. To test this, an AA to CG base-substitution mutation at position 137–138 (downstream of the startcodon) was constructed (see ‘Materials and Methods’ section and Supplementary Figure S4). When measuring β-galactosidase protein expression, it was observed that the prfA20Mut-lacZ mutant construct displayed a level dramatically reduced compared to the wild-type prfA20-lacZ (Figure 6). To avoid possible effects of the reporter RNA in the prfA1, prfA4 and prfA9 constructs, the subsequent experiments were carried out using only the prfA20 and the prfA20Mut constructs.

To examine in vitro if the structure within the coding region was affected between the prfA20-lacZ and the prfA20Mut-lacZ transcripts, an RNaseT1 structural probing assay was undertaken. During these conditions, RNaseT1 recognizes unpaired guanine bases. From the data (Figure 7), it was evident that the overall structure of the thermosensor did not differ between the prfA20-lacZ and the prfA20Mut-lacZ transcripts, in agreement with results from a previous RNA-probing experiment (3). However importantly, a dramatic difference was observed at the hairpin loop predicted to be downstream of the AUG start codon. For the prfA20-lacZ transcript, the guanine bases at positions 128, 131 and 134 were equally unpaired, indicating an unstructured hairpin loop. In contrast, at the equivalent region of the prfA20Mut-lacZ transcript, only the guanine base at position 128 was unpaired, suggesting a rigid inflexible hairpin with a short loop.

A weak secondary structure within the prfA coding RNA increases ribosome binding

A plausible explanation of the higher expression in the prfA20-lacZ construct compared to the prfA20Mut-lacZ construct would be that the ribosome binds more strongly to the SD-region of prfA20-lacZ than prfA20Mut-lacZ. To test this, toe-print experiments were conducted, by analysing the capability of ribosome binding to in vitro synthesized prfA20-lacZ or prfA20Mut-lacZ RNA samples (Supplementary Figure S5). Our data indicate that the
ribosome indeed binds the SD-region of the \textit{prfA}_{20}-\textit{lacZ} RNA more strongly than it binds the \textit{prfA}_{20\text{Mut}}-\textit{lacZ} RNA.

**DISCUSSION**

In this study, we show that the 5'-end of the \textit{prfA}-coding RNA is important for its expression. Our work shows that the ribosome requires an unstructured RNA, within the first 20 bases downstream of the AUG start codon for efficient binding and translation initiation. Stabilizing this structure severely impairs ribosomal interaction with the RNA leading to a decreased translation. Particularly, a hairpin-loop, located within the first nine codons must be in a flexible state to allow efficient ribosome binding. It has been suggested that a strong mRNA secondary structure in the 5'-part of the coding RNA affects expression negatively, by preventing binding of the ribosome (16,19).

We identified an inverse correlation between the PrfA-fusion protein expression levels both \textit{in vivo} and
in vitro versus the stability of the predicted mRNA secondary structures and particularly an hairpin loop located downstream of the start codon. Our results are in agreement with the ribosomal standby model (17,18,20). In the article by de Smit and van Duin (17), it was suggested that the ribosome initially binds to an unpaired region of the transcript, the standby site, instead of binding directly to the SD-region if it is occluded by a paired structure (like the thermosensor). By binding to the standby site, the ribosome can more easily compete with SD-regions trapped in secondary structures, during their time of opening (the more stable structure, the shorter the time of opening). It could therefore be hypothesized that the unstructured region downstream of AUG of prfA is a ribosomal standby site, where the ribosome can bind and ‘wait’ for the SD-region to be accessible. Once bound, the ribosome can more efficiently compete with the thermosensor structure occluding the SD-region, thereby increasing the frequency of translation initiation. If the ribosome is prevented to bind to the standby site (by mutations creating a more stable secondary structure) the binding of the ribosome to the SD will be reduced. Also, the unfolding of a structured mRNA after an initial interaction with the ribosome is important to allow the start codon to interact with the initiator tRNA (21).

Previously, the 5'-UTR of the prfA transcript has been shown to function as a thermosensor and has also been shown to be regulated by a trans-acting riboswitch (3,4). Our results suggest that the coding RNA of prfA does not participate in thermoregulation, but rather is important for efficient translation initiation. Vice versa, the RNA probing assay indicates that the thermosensor RNA does not interfere with the unstructured downstream region. This suggests that the binding of the ribosome to the SD is independent of the thermosensor.

Several alternative mechanisms were tested to determine if they could explain the expression difference detected between the prfA constructs: (i) Sprengart and colleagues (22), suggested that the presence of a downstream box (DB), located at the 5'-end of the coding-mRNAs, allows direct base-pair interactions between the DB and the 16S rRNA in the ribosome. However, no such DB-box showing complementarity against the 16S rRNA could be detected in any of our constructs (data not shown). (ii) It could be hypothesized that the shorter constructs carrying codons mainly from the MCS contain certain codons or stretches of bases that prevents maximal translation (i.e. rare codons). One method to determine codon bias is to measure the codon adaptive index [CAI, (23)]. By measuring CAI, we observed a slightly lower value for the shorter constructs compared with the longer, when the first 20 codons were analysed. However, all values were high (CAI > 0.68), arguing against an effect of rare codons (data not shown). (iii) Mechanisms involving rare/ specific codons or poly-A/multiple CAs close to the SD have been suggested by others (24–28). However, no such codons/stretch of bases could explain the varied expression levels we observe among the constructs (data not shown). The nature of our constructs (using the same insertion site and differing only in the amount of codons inserted) also argues against such mechanisms, since no difference in expression should be observed between the 4, 9 or the 20 codon constructs if any of these mechanisms would apply. (iv) When examining the constructs for the most striking favoured/disfavoured codons (29), no such codons were present within the first 60 bases of our gene-fusions.

We were surprised that the MCS of commercial plasmid vectors harbour these very strong RNA secondary structures. The efficiency of translation would probably be remarkably higher if an MCS expressing a more unstructured RNA sequence would be developed.

Expression of PrfA is subject to several layers of regulation, acting at the transcriptional, translational and at the post-translational level. The reason for this multiple levels of regulation of PrfA is obviously to maintain the level and/or activity of PrfA at an optimal level at each time-point. Absence of PrfA completely attenuates the virulence capability of *L. monocytogenes* and a deregulated PrfA expression leads to increased virulence gene expression during inappropriate conditions (i.e. low temperature, (3)). Expression of PrfA has been shown to be controlled at many steps during initiation of translation. First, an RNA thermosensor located within the prfA 5'-UTR obstructs binding of the ribosome at low temperatures. Second, a trans-acting riboswitch has been shown to down-regulate PrfA translation by binding to the thermosensor at higher temperatures. Third, in this work, we show that maximal translation of PrfA require an unstructured 5'-region of the coding mRNA.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5.

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