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The 5′ untranslated region-mediated enhancement of intracellular listeriolysin O production is required for *Listeria monocytogenes* pathogenicity

Aimee Shen and Darren E. Higgins*
Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115-6092, USA.

Summary

Listeriolysin O (LLO) and ActA are essential virulence determinants for *Listeria monocytogenes* pathogenesis. Transcription of *actA* and *hly*, encoding LLO, is regulated by PrfA and increases dramatically during intracellular infection. The 5′ untranslated regions (5′ UTRs) of *actA* and *prfA* have been shown to upregulate expression of their respective gene products. Here, we demonstrate that the *hly* 5′ UTR plays a critical role in regulating expression of LLO during intracellular infection. Deletion of the *hly* 5′ UTR, while retaining the *hly* ribosome binding site, had a moderate effect on LLO production during growth in broth culture, yet resulted in a marked decrease in LLO levels during intracellular infection. The diminished level of LLO resulted in a significant defect in bacterial cell-to-cell spread during intracellular infection and a 10-fold reduction in virulence during *in vivo* infection of mice. Insertion of the *hly* 5′ UTR sequence between a heterologous promoter and reporter gene sequences indicated that the *hly* 5′ UTR functions independent of PrfA-mediated transcription and can enhance expression of *cis*-associated genes through a mechanism that appears to act at both a post-transcriptional and translational level. The ability of the *hly* 5′ UTR to increase gene expression can be exploited to achieve PrfA-independent complementation of virulence genes and high-level expression of single copy heterologous genes in *L. monocytogenes*.

Introduction

Listeria monocytogenes is a facultative intracellular bacterial pathogen of humans and a wide variety of animal species (Vazquez-Boland *et al*., 2001). Upon entering host cells, expression of many virulence determinants is cell compartment-specific (Bubert *et al*., 1999). Expression of listeriolysin O (LLO) and PI-PLC, which mediate bacterial escape from phagocytic vacuoles, is activated at the transcriptional level within the vacuole, while expression of ActA and PC-PLC, which mediate actin-based motility and cell-to-cell spread, and escape from secondary spreading vacuoles, respectively, is activated once bacteria access the host cell cytosol (Bubert *et al*., 1999; Freitag and Jacobs, 1999; Shetron-Rama *et al*., 2002). Transcription of the genes encoding these principal virulence determinants is controlled by PrfA, a transcriptional activator that binds to 14 bp palindromic sequences present upstream of virulence gene promoters (Leimeister-Wachter *et al*., 1990; Chakraborty *et al*., 1992; Sheehan *et al*., 1995; Milohanic *et al*., 2003). During intracellular infection, transcription of *hly*, encoding LLO, is upregulated ~20-fold (Moors *et al*., 1999b). This induction of LLO expression is likely required for efficient secondary vacuole escape, as bacterial strains that produce decreased amounts of LLO during intracellular infection display defects in cell-to-cell spread (Dancz *et al*., 2002).

ActA is one of the most abundant *L. monocytogenes* proteins produced during intracellular infection (Brundage *et al*., 1993), with ActA protein levels being regulated by transcriptional and post-translational mechanisms (Brundage *et al*., 1993; Moors *et al*., 1999a,b). Transcription of *actA* is induced ~150- to 200-fold during infection of host cells (Moors *et al*., 1999b; Shetron-Rama *et al*., 2002), and small decreases in ActA protein levels dramatically alter the efficiency of cell-to-cell spread by decreasing the frequency of actin-tail formation (Smith *et al*., 1996; Moors *et al*., 1999a; Wong *et al*., 2004). However, overexpression of ActA also appears to impair cell-to-cell spread (Lauer *et al*., 2002). Similarly, increased levels of cytosolic LLO are toxic to host cells (Higgins *et al*., 1999; Decatur and Portnoy, 2000). Thus, stringent regulation of virulence gene expression is critical for productive intracellular infection by *L. monocytogenes*.

Most studies of virulence gene regulation in *L. monocytogenes* have focused on the contribution of PrfA in activating transcription. However, several studies have demonstrated that additional factors regulate expression of *L. monocytogenes* virulence determinants during intra-

Accepted 17 June, 2005. *For correspondence. E-mail dhiggins@hms.harvard.edu; Tel. (+1) 617 432 4156; Fax (+1) 617 738 7664.

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cellular infection (Moors *et al*., 1999b; Shetron-Rama *et al*., 2002; 2003). The 5′ untranslated regions (UTRs) of *prfA* and *actA* transcripts have been shown to regulate expression of their cognate gene products. The *prfA* 5′ UTR functions as a thermosensor to regulate translation of PrfA. At 30°C, the *prfA* 5′ UTR adopts a secondary structure that prevents initiation of translation on *prfA* transcripts. At 37°C, this inhibitory structure is no longer energetically favourable, thereby allowing translation of *prfA* transcripts (Johansson *et al*., 2002). Similarly, the *actA* 5′ UTR functions to maximize expression of ActA (Wong *et al*., 2004). Deletion of 128 nucleotides of the *actA* 5′ UTR, while retaining the *actA* ribosome binding site (RBS), results in a fourfold reduction in intracellular ActA levels and an inability to polymerize host actin (Wong *et al*., 2004). Although the mechanism underlying this function remains to be determined, the secondary structure of the *actA* 5′ UTR is believed to play a role in regulating ActA expression.

In this study, we examined the role of the *hly* 5′ UTR in regulating expression of LLO during extracellular growth and intracellular infection. Deletion of the *hly* 5′ UTR, while retaining the *hly* RBS, yielded a moderate change in LLO levels during growth in broth culture, yet resulted in a marked decrease in LLO production during intracellular infection. The diminished levels of LLO produced by the *hly* 5′ UTR mutant during cytosolic growth correlated with a significant defect in bacterial cell-to-cell spread in tissue culture cells and a 10-fold decrease in virulence during *in vivo* infection of mice. Fusion of the *hly* 5′ UTR to a heterologous promoter and reporter gene sequences resulted in enhanced gene expression, indicating that the *hly* 5′ UTR can function *in cis* independent of PrfA-mediated activation. Lastly, we demonstrate that the ability of the *hly* 5′ UTR to increase gene expression can be exploited to achieve PrfA-independent complementation of *L. monocytogenes* virulence genes and yield high-level expression of heterologous genes in single copy.

Results

The hly *5′ UTR is required for maximal LLO production during intracellular infection*

Prior studies have shown that the *actA* 5′ UTR functions in regulating ActA expression and is required for full virulence of *L. monocytogenes* (Wong *et al*., 2004). Given the similarities in induction of intracellular expression and the importance of ActA and LLO for *L. monocytogenes* pathogenesis (Moors *et al*., 1999b; Dancz *et al*., 2002; Wong *et al*., 2004), we sought to determine whether the *hly* 5′ UTR regulates expression of LLO. To determine the importance of the *hly* 5′ UTR, deletions within the *hly* 5′ UTR sequence were introduced into the chromosome of wild-type *L. monocytogenes* strain 10403S. *hly* is predominantly transcribed from a PrfA-dependent promoter (P_{hly}) (Domann *et al*., 1993). The PrfA-dependent promoter initiates transcription at two sites, P2 and P1, which have been mapped by primer extension to produce transcripts with a 5′ UTR of 133 and 122 nucleotides respectively (Mengaud *et al*., 1989) (Fig. 1A). A total of 101 bp of the *hly* 5′ UTR sequence were deleted from the *hly* locus to yield strain DH-L1231, which retains the PrfA-dependent P_{hly} promoter, the P2 and P1 transcription initiation sites, and the RBS (Fig. 1B).

The effect of deleting the *hly* 5′ UTR on LLO production was assessed during growth in broth culture and during intracellular infection. During growth in brain heart infusion (BHI) broth, no significant difference in bacterial growth rate was observed (data not shown), while a slight decrease (~31%) in LLO haemolytic activity present in culture supernatants was detected from strain DH-L1231 [92 ± 25 haemolytic units (HU)] relative to wild-type 10403S (134 ± 40 HU) (Fig. 2A). LLO protein levels in bacterial cell pellets and supernatant fractions of DH-L1231 cultures were also mildly diminished when compared with 10403S (Fig. 2B, BHI). Primer extension analysis indicated that *hly* transcript levels were 3.5-fold (71%)

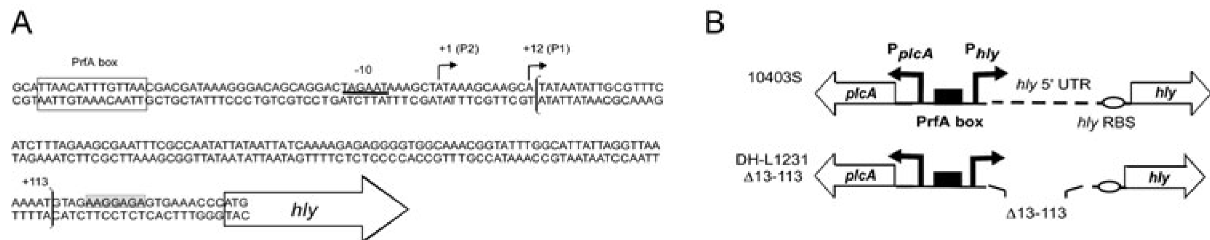

Fig. 1. Construction of a *L. monocytogenes* strain containing a deletion of the *hly* upstream region.
A. Detailed depiction of the *hly* upstream region. The PrfA-binding site (PrfA box) for the *hly* promoter is boxed, the -10 promoter element is underlined, and the two transcription initiation sites, P2 and P1, are indicated by +1 and +12 respectively. The *hly* ribosome binding site (RBS) is shaded, while the open block arrow denotes the *hly* coding region. The regions enclosed by brackets indicate nucleotides deleted from *L. monocytogenes* strain DH-L1231.
B. Schematic representation of *L. monocytogenes hly* 5′ UTR mutant strain, DH-L1231. Dark arrows denote the PrfA-dependent promoters for *hly* (P_{hly}) and *plcA* (P_{plcA}); filled rectangles, open block arrows, dashed lines, and open ovals denote the PrfA-binding site, *hly* coding region, *hly* 5′ UTR sequence, and *hly* RBS respectively.

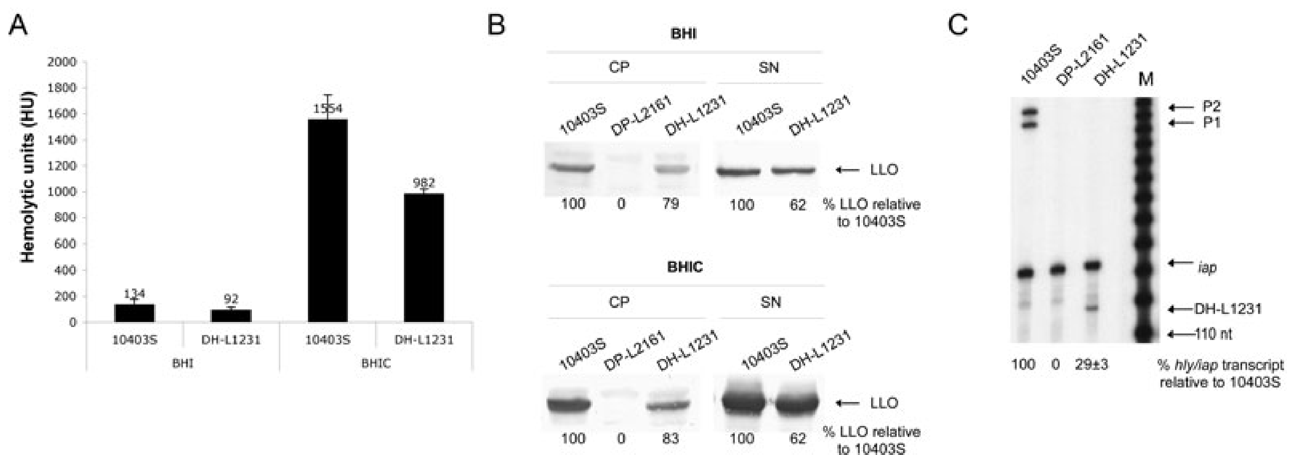

Fig. 2. Expression of LLO during growth in BHI broth.
A. Haemolytic activity of *L. monocytogenes* strains. Fourteen to 16 h cultures of wild type (10403S), LLO-negative (DP-L2161) and the *hly* 5′ UTR mutant (DH-L1231) were diluted 1:10 in BHI or BHIC broth and grown for 5 h at 37°C. Haemolytic activity (HU) present in culture supernatants was determined as described in *Experimental procedures*. The means and standard deviations of three independent experiments are shown. Haemolytic activity for DP-L2161 culture supernatants was not detected.
B. Western blot analysis of LLO expression. Aliquots of cultures used in A were centrifuged. Cell pellets (CP) were resuspended and digested with lysozyme, and supernatants (SN) were precipitated for 1 h on ice in the presence of 10% trichloroacetic acid (v/v). Samples were centrifuged and resuspended in protein sample buffer. Protein samples from a culture volume equivalent to 100 μl (CP) or 50 μl (SN) of an $OD_{600} = 1.5$ were separated by SDS-PAGE and immunoblotted using a rabbit polyclonal anti-LLO antibody. Numerical values indicate the percent band intensity relative to 10403S as determined by densitometry analysis and are representative of at least three independent experiments.
C. Primer extension analysis of *hly* transcripts. Total RNA was isolated from bacterial strains used in A that had been grown in BHI broth. 7.5 μg of total RNA was hybridized to a ^{32}P-end-labelled primer specific for *hly*. Primer extension was performed as described in *Experimental procedures*. A radiolabelled primer specific to the constitutively transcribed *iap* gene (Kohler *et al*., 1990) was simultaneously hybridized to total RNA as an internal load control. Identities of extended products are indicated. The P1 and P2 products correspond to *hly* transcripts resulting from PrfA activation of the P_{hly} promoter in 10403S. Lane M is a radiolabelled size ladder. Band intensities of probe-associated transcripts were quantified by phosphorimager analysis. Numerical values indicate the percent *hly/iap* transcript relative to 10403S and represent the mean and standard deviation of three independent experiments.

lower in DH-L1231 compared with wild-type 10403S and confirmed that transcription of *hly* initiated at the predicted P2 transcription initiation site of strain DH-L1231 (Fig. 2C) However, no *hly* transcripts were detected that initiated at the P1 transcription initiation site. This may indicate that the *hly* P1 transcription initiation site in 10403S is actually an mRNA processing site within *hly* transcripts that initiate at P2 or that DNA sequences encompassing the *hly* 5′ UTR are required for PrfA-dependent transcription initiation at the P1 site. Northern blot analysis of *hly* transcripts yielded a similar decrease in *hly* transcript levels in the *hly* 5′ UTR mutant relative to wild-type 10403S (data not shown). Thus, deletion of the *hly* 5′ UTR resulted in a moderate decrease in LLO production and a more severe decrease in *hly* transcript levels during growth in broth culture.

Despite a modest effect of the *hly* 5′ UTR on LLO levels during growth in BHI broth, we considered the possibility that deletion of the *hly* 5′ UTR might result in a greater defect in LLO production when *hly* expression is upregulated by PrfA. Treatment of BHI broth with activated charcoal (BHIC) results in activation of PrfA by chelating a compound that inhibits the activity of PrfA (Ermolaeva *et al*., 2004). Thus, during growth in BHIC, a 10- to 20-fold increase in LLO levels is observed relative to the levels produced in BHI broth (Geoffroy *et al*., 1987; Ripio *et al*., 1996). Although levels of LLO production were increased compared with growth in BHI, growth of the *hly* 5′ UTR mutant in BHIC resulted in a similar decrease (~37%) in haemolytic activity of DH-L1231 (982 ± 36 HU) relative to 10403S (1554 ± 192 HU) (Fig. 2A), without affecting bacterial growth rate (data not shown). Diminished LLO levels relative to 10403S were also observed by Western blot analysis of bacterial cell pellets and supernatant fractions of DH-L1231 (Fig. 2B, BHIC). Thus, deletion of the *hly* 5′ UTR resulted in a similar defect in LLO production during growth in PrfA-activating conditions.

As PrfA-mediated activation of virulence genes is maximal during intracellular infection, we determined the effect of deleting the *hly* 5′ UTR on LLO production during growth inside J774 host cells. Approximately 55% less secreted LLO relative to wild-type 10403S was immunoprecipitated from metabolically labelled J774 cells during infection with the *hly* 5′ UTR mutant (Fig. 3A). A greater reduction in bacterial-associated LLO levels (78%) relative to 10403S was also observed during infection of J774 cells with DH-L1231 (Fig. 3B). The decrease in LLO production by the *hly* 5′ UTR mutant relative to wild type could not be attributed to differences in intracellular growth, as DH-L1231 bacteria were recovered in similar numbers as

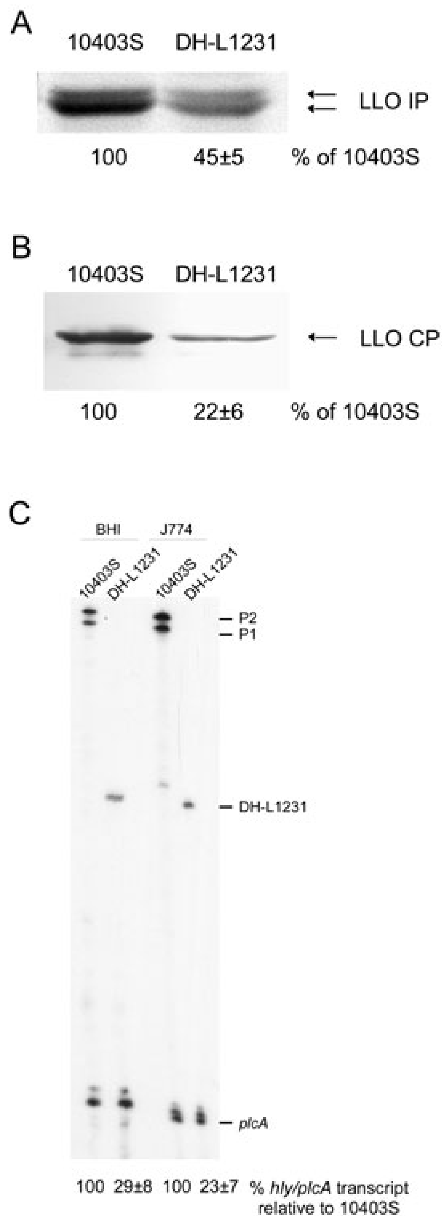

Fig. 3. The *hly* 5′ UTR is required for maximal LLO production during intracellular infection.
A. Immunoprecipitation of LLO from J774 cells infected with *L. monocytogenes*. 10403S and *hly* 5′ UTR mutant, DH-L1231, were grown in BHI broth and used to infect J774 cells as described in *Experimental procedures*. At 6 h post infection, bacterial proteins were metabolically labelled for 1 h with [^{35}S]-methionine, and LLO was immunoprecipitated from host cell lysates using monoclonal anti-LLO antibody (B3-19). Immunoprecipitated proteins (IP) were resuspended in 2× FSB and resolved by SDS-PAGE. The resulting gel was exposed to a phosphorimaging screen for 7 days and subjected to phosphorimager analyses. Numerical values indicate the percent band intensity of LLO relative to 10403S and represent the mean and standard deviation of three independent experiments. As previously shown (Moors *et al*., 1999b), two distinct species of LLO protein were detected in immunoprecipitated fractions.
B. Western blot analysis of bacterial-associated LLO during infection of J774 cells. *L. monocytogenes* strains described in A were grown in BHI broth and used to infect monolayers of J774 cells as described in *Experimental procedures*. At 7 h post infection, bacteria were pelleted from host cell lysates. Bacterial cell pellets (CP) were resuspended, digested with lysosome, and 2× FSB was added. Protein samples equivalent to 2×10^8 cfu were separated by SDS-PAGE and immunoblotted using monoclonal anti-LLO antibody (B3-19). Numerical values indicate the percent band intensity relative to 10403S as determined by densitometry analysis and represent the mean and standard deviation of three independent experiments.
C. Primer extension analysis of *hly* transcripts. A parallel infection to that described in B was used to harvest RNA from bacteria isolated from J774 cell lysates. RNA was also isolated from 10403S and DH-L1231 bacteria grown in BHI broth culture as described in Fig. 2A. Three micrograms of total RNA isolated from intracellular bacteria, and 15 μg of total RNA isolated from bacteria grown in BHI broth were hybridized to a ^{32}P-end-labelled primer specific for *hly*. Primer extension was performed as described in *Experimental procedures*. A radiolabelled primer specific to the *plcA* gene, which is divergently transcribed from *hly*, was simultaneously hybridized to total RNA as an internal load control. Identities of extended products are indicated. The P1 and P2 products correspond to *hly* transcripts that result from PrfA activation of the P_{hly} promoter in 10403S. Band intensities of probe-associated transcripts were quantified by phosphorimager analysis. Numerical values indicate the percent of *hly/plcA* transcript relative to 10403S and represent the means and standard deviations of three independent experiments.

10403S bacteria during infection of J774 cells (data not shown).

To determine whether the decrease in LLO levels detected from the *hly* 5′ UTR mutant relative to wild type during intracellular infection correlated with a decrease in *hly* transcript levels, RNA was harvested from 10403S and DH-L1231 bacteria grown inside J774 cells, and *hly* transcripts were detected directly by primer extension. During intracellular infection, DH-L1231 produced approximately fourfold less *hly* transcripts than 10403S; a similar 3.5-fold decrease in *hly* transcript levels in the *hly* 5′ UTR mutant relative to wild type was observed during growth in BHI broth (Figs 2C and 3C). Furthermore, upregulation of P_{hly} during intracellular infection did not seem to be impaired in DH-L1231, as an ~30-fold increase in *hly* transcription was observed in both 10403S and DH-L1231 upon growth in the cytosol relative to growth in BHI broth. It should be noted that fivefold more RNA was used in the extension reactions for samples isolated from bacteria grown in BHI broth than in reactions used with samples harvested from intracellular bacteria (Fig. 3C). Importantly, upregulation of the *plcA* promoter was unaffected by deletion of the *hly* 5′ UTR, despite sharing the same PrfA-box as P_{hly} (Fig. 1B). The observed ~30-fold induction of P_{hly} during intracellular infection corresponds well with the ~20-fold increase in *hly* promoter activation measured under similar conditions using transcriptional fusions (Moors *et al*., 1999b). Taken together, these results suggest that the *hly* 5′ UTR sequence is dispensable for PrfA-mediated induction of P_{hly} during intracellular infection, but is required for maximal LLO production during growth inside host cells.

The hly *5′ UTR is required for efficient cell-to-cell spread and virulence of* L. monocytogenes

We next examined whether the decrease in intracellular LLO levels observed upon deletion of the *hly* 5′ UTR translated into a defect in cell-to-cell spread and virulence of *L. monocytogenes*. Deletion of the *hly* 5′ UTR had little effect on intracellular growth of *L. monocytogenes*, because DH-L1231 grew similarly to wild-type 10403S during infection of murine bone marrow-derived macrophages (BMM) (Fig. 4A) and in J774 cells (data not shown). Furthermore, microscopic analysis of infected BMM did not indicate any significant differences in intracellular infection over the 12-h period examined in Fig. 4A. In contrast, plaquing analysis over a 72 h infection period in murine L2 fibroblasts indicated a deficiency in plaque formation for the *hly* 5′ UTR deletion mutant, as DH-L1231 yielded an average plaque size 68 ± 8% of that formed by 10403S (Fig. 4B). Prior studies from our group have shown that decreases in intracellular LLO production result in concomitant decreases in plaque size, which most likely results from a deficiency in secondary vacuole escape following cell-to-cell spread (Dancz *et al*., 2002). Thus, the *hly* 5′ UTR likely plays a role in regulating intracellular LLO production to obtain protein levels necessary for efficient cell-to-cell spread.

To assess whether the defects in cell-to-cell spread associated with deletions in the *hly* 5′ UTR would correlate with a virulence defect *in vivo*, we determined the LD_{50} value for DH-L1231 during infection of BALB/c mice. The LD_{50} for wild-type 10403S was $1–3 \times 10^4$, whereas the LD_{50} for DH-L1231 was $2–3 \times 10^5$, indicating that deletions within the *hly* 5′ UTR result in a 10-fold decrease in virulence. Taken together, these results suggest that the *hly* 5′ UTR is required for maximal expression of LLO during intracellular infection, which in turn is necessary for full virulence of *L. monocytogenes*.

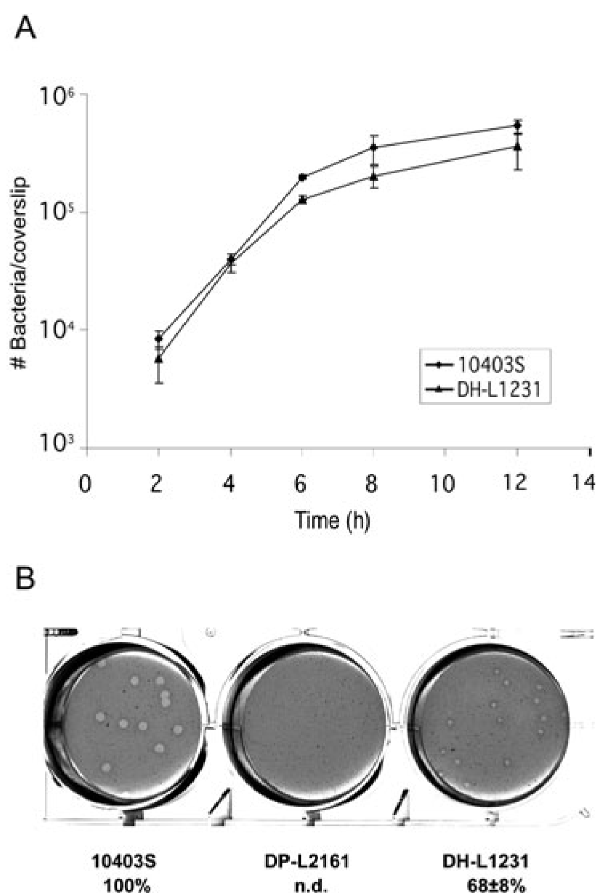

Fig. 4. The *hly* 5′ UTR is required for efficient cell-to-cell spread during intracellular infection.
A. Intracellular growth of *L. monocytogenes* in murine bone marrow-derived macrophages (BMM). Monolayers of BMM seeded onto glass coverslips were infected with *L. monocytogenes* strains at an moi of 1:3 as described in *Experimental procedures*. At the indicated times post infection, coverslips were removed and the number of intracellular bacteria determined. Data represent one of three independent experiments performed in triplicate with similar results.
B. Plaque formation in L2 fibroblasts. Wild type (10403S), LLO-negative (DP-L2161), or the *hly* 5′ UTR mutant (DH-L1231) were grown in BHI broth and added to monolayers of mouse L2 fibroblasts for 1 h. The infected monolayers were washed with PBS, and a medium-agarose overlay containing gentamicin was added to kill extracellular bacteria. Intracellular growth and cell-to-cell spread of bacteria were visualized after 72 h by the formation of clearing zones (plaques) within the L2 monolayers. The diameters of 15 plaques/sample were measured. Values are expressed as the percent diameter of plaques relative to 10403S and represent the means and standard deviations of three independent experiments. n.d., no plaques detected.

The hly *5′ UTR regulates LLO production independent of PrfA-mediated transcription of* hly

While deletions within the *hly* 5′ UTR had a detectable effect on LLO expression in BHI broth (Fig. 2A and 2B), data in Fig. 3 indicated that the *hly* 5′ UTR is required for maximal LLO expression under intracellular growth conditions where PrfA activation of *hly* expression is greatest. Although our results indicated that deletion of the *hly* 5′ UTR did not affect upregulation of P_{hly} during intracellular infection, *hly* transcripts were reduced in the *hly* 5′ UTR mutant relative to wild type regardless of the growth condition (Fig. 3C). This raised the possibility that PrfA-mediated transcription initiation from P_{hly} is dependent on DNA sequences within the *hly* 5′ UTR, especially given the absence of detectable transcript initiating from the P1 promoter in DH-L1231 (Figs 2C and 3C). To address this possibility, we uncoupled *hly* transcription from PrfA-dependent promoter activation and examined the effect of deleting the *hly* 5′ UTR on expression of LLO. Transcription of *hly* was placed under the control of the constitutive HyperSPO1 promoter (Quisel *et al*., 2001) (Fig. 5A), and *hly* constructs harbouring varying lengths of the *hly* 5′ UTR were expressed in DP-L2161, a 10403S-derived

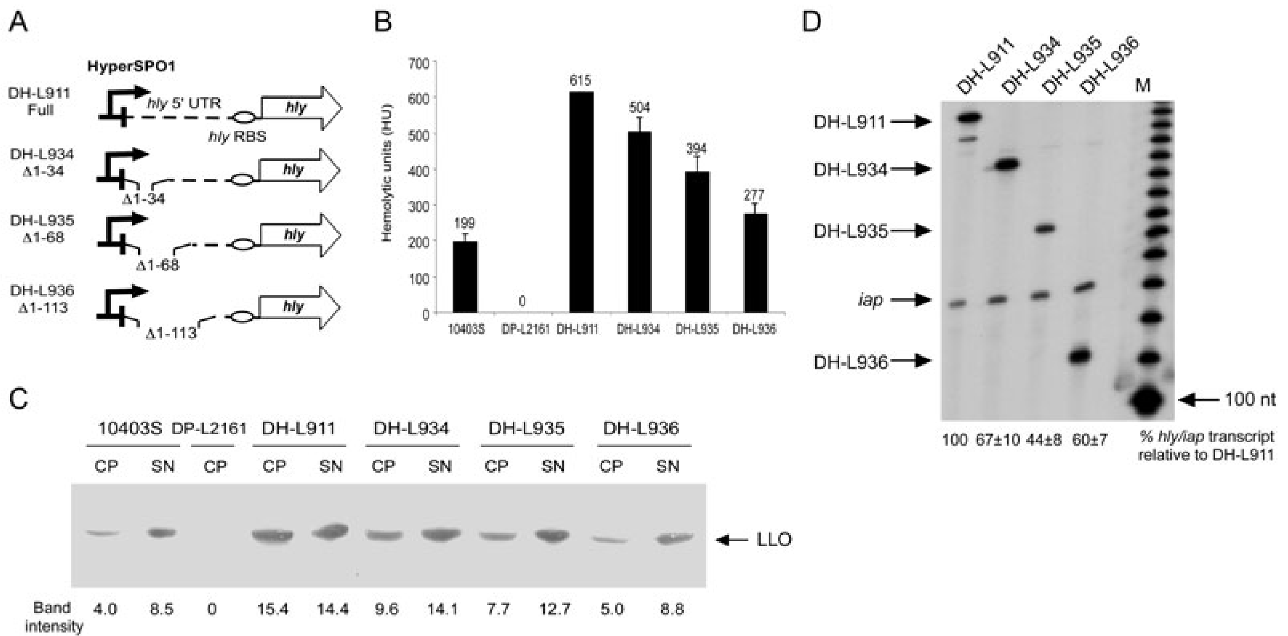

Fig. 5. The *hly* 5′ UTR can enhance expression of LLO independent of PrfA-mediated transcription of *hly*.
A. Schematic representation of HyperSPO1 promoter-controlled *hly* strains harbouring various lengths of the *hly* 5′ UTR sequence. The dark arrow, dashed line, oval and open block arrow denote the HyperSPO1 promoter, *hly* 5′ UTR sequence, *hly* RBS and *hly* coding region respectively. The regions deleted within the *hly* 5′ UTR are given relative to the P2 transcription initiation site of *hly* (Fig. 1A).
B. Haemolytic activity of *L. monocytogenes* strains. Fourteen to 16 h cultures of wild type (10403S), LLO-negative (DP-L2161) and the HyperSPO1 promoter-controlled *hly* strains were diluted 1:10 in BHI broth and grown for 5 h at 37°C. Haemolytic activity present in culture supernatants was determined as described in *Experimental procedures*. The means and standard deviations of three independent experiments are shown.
C. Quantification of LLO expression by Western blot. Aliquots of cultures used in B were centrifuged, and the cell pellets (CP) were resuspended and digested with lysozyme. Culture supernatants (SN) were precipitated for 1 h on ice in the presence of 10% trichloroacetic acid (v/v). Protein samples from a culture volume equivalent to 100 μl (CP) or 50 μl (SN) of an $OD_{600} = 1.5$ were separated by SDS-PAGE and immunoblotted with a rabbit polyclonal anti-LLO antibody. Protein band intensities were quantified by densitometry. Data shown are representative of at least three independent experiments.
D. Primer extension analysis of *hly* transcript levels. 7.5 μg of total RNA from the HyperSPO1 promoter-controlled *hly* strains was hybridized to a ^{32}P-end-labelled primer specific for *hly*. Primer extension was performed as described in *Experimental procedures*. A radiolabelled primer specific to the *iap* gene was simultaneously hybridized to total RNA as an internal load control. Identities of extended products are indicated. Band intensities of probe-associated transcripts were quantified by phosphorimager analysis. Numerical values indicate the percent *hly/iap* transcript relative to DH-L911 and represent the means and standard deviations of three independent experiments.

strain containing a deletion of the *hly* gene (Jones and Portnoy, 1994). Strain DH-L911 harboured the entire *hly* 5′ UTR (Fig. 5A) and was used as the reference strain by which to compare the effect of deletions of the *hly* 5′ UTR on LLO production. Progressive deletions of 34, 68 and 113 bp from the 5′ end of the *hly* upstream region were used to generate strains DH-L934, DH-L935 and DH-L936 respectively (Fig. 5A). The *hly* expression constructs were integrated in single copy within the ectopic $tRNA^{Arg}$ locus (Lauer *et al*., 2002) of DP-L2161.

To determine the effect of progressive deletion of the *hly* 5′ UTR on LLO production, we measured the haemolytic activity present in culture supernatants of each strain. Decreasing the length of the *hly* 5′ UTR from the 5′ end resulted in concomitant decreases in haemolytic activity. While the haemolytic activity for all of the HyperSPO1 promoter-controlled *hly* strains was greater than that observed for wild-type 10403S, the haemolytic activity of strains DH-L934, DH-L935 and DH-L936 was 1.2-, 1.6- and 2.2-fold less, respectively, than the haemolytic activity of DH-L911 (Fig. 5B). Western blot analysis of LLO protein present in bacterial cell pellets and supernatant fractions correlated with the observed haemolytic activities for each of the strains examined (Fig. 5C). Given that each progressive truncation of the *hly* 5′ UTR decreased LLO production, these results suggested that sequences from +1 through +113 of the *hly* 5′ UTR play a role in enhancing expression of LLO and can function independent of PrfA-mediated transcription initiation.

We next examined whether the reduced LLO levels observed upon deletion of the *hly* 5′ UTR correlated with a decrease in *hly* transcript levels. Primer extension analysis indicated that a general decrease in *hly* transcripts was observed upon truncation of the *hly* 5′ UTR (Fig. 5D), and confirmed that transcription of *hly* initiated at the

predicted sites within the HyperSPO1 promoter-controlled *hly* strains (Fig. 5A). DH-L934 and DH-L935 generated, respectively, 1.4-fold and 2.3-fold less *hly* transcripts than DH-L911. Similar results were obtained by Northern blot analysis (data not shown). However, a strict correlation between *hly* transcript and LLO protein levels was not observed. Although DH-L936 produced less LLO protein and haemolytic activity than DH-L935 (Fig. 5B and C), DH-L936 produced slightly more *hly* transcripts than DH-L935 (Fig. 5D). Thus, the data in Fig. 5 indicated that the *hly* 5′ UTR can mediate enhanced production of LLO independent of PrfA-regulated transcription of *hly*.

The hly *5′ UTR can enhance expression of a heterologous gene product in* L. monocytogenes

Although the *hly* 5′ UTR could enhance expression of LLO independent of PrfA-mediated transcription of *hly* (Fig. 5), it is possible that sequences within the *hly* coding region may be required for *hly* 5′ UTR-mediated enhancement of LLO expression. Alternatively, the *hly* 5′ UTR may be sufficient to indiscriminately enhance expression of *cis*-associated gene products. To distinguish between these possibilities, we generated a *L. monocytogenes* strain in which the *gfp* gene encoding green fluorescent protein (GFP) from *Aequorea victoria* was fused directly downstream of the *hly* 5′ UTR and transcribed from the HyperSPO1 promoter. The full-length *hly* 5′ UTR-*gfp* fusion was integrated in single copy within the tRNA^{Arg} locus of 10403S to yield strain DH-L1039. Additional constructs harbouring deletions within the *hly* 5′ UTR were constructed and placed under transcriptional control of the HyperSPO1 promoter to yield progressive deletions of the *hly* 5′ UTR sequence fused to *gfp*, generating strains DH-L1118, DH-L1119 and DH-L1041 respectively (Fig. 6A). The effect of progressive deletions of the 5′ end of the *hly* 5′ UTR on GFP expression was determined. Using Western blot analysis, fusion of the complete *hly* 5′ UTR to *gfp* (strain DH-L1039) resulted in a significant increase in GFP expression levels compared with strain DH-L1041,

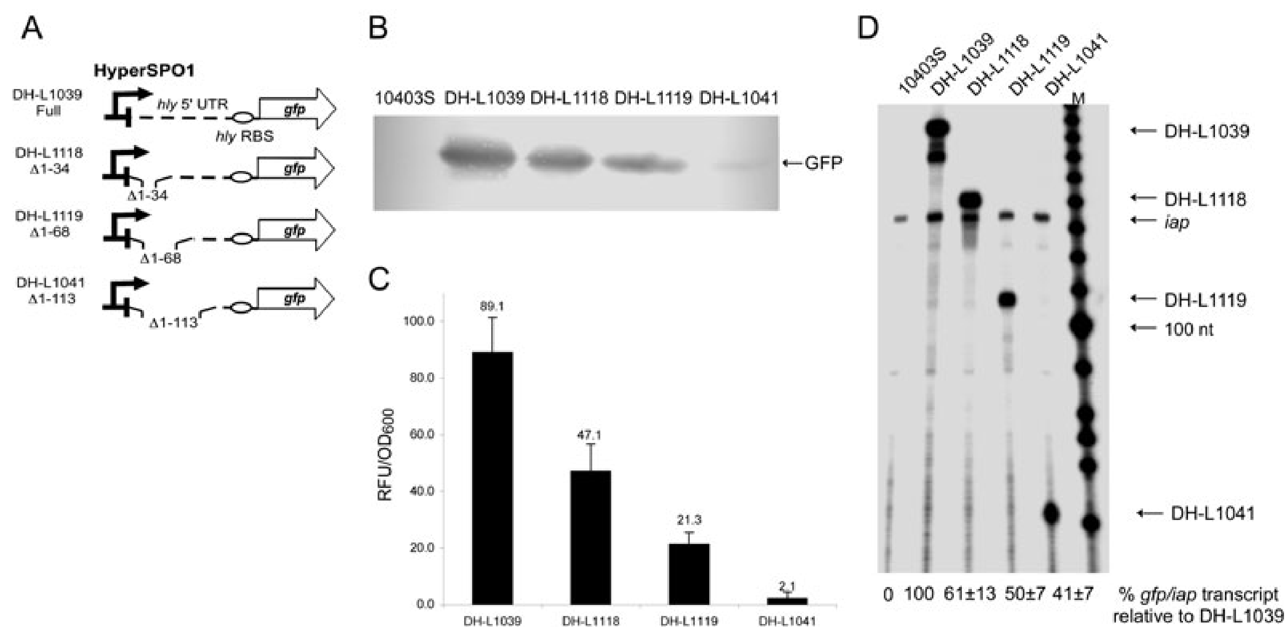

Fig. 6. *hly* 5′ UTR-mediated enhancement of GFP expression.
A. Schematic representation of HyperSPO1 promoter-controlled *gfp* strains harbouring various lengths of the *hly* 5′ UTR sequence. The dark arrow, dashed line, oval and open block arrow denote the HyperSPO1 promoter, *hly* 5′ UTR sequence, *hly* RBS and *gfpmut2* coding region respectively. The regions deleted within the *hly* 5′ UTR are indicated relative to the P2 transcription initiation site of *hly*.
B. Western blot analysis of GFP expression. Fourteen to 16 h cultures of wild type (10403S) and the HyperSPO1 promoter-controlled *gfp* strains were diluted 1:10 in BHI broth and grown for 3 h at 37°C. Aliquots of cultures were centrifuged and cell pellets were resuspended and digested with lysozyme. Protein samples from a culture volume equivalent to 100 μl of an OD_{600} = 1.0 were separated by SDS-PAGE and analysed by Western blot using a rabbit polyclonal anti-GFP antibody.
C. Fluorimetric analysis of GFP expression. Aliquots of cultures used in B were pelleted and resuspended in PBS. Relative fluorescence units of cultures was measured as described in *Experimental procedures* and normalized against OD_{600} (RFU/OD_{600}).
D. Primer extension analysis of *gfp* transcript levels. Total RNA was isolated from aliquots of cultures used in B. 7.5 μg of RNA was hybridized to a ^{32}P-end-labelled primer specific for *gfp*. Primer extension was performed as described in *Experimental procedures.* A radiolabelled primer specific to *iap* was simultaneously hybridized to total RNA as an internal load control. Identities of extended products are indicated. Lane M is a radiolabelled size ladder. Band intensities of extended transcripts were quantified by phosphorimager analysis. Numerical values indicate the percent *gfp/iap* transcript relative to DH-L1039 and represent the means and standard deviations of three independent experiments.

which harbours a 113 bp deletion of the *hly* 5′ UTR sequence (Fig. 6B). The increase in GFP protein correlated with a substantial increase in fluorescence intensity of bacteria as determined by fluorimetric analysis. Cultures of DH-L1039 generated a 43-fold higher fluorescence intensity (89 ± 12 units) compared with DH-L1041 (2 ± 2 units) (Fig. 6C). Progressive truncations from the 5′ end of the *hly* 5′ UTR resulted in concomitant decreases in GFP protein and associated fluorescence of bacteria. Fluorescence intensity of DH-L1118 (47 ± 9 units) and DH-L1119 (21 ± 4 units) were decreased approximately two- and fourfold, respectively, compared with DH-L1039 (89 ± 12 units) (Fig. 6C).

Primer extension analysis of *gfp* transcripts indicated that progressive truncation from the 5′ end of the *hly* 5′ UTR resulted in a general decrease in *gfp* transcript levels (Fig. 6D), while confirming that transcription of *gfp* initiated at the predicted sites (Fig. 6A). However, *gfp* transcript levels were not directly proportional to GFP protein levels. Whereas strain DH-L1119 produced more GFP protein and 10-fold higher fluorescence than DH-L1041 (Fig. 6B and C), *gfp* transcript levels remained similar within the two strains (Fig. 6D). Furthermore, while fluorescence intensity increased ~43-fold, respectively, in DH-L1039 compared with DH-L1041 (Fig. 6C), *gfp* transcript levels were only increased ~2.5-fold in DH-L1039 compared with DH-L1041 (Fig. 6D). This result suggests that the mechanism responsible for the significant enhancement of GFP protein production mediated by the *hly* 5′ UTR does not predominantly involve an increase in transcript levels. Fusion of full-length and truncated *hly* 5′ UTR fragments to the reporter genes *prfA* and *gus*, which encodes β-glucuronidase, yielded similar results. Significant decreases in protein levels were observed upon progressive deletion of the *hly* 5′ UTR from the 5′ end, while decreases in transcript levels were not directly proportional to the decrease in protein levels (data not shown). Collectively, these results indicated that the *hly* 5′ UTR can function *in cis* to enhance expression of a heterologous gene product through a mechanism that predominantly functions at a post-transcriptional level.

Discussion

Previous studies have implicated the 5′ UTRs of *actA* and *prfA* in regulating expression of their respective gene products during intracellular infection by *L. monocytogenes* (Johansson *et al.*, 2002; Wong *et al.*, 2004). In addition, a recent report has determined that the 5′ UTR of *inlAB* functions at a post-transcriptional level to control production of InlA and InlB, which mediate *L. monocytogenes* invasion of non-professional phagocytic host cells (Stritzker *et al.*, 2005). In our current study, we have demonstrated a role for the *hly* 5′ UTR in modulating LLO expression levels. Although deletion of the *hly* 5′ UTR had a moderate effect on LLO production during growth in broth culture, our results indicate that the *hly* 5′ UTR naturally functions to enhance LLO production during intracellular growth to levels required for full virulence of *L. monocytogenes* during *in vivo* infection. During infection of J774 cells, the *hly* 5′ UTR mutant, DH-L1231, which lacks the majority of the *hly* 5′ UTR, but retains the *hly* RBS, produced ~55% less secreted LLO and ~78% less bacterial-associated LLO relative to wild-type 10403S (Fig. 3A and B). This decrease in intracellular LLO production correlated with a 32% defect in plaque size formation during infection of L2 fibroblasts (Fig. 4B) and a 10-fold reduction in virulence in a mouse model of infection. Prior studies from our group have demonstrated that decreases in production of LLO during intracellular infection result in concomitant decreases in plaque size during infection of L2 fibroblasts (Dancz *et al.*, 2002). As no defect in intracellular growth rate was observed for strains harbouring deletions of the *hly* 5′ UTR sequence (Fig. 4A), our data suggest that intracellular LLO levels produced by a strain harbouring a deletion within the *hly* 5′ UTR are inadequate to allow efficient secondary vacuolar escape subsequent to cell-to-cell spread.

The two- to fivefold reduced levels of LLO observed in the *hly* 5′ UTR deletion mutant relative to wild type during intracellular growth correlated with an approximately fourfold decrease in *hly* transcript levels in the DH-L1231 mutant strain compared with wild type (Fig. 3C). During growth in BHI broth, *hly* transcript levels were also reduced by ~3.5-fold in DH-L1231 relative to 10403S (Figs 2C and 3C), although LLO levels were only mildly diminished (1.2- to 1.6-fold) in the *hly* 5′ UTR mutant compared with wild type (Fig. 2B). As *hly* transcription increases significantly upon intracellular growth (Moors *et al.*, 1999b), these results may indicate that the *hly* 5′ UTR increases LLO production slightly for each *hly* transcript produced, perhaps by stabilizing *hly* mRNA from degradation, and that upon upregulation of *hly* transcription, the effect of the *hly* 5′ UTR becomes amplified to result in marked changes in intracellular LLO levels. Alternatively, transcripts containing the full-length *hly* 5′ UTR may be more efficiently translated during intracellular infection relative to extracellular growth.

Although deletion of the *hly* 5′ UTR did not appear to affect PrfA-mediated activation of P_{hly} during intracellular infection (Fig. 3C), it remained possible that the DNA sequences constituting the *hly* 5′ UTR might participate in PrfA-dependent activation of the *hly* promoter. Therefore, we uncoupled transcription of *hly* from PrfA-dependence and placed transcription under the control of the constitutive HyperSPO1 promoter. Deletion of 113 bp of the *hly* upstream region within DH-L936 resulted in a twofold decrease in LLO protein and haemolytic activity

produced in broth culture relative to DH-L911, a HyperSPO1 promoter-controlled *hly* strain containing the entire *hly* 5′ UTR sequence (Fig. 5). Furthermore, truncations from the 5′ end of the *hly* 5′ UTR resulted in concomitant decreases in LLO production (Fig. 5), indicating that sequences along the entire *hly* 5′ UTR are involved in regulating LLO expression when *hly* is transcribed from the HyperSPO1 promoter. Because initiation of transcription from the HyperSPO1 promoter is presumably unaffected by *hly* 5′ UTR sequences, the differences in *hly* transcript levels observed in all strains harbouring deletions of the *hly* 5′ UTR, independent of the promoter, indicate that the *hly* 5′ UTR likely alters gene expression through a post-transcriptional mechanism. Taken together, our results suggest that the *hly* 5′ UTR may have evolved to maximize expression of LLO, perhaps by increasing the stability of native *hly* transcripts.

In addition to demonstrating a role for the *hly* 5′ UTR in regulating LLO expression, we have also shown that the *hly* 5′ UTR can function to enhance expression of other *cis*-associated genes. Fusion of the *hly* 5′ UTR to *gfp* resulted in a 43-fold increase in GFP fluorescence when the *hly* 5′ UTR-*gfp* fusion was transcribed from the HyperSPO1 promoter (Fig. 6C). Furthermore, we observed that the *hly* RBS was not specifically required for the *hly* 5′ UTR to enhance heterologous gene expression as an alternate RBS for initiation of translation could be substituted (data not shown). These results demonstrate that the *hly* 5′ UTR is sufficient to indiscriminately increase expression of downstream *cis*-associated gene products. Interestingly, the presence of the *hly* 5′ UTR enhanced expression of heterologous genes to a greater extent than for the native *hly* gene, even though the presence of the *hly* 5′ UTR increased transcript levels similarly regardless of the downstream coding sequence (Figs 5 and 6). Taken together, these results may suggest that the *hly* 5′ UTR enhances gene expression through two mechanisms, acting at a post-transcriptional and translational level. Alternatively, the ability of downstream coding sequences to modulate *hly* 5′ UTR-mediated enhancement of gene expression may suggest that sequences within the *hly* gene itself may dampen the full enhancement effect of the *hly* 5′ UTR. These internal sequences may function to reduce the translational efficiency of *hly* transcripts harbouring the *hly* 5′ UTR to prevent production of LLO to levels that may be toxic to host cells during intracellular infection, yet still allow for sufficient LLO to mediate optimal escape from secondary spreading vacuoles. This adaptation would be analogous to the presence of a PEST-like sequence within LLO that modulates intracellular protein levels to prevent toxicity to host cells (Decatur and Portnoy, 2000).

An increasing number of studies have demonstrated the importance of 5′ UTRs in regulating gene expression through diverse mechanisms (Grundy and Henkin, 2004). In this report, we have demonstrated that the *hly* 5′ UTR enhances expression of LLO during intracellular infection and have shown the ability of the *hly* 5′ UTR to enhance expression of *cis*-associated heterologous genes. The precise mechanism by which the *hly* 5′ UTR is able to mediate this effect remains to be determined, although sequences comprising the entire *hly* 5′ UTR appear to be required for maximal enhancement of protein expression. Furthermore, the mechanism by which the *hly* 5′ UTR enhances gene expression appears to be temperature and PrfA-independent, as the *hly* 5′ UTR was able to enhance expression of reporter genes expressed from the HyperSPO1 promoter independent of both growth temperature and the presence of PrfA (data not shown). Moreover, the ability of the *hly* 5′ UTR to enhance gene expression does not appear to be a general phenomenon, as the 5′ UTR of *L. monocytogenes mpl* (Vazquez-Boland *et al.*, 2001) or an artificial 5′ UTR fused to heterologous genes did not result in increased gene expression (data not shown). Lastly, we have taken advantage of the ability of the *hly* 5′ UTR sequence to indiscriminately increase expression of *cis*-associated gene products to achieve high-level expression of single-copy, chromosomal gene fusions in *L. monocytogenes*. Strains harbouring deletions of both *hly* and *prfA* could be complemented, independent of PrfA-dependent transcriptional activation, to expression levels greater than those observed for wild-type bacteria by placing *hly* 5′ UTR gene fusions under transcriptional control of the HyperSPO1 promoter (Fig. 5 and data not shown). The ability to complement virulence gene expression independent of additional regulatory factors will prove beneficial in determining the temporal or expression level requirements of *L. monocytogenes* virulence determinants during intracellular infection (Dancz *et al.*, 2002; Gründling *et al.*, 2003). Future studies to determine the role of *hly* 5′ UTR secondary structure in regulating LLO levels during extracellular growth and intracellular infection, as well as assessing the effect of the *hly* 5′ UTR on transcript stability, will yield further insights into the functional mechanism.

Experimental procedures

Bacterial and eukaryotic cell growth conditions

Bacterial strains used in this study are listed in Table 1. *L. monocytogenes* cultures were grown at 30°C in BHI broth without agitation. Where indicated, *L. monocytogenes* cultures were subsequently diluted and grown at 37°C with shaking prior to experimental analysis. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium at 37°C with shaking. All bacterial stains were stored at −80°C in BHI or LB medium supplemented with 40% glycerol. Antibiotics were used at the following concentrations: carbenicillin,

Table 1. Strains used in this study.

Strain	Description	Source or reference
L. monocytogenes strains		
10403S	Wild-type strain	Bishop and Hinrichs (1987)
DP-L2161	10403S Δ*hly*	Jones and Portnoy (1994)
DH-L1231	10403S with +13 to +113 of the *hly* 5′ UTR deleted	This study
DH-L911	DP-L2161 with pH-*hly* PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L934	DP-L2161 with pH-$hly_{\Delta 1\text{-}34}$-PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L935	DP-L2161 with pH-$hly_{\Delta 1\text{-}68}$-PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L936	DP-L2161 with pH-$hly_{\Delta 1\text{-}113}$-PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L1039	10403S with pH-*hly gfp*-PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L1118	10403S with pH-$hly_{\Delta 1\text{-}34}$-*gfp*-PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L1119	10403S with pH-$hly_{\Delta 1\text{-}68}$-*gfp*-PL3 integrated into the $tRNA^{Arg}$ locus	This study
DH-L1041	10403S with pH-$hly_{\Delta 1\text{-}113}$-*gfp*-PL3 integrated into the $tRNA^{Arg}$ locus	This study
E. coli strains		
DH-E123	pCON1 in JM109	Freitag (2000)
DH-E182	XL1-Blue [F′ *proAB* $lacI^{q}$ Δ(*lacZ*)*M15* Tn*10*] *recA1 endA1 gyrA96 thi-1 hsdR17 supE relA1 lac*	Stratagene
DH-E1232	*hly* Δ13-113-pCON1 in XL1-Blue	This study
DH-E375	CLG190 (F′ *lac pro* $lacI^{q}$) Δ(*malF*)3 Δ(*phoA*) PvuII *phoR* Δ(*lac*)X74 Δ(*ara leu*)7697 *araD*139 *galE galK pcnB zad*::Tn*10 recA*; Str^{r}	D. Boyd
DH-E474	SM10 {F^- *thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44* $M\mu^+$ C λ^- [*RP4-2* (Tc::Mu)] Km^r tra^+}	Simon *et al.* (1983)
DH-E899	pHPL3 in XL1-Blue	Gründling *et al.* (2004)
DH-E897	pH-*hly* PL3 in XL1-Blue	This study
DH-E937	pH-$hly_{\Delta 1\text{-}34}$-PL3 in XL1-Blue	This study
DH-E938	pH-$hly_{\Delta 1\text{-}68}$-PL3 in CLG190	This study
DH-E939	pH-$hly_{\Delta 1\text{-}113}$-PL3 in CLG190	This study
DH-E1038	pH-*hly gfp*-PL3 in XL1-Blue	This study
DH-E1121	pH-$hly_{\Delta 1\text{-}34}$-*gfp*-PL3 in XL1-Blue	This study
DH-E1122	pH-$hly_{\Delta 1\text{-}68}$-*gfp*-PL3 in XL1-Blue	This study
DH-E1075	pH-$hly_{\Delta 1\text{-}113}$-*gfp*-PL3 in XL1-Blue	This study
DH-E846	p2RGFP in XL1-Blue	This study

100 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1} for selection of pPL2-derived plasmids in *E. coli*, and 7.5 μg ml^{-1} or 10 μg ml^{-1} for selection of integrated pPL2 or pCON1 derivatives in *L. monocytogenes* respectively. L2 mouse fibroblast cells were grown in RPMI supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 2 mM glutamine (Mediatech, Herndon, VA). BMM were cultured as described (Portnoy *et al*., 1988). Eukaryotic cell cultures were maintained at 37°C in a 5% CO_2-air atmosphere.

Construction of a L. monocytogenes *strain containing a deletion within the* hly *5′ UTR*

Primers #42 and #343 (Table 2) and genomic DNA from wild-type *L. monocytogenes* (10403S) were used to polymerase chain reaction (PCR)-amplify an ~1 kb product harbouring the 5′ portion of the *hly* gene. Primers #342, #398 and 10403S genomic DNA were used in a PCR reaction to generate an ~700 bp product harbouring a 3′ portion of *hly*. The 5′ and 3′ PCR products were gel purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA), and used as templates for a splicing by overlap extension (SOE) PCR reaction (Horton *et al*., 1989). The flanking primers #42 and #398 were used to amplify a 1.7 kb PCR product containing a deletion of 101 bp of the *hly* 5′ UTR sequence (+13 to +113 relative to the native P2 *hly* transcription initiation site). The SOE PCR product was gel purified, digested with XbaI and KpnI and then ligated with plasmid pCON1 digested with the same restriction enzymes. The resulting plasmid, *hly* Δ13–113-pCON1, was confirmed using automated sequencing, introduced into 10403S by electroporation and allelic exchange was performed as previously described (Camilli *et al*., 1993) to generate strain DH-L1231.

Haemolytic-activity assays

Fourteen to 16 h cultures of *L. monocytogenes* strains grown in BHI were diluted 1:10 in BHI broth and grown for 5 h at 37°C. The optical density at 600 nm (OD_{600}) was determined. One millilitre aliquots were removed and the haemolytic activity present in culture supernatants was determined as described (Dancz *et al*., 2002). Haemolytic units were defined as the reciprocal of the dilution of culture supernatant that yielded 50% lysis of sheep red blood cells.

Western blot analysis of bacteria grown in BHI broth

Fourteen to 16 h cultures of *L. monocytogenes* strains grown in BHI broth were diluted 1:10 in BHI or BHIC broth and grown for 5 h at 37°C. The OD_{600} of cultures was determined. For cell pellet fractions (CP), 1 ml aliquots were removed and centrifuged. The supernatant was removed for haemolytic activity determination as described, while the bacterial cell pellets were incubated in TE-lysozyme (2 mg ml^{-1} lysozyme) buffer at 37°C in a total volume of 100 μl. Following digestion for 2 h, 100 μl of 2× final sample buffer (FSB) was added to

Table 2. Oligonucleotides used in this study.

Number	Sequence	Site[a]
42	CCTCTAGACGGGGAAGTCCATGATTAGTATGCC	XbaI
68	GCAGATGCATCCTTTGCTTCAGTTTG	–
133	CGCAGCAAATGCTGTTACCGCAATCCCAGCTGTAGCCGCG	–
168	AGATACCGGCCGATAAAGCAAGCATATAATATTGCGTT	EagI
169	AGATACCGGCCGAGAAGCGAATTTCGCCAATATTATAATTAT	EagI
205	AGATACCGGCCGAGAGAGGGGTGGCAAACGGTATT	EagI
221	AAGTCGACTTATTTGTATAGTTCATCCATGCCATG	SalI
235	AGATACCGGCCGGTAGAAGGAGAGTGAAACCC	EagI
245	GTGCGTCGTAAATAAATCTTATACAA	–
250	CGCGGATCCATTTACGAAGAGTGCAAAACAAGC	BamHI
341	ACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCAT	–
342	ATAAAGCTATAAAGCAAGCAGTAGAAGGAGAGTGAAACCC	–
343	GGGTTTCACTCTCCTTCTACTGCTTGCTTTATAGCTTTAT	–
344	GTAGAAGGAGAGTGAAACCCATGAGTAAAGGAGAAGAACT	–
345	AGTTCTTCTCCTTTACTCATGGGTTTCACTCTCCTTCTAC	–
398	GGGGTACCCTTTAAATGCTGTACCAAATTTCGC	KpnI

a. The indicated restriction endonuclease site is underlined within the oligonucleotide sequence.

CP samples. For analysis of supernatant fractions (SN), 1.2 ml aliquots were centrifuged and 1 ml of the resulting supernatant was incubated with 100 μl of 100% trichloroacetic acid (TCA) on ice. Proteins were TCA-precipitated for 1 h and then centrifuged at 13 000 *g* for 10 min. Protein samples were resuspended in 200 μl of 1× FSB containing 0.1 N NaOH. Samples in FSB were boiled for 5 min at 95°C, centrifuged for 5 min at 13 000 *g* and loaded onto denaturing 10% or 12% polyacrylamide gels. Protein samples from a culture volume equivalent to 100 μl (CP) or 50 μl (SN) of an $OD_{600} = 1.5$ were analysed for LLO. Protein samples from a culture volume equivalent to 100 μl of an $OD_{600} = 1.0$ were analysed for GFP. Western blotting was performed using rabbit polyclonal anti-LLO (Dancz *et al.*, 2002) or Living Colors A.v. peptide anti-GFP antibodies (Clontech, Palo Alto, CA). Densitometry analysis was performed using ImageQuant TL software (Amersham Biosciences, Piscataway, NJ).

RNA isolation

Fourteen to 16 h cultures of *L. monocytogenes* grown in BHI broth were diluted 1:10 in BHI broth and grown for 5 h at 37°C, while strains used for *gfp* transcript analysis were grown for 3 h at 37°C. Total RNA was isolated from 6 ml aliquots of cultures using the FastRNA ProBlue kit (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions.

Primer extension analysis

Oligonucleotide primers #68, #245, #341 and #133 were used for primer extension analysis of *hly*, *plcA, gfp* and *iap* transcripts respectively. Primer extension was performed essentially as described by (Gründling *et al.*, 2004) with the following modifications. T4 polynucleotide kinase was heat-inactivated by incubating the kinase reaction at 70°C for 10 min; 7.5 μg total RNA was used in each extension reaction, with the exception of Fig. 3C, where 15 μg total RNA was used for samples isolated in BHI broth, and 3 μg total RNA was used for samples isolated from bacteria grown intracellularly. Extended transcripts were quantified by phosphorimager analysis (Molecular Imager, Bio-Rad, Hercules, CA, and Typhoon Imager, Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation of secreted LLO during infection of J774 cells

Approximately 2.2×10^6 J774 cells were seeded on tissue-culture treated 60 mm dishes and incubated for 12–15 h. Monolayers of J774 cells were then infected in duplicate with 2.5 μl (~5×10^6 bacteria) of a PBS-washed 14–16 h culture of *L. monocytogenes* in 6 ml of DMEM. After 1 h of infection, the monolayer was washed once with PBS, pH 7.1, and 6 ml of DMEM with 5 μg ml^{-1} gentamicin was added. At 5.5 h after infection, cells were washed once with PBS, pH 7.1, and starved in methionine-free DME (Met-DME) containing 7.5% FBS, 5 μg ml^{-1} gentamicin, 30 μg ml^{-1} anisomycin, 100 μg ml^{-1} cycloheximide, and 50 μM LLnL. After 30 min, one monolayer was pulse-labelled for 1 h with 200 μCi of ^{35}S-methionine (EasyTag protein labelling mix, Perkin Elmer, Boston, MA) in 450 μl of Met-DME, while the second monolayer was mock-labelled with cold methionine at 8.5 μM in 450 μl of Met-DME for subsequent colony-forming unit (cfu) determination. Monolayers were then washed, and lysed in 0.5 ml of ice-cold RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.1% SDS] containing Complete protease inhibitor mixture (Roche, Indianapolis, IN). Nuclear insoluble material was removed by pulsing host cell lysates for 2 s at 13 000 *g*, and bacteria were isolated from the resulting supernatant by centrifugation for 7 min at 13 000 *g*. For non-radioactive samples, the resulting pellet was resuspended in 40 μl of PBS and cfu were determined. For radioactive samples, the supernatant was transferred to 250 μl of a 50% Protein A-Sepharose bead slurry preconjugated to monoclonal anti-LLO antibody B3-19. LLO was immunoprecipitated as previously described (Moors *et al.*, 1999b). Immunoprecipitated samples were boiled and resolved on an 8% polyacrylamide gel by SDS-PAGE. The resulting gel was exposed to a phosphorimager screen for 7 days and quanti-

fied by phosphorimager analysis (Typhoon Imager, Amersham Biosciences, Piscataway, NJ).

Western blot analysis and RNA isolation of bacteria grown in J774 cells

Approximately 5.0×10^6 J774 cells were seeded on tissue-culture treated 100 mm Petri dishes 12–15 h prior to infection. Monolayers of J774 cells were then infected in duplicate with 5.0 μl (~1×10^7 bacteria) of a PBS-washed 14–16 h culture of *L. monocytogenes* in 10 ml of DMEM. After 1 h of infection, the monolayers were washed once with PBS, pH 7.1, and 10 ml of DMEM with 5 μg ml^{-1} gentamicin was added. At 7 h post infection, 1 ml of ABT buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl, 0.1% Triton X-100, pH 7.0) was added to lyse J774 cells. Nuclear insoluble material was removed by pulsing the host cell lysates for 2 s at 13 000 *g*, and the bacteria were pelleted from the resulting supernatant by centrifuging for 7 min at 13 000 *g*. For one monolayer, the bacterial cell pellet was processed for RNA isolation using the FastRNA ProBlue kit (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions. For the second monolayer, the bacterial cell pellet (CP) was resuspended in TE-lysozyme (2 mg ml^{-1} lysozyme) buffer at 37°C in a total volume of 40 μl, and 2.5 μl was removed for cfu determination. Following digestion for 30 min at 37°C, 40 μl of 2× FSB was added to CP samples. Samples in FSB were boiled for 5 min at 95°C, centrifuged for 5 min at 13 000 *g*, and a sample equivalent to 2×10^8 cfu was loaded onto a 10% denaturing polyacrylamide gel. Western blot analysis was performed using monoclonal anti-LLO antibody (B3-19). Densitometry analysis was performed using ImageQuant TL software (Amersham Biosciences, Piscataway, NJ).

Intracellular growth of L. monocytogenes *in murine BMM*

Bone marrow from the femurs of adult BALB/c mice was isolated and BMM cultured as described (Portnoy *et al*., 1988). Approximately 6.6×10^5 bacteria were used to infect 2×10^6 BMM cells seeded 18 h prior onto circular 12 mm glass coverslips placed in a 60 mm Petri dish. Infection of BMM was performed in DMEM (Mediatech, Herndon, VA) supplemented with 7.5% fetal bovine serum (HyClone, Logan, UT) and 2 mM glutamine (Mediatech, Herndon, VA). Thirty minutes after addition of bacteria, BMM were washed with PBS, and DMEM containing 10 μg ml^{-1} gentamicin was added. Three coverslips were removed at appropriate time intervals and separately placed in 5 ml of sterile dH_2O in a 15 ml conical tube. Conical tubes were vortexed for 15 s to lyse BMM and dilutions of lysates were plated on LB-agar to determine the number of intracellular bacteria.

Plaque formation assays in L2 fibroblasts and determination of LD_{50} values

Assays of plaque formation within L2 cell monolayers were performed as described (Dancz *et al*., 2002). The average diameter of 15 plaques/sample for three independent experiments was determined. LD_{50} values were determined as described (Barry *et al*., 1992).

Construction of HyperSPO1 promoter-controlled hly *strains*

To construct DH-L911, harbouring the full-length *hly* 5′ UTR sequence fused to *hly*, primer pair #168 and #250 was used to PCR-amplify the *hly* gene from 10403S genomic DNA. The resulting PCR product was digested with EagI and BamHI and ligated with the pPL2-derived vector pHPL3 (Lauer *et al*., 2002; Gründling *et al*., 2004) digested with the same restriction enzymes to yield plasmid pH-*hly* PL3. To construct plasmid vectors lacking portions of the *hly* 5′ UTR sequence, each of the following forward primers, #169, #205 or #235, was used separately with reverse primer #250 and pH-*hly* PL3 as the template to PCR-amplify a DNA fragment containing the entire *hly* coding sequence with varying lengths of upstream DNA sequences. The resulting PCR products were digested with EagI and BamHI and ligated with pHPL3 digested with the same restriction enzymes. The sequence of all DNA inserts was confirmed by automated sequencing. The resulting plasmid from ligation of the PCR product generated using primer pair #169 and #250, pH-$hly_{\Delta 1-34}$-PL3, was transformed into *E. coli* strain XLI-Blue. Triparental mating (Lauer *et al*., 2002) was used to transfer pH-*hly* PL3 and pH-$hly_{\Delta 1-34}$-PL3 into DP-L2161. The resulting plasmid from ligation of the PCR product generated using primer pair #205 and #250, pH-$hly_{\Delta 1-68}$-PL3, was transformed into *E. coli* strain CLG190. The resulting plasmid from ligation of the PCR product generated using primer pair #235 and #250, pH-$hly_{\Delta 1-113}$-PL3, was also transformed into CLG190. pH-$hly_{\Delta 1-68}$-PL3 and pH-$hly_{\Delta 1-113}$-PL3 were isolated from CLG190 strains using the Qiagen Midiprep kit (Qiagen, Valencia, CA), dialysed, and transformed into electrocompetent DP-L2161 by electroporation. Integration of plasmid vectors onto the chromosome was selected as described (Lauer *et al*., 2002).

Construction of HyperSPO1 promoter-controlled gfp *strains*

Fusion of *hly* 5′ UTR sequences to *gfpmut2* was performed using SOE PCR. Primers #168 and #345 were used with genomic DNA from 10403S to generate the 5′ PCR fragment, while primers #344 and #221 were used to amplify a 3′ PCR fragment from plasmid p2RGFP harbouring the *gfpmut2* gene. The resulting PCR products were gel purified and used as templates in an SOE PCR reaction using the flanking primers #168 and #221. The final SOE PCR product containing the entire *hly* 5′ UTR sequence fused to the initiating codon of *gfpmut2* was gel purified, digested with EagI and SalI, and ligated with the pHPL3 vector digested with the same restrictions enzymes. The resulting plasmid, pH-*hly gfp*-PL3, was used as a template in PCR reactions to generate *gfp* fusions to *hly* 5′ UTRs of decreasing length. Forward primers #169, #205, or #235 were used separately in PCR reactions with reverse primer #221 and pH-*hly gfp*-PL3. PCR products were gel purified, digested with EagI and SalI, and ligated to pHPL3 digested with the same restriction enzymes to generate plasmids

pH-*hly*$_{\Delta 1-34}$-*gfp*-PL3, pH-*hly*$_{\Delta 1-68}$-*gfp*-PL3 and pH-*hly*$_{\Delta 1-113}$-*gfp*-PL3. pH-*gfp*-PL3 plasmid derivatives were verified by automated sequencing and transformed into electrocompetent 10403S by electroporation.

Analysis of GFP expression

Western blot analysis of GFP levels was performed as described, with the exception that cultures were grown for 3 h instead of 5 h. The relative fluorescence values of cultures used for Western blot analysis were also determined. One millilitre aliquots were centrifuged and bacterial pellets resuspended in 1 ml of PBS, pH 7.1. Triplicate aliquots of 200 μl were transferred to a black, polystyrene, flat bottom 96 well plate (Corning, Corning, NY) and GFP fluorescence activity determined using a SpectraMAX GeminiXS fluorimeter (Molecular Devices, Sunnyvale, CA). An excitation wavelength of 488 nm, an emission range of 525 nm, and an auto cut-off of 515 nm were used to minimize interfilter effects. Relative fluorescence of samples was determined by measuring the background fluorescence of 10403S and subtracting this value from the fluorescence units measured for 10403S strains expressing GFP to yield relative fluorescence units (RFU). RFU were normalized against OD_{600} to give RFU/OD_{600}.

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