APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2010, p. 5124–5139 Vol. 76, No. 15
0099-2240/10/$12.00 doi:10.1128/AEM.03107-09

Complementation of *Listeria monocytogenes* Null Mutants with Selected *Listeria seeligeri* Virulence Genes Suggests Functional Adaptation of Hly and PrfA and Considerable Diversification of *prfA* Regulation in *L. seeligeri*▽†

Courtney R. Lucas Stelling,[1,2] Renato H. Orsi,[2] and Martin Wiedmann[2]*

Graduate Field of Microbiology[1] and Department of Food Science,[2] Cornell University, Ithaca, New York 14853

Received 23 December 2009/Accepted 30 May 2010

While *Listeria seeligeri* and *L. monocytogenes* contain the main *Listeria* virulence gene cluster, only *L. monocytogenes* is considered an intracellular pathogen. Initial evolutionary analyses showed that the virulence genes *prfA*, *hly*, and *plcA* are conserved in *L. seeligeri*, with specific Hly and PrfA amino acid residues showing evidence for positive selection in *L. seeligeri*. Our data also show that temperature-dependent transcript patterns for *prfA*, which encodes a transcriptional regulator of virulence genes, differed between *L. monocytogenes* and *L. seeligeri*. To further investigate the divergence of virulence gene function and regulation, *L. seeligeri prfA* ($prfA_{LS}$), *hly* (hly_{LS}), and *plcA* ($plcA_{LS}$), as well as $prfA_{LS}$ constructs with different *prfA* promoter regions, were introduced into appropriate *L. monocytogenes* null mutants. Only when $prfA_{LS}$ was under the control of the *L. monocytogenes prfA* promoters (P1- and P2*prfA*) ($P1P2_{LM}$ $prfA_{LS}$) was $prfA_{LS}$ able to fully complement the $\Delta prfA_{LM}$ deletion. hly_{LS} introduced into an *L. monocytogenes* background under its native promoter showed transcript levels similar to those of hly_{LM} and was able to partially restore *L. monocytogenes* wild-type-level hemolysis and intracellular growth, even though Hly_{LM} and Hly_{LS} showed distinct patterns of cell- and supernatant-associated hemolytic activities. Our data indicate that (i) regulation of *prfA* expression differs between *L. monocytogenes* and *L. seeligeri*, although *hly* transcription is temperature dependent in both species, and (ii) PrfA and Hly functions are largely, but not fully, conserved between *L. seeligeri* and *L. monocytogenes*. Virulence gene homologues and their expression thus appear to have adapted to distinct but possibly related functions in these two species.

Listeria seeligeri is a putative nonpathogenic bacterial species in the genus *Listeria*, which includes the hemolytic species *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii* and the nonhemolytic species *L. welshimeri*, *L. innocua*, and *L. grayi*. *L. monocytogenes* causes disease in a wide range of species, including humans, while *L. ivanovii*, which affects predominantly sheep, has a narrow host range. Both of these species can also cause disease upon inoculation into laboratory animals (e.g., mice [16]) and can invade and intracellularly multiply in tissue culture cells (35). *L. seeligeri*, on the other hand, has generally not been found as a natural etiological agent of disease in animals or humans (47), does not cause disease in laboratory animals (25), and does not effectively invade and multiply in tissue culture cells (15, 25). While this species is generally considered nonpathogenic, some possible cases of human disease caused by *L. seeligeri* have been described, including a human meningitis case (48). *L. seeligeri* is commonly isolated from natural environments and has been found in some studies (51) to be the most common *Listeria* species isolated from these environments.

The apparent lack of virulence and pathogenicity for *L. seeligeri* is intriguing, as most *L. seeligeri* isolates appear to carry a homologue of the *Listeria prfA* virulence gene cluster (*p*VGC) (17, 63), which contains many of the genes required for virulence in both *L. monocytogenes* and *L. ivanovii* (29, 60). This virulence gene cluster is absent from the nonhemolytic, nonpathogenic *Listeria* spp. (i.e., *L. welshimeri*, *L. innocua*, and *L. grayi*) but is located between *prs* and *ldh* in *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri.* In *L. monocytogenes* and *L. ivanovii*, the *p*VGC is about 9 kb long and includes six major virulence genes critical for survival and multiplication inside mammalian host cells (60). In all three species this virulence gene cluster contains homologues of the *L. monocytogenes* virulence genes *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB* (29) (Fig. 1). *hly* encodes listeriolysin O (LLO) in *L. monocytogenes* and seeligeriolysin O (LSO) in *L. seeligeri*; this cholesterol-dependent cytolysin (CDC) is essential for rapid escape of *Listeria* from the host cell vacuole (1, 44). LSO has 81.5% amino acid (aa) identity to LLO, and similar transcriptional signals (including a PrfA box) upstream of *hly in L. monocytogenes* and *L. seeligeri* have been reported (18, 25). The products of *plcA* (a phosphatidylinositol-specific phospholipase C) and *plcB* (a broad-range phospholipase C) have accessory roles in escape from the host cell vacuole. *plcB*, in conjunction with *hly*, is essential for release of *Listeria* from the double-membrane vacuole that forms after cell-to-cell spread (44). In one *L. seeligeri* strain sequenced, the *p*VGC is 13 kb long and includes, in addition to six main virulence genes, a *plcB* duplication and several *L. seeligeri*-specific open reading frames (ORFs) (29). One of these ORFs, *orfE*, is located between *plcA* and *prfA* in

* Corresponding author. Mailing address: Department of Food Science, 412 Stocking Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 254-2838. Fax: (607) 254-4868. E-mail: mw16@cornell.edu.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 11 June 2010.

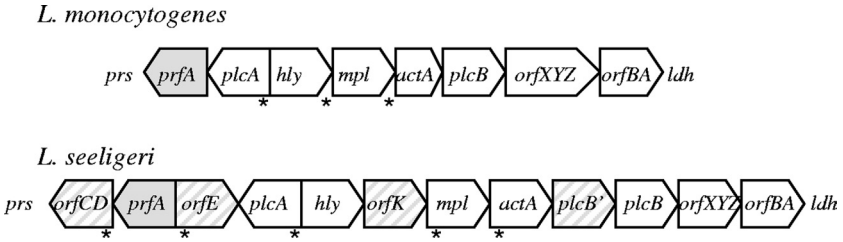

FIG. 1. Schematic of the *prfA* virulence gene cluster loci in *L. monocytogenes* and *L. seeligeri*. This cluster is located between *prs* and *ldh* in both species. *prfA*, which encodes the transcriptional regulator PrfA, is denoted by gray, and additional ORFs specific to *L. seeligeri* are denoted as stripes. Asterisks (*) denote the locations of PrfA boxes. Adapted from Kreft et al. (29).

L. seeligeri (29) and appears to be PrfA dependent. *orfE* transcription has been hypothesized to interfere with *prfA* transcription from the upstream *plcA* promoter, contributing to the inability of *L. seeligeri* to escape from the phagosome of mammalian cells (25, 29).

PrfA (positive regulatory factor A), which is also encoded by a gene (i.e., *prfA*) located in the *p*VGC, is a transcriptional regulator belonging to the Crp-Fnr family of transcriptional regulators. PrfA interacts with a 14-nucleotide (nt) palindrome DNA sequence known as the "PrfA box," located upstream of PrfA-regulated genes (14, 30, 55), including the virulence genes located in the *p*VGC. *L. seeligeri* PrfA has 73.4% amino acid identity to *L. monocytogenes* PrfA. With *L. monocytogenes*, regulation of PrfA has been shown to occur on several molecular levels (12, 13, 33, 46). At the transcriptional level, *prfA* expression is controlled by three promoter regions, including (i) two promoter regions directly upstream of *prfA* (the σ^A-dependent P1*prfA* promoter as well as P2*prfA*, which includes overlapping σ^A- and σ^B-dependent promoters), and (ii) a PrfA-dependent promoter located upstream of *plcA*, which produces a bicistronic *plcA-prfA* transcript (5). Regulation at the posttranscriptional level involves temperature-dependent translation of the P1*prfA* transcript through a hairpin that masks the Shine-Dalgarno sequence at temperatures of ≤30°C but is relieved at higher temperatures, thus facilitating higher PrfA levels at mammalian and avian body temperatures (13, 14). While it is known that PrfA can exist in active and inactive states, the mechanisms for this posttranslational regulation are still largely unknown (12, 61, 62).

Genome hybridization studies (11) and a recent genome analysis (57) have shown that *L. seeligeri* does not contain homologues of a number of internalin genes that are present in *L. monocytogenes* (e.g., *inlA* and *inlB*) and encode proteins important for invasion of different mammalian cell lines (11, 19, 39). While the absence of *inlA* and *inlB*, in particular, may explain why *L. seeligeri* has not been found to invade mammalian epithelial cell lines (11, 19, 25), additional differences between *L. seeligeri* and *L. monocytogenes* also appear to contribute to the apparent lack of virulence in *L. seeligeri*. For example, *in vitro* transcription data (33) in combination with complementation of an *L. monocytogenes prfA* null mutant with *L. seeligeri prfA* suggested that *L. seeligeri* PrfA ($PrfA_{LS}$) has a reduced ability to activate PrfA-dependent transcription in *L. monocytogenes* (compared to $PrfA_{LM}$). On the other hand, complementation of an *L. seeligeri* wild-type strain with a plasmid containing the *L. monocytogenes plcA-prfA* operon allowed expression and activation of *L. seeligeri hly*, enabling escape from the phagosome of enterocyte- and macrophage-like cells (25). While initial data thus indicate that virulence gene function and regulation differ between *L. monocytogenes* and *L. seeligeri*, our overall understanding of the diversification of virulence-associated functions between *L. monocytogenes* and *L. seeligeri* has remained limited. The goal of this study was to use a combination of (i) evolutionary analyses of *prfA*, *hly*, and *plcA*, (ii) transcriptional analyses of *prfA* and *hly* in *L. seeligeri* and *L. monocytogenes* grown under different conditions, and (iii) complementation studies involving introduction of *L. seeligeri prfA*, *hly*, and *plcA* into *L. monocytogenes* to further characterize diversification of virulence-related functions in the genus *Listeria*. We specifically focused on complementing an *L. monocytogenes* strain with selected *L. seeligeri* genes, as (i) *L. monocytogenes* contains other critical virulence genes (e.g., *inlA* and *inlB*) that are absent in *L. seeligeri*, thus allowing for evaluation of a wider range of phenotypes in complemented *L. monocytogenes* strains, and (ii) complementation of *L. seeligeri* with selected *L. monocytogenes* genes has previously been attempted (25).

MATERIALS AND METHODS

Sequencing of *L. seeligeri prfA*, *hly*, and *plcA*. To evaluate conservation and selection patterns, the *L. seeligeri* virulence genes *prfA*, *hly*, and *plcA* were PCR amplified and sequenced (see Table 1 for primers) from a set of 8 diverse *L. seeligeri* isolates (see Table 2 for isolates). Sequences were assembled and proofread using Seqman (DNAStar, Madison, WI) and were aligned in Megalign (DNAStar) using the Clustal W algorithm. PESTFind (https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm [49]) was used to identify PEST sequences in *hly*.

Positive selection analysis. Positive Darwinian selection at the DNA sequence level can be evaluated by estimating the ratio (ω) of the rate of nonsynonymous nucleotide substitutions (d_N) to that of synonymous substitutions (d_S) between homologous protein-coding gene sequences (38). An ω value of >1 suggests that the gene evolved by positive selection. Conversely, an ω value of <1 suggests that the gene evolved by negative selection, and an ω value of 1 indicates neutral evolution of the gene. Model 0 implemented in the program codeml in the software package PAML version 3.15 (66) was used to determine the average d_N/d_S ratio for *hly*, *prfA*, or *plcA* using the *L. seeligeri* sequences that were obtained here or were available in GenBank (Table 2).

Positive selection during divergence of *L. seeligeri* from other *Listeria* species (i.e., *L. monocytogenes* and *L. ivanovii*) was assessed using a branch site test (test 2) implemented in PAML 3.15 (67) as previously detailed (42). This test can detect positive selection affecting a small number of sites along prespecified branches in a phylogeny (67). These positive selection analyses were performed using alignments of *hly*, *plcA*, and *prfA* for *L. seeligeri* (Table 2), 40 *L. monocytogenes* isolates (42), and the one *L. ivanovii* isolate for which sequence data for these genes were available (strain NRRL 33021, GenBank accession no. AY510073 [64]).

Bacterial strains for mutant construction. *L. monocytogenes* 10403S (serotype 1/2a) and *L. seeligeri* FSL S4-039 were used as parent strains for mutant construction (Table 3). FSL S4-039 is an environmental isolate obtained from soil

TABLE 1. Primers used in this study[a]

Primer function or name	Sequence (5′→3′)
Amplification of *L. seeligeri hly*	
CRLLShlyF	GGGATCCGCATAGGAAAAATAATGGAGTAAACAGC
CRLLShlyR	GCGGCCGCTTATTTTATGGTGTGTGTGTTAAGCG
Amplification of *L. seeligeri plcA*	
CRLLSplcAF	GGGATCCGATTCCGAGATTTTTCGGATATATACTAG
CRLLSplcAR	GCGGCCGTCTCTCCCCTTCACTTTTTCATTCTTC
Amplification of *L. seeligeri prfA*	
CRLLSprfAF	GGGATCCTGAAACAATTAATAAAAAGCGCAAAAG
CRLLSprfAR	GCGGCCGCACATATTCCTTAAATTTTGCCTTACAAG
Amplification of *L. monocytogenes prfA*	
CRL10LMprfAFpstI	CAACTGCAGCGTACGCGTTCATGAAAATGCT
CRL2LMP1P2R	GCGGCCGGTTCGAGGATTAGGCATACTAATCATGG
Construction of the Δ*prfA* P1P2$_{LM356}$ *prfA*$_{LS}$ strain[b]	
CRL7LMP1P2F	GTCTCATCCCCCAATCGTTTTTTATCG
CRL8LSprfAR(2)	CGATAAAAAACGATTGGGGGATGATGTGAG
CRLLSprfAF	GGGATCCTGAAACAATTAATAAAAAGCGCAAAAG
Confirmation of pPL2 integration	
NC16	GTCAAAACATACGCTCTTATC
PL95	ACATAATCAGTCCAAAGTAGATGC
5′ RACE PCR for *L. seeligeri prfA*	
GSP1 CRL11LSprfA	TTATGAAAGCGCCTTTATAGTATTG
GSP2 CRL12LSprfA	TTCAGAATATCCCCGCTCTCAC
5′ RACE PCR for *L. monocytogenes prfA*	
GSP1 LM prfART	GCCTGCTCGCTAATGACTTCTA
GSP2 LM prfARACE	GGTCCCGTTCTCGCTAATACT

[a] *L. seeligeri* primers were designed using ATCC 35967 sequence data, and *L. monocytogenes* primers were designed using 10403S sequence data.
[b] Primers used to construct the Δ*prfA* P1P2$_{LM356}$ *prfA*$_{LS}$ strain include (i) CRL2LMP1P2R and CRL7LMP1P2F to amplify the *L. monocytogenes prfA* promoter region and (ii) CRL8LSprfAR(2) and CRLLSprfAF to amplify the *L. seeligeri prfA* coding sequence.

near Syracuse, NY, in 2001 (51). Previously constructed *L. monocytogenes* 10403S isogenic *prfA*, *hly*, and *plcA* null mutants (Table 3) were used as host strains for complementation experiments.

Construction of *L. monocytogenes* complementation mutants. Selected *L. seeligeri* virulence genes (i.e., *prfA*, *hly*, and *plcA*) were introduced into the appropriate *L. monocytogenes* 10403S null mutants by cloning these genes and their upstream promoter regions into the pPL2 integration vector, which integrates at tRNA^{Arg}-*attBB′* on the *L. monocytogenes* chromosome (31, 65). Briefly, PCR (primers listed in Table 1) was used to amplify the gene of interest and the appropriate promoter region for cloning into pPL2, and the pPL2 constructs were introduced into the appropriate *L. monocytogenes* mutant strains using electroporation as previously described (31). Strains generated through this approach include the *L. monocytogenes hly* and *plcA* null mutants complemented with *L. seeligeri hly* and *plcA* (including 314 and 52 nt upstream of the start codon of the respective gene); these strains are designated 10403S Δ*hly* tRNA^{Arg}::pPL2 *hly*$_{LS314}$ (Δ*hly* *hly*$_{LS314}$) and 10403S Δ*plcA* tRNA^{Arg}::pPL2 *plcA*$_{LS52}$ (Δ*plcA* *plcA*$_{LS52}$) (Table 3). In addition, we created five strains in which an *L. monocytogenes prfA* null mutant was complemented with different *prfA* alleles. Strain

TABLE 2. *L. seeligeri* isolates used for evolutionary analysis of *prfA*, *hly*, and *plcA*

Isolate no.[a]	Isolate source	Isolate source location	Sequence information was available and used for:			Source or reference for DNA sequence data
			prfA	*plcA*	*hly*	
FSL S4-035	Vegetation	Syracuse, NY	+	+	+[b]	This work
FSL S4-039	Soil	Syracuse, NY	+	+	+	This work
FSL S4-079	Vegetation	Adirondack Park, NY	+	+	+	This work
FSL S4-116	Soil	Finger Lakes National Forest, NY	+	+	+	This work
FSL S4-200	Vegetation	Catskills Park, NY	+	+	+	This work
FSL S4-212	Vegetation	Connecticut Hill, NY	+	+	+	This work
FSL S4-252	Soil	Albany, NY	+	+	+	This work
FSL S4-307	Vegetation	Catskills Park, NY	+	+	+[b]	This work
ATCC 35967[c]	Soil	Germany	+	+	+	63
SLCC 3379[c]	Soil	Germany	+	−	+	
NRRL 33019[c]	Soil	Germany	−	−	+	64

[a] Additional isolate information is available under the isolate number at www.pathogentracker.net.
[b] *hly* sequence was truncated; full gene sequence not used for positive selection analyses.
[c] Sequence data for these isolates were obtained from GenBank.

TABLE 3. Bacterial strains used in this study

Strain	Description (strain abbreviation used throughout the text)	Reference or source
10403S	*L. monocytogenes* parent strain	4
FSL S4-039	*L. seeligeri* wild-type strain	51
FSL B2-046	10403S Δ*prfA*[a]	34
FSL R3-003	10403S Δ*hly*	24
FSL R3-004	10403S Δ*plcA*	5
FSL L5-029	10403S Δ*plcA* tRNA^{Arg}::pPL2 $plcA_{LS52}$ (Δ*plcA* $plcA_{LS52}$ strain)	This work
FSL L5-030	10403S Δ*hly* tRNA^{Arg}::pPL2 hly_{LS314} (Δ*hly* hly_{LS314} strain)	This work
FSL L5-032	10403S Δ*prfA* tRNA^{Arg}::pPL2 $prfA_{LS157}$ (Δ*prfA* $prfA_{LS157}$ strain)	This work
FSL L5-162	10403S Δ*prfA* tRNA^{Arg}::pPL2 $prfA_{LS367}$ (Δ*prfA* $prfA_{LS367}$ strain)	This work
FSL L5-113	10403S Δ*prfA* tRNA^{Arg}::pPL2 $prfA_{LM356}$ (Δ*prfA* $prfA_{LM356}$ strain)	This work
FSL L5-160	10403S Δ*prfA* tRNA^{Arg}::pPL2 P*plcA* $prfA_{LM356}$ (Δ*prfA* P*plcA* $prfA_{LM356}$ strain)	This work[b]
FSL L5-112	10403S Δ*prfA* tRNA^{ARG}::pPL2 $P1P2_{LM356}$ $prfA_{LS}$ (Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain)	This work

[a] This strain carries a 339-bp in-frame deletion in *prfA*.

[b] The plasmid with the P*plcA* $prfA_{LM356}$ construct that was used to construct the pPL2 plasmid employed to generate this strain was first reported by Wong and Freitag (65).

10403S Δ*prfA* tRNA^{Arg}::pPL2 $prfA_{LS157}$ (here denoted as the Δ*prfA* $prfA_{LS157}$ strain) contains the $prfA_{LS}$ ORF and 157 nt upstream of the $prfA_{LS}$ start codon, while the Δ*prfA* tRNA^{Arg}::pPL2 $prfA_{LS367}$ (Δ*prfA* $prfA_{LS367}$) strain contains the $prfA_{LS}$ ORF and 367 nt upstream of the $prfA_{LS}$ start codon (Table 3); both include the full P1*prfA*-and-P2*prfA* (P1P2*prfA*) promoter region. Two control strains include (i) *L. monocytogenes* Δ*prfA* complemented with $prfA_{LM}$ and the 356-nt-type upstream promoter region (10403S Δ*prfA* tRNA^{Arg}::pPL2 $prfA_{LM356}$; here denoted as the Δ*prfA* $prfA_{LM356}$ strain [Table 3]) and (ii) *L. monocytogenes* Δ*prfA* complemented with $prfA_{LM}$ and its upstream promoter as well as the upstream P*plcA* promoter region (Δ*prfA* tRNA^{Arg}::pPL2 P*plcA* $prfA_{LM356}$, here denoted as the Δ*prfA* P*plcA* $prfA_{LM356}$ strain [Table 3]). Finally, we also created a pPL2 construct that contained $prfA_{LS}$ fused to a 356-nt *L. monocytogenes prfA* promoter region, which included both the P1*prfA* and P2*prfA* promoters. This chimera was constructed using splice overlap extension (SOE) PCR (21) (primers listed in Table 1) and was introduced into *L. monocytogenes* 10403S Δ*prfA* yielding the Δ*prfA* tRNA^{Arg}::pPL2 $P1P2_{LM356}$ $prfA_{LS}$ strain (the Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain [Table 3]). Correct construction and integration of all pPL2 constructs were confirmed by PCR analysis and sequencing.

Growth conditions. Unless specified otherwise, all experiments (including RNA studies) were performed using *L. monocytogenes* or *L. seeligeri* grown to stationary phase at either 16 or 37°C. An initial overnight culture grown in brain heart infusion (BHI) at 37°C was diluted 1:100 into 5 ml fresh BHI broth and grown at 37°C, with shaking to log phase (optical density at 600 nm [OD_{600}] of 0.4). This log-phase culture was then diluted 1:100 into fresh BHI, incubated with aeration (shaking at 210 rpm) or without shaking at 16 or 37°C until cells reached stationary phase (defined as an OD_{600} of 1.0 followed by 3 h of incubation for cells grown with shaking and as an OD_{600} of 0.8 followed by 3 h of incubation for cells grown without shaking).

RNA purification and quantitative reverse transcription-PCR (qRT-PCR). For RNA purification, 4 ml of culture was added to 8 ml of RNAprotect (Qiagen) and incubated at room temperature for 5 min, and cells were subsequently collected by centrifugation (at 4°C) at 5,000 × *g* for 5 min. Cell pellets were frozen at −80°C and used for total RNA extraction as previously described (34).

TaqMan qRT-PCR was used to monitor transcript levels for *hly*, *prfA*, and *plcA* in appropriate wild-type and mutant strains. While *L. monocytogenes* qRT-PCR primers and probes have previously been reported (see Table S1 in the supplemental material), primers and probes for *L. seeligeri* genes and the *prfA* chimera construct (i.e., $P1P2_{LM}$ $prfA_{LS}$) were designed using Primer Express (Applied Biosystems, Foster City, CA) (see Table S1). For normalization, transcript levels for the housekeeping genes *rpoB* and *gap* were also determined for each RNA sample, as previously described (34). qRT-PCR, including reverse transcriptase negative-control reactions, DNA standard curves, and statistical analysis, was performed as previously described (34). All qRT-PCR experiments were performed on three RNA samples representing independent biological replicates. mRNA transcript levels of target genes were normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap* (7, 27), i.e., $\{[\log_{10}$ target gene mRNA$] - [(\log_{10}$ *rpoB* mRNA $+ \log_{10}$ *gap* mRNA$)/2]\}$ as previously described (59). While some propose that normalization against transcript levels for at least three housekeeping genes is optimal (59), normalization to one housekeeping gene has been used in a number of studies of *L. monocytogenes* (20, 37, 58) and normalization to *gap* and *rpoB* transcript levels has been used in a number of previous studies (34, 40). The geometric mean of *rpoB* and *gap* transcript levels in this study showed limited variation among the different strains grown at 37°C (average 5.53, range 5.21 to 5.83), supporting the approach used here.

5′ RACE PCR. The *L. seeligeri prfA* promoter region was mapped with the 5′ rapid amplification of cDNA ends (RACE) system (Invitrogen) according to the manufacturer's protocol as previously described (26). RNA was isolated as described above from cells grown at 37°C with aeration (shaking at 210 rpm) or without aeration. Briefly, RNA was used for gene-specific first-strand cDNA synthesis, dCTP tailing, and subsequent PCR amplification using a nested gene-specific primer and a poly(G/I) primer (primers are listed in Table 1). PCR products of the appropriate size were then purified using a QIAquick gel extraction kit (Qiagen), cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen), and sequenced to identify transcriptional start sites. As gel extraction will facilitate characterization of a specific transcript, our RACE experiments are expected to map the transcriptional start site for a specific promoter.

Determination of hemolytic activity. Both supernatant- and cell-associated hemolysin activities for selected strains were determined as previously described (44), with minor modifications. Briefly, a dilution series (in phosphate-buffered saline [PBS] with 0.5 mM dithiothreitol, pH 5.8) of the supernatant fraction or a cell suspension of bacteria grown in LB-salt for 5 h at 37°C (with shaking) were prepared and incubated for 30 min at 37°C. These dilutions were then mixed with washed sheep red blood cells (RBCs) resuspended in PBS and incubated for 60 min at 37°C. To quantify the lysis of RBCs, hemoglobin release was determined by measuring the optical density (at 420 nm) of the supernatant using a Fusion Universal microplate analyzer (Packard, Meriden, CT). A hemolytic unit was defined as the reciprocal of the supernatant dilution at which 50% of the sheep red blood cells were lysed; the supernatant OD_{420} values for 50% lysis were determined as the mean of the OD_{420} values for 100% lysis (using a positive control of RBC lysed with 1% Triton X-100) and spontaneous lysis (supernatant of control RBC without Triton). Three biological replicates were performed for each assay.

Intracellular growth in macrophage-like J774 cells. Intracellular growth assays using stimulated J774 cells were performed to assess *Listeria* cell-to-cell spread in J774 cells as previously described (8), with minor modifications. Briefly, 48 h prior to the assay, J774 cells were seeded into 24-well plates at a density of 2×10^5 cells/well, using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and without antibiotics. At 24 h prior to infection, J774 cells were stimulated by addition of fresh medium containing 10 μg/ml of *Escherichia coli* 055:B5 lipopolysaccharide (Sigma). Infection was performed by adding approximately 1×10^6 CFU of *Listeria* to each well, yielding a multiplicity of infection (MOI) of approximately 1. At 30 min postinfection (p.i.), J774 cells were washed with PBS, and fresh medium containing 50 μg/ml gentamicin was added to each well to kill any extracellular bacteria. J774 cells in separate wells were lysed by addition of ice-cold sterile water at 1.5, 5, and 7 h postinfection. Intracellular *Listeria* cells were then enumerated by the plating of lysed J774 cells on BHI agar. All experiments were repeated three times.

Intracellular growth in mouse-derived bone marrow macrophage cells. For selected strains, we also performed intracellular growth assays in bone marrow macrophages (BMMØ) derived from BALB/c mice as previously described (43).

TABLE 4. Results for PAML positive selection analyses for *prfA*, *plcA*, and *hly*

Gene (no. of aa in *L. seeligeri*)	No. of syn. substitutions in *L. seeligeri* (π_{NSS})[a]	No. of nonsyn. substitutions in *L. seeligeri* (π_{NNS})[b]	d_N/d_S[c]	*P* value[d]	ω	*p*[e]	aa sites with BEB of >95%[f]
prfA (237)	18 (0.042)	3 (0.001)	0.038	0.179	56.720	0.015	107
plcA (320)	29 (0.054)	17 (0.009)	0.146	1	1.000	0.150	
hly (530)	14 (0.018)	11 (0.005)	0.184	0.004	5.050	0.048	5, 39, 67, 117

[a] π_{NSS}, nucleotide diversity (average pairwise differences per site) for synonymous (syn.) substitutions.
[b] π_{NNS}, nucleotide diversity (average pairwise differences per site) for nonsynonymous (nonsyn.) substitutions.
[c] d_N/d_S, number of nonsynonymous changes per nonsynonymous site/number of synonymous changes per synonymous site for the entire gene; calculated based on 10 *L. seeligeri* isolates for *prfA* and 9 isolates for *hly* and *plcA*.
[d] *P* values for analyses of positive selection during divergence of *L. seeligeri* from *L. monocytogenes* and *L. ivanovii*.
[e] Proportion of all amino acids falling into the class with the shown ω value of ≥1.
[f] BEB, Bayesian empirical Bayes analysis of positive selection. Identifies amino acid sites with high probability (>95%) of having evolved by positive selection during divergence of *L. seeligeri* from other *Listeria* species.

Cells were maintained for 10 days in 100-mm petri dishes in DMEM supplemented with 10% fetal calf serum (FCS), 5% horse serum, and 20% L cell-conditioned medium and cultured with penicillin and streptomycin. Intracellular growth assays of these cells were performed as described above for J774 cells.

Statistical analysis. *t* tests or one-way analysis of variance (ANOVA) with Tukey-Kramer multiple-comparison correction was performed for comparisons of means for two or more strains. All statistical analyses were performed in JMP 6 (SAS, Inc.), with *P* values of <0.05 considered significant. Exact *P* values were reported, except when *P* was <0.0001.

Nucleotide sequence accession numbers. Sequences have been deposited in GenBank under accession numbers EU755300 to EU755321.

RESULTS

PrfA, Hly, and PlcA are conserved among *L. seeligeri* isolates, but $prfA_{LS}$ and hly_{LS} show evidence of positive selection at specific codon sites. In order to initially characterize the diversification of *hly*, *prfA*, and *plcA* in *L. seeligeri*, these three genes were sequenced for 8 *L. seeligeri* strains. These sequences were analyzed together with additional *L. seeligeri* sequences available in GenBank (Table 2). PrfA, Hly (LSO), and PlcA amino acid sequences were 97 to 99% identical among *L. seeligeri* strains; the sequences showed between 3 (*prfA*) and 17 (*plcA*) nonsynonymous changes (Table 4). To further characterize the evolution of these virulence genes in *L. seeligeri*, a branch site analysis using test 2 (67) was used to determine if individual amino acids in *L. seeligeri* PrfA, Hly, and PlcA evolved by positive selection during the divergence of *L. seeligeri* from other *Listeria* species, which would provide evidence that a given gene may have adapted to divergent function in *L. seeligeri.* While no significant evidence for positive selection was found for $plcA_{LS}$ and $prfA_{LS}$ (Table 4), $PrfA_{LS}$ aa 107 showed a high probability of having evolved by positive selection (*P* of >95%[Table 4]). PrfA aa 107 is located in the β-sheet flanking the β-roll fold, a domain similar to the domain containing the cyclic AMP (cAMP) binding site in Crp regulators (62).

hly_{LS} showed strong evidence of having evolved by positive selection during the divergence of *L. seeligeri* from other *Listeria* species ($P = 0.004$). Four amino acid sites (positions 5, 39, 67, and 117) were identified as having a significant probability (*P* of >95%) of having evolved by positive selection in Hly_{LS} (Table 4). One of these amino acid residues (aa 39) was located in the PEST-like region of *L. seeligeri* Hly; a putative PEST motif has also been described for the N-terminal region of *L. monocytogenes* Hly (32, 49). A PEST motif is a region, rich in proline (P), glutamic acid (E), serine (S), and threonine (T), which, in general, can facilitate proteolysis of a protein, even though this region has been hypothesized to be important in LLO synthesis during cytosolic growth of *L. monocytogenes* (52, 53). Only 10 of the 19 aa in the *L. seeligeri* PEST-like motif were homologous to *L. monocytogenes.* The *L. seeligeri* PEST-like motif showed a considerably higher PESTfind score (15.48) than that of *L. monocytogenes* (4.74), and contains more PEST residues than the *L. monocytogenes* PEST motif. While one of the amino acid residues under positive selection (aa 5) was located in the Hly signal sequence, SignalP 3.0 (3) showed clear evidence for the presence of a signal sequence in Hly_{LS} and Hly_{LM}, with probabilities of 0.994 and 1.0, respectively.

$prfA_{LS}$ transcription in *L. seeligeri* FSL S4-039 originates from a homologue of the *L. monocytogenes* P2*prfA* promoter region. RACE PCR mapping of the *L. seeligeri* FSL S4-039 *prfA* promoter region using RNA extracted from bacteria grown at 37°C without aeration showed that $prfA_{LS}$ transcription initiated from a promoter site corresponding to the *L. monocytogenes* P2*prfA* region (Fig. 2); 13 of the 14 cloned $prfA_{LS}$ RACE PCR products mapped to a transcriptional start site that is 10 nt upstream of the −10 region in P2*prfA* (Fig. 2). *prfA* transcripts in *L. monocytogenes* 10403S grown without aeration also mapped to the P2*prfA* region (in all 8 clones sequenced) (Fig. 2). RACE PCR experiments performed using RNA extracted from *L. monocytogenes* 10403S grown at 37°C with aeration showed that transcription of $prfA_{LM}$ under these conditions initiated predominantly from the P1*prfA* promoter (only a RACE PCR product corresponding, by size, to initiation from P1*prfA* was observed, and all 4 clones sequenced mapped to an initiation site 10 nt upstream of the P1*prfA* −10 site; see Fig. S1 in the supplemental material). This finding is consistent with previous RACE PCR data for *L. monocytogenes* grown at 37°C to stationary phase with aeration; these data also showed that *prfA* transcripts initiated predominantly from the P1*prfA* region (27). On the other hand, RACE PCR results for *L. seeligeri* grown at 37°C with aeration showed that even under these conditions, *prfA* transcription predominantly initiates from the *L. seeligeri* P2*prfA* promoter (only a RACE PCR product corresponding, by size, to initiation from P2*prfA* was observed, and all 3 clones sequenced mapped to an initiation site 10 nt upstream of the P2*prfA* −10 site; see Fig. S1 in the supplemental material). Overall, these results indicate that (i) in *L. seeligeri* grown at 37°C to stationary phase, *prfA* transcription originates predominantly from the P2*prfA* region,

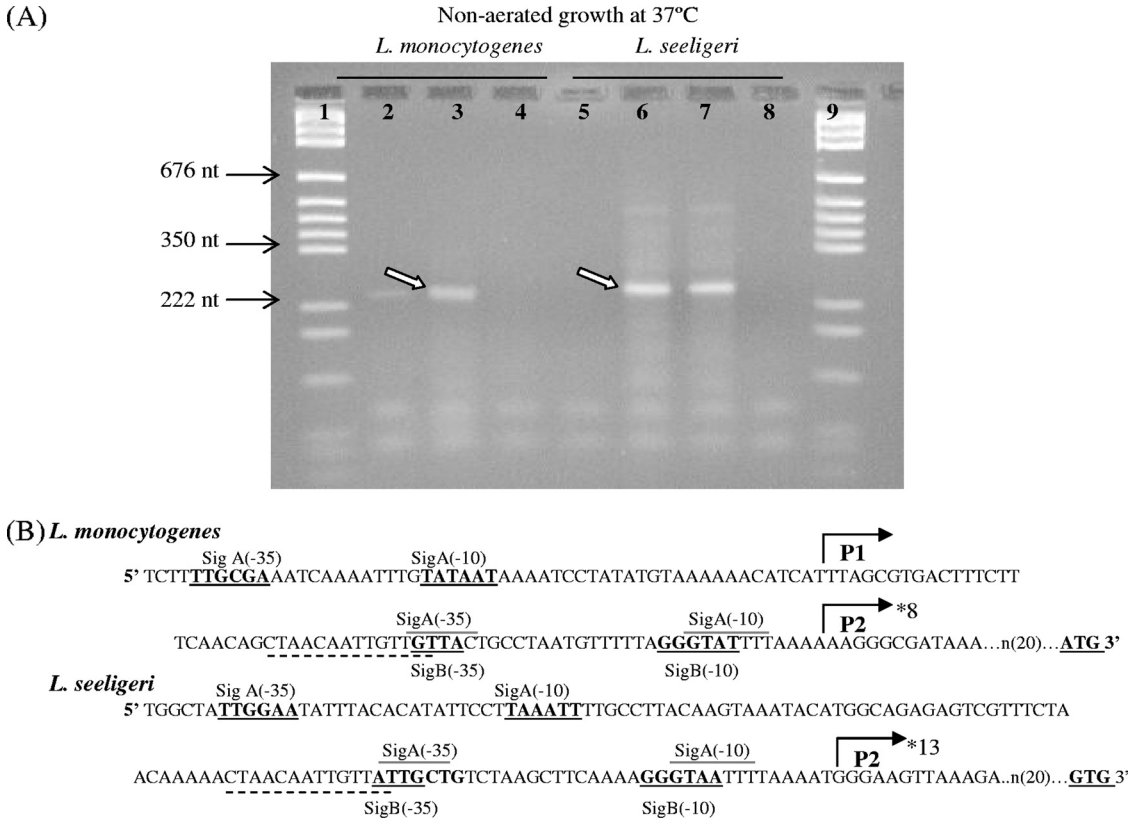

FIG. 2. Mapping of the *L. seeligeri* and *L. monocytogenes prfA* transcriptional start sites through 5′ RACE PCR on RNA isolated from bacteria grown without aeration at 37°C. (A) Agarose gel electrophoresis of 5′ RACE PCR products generated using RNA from stationary-phase *L. monocytogenes* (lanes 2 to 4) and *L. seeligeri* (lanes 5 to 8) and *prfA*-specific primers. Lanes 1 and 9, DNA marker; lanes 2 and 5, PCR on untailed *L. monocytogenes* and *L. seeligeri* cDNA, respectively (included to identify unspecific PCR products which would show up in this reaction); lane 3, PCR on tailed *L. monocytogenes* cDNA; lanes 6 and 7, PCR on tailed *L. seeligeri* cDNA (run in duplicate); lanes 4 and 8, negative PCR controls (no template). Arrows mark PCR product that was excised for cloning and sequencing to determine transcriptional start sites; the weak larger product found in *L. seeligeri* RACE PCR did not map to any apparent promoter site. (B) DNA sequence of the *prfA* promoter region in *L. monocytogenes* (strain 10403S; GenBank accession no. NZ_AARZ00000000) and *L. seeligeri* (strain FSL S4-039; determined in this study). The first (5′) nt shown here is 154 and 162 nt upstream of the start codon for *L. monocytogenes* and *L. seeligeri*, respectively; the fragments used for complementation all start upstream of the first nt shown here. The *L. monocytogenes* P1- and P2*prfA* promoters, including the σ^A- and σ^B-dependent promoters, are indicated as previously reported (13, 14, 45); −10 and −35 sequences are marked in bold and underlined; the PrfA binding box (55) is marked by a broken line (—) beneath the sequence; homologous sequences in *L. seeligeri* are also indicated in the same way. Translational start sites (ATG for *L. monocytogenes* and GTG for *L. seeligeri*) are indicated in bold and underlined. Transcriptional start sites mapped by RACE PCR in *L. seeligeri* and *L. monocytogenes* grown without aeration are indicated by an asterisk (*) (for *L. monocytogenes* grown without aeration, the P2*prfA* start site was identified in all 8 RACE PCR clones sequenced; for *L. seeligeri* grown without aeration, the P2*prfA* start site was identified in 13 of 14 RACE PCR clones sequenced).

regardless of whether cells are grown with or without aeration, and (ii) in *L. monocytogenes* grown at 37°C to stationary phase, *prfA* transcription originates predominantly from the P2*prfA* region in cells grown without aeration, while originating predominantly from P1*prfA* in cells grown with aeration, suggesting that oxygen tension affects *prfA* transcription initiation in *L. monocytogenes* but not in *L. seeligeri*.

Sequence analysis revealed both a putative σ^B- and a putative σ^A-dependent promoter in the *L. seeligeri* P2*prfA* region; the putative *L. seeligeri* σ^B promoter differs from the *L. monocytogenes* P2*prfA* σ^B promoter by 1 nt in the −10 region and 2 nt in the −35 region (Fig. 2). The putative *L. seeligeri* P2*prfA* σ^A promoter differs from the *L. monocytogenes* P2*prfA* σ^A promoter by 1 nt in the −10 region and 1 nt in the −35 region (Fig. 2). The *L. seeligeri* region corresponding to the previously reported *L. monocytogenes* P1*prfA* promoter shows a putative σ^A-dependent promoter that differs from the *L. monocytogenes* P1*prfA* σ^A promoter by 1 nt in the −10 region and 2 nt in the −35 region. The putative PrfA box upstream of the *L. monocytogenes* P1*prfA* is also largely conserved between *L. monocytogenes* 10403S and *L. seeligeri* FSL S4-039; there is only a 1-nt difference in the 3′ end of the PrfA box between these two strains (Fig. 2). Diversification in the *prfA* promoter regions thus may contribute to differences in transcriptional regulation of *prfA* between *L. monocytogenes* 10403S and *L. seeligeri* FSL S4-039.

***L. monocytogenes* and *L. seeligeri prfA* promoter regions differ in their ability to activate transcription of $prfA_{LS}$ in an *L. monocytogenes* Δ*prfA* strain.** A series of constructs with $prfA_{LS}$ and $prfA_{LM}$ under the control of different *L. monocytogenes* and *L. seeligeri prfA* promoter regions was introduced into an *L. monocytogenes* 10403S Δ*prfA* strain (Table 3) to quantify the

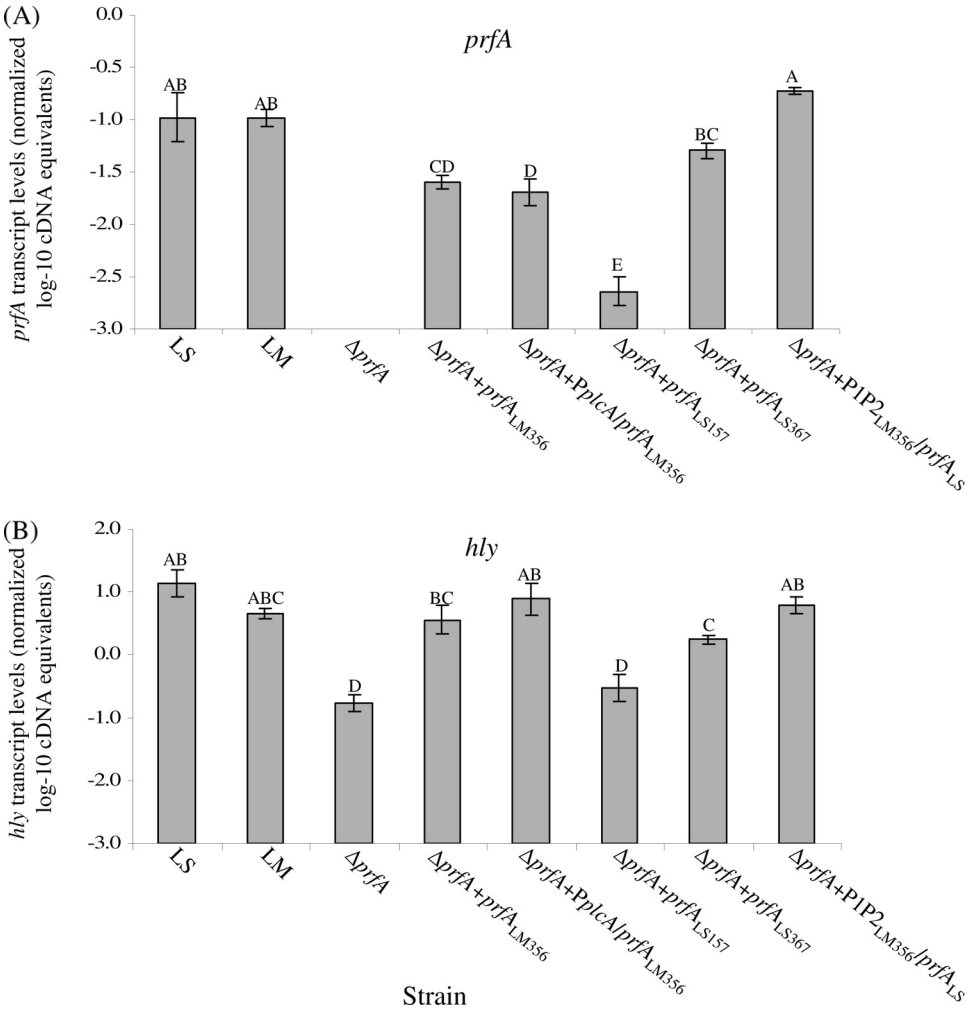

FIG. 3. Transcript levels for *prfA* (A) and *hly* (B) in *L. monocytogenes* 10403S (LM) and *L. seeligeri* FSL S4-039 (LS) wild-type strains, the *L. monocytogenes* Δ*prfA* strain, and the *L. monocytogenes* Δ*prfA* strain complemented with different pPL2 constructs carrying $prfA_{LM}$ and $prfA_{LS}$ under the control of different *prfA* promoters (strains are indicated on the *x* axis; strain designations are as detailed in Table 3 and in Materials and Methods). Transcript levels were determined by qRT-PCR and are expressed as log cDNA copy numbers/geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* (i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2]), indicated as "normalized $\log_{10}$ cDNA equivalents" on the *y* axis. All strains were grown at 37°C to stationary phase. Values shown represent the averages of qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations of three independent biological replicates. One-way ANOVA comparison with Tukey-Kramer multiple comparison correction was used to determine whether the transcript levels differed among strains; statistical groupings are shown as letters above the transcript level bars; bars that do not share a letter represent significantly different transcript levels. One-way ANOVA for both *prfA* and *hly* transcript levels showed a highly significant effect of the factor strain on transcript levels ($P < 0.0001$, for *prfA* and *hly*).

ability of *L. monocytogenes* and *L. seeligeri prfA* promoters to activate *prfA* transcription. In the control construct in which $prfA_{LM}$ is preceded by a 356-nt *L. monocytogenes* promoter region containing P1- and P2*prfA* (Δ*prfA* $prfA_{LM356}$ strain [Table 3]), *prfA* transcript levels (as determined by qRT-PCR) were significantly lower (0.62 log) (Fig. 3) than *prfA* transcript levels in the *L. monocytogenes* wild-type strain. When $prfA_{LM}$ was fused to a promoter construct containing P1- and P2*prfA* as well as the upstream P*plcA* promoter (which has been suggested to contribute to *L. monocytogenes prfA* transcription [5, 6, 36]), *prfA* transcript levels were also significantly lower (0.85 log) (Fig. 3) than *prfA* transcript levels in the *L. monocytogenes* wild-type strain. This finding is consistent with qRT-PCR data for a wild-type *L. monocytogenes* strain, which also indicated that transcription from the P*plcA* promoter has limited contributions to total *prfA* transcript levels (27). Overall, these data show that *prfA* transcription can be activated when *prfA* (with its native promoter) is inserted in *trans* in an *L. monocytogenes* Δ*prfA* strain, even though *prfA* transcript levels are slightly lower in the constructs with *prfA* in *trans* than in a strain with wild-type *prfA*.

In order to evaluate transcription of *L. seeligeri prfA* in an isogenic background, two pPL2 constructs, containing $prfA_{LS}$ as well as 157 nt or 367 nt upstream of the $prfA_{LS}$ start codon, were introduced into the *L. monocytogenes* Δ*prfA* strain (yielding the Δ*prfA* $prfA_{LS157}$ and Δ*prfA* $prfA_{LS367}$ strains, respectively); both of these constructs included the P1P2*prfA* promoters. Both the Δ*prfA* $prfA_{LS157}$ and Δ*prfA* $prfA_{LS367}$ strains showed lower *prfA* transcript levels than either wild-type *L. seeligeri* or *L. monocytogenes* grown to the same growth phase

(i.e., stationary phase) (Fig. 3). Interestingly, the Δ*prfA* $prfA_{LS367}$ strain showed significantly higher *prfA* transcript levels than the Δ*prfA* $prfA_{LS157}$ strain. These findings suggest that regions upstream of the *L. seeligeri* P1P2*prfA* region contribute to transcriptional activation of $prfA_{LS}$.

To further investigate differences between the *L. seeligeri* and *L. monocytogenes prfA* promoters and their abilities to activate *prfA* transcription, we also introduced a fusion between the *L. monocytogenes* P1*prfA*-and-P2*prfA* promoter region and $prfA_{LS}$ into the *L. monocytogenes* Δ*prfA* strain (yielding the Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain). Interestingly, the Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain showed *prfA* transcript levels that were significantly higher than those in the Δ*prfA* $prfA_{LS367}$ strain (Fig. 3) and not significantly different from the transcript levels for either the *L. monocytogenes* or *L. seeligeri* wild-type strain. These data suggest that, in an *L. monocytogenes* background, the $P1P2_{LM}$ region activates transcription of $prfA_{LS}$ more effectively than the wild-type $PprfA_{LS}$ region, further supporting that regulation of *prfA* transcription differs between *L. seeligeri* and *L. monocytogenes.* These observed differences cannot be due to differences in qRT-PCR amplification efficiencies for $prfA_{LS}$ and $prfA_{LM}$ (which were −3.48 and −3.33, respectively), as gene-specific primers and gene-specific standard curves were used to generate absolute cDNA levels for both genes (this procedure takes into account differences in amplification efficiencies).

***L. monocytogenes* strains with $prfA_{LS}$ controlled by different upstream promoter regions differ in their ability to activate *hly* transcription and in their hemolytic capability.** To evaluate the ability of *L. seeligeri* and *L. monocytogenes* PrfA, generated from the different *prfA* constructs, to activate transcription of PrfA-dependent genes, qRT-PCR was used to measure transcript levels for the PrfA-dependent gene *hly*. The *L. monocytogenes* Δ*prfA* strain showed detectable *hly* transcript levels, suggesting some PrfA-independent *hly* transcription and consistent with previous reports that showed PrfA-independent *hly* transcription (10). Complementation of the Δ*prfA* strain with $prfA_{LM}$ either under the control of the P1P2*prfA* promoter region (Δ*prfA* $prfA_{LM356}$ strain) or under the control of both the P*plcA* and P1P2*prfA* promoter regions (the Δ*prfA* P*plcA* $prfA_{LM356}$ strain) yielded strains that showed *hly* transcript levels not statistically different from the *L. monocytogenes* parent strain *hly* transcript levels (Fig. 3), even though both of these strains showed lower *prfA* transcript levels than the *L. monocytogenes* parent strain.

Consistent with the observation that $prfA_{LS}$ transcript levels were lowest in the Δ*prfA* $prfA_{LS157}$ strain, higher in the Δ*prfA* $prfA_{LS367}$ strain, and even higher in *L. monocytogenes* with $prfA_{LS}$ under the control of the $P1P2_{LM}$ promoter region (the Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain) (Fig. 3), hly_{LM} transcript levels in these three strains followed the same trend. Therefore, higher levels of $prfA_{LS}$ transcript correlate to higher levels of hly_{LM}, even though the relative changes in hly_{LM} transcript levels are smaller (compared to the changes in *prfA* transcript levels), most likely because hly_{LM} is also transcribed from a PrfA-independent promoter.

To further characterize the different *prfA* complementation mutants, we also performed semiquantitative hemolysis assays. While the *L. monocytogenes* Δ*prfA* strain showed no detectable cell-associated hemolysis, it showed low levels of supernatant-associated hemolysis (Table 5), further supporting some PrfA-independent *hly* transcription. Complementation of the Δ*prfA* strain with $prfA_{LM}$ under the control of either the P1P2*prfA* promoter region (the Δ*prfA* $prfA_{LM356}$ strain) or under the control of both the P*plcA* and P1P2*prfA* promoter regions (the Δ*prfA* P*plcA* $prfA_{LM356}$ strain) yielded strains that showed cell- and supernatant-associated hemolysis similar to that of wild-type *L. monocytogenes* (Table 5), consistent with *hly* transcript-level data. Also consistent with the *hly* transcript-level data, hemolytic activities were lowest for the Δ*prfA* $prfA_{LS157}$ strain, higher for the Δ*prfA* $prfA_{LS367}$ strain, and highest for the Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain (Table 5). Hemolysis levels for the Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain were similar to hemolysis levels in *L. monocytogenes* 10403S (Table 5), further supporting that $PrfA_{LS}$ can upregulate hly_{LM}.

TABLE 5. Hemolytic activity of whole-cell suspension and supernatant fractions of *L. monocytogenes* and *L. seeligeri* strains

Strain	Avg no. of hemolytic units for:[a]	
	Cell-associated fraction	Supernatant-associated fraction
L. monocytogenes 10403S	74.7 ± 33	37.3 ± 13
L. seeligeri FSL S4-039	16.0 ± 0	2.0 ± 0
10403S Δ*prfA* $prfA_{LS157}$	<0.5 ± 0	3.3 ± 1
10403S Δ*prfA* $prfA_{LS367}$	5.33 ± 2.3	5.3 ± 2
10403S Δ*prfA* $P1P2_{LM}$ $prfA_{LS}$	85.3 ± 37	32.0 ± 0
10403S Δ*prfA* $prfA_{LM}$	64.0 ± 0	26.7 ± 9
10403S Δ*prfA* P*plcA* $prfA_{LM}$	85.3 ± 37	32.0 ± 0
10403S Δ*hly* hly_{LS}	64 ± 0	<0.5 ± 0
10403S Δ*plcA* $plcA_{LM}$	85.3 ± 37	42.7 ± 18
10403S Δ*prfA*	<0.5 ± 0	2.7 ± 1
10403S Δ*hly*	<0.5 ± 0	<0.5 ± 0
10403S Δ*plcA*	64 ± 0	32.0 ± 18

[a] Hemolytic unit is defined as the reciprocal of the dilution at which 50% lysis of sheep red blood cells occurred; data represent the average and standard deviations for 3 independent biological replicates.

Temperature-dependent patterns of *prfA* and *hly* transcription differ between *L. seeligeri* and *L. monocytogenes*. To probe temperature-dependent transcription of *prfA* and *hly*, transcript levels for these genes were initially determined, using qRT-PCR, in *L. monocytogenes* 10403S and *L. seeligeri* FSL S4-039, both grown to stationary phase at 16 or 37°C. While *L. monocytogenes prfA* transcript levels at 16 and 37°C were not different ($P = 0.6494$), *hly* transcript levels were 2.3 log higher in *L. monocytogenes* grown at 37°C than at 16°C ($P = 0.0001$) (Fig. 4). These observations are consistent with posttranscriptional upregulation of PrfA in *L. monocytogenes* grown at 37°C, leading to increased transcription of PrfA-dependent *hly* (23, 28). In *L. seeligeri*, *prfA* transcript levels were significantly higher in bacteria grown at 37°C than in those at 16°C (1.3 log difference; $P = 0.0059$) (Fig. 4A). *L. seeligeri hly* transcripts were also significantly higher in bacteria grown at 37°C than in those at 16°C (2.3 log difference; $P = 0.0024$) (Fig. 4B). To compare levels of active PrfA in *L. monocytogenes* and *L. seeligeri* grown at different temperatures, we also calculated PrfA activity, which we defined as the ratio of normalized *hly* transcript levels to normalized *prfA* transcript levels (Fig. 4C). These data showed that PrfA activity in *L. monocytogenes* grown at 37°C is approximately 42 times higher than that in *L.*

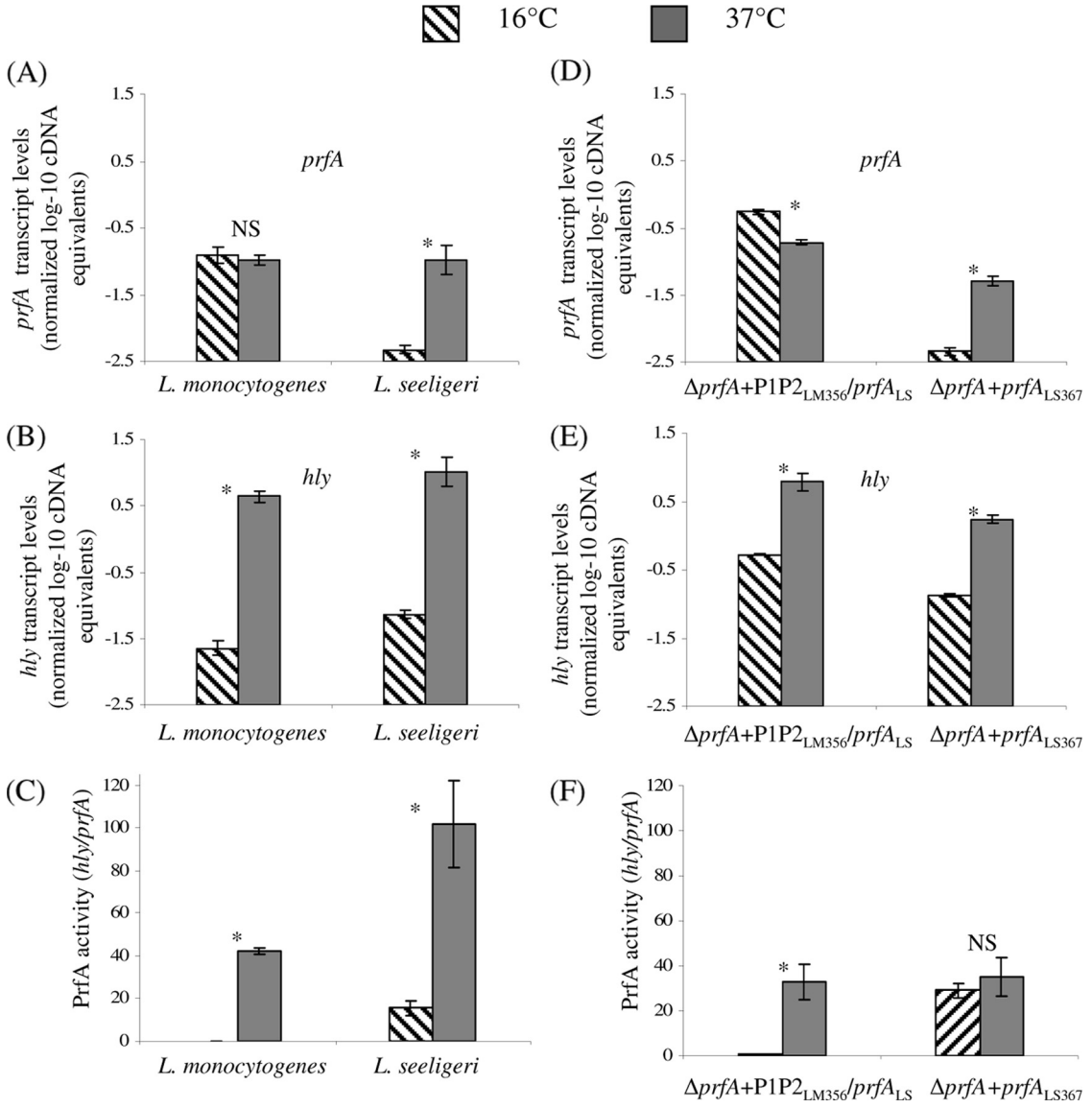

FIG. 4. Normalized, log-transformed *prfA* (A) and *hly* (B) transcript levels and PrfA activity levels (C) in wild-type *L. monocytogenes* 10403S and *L. seeligeri* FSL S4-039, and normalized, log-transformed *prfA* (D) and *hly* (E) transcript levels and PrfA activity levels (F) in *L. monocytogenes* Δ*prfA* P1P2$_{LM356}$ *prfA*$_{LS}$ and Δ*prfA prfA*$_{LS367}$, all grown to stationary phase at both 16 and 37°C. Transcript levels were determined by qRT-PCR and are expressed as log cDNA copy numbers/geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* (i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2]; indicated as "normalized log-10 cDNA equivalents" on the *y* axis). Values shown represent the averages of qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations of three independent biological replicates. "PrfA activity" is defined as the ratio of normalized *hly* transcript levels/*prfA* transcript levels, which indicates the activity of PrfA (measured as transcripts of the PrfA-dependent gene *hly*) normalized to the number of *prfA* transcripts; higher values thus indicate either enhanced translation or enhanced PrfA activity. Bars labeled with an asterisk (*) indicate transcript levels or PrfA activity levels that differed significantly between 16 and 37°C; NS indicates that no significant difference was found.

monocytogenes grown at 16°C, while PrfA activity in *L. seeligeri* grown at 37°C was only about 7-fold higher than that in *L. seeligeri* grown at 16°C. *L. seeligeri* thus shows reduced temperature-dependent activation of PrfA, while showing greater absolute PrfA activity at both 16 and 37°C than *L. monocytogenes* at the same temperatures. While PrfA activity is higher at 37°C than at 16°C in both *L. seeligeri* and *L. monocytogenes*, only *L. seeligeri* shows temperature-dependent transcription of *prfA* (with higher *prfA* transcript levels for bacteria grown at 37°C than at 16°C), suggesting differences in temperature-dependent regulation of virulence gene expression between the *L. monocytogenes* and *L. seeligeri* strains tested here.

Temperature-dependent patterns of *L. seeligeri* and *L. monocytogenes prfA* and *hly* transcription are due to differences in the *prfA* promoter sequences in these two species. To further explore temperature-dependent transcription of *L. seeligeri* and *L. monocytogenes prfA* and *hly*, transcript levels for these two genes were measured in two isogenic *L. monocytogenes* strains transcribing *prfA*$_{LS}$ under the control of either (i) the *L. seeligeri prfA* promoter region (the Δ*prfA prfA*$_{LS367}$ strain) or

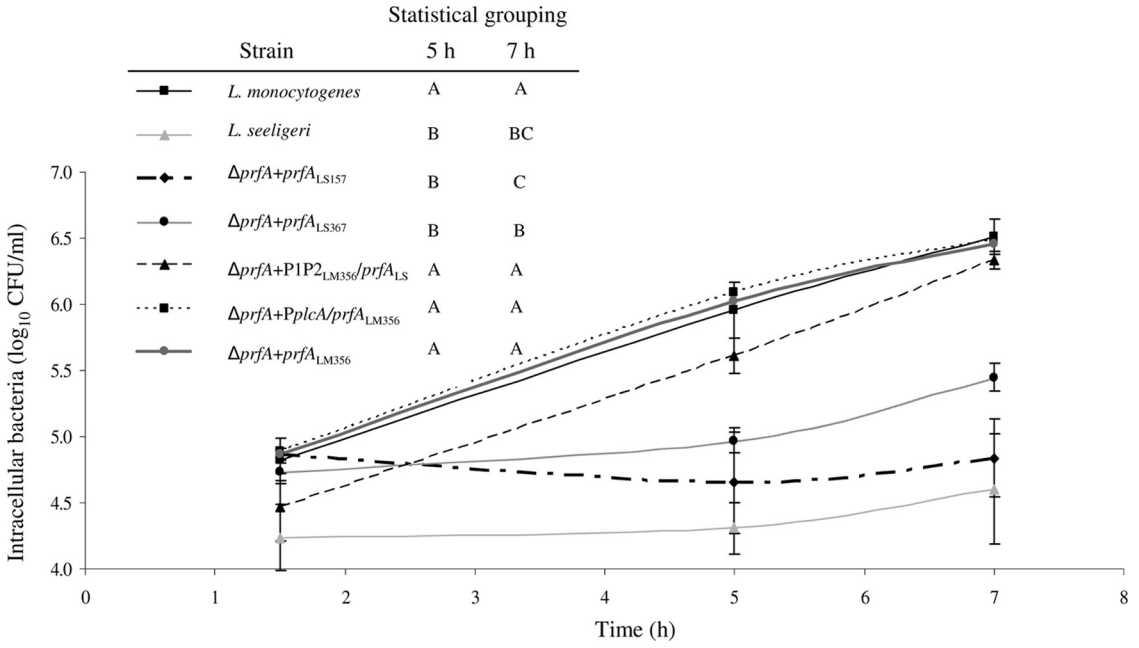

FIG. 5. Intracellular growth, in activated J774 cells, of *L. monocytogenes*, *L. seeligeri*, and *L. monocytogenes* isogenic mutants expressing $prfA_{LS}$ and $prfA_{LM}$ from different promoters (see Table 3 for strain designations). The graph shows intracellular bacterial numbers ($\log_{10}$ CFU/ml) at 1.5 h, 5 h, and 7 h postinfection; values shown represent the averages of three independent biological replicates; error bars show standard deviations of these replicates. These data were also used to calculate intracellular growth between (i) 1.5 and 5 h (i.e., [CFU/ml at 5 h] − [CFU/ml at 1.5 h]) and (ii) 1.5 and 7 h, which were used for statistical analyses; statistical analyses were performed on intracellular growth data rather than absolute intracellular bacterial numbers at different time points, as there was a significant variation between intracellular bacterial numbers for the different strains at 1.5 h p.i. Results from the statistical analyses (one-way ANOVA comparison with Tukey-Kramer multiple-comparison correction) of growth between 1.5 and 5 h, and 1.5 and 7 h are shown in the inserted table; strains that do not share a letter (e.g., "A") for a given time point show a significant difference in their intracellular growth levels between 1.5 and 5 h or 1.5 and 7 h.

(ii) the *L. monocytogenes prfA* promoter region (the Δ*prfA* P1P2$_{LM356}$ $prfA_{LS}$ strain); transcript levels were measured in these strains grown to stationary phase at 16°C or 37°C (without aeration). Overall, *prfA* and *hly* transcript patterns in these isogenic mutants were similar to transcript patterns in the corresponding wild-type strains that served as the source of the promoter in these constructs, e.g., transcript patterns for the strain transcribing $prfA_{LM}$ under the control of the *L. seeligeri prfA* promoter (Fig. 4D) were similar to the transcript patterns in the *L. seeligeri* wild-type strain (Fig. 4A). *prfA* transcript levels were lower at 37°C than at 16°C (0.45 log difference; $P < 0.0001$, two-sided *t* test) in the strain transcribing $prfA_{LS}$ from the *L. monocytogenes prfA* promoter. In the strain transcribing $prfA_{LS}$ from the *L. seeligeri prfA* promoter, *prfA* transcript levels were higher at 37°C than at 16°C (1.05 log difference; $P < 0.0001$, two-sided *t* test), further supporting that the *L. seeligeri prfA* promoter may have evolved to facilitate temperature-dependent transcription of *prfA*. For both strains, *hly* transcript levels were significantly higher in bacteria grown at 37°C than in those grown at 16°C (Fig. 4E).

For the strain transcribing $prfA_{LS}$ from the *L. monocytogenes prfA* promoter, PrfA activity was significantly higher for bacteria grown at 37°C than for those grown at 16°C (Fig. 4F), similar to the trend seen with the *L. monocytogenes* wild-type strain (Fig. 4C and F). These findings are consistent with previously reported posttranscriptional activation of PrfA in *L. monocytogenes*, i.e., through increased translation of the *prfA* transcript at 37°C compared to <30°C, due to temperature-dependent secondary structures in the region upstream of the start codon (23, 28). In the *L. monocytogenes* strain transcribing $prfA_{LS}$ from the *L. seeligeri prfA* promoter, there was no significant temperature-dependent PrfA activation, even though temperature dependence of PrfA activity was observed with the *L. seeligeri* wild-type strain, suggesting that the temperature-dependent activation of PrfA in *L. seeligeri* may depend on genetic elements outside the *prfA* fragment introduced into *L. monocytogenes* (e.g., noncoding RNAs).

$prfA_{LS}$ must be transcribed from the *L. monocytogenes prfA* promoter region to fully complement an *L. monocytogenes* Δ*prfA* strain in an intracellular growth assay in activated J774 cells. To investigate the ability of *L. seeligeri prfA* to regulate transcription of virulence genes during intracellular infection, we evaluated the ability of the different *prfA* complementation mutants (Table 3) to grow intracellularly in activated J774 cells. While the *L. monocytogenes* parent strain was clearly able to grow intracellularly (Fig. 5), consistent with previous reports (17, 25), the *L. seeligeri* parent strain showed no significant growth over time (Fig. 6), also consistent with previous data (25). These findings are also consistent with a previous study that showed a lack of proliferation of *L. seeligeri* in another macrophage cell line (9). The *L. monocytogenes* Δ*prfA* strains expressing $prfA_{LM}$ under the control of the P1*prfA*-and-P2*prfA* promoter region (the Δ*prfA* $prfA_{LM356}$ strain) or under the control of the P1P2*prfA* region and the upstream *plcA* promoter (the Δ*prfA* P*plcA* $prfA_{LM}$ strain) showed intracellular

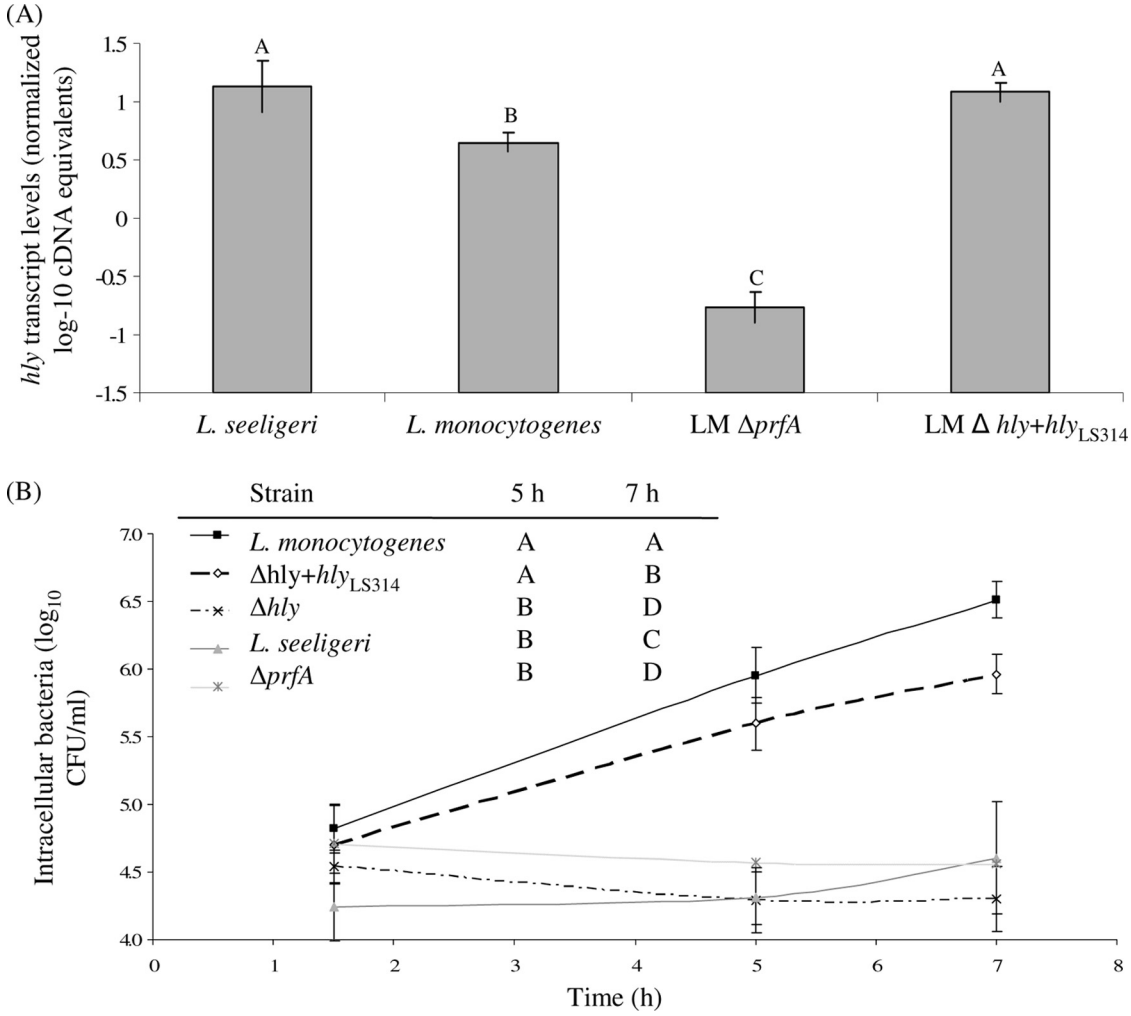

FIG. 6. *hly* transcript levels (A) and intracellular growth (B) in activated J774 cells of *L. monocytogenes*, *L. seeligeri*, and *L. monocytogenes* Δ*hly* hly_{LS314}. *hly* transcript levels were determined using qRT-PCR on RNA extracted from bacteria grown at 37°C to stationary phase; transcript levels are expressed as log cDNA copy numbers/geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* (i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2], indicated as "normalized log-10 cDNA equivalents" on the *y* axis. Values shown represent the averages of qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations of these replicates. Transcript levels and intracellular growth for *L. monocytogenes* Δ*prfA* grown under the same conditions are included as a control (these data are also shown in Fig. 4). One-way ANOVA for *hly* transcript levels showed a significant effect of the factor strain on transcript levels ($P < 0.0001$). Statistical groupings are shown as letters above the transcript level bars. Results from the statistical analyses (one-way ANOVA comparison with Tukey-Kramer multiple-comparison correction) of growth between 1.5 and 5 h, and 1.5 and 7 h are shown in the inserted table; strains that do not share a letter (e.g., "A") for a given time point show a significant difference in their intracellular growth levels (e.g., between 1.5 and 5 h or 1.5 and 7 h).

growth at 5 and 7 h that did not differ significantly from intracellular growth of the *L. monocytogenes* parent strain (Fig. 5).

The two *L. monocytogenes* strains expressing $prfA_{LS}$ under the control of *L. seeligeri* upstream promoter regions of different lengths (i.e., the Δ*prfA* $prfA_{LS157}$ and Δ*prfA* $prfA_{LS367}$ strains) both had significantly reduced intracellular growth compared to the *L. monocytogenes* parent strain. Growth of the Δ*prfA* $prfA_{LS157}$ strain was not significantly different from the intracellular growth of the *L. monocytogenes* Δ*prfA* strain, while the Δ*prfA* $prfA_{LS367}$ strain had significantly higher levels of intracellular growth at 5 and 7 h postinfection than the Δ*prfA* strain, indicating partial complementation (Fig. 5). The Δ*prfA* $P1P2_{LM356}$ $prfA_{LS}$ strain, which expresses $prfA_{LS}$ under the control of the *L. monocytogenes* P1P2*prfA* region, showed intracellular growth that was not significantly different from that of the *L. monocytogenes* parent strain (Fig. 5), suggesting that $PrfA_{LS}$, when transcribed from the *L. monocytogenes prfA* promoter, can appropriately regulate *L. monocytogenes* virulence genes essential for intracellular growth. Overall, our data suggest that the *L. seeligeri prfA* promoter region does not allow for appropriate regulation of *prfA* transcription during intracellular growth of *L. monocytogenes*.

While hly_{LS} is expressed and retains hemolytic capability when introduced into an *L. monocytogenes* Δ*hly* strain, Hly_{LS} and Hly_{LM} differ in their associations with cell and supernatant fractions. In order to evaluate the functional conservation of *L. monocytogenes* and *L. seeligeri* hemolysins, hly_{LS} (under the control of its native *L. seeligeri* promoter region) was in-

troduced into an *L. monocytogenes* 10403S Δ*hly* strain, resulting in the Δ*hly* hly_{LS314} strain. hly_{LS} transcript levels in Δ*hly* hly_{LS314} strain cells grown at 37°C to stationary phase were not significantly different from hly_{LS} transcript levels found in *L. seeligeri* but were significantly higher than hly_{LM} transcript levels found in *L. monocytogenes* 10403S grown under the same conditions (Fig. 6). These results suggest that $PrfA_{LM}$ can activate transcription of hly_{LS} utilizing its native upstream PrfA box, consistent with previous *in vitro* studies (33).

Cell-associated hemolytic activity in the Δ*hly* hly_{LS314} strain (64 hemolytic units [HU]) was similar to that in the *L. monocytogenes* parent strain (74.7 HU) but considerably higher than cell-associated hemolytic activity observed for the *L. seeligeri* parent strain (16.0 HU [Table 5]). The supernatant-associated hemolytic activities for the Δ*hly* hly_{LS314} strain (<0.5 HU) and the *L. seeligeri* parent strain (2.0 HU) were considerably lower than the supernatant-associated hemolytic activity for the *L. monocytogenes* parent strain (37.3 HU) (Table 5). The *L. monocytogenes* parent strain showed similar levels of cell- and supernatant-associated hemolysis (74.3 and 37.3 HU, respectively [Table 5]; HU are expressed as a reciprocal of the serial dilution for which 50% hemolysis was observed, with 32 and 64 being sequential dilutions), while both the *L. seeligeri* parent strain and the *L. monocytogenes* strain expressing hly_{LS} showed considerably higher cell-associated hemolytic activities than supernatant-associated hemolytic activities (Table 5). These data suggest differences in cell association between *L. seeligeri* and *L. monocytogenes* Hly.

To further evaluate the functional conservation of *L. monocytogenes* and *L. seeligeri* Hly, intracellular growth assays with the *L. monocytogenes* Δ*hly* hly_{LS314} strain were also performed (Fig. 6). While the *L. monocytogenes* Δ*hly* strain showed no intracellular growth, the *L. monocytogenes* Δ*hly* hly_{LS314} strain showed considerable intracellular growth that is significantly higher than growth of either the *L. monocytogenes* Δ*hly* or Δ*prfA* strain or *L. seeligeri* (Fig. 6). While the intracellular growth pattern of the Δ*hly* hly_{LS314} strain was similar to the growth pattern for the *L. monocytogenes* parent strain, overall growth at 5 h and 7 h postinfection was numerically slightly lower for the Δ*hly* hly_{LS314} strain compared to the *L. monocytogenes* parent strain (Fig. 6); the difference in growth at 7 h p.i. was statistically significant. These findings suggest that hly_{LS} can largely, but not completely, complement a Δhly_{LM} mutant. Partial complementation may be related to the observation that *L. seeligeri* Hly shows lower supernatant-associated hemolytic activities than *L. monocytogenes* Hly.

***plcA*$_{LS}$ cannot complement an *L. monocytogenes* 10403S Δ*plcA* strain.** To evaluate the conservation of additional *L. seeligeri* virulence genes, $plcA_{LS}$ (with its native promoter) was introduced into an *L. monocytogenes* Δ*plcA* strain, yielding the Δ*plcA* $plcA_{LS52}$ strain (Table 3). qRT-PCR analysis revealed slightly but significantly lower $plcA_{LS}$ transcript levels in the Δ*plcA* $plcA_{LS52}$ strain (grown at 37°C to stationary phase) than in the *L. seeligeri* parent strain; these transcript levels were higher than the *plcA* transcript levels in the *L. monocytogenes* parent strain (Fig. 7). Evaluation of the Δ*plcA* $plcA_{LS52}$ strain in an intracellular growth assay in primary mouse macrophage cells showed an intracellular growth pattern that did not differ significantly from that of the 10403S Δ*plcA* strain (Fig. 7). These data suggest that $plcA_{LS}$ cannot functionally complement a $plcA_{LM}$ deletion, although it cannot be excluded that complementation could occur if $plcA_{LS}$ were transcribed at higher levels in *L. monocytogenes*.

DISCUSSION

Comparative sequence analyses of *L. seeligeri prfA*, *hly*, and *plcA* and heterologous complementation studies of *L. monocytogenes* revealed that (i) the virulence genes *prfA*, *hly*, and *plcA* are conserved among *L. seeligeri* isolates but are distinct from the virulence gene homologues in *L. monocytogenes*, (ii) while regulation of *prfA* transcription has diversified between *L. monocytogenes* and *L. seeligeri*, *prfA* and *hly* transcript levels are higher in *L. seeligeri* grown at 37°C (than in those grown at 16°C), suggesting a function of *L. seeligeri* virulence genes in warm-blooded hosts, and (iii) PrfA and Hly functions are largely, but not fully, conserved between *L. seeligeri* and *L. monocytogenes*. Overall, our findings suggest that while *prfA* and *hly* have diversified in *L. seeligeri*, including in their transcriptional regulation, they have maintained similar functions, possibly involving pathogenic or commensal interactions with warm-blooded hosts.

The virulence genes *prfA*, *hly*, and *plcA* are conserved among *L. seeligeri* isolates but are distinct from the virulence gene homologues in *L. monocytogenes*. While previous studies have shown that some *L. seeligeri* strains lack the *Listeria prfA* virulence gene cluster (63), our data show that *prfA*, *hly*, and *plcA* are fairly conserved among the *L. seeligeri* strains that contain this virulence gene cluster, providing initial evidence that these genes are not pseudogenes and/or that these genes are not under neutral selection. Interestingly, patterns of conservation of the protein sequences encoded by these three genes were very similar between *L. monocytogenes* and *L. seeligeri*, with PrfA being the most conserved protein, followed by Hly, and PlcA (which represented the most diverse protein among both the *L. seeligeri* sequences analyzed here and *L. monocytogenes* sequences analyzed previously [41]). These findings are also consistent with functional studies, discussed in more detail below, which show that *L. seeligeri prfA* and *hly* can largely complement the *L. monocytogenes* Δ*prfA* and Δ*hly* null mutants, supporting considerable functional conservation of PrfA and Hly.

While considerable diversification of the overall *Listeria prfA* virulence gene cluster and the specific coding regions within this cluster has previously been reported (e.g., reference 25), no formal evolutionary analyses of the selection of virulence gene homologues during the divergence of *L. seeligeri* from other *Listeria* species have previously been reported. Our analyses showed significant evidence for positive selection in hly_{LS}. Two Hly_{LS} amino acid residues with evidence for positive selection (aa 67 and 117) are located, respectively, within putative domains 1 and 2 (50, 53), which have no clearly assigned functions. The N-terminal region of Hly, which shows the least conservation between *L. seeligeri* and *L. monocytogenes*, contains the other two amino acid residues with evidence for positive selection; aa 5 falls into the N-terminal secretion signal sequence, while aa 39 falls within a PEST-like region (32, 52), a region hypothesized to be important in LLO synthesis during cytosolic growth of *L. monocytogenes* (52, 53). Overall, our evolutionary analyses, combined with the finding that a smaller

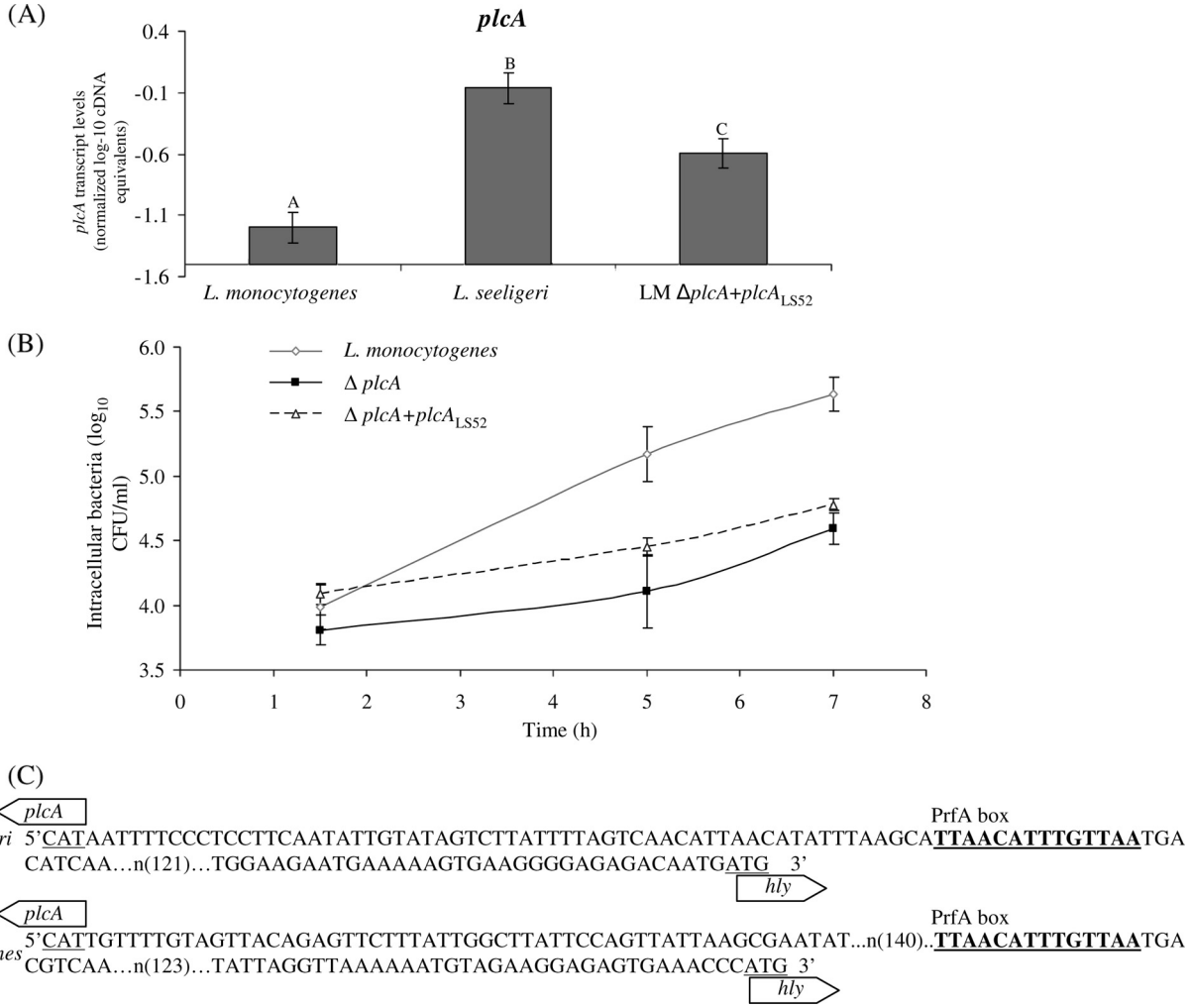

FIG. 7. *plcA* transcript levels (A) and intracellular growth (B) in primary bone marrow-derived mouse macrophage cells of *L. monocytogenes*, *L. monocytogenes* Δ*plcA*, and *L. monocytogenes* Δ*plcA* $plcA_{LS52}$ grown at 37°C. (C) DNA sequence of the intergenic region between *plcA* and *hly* in *L. seeligeri* and *L. monocytogenes*. *plcA* transcript levels were determined using qRT-PCR on RNA from bacteria grown at 37°C to stationary phase; transcript levels are expressed as log cDNA copy numbers/geometric mean of cDNA copy numbers for the housekeeping genes *rpoB* and *gap* (i.e., $\log_{10}$ target gene − [($\log_{10}$ *rpoB* + $\log_{10}$ *gap*)/2], indicated as "normalized log-10 cDNA equivalents" on the *y* axis). Values shown represent the averages of qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations of these replicates. One-way ANOVA for *plcA* transcript levels showed a significant effect of the factor strain on transcript levels ($P < 0.0001$). Statistical groupings are shown as letters above the transcript level bars.

fraction of Hly_{LS} (than of Hly_{LM}) is secreted, suggest some functional differences between the *L. seeligeri* and *L. monocytogenes* Hly proteins and a possible functional adaptation of Hly_{LS} to a distinct niche.

While regulation of *prfA* transcription has diversified between *L. monocytogenes* and *L. seeligeri*, *prfA* and *hly* transcription in *L. seeligeri* is higher at 37°C, suggesting a function of *L. seeligeri* virulence genes in warm-blooded hosts or additional environments. Our data provide clear evidence that regulation of *prfA* has diversified between *L. seeligeri* and *L. monocytogenes*, including (i) that $prfA_{LS}$ transcript levels (in an *L. monocytogenes* genetic background) when $prfA_{LS}$ is transcribed from an *L. monocytogenes prfA* promoter are higher than those when $prfA_{LS}$ is transcribed from the native *L. seeligeri* promoter, (ii) temperature-dependent *prfA* transcription in *L. seeligeri* but not *L. monocytogenes*, (iii) differential utilization of the P1- and P2*prfA* promoters depending on aeration in *L. monocytogenes* but not in *L. seeligeri*, and (iv) considerably more-pronounced temperature-dependent apparent PrfA activity in *L. monocytogenes* than in *L. seeligeri*. The observation that *prfA* transcript levels are higher in *L. monocytogenes* 10403S carrying $prfA_{LS}$ under the $P1P2_{LM}$ region than in the strain carrying $prfA_{LM}$ under the same promoter also suggests the higher transcript stability of $prfA_{LS}$ (i.e., slower transcript turnover) than of $prfA_{LM}$. While it has been well established that *L. monocytogenes prfA* transcript originating from the P1*prfA* promoter includes a thermosensor that allows for temperature-dependent *prfA* translation and facilitates induction of translation at temperatures >30°C (23), no evidence for temperature-dependent expression of virulence genes has previously been reported for *L. seeligeri*. Interestingly, the mechanism of temperature-dependent virulence gene expression in *L. seeligeri*

includes increased transcription of *prfA* itself at 37°C, which is not observed with *L. monocytogenes*. *L. seeligeri* also appears to have a mechanism for posttranscriptional temperature-dependent regulation of PrfA activity (mediated by either increased translation or activation of the translated PrfA), even though posttranscriptional temperature-dependent regulation of PrfA activity in *L. seeligeri* is less pronounced than in *L. monocytogenes*. As temperature changes can act as a signal for transition into a mammalian or avian host environment (13, 14, 23), our findings, overall, suggest that *L. seeligeri* virulence genes may play a role in facilitating interactions with a warm-blooded host.

Differences in regulation of *prfA* transcription between *L. monocytogenes* and *L. seeligeri* suggest that *L. seeligeri* may have adapted to interact with hosts other than those infected by *L. monocytogenes*. Furthermore, the observed differences in *prfA* transcriptional initiation between *L. monocytogenes* grown with and without aeration may be related to the enhanced virulence of *L. monocytogenes* grown in microaerophilic growth conditions (2), possibly as *prfA* transcription originates predominantly from P2*prfA* in cells grown without aeration, with this promoter allowing for temperature-independent translation of *prfA* transcript. The critical nature of differences in *prfA* transcription between *L. monocytogenes* and *L. seeligeri* is also supported by the observation that introduction of *L. monocytogenes prfA* (including the upstream *plcA* promoter) into *L. seeligeri* facilitated LSO synthesis and escape from the phagosome, a phenotype that was not observed with wild-type *L. seeligeri* (25).

PrfA and Hly functions are largely, but not fully, conserved between *L. seeligeri* and *L. monocytogenes*. Our data showed that expression of $prfA_{LS}$ from the *L. monocytogenes prfA* promoter was able to functionally complement an *L. monocytogenes* Δ*prfA* mutant. These findings suggest that $PrfA_{LS}$ can interact with *L. monocytogenes* PrfA boxes and upregulate *L. monocytogenes* virulence genes needed for intracellular growth and survival. Interestingly, Mauder et al. (33) found, using *in vitro* transcription assays, that $PrfA_{LS}$ has decreased binding affinity (compared to $PrfA_{LM}$) to homologous and heterologous PrfA boxes and that chromosomal replacement of $prfA_{LM}$ by $prfA_{LS}$ (also placing $prfA_{LS}$ under the control of the *L. monocytogenes prfA* promoters) only partially restores hemolytic activity and virulence in a mouse model. Apparent differences in our findings may possibly be explained by the use of different $prfA_{LS}$ genes in our studies (while the full *prfA* sequence used by Mauder et al. [33] is not available in GenBank, evaluation of primer sequences used for mutant construction suggests at least 2 aa differences between the $prfA_{LS}$ alleles used here and by Mauder et al. [33]). It is important to note, though, that in our studies, $prfA_{LS}$ transcribed from the *L. monocytogenes* promoters showed transcript levels numerically (but not statistically significantly) higher than *prfA* transcript levels in the *L. monocytogenes* wild-type strain, which could possibly help to overcome a reduced promoter affinity of $PrfA_{LS}$. Our *L. monocytogenes* strain transcribing $prfA_{LS}$ from an *L. monocytogenes* promoter also showed numerically (but not significantly) reduced intracellular numbers compared to those of the *L. monocytogenes* parent strain in the intracellular growth assay. Combined with our data that suggest positive selection of at least one PrfA residue in *L. seeligeri* and the observation that the *L. monocytogenes* and *L. seeligeri* PrfA proteins differ by 63 aa residues, it thus appears likely that $PrfA_{LS}$ shows some functional differences from $PrfA_{LM}$, including somewhat reduced affinity to PrfA promoters.

Complementation of an *L. monocytogenes* Δ*hly* strain with hly_{LS} (under the control of its native *L. seeligeri* promoter) indicated effective recognition of the *L. seeligeri hly* promoter by $PrfA_{LM}$, consistent with previous *in vitro* transcription (33) and *L. seeligeri* complementation studies (25). However, expression of Hly_{LS} in *L. monocytogenes* showed a distinct pattern of cell- and supernatant-associated hemolysis, including reduced supernatant-associated hemolysis activity compared to that of Hly_{LM}, possibly due to diversification of the signal peptide sequence. Combined with clear evidence for positive selection in hly_{LS}, our functional data suggest that Hly_{LS} has diverged functionally from Hly_{LM}, while maintaining the ability to lyse the host cell vacuole and interact with mammalian host cells. Some level of functional conservation of Hly is supported by other studies that showed that Hly_{LS} could facilitate vacuolar escape in *L. seeligeri* if hly_{LS} transcription is activated (25) and that Hly_{LS} and Hly_{LM} have identical abilities to induce gamma interferon in mouse spleen cells (22).

Conclusions. Combined with previous studies (e.g., references 25 and 33), our data suggest that virulence differences between *L. seeligeri* and *L. monocytogenes* do not simply reflect a loss of function in the *L. seeligeri* *p*VGC genes but rather reflect adaptation of *L. seeligeri* to a specific niche and/or hosts. This adaptation appears to have occurred at multiple levels, including gene loss or acquisition and diversification of virulence gene regulation as well as allelic variation and diversification of effector protein functions (e.g., hemolysin). Interestingly, the *L. seeligeri* strain tested not only showed higher virulence gene expression when grown at temperatures typical of mammalian and avian hosts but also showed a mechanism for temperature-dependent regulation (i.e., temperature-dependent regulation of *prfA* transcription) different than that for *L. monocytogenes*. While it has been suggested that maintenance of virulence genes in environmental microorganisms in general (54, 56, 68) and in *L. seeligeri* in particular may occur as a result of interaction with nonmammalian hosts (e.g., amoebae, nematodes, and insects), our data suggest that the *p*VGC virulence genes in *L. seeligeri* have adapted to function in warm-blooded hosts. Additional experiments that characterize regulation of virulence gene expression during temperature shifts will be needed, though, to provide further insight into virulence gene regulation in *L. seeligeri*. Confirmation of our findings with other *L. seeligeri* strains will also be necessary to ensure that these results are not strain specific, but pertain to *L. seeligeri* as a species.

ACKNOWLEDGMENTS

We thank Laura Goodman for initial sequencing of selected *L. seeligeri* virulence genes and Robin Yates of the David Russell lab at Cornell for help with the primary mouse macrophage work.

This project was supported by USDA Special Research grants (2003-34459-12999, 2004-34459-14296, and 2005-34459-15625).

REFERENCES

1. **Alouf, J. E.** 2001. Pore-forming bacterial protein toxins: an overview. Curr. Top. Microbiol. Immunol. **257:**1–14.
2. **Andersen, J. B., B. B. Roldgaard, B. B. Christensen, and T. R. Licht.** 2007.

Oxygen restriction increases the infective potential of *Listeria monocytogenes in vitro* in Caco-2 cells and *in vivo* in guinea pigs. BMC Microbiol. **7:**55.
3. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:**783–795.
4. **Bishop, D. K., and D. J. Hinrichs.** 1987. Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. J. Immunol. **139:**2005–2009.
5. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8:**143–157.
6. **Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.
7. **Chaturongakul, S., and K. J. Boor.** 2006. SigmaB activation under environmental and energy stress conditions in *Listeria monocytogenes*. Appl. Environ. Microbiol. **72:**5197–5203.
8. **Conte, M. P., G. Petrone, A. M. Di Biase, M. G. Ammendolia, F. Superti, and L. Seganti.** 2000. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. Microb. Pathog. **29:**137–144.
9. **Dallas, H. L., D. P. Thomas, and A. D. Hitchins.** 1996. Virulence of *Listeria monocytogenes*, *Listeria seeligeri*, and *Listeria innocua* assayed with in vitro murine macrophagocytosis. J. Food Prot. **59:**24–27.
10. **Domann, E., J. Wehland, K. Niebuhr, C. Haffner, M. Leimeister-Wachter, and T. Chakraborty.** 1993. Detection of a *prfA*-independent promoter responsible for listeriolysin gene expression in mutant *Listeria monocytogenes* strains lacking the PrfA regulator. Infect. Immun. **61:**3073–3075.
11. **Doumith, M., C. Cazalet, N. Simoes, L. Frangeul, C. Jacquet, F. Kunst, P. Martin, P. Cossart, P. Glaser, and C. Buchrieser.** 2004. New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. Infect. Immun. **72:**1072–1083.
12. **Ermolaeva, S., S. Novella, Y. Vega, M. T. Ripio, M. Scortti, and J. A. Vazquez-Boland.** 2004. Negative control of *Listeria monocytogenes* virulence genes by a diffusible autorepressor. Mol. Microbiol. **52:**601–611.
13. **Freitag, N. E., and D. A. Portnoy.** 1994. Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential *in vitro* but are redundant *in vivo*. Mol. Microbiol. **12:**845–853.
14. **Freitag, N. E., L. Rong, and D. A. Portnoy.** 1993. Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. Infect. Immun. **61:**2537–2544.
15. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55:**2822–2829.
16. **Gellin, B. G., and C. V. Broome.** 1989. Listeriosis. JAMA **261:**1313–1320.
17. **Gouin, E., J. Mengaud, and P. Cossart.** 1994. The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species. Infect. Immun. **62:**3550–3553.
18. **Haas, A., M. Dumbsky, and J. Kreft.** 1992. Listeriolysin genes: complete sequence of *ilo* from *Listeria ivanovii* and of *lso* from *Listeria seeligeri*. Biochim. Biophys. Acta **1130:**81–84.
19. **Hain, T., C. Steinweg, and T. Chakraborty.** 2006. Comparative and functional genomics of *Listeria* spp. J. Biotechnol. **126:**37–51.
20. **Hanna, S. E., and H. H. Wang.** 2006. Assessment of environmental factors on *Listeria monocytogenes* Scott A *inlA* gene expression by relative quantitative Taqman real-time reverse transcriptase PCR. J. Food Prot. **69:**2754–2757.
21. **Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease.** 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques **8:**528–535.
22. **Ito, Y., I. Kawamura, C. Kohda, H. Baba, T. Nomura, T. Kimoto, I. Watanabe, and M. Mitsuyama.** 2003. Seeligeriolysin O, a cholesterol-dependent cytolysin of *Listeria seeligeri*, induces gamma interferon from spleen cells of mice. Infect. Immun. **71:**234–241.
23. **Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer, and P. Cossart.** 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. Cell **110:**551–561.
24. **Jones, S., and Portnoy, D. A.** 1994. Small plaque mutants. Methods Enzymol. **236:**526–531.
25. **Karunasagar, I., R. Lampidis, W. Goebel, and J. Kreft.** 1997. Complementation of *Listeria seeligeri* with the *plcA-prfA* genes from *L. monocytogenes* activates transcription of seeligerolysin and leads to bacterial escape from the phagosome of infected mammalian cells. FEMS Microbiol. Lett. **146:**303–310.
26. **Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann.** 2003. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. J. Bacteriol. **185:**5722–5734.
27. **Kazmierczak, M. J., M. Wiedmann, and K. J. Boor.** 2006. Contributions of *Listeria monocytogenes* sigma B and PrfA to expression of virulence and stress response genes during extra- and intracellular growth. Microbiology **152:**1827–1838.
28. **Kreft, J., and J. A. Vazquez-Boland.** 2001. Regulation of virulence genes in *Listeria*. Int. J. Med. Microbiol. **291:**145–157.
29. **Kreft, J., J. A. Vazquez-Boland, S. Altrock, G. Dominguez-Bernal, and W. Goebel.** 2002. Pathogenicity islands and other virulence elements in *Listeria*, p. 109–125. *In* J. Hacker and J. B. Kaper (ed.), Pathogenicity islands and the evolution of pathogenic microbes, vol. 2. Springer, New York, NY.
30. **Lampidis, R., R. Gross, Z. Sokolovic, W. Goebel, and J. Kreft.** 1994. The virulence regulator protein of *Listeria ivanovii* is highly homologous to PrfA from *Listeria monocytogenes* and both belong to the Crp-Fnr family of transcription regulators. Mol. Microbiol. **13:**141–151.
31. **Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar.** 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J. Bacteriol. **184:**4177–4186.
32. **Lety, M. A., C. Frehel, I. Dubail, J. L. Beretti, S. Kayal, P. Berche, and A. Charbit.** 2001. Identification of a PEST-like motif in listeriolysin O required for phagosomal escape and for virulence in *Listeria monocytogenes*. Mol. Microbiol. **39:**1124–1139.
33. **Mauder, N., R. S. Ecke, D. I. Mertins, G. Loeffler, M. Seidel, W. Sprehe, W. Hillen, W. Goebel, and S. Muller-Altrock.** 2006. Species-specific differences in the activity of PrfA, the key regulator of listerial virulence genes. J. Bacteriol. **22:**7941–7956.
34. **McGann, P., R. Ivanek, M. Wiedmann, and K. J. Boor.** 2007. Temperature-dependent expression of *Listeria monocytogenes* internalin and internalin-like genes suggests functional diversity of these proteins among the listeriae. Appl. Environ. Microbiol. **73:**2806–2814.
35. **McLauchlin, J.** 1997. *Listeria* and listeriosis. Clin. Microbiol. Infect. **3:**484–492.
36. **Mengaud, J., M. F. Vicente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J. C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56:**766–772.
37. **Milohanic, E., P. Glaser, J. Y. Coppee, L. Frangeul, Y. Vega, J. A. Vazquez-Boland, F. Kunst, P. Cossart, and C. Buchrieser.** 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol. Microbiol. **47:**1613–1625.
38. **Nei, M., and S. Kumar.** 2000. Molecular evolution and phylogenetics, Oxford University Press, New York, NY.
39. **Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Bayles, J. B. Luchansky, and C. M. Fraser.** 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. **32:**2386–2395.
40. **Ollinger, J., B. Bowen, M. Wiedmann, K. J. Boor, and T. M. Bergholz.** 2009. *Listeria monocytogenes* σ^B modulates PrfA-mediated virulence factor expression. Infect. Immun. **77:**2113–2124.
41. **Orsi, R. H., S. B. Maron, K. K. Nightingale, M. Jerome, H. Tabor, and M. Wiedmann.** 2008. Lineage specific recombination and positive selection in coding and intragenic regions contributed to evolution of the main *Listeria monocytogenes* virulence gene cluster. Infect. Genet. Evol. **8:**566–576.
42. **Orsi, R. H., Q. Sun, and M. Wiedmann.** 2008. Genome-wide analyses reveal lineage specific contributions of positive selection and recombination to the evolution of *Listeria monocytogenes*. BMC Evol. Biol. **8:**233.
43. **Pethe, K., D. L. Swenson, S. Alonso, J. Anderson, C. Wang, and D. G. Russell.** 2004. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. Proc. Natl. Acad. Sci. U. S. A. **101:**13642–13647.
44. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.
45. **Rauch, M., Q. Luo, S. Muller-Altrock, and W. Goebel.** 2005. σ^B-dependent in vitro transcription of *prfA* and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA in vivo. J. Bacteriol. **187:**800–804.
46. **Ripio, M. T., G. Dominguez-Bernal, M. Suarez, K. Brehm, P. Berche, and J. A. Vazquez-Boland.** 1996. Transcriptional activation of virulence genes in wild-type strains of *Listeria monocytogenes* in response to a change in the extracellular medium composition. Res. Microbiol. **147:**371–384.
47. **Rocourt, J., and P. A. D. Grimont.** 1983. *Listeria welshimeri* sp. nov. and *Listeria seeligeri* sp. nov. Int. J. Syst. Bacteriol. **33:**866–869.
48. **Rocourt, J., H. Hof, A. Schrettenbrunner, R. Malinverni, and J. Bille.** 1986. Acute purulent *Listeria seeligeri* meningitis in an immunocompetent adult. Schweiz. Med. Wochenschr. **116:**248–251.
49. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:**364–368.
50. **Rossjohn, J., S. C. Feil, W. J. McKinstry, R. K. Tweten, and M. W. Parker.** 1997. Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. Cell **89:**685–692.

51. **Sauders, B.** 2005. Molecular epidemiology, diversity, distribution, and ecology of *Listeria.* Ph.D. dissertation. Cornell University, Ithaca, NY.
52. **Schnupf, P., D. A. Portnoy, and A. L. Decatur.** 2006. Phosphorylation, ubiquitination and degradation of listeriolysin O in mammalian cells: role of the PEST-like sequence. Cell. Microbiol. **8:**353–364.
53. **Schnupf, P., and D. A. Portnoy.** 2007. Listeriolysin O: a phagosome-specific lysin. Microbes Infect. **9:**1176–1187.
54. **Schulenburg, H., and J. J. Ewbank.** 2004. Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen *Serratia marcescens*. BMC Evol. Biol. **4:**49.
55. **Sheehan, B., A. Klarsfeld, T. Msadek, and P. Cossart.** 1995. Differential activation of virulence gene expression by PrfA, the *Listeria monocytogenes* virulence regulator. J. Bacteriol. **177:**6469–6476.
56. **Steenbergen, J. N., and A. Casadevall.** 2003. The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. Microbes Infect. **5:**667–675.
57. **Steinweg, C., C. T. Kuenne, A. Billion, M. A. Mraheil, E. Domann, R. Ghai, S. B. Barbuddhe, U. Karst, A. Goesmann, A. Puhler, B. Weisshaar, J. Wehland, R. Lampidis, J. Kreft, W. Goebel, T. Chakraborty, and T. Hain.** 2010. Complete genome sequence of *Listeria seeligeri*, a nonpathogenic member of the genus *Listeria*. J. Bacteriol. **192:**1473–1474.
58. **Sue, D., D. Fink, M. Wiedmann, and K. J. Boor.** 2004. σ^B-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. Microbiology **150:** 3843–3855.
59. **Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3:**research0034.1.
60. **Vázquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft.** 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14:**584–640.
61. **Vega, Y., C. Dickneite, M. T. Ripio, R. Bockmann, B. Gonzalez-Zorn, S. Novella, G. Dominguez-Bernal, W. Goebel, and J. A. Vazquez-Boland.** 1998. Functional similarities between the *Listeria monocytogenes* virulence regulator PrfA and cyclic AMP receptor protein: the PrfA* (Gly145Ser) mutation increases binding affinity for target DNA. J. Bacteriol. **180:**6655–6660.
62. **Vega, Y., M. Rauch, M. J. Banfield, S. Ermolaeva, M. Scortti, W. Goebel, and J. A. Vazquez-Boland.** 2004. New *Listeria monocytogenes prfA** mutants, transcriptional properties of PrfA* proteins and structure-function of the virulence regulator PrfA. Mol. Microbiol. **52:**1553–1565.
63. **Volokhov, D., J. George, C. Anderson, R. E. Duvall, and A. D. Hitchins.** 2006. Discovery of natural atypical nonhemolytic *Listeria seeligeri* isolates. Appl. Environ. Microbiol. **72:**2439–2448.
64. **Ward, T. J., L. Gorski, M. K. Borucki, R. E. Mandrell, J. Hutchins, and K. Pupedis.** 2004. Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes*. J. Bacteriol. **186:**4994–5002.
65. **Wong, K., and N. Freitag.** 2004. A novel mutation within the central *Listeria monocytogenes* regulator PrfA that results in constitutive expression of virulence gene products. J. Bacteriol. **186:**6265–6276.
66. **Yang, Z., R. Nielsen, N. Goldman, and A. M. Pedersen.** 2000. Codon-substitution models for heterogeneous selection pressure at amino acid sites. Genetics **155:**431–449.
67. **Zhang, J., R. Nielsen, and Z. Yang.** 2005. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol. Biol. Evol. **22:**2472–2479.
68. **Zhou, X., J. Elmose, and D. R. Call.** 2007. Interactions between the environmental pathogen *Listeria monocytogenes* and a free-living protozoan (*Acanthamoeba castellanii*). Environ. Microbiol. **9:**913–922.