Listeriolysin O as a Reporter To Identify Constitutive and In Vivo-Inducible Promoters in the Pathogen *Listeria monocytogenes*  
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"Listeria monocytogenes" is a facultative intracellular gram-positive bacterium capable of growing in the cytoplasm of infected host cells. Bacterial escape from the phagosomal vacuole of infected cells is mainly mediated by the pore-forming hemolysin listeriolysin O (LLO) encoded by *hly*. LLO-negative mutants of *L. monocytogenes* are avirulent in the mouse model. We have developed a genetic system with *hly* as a reporter gene allowing the identification of both constitutive and in vivo-inducible promoters of this pathogen. Genomic libraries were created by randomly inserting *L. monocytogenes* chromosomal fragments upstream of the promoterless *hly* gene cloned into gram-positive and gram-negative shuttle vectors and expressed in an LLO-negative mutant strain. With this *hly*-based promoter trap system, combined with access to the *L. monocytogenes* genome database, we identified 20 in vitro-transcribed genes, including genes encoding (i) *p60*, a previously known virulence gene, (ii) a putative new hemolysin, and (iii) two products of the general protein secretion pathway. By using the *hly*-based system as an in vivo expression technology tool, nine in vivo-induced loci of *L. monocytogenes* were identified, including genes encoding (i) the previously known in vivo-inducible phosphatidylinositol phospholipase C and (ii) a putative N-acetylglucosamine epimerase, possibly involved in teichoic acid biosynthesis. The use of *hly* as a reporter is a simple and powerful alternative to classical methods for transcriptional analysis to monitor promoter activity in *L. monocytogenes*.

*Listeria monocytogenes* is a gram-positive bacterium widespread in nature and responsible for sporadic severe infections in humans and other animal species (reference 3 and references therein). This pathogen is a facultative intracellular microorganism capable of invading most host cells, including epithelial cells, hepatocytes, fibroblasts, endothelial cells, and even macrophages. Each step of intracellular parasitism by *L. monocytogenes* is dependent upon the production of virulence factors (30, 34). Among the virulence factors, listeriolysin O (LLO) is an exotoxin encoded by the *hly* gene which plays a crucial role in the escape of bacteria from the phagosomal compartment. Disruption of *hly* in wild-type *L. monocytogenes* leads to a loss of hemolytic activity and a loss of virulence in the mouse model of infection (10, 13, 26). The virulence genes (*hly*, *plcA*, *plcB*, *mpl*, *actA*, *inlA*, and *inlB*) are clustered into two distinct loci on the chromosome and are controlled by a single pleiotropic regulatory activator, PrfA, which is required for virulence (6, 19, 23).

Transposon mutagenesis is the only successful strategy used so far to identify virulence genes in *L. monocytogenes*. However, it is reasonable to assume that some genes that are important in the infection process are specifically induced during host cell infection. Indeed, recent studies have shown that most PrfA-regulated virulence genes are more efficiently expressed during intracellular growth (24), and in particular, the promoters for *hly* and *plcA* are predominantly activated within the phagosomal compartment (4). This led us to investigate the use of in vivo expression technology (IVET) to identify new virulence genes of *L. monocytogenes* specifically induced within infected host cells. The general principle of IVET consists of using a promoterless reporter gene fused to random chromosomal DNA fragments of the pathogen of interest. Different types of reporter genes were developed, such as biosynthetic genes, genes conferring antibiotic resistance, genes encoding recombination enzymes, and the gene encoding green fluorescent protein. IVET has been successfully used with several bacterial pathogens (5, 12, 14, 20, 21, 22, 31, 37, 38).

The genetic system developed in this work allowed the identification of *L. monocytogenes* promoters that are either constitutive (i.e., active in bacteria grown under standard laboratory conditions and in host tissues) or specifically induced upon infection in the mouse model (in vivo). This system utilizes the plasmid-borne *hly*-encoded LLO both as an indicator of protein expression and as a promoter trap. This work was undertaken while the *Listeria* genome-sequencing project was in progress. The sequence of the genome is now complete. The availability of this source of information allowed us to identify rapidly and unambiguously all the genes corresponding to the sequences that were determined.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, media, and DNA techniques. Bacterial strains are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium, and *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth.
TABLE 1. Bacterial strains

<table>
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<th>Strain</th>
<th>Characteristics</th>
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<tbody>
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<td>High-transformation-efficiency cells</td>
<td>Invitrogen</td>
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<tr>
<td>TOP1/pCR-hly</td>
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<td>This work</td>
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L. monocytogenes

<table>
<thead>
<tr>
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<th>Characteristics</th>
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</tr>
<tr>
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<td>EGD derivative (serovar 1/2a) with a deletion of 1,080 bp in the hly gene</td>
<td>11</td>
</tr>
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Plasmid DNA from the selected pCR recombinant was prepared using Qiagen (Chatsworth, Calif.) kits.

(ii) Cloning into pTCV-1ac or pAT28 E. coli-L. monocytogenes shuttle vectors. (a) pTCV-1ac. The recombinant plasmids pCR-hly and pCR-phi-hly were cut simultaneously with BamHI and PstI restriction enzymes (NEB, Beverly, Mass.). The BamHI-PstI fragments containing the hly gene were purified on agarose gels and cloned into the low-copy-number vector pTCV-1ac (27). For this, the BamHI-PstI fragment of pTCV-1ac, containing the lacZ gene, was replaced by the BamHI-PstI fragment containing hly, yielding plasmids pTCV-hly and pTCV-phi-hly (Fig. 1).

(b) pAT28. The hly gene (with or without the promoter) was then subcloned into the high-copy-number E. coli-gram-positive-bacteria shuttle vector pAT28 (36). For this, the two pTCV recombinant plasmids were digested with BamHI and Sall (the Sall site lies directly downstream of the PstI site), and the BamHI-Sall fragment was inserted into the BamHI-Sall sites in the polylinker of pAT28, yielding plasmids pAT28-hly and pAT28-phi-hly (Fig. 1).

(iii) Generation of the libraries. Two banks were constructed, in pTCV-1ac and in pAT28-hly. In each case, L. monocytogenes (EGDwt) chromosomal DNA was partially digested by Sau3A. DNA fragments (between 0.5 and 2 kb) were sized on TAE-agarose gels and cloned into the dephosphorylated BamHI site of either pTCV-1ac or pAT28-hly. Each bank was electrophoresed into E. coli TOP10 cells (Invitrogen Corp.). Recombinant E. coli clones (5 × 10⁵ to 10⁶) were obtained. Restriction analysis of 20 different clones from each transformation confirmed that >85% of the clones had an inserted chromosomal fragment of an average size ranging between 0.8 and 2 kb. For each bank, the transformants were pooled, and the pools were grown overnight at 37°C with agitation in Luria-
Bertani and SPC medium. A plasmid preparation was made from each culture. The two plasmid banks were then transferred to *L. monocytogenes* strain EGDA<h>hly</h> by electroporation using 1 μg of plasmid per electroporation. Approximately 10<sup>4</sup> recombinant colonies were obtained for each plasmid bank, pooled, and the two pools were grown in BHI-SPC overnight at 37°C without agitation.

(iv) Screening of the libraries for in vivo-inducible genes. Two strategies were tested to identify in vivo-inducible promoters. First, each bank was subjected to successive passages in mice. With the two banks, the initial frequency of hemolytic clones (i.e., constitutive promoters) was low (1 to 5%). This frequency rose after each passage in the animal, indicating an obvious link between the expression of the cloned gene and its capacity to persist and multiply in the infected organs. For example, with the pTCV-<i>hly</i>-<i>b</i>h<sub>j</sub>h<sub>k</sub> bank, the initial frequency of hemolytic clones was 1%. This frequency rose after each passage in the animal (to 3, 7, and 14% after the first, second, and third passages, respectively), reaching 23% after the fourth passage.

A series of nonhemolytic clones were recovered from the brains of infected mice and were further analyzed. However, none of them corresponded to putative in vivo-inducible promoters (data not shown). We therefore used a second strategy to identify in vivo-inducible promoters and focused our efforts on the bank constructed in pAT28-<i>hly</i>. On horse blood agar, the <i>hly</i>-positive clones of this bank were significantly more hemolytic than in the previous one, due to the higher number of <i>hly</i> gene copies per cell, yielding a higher rate of LLO expression. Indeed, when <i>hly</i> preceded by its promoter, phyl, was carried on the low-copy-number vector (pTCV), no detectable hemolytic activity could be recorded in bacterial culture supernatants, while with the high-copy-number vector (pAT28), the hemolytic activity recorded was fourfold higher than that in EGD<i>D</i>hly. The two <i>hly</i>-positive clones could be visualized on blood agar: nonhemolytic or very weakly hemolytic clones and highly hemolytic clones. Only the nonhemolytic or very weakly hemolytic clones were chosen for the in vivo screening.

One hundred pools of 10 different clones each (i.e., a total of 1,000 clones) were prepared and tested in vivo. For each pool, 2 × 10<sup>8</sup> bacteria (from nonagitated overnight cultures grown at 37°C) were used per inoculation (two mice were infected per pool). Each clone within the pool was thus represented approximately 2 × 10<sup>8</sup> times. Only 9 pools out of 100 tested killed the infected mice or made them visibly ill within 3 to 10 days after injection. These nine pools were further analyzed. For this, the 10 clones from each pool were individually inoculated into mice (two mice per clone at 2 × 10<sup>8</sup> bacteria per mouse). The nine clones inducing death or severe illness 3 to 4 days after injection were further studied.

The hemolytic activity in culture supernatants from these nine strains was checked on horse red blood cells (see “Hemolysis” below). None of them showed any detectable activity.

(v) Control strains. For the pTCV-<i>hly</i>-derived bank, we used as a positive control EGDA<h>hly</h> carrying pTCP-<i>hph</i>-<i>hly</i>-<i>b</i>h<sub>j</sub>h<sub>k</sub> (phyl preceded by its natural promoter). The resulting strain became weakly hemolytic on blood agar and regained in vivo virulence (Fig. 2). For the pAT28-<i>hly</i>-derived bank, the positive control was EGD<i>D</i>hly carrying pAT28-<i>hph</i>-<i>hly</i>-<i>b</i>h<sub>j</sub>h<sub>k</sub>. The resulting strain became highly hemolytic and regained in vivo virulence (Fig. 2). The recipient strain, EGD<i>D</i>hly, without a plasmid was used as a negative control.

Hemolysis. Hemolytic phenotypes were visualized by spreading bacteria onto horse blood agar plates. Mouse, C57Bl/6J, mice were kept at the animal facility of the Pasteur Institute, where they were fed twice a day (8 g/day) and were used at 10 weeks of age. The mice were kept at 14°C, with a 12-h light-dark cycle. Hemolytic activity was also measured by lysis of horse red blood cells on supernatants from cultures in exponential growth phase in BHI-SPC at 37°C. All the cultures were adjusted to an optical density at 600 nm of 0.6 before supernatant collection. Assays were carried out as described previously (9) at pH 6. Hemolytic activity was expressed as the reciprocal of the dilution of culture supernatant (40 μl) required to lyse 50% of horse erythrocytes.

Infection of mice. Six- to 8-week-old pathogen-free ICR female Swiss mice (Janvier, Le Genest St. Isle, France) were used. For the kinetics of infection, groups of five mice were inoculated intravenously in the lateral tail vein. Organs (spleen and brain) were aseptically removed and separately homogenized in 0.15 M NaCl. Bacterial numbers in the organ homogenates were determined at various intervals on BH plates containing appropriate antibiotics. In the absence of SPC selection, PAT28-derived constructions appeared fully stable in culture (Fig. 2A). In contrast, in vivo instability was observed (not shown). Therefore, all the subsequent in vivo studies were carried out on animals pretreated with SPC (1 mg of SPC per mouse twice a day). For the pTCP-derived constructions, in vitro instability had already been observed (Fig. 2A). In vivo studies were carried out on animals that were pretreated with kanamycin (at a dose of 600 μg per mouse twice a day).

Sequence analysis of the inserted fragments and *Listeria* genome database. Two different pairs of primers, flanking the BamHI cloning site and denoted p1-nP for pTCV-<i>hly</i>-derived plasmids and p2-nP for pAT28-<i>hly</i>-derived plasmids, were used to amplify and sequence the chromosomal DNA fragments inserted in two classes of plasmids (Fig. 1). The sequences of the primers were: p1, 5′-TTTAAATCTTATTTTACTCA3′; p2, 5′-ACGGGAAACCTGATTTTACC3′; and pN, 5′-ACATGTTTTTTTATTACGATCAAAAA3′. The PCR products were sequenced with the automated Prism 310 sequencer (Perkin-Elmer, Applied Biosystems). The DNA sequence of each fragment cloned in p1-nP was launched in the complete 2,900,000-bp *Listeria* genome database (BLASTn search). Searches were performed via the Internet with BLAST soft-

RESULTS

Construction and screening of *L. monocytogenes* genomic libraries. Two banks were created by fusing a promoterless copy of <i>hly</i> to random *L. monocytogenes* chromosomal fragments in gram-negative and gram-positive shuttle plasmids: pTCV-<i>hly</i> (low copy) and pAT28-<i>hly</i> (high copy). The two banks were first constructed in <i>E. coli</i> and then transferred by electroporation into EGDA<h>hly</h>, a nonhemolytic derivative of EGD<i>D</i>hly (Fig. 1) (see Materials and Methods). As shown in Fig. 2, the non-hemolytic control strain EGD<i>D</i>hly, inoculated intravenously (2 × 10<sup>8</sup> bacteria/mouse), was avirulent and was rapidly eliminated from the spleens of infected mice. As previously reported, the LLO-negative strains are approximately 5 log units less virulent than the wild-type strains in the mouse model (8, 10, 26); the lethal infecting dose ranged between 10<sup>3.3</sup> and 10<sup>5.8</sup> for EGD and LO28, respectively (3).

Virulence was restored in EGD<i>D</i>hly transformed with each plasmid vector harboring the <i>hly</i> gene under the control of its natural promoter (at a 100-fold-lower dose, i.e., 2 × 10<sup>7</sup> bacteria/mouse). Transformed hemolytic bacteria (Fig. 2) rapidly grew in the spleens of infected mice, ultimately resulting in the deaths of the mice within 2 to 4 days.

Constitutive promoters were screened from the two banks after spreading them onto blood agar plates and selection of hemolytic colonies. In vivo-inducible promoters were selected after passage in mice of the bank constructed in pAT28-<i>hly</i>. A total of 1,000 nonhemolytic or very weakly hemolytic clones were tested in the in vivo screening (see Materials and Methods for details). First, 100 pools of 10 different clones each were generated and tested in mice (for each pool, a dose of 2 × 10<sup>8</sup> bacteria was injected per mouse). Under these conditions, only 9 pools out of 100 tested killed the infected mice or made them visibly ill within 3 to 10 days after injection. These nine pools were then further analyzed: each clone was individually inoculated into mice. Nine in vivo-inducible clones inducing death or severe illness 3 to 4 days after injection were identified by this procedure.

Constitutive promoters of *L. monocytogenes*. The promoters allowing secretion of LLO by bacteria grown under standard laboratory conditions were called constitutive (i.e., the hemolytic clones on blood agar plates) (Table 2). Clones expressing LLO constitutively were obtained either directly (11 of 20) or after passage of the banks in mice, spreading of the infected organs on blood agar, and selection of highly hemolytic colonies (9 of 20). The products of the 20 ORFs located downstream of the identified promoter are listed alphabetically below (designated by the name of the ORF product in the databases with the highest similarity). When no homologous protein was found, it was named Orf<i>n</i>, where <i>n</i> is the number of predicted residues. Only one sequence (denoted Iap) corresponded to a previously identified *L. monocytogenes* protein.

From the pTCV-<i>hly</i>-derived bank, the cloned fragments comprised the promoter regions of the 13 putative genes encoding the following proteins: Asd, a protein homologous to an aspartate semialdehyd dehydrogenase of *Bacillus subtilis* which is involved in cell wall biosynthesis and thus may be important for bacterial virulence; CydA, a protein highly similar to a putative cytochrome oxdase of *B. subtilis*; Iap, a major...
virulence-associated extracellular protein of L. monocytogenes, also reported as a murein hydrolyase (16, 39); LaaB, a protein having some similarities to a putative transcriptional regulator of Lactobacillus sakei; LepS, a protein homologous to the LepS signal peptidase (Spase) of B. subtilis (this ORF was preceded by two ORFs encoding 188 and 189 amino acids, respectively, also sharing significant similarities with one another [Fig. 3] [see Discussion]; examination of the sequence preceding the predicted AUG initiation codon of orf140 suggests that the translational start site probably lies upstream [indeed, 48 in-frame triplets precede the AUG codon, and this upstream translated portion presents similarities with the corresponding N-terminal portions of the two other ORFs]); Maa, a protein homologous to a putative maltose acetyltransferase of E. coli; RluB, a protein highly similar to a putative pseudouridylylate synthase of B. subtilis; SecY, a protein homologous to the SecY preprotein translocases of Bacillus licheniformis and B. subtilis (61% identity) of identical sizes (32, 33); ValS, a protein highly similar to the valyl-tRNA synthetase of B. subtilis; YhiD, a protein homologous to a putative Mg$^{2+}$ ATPase of E. coli; YqgU, a protein showing only a weak similarity to a putative lipoprotein of B. subtilis; YrvN, a protein highly similar to a
putative helicase of B. subtilis; and YtgI, a protein highly similar to a putative thiol-peroxydase of B. subtilis.

From the pAT28-hly-derived bank, the cloned fragments comprised the promoter regions of seven putative genes encoding the following proteins: BglI, a protein highly similar to the 6-phospho-β-glucosidase of B. subtilis; FnR, a protein showing similarities to a putative transcriptional regulator from Aquifex aeolicus belonging to the Crp/Fnr family; LacD, a protein showing significant similarities to the tagatose 1,6-diphosphate aldolase of Staphylococcus aureus; PatB, a protein homologous to a putative aminotransferase from B. subtilis and to a 399-amino-acid hemolysin from Treponema denticola (32% identity; 51% similarity) (alignment of the sequences of the different amino transferases with the T. denticola hemolysin revealed 13 invariant residues [7], twelve of which are conserved in the L. monocytogenes protein [data not shown]; examination of the deduced DNA sequence of the L. monocytogenes gene and alignments with protein homologues strongly suggests that the translational initiation codon is a UUG [38] located 32 codons upstream of the first AUG [not shown]; the protein thus likely comprises 388 residues); ThrS, a protein highly similar to the thereonyl-tRNA synthetase 1 of B. subtilis; YtmB, a protein similar to YtmB of B. subtilis of unknown function; and Orf243, a protein without similarity to any protein sequences in the databases.

In vivo-inducible promoters. Among the in vivo-inducible sequences identified, three corresponded to promoters of genes determining identified ORFs inserted in the correct orientation with respect to the hly gene. The first clone (pa428) encompassed the promoter of plcA encoding phosphatidylinositol phospholipase C (PI-PLC), a major virulence factor of L. monocytogenes. The 1.5-kb DNA fragment cloned upstream of hly comprised the plcA promoter, the plcA gene, and the prfA promoter region, including the first 92 bp of the prfA gene. The second clone (pa364) comprised the promoter region of a protein homologous to the family of N-acetylglucosamine epimerases (Fig. 4), including YvyH of B. subtilis, Cps19ka of Streptococcus pneumoniae (58% identity), and Cap5P of S. aureus (62% identity). Examination of the deduced DNA sequence of the L. monocytogenes gene and alignments with the protein homologues strongly suggests (as for the patB sequence discussed above) that the translational initiation codon is a UUG located 6 triplets upstream of the AUG and preceded at optimal distance by a classical Shine-Dalgarno sequence (not shown). The L. monocytogenes epimerase thus likely contains 379 residues. The third clone (pa394) comprised the pro-
moter region of a short protein of unknown function (designated Orf104) having similarities to a putative protein of the archae bacterium *Aeropyrus pernix*, which possesses a putative membrane lipoprotein lipid attachment site.

In two cases, the fused fragment belonged to an internal portion of a structural gene but in the direction opposite to *hly*: clone pa303 comprised the proximal part of a gene encoding a protein having similarities to the MviM virulence factor of *Salmonella enterica* serovar Typhimurium (18); clone pa762 comprised the proximal part of a gene encoding a protein having similarities to a PhoR-like protein of *B. subtilis* (30). In four constructs (clones pa393, pa669, pa769, and pa896), no ORF encoding more than 50 residues could be identified in the corresponding region of the *Listeria* genome. However, in those four cases, putative promoters were predicted in the fused sequences by their significant scores (0.99 or 1 of 1) with the Promoter Prediction by Neural Network program (available at http://www.dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search).

**Kinetics of bacterial growth in organs of mice infected by transformed *Listeria* harboring in-vivo-inducible promoters.**

The kinetics of bacterial growth was followed in mice infected with EGD*dhly* transformed with pAT28-*hly* harboring in-vivo-inducible promoters from *plcA*, *yvyH*, or *orf104* compared with EGD*dhly*, used as a negative control. Mice were inoculated with $1.5 \times 10^8$ bacteria, and bacterial survival was followed in the spleen and the brain over a 4-day period (five mice per day for each strain). As shown in Fig. 5, bacteria from EGD*dhly* were rapidly destroyed in the spleen and failed to infect the brain. In contrast, when *hly* was under the control of the *yvyH* promoter, the mice died 4 days after infection. When *hly* was under the control of the *plcA* (and *prfA*) promoter, the mice survived. However, bacterial counts were significantly higher 2 days after infection (2 to 3 log higher) in the spleen compared to the control (Fig. 5), and bacteria were found in the brain. When *hly* was under the control of the promoter of *orf104*, the mice also survived. Although bacterial counts in the spleens were almost identical to those recorded with the negative con-
trol, the mutant bacteria were able to infect the brain and persisted for 3 days before being cleared. This result clearly confirmed that these three promoters were active in vivo.

**DISCUSSION**

We designed a genetic system based on the utilization of the plasmid-borne *hly*-encoded LLO as an indicator of protein expression and as a promoter trap in *L. monocytogenes*. Combined with access to the recently completed *Listeria* genome sequence, this *hly*-based system constituted (i) a simple and powerful alternative to classical methods for transcriptional analysis of constitutive promoters (hemolytic activity, which reflects *hly* transcription and translation, can be easily visualized on plates or quantified on erythrocytes) and (ii) a new IVET tool for the selection of in vivo-inducible loci in *L. monocytogenes*. For both classes of promoters, among the identified sequences were previously known virulence genes of *L. monocytogenes*, confirming the efficiency of the system.

**Constitutive promoters.** Twenty different constitutive promoters were identified, including that of *iap*, encoding the internalin-associated protein p60 (in contrast to most other virulence genes of *L. monocytogenes*, this promoter is PrfA independent) (4, 16), and those of housekeeping genes, metabolic and biosynthetic genes, and putative transcriptional regulators. Of particular interest, a putative new *L. monocytogenes* hemolysin gene was also identified. The protein is similar to a hemolysin of *T. denticola* (7) which has significant similarities to members of the aminotransferase family. Interestingly, among the other constitutive promoters, we found two key
elements of the general secretion machinery of _L. monocytogenes_: a cluster of leader peptidase genes and the secY gene. In _B. subtilis_, most genes for components of the protein secretion machinery are present in only one copy (17), despite the fact that this bacterium has a large capacity for protein secretion. The only known exception concerns the genes for type I Spases. Indeed, while in many eubacteria one Spase seems to be sufficient for the processing of secretory preproteins, in _B. subtilis_ up to seven Spase I proteins have been identified so far. In contrast to _E. coli_, where it has been demonstrated that Spase activity is essential for cell growth, in _B. subtilis_, none of the sip genes is essential by itself. However, a specific combination of mutations in these genes is lethal (reference 35 and references therein). As shown in Table 2, _orf140_ encodes a protein homologous to LepS signal peptidase belonging to the Spase I family. Its promoter region directed the expression of LLO in vitro, suggesting that this protein is expressed in _L. monocytogenes_. Examination of the region directly upstream of _orf140_ revealed the presence of two genes also encoding puta-tive Spase-like proteins. As shown in Fig. 3, the alignment of the three consecutive ORFs revealed significant amino acid conservation. Furthermore, all three ORF products possessed the conserved serine, lysine, and aspartate residues essential for Spase activity, favoring the idea that they are indeed func-tional. The participation of each of the three Spases in protein secretion (including secretion of virulence factors) will have to be addressed experimentally. The other gene implicated in the secretion machinery that was identified was secY, encoding the prepore translocase SecY. The protein of _L. monocytogenes_ is composed of 431 residues. Sequence conservation between _L. monocytogenes_ and _B. subtilis_ SecY proteins was uniformly distributed (data not shown), reflecting probable high functional similarities. Notably, as in _B. subtilis_, no other SecY homologue was found in the _Listeria_ database.

**In vivo-inducible loci.** The _hly_-based IVET system was spe-cifically devised to identify promoters expressed within the host cell phagosome. Indeed, only the bacteria able to express the _hly_-encoded LLO in the phagosomal compartment will be able to escape from the phagosome and therefore survive and multiply in the cytoplasm of infected cells. This also represents the major limitation of the system, since promoters that would be induced in later stages of the infectious process could not be identified. Another limitation of the system is its reliance on the use of a multicopy plasmid to carry the reporter gene, which could lead, in some cases, to the titration of regulatory factors required for efficient promoter expression.

Among the nine in vivo-inducible loci identified, we found the promoter of _plcA_, encoding PI-PLC. The fused fragment contained both the _plcA_ gene preceded by its promoter and the promoter of _prfA_. In perfect agreement with this result, it has very recently been demonstrated that the promoter for _plcA_ was predominantly activated within the phagosomal compart-ment while the levels of _prfA_ transcripts present in intracellular bacteria remained low (4). We then identified the promoter of a gene encoding a protein belonging to the family of _N_-acetylglucosamine epimerases ( _yvyH_ [Fig. 4]). In _B. subtilis_, _YvyH_ is likely involved in the synthesis of the ManNAc-containing link-age unit between peptidoglycan and glycerol teichoic acid. In _S. aureus_, cap5p-encoded UDP-GlcNac2-epimerase enzymatic activity was demonstrated recently (15). This enzyme, which converts UDP-GlcNAc to _N_-acetylmannosamine, is involved in capsule biosynthesis. While _S. aureus_ possesses an additional gene, _mnaA_, encoding UDP-GlcNac2-epimerase (15), no addi-tional _YvyH_ homologue could be found (by tBLASTn search) in the _Listeria_ genome database. Another inducible promoter was located upstream of an _ORF_ encoding 104 residues hav-ing some similarities to a putative lipoprotein. The kinetics of bacterial growth in organs demonstrated that the _yvyH_, _plcA_, and _orf104_ promoters allowed the in vivo-inducible expression of _hly_-encoded LLO (Fig. 5). Induced expression of _hly_ under the control of the _yvyH_ promoter was higher than under those of _plcA_ (and _prfA_) and _orf104_. At this stage, the difference in virulence among the three constructs cannot be attributed to a difference in promoter strength and might simply be due to a less favorable positioning of the promoter sequence with re-spect to _hly_ in the construct. Further characterization of the in vivo-inducible locus _yvyH_ will be undertaken to determine its role in bacterial virulence. Five additional fusions with the in vivo-inducible phenotype were also identified. In two cases, the fragments fused to _hly_ corresponded to the proximal portions of genes. However the polarity of transcription was in the orientation opposite to that of _hly_. We checked whether there was a divergently transcribed gene immediately upstream of these two promoters. In both cases, in the _Listeria_ chromosome, the preceding gene was in the same orientation.
type of fusion has already been obtained repeatedly with other IVET systems (37), and it was speculated that such fusions might generate antisense transcripts acting to downregulate the in vivo expression of the corresponding genes. Interest-ingly, one of the two sequences corresponds to a PhoR homolog, a member of the two-component regulatory system PhoP-PhoR involved in the regulation of alkaline phosphatase genes in response to environmental signals. In B. subtilis, it has been shown recently (28) that the PhoP-PhoR system is in- volved in the control of the biosynthesis of teichoic acid, a key component of the cell walls of gram-positive bacteria. Thus, regulation of PhoR expression in vivo might be relevant for bacterial adaptation to intracellular life. In four cases, no ORF could be identified, but putative promoters were predicted within the fused sequences.

In summary, we showed that the hly-based promoter trap constituted a dual system to identify both constitutive and in vivo-inducible promoters in L. monocytogenes. This study was not exhaustive and could be extended in the future, for example, by monitoring the activity of predicted in silico promoters.

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