INFECTION AND IMMUNITY, June 2000, p. 3242–3250 Vol. 68, No. 6
0019-9567/00/$04.00+0

Listeriolysin O as a Reporter To Identify Constitutive and In Vivo-Inducible Promoters in the Pathogen *Listeria monocytogenes*

IHARILALAO DUBAIL, PATRICK BERCHE, THE EUROPEAN *LISTERIA* GENOME CONSORTIUM,† AND ALAIN CHARBIT*

Laboratoire de Microbiologie, INSERM U-411, Faculté de Médecine Necker, 75730 Paris Cedex 15, France

Received 3 February 2000/Returned for modification 8 March 2000/Accepted 16 March 2000

***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 proteins 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

* Corresponding author. Mailing address: INSERM U411, Faculté de Médecine Necker, 156 rue de Vaugirard, 75730 Paris Cedex 15, France. Phone: 33 1-40 61 53 76. Fax: 33 1-40 61 55 92. E-mail: charbit@necker.fr.

† The European Listeria Genome Consortium is composed of Philippe Glaser, Alexandra Amend, Fernando Baquero-Mochales, Patrick Berche, Helmut Bloecker, Petra Brandt, Carmen Buchrieser, Trinad Chakraborty, Alain Charbit, Elisabeth Couvé, Antoine de Daruvar, Pierre Dehoux, Eugen Domann, Gustavo Dominguez-Bernal, Lionel Durant, Karl-Dieter Entian, Lionel Frangeul, Hafida Fsihi, Francisco Garcia del Portillo, Patricia Garrido, Werner Goebel, Nuria Gomez-Lopez, Torsten Hain, Joerg Hauf, David Jackson, Jurgen Kreft, Frank Kunst, Jorge Mata-Vicente, Eva Ng, Gabriele Nordsiek, Jose Claudio Perez-Diaz, Bettina Remmel, Matthias Rose, Christophe Rusniok, Thomas Schlueter, Jose-Antonio Vazquez-Boland, Hartmut Voss, Jurgen Wehland, and Pascale Cossart.

TABLE 1. Bacterial strains

Strain	Characteristics	Reference or source
E. coli		
TOP10	High-transformation-efficiency cells	Invitrogen
TOP10/pCR-*hly*	TOP10 transformed with pCR-*hly*	This work
TOP10/pCR-p*hly-hly*	TOP10 transformed with pCR-p*hly-hly*	This work
TOP10/pAT28-*hly*	TOP10 transformed with pAT28-*hly*	This work
TOP10/pTCV-*hly*	TOP10 transformed with pTCV-*hly*	This work
TOP10/pAT28-p*hly-hly*	TOP10 transformed with pAT28-p*hly-hly*	This work
TOP10/pTCV-p*hly-hly*	TOP10 transformed with pTCV-p*hly-hly*	This work
L. monocytogenes		
EGDwt	Wild-type strain, serovar 1/2a	10
EGDΔ*hly*	EGD derivative (serovar 1/2a) with a deletion of 1,080 bp in the *hly* gene	11
EGDΔ*hly*/pAT28-*hly*	EGDΔ*hly* transformed with pAT28-*hly*	This work
EGDΔ*hly*/pAT28-p*hly-hly*	EGDΔ*hly* transformed with pAT28-p*hly-hly*	This work
EGDΔ*hly*/pTCV-*hly*	EGDΔ*hly* transformed with pTCV-*hly*	This work
EGDΔ*hly*/pTCV-p*hly-hly*	EGDΔ*hly* transformed with pTCV-p*hly-hly*	This work

(Difco Laboratories, Detroit, Mich.) at 37°C. The wild-type virulent strain of *L. monocytogenes* EGD (denoted EGDwt below) belongs to the serovar 1/2a (10). EGDΔ*hly* serotype 1/2a (kindly provided by T. Chakraborty) is a derivative of EGDwt which contains an in-frame chromosomal deletion of 1,080 bp in the *hly* gene (11). EGDΔ*hly* was transformed with the different recombinant plasmids by electroporation as previously described (25). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and spectinomycin (SPC), 60 μg ml^{-1}.

Chromosomal DNA and plasmid isolation, restriction enzyme analyses, and amplification by PCR were performed according to standard protocols (2, 29).

Construction and analysis of *L. monocytogenes* genomic libraries. (i) PCR amplification of the *hly* gene. The wild-type *hly* gene preceded by its ribosome binding site was amplified by PCR from *L. monocytogenes* (EGDwt) genomic DNA using the following primer pair: 5′CCGGATCCTGTAGAAGGAGAG TGAAACCC**ATG**3′ (the ATG start codon is in boldface characters) and 5′CC CTGCAGACAATTATTCGATTGGATTATCTAC3′. The wild-type *hly* gene with its upstream promoter region (denoted *hlyp*) was amplified using the following primer pair: 5′CCGGATCCCTTAAAGTGACTTTTATGTTGAGGC A3′ and 5′CCCTGCAGACAATTATTCGATTGGATTATCTAC3′. The primers were designed to generate cohesive restriction sites (underlined) for *Bam*HI (5′ end) and *Pst*I (3′ end), respectively. Oligonucleotides were synthesized by Genset (Paris, France). The ampliTaq DNA polymerase of *Thermus aquaticus* from Perkin-Elmer (Branchburg, N.J.) was used. The amplified double-stranded DNA fragments were first cloned into the pCR cloning vector using the TA cloning kit (Invitrogen Corp., San Diego, Calif.). The clones with the inserted PCR product were selected by restriction analysis, using standard procedures. Plasmid DNA from the selected pCR recombinant was prepared using Qiagen (Chatsworth, Calif.) kits.

(ii) Subcloning into pTCV-*lac* or pAT28 *E. coli-L. monocytogenes* shuttle vectors. (a) pTCV-*lac*. The recombinant plasmids pCR-*hly* and pCR-p*hly-hly* were cut simultaneously with *Bam*HI and *Pst*I restriction enzymes (NEN, Beverly, Mass.). The *Bam*HI-*Pst*I fragments containing the *hly* gene were purified on agarose gels and subcloned into the low-copy-number vector pTCV-*lac* (27). For this, the *Bam*HI-*Pst*I fragment of pTCV-*lac*, containing the *lacZ* gene, was replaced by the *Bam*HI-*Pst*I fragment containing *hly*, yielding plasmids pTCV-*hly* and pTCV-p*hly-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 *Bam*HI and *Sal*I (the *Sal*I site lies directly downstream of the *Pst*I site), and the *Bam*HI-*Sal*I fragment was inserted into the *Bam*HI–SalI sites in the polylinker of pAT28, yielding plasmids pAT28-*hly* and pAT28-p*hly-hly*) (Fig. 1).

(iii) Generation of the libraries. Two banks were constructed, in pTCV-*hly* and in pAT28-*hly*. In each case, *L. monocytogenes* (EGDwt) chromosomal DNA was partially digested by *Sau*3A. DNA fragments (between 0.5 and 2 kb) were sized on TAE-agarose gels and cloned into the dephosphorylated *Bam*HI site of either pTCV-*hly* or pAT28-*hly*. Each bank was electroporated into *E. coli* TOP10 cells (Invitrogen Corp.). Recombinant *E. coli* clones (5×10^3 to 10^4) 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-

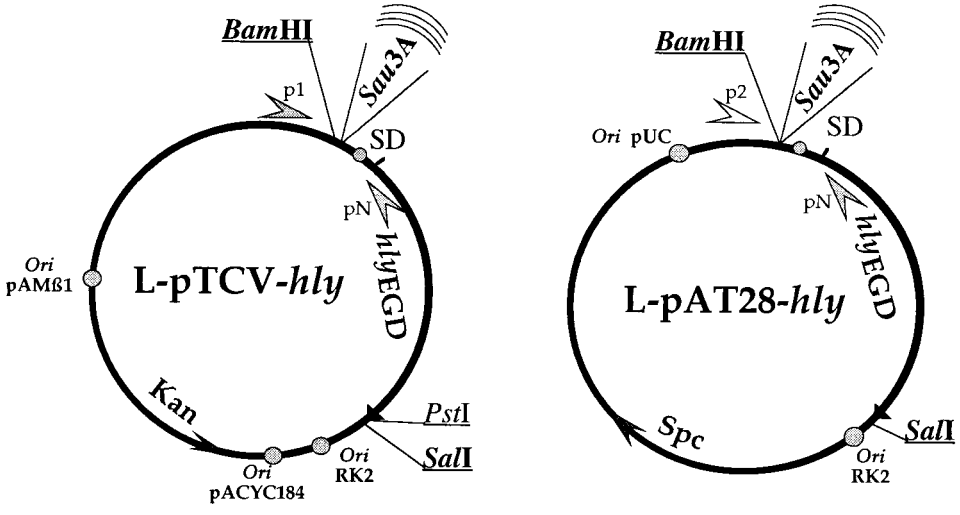

FIG. 1. Construction of libraries. The arrows flanking the *Bam*HI cloning site, denoted p1-pN for the library in pTCV-*hly* and p2-pN for the library in pAT28-*hly*, correspond to the pairs of primers used to amplify and sequence the chromosomal DNA fragments inserted upstream of *hly*. The *Bam*HI and *Sal*I sites flanking the *hly* gene are underlined.

Bertani–SPC medium. A plasmid preparation was made from each culture. The two plasmid banks were then transferred to *L. monocytogenes* strain EGDΔ*hly* by electroporation using 1 μg of plasmid per electroporation. Approximately 10^4 recombinant *Listeria* were obtained for each bank. The transformants were 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 LLO and the capacity to persist and multiply in the infected organs. For example, with the pTCV-*hly* 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-*hly*. On horse blood agar, the *hly*-positive clones of this bank were significantly more hemolytic than in the previous one, due to the higher number of *hly* gene copies per cell, yielding a higher rate of LLO expression. Indeed, when *hly* preceded by its promoter, p*hly*, 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 EGDwt. Two classes of 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, 2×10^8 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 2×10^7 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 2×10^8 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-*hly*-derived bank, we used as a positive control EGDΔ*hly* carrying pTCV-p*hly-hly* (*hly* preceded by its natural promoter). The resulting strain became weakly hemolytic on blood agar and regained in vivo virulence (Fig. 2). For the pAT28-*hly*-derived bank, the positive control was EGDΔ*hly* carrying pAT28-p*hly-hly*. The resulting strain became highly hemolytic and regained in vivo virulence (Fig. 2). The recipient strain, EGDΔ*hly*, without a plasmid was used as a negative control.

Hemolysis. Hemolytic phenotypes were visualized by spreading bacteria onto horse blood agar (BioMerieux, Marcy l'Etoile, France). 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 Geneset 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 BHI 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 pTCV-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 *Bam*HI cloning site and denoted p1-pN for pTCV-*hly*-derived plasmids and p2-pN for pAT28-*hly*-derived plasmids, were used to amplify and sequence the chromosomal DNA fragments inserted upstream of *hly* (Fig. 1). The sequences of the primers were as follows: p1, 5′GTTGAATAACACTTATTCCTATC3′; p2, 5′CAGGAAACAGCTATG ACC3′; and pN, 5′TACTTTTTTTATTACGATCAAAAA3′. The PCR products were sequenced with the automated Prism 310 sequencer (Perkin-Elmer, Applied Biosystems). Then, the DNA sequence of each fragment cloned upstream of *hly* was launched in the complete 2,900,000-bp *Listeria* genome database (BLASTn search). Searches were performed via the Internet with BLAST software (1) from the National Center for Biotechnology Information home page (http://www.ncbi.nlm.gov/BLAST/). The region of the *Listeria* genome containing the sequenced DNA was then analyzed with the program ORFfinder (http://www.ncbi.nlm.nih.gov/gorf/) to identify the open reading frame (ORF)—or N-terminal portions—comprised within the cloned fragment. The identified ORFs were then launched in the general databases (nonredundant BLASTp search).

RESULTS

Construction and screening of *L. monocytogenes* genomic libraries. Two banks were created by fusing a promoterless copy of *hly* to random *L. monocytogenes* chromosomal fragments in gram-negative and gram-positive shuttle plasmids: pTCV-*hly* (low copy) and pAT28-*hly* (high copy). The two banks were first constructed in *E. coli* and then transferred by electroporation into EGDΔ*hly*, a nonhemolytic derivative of EGDwt (Fig. 1) (see Materials and Methods). As shown in Fig. 2, the non-hemolytic control strain EGDΔ*hly*, inoculated intravenously (2×10^9 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 $10^{4.5}$ and $10^{5.8}$ for EGD and LO28, respectively (3).

Virulence was restored in EGDΔ*hly* transformed with each plasmid vector harboring the *hly* gene under the control of its natural promoter (at a 100-fold-lower dose, i.e., 2×10^7 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-*hly*. 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 2×10^8 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*n*, where *n* is the number of predicted residues. Only one sequence (denoted Iap) corresponded to a previously identified *L. monocytogenes* protein.

From the pTCV-*hly*-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 semialdehyde 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 oxydase of *B. subtilis*; Iap, a major

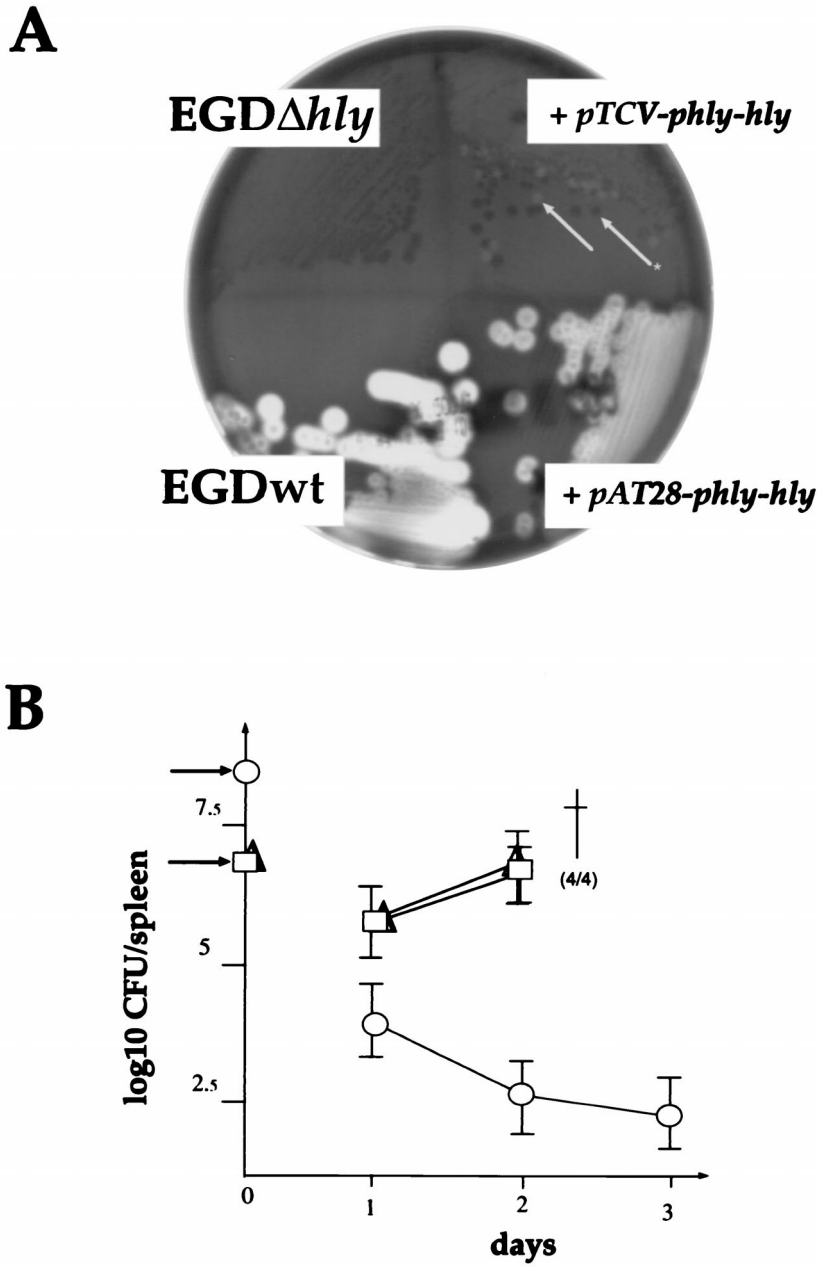

FIG. 2. Complementation. (A) In vitro complementation. The hemolytic phenotype of *L. monocytogenes* colonies was visualized on horse blood agar plates after 24 h of incubation at 37°C. The two strains denoted + *pTCV*-p*phly-hly* and + *pAT28*-p*hly-hly* correspond to EGDΔ*hly* transformed with the corresponding plasmids. The arrows show the two types of colonies (Hly^+ and Hly^-) observed with the strain transformed with pTCV-p*hly-hly* in the absence of antibiotic selection (Hly^- is indicated by a star). (B) In vivo complementation. The kinetics of infection was followed in the spleens of infected mice. With the negative control, EGDΔ*hly* (○), mice were inoculated with 2×10^9 bacteria/mouse (indicated by an arrow to the left of the ordinate); with the two transformed derivatives, mice were inoculated with 2×10^7 bacteria/mouse (arrow) (□, EGDΔ*hly* transformed with pTCV-p*hly-hly*; △, EGDΔ*hly* transformed with pAT28-p*hly-hly*). †, death; four mice out four infected died.

virulence-associated extracellular protein of *L. monocytogenes*, also reported as a murien 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 pseudouridylate 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

TABLE 2. *L. monocytogenes* loci identified

Clone[a]	Size of ORF product[b]	Similar proteins[c]	Similarity[d]		Function[e]
			% Id.	% Sim.	
Constitutive promoters					
pt11	130	LaaB; *L. sakei*	27	54	Transcriptional regulator
pt16	140	LepS; *B. subtilis*	46	61	Signal peptidase
pt15	199	Maa; *E. coli*	65	76	Maltose-acetyltransferase
pt24	883	ValS; *B. subtilis*	74	87	Valyl-tRNA synthetase
pt7	220	YhiD; *E. coli*	46	61	Mg^{2+} ATPase
pt19	363	YqgU; *B. subtilis*	22	41	Lipoprotein
pt32	436	YrvN; *B. subtilis*	72	81	Helicase
pt31	165	YtgI; *B. subtilis*	63	76	Peroxidase
pa5	338	LacD; *S. aureus*	55	75	Tagatose 1,6-diphosphate aldolase
pa308	80	YtmB; *B. subtilis*	60	76	Unknown
pa4	243	Orf243			Unknown
ptS2	346	Asd; *B. subtilis*	69	82	Aspartate semialdehyde dehydrogenase
ptS8	438	CydA; *B. subtilis*	70	82	Cytochrome oxidase
ptS1	484	Iap; *L. monocytogenes*	100	100	Internalin-associated protein
ptS3	244	RluB; *B. subtilis*	67	85	Pseudouridine synthetase
ptS4	431	SecY; *B. licheniformis*	68	80	Preprotein translocase
paS8	512	BglI; *B. subtilis*	62	75	β-Glucosidase
paS3	219	FnR; *A. aeolicus*	26	44	Transcriptional regulator (Crp/Fnr family)
paB13	388	PatB; *B. subtilis*	38	62	Hemolysine
paS10	640	ThrS; *B. subtilis*	75	86	Threonine-tRNA synthetase
In vivo-inducible promoters					
pa428	317	PlcA; *L. monocytogenes*	100	100	PI-PLC
pa364	373	YvyH; *B. subtilis*	62	78	*N*-acetyl-glucosamine epimerase
pa394	104	Orf104	38	47	Lipoprotein
pa762	591	PhoR (opposite to *hly*)	31	52	Transcriptional regulator
pa303	301	MviM (opposite to *hly*)	37	48	Virulence factor
pa393					
pa669					
pa769					
pa896					

[a] The clone numbers are preceded by pt for pTCV-derived or pa for pAT28-derived sequences. The pTCV-derived clones (listed alphabetically) are followed by the pAT28-derived clones. Constitutive clones were subdivided in two classes based on whether they were identified as being expressed on blood agar alone (above the line) or as selected in vivo and expressed on blood agar (below the line). A number preceded by a letter means that the clone was screened on blood agar from the spleens (S) or the brains (B) of infected animals.

[b] Size in amino acids of the ORF product encoded by the gene located downstream of the identified promoter.

[c] Name of the ORF product in the databases sharing the highest similarity with the identified ORF product.

[d] Similarity with the identified ORF product. Id., identity; Sim., similarity.

[e] Predicted (on the basis of sequence similarity) or identified function for the ORF product.

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 of *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 threonyl-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-

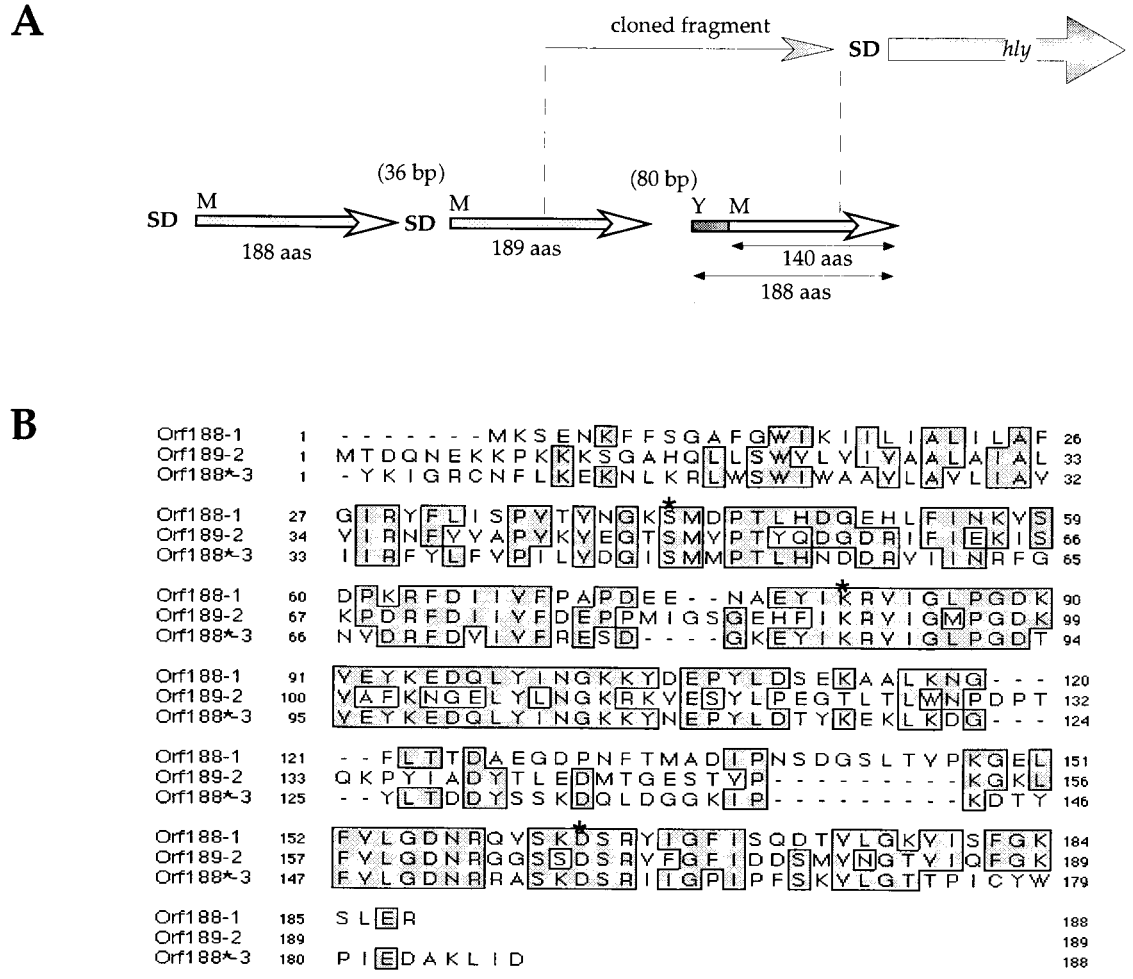

FIG. 3. The leader peptidase cluster. (A) Schematic representation of the *L. monocytogenes* genetic cluster. The upper part indicates the approximate size of the fragment cloned upstream of *hly*. The lower part represents the three consecutive ORFs. The numbers in parentheses indicate the numbers of base pairs between the stop codon of one ORF and the start codon of the following one. SD, Shine-Dalgarno consensus sequence; M, putative N-terminal methionine residue; Y, putative N-terminal tyrosine residue. For the third ORF, the N-terminal methionine residue is preceded by 48 residues (indicated by dark shading). (B) Multiple sequence alignment of the three ORF products. Multiple sequence alignment was performed using the CLUSTALw algorithm (available at http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl). Identical residues are boxed. Orf188-1 corresponds to the product of the first ORF, Orf189-2 corresponds to the product of the second ORF, and Orf188*-*3 corresponds to product of the third ORF (188* refers to the maximal predicted size, comprising the 140 amino acids starting at the first methionine plus the 48 preceding residues). The stars above residues S, K, and D indicate the conserved residues essential for leader peptidase activity.

moter region of a short protein of unknown function (designated Orf104) having similarities to a putative protein of the archaebacterium *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 that of *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 pro gram (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 EGDΔ*hly* transformed with pAT28-*hly* harboring in vivo-inducible promoters from *plcA*, *yvyH*, or *orf104* compared with EGDΔ*hly*, used as a negative control. Mice were inoculated with 1.5×10^8 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 EGDΔ*hly* 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-

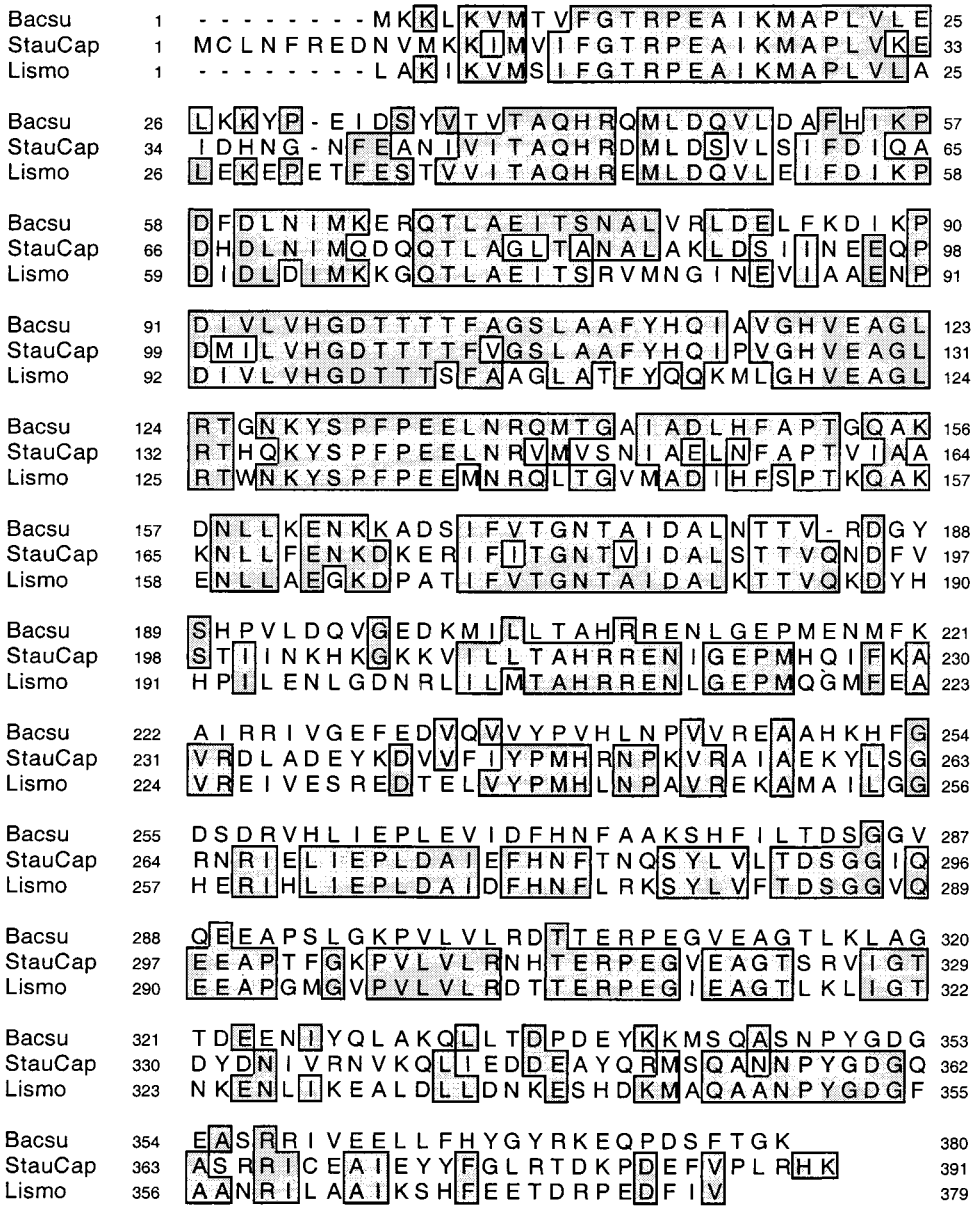

FIG. 4. Multiple sequence alignment of *L. monocytogenes* (Lismo) sequence with those of *B. subtilis* (Bacsu) and *S. aureus* (StauCap) *N*-acetyl glucosamine epimerases. Alignment was performed using CLUSTALw. Identical residues are boxed. Bacsu, YvyH of *B. subtilis* (380 amino acids [aa]); StauCap, Cap5p protein of *S. aureus* (391 aa).

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

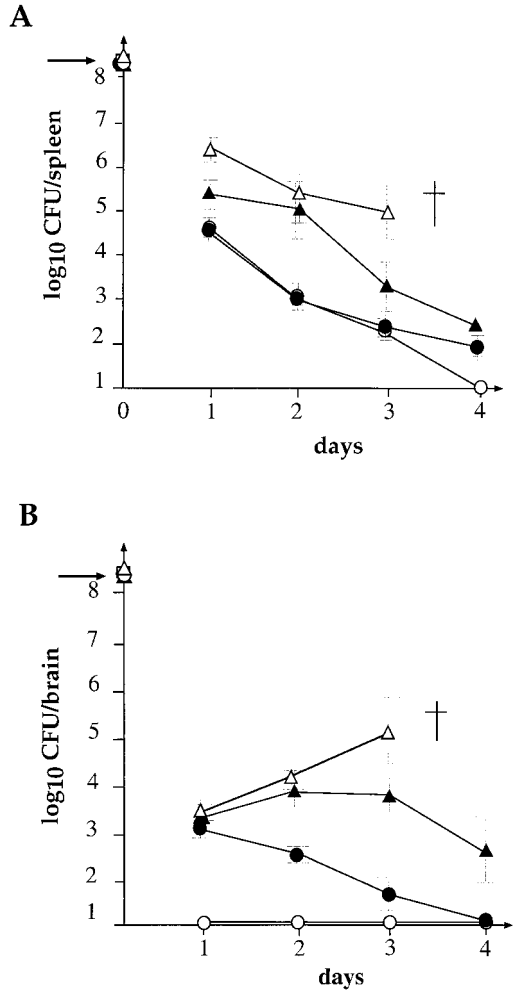

FIG. 5. In vivo survival of the in vivo-inducible strains. The kinetics of bacterial growth was followed in organs of mice infected with EGDΔ*hly* transformed with pAT28-*hly* harboring in vivo-inducible promoters from *plcA* or *yvyH* compared with EGDΔ*hly* as a negative control. Mice were inoculated with 1.5×10^8 bacteria (indicated by an arrow to the left of the ordinate). Bacterial survival was followed in the spleen (A) and the brain (B) over a 4-day period. ○, EGDΔ*hly*; ▲, EGDΔ*hly* transformed with pAT28-*pplcA-hly*; △, EGDΔ*hly* transformed with pAT28-*pyvyH-hly*; ●, EGDΔ*hly* transformed with pAT28-*porf104-hly*; †, death. The error bars represent standard deviations.

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 putative 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 functional. 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 preprotein 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 specifically 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 compartment 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 linkage 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 additional 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 having 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 respect 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. This

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. Interestingly, one of the two sequences corresponds to a PhoR homologue, 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 involved 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.

ACKNOWLEDGMENTS

We thank T. Chakraborty (Giesen, Germany) for the gift of EGDΔ*hly*. We thank Colin Tinsley for careful reading of the manuscript.

This work was supported by CNRS, INSERM, and University Paris V.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Wiley Interscience, New York, N.Y.
3. **Berche, P.** 1995. Bacteremia is required for invasion of the murine central nervous system by *Listeria monocytogenes*. Microb. Pathog. **18:**323–336.
4. **Bubert, A., Z. Sokolovic, S. K. Chun, L. Papatheodorou, A. Simm, and W. Goebel.** 1999. Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. Mol. Gen. Genet. **261:**323–336.
5. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18:** 671–683.
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. **Chu, L., A. Burgum, D. Kolodrubetz, and S. C. Holt.** 1995. The 46-kilodalton-hemolysin gene from *Treponema denticola* encodes a novel hemolysin homologous to aminotransferases. Infect. Immun. **63:**4448–4455.
8. **Cossart, P., M. F. Vicente, J. Mengaud, F. Baquero, J. C. Perez-Diaz, and P. Berche.** 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect. Immun. **57:**3629–3636.
9. **Darji, A., T. Chakraborty, K. Niebuhr, N. Tsonis, J. Wehland, and S. Weiss.** 1995. Hyperexpression of listeriolysin in the nonpathogenic species *Listeria innocua* and high yield purification. J. Biotechnol. **43:**205–212.
10. **Gaillard, J. L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52:**50–55.
11. **Guzman, C. A., M. Rohde, T. Chakraborty, E. Domann, M. Hudel, J. Wehland, and K. N. Timmis.** 1995. Interaction of *Listeria monocytogenes* with mouse dendritic cells. Infect. Immun. **63:**3665–3673.
12. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by in vivo gene expression. Proc. Natl. Acad. Sci. USA **94:**934–939.
13. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **169:**1291–1297.
14. **Kili, A. O., M. C. Herzberg, M. W. Meyer, X. Zhao, and L. Tao.** 1999. Streptococcal reporter gene-fusion vector for identification of in vivo expressed genes. Plasmid **42:**67–72.
15. **Kiser, K. B., N. Bhasin, L. Deng, and J. C. Lee.** 1999. *Staphylococcus aureus* cap5P encodes a UDP-*N*-acetylglucosamine 2-epimerase with functional redundancy. J. Bacteriol. **181:**4818–4824.
16. **Kohler, S., A. Bubert, M. Vogel, and W. Goebel.** 1991. Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. J. Bacteriol. **173:**4668–4674.
17. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
18. **Kutsukake, K., T. Okada, T. Yokoseki, and T. Iino.** 1994. Sequence analysis of the *flgA* gene and its adjacent region in *Salmonella typhimurium*, and identification of another flagellar gene, *flgN*. Gene **143:**49–54.
19. **Leimeister-Wachter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87:**8336–8340.
20. **Lowe, A. M., D. T. Beattie, and R. L. Deresiewicz.** 1998. Identification of novel staphylococcal virulence genes by in vivo expression technology. Mol. Microbiol. **27:**967–976.
21. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
22. **Mahan, M. J., J. W. Tobias, J. M. Slauch, P. C. Hanna, R. J. Collier, and J. J. Mekalanos.** 1995. Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. Proc. Natl. Acad. Sci. USA **92:**669–673.
23. **Mengaud, J., S. Dramsi, E. Gouin, J. A. Vazquez-Boland, G. Milon, and P. Cossart.** 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol. Microbiol. **5:**2273–2283.
24. **Moors, M. A., B. Levitt, P. Youngman, and D. A. Portnoy.** 1999. Expression of listeriolysin O and ActA by intracellular and extracellular *Listeria monocytogenes*. Infect. Immun. **67:**131–139.
25. **Park, S. F., and G. S. Stewart.** 1990. High efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. Gene **94:**129–132.
26. **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.
27. **Poyart, C., and P. Trieu-Cuot.** 1997. A broad host-range mobilizable shuttle vector for the construction of transcriptional fusions to β-galactosidase in Gram-positive bacteria. FEMS Microbiol. Lett. **156:**193–198.
28. **Qi, Y., and F. M. Hulett.** 1998. Role of Pho-P in transcriptional regulation of genes involved in cell wall anionic polymer biosynthesis in *Bacillus subtilis*. J. Bacteriol. **180:**4007–4010.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Expression of cloned genes in *Escherichia coli*, p. 17.37–17.41. *In* J. Sambrook, E. F. Fritsch, and T. Maniatis (ed.), Molecular cloning: a laboratory manual., 2nd ed., vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Sheehan, B., C. Kocks, S. Dramsi, E. Gouin, A. D. Klarsfeld, J. Mengaud, and P. Cossart.** 1994. Molecular and genetic determinants of the *Listeria monocytogenes* infectious process. Curr. Top. Microbiol. Immunol. **192:**187–216.
31. **Staib, P., M. Kretschmar, T. Nichterlein, G. Kohler, S. Michel, H. Hof, J. Hacker, and J. Morschhauser.** 1999. Host-induced, stage-specific virulence gene activation in *Candida albicans* during infection. Mol. Microbiol. **32:**533–546.
32. **Suh, J. W., S. A. Boylan, S. H. Oh, and C. W. Price.** 1996. Genetic and transcriptional organization of the *Bacillus subtilis spc*-α region. Gene **169:**17–23.
33. **Suh, J. W., S. A. Boylan, S. M. Thomas, K. M. Dolan, D. B. Oliver, and C. W. Price.** 1990. Isolation of a *secY* homologue from *Bacillus subtilis*: evidence for a common protein export pathway in eubacteria. Mol. Microbiol. **4:**305–314.
34. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite *Listeria monocytogenes*. J. Cell Biol. **109:**1597–1608.
35. **Tjalsma, H., A. Bolhuis, M. L. van Roosmalen, T. Wiegert, W. Schumann, C. P. Broekhuizen, W. J. Quax, G. Venema, S. Bron, and J. M. van Dijl.** 1998. Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis*: identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases. Genes Dev. **12:**2318–2331.
36. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1990. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in Gram-positive bacteria. Nucleic Acids Res. **18:**4296.
37. **Valdivia, R. H., and S. Falkow.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. **22:**367–378.
38. **Wang, J., A. Mushegian, S. Lory, and S. Jin.** 1996. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by in vivo selection. Proc. Natl. Acad. Sci. USA **93:**10434–10439.
39. **Wuenscher, M. D., S. Kohler, A. Bubert, U. Gerike, and W. Goebel.** 1993. The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. J. Bacteriol. **175:**3491–3501.

Editor: D. L. Burns