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Listeria monocytogenes p60 Supports Host Cell Invasion by and In Vivo Survival of Attenuated *Salmonella typhimurium*

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The extracellular protein p60 is a major virulence factor of the intracellular bacterium *Listeria monocytogenes*. Its roles in pathogen survival in vivo and host cell invasion in vitro were studied. To this end, *Salmonella typhimurium* SL7207 was used as carrier for secreted p60-HlyA fusion protein by *Escherichia coli* HlyB and HlyD transport proteins. C57BL/6 mice infected intravenously with this strain suffered from increased bacterial numbers in livers and spleens compared with the p60-nonexpressing control strain, but only transiently. In vitro experiments showed that p60 promotes invasion of recombinant *S. typhimurium* SL7207 p60 into hepatocytes and resting macrophages independent from complement. Moreover, the uptake of wild-type *L. monocytogenes* EGD and *L. monocytogenes* BUG 8, an internalin-deficient strain, into hepatocytes was partially blocked by anti-p60 antibodies. The impaired invasion of dissociated bacterial chains of *L. monocytogenes* RIII, a p60 expression mutant, into hepatocytes and macrophages was partially restored by addition of p60- or p60-HlyA-enriched bacterial supernatants. These data suggest that the *L. monocytogenes* surface-associated proteins, p60 and internalin, act in concert to achieve optimal uptake into nonprofessional phagocytes and macrophages. Together, these experiments reveal a substantial impact of p60 on cell invasion and virulence and thus emphasize the importance of the intracellular habitat for survival of *L. monocytogenes* in the host.

Listeria monocytogenes, the etiologic agent of listeriosis in humans and animals, and *Salmonella typhimurium*, a mouse pathogen of the typhoid-paratyphoid group, are intracellular bacteria (25). These pathogens are endowed with distinct features, which secure life in unique, intracellular niches of the host. Both nonprofessional phagocytic cells, e.g., hepatocytes and epithelial cells, and professional phagocytes, i.e., macrophages, are used as habitats which facilitate microbial survival in the host. Penetration of *L. monocytogenes* into cultured epithelial cells is promoted by the gene locus *inlA*, which has been identified by transposon mutagenesis (14). Its gene product, internalin, is a repeat protein reminiscent of surface antigens of gram-positive cocci (14). Internalin appears to be attached to the bacterial cell wall via its hydrophobic C-terminal part. In addition, a 60-kDa protein (p60), a major extracellular product of all isolates of *L. monocytogenes* (3), probably contributes to the invasion of epithelial cells (29). A spontaneously occurring mutant of *L. monocytogenes*, strain RIII, which shows decreased expression of p60, fails to enter nonprofessional phagocytes and is avirulent for mice (29, 33). Pretreatment of RIII mutants with partially purified p60 causes disaggregation of bacterial chains and restores invasiveness (29). The *iap* gene, encoding p60 of *L. monocytogenes*, has recently been cloned and sequenced (28). The p60 protein shows sequence similarity with the repeat domain of an autolysin of *Streptococcus faecalis* (39). Expression of the *iap* gene by *L. monocytogenes* and *Bacillus subtilis* DB104 causes bacteriolysis, suggesting that p60 acts as a murine hydrolase which is involved in cell division (39). A concomitant role of p60 in adhesion and bacteriolysis is not obvious and requires further clarification. Destruction of the *iap* gene in *L. monocytogenes* by sequence-specific recombination is lethal (39). Therefore, expression of biologically active p60 in an appropriate carrier system, such as the attenuated *S. typhimurium* SL7207 *aroA* mutant (21), could provide a helpful tool for studying the role of p60 in pathogenesis. Attenuated strains of *S. typhimurium* have received increasing attention because of their potential use as live vaccines against virulent *Salmonella* infections and also as live vaccine vectors expressing cloned genes encoding protective protein antigens from unrelated pathogens (22). Growth of auxotrophic mutants of *S. typhimurium* carrying nonreverting deletions in the *aroA* gene depends on certain aromatic compounds. These deletion mutants have served as effective vaccines in several infection models (19, 22).

Using recombinant *S. typhimurium* SL7207 as a carrier and the HlyB-HlyD transport system from *Escherichia coli* for secretion of a p60-hemolysin (HlyA) fusion protein, we found a major impact of p60 from *L. monocytogenes* on bacterial invasion in vivo and in vitro. First, the load of *S. typhimurium* SL7207 expressing p60-HlyA (SL7207 p60) in C57BL/6 mice was increased. Second, elevated in vitro invasion of macrophages and hepatocytes by SL7207 p60 supports a central role of this protein in bacterial internalization.

MATERIALS AND METHODS

Mice. C57BL/6 mice were kept under specific-pathogen-free conditions in isolators and fed autoclaved food and water ad libitum at the central animal facilities of the University of Ulm. In a given experiment, mice were age and sex matched.

Construction and expression analysis of *S. typhimurium* SL7207 p60. Sense primer M430 (5′–*Nsi*I site–*iap* DNA sequence positions 531 to 544–TC CGATATGCATCCACTGTAGTAGTCG) and antisense primer M429 (5′–*Nsi*I site–*iap* DNA sequence positions 1885 to 1897–AAGTTAATGCATATATCGC GACCGAAGCC) were used to amplify by PCR (24) a 1.4-kb DNA fragment representing the *iap* gene from plasmid pSK5 (28). PCRs were performed in a Thermal Cycler 60 (Biometra, Göttingen, Germany) for 30 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min. After purification with the GeneClean Kit (Bio101, La Jolla, Calif.) and digestion with the *Nsi*I restriction enzyme, the DNA fragment, carrying the *iap* gene without the N-terminal signal sequence,

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was inserted into the single *Nsi*I site (DNA sequence positions 1418 to 4208 [20]) of the export vector pANN202-812ΔNsiIΔClaI (31b, 36). The resulting plasmid, pIPH1, carried the *iap-hlyA* fusion gene and the functional *hlyB* and *hlyD* genes required for HlyA protein export in *Salmonella* sp. and *E. coli*. Both plasmids, pANN202-812ΔNsiIΔClaI (control) and pIPH1 (p60), were transformed into *S. typhimurium* SL7207. The bacterial cell extracts and culture supernatant samples from the SL7207 strains, SL7207 control and SL7207 p60, were prepared as previously described (18). Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (31) and transferred to nitrocellulose filters (35). Proteins reacting with polyclonal rabbit anti-p60 antibodies (17) were visualized by the horseradish peroxidase reaction with 0.015% hydrogen peroxide and 4-chloro-1-naphthol.

Enzymes. Restriction endonucleases, *Taq* DNA polymerase, and T4 ligase were obtained from Boehringer (Mannheim, Germany). The reaction conditions were those described by the manufacturer.

Microorganisms. *L. monocytogenes* EGD Sv 1/2a was originally obtained from G. B. Mackaness. *L. monocytogenes* RIII (SLCC 5779), a rough mutant strain, was originally obtained from J. Potel, Institute for Medical Microbiology, Medical Academy, Hannover, Germany. *L. monocytogenes* EGD Sm^r BUG 8, the Tn*1545*-induced internalin (*inlA*) mutant strain, was a kind gift from P. Cossart, Institut Pasteur, Paris, France (6, 14). *Listeria* spp. were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C. Strain SL7207 is *S. typhimurium* 2337-65 (WRAY) *hisG46* del*407 aroA544*::Tn*10* Tc^s and was kindly provided by B. Stocker, Department of Medical Microbiology, Stanford University, Palo Alto, Calif. (21). The *S. typhimurium* strains SL7207 pANN202-812ΔNsiIΔClaI (termed SL7207 control) and SL7207 pIPH-1 (termed SL7207 p60) were grown in 2× yeast tryptone medium (Difco) supplemented with 10 μg of *p*-aminobenzoic acid (PABA; Sigma, Deisenhofen, Germany) per ml, 10 μg of 2,3-dihydroxybenzoic acid (DHB; Sigma) per ml, and 100 μg of ampicillin (Sigma) per ml at 37°C. Both recombinant *S. typhimurium* strains, SL7207 p60 and SL7207 control, showed similar growth rates in broth and on agar.

Infection studies. Mice at 8 to 10 weeks of age were infected intravenously (i.v.) with *S. typhimurium* SL7207 recombinant strains at 5×10^5 bacteria per 200 μl. CFU in spleens, livers, and mesenterial lymph nodes (MLNs) of infected mice were determined by plating serial dilutions of organ homogenates on nutrient agar containing 10 μg of PABA per ml, 10 μg of DHB per ml, and 100 μg ampicillin per ml. *L. monocytogenes* bacteria were recovered from organ homogenates by plating on tryptic soy agar (GIBCO BRL, Eggenstein, Germany) supplemented with 10 μg of kanamycin (Sigma) per ml in the case of *L. monocytogenes* BUG 8.

Cell lines. The mouse liver cell line ATCC TIB75, a transformed cell line derived from embryonic hepatocytes, was cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL) with 10% fetal calf serum (FCS) (34a). Bone marrow-derived macrophages (BMM) from the femora of 8- to 12-week-old C57BL/6 mice were used as resting macrophages (12). They were cultured in DMEM (GIBCO BRL) supplemented with 10% FCS, 5% horse serum, and 30% conditioned medium from L929 cells at 37°C in CO_2 for 9 days as described previously (11).

Invasion of hepatocytes and BMM by bacteria. Hepatocytes or BMM were seeded into 96-well flat-bottom microculture plates in DMEM supplemented with 10% FCS, without antibiotics, at 10^5 cells per well. Cells were infected with the *L. monocytogenes* strain EGD, BUG 8, or RIII or with the *S. typhimurium* strain SL7207 control or SL7207 p60 at various infection rates (indicated in the figure legends). At 60 min postinfection (p.i.) (hepatocytes) or 30 min p.i. (macrophages), gentamicin sulfate (10 μg/ml for *L. monocytogenes* and 200 μg/ml for *S. typhimurium*) was added to kill extracellular bacteria. Drevets et al. (8) reported intracellular killing of *L. monocytogenes* by gentamicin. In contrast, we did not detect any intracellular bacterial growth inhibition, although high concentrations of gentamicin were used in the *Salmonella* invasion assays. Cells were washed three times with phosphate-buffered saline (PBS) and lysed by treatment with 0.1% saponine dissolved in PBS at the time points indicated in the figures. Bacteria from five wells were pooled, serial dilutions were plated onto Trypticase soy plates (Difco) or 2× yeast tryptone broth (including 10 μg each of PABA and DHB per ml), and CFU were determined. For blocking of invasion by *L. monocytogenes* EGD and BUG 8, bacteria were preincubated with polyclonal rabbit anti-p60 antibodies (final dilution, 1:400) at 4°C for 30 min prior to hepatocyte infection. Normal rabbit immunoglobulins G (Dianova, Hamburg, Germany) were used as the isotype control. The influence of mouse serum on bacterial invasion into macrophages was analyzed by addition of 10% mouse serum to bacteria at 4°C for 30 min prior to infection. The contribution of p60 or p60-HlyA proteins on *L. monocytogenes* RIII invasion of hepatocytes and BMM was studied by the addition of fivefold-concentrated, bacterium-free supernatants from *L. monocytogenes* M3 (Hly^- [29]) or from *S. typhimurium* SL7207 p60 prior to (1 volume of fivefold-concentrated supernatants incubated overnight at 4°C) and during (1/4 volume of fivefold-concentrated supernatants) infection. Supernatants from the *S. typhimurium* SL7207 control strain were used as a p60-HlyA-negative *Salmonella* control. Ultrasonication of *L. monocytogenes* RIII was performed as previously described (29). The CFU-counting procedure for *L. monocytogenes* RIII-infected cell types is the same as that described above.

RESULTS

Construction and characterization of recombinant *S. typhimurium* SL7207 expressing p60 from *L. monocytogenes*. The *S. typhimurium* SL7207 strain was used as carrier for listerial p60 to study the impact of this protein on bacterial virulence independent from other gene products of *L. monocytogenes* (32). In order to mimic cell surface and extracellular localization of p60 in the gram-negative *Salmonella* sp. as it occurs in *L. monocytogenes*, we used the HlyB-HlyD export system as described previously (17, 37). We used the plasmid pANN202-812ΔNsiIΔClaI (31b, 36), carrying the entire hemolysin operon except for *hlyA* from *E. coli*, as a vector for the construction of an *iap-hlyA* gene fusion. At the N-terminal end, the corresponding hybrid protein p60-HlyA contains 34 amino acids specific for HlyA, and at the carboxy terminus, it contains 61 amino acids representing the transport signal of HlyA (18). The translocation signal allowed recognition by the required transport proteins, HlyB and HlyD, which were also encoded by the same recombinant construct outlined in Fig. 1A, now termed pIPH-1. The p60-HlyA fusion protein carries the complete amino acid sequence for p60 but lacks the N-terminal signal peptide of p60 in order to prevent competition with the general export pathway (16). The p60-HlyA fusion protein, 66 kDa in size, was detected in the supernatants of *S. typhimurium* SL7207 p60 by immunoblotting with polyclonal antibodies raised against p60 (Fig. 1B).

Elevated numbers of *S. typhimurium* SL7207 p60 in C57BL/6 mice. In order to characterize the impact of p60 on bacterial growth in infected organs, we studied the course of *S. typhimurium* SL7207 p60 infection through the i.v. or oral route in C57BL/6 mice. Results shown in Fig. 2 reveal that mice infected i.v. with recombinant *S. typhimurium* SL7207 p60 suffered from significantly higher bacterial numbers (ca. $\log_{10}$ 2.5 CFU) in livers and spleens at days 3 and 8 p.i. compared with mice infected with *S. typhimurium* SL7207 control. However, at 3 weeks p.i., bacterial numbers in infected organs were comparable for both microbes, SL7207 p60 and SL7207 control (Fig. 2). In contrast, at days 1, 3, and 7 after oral application of 5×10^9 bacteria, numbers of *S. typhimurium* SL7207 p60 and SL7207 control microorganisms in MLNs were in the same order of magnitude (Table 1). Interestingly, under similar experimental conditions with kanamycin selection, the bacterial burden of *L. monocytogenes* BUG 8 in livers, spleens, and MLNs was comparable to that of wild-type *L. monocytogenes* EGD (Table 1). The BUG 8 mutant strain is p60 positive and *inlA* negative and expresses impaired invasion capacity for epithelial cells (14). The potential loss of the recombinant plasmids from *S. typhimurium* in vivo was assessed by plating organ homogenates on selective (ampicillin) and nonselective agar. Approximately 90 to 100% of bacteria still harbored the plasmids pIPH-1 and pANN202-812ΔNsiIΔClaI by day 21 p.i. We conclude that p60 plays a profound but transient role in bacterial survival in the host and hence represents a virulence factor of *L. monocytogenes*. In addition, these data do not confirm participation of the *inlA* gene product in listerial invasion through the intestine and do not directly exclude such a function for p60.

Increased invasion of murine hepatocytes and macrophages by *S. typhimurium* SL7207 p60. On the basis of our in vivo findings that p60 increased the survival and replication of *S. typhimurium* early during infection, we assessed whether this feature was related to increased invasion of host cells in vitro. Because hepatocytes and resident macrophages (Kupffer cells in the liver) serve as preferential habitat for both *S. typhimurium* and *L. monocytogenes* (5, 34a, 38), we performed in-

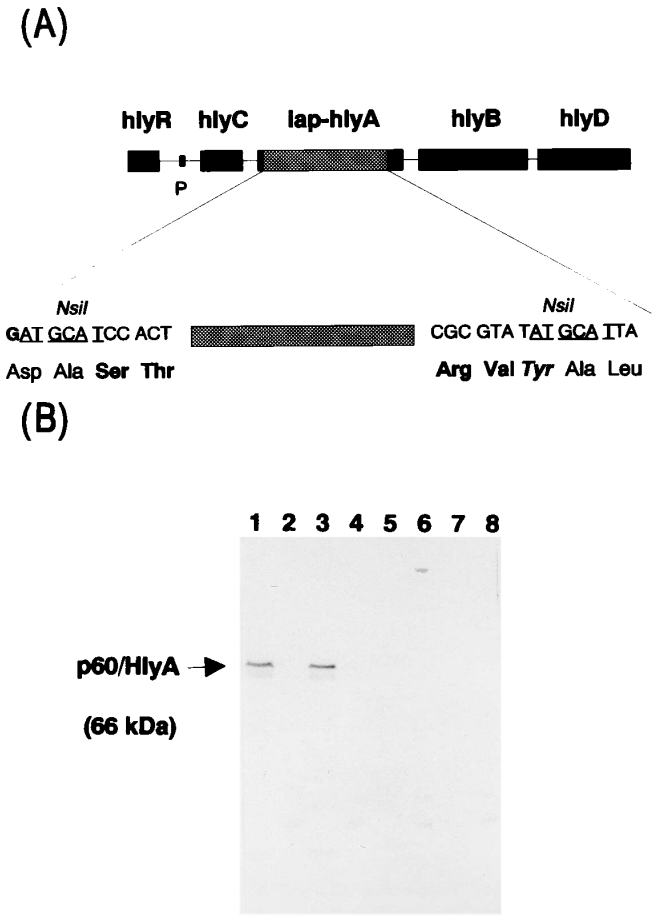

FIG. 1. (A) Schematic representation of the secretion plasmid pIPH-1. The coding frame at the fusion sites is indicated. p60-derived amino acids (boldface letters), HlyA-derived amino acids (lightface letters), and an unrelated amino acid (italics) are shown. *hlyC*, *hlyA*, *hlyB*, and *hlyD* make up the hemolysin gene cluster from *E. coli* pHly152, *hlyR* is an upstream regulation element, P is the promoter, and *iap-hlyA* is a gene fusion. *Nsi*I, restriction sites (DNA sequence positions 1418 and 4208 [18]). (B) Identification of the p60-HlyA fusion protein by immunoblotting with polyclonal rabbit anti-p60 antibodies. *S. typhimurium* SL7207 carrying the plasmid pIPH-1 (SL7207 p60; lanes 1 to 4) or pANN202-812ΔNsiIΔClaI (SL7207 control; lanes 5 to 8) was grown in 2× yeast tryptone medium supplemented with DHB and PABA to a cell density of 2×10^8 per ml (lanes 1, 2, 5, and 6) or 7×10^8/ml (lanes 3, 4, 7, and 8) and used to prepare cellular (lanes 2, 4, 6, and 8) and extracellular proteins (lanes 1, 3, 5, and 7) (see Materials and Methods).

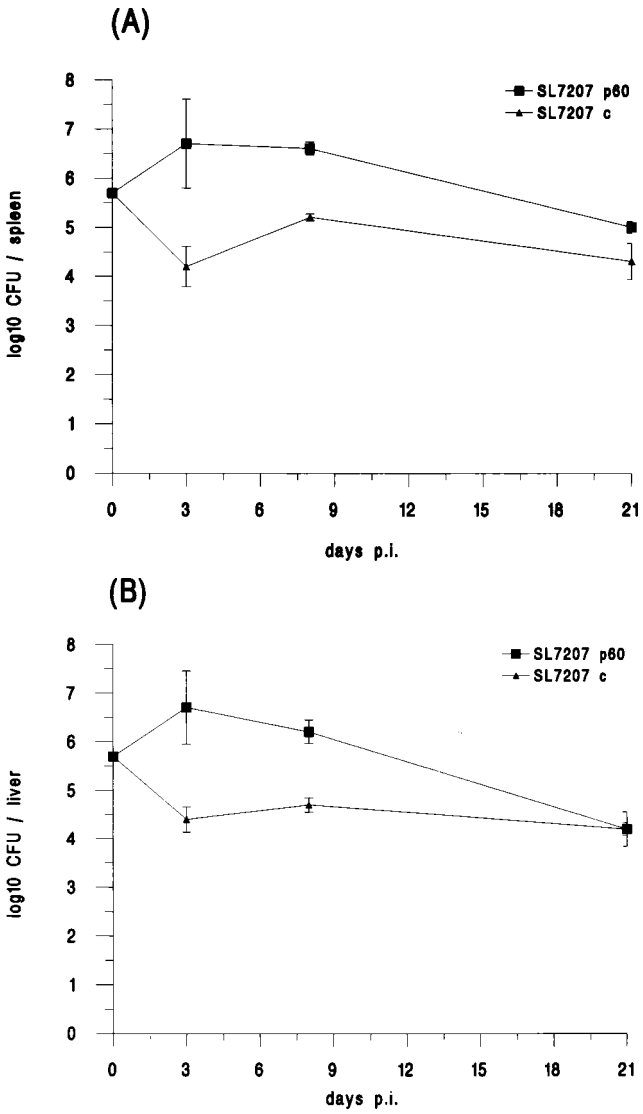

FIG. 2. Course of *S. typhimurium* SL7207 p60 infection in C57BL/6 mice. The numbers of recovered bacterial colonies from spleens (A) and livers (B) of mice after i.v. infection with 5×10^5 SL7207 control (▲) or SL7207 p60 (■) cells. Each point represents the geometric mean of three experiments with four or five mice per group. Vertical bars show the standard deviation of the geometric mean.

vasion studies with these two cell types. We used BMM as a source of resting macrophages (12). As a source of hepatocytes, the murine hepatocyte cell line ATCC TIB75 was used. Hepatocytes or BMM (10^5 cells per well) were seeded into 96-well flat-bottom microtiter plates and incubated at 37°C overnight. *S. typhimurium* SL7207 control or SL7207 p60 bacteria were added at the bacterium/cell ratios indicated in the figure legends, and external bacteria were killed by gentamicin treatment. As shown in Fig. 3A, the capacity of *S. typhimurium* SL7207 p60 to invade hepatocytes was increased 10- to 40-fold (depending on the hepatocyte/bacterium ratio) compared with the SL7207 control. For macrophages, the invasion rate of *S. typhimurium* SL7207 p60 was more than 100-fold higher than that of SL7207 control (Fig. 3B). Addition of 10% mouse serum to *S. typhimurium* SL7207 control and SL7207 p60 prior to macrophage infection slightly elevated phagocytosis of both strains, suggesting that complement receptor-mediated uptake was p60 independent. We conclude that p60 promotes invasion of both professional and nonprofessional phagocytes by *Salmonella* spp. in the absence of complement-facilitated uptake.

Partially purified p60-HlyA fusion protein restores invasiveness of the *L. monocytogenes* RIII strain. In order to stress the functional activity of the p60-HlyA hybrid protein during listerial uptake, we determined invasion rates of the p60-expression mutant *L. monocytogenes* RIII into the murine hepatocyte cell line (ATCC TIB75) and BMM in the presence of p60-HlyA-enriched supernatants. The *S. typhimurium* SL7207 p60 strain or the nonhemolytic *L. monocytogenes* M3 mutant (30), respectively, was used as the source for enrichment of p60-HlyA or p60 proteins in the supernatants of bacterial cultures. The supernatant of *S. typhimurium* SL7207 control served as the p60-HlyA-negative control in the invasion assay. As shown in Fig. 4A, *L. monocytogenes* RIII bacterial chains and single

TABLE 1. Growth of *S. typhimurium* SL7207 p60 or *L. monocytogenes* BUG 8 in C57BL/6 mice after oral infection

Bacterial strain	Day p.i.	Log_{10} CFU (mean ± SD) in:		
		Spleens	Livers	MLNs
S. typhimurium[a]				
SL7207 p60	1	<2	<2	3.7 ± 0.2
	3	<2	<2	4.05 ± 0.3
	7	<2	<2	4.4 ± 0.2
SL7207 control	1	<2	<2	3.5 ± 0.2
	3	<2	<2	3.8 ± 0.2
	7	<2	<2	4.4 ± 0.1
L. monocytogenes[b]				
BUG 8	1	2.7 ± 0.4	2.9 ± 0.8	2.6 ± 0.3
	5	3.6 ± 0.1	4.5 ± 1.5	2.3 ± 0.3
EGD	1	3.3 ± 0.2	2.4 ± 0.5	2.5 ± 0.7
	5	4.2 ± 0.8	3.7 ± 0.2	2.6 ± 0.3

[a] Five mice per group were inoculated per os with 5×10^9 *Salmonella* cells. The CFU differences between SL7207 p60- and SL7208 control-infected mice were not statistically significant ($P > 0.05$, Student's t test).

[b] Five mice per group were infected per os with 2×10^8 *Listeria* cells. The CFU differences between BUG 8- and EGD-infected mice were not statistically significant ($P > 0.05$, Student's t test).

RIII bacteria after ultrasonication of RIII chains failed to invade hepatocytes. In contrast, *L. monocytogenes* RIII cells were partially able to enter BMM but only as long bacterial chains (Fig. 4B). Addition of p60 or p60-HlyA prior to (1 volume of fivefold-concentrated supernatant incubated overnight at 4°C) and during (1/4 volume of fivefold-concentrated supernatant) to single-cell suspensions of RIII bacteria partially restored listerial uptake by both host cell types (Fig. 4). In contrast, p60-HlyA-negative supernatants from *S. typhimurium* SL7207 control bacterial cultures did not induce *L. monocytogenes* RIII invasion in our system (data not shown). We conclude that p60-HlyA secreted by *S. typhimurium* SL7207 p60 exhibits the same functions as the original p60 from *L. monocytogenes* in listerial invasion.

Anti-p60 antibodies block hepatocyte uptake of *L. monocytogenes* EGD and BUG 8. Gaillard et al. (14) described a Tn*1545*-induced *L. monocytogenes* mutant strain, BUG 8, with defective entry into various epithelial cell lines, e.g., Caco-2, HT-29, HeLa, and HEp-2. We tested the capacity of this internalin-deficient *L. monocytogenes* BUG 8 strain to invade the murine hepatocyte cell line (ATCC TIB75). As shown in Fig. 5, the invasion of hepatocytes by *L. monocytogenes* BUG 8 was reduced to 2 to 29% (depending on the infection ratio) of the invasion rate determined for *L. monocytogenes* EGD. In contrast, relative uptake of *L. monocytogenes* BUG 8 into macrophages corresponded to about 65% of invasion rates established for *L. monocytogenes* EGD bacteria (data not shown). Thus, internalin mainly participated in invasion of hepatocytes. In order to determine the role of p60 in internalin-independent entry of *L. monocytogenes* BUG 8 into hepatocytes, polyclonal rabbit anti-p60 antibodies were added to BUG 8 bacteria 30 min prior to cell infection. The invasion assay was performed with the *L. monocytogenes* strains EGD and BUG 8 at hepatocyte/bacterial cell ratios of 1:20 and 1:50 (Fig. 5). Invasion of hepatocytes by BUG 8 organisms was markedly inhibited by the anti-p60 antibodies. Inhibition ranged from 80 to 90% at hepatocyte/bacterial cell ratios of 1:20 and 1:50, respectively, and *L. monocytogenes* EGD invasion was blocked by ca. 60%. We take these data as further evidence for a direct and internalin-independent contribution of p60 to host cell invasion.

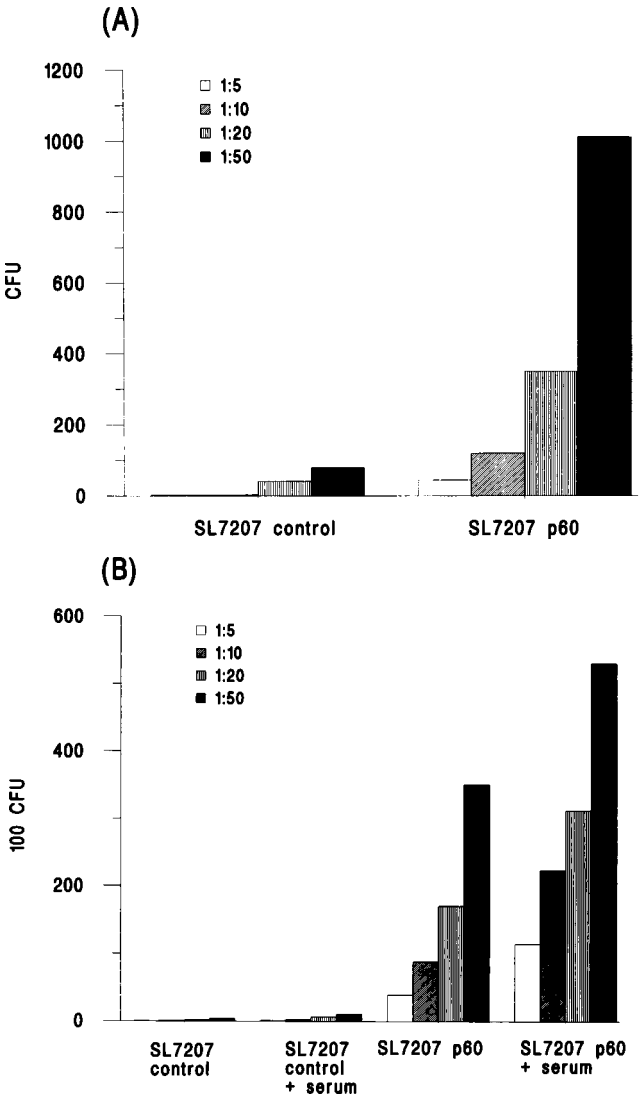

FIG. 3. Uptake of *S. typhimurium* SL7207 p60 by hepatocytes and BMM. (A) Invasion of a murine hepatocyte cell line (ATCC TIB75) by *S. typhimurium* SL7207 p60 and SL7207 control with different hepatocyte/bacterial cell ratios as indicated in the figure. Internalized bacterial numbers 60 min p.i. are expressed on a linear scale of CFU per 10^5 hepatocytes. (B) Uptake of *S. typhimurium* SL7207 p60 and SL7207 control cells by BMM in the presence or absence of murine serum. The infection doses (macrophage/bacterial cell ratios) are shown outlined in the figure. The CFU per 10^5 BMM values for recovered intracellular bacteria are given on a linear scale. Experiments were repeated three times with similar results.

DISCUSSION

In the experiments described here, an attenuated *aroA* deletion mutant of *S. typhimurium*, SL7207, combined with the improved HlyB-HlyD translocation system (17, 18) was used as a powerful vehicle for mimicking compartmentalization of p60 protein from *L. monocytogenes*. Using this carrier system, we obtained strong evidence for a crucial impact of *L. monocytogenes* p60 in host cell invasion and in survival in mice. The recombinant *S. typhimurium* strain efficiently expressed and secreted p60 as a 66-kDa p60-HlyA fusion protein. However, small amounts of this antigen remained cell associated, probably attached to the outer surface of the bacterial membrane (15a, 17).

Our in vivo studies reveal that the p60-HlyA fusion protein

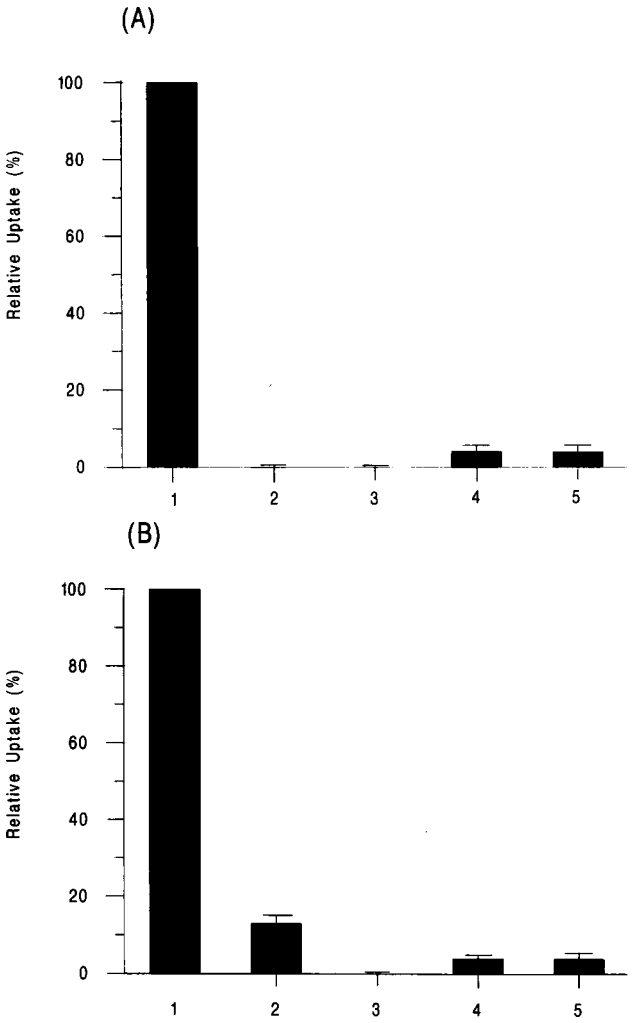

FIG. 4. Functional comparison of p60-HlyA and p60 in *L. monocytogenes* RIII invasion. Reconstitution of invasiveness of *L. monocytogenes* RIII into a hepatocyte cell line (A) and BMM (B) by treatment with p60 (concentrated supernatant of *L. monocytogenes* M3) or p60-HlyA (concentrated supernatant of *S. typhimurium* SL7207 p60) in a cell/bacterial cell ratio of 1:5. The efficiency of uptake for *L. monocytogenes* EGD was set at 100% (lane 1); lane 2, *L. monocytogenes* RIII; lane 3, *L. monocytogenes* RIII ultrasonicated for 60 s; lane 4, *L. monocytogenes* RIII ultrasonicated plus p60; lane 5, *L. monocytogenes* RIII ultrasonicated plus p60-HlyA. Each column represents the mean value + the standard deviation from three independent experiments. The differences in group two between BMM and hepatocytes are statistically significant ($P < 0.05$; Student's t test).

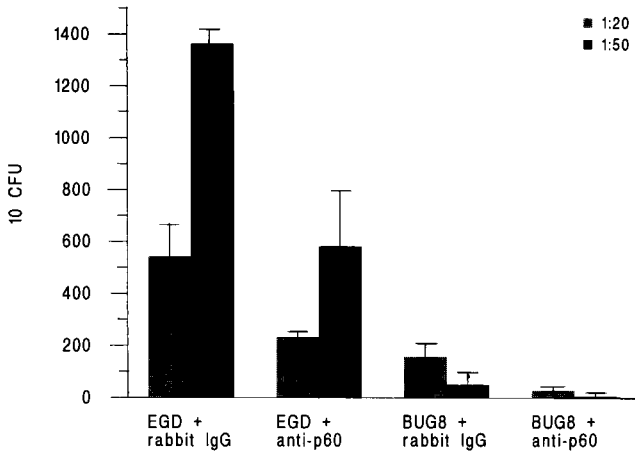

FIG. 5. Contribution of p60 to hepatocyte invasion by *L. monocytogenes*. Blocking of *L. monocytogenes* BUG 8 and EGD uptake into a murine hepatocyte cell line (ATCC TIB75) (at the hepatocyte/bacterial cell ratios indicated in the figure) by rabbit polyclonal anti-p60 antibodies. Normal rabbit immunoglobulin G antibodies were used as a control. The CFU per 10^5 hepatocytes 60 min p.i. are shown on a linear scale. Experiments were performed three times with similar results. Results are means + standard deviations. The differences between the immunoglobulin G-treated control group and the anti-p60-treated group are significant ($P < 0.05$, Student's t test).

not only serves as a surface-expressed antigen of attenuated *S. typhimurium* SL7207 p60 but simultaneously promotes bacterial growth early in infection. Although p60 is expressed as a hybrid protein with N- and C-terminal amino acid sequences derived from HlyA, it contributed to the in vivo survival of *S. typhimurium*. Mice infected i.v. with *S. typhimurium* SL7207 p60 suffered from higher bacterial loads in livers and spleens at days 3 and 8 p.i. than did mice infected with the SL7207 control strain. Oral ingestion of *S. typhimurium* SL7207 p60 did not lead to significant differences of bacterial numbers in MLNs at day 1, 3, or 7 p.i. compared with SL7207 control. These data suggest that p60-mediated uptake pathways are not relevant for *Salmonella* invasion via M cells or columnar epithelial cells in the intestine (24a, 26). Evidence that mucosal penetration by *L. monocytogenes* into the host is different from that by *Salmonella* sp. exists. Listerial invasion seems not to be restricted to M cells and appears to progress through epithelial cells above Peyer's patches (31a). Oral infection performed with *L. monocytogenes* BUG 8, a p60-secreting, internalin-deficient, and in vitro-noninvasive strain for epithelial cell lines (14), and with wild-type *L. monocytogenes* EGD also revealed no invasion differences from the intestine.

Although p60-HlyA had no influence on the kinetics of *S. typhimurium* infection through the intestine, our data do not exclude a p60 contribution to listerial invasion after oral infection. Nevertheless, our study raises the possibility that an additional virulence factor(s) which participates in uptake of intestinal *L. monocytogenes* exists.

The data given in Table 2, which show that *S. typhimurium* SL7207 p60 uptake into hepatocytes and macrophages is drastically enhanced compared with the p60-nonexpressing SL7207 control strain, support our in vivo findings. Both cell types serve as a habitat for *L. monocytogenes* and *S. typhimurium*. Note that the most dominant induction of *S. typhimurium* SL7207 p60 invasion was observed with BMM. Furthermore, opsonization of bacteria with 10% mouse serum prior to macrophage infection increased invasion only slightly. In addition, we showed that the p60-HlyA fusion protein exhibits the same functional properties as p60 of *L. monocytogenes* in the invasion process. Thus, we conclude that p60-HlyA and p60 promote *Salmonella* and *Listeria* invasion of professional phagocytes independent from complement-facilitated uptake (1, 7).

Besides p60, a second cell surface-associated protein, internalin (6, 14), is involved in the invasion of hepatocytes and macrophages by *L. monocytogenes*. Our data from *L. monocytogenes* BUG 8 and RIII invasion assays suggest that both proteins have to be coexpressed to promote efficient internalization into hepatocytes and macrophages. Therefore, it appears that both internalin and p60 contribute to invasion of nonphagocytic and phagocytic cells in vitro. Nevertheless, internalin had only a minor impact on invasion into macrophages.

Invasion of epithelial cells, e.g., Henle-407 cells, by *S. typhi-*

TABLE 2. Increase or decrease of host cell invasion by *S. typhimurium* SL7207 p60 and *L. monocytogenes* EGD, RIII, and BUG 8

Bacterial strain	Change[a] of level of invasion (direction) of:	
	Hepatocyte cell line (ATCC TIB75)	BMM
L. monocytogenes		
RIII[b]	0 (↓)	13 (↓)
RIII[c]	0 (↓)	0 (↓)
RIII + p60-HlyA[c,d]	4 (↓)	4 (↓)
RIII + p60[c,d]	4 (↓)	4 (↓)
BUG 8[b]	2–29 (↓)	65 (↓)
BUG 8 + anti-p60[e]	0.3–5 (↓)	ND[f]
EGD + anti-p60[e]	43 (↓)	ND
S. typhimurium SL7207 p60[g]	10- to 40-fold (↑)	93- to 110-fold (↑)

[a] Percent change, unless specified as fold. Downward-pointing arrow, loss; upward-pointing arrow, gain. All experiments were performed three times with comparable results. The range of values is explained by the difference in infection doses used in the experiments.

[b] Compared with rate of wild-type *L. monocytogenes* EGD invasion.

[c] Invasion rate of ultrasonicated *L. monocytogenes* RIII bacteria compared with that of *L. monocytogenes* EGD.

[d] Addition of 1 volume of fivefold-concentrated p60-HlyA- or p60-containing supernatant to a single-cell suspension of *L. monocytogenes* RIII bacteria incubated overnight at 4°C and of 1/4 volume of the same supernatant preparation during infection.

[e] Percent reduction of bacterial numbers compared with that of rabbit immunoglobulin G-treated microorganisms (see Materials and Methods).

[f] ND, not done.

[g] Judged by comparison with *S. typhimurium* SL7207 control internalization.

murium is thought to be receptor mediated. Bacterial internalization is accompanied by tyrosine phosphorylation of the epidermal growth factor (EGF) receptor (15). Consistent with this notion, an isogenic strain of *S. typhimurium* with defective tyrosine phosphorylation and invasion carries a mutation in the *invA* gene.

However, *S. typhimurium* is capable of entering into a number of cultured mammalian cells, including the murine hepatocyte cell line ATCC TIB75 used here, which do not express the EGF receptor (2, 17a). Hence, *S. typhimurium* can stimulate more than one signal transduction pathway to promote entry into mammalian cells. We assume that p60 stimulated EGF receptor-independent uptake of *S. typhimurium* because it promoted entry of *S. typhimurium* SL7207 p60 into hepatocytes, which do not express EGF receptors. Therefore the question of how p60 facilitates entry into professional and nonprofessional phagocytic cells arises.

We assume a more general mechanism for the induction of phagocytosis by unique structural features of the p60 protein. Bubert et al. (3) identified sequence homology between the C-terminal part of p60 and the C-terminal half of an *Enterococcus faecium* protein, p54 (13). The p54 protein can be detected in cell wall preparations and exhibits low homology to M proteins of group A streptococci. It exclusively contains 12 negatively charged residues in the surroundings of and within the TN repeat domain (sequence positions 281 to 398 [13]). In contrast, p60 from *L. monocytogenes* carries three positively charged amino acids, but no negatively charged residues, close by and within the TN repeat region (sequence positions 305 to 374 [28]). The net charges of the two proteins are −20 for p54 and +11 for p60. This striking difference in the distribution of charges to distinct protein regions represents a hint for the possible mechanism of p60-facilitated endocytosis. The structurally embedded, positively charged amino acids in p60 could improve interactions between the bacteria and the negatively charged surface molecules of phagocytic cells via electrostatic attraction. Conversely, the negative charges of the M protein cause electrostatic repulsions which interfere with the uptake of streptococci by phagocytes (10, 23).

In conclusion, our data have provided further information about virulence strategies of *L. monocytogenes*. The striking impact of p60 both on host cell invasion in vitro and on mouse virulence in vivo emphasizes the central role of the intracellular habitat for *L. monocytogenes* survival in the host. Moreover, it appears that p60 also contributes to host invasion through the natural port of entry, the intestinal epithelium.

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