INFECTION AND IMMUNITY, Dec. 1994, p. 5608–5613
0019-9567/94/$04.00+0

Vol. 62, No. 12

Characterization of *Listeria monocytogenes* Pathogenesis in a Strain Expressing Perfringolysin O in Place of Listeriolysin O

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Received 18 July 1994/Returned for modification 13 September 1994/Accepted 28 September 1994

Listeriolysin O (LLO) is a pore-forming cytolysin that enables *Listeria monocytogenes* to escape from a host cell vacuole. The structural gene for the related cytolysin perfringolysin O (*pfo*) was cloned downstream from the promoter for *hly*, the gene encoding LLO, both on a plasmid and on the *L. monocytogenes* chromosome. Both strains secreted active PFO, although regulation was not identical to that of LLO. The chromosomal PFO-expressing strain was characterized for intracellular growth and cell-to-cell spread. It escaped from a host cell vacuole with 64% efficiency compared with the wild type as determined by immunofluorescent staining of bacteria for F-actin, a marker for entry into the cytoplasm. In addition, it replicated intracellularly with a doubling time similar to that of the wild type for 5 h, after which growth was aborted because of a cytotoxic effect on the host cell and influx of extracellular gentamicin. The chromosomal PFO strain was able to plaque in mouse L_2 fibroblasts, but it did so at 20% efficiency compared with the wild type and the plaques were significantly smaller. Both strains expressing PFO were completely avirulent in mice. These results indicate that PFO can mediate escape from a host cell vacuole but cannot complement an *hly* deletion strain for virulence.

Listeria monocytogenes is a gram-positive facultative intracellular pathogen that causes disease primarily in immunocompromised hosts, pregnant women, and neonates, resulting in bacteremia, spontaneous abortion, meningitis, or meningoencephalitis (11, 14). During the course of infection, *L. monocytogenes* escapes from a host cell vacuole, grows in the cytoplasm, and exploits a host system of actin-based motility to ultimately spread to adjacent cells (21, 31).

Lysis of the host cell vacuole is mediated in large part by the thiol-activated cytolysin listeriolysin O (LLO) (4, 12, 31). Transposon insertions in *hly*, the structural gene encoding LLO, result in a nonhemolytic phenotype, complete avirulence, and, for the most part, the inability to lyse the host vacuole (13, 18, 19, 25). Introduction of *hly* on a plasmid restores both hemolytic capacity and virulence (8).

Thiol-activated hemolysins, so named for the unique cysteine contained in a highly conserved undecapeptide, have been identified in 19 species of gram-positive bacteria, including streptolysin O produced by *Streptococcus pyogenes*, pneumolysin O produced by *Streptococcus pneumoniae*, and perfringolysin O (PFO) produced by *Clostridium perfringens* (1, 29). LLO, however, is the only one that is produced by an intracellular bacterium. We are interested in determining the particular features of LLO that allow it to function in an intracellular milieu.

It has been previously shown that *Bacillus subtilis* expressing either LLO or PFO was able to lyse the host cell vacuole and grow in the cytoplasm (4, 26). Although *B. subtilis* expressing PFO was able to lyse the vacuolar membrane, there was evidence that PFO, but not LLO, might be exerting a deleterious effect on the host cell (26). This led to the model that LLO is unique not in its ability to lyse the host cell vacuole but in its relative lack of cytotoxicity. The only known difference between the two cytolysins is the relative lack of activity of LLO, compared with PFO, at pH 7.0, whereas both are active at an acidic pH (15, 26). Conceivably the low pH optimum of LLO might represent a protective mechanism to prevent deleterious effects on the cytoplasmic membrane of the host cell by LLO.

In this study, we cloned the structural gene for PFO into *L. monocytogenes* under control of the *hly* promoter. PFO was chosen because the specific activities of LLO and PFO are almost identical, whereas the specific activity of streptolysin O is 10-fold lower than that of LLO (26). The overall amino acid identity between PFO and LLO is 43% (20, 32). Our results indicate that PFO is not able to complement LLO for virulence despite being able to complement for hemolytic activity and lysis of the vacuole. Furthermore, our data suggest that this may be due to a cytotoxic effect exerted by PFO on the host cell.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The parental wild-type *L. monocytogenes* strain used in all of the studies was 10403S, which belongs to serotype 1 and is resistant to streptomycin in concentrations of 1 mg/ml (5). Bacteria were grown in either brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.), Luria-Bertani medium (LB), or LB with 0.4% glucose buffered to pH 7.4 with 77 mM K_2HPO_4, 7.6 mM ammonium sulfate, and 1.7 mM sodium citrate (LB/Glc) as specified. Broth cultures were grown as follows. A 2-ml culture of the appropriate medium was inoculated from a BHI plate and grown to stationary phase overnight at 30°C with the culture tube lying flat. Cultures were diluted 1:10 in the same medium and grown at 37°C with vigorous shaking for 5 h (i.e., until early stationary phase). Permanent stock cultures of the bacterial strains were stored at −70°C in a 50% LB–glycerol solution. For routine use, *L. monocytogenes* strains were kept at room temperature on BHI-agar plates.

Construction of bacterial strains. The *Escherichia coli* host

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strain used for all plasmid constructions was JM109 (New England Biolabs, Beverly, Mass.).

(i) Construction of plasmid-based strains. Cloning in *E. coli* was accomplished with the shuttle vector pAM401, which is capable of replication in both gram-positive and gram-negative bacteria (34). Selection was with chloramphenicol (20 μg/ml) in both gram-positive and gram-negative bacteria. Cloning of the plasmid-based PFO strain was performed with a two-part ligation. In the initial step, the promoter and regulatory palindrome for *hly* were amplified by PCR with the primers 5′GGGTCGACTCCTTTGATTAGTATATTCCT3′ and 5′GGGGATCCTAACCTAATAATGCCAAATAC3′ (Operon Technologies, Alameda, Calif.), which were designed to generate sticky end restriction sites for *Sal*I and *Bam*HI, respectively. The 200-bp fragment was cloned into pAM401 digested with the appropriate restriction enzymes to generate pDP1652. In the second step, the structural gene and ribosome binding site for PFO were amplified from *C. perfringens* chromosomal DNA with the PCR primers 5′GGGGATCCTTAACAAATGAGGGGAAAATTA3′ and 5′GGTCTAGAACTTTATTATAACTCTAAAATATG3′, which contained restriction sites for the enzymes *Bam*HI and *Xba*I, respectively. The 1.6-kb fragment was cloned into pDP1652 to generate pDP1868. DP-L1044, a nonhemolytic Tn*917-lac* mutant of wild-type *L. monocytogenes* (30), was transformed by electroporation with pDP1868 to yield DP-L1875. DP-L1876 was obtained by electroporation of DP-L1044 with a plasmid containing the entire regulatory region and structural gene for *hly* on a *Sau* 96 fragment (8) cloned in pAM401.

(ii) Construction of *L. monocytogenes*::*pfo*. Cloning in *E. coli* was accomplished with the plasmid pKSV7, which is a shuttle vector capable of replication in *E. coli* and temperature-sensitive replication in *L. monocytogenes* as well as other gram-positive bacteria (28). pKSV7 was maintained in *E. coli* in the presence of 50 μg of ampicillin per ml.

The construction of the chromosomal PFO-expressing strain was accomplished in two steps. The first step involved construction of a *hly* deletion strain as follows. A 500-bp fragment from bp 962 to 1463 (20), which is upstream from the Shine-Dalgarno sequence for the *hly* gene, was amplified from *L. monocytogenes* chromosomal DNA by PCR with the primers 5′GGGAATTCAATTGTTGATACAATGACATC3′ and 5′GGGATCCTAACCTAATAATGCCAAATAC3′ (Operon Technologies). The primers were designed to generate sticky end restriction sites for *Eco*RI and *Bam*HI, respectively. A second 500-bp fragment at the 3′ end of the *hly* gene from bp 3029 to 3529 was similarly amplified with the primers 5′GGGGATCCCCACGCTTTATCCGAAATAT3′ and 5′GGCTGCAGGGTCTTTTTGGCTTGTGTAT3′. These primers were designed to generate restriction sites for *Bam*HI and *Pst*I, respectively. The inserts were then ligated into pKSV7 digested with the appropriate restriction enzymes in a two-step ligation to yield pDP2154. By using this construct, allelic exchange was performed as described by Camilli et al. (7) to generate the *hly* deletion strain. Nonhemolytic colonies were identified on 5% blood–LB-agar plates.

In the next step, the gene for PFO, *pfo*, was recombined into the chromosome of the *hly* deletion strain, DP-L2161, again by homologous recombination. The structural gene for PFO (32) was amplified from *C. perfringens* chromosomal DNA (provided by R. Tweten) by PCR with the primers 5′GGGGATCCTTAACAAATGAGGGAAAATTA3′ and 5′GGGGATCCACTTTATTATAACTCTAAAATATG3′. These primers were both designed to generate sites for *Bam*HI. The purified PCR product was then ligated into pDP2154 digested with *Bam*HI to yield pDP2169. The *hly* deletion strain was transformed with pDP2169 by electroporation to yield strain DP-L2219. Recombination into the chromosome was accomplished by allelic exchange (7). Allelic exchange of *pfo* for *hly* was corroborated by Southern blotting (27). The resulting strain, DP-L2221, had *pfo* cloned into the *L. monocytogenes* chromosome immediately downstream from the promoter for *hly*.

Tissue culture cells and growth media. The mouse macrophagelike cell line J774, primary bone marrow macrophages, and L_2 fibroblasts were propagated as previously described (30).

Hemolytic activity. A 1-ml aliquot of the bacterial culture, grown as described above, was subjected to centrifugation in a microcentrifuge (14,000 × *g*) for 1 min. The supernatant was saved for assay. Assays were carried out at pH 5.6 as previously described (25) and at pH 7.4 by adjusting the PBS-cysteine buffer to pH 7.4 with 2 N NaOH. Hemolytic activity is expressed as the reciprocal of the dilution of culture supernatant required to lyse 50% of sheep erythrocytes.

SDS-PAGE. Bacterial culture supernatants were precipitated with 10% trichloroacetic acid and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7% polyacrylamide) with protein molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) as previously described (25). Gels were subsequently stained with 0.05% Coomassie brilliant blue R.

Intracellular growth curves. Intracellular growth curves in J774 cells were performed as previously described (25) with either high (25 μg/ml) or low (5 μg/ml) concentrations of gentamicin as indicated in the text. The growth curve without gentamicin was performed by adding gentamicin to 25 μg/ml 1 h after infection, washing the cells three times with phosphate-buffered saline (PBS), and adding fresh medium prewarmed to 37°C 1 h later.

Determination of plaque formation in L_2 fibroblasts. Determination of plaque formation in mouse L_2 fibroblasts was performed as previously described (30) with the exception that a 10-fold higher inoculum was used for the chromosomal PFO strain than for wild-type *L. monocytogenes*.

LD_{50} determination. Fifty percent lethal doses (LD_{50}s) were determined after tail vein injection of BALB/c mice (Charles River) as previously described (25).

Immunofluorescence. The percentage of bacteria that had escaped from the vacuole was indirectly determined on the basis of the percentage of bacteria coated with actin filaments as determined by phalloidin staining (9, 10). Bone marrow-derived macrophages were grown and harvested as described previously (25) and seeded onto glass coverslips in 35-mm-diameter dishes at 7.5×10^5 cells per dish in the absence of macrophage growth factor, CSF-1, on the night prior to infection. The macrophages were infected with mid-log phase bacteria (optical density at 600 nm, 0.8) such that each cell contained approximately 10 to 15 bacteria after a 30-min infection. After 90 min, the bacteria were fixed with formalin (3.2% electron microscopy-grade formaldehyde in PBS) for 15 min at room temperature. The coverslips were then washed three times with TBS-Tx (150 mM NaCl, 20 mM Tris [pH 7.4], 0.1% Triton X-100). After the final wash, the coverslips were incubated in antibody dilution buffer for 5 min at room temperature. Intracellular bacteria were stained by indirect immunofluorescence with *Listeria* O rabbit antiserum (Difco) at a 1:320 ratio in antibody dilution buffer (TBS-Tx plus 1% bovine serum albumin) for 1 h at room temperature. The coverslips were then washed thoroughly with TBS-Tx and incubated with antibody dilution buffer containing fluorescein isothiocyanate-labelled goat anti-rabbit immunoglobulin G secondary antibody at a 1:160 ratio (Sigma Chemical Co., St.

TABLE 1. Relevant characteristics of *L. monocytogenes* strains used in this study

Strain	Genotype	LD_{50}	Source or reference
10403S	Wild type	2×10^4	5
DP-L2161	Δ*hly*	2×10^9	This study
DP-L2221	DP-L2161::*pfo*	1.5×10^9	This study
DP-L1044	*hly*::Tn*917-lac*	1×10^9	2
DP-L1875	DP-L1044 (pAM401-*pfo*)	5×10^8	This study
DP-L1876	DP-L1044 (pAM401-*hly*)	2×10^5	This study

Louis, Mo.) and tetramethylrhodamine isothiocyanate phalloidin at a 1:20 ratio (Molecular Probes, Inc., Eugene, Oreg.). Coverslips were mounted in Testog FITC-Guard (Testog, Inc., Chicago, Ill.). At least 100 bacteria were counted for each sample (approximately 10 cells picked at random from three different fields) as determined by labelling with fluorescein isothiocyanate. The number of bacteria colabelled with tetramethylrhodamine isothiocyanate was then determined to yield the percentage of total bacteria which were coated with actin filaments and therefore present in the cytoplasm. The results presented represent the averages of two experiments.

DNA sequence analysis. The accuracy of both PFO constructs was confirmed by DNA sequence analysis. Dideoxy sequencing of double-stranded plasmid DNA was performed as recommended by the manufacturer with a Sequenase version 2.0 kit (U.S. Biochemical Corporation, Cleveland, Ohio).

RESULTS

Cloning of PFO into *L. monocytogenes*. The primary issue to be addressed by this study was whether LLO could be functionally replaced by PFO in *L. monocytogenes*. Two complementary strategies were taken to examine this question: a plasmid-based system and a chromosomally based system. In the former approach, the structural gene for PFO was cloned downstream from the *hly* promoter in the shuttle plasmid pAM401 and transformed into a nonhemolytic mutant of *L. monocytogenes*. As a control, the structural gene for LLO was similarly cloned into pAM401 and transformed into the same nonhemolytic strain. The second approach was designed to replace *hly* with *pfo* on the chromosome in *L. monocytogenes*. This was accomplished with a system of allelic exchange as outlined in Materials and Methods. Relevant strains are listed in Table 1.

Hemolytic activity. The regulated expression of the cloned gene products was examined by assaying the hemolytic activity of bacterial culture supernatants (Table 2). As expected, the strain containing the *hly* deletion had no detectable hemolytic activity. Both of the plasmid-based strains for PFO and LLO expressed levels of activity similar to wild-type *L. monocytogenes* when grown in LB or BHI. When present in a single copy, however, the PFO-expressing strain had levels similar to those of the wild type in BHI but produced only 1/10th the activity of wild-type *L. monocytogenes* when grown in LB. The reduction in hemolytic activity secreted by the chromosomally based PFO strain was clearly due to a reduction in the amount of hemolysin present, as documented by SDS-PAGE (Fig. 1). The activity of LLO, but not PFO, was depressed at pH 7.4, which is consistent with the known pH optima of the two cytolysins (data not shown). The hemolytic activity of both LLO and PFO was repressed after growth of the bacteria in the presence of glucose; however, this repression was less marked for the chromosomal PFO strain than for all others. These results suggest that some, but not all, levels of regulation have been maintained in the chromosomally based PFO strain.

TABLE 2. Hemolytic activity[a]

Strain	Hemolytic activity[a]		
	LB	LB/Glc	BHI
10403S	284	13.3	24
DP-L2161	0	0	0
DP-L2221	27	11.4	18
DP-L1044	0	0	0
DP-L1875	231	61	102
DP-L1876	178	61	100

[a] Expressed as the reciprocal of the dilution at which there is 50% lysis of sheep erythrocytes observed at pH 5.6. Data were normalized to a bacterial optical density at 600 nm of 1.0.

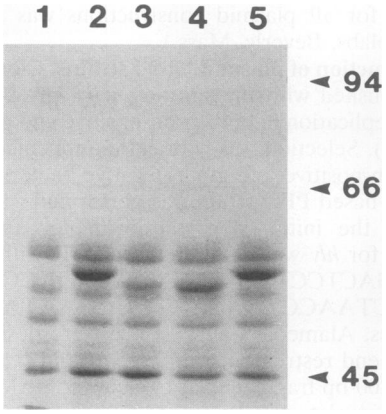

FIG. 1. SDS-PAGE of *L. monocytogenes*-secreted proteins. Proteins were precipitated from culture supernatants with 10% trichloroacetic acid and separated by SDS–7% polyacrylamide gel electrophoresis. Each lane represents approximately 4 ml of culture supernatant. Lanes: 1, DP-L2161 (*L. monocytogenes hly* deletion strain); 2, *L. monocytogenes* 10403S; 3, DP-L2221 (*L. monocytogenes* strain expressing PFO from the chromosome); 4, DP-L1875 (*L. monocytogenes* strain expressing PFO from a plasmid); 5, DP-L1876 (*L. monocytogenes* strain expressing LLO from a plasmid). The molecular mass in kilodaltons is indicated at the right margin.

Intracellular growth. We examined the ability of the strains to grow inside the mouse macrophagelike cell line J774. When PFO was expressed in a single copy, the bacteria were able to grow inside J774 cells. However, after 4 h, the intracellular growth rate of the PFO-expressing strain was decreased in the presence of gentamicin in a dose-dependent fashion (Fig. 2A). In the absence of gentamicin, both the wild-type and the PFO-expressing strains grew with similar doubling times. Most strikingly, in the presence of high levels of gentamicin, there was rapid killing of the strain expressing PFO from a plasmid (Fig. 2B). Furthermore, cells infected with the PFO-expressing bacteria, but not wild type, developed dark pyknotic nuclei and cytoplasmic disruption indicative of cytotoxicity (Fig. 3). These data are consistent with the hypothesis that PFO induces cytotoxicity in the host cell, resulting in entry of extracellular gentamicin and bacterial death.

Lysis of the vacuole. It is clear from the results presented above that *L. monocytogenes* expressing PFO can replicate in the mouse macrophagelike J774 cell line. Next, we used indirect immunofluorescence to compare the percentages of wild-type versus PFO-expressing bacteria that could be found

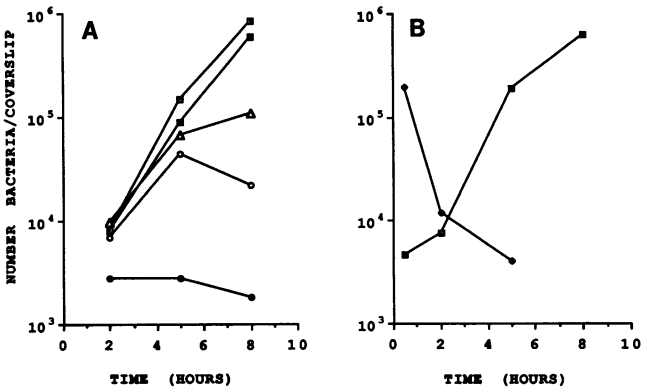

FIG. 2. Growth of *L. monocytogenes* strains in the mouse macrophagelike cell line J774. (A) ⊡, *L. monocytogenes* 10403S with 25 μg of gentamicin per ml; ■, DP-L2221 in the absence of gentamicin; △, DP-L2221 with 5 μg of gentamicin per ml; ○, DP-L2221 with 25 μg of gentamicin per ml; ●, *hly* deletion strain DP-L2161 with 25 μg of gentamicin per ml. (B) ⊡, DP-L1876 expressing LLO from a plasmid; ■, DP-L1875 expressing PFO from a plasmid. A 20-fold higher inoculum was used for DP-L1875 than for DP-L1876. Both growth curves were determined in the presence of 25 μg of gentamicin per ml.

in the cytoplasm 90 min after infection of bone marrow-derived macrophages. Bacterial association with F-actin was used as an indication of entry into the cytoplasm (9, 10). For the PFO-expressing strain, 36% ± 6% of the organisms are found in the cytoplasm at 90 min as opposed to 56% ± 9% of the wild type. The negative control for this experiment was the *hly* deletion strain DP-L2161, for which 0% bacteria were found in the cytoplasm. Because some bacteria at early time points may have just entered the cytoplasm and may not yet be coated with F-actin, this number is likely an underestimate of the actual number of cytoplasmic bacteria, but it is very close to the number determined for the wild type by quantitative electron microscopy (7).

Cell-to-cell spread. The ability of the chromosomal PFO strain to lyse the host cell vacuole and initially grow and spread from cell to cell to some extent was evident by light microscopic examination of stained coverslips (Fig. 3). Bacteria were found in pseudopodlike projections characteristic of infection with wild-type *L. monocytogenes*. This finding was further corroborated by the ability of these bacteria to form plaques in monolayers of mouse L_2 fibroblasts. The plaques formed by the chromosomal PFO-expressing strain were considerably smaller than those formed by wild-type *L. monocytogenes* (Fig. 4), however, which is consistent with a spreading defect due to death of the bacteria before cell-to-cell spread. The efficiency with which the chromosomal PFO strain formed plaques was also reduced to 20% of that of the wild type. The plasmid-based PFO-expressing strain did not form detectable plaques

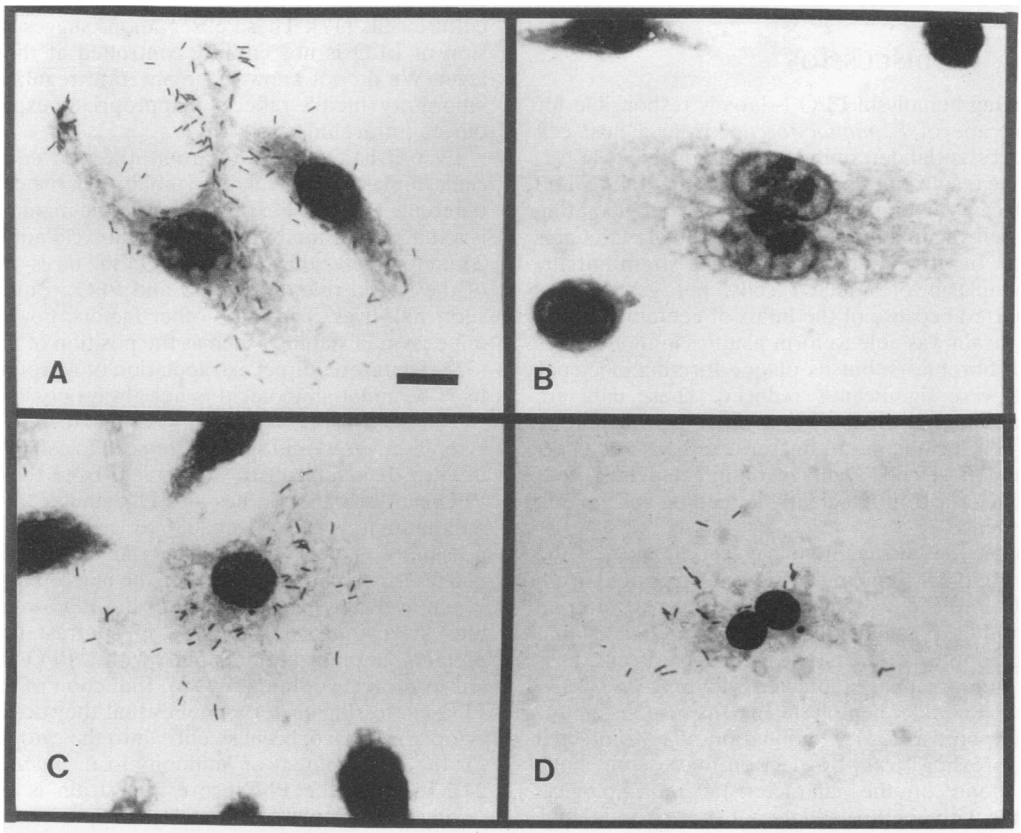

FIG. 3. Light micrographs of *L. monocytogenes* strains in J774 cells 5 h after infection. (A) Cells infected with wild-type *L. monocytogenes* 10403S. (B) Cells infected with *hly* deletion strain DP-L2161. (C and D) Cells infected with *L. monocytogenes* DP-L2221 expressing PFO from the chromosome. Approximately 80% of the cells infected with DP-L2221 resemble the cells depicted in panel C 5 h after infection; the other 20% resemble the cells depicted in panel D. The scale bar in panel A is 10 μm long.

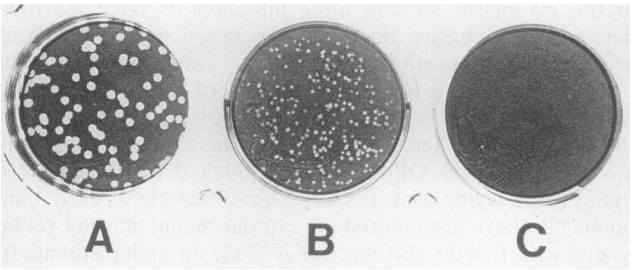

FIG. 4. Plaque formation of *L. monocytogenes* strains in L_2 fibroblasts. (A) 10403S. (B) DP-L2221. (C) DP-L2161.

(data not shown). One possible explanation for this is that the plasmid-based strain killed the host cell more quickly than the chromosomal PFO-expressing strain and was therefore rapidly killed by the influx of gentamicin present in the first agarose overlay.

Effect on virulence. The virulence of the five strains was evaluated after intravenous infection in mice. As previously reported (2, 25), the LLO deletion strains are approximately 5 logs less virulent than the wild-type strains. As shown by Cossart et al. (8), the presence of LLO on a plasmid increased virulence by 4 logs. The inability of LLO on a plasmid to restore the bacteria to full virulence may result from the fact that the *hly* promoter is present in multiple copies (7). In contrast, the strains expressing PFO either from a plasmid or the chromosome were avirulent. Therefore, PFO was unable to complement LLO for virulence in a mouse model of infection.

DISCUSSION

The pore-forming hemolysin LLO is largely responsible for mediating the escape of *L. monocytogenes* from a host cell vacuole and is an essential determinant of pathogenicity (8, 12, 13, 18, 25, 31). The results of this study indicate that the related hemolysin PFO can functionally replace LLO in mediating lysis of the host cell vacuole but is absolutely unable to confer virulence in mice. In vitro, the PFO-expressing strain initially grew in the cytoplasm of infected cells, but growth was subsequently aborted because of the influx of gentamicin. The PFO-expressing strain was able to form plaques in monolayers of mouse L_2 cell fibroblasts, but its plaque-forming efficiency and plaque size were significantly reduced. These data are consistent with a model in which PFO is capable of mediating lysis of the host cell vacuole but ultimately exerts a toxic effect upon the infected host cell. Damage of infected host cells would prevent bacteria from efficiently spreading cell to cell, resulting in avirulence.

It is not known if the host cell damage seen in some of the cells infected with the PFO-expressing strain resulted from insertion of PFO into the cytoplasmic membrane or internal membranes. Another possible mechanism of action is that PFO is inducing apoptosis in some of the infected cells, as is known to occur in macrophages infected with *Shigella flexneri* (36). The *S. flexneri* contact hemolysin IpaB is responsible for the induction of apoptosis (35). Examination of cells infected with the PFO-expressing strain by electron microscopy, however, revealed none of the characteristic morphological changes associated with apoptosis (data not shown) and therefore seemed to represent true cytotoxicity.

It is unclear at this point what particular properties allow LLO but not PFO to mediate *L. monocytogenes* virulence. The most striking difference between LLO and PFO is in their pH optima (15, 25). LLO has a pH optimum in the acidic range, while PFO is equally active at both acidic and neutral pHs. Accordingly, one interpretation of our results is that released LLO is relatively inactive in the cytoplasm, while PFO is fully active. The low pH optimum of LLO may represent a protective mechanism to prevent host cell damage in the cytoplasm. Conceivably, *L. monocytogenes* has adopted a strategy used by eucaryotic cells to compartmentalize the potentially toxic activity of lysosomal acid hydrolases.

The regulation of LLO is clearly complex, and it has not been established whether LLO is normally expressed by *L. monocytogenes* during intracytoplasmic growth. There is, however, indirect immunological evidence that LLO is continually secreted in the cytoplasm of infected cells (23). In this study, we attempted to preserve normal transcriptional regulation by cloning the structural gene and ribosome binding site for PFO downstream from the *hly* promoter. Nevertheless, it is still possible that our results can be explained by the inappropriate expression of PFO. As described above, although the expression of PFO was the same as that of LLO after growth in BHI, it was significantly lower after growth in LB. Furthermore, PFO was maximally expressed during mid-logarithmic growth, while LLO was expressed during late logarithmic growth (17). It is possible that the aberrant regulation of PFO was due to our cloning strategy, which changed the number of nucleotides between the *hly* promoter and the PFO ribosome binding site. Therefore, we recloned PFO into *L. monocytogenes* by using SOE-ing PCR (16). This technique enabled us to use the LLO ribosome binding site and to preserve spacing exactly. This new strain, however, behaved exactly the same as the original, in terms of both hemolysin production and growth in tissue culture cells (17). These observations suggest that the expression of LLO is not entirely controlled at the transcriptional level. We do not know if the aberrant regulation observed in laboratory media reflects inappropriate expression of PFO during intracellular growth.

Even if LLO and PFO are synthesized equally in the host cell cytoplasm, their relative stabilities in the cytoplasm may be different. The half-life of proteins in the mammalian cytoplasm is a function of the N-terminal amino acid and can range from 2 min to greater than 20 h (33). On the basis of the predictions of the N-end rule, both LLO and PFO would have relatively short half-lives. There are other factors, however, that determine protein stability, such as the position of an internal lysine (33). Therefore, direct extrapolation of a protein half-life from its N-terminal amino acid is not always possible.

The PFO-expressing strain is able to enter the host cell cytoplasm, grow, and spread from cell to cell to a limited extent in vitro. It is difficult to extrapolate from these data why the PFO-expressing strain has an LD_{50} similar to that of an LLO null mutant, which is unable to grow in cells at all. One possibility is that, unlike the bone marrow-derived macrophages used in our experiments, the macrophages encountered in the liver may prevent entry of the PFO-expressing bacteria into the cytoplasm. However, in contrast to LLO deletion mutants, immunization of mice with a PFO-expressing strain led to protective immunity (6). Induction of immunity by the PFO-expressing bacteria implies that they do indeed enter the cytoplasm in vivo, because entry into the cytoplasm is required for the development of immunity to *L. monocytogenes* (3, 22, 24). In vitro, the PFO-expressing strain is eventually killed during intracytoplasmic growth in the presence of extracellular gentamicin. Presumably, PFO is causing the host cell to become permeable to the extracellular gentamicin. We speculate that, in vivo, bactericidal factors in serum may enter damaged infected cells and kill PFO-expressing bacteria.

A determinant of pathogenicity as critical but as potentially toxic as LLO would logically have a number of fail-safe mechanisms. Indeed, the results of this study are consistent with a model in which regulation of LLO occurs at a number of posttranscriptional levels, including pH optimum and, perhaps, protein stability. We intend to evaluate which factors are most important by selecting for mutants in PFO that confer wild-type levels of intracellular *L. monocytogenes* growth and pathogenicity.

ACKNOWLEDGMENTS

We thank A. Bouwer and D. Hinrichs for the animal studies.

This work was supported by National Institutes of Health grant AI-26919 (D.A.P.) and by Physician Scientist Award AI-01031 (S.J.).

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