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Capacity of Listeriolysin O, Streptolysin O, and Perfringolysin O To Mediate Growth of *Bacillus subtilis* within Mammalian Cells

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The *Listeria monocytogenes* hemolysin listeriolysin O (LLO) plays a major role in mediating the escape of *L. monocytogenes* from a vacuolar compartment. In a previous report, it was shown that *Bacillus subtilis* expressing LLO could escape from a host vacuolar compartment and grow in the cytoplasm (J. Bielecki, P. Youngman, P. Connelly, and D. A. Portnoy, Nature [London] 345:175–176, 1990). In the present study, two related thiol-activated hemolysins, streptolysin O (SLO) and perfringolysin O (PFO), were expressed in *B. subtilis* and their ability to mediate intracellular growth was monitored by visual inspection and by assaying for CFU. Like LLO, PFO was active within the vacuolar environment, whereas SLO showed negligible activity. However, expression of PFO seemed to damage the host cells. The pH of the vacuole probably had little to do with these results, since all three hemolysins showed full or enhanced activity at pH 5.5, although LLO showed greatly reduced activity at pH 7. In addition, neutralization of the pH within host vacuoles by using weak bases had little effect on the lysis of the vacuole. The lack of SLO activity is probably caused by its lower specific activity; the purified protein had 10-fold less activity on a molar basis. These results suggest that LLO is not unique in its capacity to mediate intracellular growth of *B. subtilis*.

The thiol-activated (oxygen-labile) cytolysins are a family of related hemolysins that are expressed by 15 diverse species of gram-positive bacteria (9, 35). Representatives from four different genera include streptolysin O (SLO), pneumolysin, perfringolysin O (PFO), cerolysin, and listeriolysin O (LLO), produced by *Streptococcus pyogenes*, *S. pneumoniae*, *Clostridium perfringens*, *Bacillus cereus*, and *Listeria monocytogenes*, respectively. Common features shared among these hemolysins include inhibition by free cholesterol, the presumed receptor, and the presence of a single unique cysteine that renders the hemolysins susceptible to reversible inactivation by oxidation. Their mechanism of action, based on the properties of SLO, is thought to involve the binding of monomers to membrane cholesterol followed by oligomerization of 20 to 80 monomers into ring and arclike structures (7). There is evidence that PFO induces colloid osmotic lysis, presumably via PFO-induced membrane pores (16).

There is direct and suggestive evidence that thiol-activated hemolysins play a role in the pathogenicity of *S. pyogenes*, *S. pneumoniae*, and *C. perfringens* (5, 9, 36, 40). However, the most compelling evidence for a direct role of a thiol-activated hemolysin in pathogenicity comes from studies on the role of LLO in the pathogenesis of *L. monocytogenes* infection. First, transposon insertion mutants devoid of LLO activity are completely avirulent in a murine model of infection (13, 19, 32). Introduction of the gene encoding LLO into a strain containing a structural gene insertion restores virulence (11). Second, nonhemolytic mutants are for the most part defective for intracellular growth in vitro (12, 23, 32) and reside within host vacuoles, whereas the parent strain grows directly in the host cytoplasm (12, 37). Third, *Bacillus subtilis* expressing LLO can lyse the phagocytic vacuole and grow directly in the cytoplasm (8). The latter result was documented with light and electron microscopy and by enumeration of CFU. Taken together, these results indicate that LLO is an essential determinant of pathogenesis and is largely responsible for the ability of *L. monocytogenes* to lyse the phagocytic vacuole.

The question that led to the present study was, what, if anything, is unique about LLO that allows it to function within a vacuolar environment? The related hemolysins SLO, PFO, and pneumolysin are thought to play roles in pathogenicity, although, unlike *L. monocytogenes*, the host bacteria are extracellular pathogens. However, the possibility cannot be entirely excluded that these bacteria occasionally grow intracellularly. Therefore, to begin to address these issues, the structural genes encoding SLO and PFO were expressed in *B. subtilis* and compared with LLO with regard to their capacity to promote growth of *B. subtilis* within a macrophage cell line. The results suggest that LLO is not unique in its ability to mediate lysis of the host vacuole; *B. subtilis* strains expressing PFO, but not those expressing SLO, grew within a macrophage cell line.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The primary *B. subtilis* strain used in this study was derived from an asporogenic derivative of strain ZB307 (44) containing a *spoIIE*::Tn*917*ΩHU181::*lac55* insertion and designated MB4. ZB307 also contains an SPβ prophage containing pBR322 sequences. *B. subtilis* BD1809 (*his leu met*) contains a *spo0A*::*cat* deletion and was provided by D. Dubnau. Strains DP-B980, DP-B1267, and DP-B1512 are derivatives of MB4 with single copies of *hly* from *L. monocytogenes*, *slo* from *S. pyogenes*, and *pfo* from *C. perfringens*, respectively,

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cloned into pAG58-ble-1 and integrated into the SPβ prophage as described previously (8). Strain DP-B1066 is a control strain in which the vector alone is integrated into the SPβ prophage. *B. subtilis* DP-B1313 is strain BD1809 transduced with SPβ containing pAG58-ble-1-*slo* from strain DP-B1267. The rationale for using a *spo0A* strain was that it secretes less proteolytic activity than a *spo2E* strain does. The *Escherichia coli* host for recombinant DNA was DH5α-MCR.

Bacteria were grown on brain heart infusion (BHI) agar and broth (Difco Laboratories, Detroit, Mich.). Stock cultures were kept as suspensions of cells at −70°C in 50% glycerol. For routine use, *B. subtilis* strains were maintained on BHI agar at room temperature and passaged every 2 days. For growth of liquid cultures, logarithmic-stage bacteria were scraped from an agar plate and inoculated into BHI broth at an optical density at 600 nm of 0.01. Cultures were grown in 10 ml of BHI in 250-ml flasks with vigorous shaking at 37°C and harvested when the optical density at 600 nm reached between 0.8 and 1.2. Cultures were never placed at 4°C to avoid lysis of the asporogenic mutant strains of *B. subtilis*. Phleomycin (Bristol Laboratories, Syracuse, N.Y.) was used at 800 ng/ml. Isopropyl-β-D-thiogalactoside (IPTG) was used at a final concentration of 1 mM unless otherwise specified.

Tissue culture cells and growth medium. The mouse macrophagelike cell line J774, the human epithelial cell line Henle 407, and primary cultures of mouse bone marrow-derived macrophages were propagated as previously described (32).

Plasmid construction, transformation, and transduction of *B. subtilis*. All plasmid constructs were derived from pAG58-ble-1 (43). This vector is an expression vector containing the IPTG-inducible promoter p_{spac}. It can replicate in *E. coli* and integrate stably into the *B. subtilis* chromosome at sites of homology provided by an SPβ prophage. The cloning of *hly*, the structural gene encoding LLO, was described previously (8). Plasmid pMK206 (31) was the source of *slo*. A 2-kb *Bam*HI-*Eco*RI fragment treated with Klenow fragment to produce flush ends was ligated into *Hin*dIII-digested pAG58-ble-1 that was treated with Klenow fragment. A clone containing the insert in the correct orientation was verified by restriction enzyme analysis. The *pfo* gene (bases 1 through 1675 [39]) was amplified from *C. perfringens* chromosomal DNA with the polymerase chain reaction and ligated into pAG58-ble-1 digested with *Sal*I and *Xba*I. The *Sal*I and *Xba*I restriction sites of the polymerase chain reaction-amplified product were created as noncomplementary ends of the amplification primers. The polymerase chain reaction primers used were 5′-GGTCTAGACCCAACCCTA TTAAGTTTTTA-3′ and 5′-GGGTCGACACATTATAACT CTAAAATATC-3′.

Plasmids were introduced into *B. subtilis* by transformation of naturally competent cells (1).

SPβ containing pAG58-ble-1 was thermally induced and transduced into *B. subtilis* BD1809 as previously described (22).

Hemolytic activity produced by *B. subtilis* strains. A sample (1 ml) of culture grown as described above was subjected to centrifugation for 1 min in a microcentrifuge (14,000 × *g*), and the supernatant fluid was assayed for hemolytic activity immediately. Hemolytic activity was determined at pH 5.8 as previously described (32), except that hemolytic units were expressed as the reciprocal of the dilution of bacterial supernatant fluid showing 50% lysis of sheep erythrocytes.

Intracellular growth assay. Intracellular growth in J774 cells was similar to that described for *L. monocytogenes* with minor modifications. J774 cells (2×10^6) were deposited onto round coverslips (12 by 1 mm; Propper Manufacturing Co. Inc., Long Island City, N.Y.) in 60-mm petri dishes the evening before use and incubated in 6 ml of the appropriate medium without antibiotics. Bacteria grown to an optical density at 600 nm of 1.0 in the presence of 1.0 mM IPTG as described above were washed once with phosphate-buffered saline (PBS; pH 7.4) and used immediately to infect monolayers. For growth curves, monolayers were infected with 5 μl of the washed culture for 30 min in the presence of 1 mM IPTG and then washed three times with 37°C PBS. For the 30-min time point, coverslips were deposited, in triplicate, into 5 ml of sterile distilled water in 15-ml conical tubes. After mixing vigorously for 15 s to lyse the infected cells, dilutions were plated onto BHI agar. This time point represents cell-associated bacteria and does not discriminate between intracellular bacteria and those merely attached. Five milliliters of 37°C medium without IPTG was added to the remaining coverslips. After 30 min, gentamicin sulfate was added to a final concentration of 5 μg/ml. This concentration of gentamicin is bactericidal for extracellular bacteria without affecting intracellular bacteria. At each subsequent time point, triplicate coverslips were treated as described above for the 30-min time point. The data presented in Fig. 3 and 6 represent the averages of the number of bacteria from three coverslips. It should be noted that the results in Fig. 3 were essentially identical even when gentamicin was completely omitted from the experiment; *B. subtilis* grew at a very slow rate in the tissue culture medium. During experiments designed to evaluate the role of vacuolar pH, NH_4Cl was used at a concentration of 20 mM and was present in the medium and in the PBS from the beginning of the infection.

Infection of bone marrow-derived macrophages was identical to that for the J774 cells and was described previously (32).

SDS-PAGE and Western immunoblotting. Trichloroacetic acid precipitation of culture supernatant fluid and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7% polyacrylamide) was performed as previously described (32). Gels were stained in 0.05% Coomassie brilliant blue R. Protein molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were used.

For Western blotting, proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, N.H.) with a TRANSPHOR electroblotter (Hoefer Scientific Instruments, San Francisco, Calif.). All subsequent steps were performed in 50 mM Tris (pH 7.5)–2 mM EDTA–0.15 M NaCl–0.5% Nonidet P-40–1% calf serum. The nitrocellulose filter was first treated overnight in 1% bovine serum albumin and then incubated for 1 h with a mouse anti-LLO monoclonal antibody (kindly provided by William Bibb) and a horse antiserum to SLO (kindly provided by Gordon Dougan). The nitrocellulose filter was washed and reacted with ^{125}I-labeled protein A and ^{125}I-labeled sheep anti-mouse immunoglobulin G (Amersham Corp., Arlington Heights, Ill.). After extensive washing, the filter was exposed to X-ray film at −70°C in the presence of two intensifying screens.

Purification of LLO and recombinant derived PFO. PFO was purified as previously described (38) from *E. coli* JM109 containing the plasmid pRT1B. LLO was purified by a method similar to that described for the purification of PFO from *C. perfringens* (37a, 38). Pure recombinant-derived SLO was purified as previously described (31). Purified proteins were frozen at −70°C in the presence of 20%

TABLE 1. Bacterial strains and hemolytic activity

Strain[a]	Relevant genotype or description[b]	Hemolytic titer[c]
DP-B1066	Vector alone	UD
DP-B980	*hly*	80–160
DP-B980 (10 μM IPTG)	*hly*	10–20
DP-B1267	*slo*	5–10
DP-B1313	*slo*	20–30
DP-B1512	*pfo*	160–320

[a] All strains are derivatives of *B. subtilis* ZB307 (44) containing a *spoIIE*::Tn*917* insertion except DP-B1313, which is a derivative of *B. subtilis* BD1809.

[b] All strains contain plasmid pAG58-ble-1 (vector) with the indicated insert.

[c] Expressed as the reciprocal of the dilution at which 50% lysis of the sheep erthrocytes was observed at pH 5.6. Cultures were grown in BHI broth to an optical density at 600 nm of 1.0 in the presence of 1 mM IPTG unless otherwise indicated. UD, undetectable.

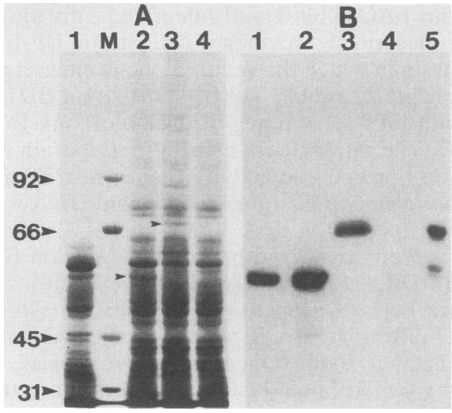

FIG. 1. SDS-PAGE and Western blotting of secreted polypeptides. (A) Proteins were precipitated from culture supernatant fluids with 10% trichloroacetic acid and separated on an SDS–7% polyacrylamide gel. Each lane represents approximately 2 ml of culture supernatant fluid. Lanes: 1, *L. monocytogenes* 10403S; M, molecular mass standards (masses in kilodaltons are shown to the left); 2, *B. subtilis* DP-B980 expressing LLO; 3, *B. subtilis* DP-B1313 expressing SLO; 4, *B. subtilis* DP-L1066 with vector alone. The arrowheads point to LLO in lane 2 and SLO in lane 3. (B) Autoradiograph of a Western blot after incubation with ^{125}I-labeled protein A. Lanes 1 through 4 are the same as in panel A; lane 5 contains purified SLO obtained from Sigma.

glycerol until used. The recombinant-derived PFO and SLO have been shown to be similar in activity to native toxins derived from each bacterial species (20, 38).

Hemolytic activity of the purified hemolysins. The relative hemolytic activities of the hemolysins were determined by right-angle scatter analysis as previously described (17). The basis of this analysis relies on the differences in the right-angle scatter of intact and lysed erythrocytes. The kinetics of the decrease in right-angle scatter has been shown to correlate with lysis-induced hemoglobin release from PFO-treated erythrocytes (17). Briefly, washed human erythrocytes were prepared by repeated dilution and centrifugation (5,000 × *g*) of the erythrocytes in buffer (10 mM sodium phosphate [pH 7.0], 145 mM NaCl, 5 mM KCl, 100 mg of gelatin [Difco] per ml, 1 mM dithiothreitol, 1 mM glucose) until the supernatant was substantially free of hemoglobin. Dynamic changes in the right-angle scatter of erythrocytes undergoing lysis was measured with a SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, Ill.) equipped for individual sample cell stirring and temperature control. The excitation and emission monochromators were set to 590 nm with a slit width of 2 nm. A 1-cm-path-length quartz cuvette containing a final reaction volume of 2 ml was used for all experiments. For each experiment, 10^8 erythrocytes were added to buffer that had been adjusted to the desired pH with HCl. The final reaction volume in the cuvette was maintained at 2 ml, and the temperature was set at 30°C. Data acquisition was initiated, and the desired toxin was injected after 25 s. Hemolysis was then followed by the right-angle scatter at 590 nm. The relative activity of each toxin was based on the time required to achieve 50% hemolysis (17).

RESULTS

Expression of LLO, PFO, and SLO by *B. subtilis*. The genes encoding PFO and SLO were cloned downstream from the IPTG-inducible promoter p_{spac} present in plasmid pAG58-ble-1 as previously described for LLO (8). Each plasmid was integrated into the *B. subtilis* chromosome by homologous recombination. The resulting *B. subtilis* strains containing *hly* and *pfo* secreted relatively high levels of hemolytic activity after IPTG induction, whereas the strain containing *slo* expressed about 10-fold less activity (Table 1). Introduction of *slo* into a *spo0A* strain resulted in enhanced hemolytic expression, but this strain still secreted significantly less activity than that seen with *B. subtilis* expressing either LLO or PFO. The lack of SLO activity was not caused by lack of expression, since a polypeptide that comigrated with authentic SLO and reacted with anti-SLO serum was secreted (Fig. 1). The apparent differences in levels of expression did not seem sufficient to account for the 10-fold difference in hemolytic activity (Fig. 1). Therefore, the secreted SLO had an inherently lower specific activity and/or was more susceptible to inactivation.

To evaluate the relative activities of the three hemolysins under study, the purified hemolysins were compared for hemolytic activity by using a method that monitors erythrocyte lysis by measuring a decrease in right-angle scatter (16, 17). This is a very rapid and sensitive assay that reduces the potentially deleterious preincubation period used in standard assays. By using this method with purified proteins, LLO and PFO were found to be approximately 10 times more active than SLO on a molar basis (Fig. 2). Therefore, the lower amounts of hemolytic activity secreted by *B. subtilis* containing *slo* probably reflect the lower specific activity of SLO. However, these results are based on hemolytic activity on human erythrocytes and do not necessarily reflect activity on other substrates.

Fate of *B. subtilis* expressing LLO, SLO, and PFO inside J774 cells. We previously reported that *B. subtilis* expressing LLO can grow in the cytoplasm of J774 macrophagelike cells (8). This cell line was chosen because it is phagocytic and relatively incapable of bactericidal activity against *L. monocytogenes* or *E. coli* K-12 (31a). Attempts to grow *B. subtilis* expressing LLO in human epithelial Henle 407 cells failed (data not shown), presumably because the *B. subtilis* strain is not invasive and the hemolysin does not mediate internalization (23, 32).

The fate of *B. subtilis* strains expressing the various hemolysins within J774 cells was monitored by measuring the change in the number of CFU over 5.5 h (Fig. 3) and by light microscopic inspection of stained coverslips (Fig. 4). It is clear that expression of both LLO and PFO was sufficient

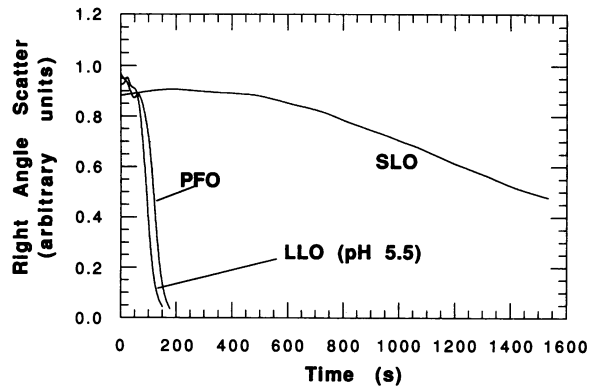

FIG. 2. Maximal hemolytic activities of LLO, PFO, and SLO. The relative activities of the three hemolysins are shown. In each case 9.4×10^{-13} mole of each toxin was injected into a cuvette containing a total of 2×10^8 erythrocytes. Hemolysis was monitored as the decrease in right-angle scatter. PFO and SLO assays were carried out at pH 7.0, whereas the LLO assay was carried out at pH 5.5.

to mediate growth of the *B. subtilis* strain within the J774 cells (Fig. 3). Based on the previous study (8) and the data in Fig. 4, it was presumed that growth was occurring in the host cytoplasm, although this was not directly demonstrated. In contrast, *B. subtilis* containing the vector alone or SLO showed a steady decline in the number of CFU, presumably because the bacteria are unable to escape from the vacuole. Examination of the infected cells by light microscopy revealed that, although LLO and PFO both mediated intracellular growth, PFO apparently mediated damage of the host cells after heavy infection (Fig. 4E). *B. subtilis* expressing SLO for the most part was unable to grow; occasionally, a cell could be identified by light microscopy in which the SLO-expressing strain had grown, but this was a rare event (Fig. 4H).

There is an apparent discrepancy between Fig. 3 and 4 with regard to the extent of bacterial multiplication. Examination of the growth curves (Fig. 3) reveals that the *B. subtilis* strains expressing LLO or PFO doubled approximately three times. In contrast, visual inspection of infected cells consistently showed more than 50 bacteria per cell after 5 h (Fig. 4D), which would result from six bacterial generations. It is unlikely that this descrepancy reflects intracellular suseptibility to gentamicin, since complete omission of gentamicin had little measurable effect (data not shown). The most likely explanation is that the CFU shown in Fig. 3 represent the sum of bacterial growth within the cytoplasm, bacterial killing within the vacuole, and perhaps the loss of some cells from the coverslips. Therefore, the number of CFU represents an underestimate of the actual degree of bacterial replication.

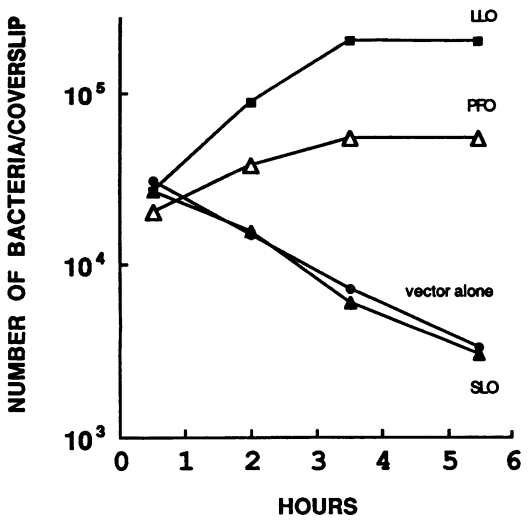

FIG. 3. Fate of *B. subtilis* within the J774 macrophagelike cell line. ■, DP-B980; △, DP-B1512; ▲, DP-B1313; ●, DP-B1066.

There are at least three explanations for the inability of SLO to mediate intracellular growth. First, as shown above, SLO is 10-fold less active than the other two hemolysins and may therefore not be made in sufficient quantity to lyse the vacuole. Second, perhaps SLO is less stable than the other two hemolysins within the vacuolar environment. Third, SLO is present in sufficient quantity but is unable to function within the vacuolar environment. To address this issue, we evaluated the growth of *B. subtilis* expressing LLO within J774 cells after growth in the presence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) at 10 μM, a concentration that limits hemolytic expression to levels less than that in the SLO-expressing strain (Table 1). With this low level of IPTG, only 10 U of hemolytic activity per ml is detected (the normal amount is 80 to 160 U/ml). Under these conditions, the extent of intracellular growth was slightly limited but was still clearly higher than that for the SLO-expressing strain (data not shown).

Effect of pH on intracellular growth and hemolytic activity. SLO is reported to exhibit significantly less activity at pH 5.5 than at pH 7.0 (14). A plausible explanation for the inability of SLO to mediate lysis of the host vacuole is the potentially acidic pH of the vacuolar environment. Therefore, we repeated the intracellular growth curves in the presence of NH_4Cl, a reagent known to raise the pH of acidic vacuolar compartments (26). The results suggest that NH_4Cl did have a slight effect (data not shown), but visual inspection of the infected cells showed that even in the presence of the weak base, the mediation of intracellular growth of the SLO-expressing *B. subtilis* was still comparatively ineffective.

Like SLO, PFO was also reported to be less active at pH 5.5 (14). However, as mentioned above, PFO was essentially as effective as LLO at mediating growth of *B. subtilis*. Therefore, we decided to reevaluate the pH requirements of the three hemolysins by using the method of right-angle scatter. The results confirmed the previous report that LLO is significantly more active at pH 5.5 than at 7.0 (Fig. 5A). However, in contrast to the previous report, SLO and PFO have almost identical activities at pH 5.5 and 7.0 (Fig. 5B and C). Last, pneumolysin showed the same activities at pH 5.5 and pH 7.0 (data not shown).

Fate of *B. subtilis* expressing LLO, PFO, and SLO in bone marrow-derived macrophages. As mentioned above, the J774 macrophagelike cell line is relatively nonbactericidal. Therefore, we used primary cultures of bone marrow-derived macrophages to evaluate the ability of the thiol-activated hemolysins to mediate growth in a macrophage with bactericidal capacity. In initial experiments, there was clearly an extreme degree of bactericidal activity against all of the *B. subtilis* strains (data not shown). However, visual inspection showed a difference; macrophages infected with *B. subtilis* expressing either LLO or PFO appeared to have more intracellular bacteria, but in many cases the macrophage appeared to have been killed. Therefore, we repeated the

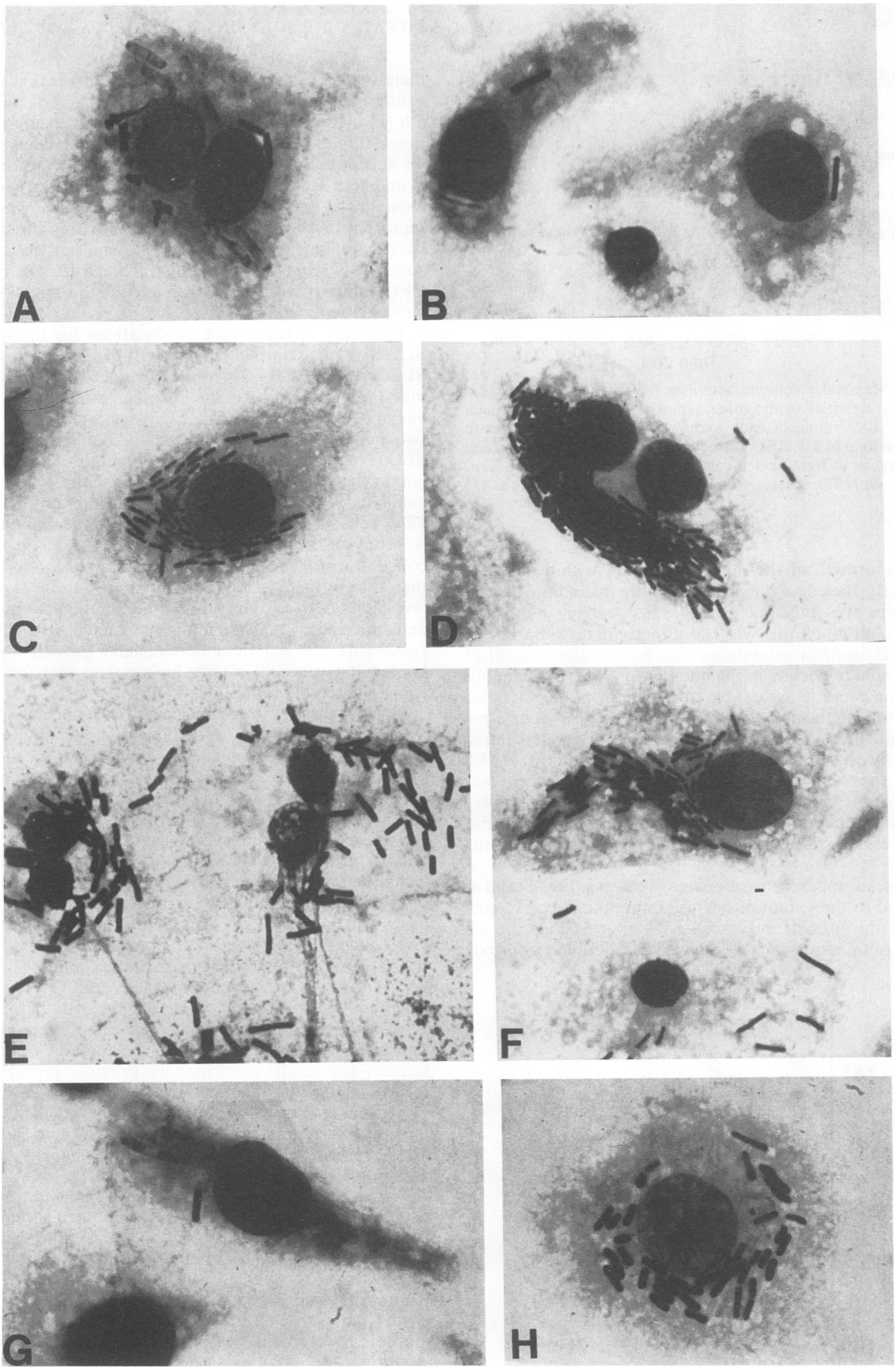

FIG. 4. Light micrographs of *B. subtilis* within J774 cells. (A) Cells infected with a high dose of DP-B1066 containing the vector alone after 3 h; (B) DP-B1066 after 5 h; (C) DP-B980 expressing LLO after 3 h; (D) DP-B980 after 5 h; (E) cells infected with a high dose of DP-B1512 expressing PFO after 3 h; (F) DP-B1512 after 5 h; (G) cells infected with a high dose of DP-1313 after 3 h; (H) DP-1313 after 5 h. Panel H represents a very rare cell; the other panels are representative.

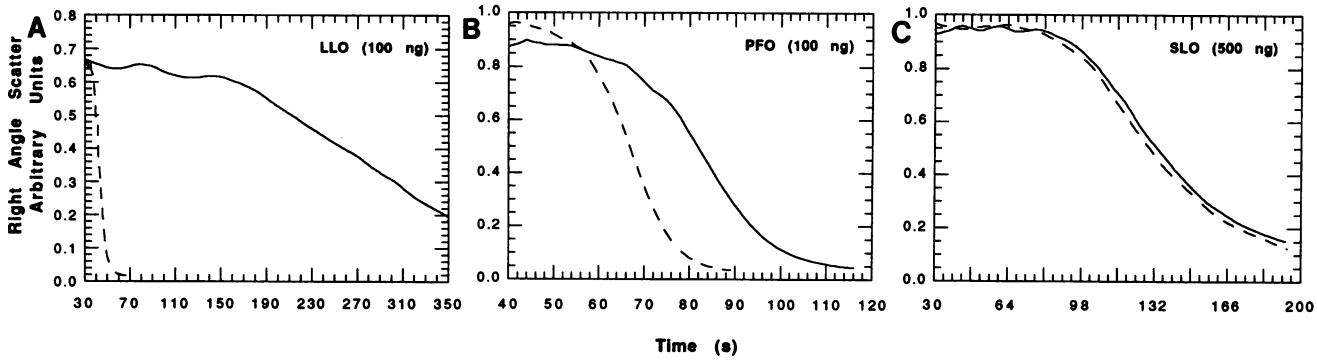

FIG. 5. Hemolytic activities of LLO (A), PFO (B), and SLO (C) at pH 7.0 and 5.5 were compared by right-angle scatter analysis. The dashed lines represent the activity at pH 5.5 and the solid lines represent the activity at pH 7.0. Five times more SLO was used to achieve a reasonable level of activity, since it is at least 10 times less active than either LLO or PFO.

growth curve experiments without the addition of gentamicin (Fig. 6). Whereas *B. subtilis* cells containing SLO or the vector alone were killed, *B. subtilis* cells expressing LLO or PFO showed little change in the number of CFU, which likely reflected the sum of the number of bacteria that were killed and the number of those that grew.

DISCUSSION

In a previous study, we showed that expression of the *L. monocytogenes* hemolysin in *B. subtilis* converted this nonpathogenic soil bacterium into a parasite capable of growth in the cytoplasm of mammalian cells (8). This result was consistent with previous data suggesting that the role of LLO was to lyse the phagocytic vacuole and thus allow *L. monocytogenes* access to the mammalian cytoplasm (12, 29, 32, 37). Also consistent with this model, LLO was reported to be unique among the thiol-activated hemolysins in that it has an acidic pH optimum (14). Most of the other thiol-activated hemolysins are expressed by bacteria that are thought to be exclusively extracellular pathogens, e.g., *S. pyogenes*, *S. pneumoniae*, and *C. perfringens*. Based on all of these criteria, it was our hypothesis that *B. subtilis* expressing the hemolysins from these other pathogens would not grow in the mammalian cell cytoplasm. Indeed, *B. subtilis* expressing SLO was incapable of growth within the mammalian cells. In contrast, *B. subtilis* expressing PFO grew within the J774 cells almost as well as *B. subtilis*

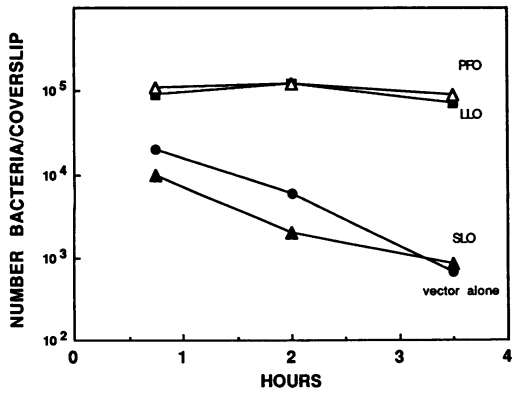

FIG. 6. Fate of *B. subtilis* within bone marrow-derived macrophages in the absence of gentamicin. The symbols are as in Fig. 3.

expressing LLO. Therefore, LLO is not the only member of the thiol-activated family of hemolysins that can mediate intracellular growth when expressed by *B. subtilis*..

Both SLO and PFO are thought to play a role in pathogenesis by affecting neutrophil function at sublytic concentrations (36, 40). This is consistent with the extracellular nature of *S. pyogenes* and *C. perfringens*. Thus the observation that SLO and PFO are fully active at both pH 7.0 and 5.5 is consistent with their activity extracellularly or within an acidic host compartment. In contrast, LLO has a pH optimum of approximately 5.5. This is consistent with the model that LLO acts exclusively within a potentially acidic phagosome. However, the intracellular pH of host vacuoles containing *L. monocytogenes* has not been reported. Also, the addition of weak bases such as chloroquine and ammonium chloride during infection has little or no measurable effect on the growth of intracellular *L. monocytogenes* within J774 cells (31a). Therefore, it is premature to conclude that *L. monocytogenes* resides within an acidic compartment before its escape into the cytoplasm. It is even possible that LLO-induced pore formation may prevent acidification of the vacuole and/or phagolysosome fusion. Perhaps what is more important about the low pH optimum of LLO is that it suggests a mechanism by which the host cell can be protected from the potentially deleterious effects of released LLO in the cytoplasm. Indeed, *B. subtilis* expressing PFO appeared to damage the host cell (Fig. 4E). Whether this reflects pore-forming activity within the cytoplasm is not yet known.

A fundemental question is how LLO and PFO actually mediate lysis of the host vacuole. Lysis of erythrocytes is probably mediated by the introduction of pores resulting in colloid osmotic lysis of erythrocytes (16). However, it seems unlikely that a cellular vacuole has a higher osmotic strength than the cytoplasm. Therefore, the vacuole would not lyse by a colloid osmotic mechanism. This would suggest that LLO may not be acting alone to lyse the vacuole. In the case of *L. monocytogenes*, there are two distinct phospholipases C (3, 10, 15, 24, 27) that may act in concert with LLO to lyse the vacuole. The possibility that a *B. subtilis* secretion product, perhaps a protease or phospholipase, also acts in concert with the hemolysin to lyse the host vacuole cannot be ruled out. Thus one role for the hemolysin may be to insert pores into the vacuole, thereby allowing for the entry of nutrients. This might permit bacterial growth and the production of another required factor(s). It is also possible that merely the act of bacterial growth could result in the

rupture of the host vacuole. However, in the case of wild-type *L. monocytogenes*, individual bacteria can be easily visualized in ruptured vacuoles (37). Thus, the precise role of the hemolysin in mediating rupture of the vacuole is still not known.

The results of this study indicate that SLO, unlike LLO and PFO, is relatively ineffective at mediating growth of *B. subtilis* in the J774 cells. One explanation was provided in that the specific activity of the purified SLO for human erythrocytes was only 1/10 that of the other two hemolysins. Another possible explanation is that the activity of SLO on mouse erythrocytes is lower than that on human erythrocytes (35). This may reflect something inherently different in the structure of SLO, relative instability, or substrate specificity. Perhaps SLO exerts its effects without lysis of host cells; indeed, SLO is now widely used as a reagent to permeabilize eucaryotic cells without causing lysis (34). Examination of the amino acid sequence of these hemolysins reveals that SLO has a 48-amino-acid N-terminal extension that is not present in LLO and a 66-amino-acid extension that is not present in PFO (21, 28, 39). This amino-terminal extension of SLO is very susceptible to proteolysis, resulting in a truncated form of SLO that still retains activity (6). Perhaps the recombinant molecule secreted by *B. subtilis* is not processed, as is suggested by our Western blot analysis, and this results in its instability. Alternatively, it may be processed inappropriately. Further structure-function analysis is required to resolve this issue.

The ability of *B. subtilis* to grow within mammalian cells is clearly cell specific. The primary host cell used in this study was the macrophagelike cell line J774. This cell line was chosen because it is phagocytic, thereby bypassing the need for bacterially mediated internalization. Also, it is relatively nonbactericidal against *L. monocytogenes*, although it killed *B. subtilis* to some extent. Use of this cell line was informative, but it does not necessarily mimic the interaction with primary macrophages. Accordingly, the fate of *B. subtilis* expressing the thiol-activated hemolysins was examined in primary cultures of bone marrow macrophages. In these cells, the outcome of the interaction was different than that seen in J774 cells. In the presence of gentamicin, *B. subtilis* cells expressing any of the hemolysins were killed. However, in the absence of gentamicin, the number of viable *B. subtilis* bacteria expressing either LLO or PFO was 100 times greater than the number of bacteria expressing SLO or containing vector alone. These results suggest that LLO and PFO promote bacterial intracellular growth but may allow the entrance of gentamicin into the macrophage by either killing or permeabilizing the cell. A possible explanation for this result is that bacterial escape from phagolysosomes results in release of lysosomal enzymes into the host cytoplasm, which is detrimental. Alternatively, primary macrophages are more susceptible than J774 cells to hemolysin-mediated permeabilization. It is tempting to speculate that these results could have in vivo relevance. Perhaps, after internalization by macrophages or neutrophils, *S. pyogenes* or *C. perfringenes* kills the host cell and utilizes the nutrients provided by the killed cell.

The production of hemolytic activity is widespread among bacteria (7, 41). From the results of this study, an obvious question is, do any of these hemolysins mediate intracellular growth? Indeed, other intracytoplasmic pathogens, including *Shigella* spp., *Rickettsia* spp., and *Trypanosoma cruzi*, express hemolytic activity that is thought to mediate lysis of the host vacuole (2, 33, 42). In the case of *T. cruzi*, the hemolysin is an acid-activatible pore-forming molecule. Thus it certainly seems reasonable that other pathogens may use a similar strategy. However, it should be pointed out that, at least for *L. monocytogenes* and *S. flexneri*, entry into the cytoplasm alone is not sufficient for pathogenicity. Both of these pathogens have evolved sophisticated mechanisms for cell-to-cell spread that involves utilization of host actin filaments (4, 18, 25, 29, 30, 37). Thus, the ability to enter the cytoplasm and grow is only one step, albeit an important one, in the evolution of an intracytoplasmic parasite.

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REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Andrews, N. W., C. K. Abrams, S. L. Slatin, and G. Griffiths.** 1990. A *T. Cruzi*-secreted protein immunologically related to the complement component C9: evidence for membrane pore-forming activity at low pH. Cell **61**:1277–1287.
3. **Barclay, R., D. R. Threlfall, and I. Leighton.** 1989. Haemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*. Med. Microbiol. **30**:111–118.
4. **Bernardini, M. L., J. Mounier, H. d'Hauterville, M. Coquis-Rondon, and P. J. Sansonetti.** 1989. *icsA*, a plasmid locus of *Shigella flexneri*, governs bacterial intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA **86**:3867–3871.
5. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect. Immun. **57**:2037–2042.
6. **Bhakdi, S., M. Roth, A. Sziegoleit, and J. Tranum-Jensen.** 1984. Isolation and identification of two hemolytic forms of streptolysin O. Infect. Immun. **46**:394–400.
7. **Bhakdi, S., and J. Tranum-Jensen.** 1986. Membrane damage by pore-forming bacterial cytolysins. Microb. Pathog. **1**:5–14.
8. **Bielecki, J., P. Youngman, P. Connelly, and D. A. Portnoy.** 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammlian cells. Nature (London) **345**:175–176.
9. **Boulnois, G. J., J. C. Paton, T. J. Mitchell, and P. W. Andrew.** 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. Mol. Microbiol. **5**:2611–2616.
10. **Camilli, A., H. Goldfine, and D. A. Portnoy.** 1991. *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. J. Exp. Med. **173**:751–754.
11. **Cossart, P., M. F. Vincente, 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.
12. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55**:2822–2829.
13. **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.
14. **Geoffroy, C., J. L. Gaillard, J. E. Alouf, and P. Berche.** 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. **55**:1641–1646.
15. **Geoffroy, C., J. Raveneau, J. Beretti, A. Lecroisey, J. Vazques-Boland, J. E. Alouf, and P. Berche.** 1991. Purification and characterization of an extracellular 29-kilodalton phospholipase

C from *Listeria monocytogenes*. Infect. Immun. **59:**2382–2388.
16. **Harris, R. W., P. J. Sims, and R. K. Tweten.** 1991. Evidence that *Clostridium perfringens* theta-toxin induces colloid-osmotic lysis of erythrocytes. Infect. Immun. **59:**2499–2501.
17. **Harris, R. W., P. J. Sims, and R. K. Tweten.** 1991. Kinetic aspects of the aggregation of *Clostridium perfringens* toxin on erthrocyte membranes: a fluorescence energy transfer study. J. Biol. Chem. **266:**6936–6941.
18. **Havell, E. A.** 1986. Synthesis and secretion of interferon by murine fibroblasts in response to intracellular *Listeria monocytogenes*. Infect. Immun. **54:**787–792.
19. **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.
20. **Kehoe, M., and K. N. Timmis.** 1984. Cloning and expression in *Escherichia coli* of the streptolysin O determinant from *Streptococcus pyogenes*: characterization of the cloned streptolysin O determinant and demonstration of the absence of substantial homology with determinants of other thiol-activated toxins. Infect. Immun. **43:**804–810.
21. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging thiol-activated toxins. Infect. Immun. **55:**3228–3232.
22. **Kenney, T. J., and C. P. J. Moran.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169:**3329–3339.
23. **Kuhn, M., S. Kathariou, and W. Goebel.** 1988. Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. Infect. Immun. **56:**79–82.
24. **Leimeister-Wachter, M., E. Domann, and T. Chakraborty.** 1991. Detection of a gene encoding a phospatidylinositol specific phospholipase C that is coordinately expressed with listeriolysin in *Listeria monocytogenes*. Mol. Microbiol. **5:**361–366.
25. **Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa.** 1986. A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *S. flexneri* 2a. Cell **46:**551–555.
26. **Maxfield, F. R.** 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. **95:**676–681.
27. **Mengaud, J., C. Braun-Breton, and P. Cossart.** 1991. Identification of phosphatidylinositol-specific phospholipase C activity in *Listeria monocytogenes*: a novel type of virulence factor? Mol. Microbiol. **5:**367–372.
28. **Mengaud, J., M. Vincente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56:**766–772.
29. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. **58:**1048–1058.
30. **Pal, T., J. W. Newland, B. D. Tall, S. B. Formal, and T. L. Hale.** 1989. Intracellular spread of *Shigella flexneri* associated with the *kcpA* locus and a 140-kilodalton protein. Infect. Immun. **57:**477–486.
31. **Pinkney, M., E. Beachey, and M. Kehoe.** 1989. The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. Infect. Immun. **57:**2553–2558.
31a. **Portnoy, D. A.** Unpublished data.
32. **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.
33. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51:**461–469.
34. **Schulz, I.** 1990. Permeabilizing cells: some methods and applications for the study of intracellular processes. Methods Enzymol. **192:**280–300.
35. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins, p. 129–183. *In* J. Jeljaszewicz and T. Wasstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
36. **Stevens, D. L., J. Mitten, and C. Henry.** 1987. Effects of α and θ toxins from *Clostridium perfringens* on human polymorphonuclear leukocytes. J. Infect. Dis. **156:**324–333.
37. **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.
37a. **Tweten, R. K.** Unpublished data.
38. **Tweten, R. K.** 1988. Cloning and expression in *Escherichia coli* of the perfringolysin O (theta-toxin) gene from *Clostridium perfringens* and characterization of the gene product. Infect. Immun. **56:**3228–3234.
39. **Tweten, R. K.** 1988. Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin O and pneumolysin. Infect. Immun. **56:**3235–3240.
40. **Van Epps, D. E., and B. R. Andersen.** 1974. Streptolysin O inhibition of neutrophil chemotaxis and mobility: nonimmune phenomenon with species specificity. Infect. Immun. **9:**27–33.
41. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5:**521–528.
42. **Winkler, H. H.** 1990. *Rickettsia* species (as organisms). Annu. Rev. Microbiol. **44:**131–153.
43. **Youngman, P., H. Poth, B. Green, K. York, G. Olmedo, and K. Smith.** 1989. Methods for genetic manipulation, cloning, and functional analysis of sporulation genes in *Bacillus subtilis*, p. 65–87. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
44. **Zuber, P., and R. Losick.** 1987. Role of abrB in spo0A- and spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169:**2223–2230.