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Phospholipase A of *Yersinia enterocolitica* Contributes to Pathogenesis in a Mouse Model

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Some isolates of *Yersinia enterocolitica* exhibit phospholipase activity, which has been linked to lecithin-dependent hemolysis (M. Tsubokura, K. Otsoki, I. Shimohira, and H. Yamamoto, Infect. Immun. 25:939–942, 1979). A gene encoding *Y. enterocolitica* phospholipase was identified, and analysis of the nucleotide sequence revealed two tandemly transcribed open reading frames. The first, *yplA*, has 74% identity and 85% similarity to the phospholipase A found in *Serratia liquefaciens*. Though the other, *yplB*, was less similar to the downstream accessory protein found in *S. liquefaciens*, the organization in both species is similar. Subsequently, a *yplA*-null *Y. enterocolitica* strain, YEDS10, was constructed and demonstrated to be phospholipase negative by plate and spectrophotometric assays. To ascertain whether the phospholipase has a role in pathogenesis, YEDS10 was tested in the mouse model. In experiments with perorally infected BALB/c mice, fewer YEDS10 organisms were recovered from the mesenteric lymph nodes and Peyer's patches (PP) than the parental strain at 3 or 5 days postinfection. Furthermore, bowel tissue and PP infected with YEDS10 appeared to be less inflamed than those infected with the parental strain. When extremely high doses of both the parental and YEDS10 strains were given, similar numbers of viable bacteria were recovered from the PP and mesenteric lymph nodes on day 3. However, the numbers of foci and the extent of inflammation and necrosis within them were noticeably less for YEDS10 compared to the parental strain. Together these findings suggest that *Y. enterocolitica* produces a phospholipase A which has a role in pathogenesis.

Phospholipases (PL) are considered virulence factors for bacterial species that cause disparate disease syndromes, ranging from infections causing massive tissue destruction, such as gas gangrene and *Pseudomonas aeruginosa* infections, to foodborne listeriosis. The best characterized PL are predominantly the phospholipases C (PLC), which hydrolyze phospholipids to release the phospho-head group and diacylglycerol (DAG), and include the *Clostridium perfringens* alpha-toxin, the two *P. aeruginosa* PLC, *Staphylococcus aureus* beta-toxin, and the two *Listeria monocytogenes* PLC (reviewed in references 48 and 51). Many of the PLC are hemolytic and are thought to directly cause tissue destruction, destabilizing host cellular membranes by hydrolyzing membrane phospholipids. Though one of several toxins secreted by *C. perfringens*, alpha-toxin is the primary virulence factor in models of gas gangrene: injection of purified alpha-toxin reproduces many features of *C. perfringens*-induced shock in rabbits, and an alpha-toxin null mutant has reduced virulence in the mouse myonecrosis model (3, 50). Rather than wholesale destruction of membranes, the two *L. monocytogenes* PLC (with listeriolysin O) apparently disrupt specific intracellular vacuoles yet preserve host cell integrity (8, 47). Thus, PLC produced by bacterial pathogens have been shown to disrupt host cell membranes; their actions range from wholesale cytolysis to controlled destruction of intracellular vacuoles.

In addition, bacterial PLC also have the potential to exert profound effects on the host indirectly by the production of lipid second messengers that modulate host cell signaling pathways. Normally, hormones stimulate host PLC which hydrolyze phosphatidylinositol releasing DAG into the cytoplasm, and this in turn induces protein kinase C (PKC) to modulate host cell metabolism and growth (14). Consequently, the PLC of the intracellular pathogen *Legionella pneumophila* was proposed to modulate neutrophil activation via release of DAG (13). In support of the hypothesis that bacterial PLC can modulate host cell signaling, treatment with exogenous bacterial PLC was shown to reduce O_2 production from human polymorphonuclear lymphocytes (PMN) (52) and to induce tissue destructive matrix metalloproteinase secretion from human epithelial cells, both presumably by PKC induction via DAG (17). Moreover, the products of further DAG degradation by endogenous cytoplasmic enzymes induce the arachidonic acid cascade, stimulating the production of leukotrienes that increase vascular permeability or potent inflammatory mediators such as prostaglandins and thromboxanes (51). Preparations of *C. perfringens* alpha-toxin were shown to induce the inflammatory response via induction of the arachidonic acid cascade (18), and the hemolytic *P. aeruginosa* PLC stimulated the production of inflammatory mediators and increased vascular permeability (31). Evidently, the products of bacterial PLC activity can modulate host cell signaling pathways by induction of PKC and the arachidonic acid cascade.

Another class of PL, endogenous host phospholipases A (PLA), directly stimulates the arachidonic acid cascade by releasing fatty acids, including arachidonic acid, from the glycerol backbone of phospholipids. None of the few bacterial PLA implicated in pathogenesis have been conclusively linked to the induction of the arachidonic cascade. However, these PLA have not been as thoroughly studied as bacterial PLC, and most PLA have not been tested in an animal model. Nevertheless, the injection of *Salmonella newport* PLA into rabbit

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ligated ileal loops induced similar levels of fluid accumulation, desquamation, and mononuclear cell infiltration as did injection with bacteria (35). In contrast, a PL mutant strain of *Vibrio cholerae* was found to induce similar amounts of fluid accumulation in rabbit ligated ileal loops compared to the parent strain (16). Indeed, the only data suggesting a role in pathogenesis for many bacterial PLA, including the PLA from *Vibrio parahaemolyticus* (43), *Rickettsia prowazekii* (59), and *Campylobacter coli* (23), have been hemolytic activity often due to the accumulation of the product, lysophospholipids, which destabilize membranes. In contrast, *Helicobacter pylori* PLA has been implicated in ulcer formation through its destruction of the essential, protective mucus layer which exposes the gastric epithelium to hemolytic lysophospholipids, degradative enzymes, and low pH (45). Though supporting data are somewhat limited in general, some bacterial PLA are considered virulence factors that seem to fulfill other functions besides disrupting cellular membranes. Yet the potential for bacterial PLA to induce the arachidonic acid cascade has not been addressed.

Previous work by Tsubokura et al. (53) demonstrated lecithin-dependent (phosphatidylcholine) hemolytic activity in some strains of *Y. enterocolitica*. Partially pure preparations of the lecithin-dependent hemolysin produced lysophosphatidylcholine and fatty acids from lecithin, suggesting it was a phospholipase A. However, the significance of this PL activity in the *Y. enterocolitica* disease process had not been examined. The capacity to engineer specific mutants in *Y. enterocolitica* and to test these mutants in an animal model affords an opportunity to assess the role of the PL in pathogenesis. *Y. enterocolitica* infections in the mouse model closely mirror the progress of the disease in humans. After intragastric inoculation, yersiniae pass through the gastrointestinal tract to the lower ileum and penetrate the epithelial layer through the follicle-associated epithelium overlying the Peyer's patches (PP), the unencapsulated lymphoid tissue located in the wall of the intestine. Aggregates or microcolonies of bacteria first become evident by 17 h postinfection in the dome of PP near an intact epithelial cell layer. PMN are recruited to the sites of infection within 24 h, and PMN and macrophages are found surrounding the masses of extracellular bacteria. The response to infecting yersiniae is similar in mesenteric lymph nodes (MLN); the infiltrate is predominantly PMN with some macrophages (2, 10). The inflammatory lesions progress to form pyogenic abscesses in both the PP and MLN.

To assess whether the *Y. enterocolitica* phospholipase plays a role in bacterial pathogenesis and in the inflammatory process observed in humans and mice, the gene encoding the PL was identified, the PL activity was confirmed, and a *Y. enterocolitica* PL null mutant was constructed. The progression of peroral infection of mice demonstrated that viable mutant yersiniae were not as readily recovered from PP and MLN in high numbers nor was there as much inflammation and necrosis in PP and MLN as in tissues infected with the PL-positive parental strain. Thus, PL activity in *Y. enterocolitica* was shown to promote colonization of PP and MLN and to increase the pathologic changes resulting from the host inflammatory response.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this study include the virulent American strain, *Y. enterocolitica* 8081v (39), the restriction minus derivative JB580 (27), and its derivative YEDS10 (all of which are pYV^+) and the strains listed in Table 1. The pLAFR2- and pUC19-based plasmids were maintained in *Escherichia coli* LE392 and DH5α. The pEP185.2-based plasmids, which have *ori*R6K and are mobilizable, were maintained in *E. coli* SY17-1 λpir (28, 44). *Y. enterocolitica* and *E. coli* strains were grown in Luria-Bertani (LB) broth, on LB agar plates, or on MacConkey agar base supplemented with 1% Tween 80 or 0.2% egg yolk lecithin and 1 mM $CaCl_2$ at 26 or 37°C with aeration. The basis for the enzymatic plate assays was the precipitation of calcium salts of free fatty acids produced from the substrates Tween 80 or lecithin by the action of lipases-PL or PL alone, respectively. Tween 80 is not a true phospholipid or lipid substrate, but its solubility in aqueous solutions makes it easier to use. Antibiotics were used in the following concentrations where appropriate: ampicillin, 100 μg/ml; chloramphenicol, 50 or 25 μg/ml; nalidixic acid, 20 μg/ml; erythromycin, 100 μg/ml; and tetracycline, 15 μg/ml.

Subcloning and Tn*Max2* mutagenesis. A pLAFR2 library was constructed with chromosomal DNA from strain 8081v as described previously (4). The library was screened on MacConkey plates supplemented with 1% Tween 80 and $CaCl_2$. Cosmids and plasmids were purified by the alkaline lysis method (29) or with the Wizard Minipreps (Promega, Madison, Wis.). The Tween plate positive cosmid, pVM200, was digested with *Hin*dIII, and the fragments were ligated into the *Hin*dIII site of pLAFR2. Ligation reactions were electroporated into aliquots of washed *E. coli* LE392 or DH5α by using a Gene Pulser (Bio-Rad) according to the manufacturer's instructions. Subclone pVM202 was isolated on the basis of activity on Tween plates and carried a 6.0-kb insert. The 6.0-kb fragment was excised with *Hin*dIII and ligated into pUC19. The resulting plasmid, pDHS20, was thoroughly mapped, and subclones were generated with several restriction enzymes, including *Sph*I, *Ava*I, *Kpn*I, and *Acc*I. Subclones were tested on Tween plates to identify the region of interest (some of these are depicted in Fig. 1). Tn*Max2* (Ery^r) insertions into pDHS20 were generated as described by Haas et al. (24). Briefly, transposition was induced in *E. coli* E131 (pTn*Max2*, pDHS20) by the addition of 100 μM isopropyl-β-D-thiogalactopyranoside, and the plasmids were purified. The plasmid preparations were electroporated into DH5α, which does not support pTn*Max2* replication, and Ery^r transformants were selected to recover pDHS20 with transposon insertions. The position of the Tn*Max2* insertion in selected transformants was mapped by using restriction enzymes.

Sequence determination and analysis. The template plasmids, pUC19-derived subclones and Tn*Max2* insertions in pDHS20, were prepared by the alkaline lysis method (29) and followed by polyethylene glycol precipitation (1). The plasmids were sequenced by the dideoxy method as modified for use with Sequenase (U.S. Biochemicals) by using M13 forward and reverse primers (New England Biochemicals) and primers to either end of Tn*Max2* (5′-CCTGACAGAAATGG C-3′ and 5′-CCTAAAGGGATCCAAAAGCT-3′), and novel primers were synthesized as needed. [^{33}P]dATP was purchased from Andotek (Irvine, Calif.). Both sequence strands were assembled and analyzed by using the programs compiled in the Wisconsin Sequence Analysis Package (GCG, Madison, Wis.).

Southern analysis. Chromosomal DNA was purified from *Yersinia* strains as previously described (30) and was digested with *Hin*dIII. The restriction digests were separated by electrophoresis on a 0.7% agarose gel and transferred to nitrocellulose by the method of Southern (49). The *yplA* probe was generated by random primer labeling with [α-^{32}P]dCTP (15) after the fragment was excised from a plasmid and separated by electrophoresis in an agarose gel (Fig. 1). Medium stringency hybridizations and washes were performed essentially as described by Maniatis et al. (29).

Creation of a PL-negative mutant. Based on sequence information, opposing primers were synthesized well within the coding region, with the 3′ primer just downstream of the serine active site consensus sequence (5′-ATAGATCCGGC GAGCCTATC-3′ and 5′-AGTGCATCGACCAAATGCC-3′). Upon PCR amplification, a 300-bp fragment completely internal to the structural part of the *yplA* gene was produced. This fragment was ligated into a suicide vector, pEP185.2 (27). This resulting plasmid, pDHS27, was mated from SY17-1 λpir into PL-positive *Y. enterocolitica* JB580 essentially as described previously (44), and Cm^r exconjugates were selected on M63 minimal plates supplemented with 0.2% glucose, 0.0001% vitamin B_1, 20 μg of nalidixic acid per ml, and 25 μg of chloramphenicol per ml. Mutants were analyzed by Southern hybridization with *yplA* as a probe, and the expected hybridizing fragments of 5.6 and 4.8 kb confirmed that pEP185.2 was inserted into *yplA*, interrupting the gene just after the sequence encoding the lipase serine active site consensus. The mutant, YEDS10, was found to be PL negative on lecithin and Tween plates and by the spectrophotometric assay as described below.

PLA assay. *Y. enterocolitica* strains were incubated in LB broth for 24 h at 26°C with vigorous shaking. Crude preparations of protein were made by the addition of sodium dodecyl sulfate to 0.1%, and the cells were lysed by sonication at 4°C with a microprobe tip with four 15-s pulses at the maximum setting. Cell wall debris was removed by centrifugation in a microfuge at 4°C for 1 min at maximum speed. Assays with the PLA substrate 4-nitro-3-(octanoyloxy)benzoic acid (0.25 mg/ml) and 50 μl of crude lysates were carried out at 26°C in duplicate samples, one with 10 mM Ca^{2+} and the other with 1 mM EDTA, by a modification of the method of Cho and Kezdy (12). The value for activity was calculated by the change of absorbance at 410 nm (corrected for breakdown of substrate) over time per milligram of total bacterial protein as determined by the Bio-Rad DC protein assay.

Mouse model. *Y. enterocolitica* JB580 and YEDS10 were grown at 26°C overnight with aeration in LB broth, washed with phosphate-buffered saline (PBS), and diluted to the appropriate dose in sterile PBS. Virus-free, 6- to 8-week-old female BALB/c mice were infected intragastrically with 0.2 ml of bacterial suspension at various doses. At days 3 and 5 postinfection, mice were sacrificed by

TABLE 1. Comparison of Southern hybridization results and activities on lecithin and Tween 80 plates of various *Yersinia* species

Bacterial strain	Hybridizing band (kb)	Precipitation on plates with:		Reference or source[a]
		0.2% Lecithin	1% Tween 80	
Various *Yersinia* strains				
Y. enterocolitica 8081c (pYV$^-$) Biol 1	6.0	++	++	39
Y. enterocolitica 8081v (pYV$^+$) Biol 1	6.0	++	++	39
Y. enterocolitica YEDS10	4.8, 5.6	−	−	This study
Y. pestis EV76-6 (pYV$^-$)[a]	−	−	−	38
Y. pseudotuberculosis YPIII$^+$ (pYV$^+$)	−	−	−	19
Y. pseudotuberculosis YPIII$^-$ (pYV$^-$)	−	−	−	6
Y. pseudotuberculosis K286[b]	−	−	−	32
Y. kristensenii	−	−	−	W. Hill, FDA
Y. frederikensenii	−	−	−	W. Hill, FDA
Y. frederikensenii[b] (chronic diarrhea, weight loss)	3.0	++	++	M. Cafferkey
Y. rohdei[c]	−	−	−	CDC
Y. aldovae	−	+	+	CDC
Y. intermedia	6.0	+	+	W. Hill, FDA
Assorted *Y. enterocolitica* strains[d]				
O:27	6.0	+	+	CDC
O:20	7.5	+	+	CDC
O:21	6.0	+	+	CDC
O:18	7.5	+	+	CDC
O:4,32	7.5	+	+	CDC
O:5,27	7.3, 8.4	−	−	CDC
Y. enterocolitica clinical isolates				
Biol 1, O:6,30[e] (Crohn's disease)	6.0	++	++	M. Cafferkey
Biol 1, O:9[e] (colitis and performation)	3.0	+	+/−[g]	M. Cafferkey
Biol 1, O:9[e] (septicemia)	3.0	++	++	M. Cafferkey
Biol 1, O:3[e] (acute diarrhea)	6.0	++	++	M. Cafferkey
Biol 3, O:3 (acute appendicitis)	7.3	−	−	M. Cafferkey
Biol 3, O:3[f] (acute colitis)	7.3	−	−	M. Cafferkey
Biol 4, O:3 (acute diarrhea)	8.6	−	−	M. Cafferkey
Biol 4, O:3 (mesenteric adenitis)	8.6	−	−	M. Cafferkey
Biol 4, O:3 (acute terminal ilietis)	8.6	−	−	M. Cafferkey

[a] *Y. pestis* EV76-6 grew poorly on both media.
[b] Clinical isolate.
[c] Two isolates (from dog and human stool samples) were tested.
[d] All CDC reference strains.
[e] Isolate is pYV$^-$.
[f] Two of three isolates of this biogroup and O antigen type were pYV$^+$; each was determined to have caused acute colitis.
[g] Initial test was negative; freezer stock retested on new plates showed faint precipitate.

cervical dislocation, and the 8 PP nearest the ileocecal junction were excised. Alternate PP were set aside for obtaining viable bacterial counts or immediately fixed in formaldehyde for paraffin embedding and sectioning. All the MLN were harvested and divided equally for the determination of viable *Y. enterocolitica* counts and for histological examination. For viable bacteria counts, the tissue samples were placed in 0.5 ml of sterile PBS, macerated, serially diluted in 10-fold steps, and plated on LB with nalidixic acid or chloramphenicol or on MacConkey-lecithin plates. Colonies were counted after 48 h, at which time lecithinase activity could be detected in *Y. enterocolitica* JB580. During the course of the experiment the mutant YEDS10 remained Cm^r and negative on Tween 80 or lecithin plates.

For histological examination, samples were immediately fixed with 3.7% formaldehyde in PBS for a minimum of 48 h, placed in cassettes, and processed for paraffin embedding (40). Samples were embedded in paraffin, sectioned on a microtome, and stained with hematoxylin and eosin as described previously (37). Each individual PP or MLN in the stained sections was scored as having either areas of inflammation or inflammation with necrosis. The size of the lesions was not considered in the scoring used in Fig. 5, and only tissues with no discernible pathology were scored as normal.

Nucleotide sequence accession number. The sequence including *yplA* and *yplB* has been assigned GenBank accession no. AF0678496.

RESULTS

Identification of a locus encoding *Y. enterocolitica* lecithinase. Earlier work by Tsubokura et al. (53) demonstrated that some *Y. enterocolitica* strains produced a PL which promoted lecithin-dependent (phosphatidylcholine) hemolysis. Initially, *Y. enterocolitica* strains were tested on egg yolk plates; subsequently, activity was determined with Tween 80 plates. Since Tween 80 is neither a phospholipid nor a triacylglyceride lipid, the detected enzymatic activity could be either a PLA (or PLB) or (triacylglyceride) lipase. However, the previous determination that *Y. enterocolitica* produced lecithinase activity suggests that the Tween 80 hydrolysis was due specifically to PL activity.

Before identifying the gene encoding the presumptive PL, *Y. enterocolitica* was tested by inoculation of Tween 80 plates to confirm that the strain produced the lecithinase. *Y. enterocolitica* 8081 and JB580 with or without the virulence plasmid were found to be positive on plates when incubated at 26°C but not when incubated at 37°C. Subsequently, a cosmid (pLAFR2) library made from *Y. enterocolitica* 8081v was screened for activity on Tween 80 plates incubated at 26°C; *E. coli* LE392(pLAFR2) was negative on the plates under all conditions tested even after prolonged incubation. A recombinant cosmid in LE392 was isolated which was positive for activity on the plates; the cosmid, pVM200, had an insert of approximately 30 kb. A 6.0-kb *Hin*dIII fragment (pVM202) was ligated back into pLAFR2 and was found to be sufficient to

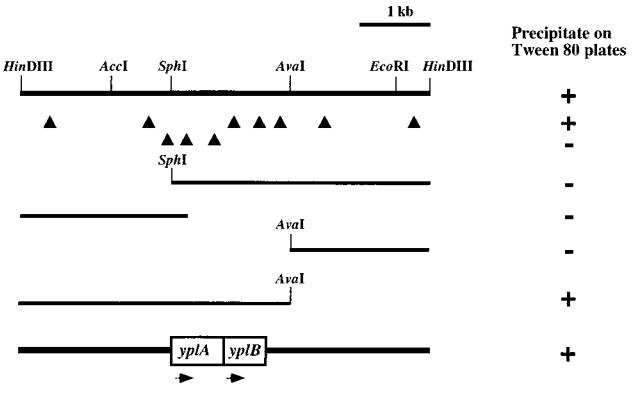


FIG. 1. Analysis of the *yplA yplB* region of *Y. enterocolitica*. The top line depicts a restriction map of the 6-kb fragment, and below are shown the locations and directions of transcription of the ORFs with the Southern hybridization probe indicated by the line below. The top row of triangles indicate the positions of representative single transposon insertions into pDHS20 which were still active on Tween plates, as indicated in the righthand column. The row of three triangles underneath indicates the position of the cluster of single transposon insertions which eliminated PL activity. Below the triangles are a few examples of subcloned fragments. The ability of each construct to confer PL activity to *E. coli* is indicated in the righthand column.

make *E. coli* positive on Tween 80 plates. This 6.0-kb fragment was subcloned into pUC19; the resultant strain LE392(pDHS20) demonstrated greater activity on the Tween plates than did the cosmid constructs and was stably maintained in spite of its high copy number. Plasmid pDHS20 was further subcloned and assayed to determine the smallest region sufficient to produce PL activity (Fig. 1). Concurrently, 36 independent transposon insertions in pDHS20 were mapped and also tested for their activity on the plates. A cluster of Tn*Max2* insertions that eliminated activity clearly defined the region necessary to produce PL activity; this was supported by the results from the experiments with the subclones (Fig. 1).

Sequencing and analysis of *yplA* and *yplB*. Subsequently, a 2-kb fragment encompassing the region of interest was sequenced by using the transposon insertions and the pUC19 subclones as templates. Two open reading frames (ORF) were found in the sequence: both ORF apparently are transcribed in the same direction, with the coding regions slightly overlapping by 8 bp at the end of one and the beginning of the next ORF, *yplB* (Fig. 1). The first ORF, *yplA*, is larger and spans at least 798 bp, encoding 266 amino acids (see below); the downstream *yplB* spans 603 bp, encoding 200 amino acids. After the transposon insertions and subcloned fragments maintained in *E. coli* LE392 on Tween 80 plates were compared, an intact *yplA* ORF was clearly implicated as necessary for activity. Only transposon insertions in the coding sequence of *yplA*, but not in that of *yplB*, eliminated expression of PL activity in *E. coli*.

BLAST database searches (GCG and GenBank) with the predicted amino acid sequence of YplA found *S. liquefaciens* PLA (PhlA) to be most similar (Fig. 2). There was 74% identity and 84% similarity over 244 residues between the predicted primary sequences of *Y. enterocolitica* YplA and *S. liquefaciens* PhlA (22). The N-terminal quarter of the polypeptides had small stretches with less similarity. The predicted *Y. enterocolitica* protein also has 77% identity and 90% similarity over 217 residues (with a 25-residue gap) to an uncharacterized *Serratia proteomaculans* "lipase" sequence, which is probably the homolog of the *S. liquefaciens* gene (GenBank Z19596 [46]). The predicted primary sequences were 319 and 255 residues for the *S. liquefaciens* and *S. proteomaculans* proteins, respectively, which is similar to the size predicted for YplA. Moreover, all three peptide sequences contained a match to the consensus for the lipase active site. The primary sequence of YplA is LVITGHSLGG (invariant [boldface], conserved [underscored] residues) in the putative active site (MOTIFS; GCG). The active site serine (see above) is generally found in bacterial lipases, but most bacterial PLA do not match the consensus well (e.g., *Proteus vulgaris* [7] and *E. coli* [26]). Interestingly, no other bacterial PL or lipases had significant similarity to the *Y. enterocolitica* sequence as determined by the BLAST program.

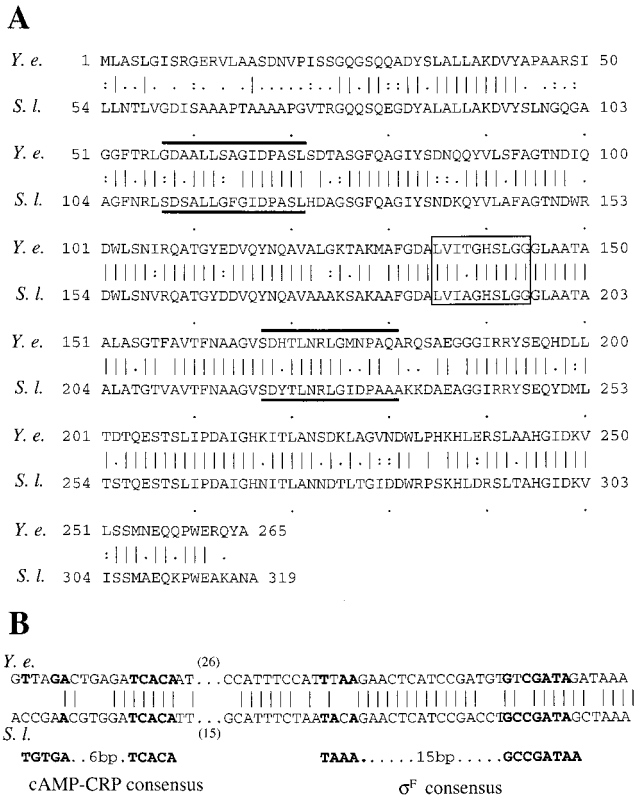


FIG. 2. Amino acid and putative promoter region alignments of *Y. enterocolitica* and *S. liquefaciens* PL. (A) Alignment of predicted amino acid sequence of *Y. enterocolitica* YplA (top line) to PhlA starting at residue 54 from *S. liquefaciens*; identical residues are indicated by vertical lines, and similar residues are indicated by periods or semicolons (Bestfit; GCG). The match to the lipase serine active site consensus is boxed, and the 15-residue repeats are indicated by the heavy lines. The consensus sequence derived from the *Serratia* PhlA was reported to be SDXXLXXXGIDPAAA (21). (B) Alignment of the nucleotide sequence 5′ to *yplA* (top line) and *phlA* (including pA promoter; bottom line) (BLAST and Compare; GCG). Matches to the CRP-cAMP and σ^F consensus are indicated by boldface letters, and their consensus sequences are listed below the aligned *Serratia* and *Yersinia* sequences.

Examination of the predicted amino acid sequence revealed three potential N-terminal methionines upstream from the region of similarity to the *Serratia* PLA. However, two of them (the most proximal and distal) were located before a hydrophobic segment, though both lacked N-terminal basic residues. The *S. liquefaciens* PLA also has a hydrophobic N terminus lacking any basic residues, yet it has been determined that the protein is secreted (21). Another notable feature was that the active site serine consensus peptide was found within a 40-residue hydrophobic stretch. A hydrophobic region near the active site is thought to promote interaction between the enzyme and the substrate, whether it is lipid or the hydrophobic

part of a phospholipid. Furthermore, when the predicted *Yersinia* sequence was compared to itself, a 15-residue repeated region was identified (Fig. 2). Similar peptide repeats were found in comparisons to the *S. liquefaciens* PLA. Peptide repeats had been noted in *S. liquefaciens* PLA, and it was hypothesized that these regions were involved in binding to an accessory protein which modulates the activity of the enzyme (21). The similarity in primary sequence and organization to the *S. liquefaciens* PLA supports the hypothesis that *yplA* encodes a *Y. enterocolitica* PL.

The downstream *yplB* found in the *Y. enterocolitica* sequence presented here has 44% identity and 63% similarity over 187 of its amino acid residues to the accessory protein, PhlB, of *S. liquefaciens* (20). The corresponding region was not sequenced from *S. proteomaculans* (46). Furthermore, the arrangements of the *phlAB* locus of *S. liquefaciens* and the *Y. enterocolitica* sequence were found to be very similar; in both, the genes encoding the PL (*phlA* and *yplA*, respectively), were located upstream of and overlapped with the accessory genes (*phlB* and *yplB*, respectively). Apparently, YplB was dispensable for lecithinase activity when transformed into *E. coli*, since Tn*Max2* insertions into *yplB* were still active on Tween 80 plates. Similarly, the *S. liquefaciens phlB* was not necessary for the expression of PLA activity in *E. coli*, but it was required in *S. liquefaciens* to maintain cell viability by preventing potentially deleterious degradation of inner membrane phospholipids (21).

Comparisons between the *Serratia phlAB* locus and *Y. enterocolitica yplAB* at the nucleotide level demonstrated two ~43-bp regions of similarity (Compare [window, 10; stringency, 6.0]; GCG) coinciding with the 5′ region of *phlA* that include the primary promoter, pA (putative −35 and −10 regions and the transcription start site), and the putative ribosome binding site and N-terminal coding region (promoter sequence [see Fig. 2B]). Examination of the *Y. enterocolitica* sequence in that region, TAA(N_{15})GTCGATAG revealed good agreement with the σ^F consensus, TAAA(N_{15})GCCGATAA (conserved residues indicated by underscore). The σ^F of *E. coli* directs the transcription of flagellar genes, including flagellin, motility, and chemotaxis genes, and has been shown to promote RNA polymerase binding to the listed consensus sequence (reviewed in reference 25). Furthermore, 26 bp upstream of the pA promoter in *S. liquefaciens* was a sequence that resembles the *E. coli* cyclic AMP receptor protein-cyclic AMP complex (CRP-cAMP) consensus sequence: TGTGA(N_6)TCACA. About 40 bp upstream of the putative σ^F consensus in *Y. enterocolitica* is the sequence TTAGA(N_6)TCACA, which has better agreement with the CRP-cAMP consensus than the *S. liquefaciens* sequence, CCGAA(N_6)TCACA. Whether these sequences in the 5′ region function as a promoter or an operator in *Y. enterocolitica* is beyond the scope of this study. However, cultures grown on plates with added glucose have less PL activity than those without the added glucose, suggesting that the enzyme is downregulated in the presence of glucose, i.e., it is catabolite repressed.

PL assays. The similarity of *yplA* to the *S. liquefaciens phlA* and the finding of lecithinase activity in *Y. enterocolitica* suggested that it encoded a PL rather than a lipase. To determine the validity of this hypothesis, both *Y. enterocolitica* and *E. coli* strains were subjected to several PL assays. First, a true phospholipid substrate, phosphatidylcholine (lecithin) was substituted for Tween 80 in the plate assay to confirm that the *Y. enterocolitica* lecithinase (and not a lipase) was being assayed. The ability to hydrolyze Tween 80, an oleic acid ester, suggested that the *Y. enterocolitica* lecithinase is a PLA (or PLB) rather than a PLC or PLD, which cleave phospholipids at the polar head groups and do not release free fatty acids. *Y. enterocolitica* 8081v (and the pYV^- derivative) and *E. coli* transformed with intact *yplA* on pLAFR2 or pUC19 derivatives demonstrated PL activity on phosphatidylcholine plates (Fig. 1). Plasmid constructions in which Tn*Max2* had inserted into the coding region of *yplA* failed to confer the activity to *E. coli*. Thus, the results on activity plates with strains of *E. coli* and *Y. enterocolitica* were the same regardless of whether the substrate was Tween 80 or phosphatidylcholine.

Freshly grown cultures of *Y. enterocolitica* were tested for the expression of PLA in a spectrophotometric assay by using 4-nitro-3-(octanoyloxy)benzoic acid, a compound designed specifically to measure the activity of PLA_2 (12). Preparations from 20- to 24-h *Y. enterocolitica* cultures demonstrated an average activity of 0.0117 s^{-1} mg^{-1} regardless of the presence of pYV. The activity was dependent on the presence of Ca^{2+} in the reaction buffer, which is also true for most PLA enzymes. Though crude extracts were prepared from *E. coli* strains, activity was not detected even though the strains were found to be positive by the plate assay. *E. coli* strains may not produce as much PL as *Y. enterocolitica*. Considered together, these data suggest that *yplA* encodes a PLA rather than a lipase.

Southern analysis of chromosomal digests of various *Yersinia* spp. To determine the distribution of *yplA* among *Yersinia* spp. chromosomal DNA was prepared from a variety of *Yersinia* spp., including both pathogens and a few environmental species. In addition, DNA was prepared from a bank of different serotypes of *Y. enterocolitica* and a number of *Y. enterocolitica* clinical isolates. The chromosomal DNA was examined by Southern hybridization by using a fragment of the 8081v *yplA* as the probe (Fig. 1). The strains were also grown on 1% Tween 80 and 0.2% lecithin plates to determine if a functional PLA was produced. Other than *Y. enterocolitica*, only *Y. aldovae*, *Y. intermedia*, and the *Y. frederikensenii* clinical isolate demonstrated activity on the plates (Table 1). The chromosomal DNA from *Y. intermedia* and the *Y. frederikensenii* clinical isolate demonstrated hybridizing bands upon Southern hybridization under conditions of medium stringency, whereas DNA from *Y. aldovae* or a type strain of *Y. frederikensenii* lacked hybridizing bands. The two other pathogenic species, *Y. pseudotuberculosis* and *Y. pestis*, did not demonstrate activity on plates; this was expected, since the production of PLA activity has been reported to be restricted to *Y. enterocolitica*. They also lacked a hybridizing band on Southern analysis, suggesting that among pathogenic *Yersinia* spp., *yplA* is confined to *Y. enterocolitica*. All of the *Y. enterocolitica* strains hybridized to the *yplA* probe. As expected, the majority of *Y. enterocolitica* biotype 1 strains produced detectable PL activity on lecithin plates. The biotyping scheme developed by Wauters uses activity on Tween plates as the distinguishing trait for biotype 1; all other biotypes are negative in the assay (57, 58). The clinical isolates which hybridized but did not show PLA activity were all serotype O:3 of biotypes 3 and 4. Therefore, the sequences encoding PL activity were found in all of the *Y. enterocolitica* strains examined, including the clinical isolates, although expression of the activity in vitro was not detected in the clinical isolates other than biotype 1.

Construction of a PL null *Y. enterocolitica*. To ascertain whether YplA contributes to the virulence of *Y. enterocolitica* in the mouse model, a *Y. enterocolitica yplA* mutant strain was constructed. The resultant *Y. enterocolitica* strain, YEDS10, has a plasmid inserted just downstream of the active site consensus sequence, truncating the polypeptide product by 120 residues. Southern analysis of chromosomal *Hin*dIII digests of

YEDS10 compared with the parent strain confirmed the insertion of sequences within *yplA*; the *yplA* probe hybridized to two fragments of the predicted size, 4.8 and 5.6 kb, rather than one 6-kb fragment from JB580. The strain, YEDS10, was tested on Tween 80 and lecithin media and found to be negative for PL activity. In addition, crude extracts of the parental *Y. enterocolitica* strain JB580 and the mutant were tested by a spectrophotometric assay for PLA activity. JB580 was found to have detectable PL activity by this assay, whereas YEDS10 did not (data not shown).

Kinetics of infection of mouse model. After the peroral inoculation of mice, *Y. enterocolitica* infection proceeds from the lumen through the epithelium of the lower small intestine to the PP, proceeding from there to the MLN and, with sufficient inoculum, from there to the bloodstream and deeper tissues, i.e., the spleen, liver, and lungs (11). Previous work with the perorally infected mouse model has shown that $>10^6$ CFU/g of tissue of viable yersiniae can be recovered at 18 h postinoculation (p.i.) from PP. Colonization of PP reaches consistently high levels ($>10^6$ CFU/g) at day 3 or 4 p.i., stays above 10^6 CFU/g, and then decreases between days 10 and 14 p.i. (36). Likewise, MLN may have recoverable counts at day 3 p.i., depending on the inoculum size, but they are generally colonized by virulent *Y. enterocolitica* by day 5. However, recovery of viable bacteria from the spleen and liver is inconsistent and sporadic with respect to the day p.i. at which the counts are recovered and the levels of infection are achieved. Therefore, a modified infection kinetics assay was devised in which the mutant strain, YEDS10, was inoculated at a variety of doses in parallel to similar doses of the parental strain, *Y. enterocolitica* JB580. In several experiments, at day 3 or 5 p.i., the PP and MLN were removed, and the viable bacteria were recovered and quantitated (Fig. 3 and 4).

At doses of approximately 10^8 CFU/mouse, the parental strain colonized the PP and MLN to $>10^7$ CFU/g at days 3 and 5 p.i., but significantly fewer YEDS10 were recovered on both days from all but one mouse (Fig. 3, mice 1 and 2, and Fig. 4B, for example). At the 10-fold-lower dose, the virulent JB580 strain consistently colonized the PP on day 5 but not the MLN (Fig. 3, mice 3 through 7). The differences between the mutant and parental strains are apparent at day 5, since fewer mutant yersiniae were recovered from the PP of mice at that time. Though the amount of JB580 recovered from MLN varied at day 5 p.i., none of the MLN of any mice inoculated with the lower dose were colonized by the mutant strain (Fig. 3, mice 3 through 7). At extremely high doses of approximately 10^9 CFU/mouse, viable yersiniae, both strains YEDS10 and JB580, were retrieved from the PP and MLN at days 3 and 5 p.i. (Fig. 4). However, the counts were lower for YEDS10 than for JB580. Also, it appeared that YEDS10 might be cleared from some MLN on day 5, since there were no recoverable counts in the MLN from some mice (note the limit of detection ranges from 200 to 500 CFU/g; Fig. 4, mice 4 and 5). Clearly, the mutant is not as adept at colonizing either the PP or the MLN as the parent strain. Whether the mutant is less capable of establishing infection in the tissue or of multiplying within the tissue has not been established. It is possible that the mutation in YEDS10 might exert a polar effect on nearby genes. To eliminate that possibility, a functional plasmid-encoded *yplA* gene could be introduced into YEDS10 in an attempt to complement the mutation. However, previous experience has found that plasmids are difficult to maintain during an extended infection in mice, and even the vectors alone reduce the virulence of *Y. enterocolitica* strains.

Histological examination of inflammatory response in mouse tissue. Upon dissection it was noted that the degree of gross inflammation of the intestine was clearly less in mice infected with YEDS10 than in those infected with the parental strain. PP from mice infected with JB580 were frequently enlarged and often abscessed, as had been reported previously (9), but the PP from mice infected with the mutant were often smaller and sometimes appeared to be as small as in a mock-infected mouse. To confirm this observation, additional experiments were conducted in which samples were prepared for histological examination. Samples were prepared for histology from the same mice given the lower inoculum from which viable bacterial counts were obtained (Fig. 3, mice 3 through 7). The hematoxylin-and-eosin-stained paraffin sections of PP, MLN, and ileal bowel tissue dissected at day 5 p.i. were scored by a trained pathologist as having no visible pathology, inflammation, or inflammation with necrosis (Fig. 5A). Note that the scoring system used to derive Fig. 5 did not consider the size of the lesion or the numbers of foci of inflammation or necrosis, merely the presence of any inflammation or necrosis in each tissue. The results from this experiment demonstrated that even though significant numbers of viable bacteria were recovered at day 5 p.i. from the PP of some mice infected with

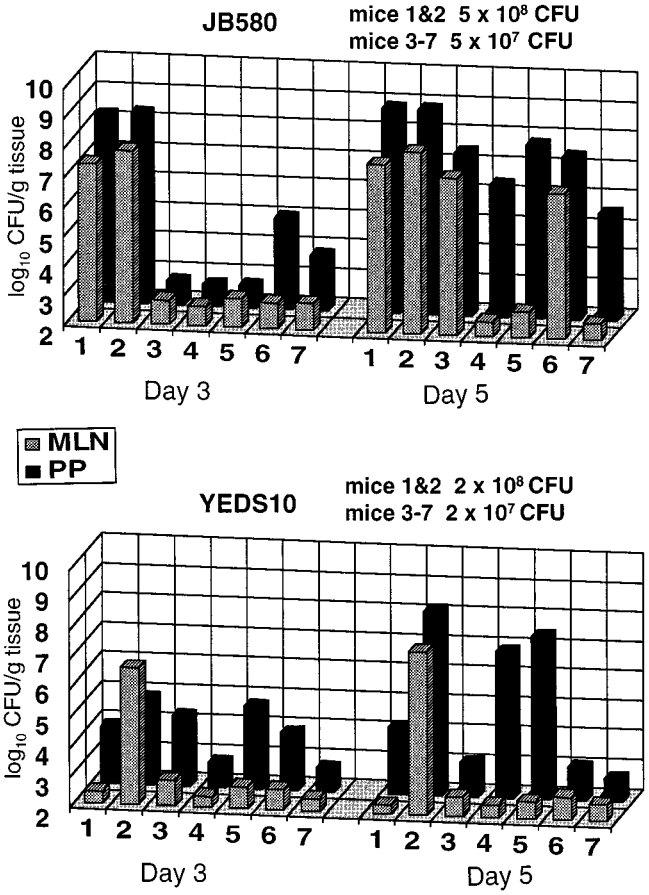


FIG. 3. Kinetics of infection of PP and MLN by the PL null mutant, YEDS10, compared to wild-type (JB580) *Y. enterocolitica*. The numbers of CFU recovered per gram of tissue are recorded on the vertical axis for PP (solid columns) and MLN (stippled columns). The tissues from the same mouse are arranged in each numbered row, and each day 7 mice infected per strain were sacrificed. The mice labeled 1 and 2 for each day p.i. (days 3 and 5 p.i.) were each given a 10-fold-higher inoculum than were mice 3 through 7. The limits of detection vary with the weight of tissue from 200 to 850 CFU/g; therefore, very short columns indicate that either no or only a few bacteria were recovered.

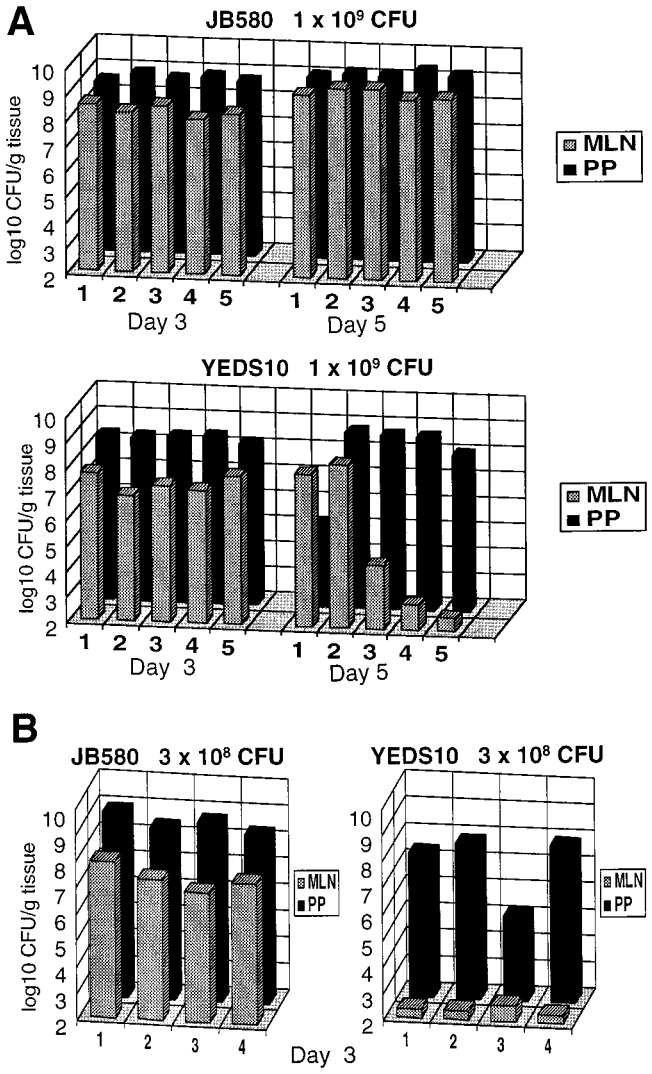


FIG. 4. Kinetics of infection with 10^9 (A) and 3×10^8 (B) CFU of YEDS10 and JB580. As in Fig. 3, the numbers of CFU recovered per gram of tissue are recorded on the vertical axis for PP (solid columns) and MLN (stippled columns). For each strain, four or five mice given the same inocula were sacrificed each day. The limits of detection vary with the weight of tissue from 200 to 850 CFU/g; therefore, very short columns indicate that either no or only a few bacteria were recovered.

YEDS10 (Fig. 3), none of the PP tissues demonstrated any detectable inflammation or necrosis (Fig. 5A).

Obviously, if the YEDS10 has not established an infection in the tissue it would not be surprising if there was little or no inflammation. A better comparison in the extent of the inflammatory response between the strains would be achieved if approximately equal levels of the mutant and wild-type strains were recovered from the tissues. Therefore, the peroral inoculum given to mice was increased to see if the colonization defect of YEDS10 could be overcome (Fig. 4). At doses of approximately 10^9 CFU/mouse, both strains consistently colonized the PP to quite high levels, i.e., $>10^7$ CFU/g of tissue (Fig. 4A). Upon dissection of the mice the PP were observed to be abscessed and very enlarged. Even at a high dose, colonization of the MLN by YEDS10 was not uniformly high, though some mice had very high levels of recoverable bacteria. In contrast, mice infected with JB580 were colonized to high levels ($>10^8$ CFU/g) in both PP and MLN at both days 3 and 5 p.i. Parallel tissue samples from these mice were prepared for histology, and the sections were scored as described above (Fig. 5B). Clearly, there is a significant difference in the amount of inflammation and necrosis in tissues infected with the *yplA* mutant strain; by day 5 all PP and MLN tissues from mice infected with the parental strain displayed both inflammation and necrosis, yet some of the tissues infected with the mutant displayed neither inflammation nor necrosis (Fig. 6D and J). Furthermore, upon examination it was apparent that areas of inflammation and inflammation with necrosis were larger in tissues infected with the parental strain than in tissues infected with YEDS10 (Fig. 6).

The *Y. enterocolitica* infections resulted in the same pathology that had been noted before (9, 37) (Fig. 6). The inflammatory foci have PMN as the predominant infiltrating cell, and these foci first appear in the lymphoid parenchyma. The nuclei of the large underlying mononuclear cells are farther apart, a finding characteristic of inflammation. Foci that have progressed to necrosis show the characteristics of inflammation in the periphery; PMN are present, and the nuclei more dispersed, but there are aggregates of undefined material, probably cell debris surrounded by PMN. Previous work had demonstrated that the centers of necrotic foci containing the cell debris stain positively for bacteria (37). Sections of infected MLN showed similar pathology, i.e., inflammation leading to necrosis and the influx of PMN. No granulomatous changes were noted in any tissue as had been reported by Une in studies with a rabbit model for *Y. enterocolitica* infection (54).

In addition, severe inflammatory reactions had spread to the small intestinal tissue of mice infected with the wild-type *Y. enterocolitica*. Upon dissection of the mice, small abscesses were observed throughout the walls of the ileum and cecum. Tissue sections from the small intestine demonstrated that areas of inflammation and necrosis could be found between the seemingly intact epithelium and the muscle layer within the lamina propria of mice infected with *Y. enterocolitica* (Fig. 5 and 6). Whether these originate in areas with unstructured aggregates of lymphocytes or in nonlymphocyte cells is not known. In conclusion, *Y. enterocolitica* YEDS10 with a disruption in *yplA* induced either a reduced or a delayed inflammatory process.

DISCUSSION

The human pathogens, *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*, are characterized by their predilection for infecting and multiplying within lymphoid tissues. These pathogenic bacteria survive in and multiply within tissues specifically designed to filter out and destroy microbes and/or initiate the immune response to foreign antigens. Data have been accumulating that *Yersinia* virulence factors modulate the immune response both at the cellular level and more broadly by affecting cytokine production. Several *Yersinia* virulence genes have been identified which promote survival during encounters with professional phagocytes by interfering with phagocytosis, reducing the oxidative burst, and destroying the phagocytes by inducing apoptosis (33, 41). In addition, the data suggest that pathogenic yersiniae can lessen the local immune response by reducing the secretion of chemoattractant and proinflammatory cytokines, tumor necrosis factor alpha (5), gamma interferon (34), and interleukin-8 (42). However, neutralizing antibodies to gamma interferon and tumor necrosis factor alpha did not reduce the influx of PMN and monocytes to PP and MLN (2). Furthermore, *Y. enterocolitica* infections

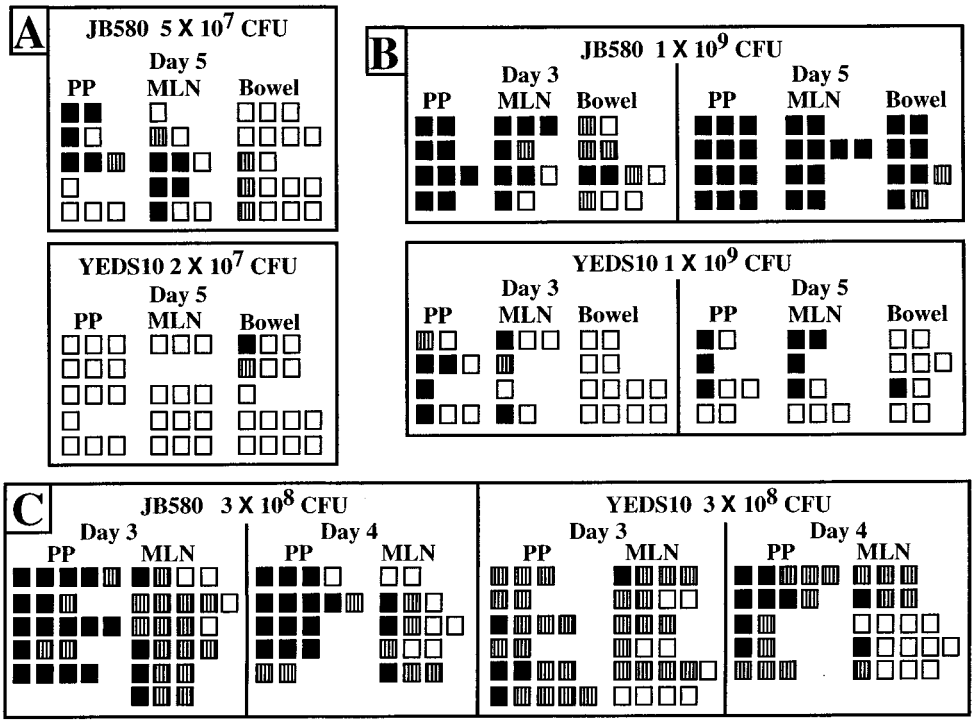


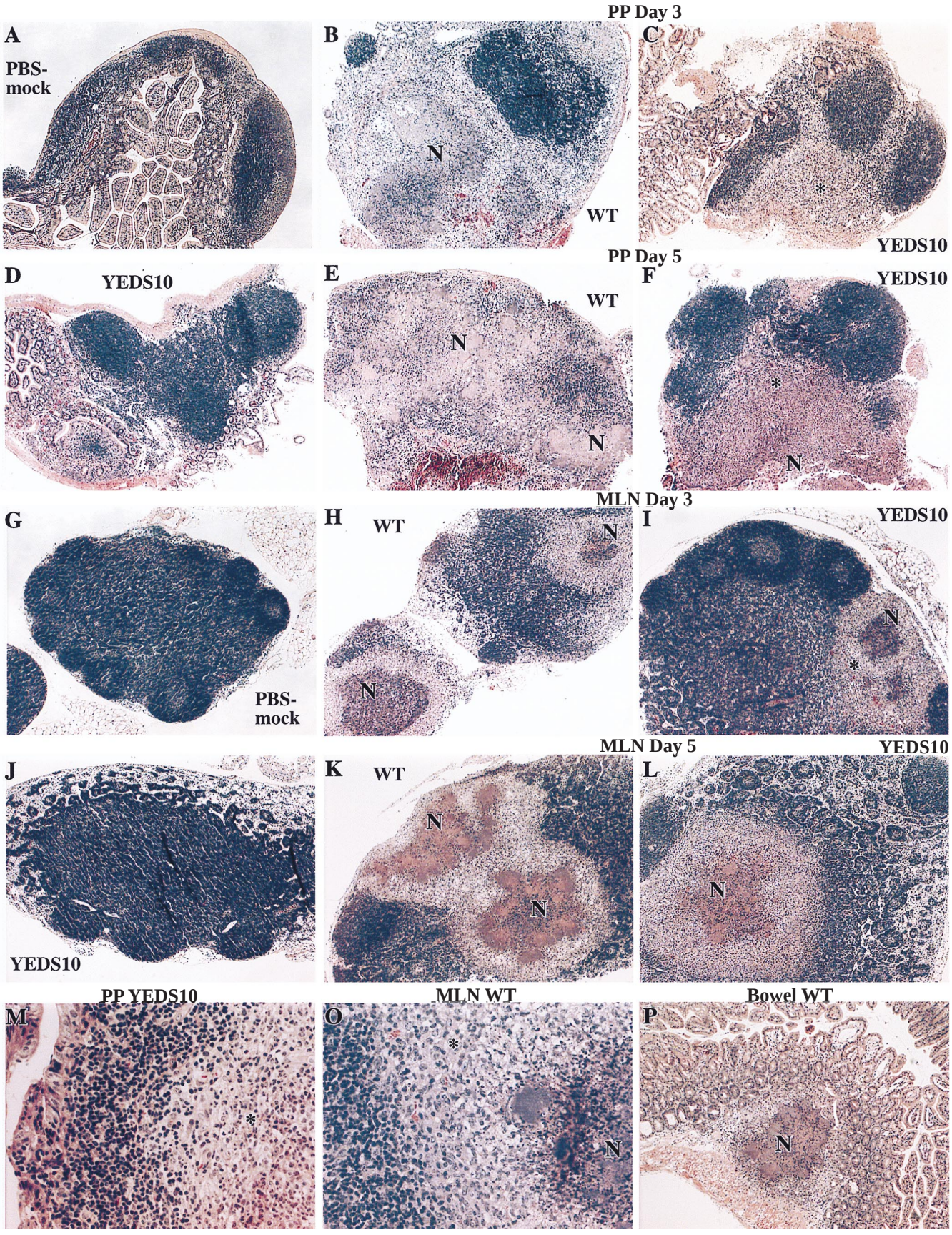
FIG. 5. Comparison of pathology of mouse tissues infected with YEDS10 and JB580. Samples of PP, MLN, and small intestine (bowel) were fixed, sectioned, stained, and scored positively for inflammation (striped box), inflammation with necrosis (solid box), or no pathology (open box). Each piece of tissue was scored as having the most serious pathology evident, regardless of the size or number of lesions. Boxes in a row represent tissues taken from a single mouse, and the number of boxes indicates each individual PP, MLN, or piece of bowel taken from that mouse. Equal numbers of tissue samples were taken where possible, but the very small tissue pieces were sometimes lost during processing. (A) Results from tissue samples taken at day 5 p.i. from mice 3 through 7 in the same experiment in which the bacterial counts are recorded for Fig. 3. (B) Histology results from four of the five mice for which bacterial counts were determined on both days 3 and 5 p.i. in Fig. 4. (C) Results from another experiment in which the numbers of CFU recovered were not determined from the PP and MLN tissue prepared for histology. On day 3 p.i., tissues from four additional mice of each group were used to determine the numbers of CFU per gram of tissue (Fig. 4B).

progress to form pyogenic lesions with necrosis in PP and MLN, indicating a sustained inflammatory response (10).

Bacterial PL can act as virulence factors directly by hydrolyzing host membrane phospholipids and indirectly by modulating the inflammatory response via the production of lipid second messengers. These indirect effects have only been demonstrated for some bacterial PLC (13, 18, 31). Bacterial PLA have not yet been shown to subvert signaling pathways, though endogenous host PLA directly initiate the arachidonic acid cascade, thus inducing the inflammatory response. Results from ligated ileal loop experiments with *S. newport* and its purified PLA have suggested a role for the PLA in pathogenesis (35). Indeed, the increased fluid accumulation and the pathologic changes seen when purified PLA was injected may indicate that the inflammatory response was induced.

In the present study we describe the isolation and identification of *yplA* encoding PLA activity, which has been implicated in lecithin-dependent hemolysis by some *Y. enterocolitica* strains. The predicted amino acid sequence of *Y. enterocolitica* YplA has striking similarity to that of *S. liquefaciens* PLA (22) and an uncharacterized "lipase" from *S. proteomaculans* (46). Notably, both of the *Serratia* primary sequences and YplA contain the consensus for a serine active site typically found in lipases. Both PLA and triacylglyceride lipases hydrolyze a fatty acid from the glycerol backbone; indeed, a few enzymes have the capacity to hydrolyze both polar phospholipids and triacylglyceride lipids (56). The products of *Serratia* PhlA hydrolysis of specific phospholipids have been identified as fatty acids by chromatography, thus confirming that the enzyme is a PLA (22). Given the similarity of YplA to the primary sequence of the *Serratia* PLA, the production of PL activity by *Y. enterocolitica* was confirmed by testing on lecithin plates and by spectrophotometric PLA assay. This is consistent with previous studies with *Y. enterocolitica* that demonstrated a partially pu-

FIG. 6. Pathology in mouse tissues stained with hematoxylin and eosin. The lighter pink staining areas of inflammation are indicated by "∗," and the areas of necrosis are indicated by "N." (A to C) PP removed day 3 p.i.: a PBS mock-infected control (A), depicting the normal architecture, with dark-blue-staining lymphoid aggregates with ileal villi in the center; tissue infected with JB580 (B), showing a large area of necrosis with acellular areas devoid of intact, blue-staining nuclei and some normal lymphoid cells with closely packed, blue-staining nuclei; and tissue infected with YEDS10 (C), showing an area of inflammation (which appears pink due to the staining of the cytoplasm) surrounded with three normal-looking lymphoid aggregates stained dark blue. (D to F) PP removed at day 5 p.i. infected with JB580 (E) and YEDS10 (D and F). The PP in panel E is enlarged and completely necrotic, whereas the PP in panel D has no visible pathology; there are normal areas adjacent to the large area of inflammation with some necrosis in panel F. (G to I) MLN removed at day 3 p.i. from a PBS mock-infected control (G), tissue infected with JB580 (H), and tissue infected with YEDS10 (I). Note that the lesions of inflammation and necrosis appear smaller in panel I. (J to L) MLN removed at day 5 p.i. infected with JB580 (K) and YEDS10 (J and L). The MLN in panel J has no visible pathology, unlike the distinct areas of necrosis surrounded by inflammation seen in panels K and L. (M and O) Higher magnification (×180) of inflammation (right side) in PP infected with YEDS10 (M) and of necrosis with inflammation in MLN infected with JB580 (O). (P) Abscess in the wall of the ileum infected with JB580 with necrosis in the center, with a seemingly intact mucosal epithelium and villus structure. Magnification (panels A through L, panel P), ×36.


PP Day 3
A
PBS-mock
B
N
WT
C
*
YEDS10
PP Day 5
D
YEDS10
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N
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YEDS10
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MLN Day 3
G
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rified lecithinase activity produced lysophospholipids and fatty acids (53) and showing that *Y. enterocolitica* extracts released radiolabeled fatty acid from specific phospholipid derivatives (7).

Among the pathogenic *Yersinia* spp., lecithinase activity has only been associated with some strains of *Y. enterocolitica*, including the virulent American strains. The distribution of *yplA* in different *Yersinia* spp. and a variety of *Y. enterocolitica* isolates was determined by Southern analysis; the strains were tested concurrently on Tween 80 and lecithin plates. As expected, the *Y. pestis* and *Y. pseudotuberculosis* strains tested did not show activity on plates nor did the *yplA* probe hybridize to the chromosomal DNA. Of the nonpathogenic species only *Y. aldovae* and *Y. intermedia* hydrolyzed Tween 80 and lecithin, and a faint hybridizing fragment was detected in the *Y. intermedia* chromosome. Interestingly, all *Y. enterocolitica* strains displayed hybridizing bands regardless of whether PL activity was detected under laboratory conditions or not. For those *Y. enterocolitica* strains for which enzymatic activity was not detected, it is not clear whether they produce too little YplA to be detected, whether the gene is not functional, or whether the gene is not expressed under the conditions examined. All the biotype 1 strains had detectable PL activity, including the clinical isolates.

A *Y. enterocolitica* strain was created with a disruption in *yplA* which eliminated PLA activity. This strain was tested for kinetics of colonization of the PP and MLN in perorally infected mice. When compared to the parental strain the *Y. enterocolitica* mutant, YEDS10, failed to either establish an infection of the PP and MLN or to reach similar levels of colonization unless infected with extremely high doses. YEDS10 might be delayed in reaching the tissues such that it fails to colonize as early as did the wild-type strain, and thus bacterial multiplication in microcolonies lags behind at day 3 or 5 p.i. Alternatively, the mutant may require a greater inoculum to overcome a threshold and establish infection of the tissues; the immune system may destroy the first viable yersiniae that reach the tissue, but when more yersiniae invade the defenses are overwhelmed. The fact that increasing the inoculum to 10^9 CFU could overcome the defect of the mutant so that similar levels of viable yersiniae were recovered as with the parental strain supports the idea of an inoculum threshold. How might a PL function to promote the colonization of *Y. enterocolitica*? First, the PL could promote bacterial penetration of tissue by disrupting host cell membranes and allowing bacteria to pass between host cells. Second, the PL might promote bacterial survival by disrupting PMN and macrophage membranes and so hamper the cellular immune defenses. Some support for the hypothesis that the *Y. enterocolitica* PL might perturb the eukaryotic membranes was provided by the work of Tsubokura et al. (53), who demonstrated lecithin-dependent hemolysis by a partially purified *Y. enterocolitica* lecithinase. Thirdly, the PL might promote bacterial growth by providing nutrients in the form of fatty acids for biosynthesis or metabolism.

An observation that mice infected with the mutant had less gross inflammation of the intestinal tissues led to an examination of the inflammatory changes in the tissues. Even when the colonization defect of the mutant was overcome by infection with a greater inoculum, an examination of the tissues revealed a less extensive inflammatory response in mice infected with YEDS10. Perhaps this result could be partly explained if YEDS10 infection lags behind the parental strain, JB580, in the PP and MLN, since the foci of inflammation with or without necrosis were fewer and smaller in tissues infected with the mutant. However, in mice from which $\geq 10^8$ CFU of YEDS10 per g were recovered, some PP and MLN histology samples did not even demonstrate inflammation; yet in tissues where similar numbers of JB580 were recovered most or all of the samples showed inflammation and necrosis. It does not seem likely that such numbers of viable yersiniae could be achieved in tissues without triggering at least some inflammation.

The finding that *Y. enterocolitica* YEDS10, which lacks a PL, induces less inflammation in mouse tissues leads to a rather tantalizing speculation. Endogenous mammalian PLA_2 are induced by cytokines and are an integral part of the inflammatory cascade, leading to the release of arachidonic acid and then to the production of proinflammatory and chemoattractant eicosanoids (55). Could a *Y. enterocolitica* PLA lead to an overstimulation of the inflammatory cascade? Future investigation will be necessary to determine if YplA hydrolyzes arachidonic acid from specific phospholipids and if injection of the protein itself can induce an inflammatory response. It might seem unlikely given that the data suggest that virulent *Y. enterocolitica* reduces the secretion of proinflammatory cytokines, thus dampening the immune response. Perhaps various *Yersinia* factors modulate the immune response in different ways at different times, presumably to enhance their survival.

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