INFECTION AND IMMUNITY, Mar. 1997, p. 924–930 Vol. 65, No. 3
0019-9567/97/$04.00+0

Effect of *Yersinia pestis* YopM on Experimental Plague

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Received 23 August 1996/Returned for modification 16 October 1996/Accepted 26 November 1996

YopM of *Yersinia pestis* has previously been shown to be necessary for full virulence in mice and to be able to bind human α-thrombin. This activity prompted the hypothesis that YopM, functioning extracellularly during plague, might be accessible to neutralization by antibody and hence might be a protective antigen. This study tested this hypothesis and found that YopM was not protective, either by passive or active immunization, in inbred or outbred mice. These findings showed that either YopM-specific antibody does not have access to YopM during experimental plague or the function of extracellular YopM is not neutralizable by antibody. Exogenously supplied YopM partially restored virulence to a YopM$^-$ strain of *Y. pestis* while having no effect on lethality of *Listeria monocytogenes*. These findings indicate that YopM does not significantly alter host defenses important for resistance against heterologous infection (*Listeria monocytogenes*) but raise the possibility that YopM has a minor extracellular function specific to homologous infection (*Y. pestis*).

The causative agent of bubonic plague, *Yersinia pestis*, has a 75-kbp low-calcium response (LCR) plasmid that encodes a set of about 10 secreted proteins called Yops (*Yersinia* outer proteins) and the secreted V antigen (LcrV) (37). In vitro, these proteins are maximally expressed and secreted into the culture medium at 37°C in the absence of Ca^{2+} (37, 38). In tissue culture infection models, several of the Yops (YopE, YopH, and YpkA) have been shown to be vectorially translocated from yersiniae adherent to epithelial or macrophage-like cell lines into the eucaryotic cell, where they exert cytotoxic or enzymatic activities that derange the mobilization of effective defenses against the infection (8, 22, 27, 33, 37). It is generally believed that these and perhaps also other Yops have exclusive intracellular roles in vivo, functioning to neutralize innate defenses when yersiniae contact phagocytic cells. This direct targeting of Yops into the eucaryotic cell would have the consequence that these virulence proteins would bypass exposure to the potent plasminogen activator protease (Pla) located on the surface of *Y. pestis* and avoid neutralization by antibody (37).

However, at least one of the secreted LCR virulence proteins, V antigen, is accessible to antibody because it is a protective antigen by passive immunization (19, 37). Antibody is believed to act by neutralizing a strong downmodulatory effect that LcrV has on secretion of gamma interferon and tumor necrosis factor alpha early in infection (20). This immunomodulatory effect is exhibited directly by LcrV without the necessity of other *Yersinia* proteins as cofactors (21). Accordingly, LcrV, which is secreted in culture medium in vitro, probably is released into the extracellular fluid in vivo following its secretion to the bacterial surface, but this has not yet been formally proven. In keeping with an extracellular targeting, LcrV is much less susceptible to degradation by Pla than are the vectorially translocated Yops (28). Other than LcrV, only two other major LCR proteins are stably secreted in vitro by *Y. pestis* into culture medium: LcrE (also called YopN), which acts at the bacterial surface to regulate secretion of LcrV and Yops in response to Ca^{2+} and presumably also in response to contact with eucaryotic cells (35, 38), and YopM (35).

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Our lab initiated the characterization of YopM's role in *Yersinia* pathogenesis, because its relative resistance to Pla and very strongly acidic character suggested that this Yop might function differently from others (12). The ca. 42-kDa YopM is necessary for full virulence of *Y. pestis* in a mouse model of plague (13). The finding that the YopM sequence had significant homology to a platelet surface protein that binds α-thrombin prompted tests that demonstrated YopM's ability to bind α-thrombin (but not prothrombin) in vitro (12, 13, 26). In a functional test, YopM present at a 1:1 molar ratio with α-thrombin was able to block the thrombin-elicited platelet activation, but it did not affect activation of platelets by other agonists (13, 26). These findings suggested that YopM might function extracellularly by sequestering α-thrombin as it is generated in a focus of infection, thereby preventing a maximal local inflammatory response (12, 26). This model raised the possibility that YopM's function, like that of V antigen, might be neutralizable by antibody and that YopM might be a second LCR-related protective antigen.

Presently available plague vaccines are attenuated live *Y. pestis* or a suspension of formalin-killed bacteria and are highly reactogenic (14, 16, 17). If anti-YopM antibodies afford a significant degree of protection against *Y. pestis* infection, YopM would be useful in a subunit plague vaccine with other yersinial proteins such as LcrV and the capsular protein F1, which also is protective against experimental plague (2, 15, 29). Therefore, the purpose of the present study was to evaluate the immunogenicity and protective capacity of YopM in a mouse model of plague. The findings of this study also have implications for our picture of the function of YopM in pathogenesis.

MATERIALS AND METHODS

Bacteria. Mouse challenge studies used conditionally virulent *Y. pestis* KIM5 (from R. Brubaker, Michigan State University), which produces YopM, the YopM$^-$ mutant *Y. pestis* KIM5-3233 carrying an insertion mutation that completely abolishes expression of YopM (*yopM*::*lacZYA*) (13), and *Listeria monocytogenes* EGD (from Donald A. Cohen, University of Kentucky). All *Y. pestis* strains used in this study were Pgm$^-$ and hence avirulent from a peripheral route of challenge but fully virulent by the intravenous (i.v.) route (39). They contained the three native *Y. pestis* plasmids: the LCR plasmid pCD1 (5, 7), the Pla-encoding pPCP1 (32), and the capsular protein-encoding pMT1 (24). Other *Yersinia* strains used during characterization of YopM preparations were *Y. pestis* KIM6 (pCD1$^-$; from R. Brubaker), *Y. pestis* KIM5-3169.5 (pCD1 *yopH*::Mu *d*I1734) (25), and *Y. pseudotuberculosis* 43 (serotype III, lacks its LCR plasmid; from R. Brubaker). *Y. pseudotuberculosis* 43 containing the *Y. pestis* LCR plasmid carrying a mutation in YopH (pCD1 *yopH*::Mu *d*I1734) and plasmid pBS10,

which overexpresses YopM, was used to produce YopM in large amounts (26). *Escherichia coli* DH5α (Gibco BRL, Grand Island, N.Y.) containing pHTV carrying the *Y. pestis lcrV* sequence cloned into the pPROEX-1 expression vector (Gibco BRL) was used to express histidine-tagged LcrV (HTV), which served as a positive control antigen in this study. pHTV produces full-length LcrV having a 19-residue leader containing six His molecules fused to its N terminus. The construction and characterization of pHTV will be detailed elsewhere (6).

Animals. Female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Ind., or Jackson Laboratories, Bar Harbor, Maine), 6 to 13 weeks of age, or female Swiss Webster mice (Harlan Sprague-Dawley), 5 to 8 weeks of age, were used in immunization and challenge studies. Twelve-week-old female New Zealand White rabbits (Myrtle's Rabbitry, Topson Station, Tenn.) were used to produce antibodies to purified YopM.

Purification of YopM. *Y. pseudotuberculosis* 43 (pCD1 *yopH*::Mu *d*I1734 pBS10) was induced to maximally express YopM by initial growth at 26°C in the defined medium TMH, not supplemented with Ca^{2+}, followed by a shift to 37°C for the remainder of the growth period (36). Ten-liter cultures were grown in a Magnaferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) with an 11-liter working volume. We increased the amount of potassium gluconate in the TMH medium 10-fold to 100 mM to have a sufficient amount of carbon source for a high-density culture to prevent a shift to metabolism of amino acids in the medium with a concomitant rise in culture pH: proteins in culture supernatant precipitated at a pH of >9. Six to 18.5 h after the temperature shift, culture supernatant proteins were separated from cells by passage over a 5-m^2, tangential-flow, 0.45-μm-pore-size microporous membrane cassette in a Pellicon concentrator (Millipore Corp., Bedford, Mass.) and concentrated to ca. 500 ml by ultrafiltration via a Millipore Minitan tangential-flow concentrator fit with a 30,000-Da-cutoff ultrafilter. Supernatant proteins retained by the filter were then precipitated overnight at 4°C with ammonium sulfate at a final saturation of 70%, dialyzed against imidazole buffer (50 mM imidazole, 1 mM Na_2EDTA, 0.1% [vol/vol] β-mercaptoethanol [pH 7.5]), and then applied to a DEAE-Sephacel (Pharmacia LKB, Piscataway, N.J.) column equilibrated in the same buffer (26). The column was washed with 5 volumes of imidazole buffer followed by elution of bound proteins (including YopM) with a linear (0 to 0.5 M) gradient of NaCl in the same buffer or by first eluting lightly bound proteins with 0.2 M NaCl, followed by elution of YopM with a gradient of 0.2 to 0.5 M NaCl. Column fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 12.5% (wt/vol) acrylamide gels (10) followed by silver staining (PhastSystem; Pharmacia) and by testing on immunoblots prepared as previously described (23). Electrophoresis molecular weight markers were obtained from Pharmacia (nonstained high-molecular-weight series) and from Bio-Rad Laboratories, Inc., Hercules, Calif. (prestained high-molecular-weight series). YopM elutes as a broad, asymmetric peak, with the highest concentration of YopM being eluted at 0.35 M NaCl. YopM-containing peak and higher-salt-eluted, trailing fractions were pooled separately, dialyzed against 0.05 M ammonium bicarbonate buffer (pH 7.4), lyophilized, and stored desiccated at −20°C. The concentrations of YopM and of protein in all other protein preparations in this study were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Cross-linking with disuccinimidyl suberate (Pierce) to characterize YopM was done by Skrzypek and Straley (30, 31) as previously described (26).

In the experiments shown in Fig. 1, where YopM purity was assessed, several *Yersinia* strains were grown as described above but in small cultures (e.g., 30 ml). These were processed to obtain extracellular and soluble cellular (cytoplasmic plus periplasmic) proteins as previously described (26) except that extracellular proteins were precipitated with trichloroacetic acid (5%, vol/vol) instead of ammonium sulfate, and the pelleted proteins were neutralized with 1 M NaOH before being suspended in electrophoresis sample buffer.

Removal of endotoxin. Endotoxin was removed from YopM preparations by using End-X B52 beads (Associates of Cape Cod, Inc., Woods Hole, Mass.) equilibrated with pyrogen-free phosphate-buffered saline (PF-PBS; pH 7.4) containing 0.1% (wt/vol) sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.). PF-PBS was prepared from the addition of one package of BupH PBS (Pierce) to 500 ml of nonpyrogenic water (Baxter Healthcare Corp., Deerfield, Ill.). Sodium deoxycholate was subsequently removed from the YopM preparation by dialysis against PF-PBS. Endotoxin levels in PF-PBS and in YopM preparations before and after End-X B52 treatment were determined by chromogenic *Limulus* amoebocyte lysate assay (LAL; BioWhittaker Inc., Walkersville, Md.), and YopM was given to mice only if the endotoxin concentration was ≤1 ng per 40 μg of YopM protein.

Purification of HTV. *E. coli* DH5α(pHTV) was grown in 200 ml of LB broth (18) containing ampicillin (100 μg/ml) at 37°C to an A_{620} of 0.8. Production of HTV was induced with 0.5 mM isopropylthio-β-D-galactoside (IPTG; Gibco BRL) followed by a further 3 h of incubation at 37°C. The cells were pelleted, resuspended in nickel-nitrilotriacetic acid column buffer A (Gibco BRL protocol), and lysed by explosive decompression in a French press, after which insoluble fragments and unlysed cells were removed by ultracentrifugation. HTV was purified from the supernatant by using a 1-ml nickel-nitrilotriacetic acid column (Qiagen Inc., Chatsworth, Calif.) according to the suggested protocol (Gibco BRL). Its purity was estimated by SDS-PAGE on a 12.5% (wt/vol) gel followed by silver staining. Endotoxin concentrations were confirmed by LAL assay to be ≤1 ng per 40 μg of protein.

Antibody reagents produced in rabbits. Two New Zealand White rabbits were immunized subcutaneously (s.c.) as previously described (23) with 100 μg of purified YopM in PBS (pH 7.4) emulsified 1:1 with Freund's adjuvant (FA; Difco Laboratories, Detroit, Mich.) and boosted bimonthly s.c. with 100 μg of YopM in PBS-FA. In this and all other uses of FA, the first dose contained the complete adjuvant, while subsequent doses contained incomplete adjuvant (Difco). YopM serum antibody titers were monitored by enzyme-linked immunosorbent assay (ELISA), and the specificity of the antiserum was determined by Western blotting of *Y. pestis* cell extracts (data not shown). Anti-HTV (α-HTV) antibodies were produced similarly (6). Three antisera raised previously were used to determine the purity of the YopM preparations: α-MC-term., against a peptide derived from the 12 C-terminal residues (356 to 367) of YopM (26); α-M-int., against a sequence present twice near the center of YopM (12, 26); and α-ECP, against total proteins in the culture supernatant of *Y. pseudotuberculosis* 43(pCD1 *yopKL*::Mu *d*1 [Ap^r *lac*]). α-ECP antibody was partially purified by ammonium sulfate precipitation followed by dialysis against PBS. Immunoglobulin G (IgG) from other rabbit antisera was affinity purified by chromatography on protein A-Sepharose CL-4B (Sigma) or on an Affi-Prep (Bio-Rad)–protein A column as recommended by the manufacturer.

Mouse α-YopM and anti-bovine serum albumin (α-BSA) serum and ascites. Female BALB/c mice, 12 to 13 weeks of age, were bled (day 0) and intraperitoneally (i.p.) given 40 μg of YopM in PF-PBS–FA or PF-PBS–FA alone on days 2, 16, and 37. Seven to 14 days later, they were injected i.p. with 300 μl of Pristane (2,6,10,14-tetramethylpentadecane; Sigma). Approximately 1 week later, mice were injected i.p. with 1.2×10^6 XS63 cells, a nonsecreting mouse myeloma cell line (American Type Culture Collection, Rockville, Md.), in Hanks' balanced salt solution (Gibco BRL). Ascites developed within 3 weeks after injection of the myeloma cells and was collected as it formed by using a 20-gauge needle and a 10-ml syringe. In the process of raising mouse anti-YopM (α-YopM) antibody, we used these mice to compare the efficacy of two adjuvants, FA and Adjuvax (AV; Alpha Beta Technology, Worcester, Mass.). AV was added as a powder to YopM in PBS and mixed by multiple draws through an 18-gauge needle. Five mice received YopM-AV, and five received PBS-AV. After the YopM-immunized (AV-treated) group of mice reached peak α-YopM titers, all of the AV-treated mice were primed with Pristane and subsequently handled as for FA-treated mice. Ascitic fluid was centrifuged to remove cells and debris.

Additional mice were immunized twice, 2 weeks apart, with 40 μg of BSA (fraction V; Sigma) or 40 μg of YopM in PBS-FA or with PBS-FA alone. These mice were bled biweekly from the tail vein (ca. 0.5 ml of blood per sample), and the YopM antiserum was later combined with the ascites, as it proved to have a 10-fold-higher concentration of α-YopM antibody than did ascites.

IgG from the ascites and sera was semipurified by affinity chromatography on Affi-Prep (Bio-Rad)–protein A columns as suggested by the manufacturer.

ELISA for α-YopM, α-HTV, and α-BSA antibody levels in sera. Flat-bottom, 96-well Nunc Maxisorp immunoplates (Fisher Scientific, Pittsburgh, Pa.) were coated with 100 μl of the desired protein solution (4 μg/ml in PBS) at room temperature (RT) for 2 h (or overnight at 4°C). The wells were blocked with skim milk (200 μl of a 2% [wt/vol] solution in PBS per well, 2 h at RT or 4°C overnight) and washed thrice, 3 min each time, with PBS containing 0.05% (vol/vol) Tween 20 (Fisher) and 0.02% (wt/vol) sodium azide (Sigma) (PBS-TA). Test sera or ascites (primary antibody source) was serially diluted in PBS-TA, and 100 μl of each dilution was added to duplicate wells for 2 h at RT. One hundred microliters of PBS-TA alone was added to control wells. The plates were then washed and coated for 2 h at RT with alkaline phosphatase-labeled secondary antibody: goat anti-mouse IgG γ chain diluted 1:20,000, goat anti-rabbit IgG diluted 1:1,500, or goat anti-human IgG γ chain diluted 1:20,000 (Sigma). The wells were washed, 100 μl of Sigma Fast substrate (*p*-nitrophenyl phosphate) was added to each well, the plates were incubated at RT for 15 min, and the reaction was stopped by the addition of 25 μl of 3 N NaOH to each well. A_{405} was measured with a ν Max kinetic microplate reader (Molecular Devices Corp., Menlo Park, Calif.). Average absorbance values were determined for each dilution of each serum sample, and the antibody titer of each sample was calculated to be the highest dilution at which the absorbance value was greater than two times the standard deviation from the average absorbance of control wells.

Quantitation of YopM-specific Ig isotypes and subclasses following active immunization with YopM. Nunc Maxisorp immunoplates (Fisher) were coated with YopM, blocked with 2% (wt/vol) skim milk, and washed as described above. Tenfold dilutions of YopM-specific mouse sera were made in PBS-TA, and 100-μl aliquots were added to duplicate wells for 2 h at RT. The wells were washed, and alkaline phosphatase-labeled anti-mouse isotype-specific antibody (diluted 1:500 in PBS-TA; Fisher) was added to each well for 2 h at RT. The wells were washed, and the rest of the assay was done as described above. Ig isotype and subclass concentrations (in micrograms per milliliter) were calculated from standard curves of Ig concentration versus A_{405} values (determined by using Ig standards [Fisher]).

Passive immunization of inbred (BALB/c) and outbred (Swiss Webster) mice followed by challenge with *Y. pestis* KIM5. Female BALB/c mice (6 to 8 weeks old) were passively immunized i.p. with a single dose of 500 μg of mouse α-YopM IgG or negative control (α-BSA) IgG in 500 μl of PF-PBS. Control mice received 500 μl of PF-PBS i.p. Twenty-four hours after passive immunization, the mice were bled, and pooled serum samples were tested by ELISA to

determine that α-YopM or α-BSA antibodies had entered the serum. Forty-eight hours after immunization, groups of 10 mice were challenged i.v. via the retro-orbital sinus with decimally increasing doses of *Y. pestis* KIM5 (10^1 to 10^5 CFU) in 100 μl of PF-PBS. In these and all other challenge experiments, the actual CFU values given were confirmed by plating. The mice were observed for 17 days after challenge, and the average doses required to kill 50% of the mice (LD_{50}) for the treatment groups were calculated (24).

Female Swiss Webster mice were immunized as described above with α-YopM or positive control (α-HTV) IgG in PF-PBS, while control mice were given PF-PBS. Twenty-four hours later, they were assessed for α-YopM and α-HTV antibody as described above. Forty-eight hours after immunization, groups of five mice were challenged i.v. with 10^1 to 10^4 CFU of *Y. pestis* KIM5 and observed for 14 days, and LD_{50}s were determined.

Active immunization of inbred and outbred mice followed by challenge with *Y. pestis* KIM5. In a preliminary test to choose an appropriate immunization route, 8- to 12-week-old female BALB/c mice were given three biweekly 40-μg doses of YopM in PF-PBS emulsified 1:1 with FA. Two routes were compared: i.p. (dose in 200 μl) and s.c. (100 μl at each of two sites on the back). There were 20 mice for each route; 10 received YopM-FA, and 10 received PBS-FA.

For challenge with *Y. pestis*, 8-week-old female BALB/c mice were immunized i.p. biweekly for 6 weeks with 0.2 ml containing 40 μg of YopM in PF-PBS emulsified 1:1 with FA or PF-PBS–FA alone (control mice). Unless otherwise specified, titers of relevant antibodies (in this case, α-YopM) were assessed in active immunization experiments by ELISA 1 day before each immunization and again 1 day before challenge. Eighteen days after the third and final immunization, groups of five mice were challenged i.v. with decimally increasing doses (10^1 to 10^7 CFU) of *Y. pestis* KIM5 in 100 μl of PF-PBS. The mice were observed for 24 days postchallenge, and LD_{50}s were determined.

In a second experiment, 6- to 8-week-old female BALB/c mice were immunized twice, 2 weeks apart, with a reduced amount of YopM (20 μg) in PF-PBS–FA. A second, positive control group of mice was immunized with 20 μg of HTV by the same regimen. Negative control mice received PF-PBS–FA or PF-PBS alone. Groups of 10 mice in each of the treatment categories were challenged i.v. with 10^1 to 10^7 CFU of *Y. pestis* KIM5 1 month after the second immunization. The mice were observed for 24 days postchallenge, and LD_{50}s were determined.

A third experiment involved the i.p. immunization of 6- to 8-week-old female Swiss Webster mice twice, 2 weeks apart, with 20 μg of YopM-FA or HTV-FA, while negative control mice were injected twice with PF-PBS–FA. Serum α-YopM and α-HTV titers were quantitated 2 weeks after the second immunization. One month after the second immunization, groups of five mice were challenged i.v. with 10^1 to 10^5 CFU of *Y. pestis* KIM5. The mice were observed for 17 days postchallenge, and LD_{50}s were determined.

Effect of exogenous YopM on *Y. pestis* and *L. monocytogenes* challenge in outbred mice. On day 1, groups of 10 5- to 6-week-old female Swiss Webster mice were challenged i.v. via the retro-orbital sinus with 10^1 to 10^4 CFU of $YopM^-$ *Y. pestis* KIM5-3233 or 10^1 to 10^4 CFU of *L. monocytogenes* EGD in 100 μl of PF-PBS. Approximately 2 h later, five mice for each challenge dose were administered PF-PBS, and the other five mice received 100 μg YopM in 100 μl of PF-PBS, given in the other eye. On day 2, the YopM-supplemented mice received YopM i.v., while on days 3, 4, 5, and 6 postinfection, they received i.p. injections of 100 μg of YopM in 100 μl of PF-PBS. This test was made twice with similar results, once using YopM not cleaned of endotoxin; the data shown in Table 3 are for endotoxin-free YopM.

To assess the effect of antibody on the action of exogenous YopM, 40 mice were injected i.p. with 500 μg of rabbit α-YopM antibody or irrelevant rabbit antibody (from preimmune serum) on days 0 and 2. On day 1, groups of 10 mice were challenged retro-orbitally with 10^1 to 10^4 CFU of $YopM^-$ *Y. pestis* KIM5-3233 and then were given YopM or PBS in the other eye as described above. On day 2, both YopM and antibody were administered i.p. To minimize interaction of YopM and antibody in the peritoneum, these proteins were administered 6 h apart. Additional groups of five control mice were given either PF-PBS or YopM but were not challenged. The LD_{50} and mean time to death for each treatment group were determined.

Convalescent plague sera. Three samples of convalescent plague serum were kindly provided by T. Quan (Centers for Disease Control and Prevention, Fort Collins, Colo.). Their sample designations and passive hemagglutination titers (3, 40) against *Y. pestis* capsular antigen (fraction 1 antigen) were as follows: TX84-220, 1:32; 80NM 912, 1:512; and 77NM 697C, 1:32. These sera were tested for YopM and LcrV serum antibody titers by the indirect ELISA method.

RESULTS

Purity of YopM and HTV. A *Y. pseudotuberculosis* strain which overexpressed YopM had been constructed earlier for YopM purification, as *Y. pseudotuberculosis* is less likely than *Y. pestis* to lyse during growth, and *Y. pseudotuberculosis* lacks the Pla protease which could contribute to YopM degradation (26) even though YopM is relatively resistant to Pla's proteolytic activity. In this study, we scaled up YopM production. After anion-exchange chromatography with a linear salt gradient, the peak YopM-containing fractions had a significantly more complex protein pattern than previously seen (27), with many YopM-cross-reactive bands smaller than the ca. 42-kDa main band of YopM plus a few larger species. The discrepancy came from the use of silver staining instead of Coomassie blue staining in SDS-PAGE and a high-titer rabbit α-YopM antibody preparation raised against the whole YopM protein as the primary antibody instead of the antipeptide antibody directed against the C-terminal 12 residues of YopM (α-MC-term.) in immunoblots. Our peak YopM-containing fractions clearly appeared very heterogeneous. In total, the extra bands constituted less than ca. 10% of the protein and were not visible in a Coomassie blue-stained gel unless many micrograms of YopM were loaded in the lane. Fractions that eluted with higher salt concentrations had a progressively simpler electrophoretic profile, ultimately giving a single band. However, there was not enough of these trailing fractions for the present study, and we had to determine the source of the extra bands in peak fractions before using this YopM to immunize mice.

Peak fractions from DEAE chromatography were subjected to additional fractionation procedures: centrifugation over a 30,000-Da-cutoff filter, gel filtration by high-pressure liquid chromatography or conventional chromatography (Superose-12 or Sepharose CL-6B, respectively), and hydrophobic interaction chromatography (butyl-Sepharose), without removing the extra bands seen after SDS-PAGE (30, 31). The extra bands were determined in a series of tests to be derived from YopM. Figure 1 illustrates two of these tests. We prepared immunoblots in which YopM was detected by using the α-MC-term. antibody preparation used earlier (26) and an antipeptide antibody raised against an internal epitope on YopM, α-M-int. (Fig. 1A and B). Both of these antibody preparations were highly specific for YopM and show no significant reactions with proteins from the soluble cellular or extracellular proteins of several *Yersinia* strains lacking YopM, including the *Y. pseudotuberculosis* host used in our work for overexpression of YopM (Fig. 1A and B, lanes 1 to 3). The only protein recognized in either the soluble or extracellular protein fractions of any of the $YopM^+$ bacteria was YopM (lanes 4 to 6). The multiple YopM bands were visualized with the internally reactive antibody α-M-int. (Fig. 1B), whereas mainly full-length YopM and some minor higher-molecular-weight forms were seen with the C-terminally reactive antibody α-MC-term. (Fig. 1A). This result indicates that the smaller bands represent YopM from which various amounts have been removed from the C terminus. The minor higher-molecular-weight species could represent various aggregates of both processed and unprocessed YopM. Putative YopM dimerization was noted earlier (26), and dimers and trimers were variably obtained in our chromatography and native gel electrophoresis experiments characterizing YopM fractions (34). Such aggregates, containing at least one intact C terminus, would be recognizable by the C-terminally reactive antibody. In other tests, when peak-fraction YopM was subjected to chemical cross-linking, the smaller bands disappeared: only monomer (42 kDa) and traces of higher-molecular-weight forms were seen in gels prepared by SDS-PAGE followed by Western analysis using the internally reactive antibody or strong rabbit antibodies raised against the whole YopM protein (30). This result indicates that in the native material of peak fractions, the species that migrate as the smaller bands are closely associated with their C termini so that these can be cross-linked to give a normal-size YopM upon denaturation with SDS and β-mercaptoethanol. Accordingly, native chromatographic procedures were unsuccessful in eliminating the smaller bands from peak YopM fractions

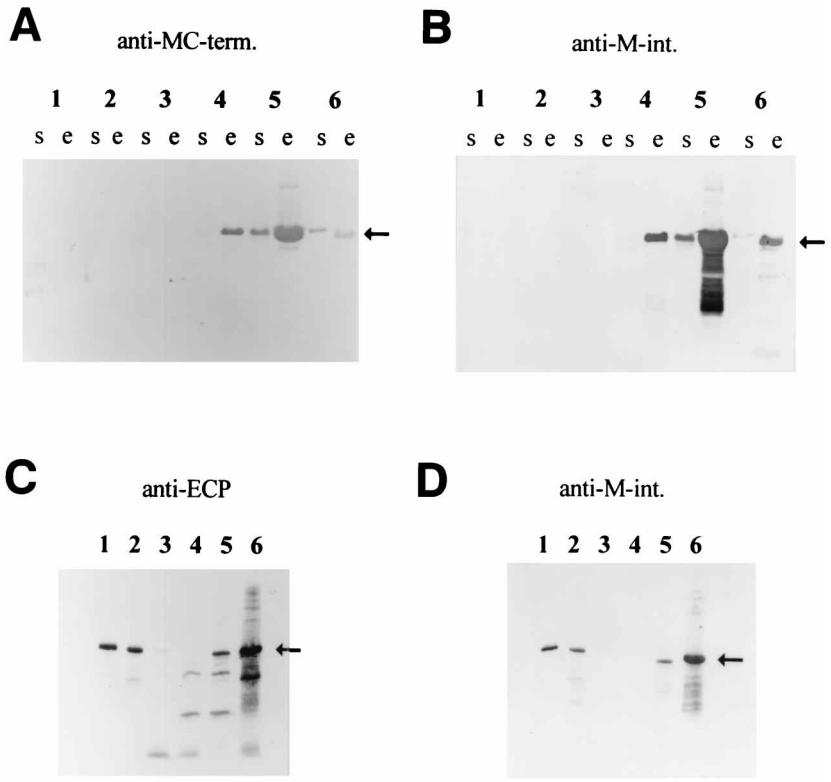

FIG. 1. Immunoblot analysis of *Yersinia* fractions containing or lacking YopM. In panel A, the primary antibody was the C-terminally reactive α-MC-term.; in panel B, the primary antibody was the internally reactive α-M-int. s, soluble cellular fraction (cytoplasm plus periplasm); e, extracellular protein fraction (cell-free supernatant). Lanes: 1, LCR^- (and hence $YopM^-$) *Y. pseudotuberculosis* 43; 2, *Y. pestis* KIM6 ($pCD1^-$ and hence $YopM^-$); 3, $YopM^-$ *Y. pestis* KIM5-3233; 4, *Y. pestis* KIM5-3169.5 (pCD1 *yopH*::Mu *d*I1734; $YopH^-$ $YopM^+$); 5, *Y. pseudotuberculosis* 43 (pCD1 *yopH*::Mu *d*I1734 pBS10; $YopH^-$ $YopM^+$); 6, *Y. pestis* KIM5 ($YopM^+$). In panels C and D, the primary antibody was α-ECP directed against a mixture of *Yersinia* secreted proteins (C) or α-M-int. (D). Lanes: 1, trailing YopM fraction; 2, peak YopM fraction. Other lanes contained crude preparations of secreted proteins: 3, *Y. pseudotuberculosis* 43 ($YopM^-$); 4, *Y. pestis* KIM5-3233 ($YopM^-$); 5, *Y. pestis* KIM5 ($YopM^+$); 6, *Y. pseudotuberculosis* 43 (pCD1 *yopH*::Mu *d*I1734 pBS10; $YopH^-$ $YopM^+$).

(data not shown), because these bands come from some of the YopM that is present as a full-sized but nicked protein, and the pieces stay together until they are dissociated by boiling in SDS-PAGE sample buffer.

Figure 1C and D show that the shoulder/trailing YopM fractions gave one band in an immunoblot when probed with α-ECP (raised against secreted proteins of *Y. pseudotuberculosis* carrying the $YopKL^-$ LCR plasmid of *Y. pestis*); the peak fraction gave the same pattern as when probed with α-M-int. These findings showed that both peak and shoulder/trailing fractions were not detectably contaminated with other extracellular proteins.

We believed that all of these pure YopM fractions were suitable for immunization of mice, as the mice process the protein anyway, and the nicked form is only about 10% of the total. However, we did do one direct comparison of the immunogenicity and protectiveness of nicked (a peak fraction) and intact (a trailing fraction) YopM in BALB/c mice and found no difference in prechallenge α-YopM antibody titers (10^5 for both intact and nicked YopM) or LD_{50} for the two sets of immunized mice (10^3 for intact YopM and 6×10^2 for nicked YopM). Hence, we will not specify the YopM fractions used for the remainder of tests in this study.

Endotoxin contamination was a significant concern. After anion-exchange chromatography, our YopM preparations had microgram quantities of endotoxin contaminating milligram quantities of YopM as determined by LAL assay (data not shown). Several endotoxin removal methods were tried: use of Detoxi-Gel (Pierce), gel filtration on Sepharose CL-6B (Pharmacia Biotech, Piscataway, N.J.), and use of Sartorius (Edgewood, N.Y.) Q15 strongly basic anion-exchanger syringe filters; however, they consistently retained significant amounts of YopM as well as endotoxin. Only the End-X B52 system, which consists of endotoxin-neutralizing protein covalently coupled to beads, was found to remove endotoxin without also removing protein. However, we had to add a low concentration of deoxycholate to dissociate YopM from endotoxin. Although this system works extremely well, the End-X B52 resin cannot be regenerated and the YopM sample requires dialysis to remove deoxycholate.

HTV gave one band plus a minor degradation product on SDS-PAGE with silver staining and contained >95% intact LcrV (data not shown).

Immunization conditions. Initial tests determined the optimum adjuvant, route of inoculation, and time course of antibody response for active immunization and persistence of injected antibody for passive immunization. BALB/c mice immunized i.p. by using FA reached their peak anti-YopM titer after a single immunization, by day 15. Mice receiving AV eventually attained titers matching those of FA-treated mice, but only after 50 days. Thus, we chose FA for the rest of this study.

BALB/c mice were immunized three times at biweekly intervals with 40-μg doses of YopM plus FA via the i.p. or s.c. route. At 15 days, the anti-YopM titer for the s.c. route was 100-fold lower than that for the i.p. route, but by 29 days, the

TABLE 1. Passive immunization of BALB/c and Swiss Webster mice followed by challenge with *Y. pestis* KIM5

Mouse strain	Immunogen	μg of IgG/ mouse	Serum antibody titer		LD_{50} (CFU/mouse)
			YopM	LcrV	
BALB/c	PBS	0	$\leq 10^1$	NA[a]	4.5×10^1
	α-BSA IgG[b]	500	$\leq 10^1$	NA	2.6×10^2
	α-YopM IgG[b]	500	10^4	NA	3.0×10^2
Swiss Webster	PBS	0	$\leq 10^1$	$\leq 10^1$	1.5×10^1
	α-HTV IgG[c]	500	$\leq 10^1$	10^4	$>1.0 \times 10^4$
	α-YopM IgG[c]	500	10^4	$\leq 10^1$	$<1.0 \times 10^1$

[a] NA, not applicable.
[b] Mouse IgG.
[c] Rabbit IgG.

titers were comparable (ca. 10^5). Immunization by the s.c. route elicited amounts of the various isotypes and subclasses of Ig comparable to those found for the i.p. route. As expected for immunization with a soluble antigen, serum IgG1 concentrations greatly exceeded those of IgG2b, IgG2a, IgG3, IgM, and IgA (e.g., on day 42 after the first i.p. immunization, the respective amounts were 11, 0.9, 0.15, 0.15, 0.022, and 0.013 mg/ml). We chose i.p. as the route of antigen administration for the remainder of the work, because this route provides the quickest and strongest antibody response and because it makes sense to use a more central (i.p.) than peripheral (s.c.) immunization route for our particular plague model, as we must challenge i.v. with Pgm^- *Y. pestis* KIM.

To test for persistence in the circulation of α-YopM antibodies given by passive immunization, we gave two groups of five mice, by the i.p. route, 500 μl of ascites from YopM-immunized mice, bled them 1 day prior to passive transfer and at days 1, 6, 9, 16, and 23, and measured the α-YopM Ig levels in pooled sera for each group at each time point. The two groups gave similar results, showing that the serum antibody was already maximal at day 1, stayed constant through day 6, declined slightly on day 9, and declined to just under half the maximal level by day 16 and to about a third of the maximal level by day 23. This result showed that a single antibody dose would be adequate for our passive protection tests, as mice typically die from *Y. pestis* KIM5 challenge between days 4 and 7.

Passive immunization and challenge of inbred and outbred mice. To determine if antibody alone could mediate protection against *Y. pestis* challenge, IgG from mice immunized with YopM or BSA was passively transferred to naive inbred animals. Following challenge with *Y. pestis* KIM5, there were no significant differences in CFU of *Y. pestis* required to kill 50% of the animals in any of the three groups, indicating that YopM antibody on its own is not protective (Table 1). However, there appeared to be a slight increase in the LD_{50}s for animals immunized with α-BSA or α-YopM antibody, indicating a small nonspecific protective effect of antibody per se (Table 1).

Outbred (Swiss Webster) mice were also examined to determine if passive immunization against YopM was protective. However, the presence of α-YopM antibody also did not increase the LD_{50} in these passively immunized mice compared to control animals (Table 1). Interestingly, 60% of mice given α-HTV antibody were protected at a challenge dose of 10^4, while all mice at this same dose in the two other treatment groups had died by day 7 postchallenge (data not shown), indicating that antibody against LcrV is protective (as previously shown [19]) and showing that mice can be protected against experimental plague by our immunization protocol.

Active immunization and challenge of inbred and outbred mice. We next tested the protective efficacy of active immunization, as this protocol might prime effector cells which may be required in addition to YopM-specific antibody to protect against *Y. pestis* challenge.

In the first study, although the α-YopM antibody titer in pooled serum from immunized BALB/c mice was 10^5 by ELISA at the time of challenge, the LD_{50} in the YopM-immunized group of mice was not significantly different from that of the PBS-treated group following challenge with *Y. pestis* KIM5 (Table 2), indicating that YopM is not protective by active immunization.

We were concerned that the high dose of YopM given for immunization might not have been completely eliminated by the mice by 18 days after the final boost (at time of challenge) and might have had a residual effect on host defenses at challenge. Accordingly, we modified the immunization protocol to give a smaller amount of YopM immunogen with only one boost and a longer time period (1 month) between the last immunization and challenge. As with the first study, the α-YopM titers were $\geq 10^5$ in pooled immune mouse serum; however, upon challenge there was no significant difference in LD_{50}s when immunized and control mice were compared (Table 2), again supporting the finding that active immunization with YopM is not protective. FA appeared to have a slight nonspecific protective effect (Table 2).

Because BALB/c mice as a group possess only one allotype ($H\text{-}2^d$) of the major histocompatibility complex class II locus required for antigen presentation (8), they are limited in their repertoire of presentable peptides and may not effectively present YopM peptide fragments to immune effector cells. Therefore, we immunized and challenged outbred mice (Swiss Webster), which as a group would possess a spectrum of major histocompatibility complex loci and should be able to present a wider variety of peptides to effector cells. Before challenge, YopM-immunized mice collectively had a specific serum antibody titer of 10^5, and HTV-immunized animals had a pooled α-HTV serum antibody titer of $>10^5$. Similar to the findings of the first experiment, active immunization with YopM did not significantly increase the LD_{50}s for these mice over those for controls (Table 2). Mice immunized with HTV demonstrated a significant degree of resistance to challenge, as all mice survived even the highest challenge dose, indicating, as previously shown (1, 11, 19, 21, 41), that LcrV is a protective antigen.

TABLE 2. Active immunization of BALB/c and Swiss Webster mice followed by challenge with *Y. pestis* KIM5

Mouse strain	Expt	Immunogen	μg of immunogen/ mouse	Serum antibody titer		LD_{50} (CFU/mouse)
				YopM	LcrV	
BALB/c	1	PBS + FA	NA[a]	$\leq 10^1$	NA	4.5×10^3[b]
	1	YopM + FA	40	10^5	NA	1.4×10^3[b]
	2	PBS	NA	$\leq 10^1$	NA	$<1.0 \times 10^1$
	2	PBS + FA	NA	$\leq 10^1$	NA	1.0×10^1
	2	YopM + FA	20	10^5	NA	1.2×10^1
Swiss Webster	3	PBS + FA	NA	$\leq 10^1$	$\leq 10^1$	$<1.0 \times 10^1$
	3	YopM + FA	20	10^5	$\leq 10^1$	3.0×10^1
	3	HTV + FA	20	$\leq 10^1$	$>10^5$	$>1.0 \times 10^5$

[a] NA, not applicable.
[b] The LD_{50}s for this experiment were larger than for others with BALB/c mice, probably because the mice were older at the start of the experiment and significantly older once challenged, due to a more prolonged immunization regimen than in other experiments.

TABLE 3. Effects of exogenous YopM on virulence of *Y. pestis* and *L. monocytogenes*

Bacterium	Exogenous YopM[a]	Antibody[b]	MTD[c] (days)	LD_{50} (CFU/mouse)
Y. pestis KIM5-3233[d]	−	None	6	7×10^2
	+	None	5[e]	2×10^2
	+	α-YopM	6	5×10^2
	+	Irrelevant		3×10^3
L. monocytogenes EGD	−	None	3	4×10^2
	+	None	3	5×10^2

[a] One hundred micrograms of YopM was administered (i.v. or i.p.) in 100 μl of PF-PBS on days 0 through 5.

[b] Five hundred micrograms of protein A-purified rabbit antibody was administered i.p. in 500 μl of PF-PBS on days 0 and 2. Irrelevant antibody was purified from preimmune serum samples.

[c] MTD, mean time to death for mice given the dose that was at least 10-fold above the calculated LD_{50} (10^4 bacteria).

[d] YopM$^-$ mutant.

[e] Not significantly different from *Y. pestis* KIM5-3233 without exogenous YopM at P of <0.05.

Effect of exogenous YopM on *Y. pestis* and *L. monocytogenes* challenge in mice. Because it was conceivable that YopM could have an extracellular antihost role without being neutralizable by antibody, we tested whether exogenously supplied YopM exacerbated infections by homologous (*Yersinia*) and heterologous (*Listeria*) pathogens. Because only a few *Y. pestis* KIM5 bacteria kill mice (Tables 1 and 2), we would not expect to measure reliably an exacerbating effect of YopM treatment on infection by this strain. Accordingly, we used the YopM$^-$ *Y. pestis* KIM5-3233, previously shown to be attenuated in BALB/c mice (13), and tested for the ability of exogenous YopM to reconstitute virulence. YopM treatment did not exacerbate a *Listeria* infection (Table 3). However, YopM did enhance the virulence of the YopM$^-$ *Y. pestis* to a degree, causing a more than threefold decrease in the LD_{50}. This effect was masked by the nonspecific protective effect of large doses of either specific or irrelevant antibody (also see Table 1), and so we were not able to determine whether α-YopM antibody can neutralize exogenous YopM's virulence-enhancing effect. These findings indicate that YopM does not act by itself to counteract host defenses important for resistance to *Listeria* but that exogenously supplied YopM might have a virulence-promoting effect on *Yersinia*. This inference raises the possibility that YopM has a minor extracellular function.

Human plague convalescent sera. By way of relating our study more directly to its potential relevance for humans, we determined if three samples of human plague convalescent serum, known by a standard passive hemagglutination test to have specific reactivity for the capsular protein F1, would also have reactivity for YopM and LcrV. All of the samples had reactivity against YopM (titers of 1:800 to 1:1,600 by ELISA) and LcrV (titers of 1:1,600 to ≥1:3,200 by ELISA). The serum sample with the highest agglutination titer to F1 (1:512) also had the highest antibody titer to LcrV (≥1:3,200 [highest dilution tested]) but had a lower titer to YopM (1:800). In contrast, the serum sample with the highest titer to YopM (1:1,600) had a relatively lower titer to LcrV (1:1,600) and a low agglutination titer to F1 (1:32).

DISCUSSION

In this study, we sought to determine if immunization against YopM is protective against challenge with *Y. pestis*. Antibodies to YopM were hypothesized to be protective, because YopM has an in vitro activity, thrombin binding, that is compatible with an extracellular location for the protein during an infection and because YopM is necessary for full virulence in mice (13). To test our hypothesis, we used an i.v. challenge model of systemic *Y. pestis* infection in mice.

We found that YopM is highly immunogenic in mice and rabbits. As with the known protective antigens F1 and V antigen, it also is expressed sufficiently during human plague to elicit a specific immune response. We speculate that YopM is sufficiently present in infection of humans to exert its virulence effects.

However, neither passive immunization of mice with antibody from YopM-immunized mice or rabbits nor active immunization with YopM protected against *Y. pestis* challenge. Accordingly, the hypothesis was not supported: YopM is not a protective antigen. In contrast, we found that both passive and active immunization against LcrV (HTV) were protective in our mouse plague model. As seen previously, active immunization against LcrV appeared to convey strong protection (11, 21), while passive immunization conveyed only partial protection (19), indicating that specific host factors, in addition to antibody, are required for full protection against *Y. pestis* challenge in mice.

It is possible that YopM is not neutralized by antibody because its main target in the host is intracellular. In vivo, YopM might enter host cells by the well-established vectorial translocation mechanism that functions for YopE, YopH, and YpkA and thereby be sequestered from access to antibody. Indeed, recent tests with chimeric proteins consisting of N-terminal portions of YopM fused to the adenylate cyclase domain of the *Bordetella pertussis* hemolysin-adenylate cyclase showed that some of the chimeric YopM is translocated into cells (4). Moreover, findings from our laboratory show that intact YopM is mostly targeted directly into cells, with a small amount consistently found in the supernatants of the infected cells (31). Accordingly, the significance of intracellularly targeted YopM is unresolved, and an extracellular site of YopM function cannot be ruled out.

It is possible that YopM's virulence mechanism is not neutralizable by antibody, even though YopM might have an extracellular function. For example, if YopM has an environmentally modulated conformation, its putative active site might never be accessible to antibody because it is sequestered within the protein. We were intrigued by the finding that YopM could partially restore virulence to the YopM$^-$ *Y. pestis* strain without exacerbating a *Listeria* infection. This finding suggests that YopM may have a virulence-related effect from an extracellular location and that its effect seems to be *Yersinia* specific, perhaps because YopM acts in concert with another *Yersinia* component. If added YopM needs to interact with yersiniae to exert its effect, the failure of exogenous YopM to restore complete virulence to the YopM$^-$ *Y. pestis* could have been due to the inability of the added YopM to reach foci of infection in sufficient concentration.

Our previous (26) and recent (30) studies of thrombin binding by YopM are consistent with a major virulence role of YopM other than thrombin sequestration, as YopM's interaction with thrombin appears to be relatively weak. This does not rule out a possible locally high and effective YopM concentration at a focus of infection but does raise the issue of possible other targets for YopM, including intracellular ones (31). We are currently examining the fates of exogenously added YopM and of YopM secreted by yersiniae to clarify the functions of this important *Yersinia* virulence protein.

ACKNOWLEDGMENTS

We thank Thomas Stoss for his assistance with all aspects of the animal work involved in this study. We thank Ken Fields (this laboratory) for supplying the HTV clone and α-HTV rabbit antiserum and Wendi Gardner and Tom Stoss for helping prepare the α-YopM rabbit antibody.

This project was supported by Collaborative Agreement DAMD17-94-J-4013 from the Department of the Army, USAMRDC. The production of HTV and all rabbit antibodies (α-HTV and α-YopM) was done in the course of a project supported by NIAID (grant 21017).

REFERENCES

1. **Anderson, G. W., Jr., S. E. C. Leary, E. D. Williamson, R. W. Titball, S. L. Welkos, P. L. Worsham, and A. M. Friedlander.** 1996. Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of *Yersinia pestis*. Infect. Immun. **64:**4580–4585.
2. **Andrews, G. P., D. G. Heath, G. W. Anderson, Jr., S. L. Welkos, and A. M. Friedlander.** 1996. Fraction 1 capsular antigen (F1) purification from *Yersinia pestis* CO92 and from an *Escherichia coli* recombinant strain and efficacy against lethal plague challenge. Infect. Immun. **64:**2180–2187.
3. **Bahmanyar, M., and D. C. Cavanaugh.** 1976. Plague manual, p. 25–34. World Health Organization, Geneva, Switzerland.
4. **Boland, A., M.-P. Sory, M. Iriarte, C. Kerbourch, P. Wattiau, and G. R. Cornelis.** 1996. Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB,D,N delivery apparatus. EMBO J. **15:**5191–5201.
5. **Ferber, D. M., and R. R. Brubaker.** 1981. Plasmids in *Yersinia pestis*. Infect. Immun. **27:**839–841.
6. **Fields, K. A., and S. C. Straley.** Unpublished data.
7. **Goguen, J. D., J. Yother, and S. C. Straley.** 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mu*d*1 (Ap *lac*) insertion mutants. J. Bacteriol. **160:**842–848.
8. **Håkansson, S., E. E. Galyov, R. Rosqvist, and H. Wolf-Watz.** 1996. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. Mol. Microbiol. **20:** 593–603.
9. **Hansen, T. H., and D. H. Sachs.** 1989. The major histocompatibility complex, p. 445–487. *In* W. E. Paul (ed.), Fundamental immunology. Raven Press, New York. N.Y.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
11. **Leary, S. E. C., E. D. Williamson, K. F. Griffin, P. Russell, S. M. Eley, and R. W. Titball.** 1995. Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. Infect. Immun. **63:**2854–2858.
12. **Leung, K. Y., and S. C. Straley.** 1989. The *yopM* gene of *Yersinia pestis* encodes a released protein having homology with the human platelet surface protein GPIbα. J. Bacteriol. **171:**4623–4632.
13. **Leung, K. Y., B. S. Reisner, and S. C. Straley.** 1990. YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. Infect. Immun. **58:**3262–3271.
14. **Marshall, J. D., Jr., P. J. Bartelloni, D. C. Cavenaugh, P. J. Kadull, and K. F. Meyer.** 1974. Plague immunization. II. Relation of adverse clinical reactions to multiple immunizations with killed vaccine. J. Infect. Dis. **129**(Suppl.)**:**19–25.
15. **Meyer, K. F., J. A. Hightower, and F. R. McCrumb.** 1974. Plague immunization. VI. Vaccination with the fraction I antigen of *Yersinia pestis*. J. Infect. Dis. **129**(Suppl.)**:**41–45.
16. **Meyer, K. F.** 1970. Effectiveness of live or killed plague vaccines in man. Bull. W.H.O. **42:**653–666.
17. **Meyer, K. F., D. C. Cavanaugh, P. J. Bartelloni, and J. D. Marshall, Jr.** 1974. Plague immunization. I. Past and present trends. J. Infect. **129**(Suppl.)**:**13–18.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Motin, V. L., R. Nakajima, G. B. Smirnow, and R. R. Brubaker.** 1994. Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. Infect. Immun. **62:**4192–4201.
20. **Nakajima, R., and R. R. Brubaker.** 1993. Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. Infect. Immun. **61:**23–31.
21. **Nakajima, R., V. L. Motin, and R. R. Brubaker.** 1995. Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. Infect. Immun. **63:**3021–3129.
22. **Persson, C., R. Nordfelth, A. Holmström, S. Håkansson, R. Rosqvist, and H. Wolf-Watz.** 1995. Cell surface-bound *Yersinia* translocate the protein tyrosine-phosphatase YopH by a polarized mechanism into the target cell. Mol. Microbiol. **18:**135–150.
23. **Plano, G. V., S. S. Barve, and S. C. Straley.** 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. **173:**7293–7303.
24. **Protsenko, O. A., P. L. Anisimov, O. T. Mozharov, N. P. Konnov, Y. A. Popov, and A. M. Kokooshkin.** 1983. Detection and characterization of *Yersinia pestis* plasmids determining pesticin I, fraction I antigen, and "mouse" toxin synthesis. Genetica **19:**1081–1090.
25. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
26. **Reisner, B. S., and S. C. Straley.** 1992. *Yersinia pestis* YopM: thrombin binding and overexpression. Infect. Immun. **60:**5242–5252.
27. **Rosqvist, R., K.-E. Magnusson, and H. Wolf-Watz.** 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. **13:**964–972.
28. **Sample, A. K., and R. R. Brubaker.** 1987. Post-translational regulation of Lcr plasmid-mediated peptides in pesticinogenic *Yersinia pestis*. Microb. Pathog. **3:**239–248.
29. **Simpson, W. J., R. E. Thomas, and T. G. Schwan.** 1990. Recombinant capsular antigen (fraction 1) from *Yersinia pestis* induces a protective antibody response in BALB/c mice. Am. J. Trop. Med. Hyg. **43:**389–396.
30. **Skrzypek, E., and S. C. Straley.** 1996. Interaction between *Yersinia pestis* YopM protein and human α-thrombin. Thrombosis Res. **84:**33–43.
31. **Skrzypek, E., and S. C. Straley.** Unpublished data.
32. **Sodeinde, O. A., and J. D. Goguen.** 1988. Genetic analysis of the 9.5-kilobase virulence plasmid of *Yersinia pestis*. Infect. Immun. **56:**2743–2748.
33. **Sory, M.-P., A. Boland, I. Lambermont, and G. R. Cornelis.** 1995. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cya*A gene fusion approach. Proc. Natl. Acad. Sci. USA **92:**11998–12002.
34. **Straley, S. C.** Unpublished data.
35. **Straley, S. C.** 1988. The plasmid-encoded outer-membrane proteins of *Yersinia pestis*. Rev. Infect. Dis. **10:**S323–S326.
36. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51:**445–454.
37. **Straley, S. C., E. Skrzypek, G. V. Plano, and J. B. Bliska.** 1993. Yops of *Yersinia* spp. pathogenic for humans. Infect. Immun. **61:**3105–3110.
38. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol. Microbiol. **8:** 1005–1010.
39. **Une, T., and R. R. Brubaker.** 1984. In vivo comparison of avirulent Vwa^- and Pgm^- or Pst^r phenotypes of yersiniae. Infect. Immun. **43:**895–900.
40. **Williams, J. E., and D. C. Cavanaugh.** 1979. Measuring the efficacy of vaccination in affording protection against plague. Bull. W.H.O. **57:**309–313.
41. **Williamson, E. D., S. M. Eley, K. F. Griffin, M. Green, P. Russell, S. E. C. Leary, P. C. F. Oyston, T. Easterbrook, K. M. Reddin, A. Robinson, and R. W. Titball.** 1995. A new improved sub-unit vaccine for plague: the basis of protection. FEMS Immunol. Med. Microbiol. **12:**223–230.

Editor: R. E. McCallum