INFECTION AND IMMUNITY, June 1985, p. 676–685
0019-9567/85/060676-10$02.00/0

Vol. 48, No. 3

Immune Response to Plasmid- and Chromosome-Encoded *Yersinia* Antigens

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The immune response of humans and mice to temperature-specific, plasmid- or chromosome-encoded proteins of *Yersinia pestis* and *Yersinia enterocolitica* was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Extracts from *Y. pestis* and *Y. enterocolitica* strains with and without the virulence plasmids pYV019 and pYV8081, respectively, were resolved by denaturing electrophoresis, and the major antigens were visualized with sera from convalescing plague patients, individuals immunized with plague vaccine, and mice and rabbits immunized with avirulent live yersiniae. The *Y. pestis* grown in vitro in this study did not express detectable amounts of plasmid-encoded antigens. The sera from plague patients recognized *Y. pestis* and *Y. enterocolitica* antigens ranging from 15 to 72 kilodaltons (kDa), whereas sera from immunized subjects recognized four antigenic components in *Y. pestis* ranging from 17 to 64 kDa and five antigens in *Y. enterocolitica* ranging from 16 to 68 kDa. Sera from mice reacted with 7 antigens in *Y. pestis* and 12 antigens in *Y. enterocolitica* ranging from 14 to 68 kDa, and sera from rabbits reacted with 7 and 10 antigens in *Y. pestis* and *Y. enterocolitica*, respectively. All of the plague patient sera, as well as the sera from immunized mice and rabbits, reacted with a 22-kDa *Y. enterocolitica* plasmid-associated polypeptide, and five of the patient sera also recognized a *Y. enterocolitica* plasmid-associated 31-kDa protein. The results indicate a common immune response to at least these two plasmid-specified *Yersinia* outer membrane proteins. *Y. pestis* apparently expresses these components only in vivo, and in vitro, *Y. enterocolitica* expresses a greater number of plasmid-associated antigens than does *Y. pestis*.

The genus *Yersinia* comprises three species which are capable of causing invasive disease in humans and other mammals, i.e., *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (2). *Y. pestis* is the causative agent of bubonic plague, the hallmark of which is the development of a bubo, a massive inflammatory response in the lymph node which drains the site of inoculation, usually via a flea bite. If the infection is widely disseminated, it can spread to the lungs, causing pneumonic plague, and in the terminal stages of the disease, septicemia develops.

The virulence of *Yersinia* species is associated with a 40- to 45-megadalton (MDa) plasmid (7–10, 25, 26). It has been difficult to study the major virulence-associated antigens because there are some differences in the antigens expressed in cells grown in vivo and in vitro. The expression of the virulence antigens in different *Yersinia* species grown in vitro is affected by growth temperature, Ca^{2+}, and other nutrients.

Five determinants of virulence have been described for *Y. pestis* (2), including the V and W antigens (3) and "fraction 1," which is a protein-lipid-polysaccharide complex. *Y. enterocolitica* and *Y. pseudotuberculosis* grown in vitro at 37°C in Ca^{2+}-deficient medium express at least three novel plasmid-associated outer membrane polypeptides of 45, 35, and 23 kilodaltons (kDa) (17, 22). Bölin et al. demonstrated that *Y. pseudotuberculosis* and *Y. enterocolitica* also express a plasmid-associated outer membrane protein of approximately 140 kDa, which they termed protein 1 (1). *Y. pestis* does not express the plasmid-associated outer membrane proteins in vitro (17). Portnoy et al., comparing the plasmid DNAs from the three *Yersinia* species, showed that each plasmid has sufficient coding capacity to express these novel, temperature-specific outer membrane proteins (18).

The purpose of the current work was to determine which plasmid- and chromosome-encoded antigens elicit immune responses in rabbits, mice, and human subjects who had been immunized with the currently available plague vaccine or who had contracted bubonic plague. To do this work, *Y. pestis*, *Y. enterocolitica*, and *Escherichia coli* strains with and without the 40-MDa *Y. pestis* virulence-associated plasmid were grown under conditions which were permissive or restrictive for the expression of plasmid-specified proteins. Extracts were resolved by denaturing electrophoresis, and the major antigens were visualized by immunoblotting with sera from the test animals and human subjects.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in these studies, together with their properties and characteristics with respect to virulence, are listed in Table 1. Bacteria were grown at either 28 or 37°C in heart infusion broth supplemented with 3 mM calcium chloride, 20 mM magnesium chloride, and 0.2% xylose (Difco Laboratories). When the organisms were grown at 37°C, the heart infusion broth also contained 0.02% sodium oxalate, a calcium-chelating agent which allows the expression of the Ca^{2+}-dependence phenotype (15).

Production of antisera. Five- to six-week-old outbred Swiss-Webster female mice (NAMRU strain, Naval Biosciences Laboratory, Oakland, Calif.) were used for these experiments. Thirty mice were injected intraperitoneally with 10^4 live *Y. pestis* EV76 or *Y. pestis* 195-P2 organisms. Three doses were administered 1 week apart, and the sera were collected 1 week after the last injection. Injection 1 was given with complete Freund adjuvant, and injections 2 and 3 were administered with incomplete Freund adjuvant. The mice were exsanguinated by heart puncture, and the sera

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TABLE 1. Bacterial strains and plasmids

Organism and strain	Plasmid[a]	Relevant properties[b]	Source[c]
Y. pestis			
EV76	pYV019	Avirulent (pigmentation mutant)	1
195-P1		Avirulent	2
195-P2	pYV019::IS*100*	Avirulent (insertion abolishing Ca^{2+} dependence)	2
Y. enterocolitica			
8081	pYV8081	Virulent	1
8081c		Avirulent	1
E. coli			
LE392	pYV019::Tn*5*	In vitro transformant	1
LE392			1

[a] pYV019 and pYV8081 are designations given to the naturally occurring plasmids in *Y. pestis* and *Y. enterocolitica*, respectively.

[b] Virulence in *Yersinia* species is associated with the following properties: expression of fraction 1 (capsular antigen), pesticin, and toxin secretion, and dependence on Ca^{2+} for growth.

[c] (1) D. A. Portnoy and S. Falkow, Department of Microbiology, Stanford University School of Medicine, Stanford, Calif. (2) Reference collection, University of California Naval Biosciences Laboratory, Oakland, Calif.

were pooled and filtered consecutively through 0.45- and 0.22-μm membrane filters (Millipore Corp.).

Antiserum to *Y. enterocolitica* 9576, produced in rabbits by R. J. Martinez (University of California, Los Angeles), was the gift of D. Portnoy (Stanford University).

Human sera. Antisera from 13 plague patients between 3 and 52 years of age, drawn at intervals ranging from 1 day to 2 months after the onset of symptoms, were provided by T. J. Quan of the plague branch at the Centers for Disease Control, Fort Collins, Colo. Sera were collected from two patients in the acute and convalescent phases of bubonic plague. Convalescent-phase sera were collected from one subject with septicemic plague, one subject with bubonic plague and secondary pneumonia, and nine patients who had bubonic plague with no secondary complications. Specific antibody titers in the sera were measured by the passive hemagglutination assay (PHA) (24), and the endpoints were given as reciprocals of the dilution.

Sera were collected from five laboratory workers before and after a standard course of immunization with commercial plague vaccine (Cutter Laboratories), and their titers were measured by the PHA assay. The titers of all preimmunization sera were less than 4, whereas titers after immunization varied from 4 to 32.

Antigen preparation for SDS-polyacrylamide gel electrophoresis (PAGE). (i) Whole-cell extracts. Organisms from each strain were grown overnight at 28°C in 10 ml of heart infusion broth containing the supplements described above. After the incubation, the organisms in 5 ml of broth were collected by centrifugation (2,000 rpm, 5 to 10 min) in a model R size 2 IEC centrifuge. The supernatant fluids were discarded, and the pellets were mixed with an equal volume of distilled water and 2× sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris-hydrochloride [pH 6.8], 5% SDS, 0.36 M 2-mercaptoethanol, 22.5% [vol/vol] glycerol, and 0.001% bromophenol blue) and boiled for 10 min. The remaining cells were transferred to fresh heart infusion broth at a 1:10 dilution in Ca^{2+}-deficient medium containing sodium oxalate and placed on a shaker for 6 to 8 h at 37°C, and a lysate was prepared as described above. To ensure that all of the organisms were killed by being boiled in the sample buffer, blood agar plates were streaked and determined to be negative for growth before the boiled samples were transferred out of P3 containment.

(ii) Outer membrane extracts. Outer membrane proteins were prepared as described by Portnoy et al. (18). 50-ml volumes of *Y. enterocolitica* 8081 and *Y. enterocolitica* 8081c cells were grown at 28 or 37°C to stationary phase in heart infusion broth containing the supplements described above. The cultures were subjected to centrifugation at 8,000 rpm, and the bacterial sediment was suspended in 10 mM Tris–5 mM $MgCl_2$, pH 7.5. The bacteria were lysed by sonication, and unbroken cells were removed by a low-speed centrifugation at 2,700 rpm for 20 min, after which the supernatant fluid was sedimented by a high-speed centrifugation at 17,000 rpm for 60 min. The supernatant fluid was discarded, the tubes were allowed to dry, and the pellet was suspended in 10 mM Tris (pH 8)–5 mM $MgCl_2$–2% Triton X-100 and allowed to incubate at room temperature for 20 min. The sample was then subjected to centrifugation at 17,000 rpm for 60 min at 4°C, and the pellet was suspended in electrophoresis sample buffer as described above and subjected to SDS-PAGE. A preparation of *Y. pseudotuberculosis* III/pIBI outer membrane proteins, made and characterized by H. Wolf-Watz by the identical procedure, was generously provided by him.

SDS-PAGE. SDS-PAGE was performed in a discontinuous system (12) with a 12.5% polyacrylamide slab gel (14 by 16 cm; Protean Slab Gel, Bio-Rad Laboratories) with a 4.75% polyacrylamide stacking gel. To detect high-molecular-weight components of the bacteria, 10% to 15% polyacrylamide gradient gels were used with a stacking gel of 4.75% polyacrylamide. Samples of approximately 10^8 organisms in 0.1 ml were boiled for 5 min immediately before use and applied to the gel. A mixture of molecular weight standards (Sigma Chemical Co.) was prepared at a concentration of 4 mg/ml in SDS sample buffer, and approximately 10 μl was applied to the gel. The standards were: bovine serum albumin (64 kDa), ovalbumin (44 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), β-lactalbumin (18 kDa), and lysozyme (14 kDa). Electrophoresis was performed at a constant current of 15 mA per slab for the first 1.5 h until the bromophenol blue entered the 12.5% polyacrylamide gel and then at 25 mA per slab for an additional 4.5 h. Gels were either silver stained or subjected to immunoblotting. Molecular weights were estimated from a linear least-squares fit of the logarithm of molecular weight versus relative mobility of the standards. The standard error of the estimate for these plots was generally less than or equal to ±2 kDa.

Silver stain. Silver staining was performed by a modification of the method of Burk et al. (5). The gels were rinsed in distilled water, gently shaken for 1 h at room temperature in a Coomassie blue stain-formaldehyde fixative consisting of 25.7% (vol/vol) ethanol, 5.3% (vol/vol) formaldehyde, and 0.095% Coomassie blue R-250 in distilled water, and then destained overnight in a solution of 25% (vol/vol) ethanol and 0.37% (vol/vol) formaldehyde in distilled water. The gels were rinsed for 4 h with a continuous flow of distilled water and then stained with the silver stain (2.5 ml of concentrated NH_4OH and 8 ml of 20% $AgNO_3$ in 189 ml of 0.022 N NaOH) for 15 min with mild agitation. The gels were rinsed in distilled water for 1 h as described above, and then the stain was developed with 0.05% citric acid–0.007% formaldehyde. The developing reaction was stopped by immersing the gels in 0.175% methylamine–0.05% citric acid.

Immunoblot analysis. Western blot analysis of antigens

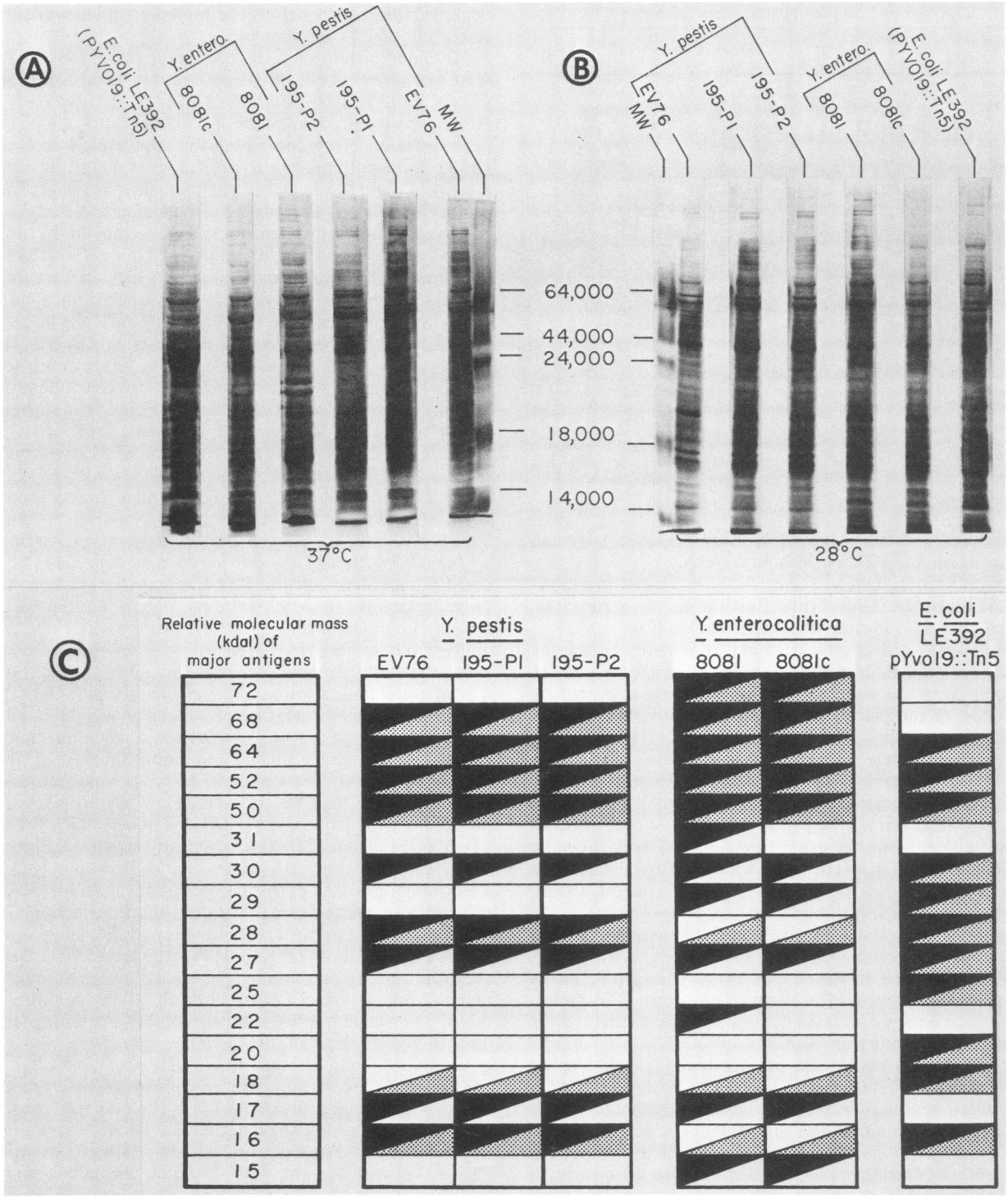

FIG. 1. Distribution of the major proteins from extracts of *Y. pestis*, *Y. enterocolitica*, and *E. coli* strains, visualized by the silver stain. (A) Silver-stained acrylamide gel of cultures grown at 37°C. (B) Silver-stained acrylamide gel of cultures grown at 28°C. (C) Schematic of the silver-stained gels; the triangles represent major bands in extracts of cultures grown at 37 (solid) or 28°C (dotted).

was performed by the methods of Burnette (6) and Towbin et al. (23). After electrophoresis, the gels were removed and the separated bacterial components were electrophoretically transferred to nitrocellulose paper (BA-85, Schleicher & Schuell, Inc.; 0.45-μm pore size). The gel was placed on a sheet of nitrocellulose cut to the same size and subjected to electrophoresis overnight at 100 mA in 25 mM Tris base, 192 mM glycine, and 20% methanol in a Transblot apparatus (Bio-Rad). After transfer, the nitrocellulose blots were shaken for 1.5 h at 37°C in a blocking buffer (phosphate-buffered saline containing 3% fraction V bovine serum albumin and 10% heat-inactivated calf serum) to block nonspecific protein binding. The blots were then incubated for 2 h at room temperature with the human, mouse, or rabbit antisera

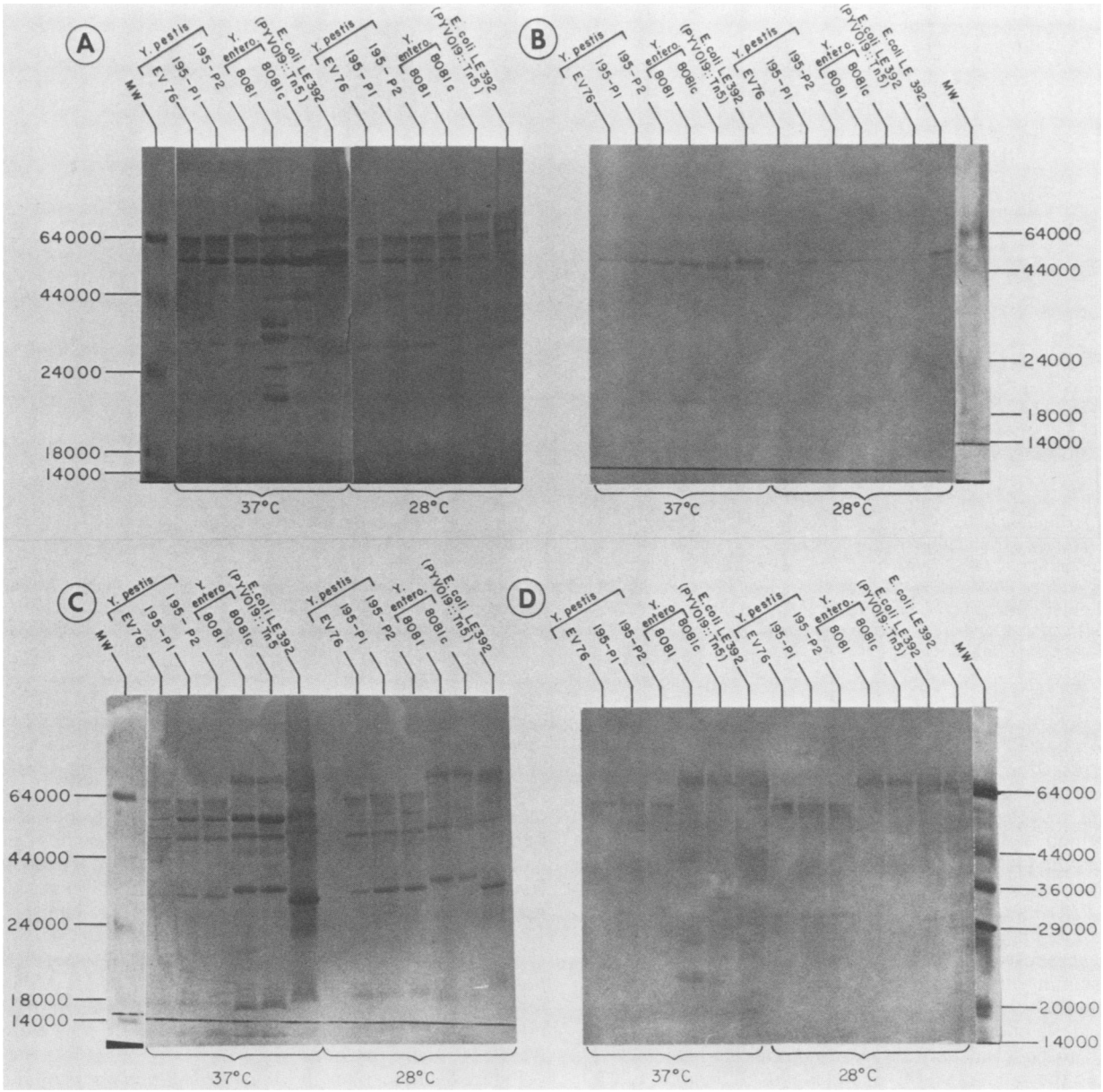

FIG. 2. Immunoblots of *Yersinia* and *E. coli* extracts prepared at the temperatures indicated and challenged with the human plague sera listed in Fig. 4. (A) Human convalescent-phase serum 7c with a PHA titer of 256. (B) Human acute-phase serum 4a with a PHA titer of 32. (C) Human convalescent-phase serum 2c with a PHA titer of 32. (D) Human convalescent-phase serum 1c with a PHA titer of 0. Molecular weight markers (MW) were stained with amido black as described in the text.

to *Yersinia* strains (diluted 1:100 to 1:500 in blocking buffer), washed at room temperature by shaking with four changes of phosphate-buffered saline containing 0.3% fraction V bovine serum albumin, 15 min per wash, then shaken for 1.5 h at room temperature in peroxidase-conjugated goat anti-human, goat anti-mouse, or goat anti-rabbit immunoglobulin (1:1,000 in blocking buffer; Cappel Laboratories, Inc.), and finally washed in phosphate-buffered saline containing bovine serum albumin as described above. The antigen-antibody complexes were visualized by the addition of a substrate solution (0.02% 4-chloro-1-napthol and 0.005% hydrogen peroxide in 0.075 M Tris-hydrochloride buffer [pH 7.4]). Immunoblots subjected to each step except incubation with the *Yersinia*-directed antibodies served as negative controls to ensure that adventitious binding by the other reagents was not occurring. No bands were detected after the addition of the substrate solution to these blots. Molecular weight marker lanes were stained separately for 5 to 10 min with 0.1% amido black in 10% acetic acid–45% methanol and then destained in several changes of 10% acetic acid–25% isopropanol.

RESULTS

Bacterial strains. Seven bacterial strains were used for the immunoblot analyses. Because some of the plasmid-associated proteins are expressed at 37 but not at 28°C, samples of each strain were grown at both temperatures. *Y. enterocolitica* 8081, which contains the intact 40-MDa plasmid, was

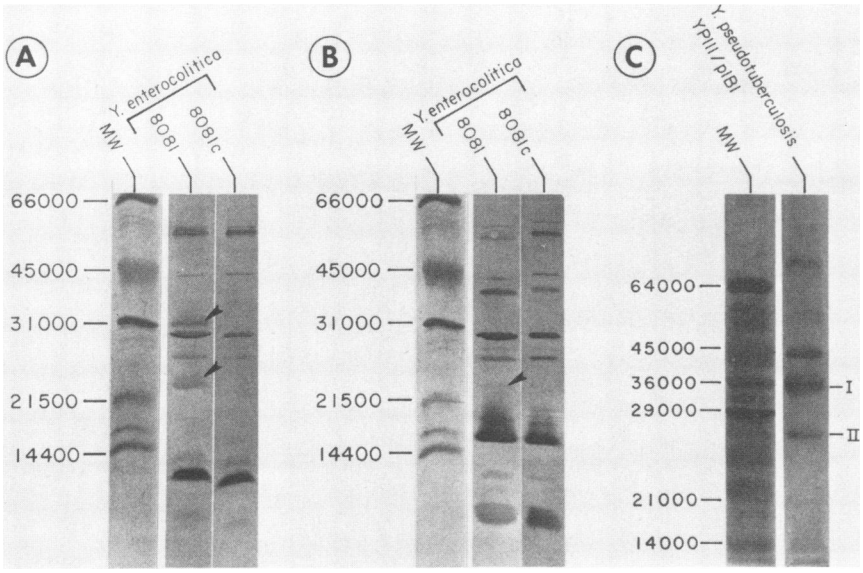

FIG. 3. Immunoblots of *Y. enterocolitica* 8081, *Y. enterocolitica* 8081c, and *Y. pseudotuberculosis* III/pIBI outer membrane protein extracts prepared from cultures grown at 37°C as described in the text. The outer membrane proteins were challenged with the human plague sera listed in Fig. 4. (A) *Y. enterocolitica* 8081 and 8081c outer membrane proteins challenged with human convalescent-phase serum 7c. (B) *Y. enterocolitica* 8081 and 8081c outer membrane proteins challenged with human convalescent-phase serum 2c. (C) *Y. pseudotuberculosis* III/pIBI outer membrane proteins challenged with human convalescent-phase serum 7c. Bands I and II have molecular weights identical to those of YOPs 4 and 5, respectively, as defined by Wolf-Watz. The arrows indicate immunoreactive proteins found only in the plasmid-carrying strains.

used to determine whether the sera from infected humans or mice recognized any plasmid-associated proteins which are not expressed in vitro by *Y. pestis*. *E. coli* LE392 was used as a control strain to determine which *Yersinia* antigens were common to other gram-negative bacteria. *E. coli* LE392 transformed with pYV019::Tn*5* was used to determine whether the 40-MDa *Yersinia* plasmid-encoded proteins could be expressed in *E. coli*.

SDS-PAGE analysis of major polypeptides from whole-cell lysates. The major polypeptides of the *Yersinia* and *E. coli* strains were resolved by denaturing PAGE and visualized by silver staining. The protein profile of each strain is represented in Fig. 1. *Y. pestis* EV76, 195-P1, and 195-P2 grown at 37°C had identical patterns of nine polypeptides ranging from 16 to 68 kDa, and *Y. enterocolitica* 8081 and 8081c exhibited the same nine polypeptides plus four more, of 15, 22, 31, and 72 kDa. Of these polypeptides, proteins of 15 and 72 kDa were present in both *Y. enterocolitica* strains, whereas the 22- and 31-kDa proteins were expressed only in the virulent, plasmid-carrying 8081 strain.

Expression of major *Yersinia* proteins as a function of growth temperature. Most of the proteins common to *Y. pestis* and *Y. enterocolitica* were expressed equally well at 28 and 37°C. The *E. coli* LE392(pYV019::Tn*5*) and *E. coli* LE392 control strains also exhibited identical protein patterns at both growth temperatures. However, in the three *Y. pestis* strains and both *Y. enterocolitica* strains, the major 17- and 30-kDa proteins were expressed only at 37°C, and an 18-kDa protein was only observed in the 28°C cultures. The 16- and 28-kDa proteins common to both species were expressed in *Y. pestis* strains grown at either temperature, but they were expressed in *Y. enterocolitica* only in cultures grown at 28°C. Furthermore, the 22- and 31-kDa proteins unique to plasmid-carrying *Y. enterocolitica* 8081 were expressed only at 37°C.

***Yersinia* antibodies in human acute- and convalescent-phase sera.** Antibodies against the *Yersinia* species antigens were detected by the immunoblot technique. Representative blots are shown in Fig. 2 and 3, and the data for all plague patients are summarized in Fig. 4. The two acute-phase sera recognized only the 52- and 68-kDa proteins of *Y. pestis*, but various convalescent-phase sera reacted with as many as 10 antigens ranging from 15 to 68 kDa. In addition, two convalescent-phase sera which did not have detectable PHA titers reacted with *Yersinia* antigens on the blots (Fig. 2 and 4). All 13 sera recognized the same set of antigens in extracts from each of the *Y. pestis* strains; i.e., no plasmid-specified *Y. pestis* antigens were detected in this experiment. Although fewer antigenic bands were observed in the *Y. pestis* cultures grown at 28°C than in those grown at 37°C, the patterns were essentially the same.

Figure 5 summarizes the *Y. enterocolitica* antigens recognized by the plague patient sera used in the experiments described above. Various sera recognized from two to nine antigens of between 16 and 72 kDa in the strains grown at 37°C and similar but fewer antigenic components in *Y. enterocolitica* grown at 28°C. The acute-phase sera reacted with a 52-kDa component in both *Y. enterocolitica* strains which is likely to be identical to the component recognized in *Y. pestis*.

In the *E. coli* LE392(pYV019::Tn*5*) strain as well as in the control strain lacking the virulence-associated plasmid, the acute-phase sera recognized only the 52-kDa antigen, whereas the convalescent-phase sera recognized five major antigenic components (of 68, 64, 52, 28, and 16.5 kDa) likely to be similar or identical to those from the *Y. pestis* and *Y. enterocolitica* strains (Fig. 5).

Plasmid-specified proteins recognized by human acute- and convalescent-phase sera. Since, as previously stated, the *Y. pestis* cultured in vitro for these experiments did not express detectable amounts of plasmid-encoded proteins, reactions of the patient sera were compared on immunoblots of

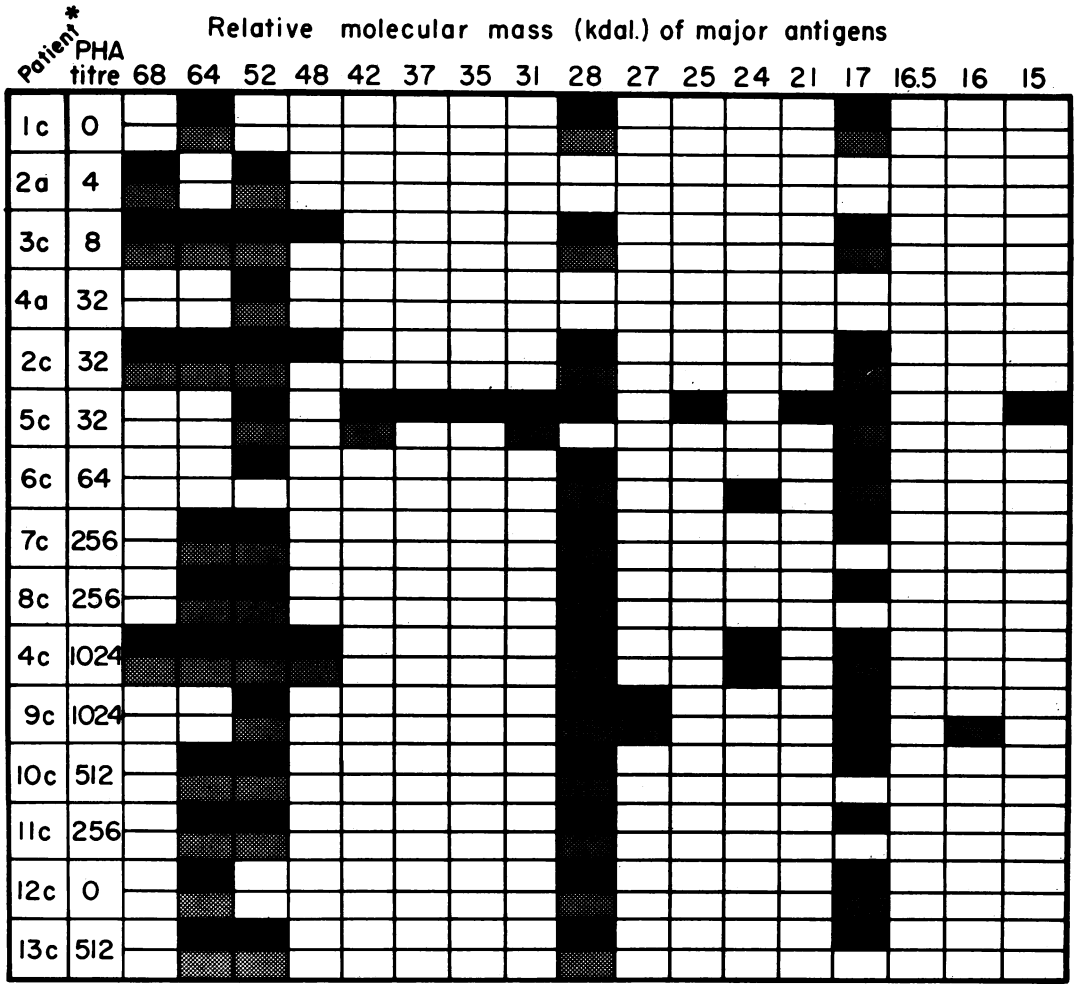

FIG. 4. Immunoblots of *Y. pestis* EV76, 195-P1, and 195-P2 were challenged with sera from patients in the acute or convalescent phase of bubonic plague. For patients 2 and 4, acute (a) and convalescent (c) sera were tested. Solid or dotted rectangles represent antigens recognized by the antisera after the organisms were grown at 37 or 28°C, respectively.

extracts from the *Y. enterocolitica* and *E. coli* strains with and without a functional pYV019. The two acute-phase sera reacted with the 22-kDa plasmid-specified protein in *Y. enterocolitica* 8081, as well as with the 52-kDa component of *Y. pestis* and *Y. enterocolitica* which appears not to be encoded by pYV019. All of the convalescent-phase sera, including those without a PHA titer, recognized the 22-kDa protein, and 5 of the 13 sera also reacted with the 31-kDa plasmid-specified protein in *Y. enterocolitica* 8081 grown at 37°C (Fig. 5). These two antigens were not observed in the plasmid-cured *Y. enterocolitica* strain or the plasmid-carrying culture grown at 28°C.

H. Wolf-Watz and I. Bolin recently proposed (personal communication) a new nomenclature in which the plasmid-encoded proteins are identified as YOPs (*Yersinia* outer membrane proteins) 1 through 5. Since *Y. pestis* does not express the outer membrane proteins in vitro and since the individual YOPs have not been purified and characterized, this hypothesis could be tested only by comparing the patterns of polypeptide bands in outer membrane preparations from *Y. enterocolitica* and *Y. pseudotuberculosis* strains that express and do not express the polypeptide bands originally defined as YOPs. To do this we subjected outer membrane preparations to SDS-PAGE and immunoblotting, and we challenged the blots with human convalescent-phase sera 7c and 2c. Sera 7c and 2c recognized eight bands and seven bands, respectively, in the outer membrane preparation from *Y. enterocolitica* and six bands in the preparation from strain 8081c. The additional immunoreactive bands in the extract from the plasmid-carrying strain had molecular sizes of 22 and 31 kDa, identical to those defined by Wolf-Watz and Bolin for YOPs 4 and 5, respectively, in these strains (Fig. 3A and B). In a separate experiment, we subjected an outer membrane preparation of plasmid-containing *Y. pseudotuberculosis* III/pIBI to SDS-PAGE and immunoblotting. One human convalescent-phase serum which recognized the 22- and 31-kDa *Y. enterocolitica* antigens reacted with four major bands in this *Y. pseudotuberculosis* outer membrane extract. Two of these bands had apparent molecular weights of 24 and 36 kDa, identical to the weights reported by Wolf-Watz for YOPs 4 and 5 (Fig. 3C).

***Yersinia* antibodies in sera from immunized laboratory personnel.** Sera taken from subjects before immunization did not react with any antigens on blots of *Yersinia* strains grown at 37°C, but the five postvaccination sera recognized what appear to be common antigenic components of 64, 52, 28, and 17 kDa in *Y. pestis*; 68, 52, 29, and 16 kDa in *Y.*

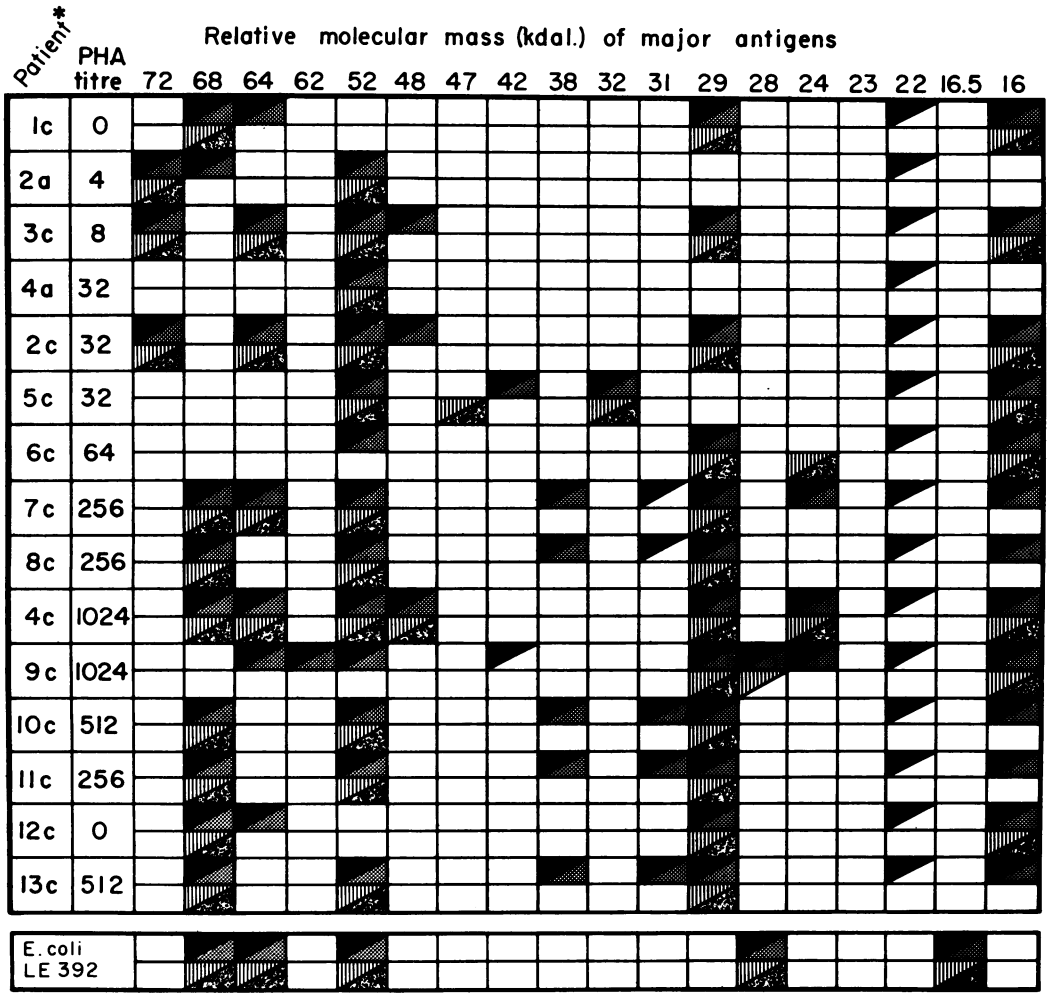

FIG. 5. *Y. enterocolitica* and *E. coli* antigens recognized by human plague sera. Reactions with the acute- and convalescent-phase sera are noted by a and c, respectively. The panels for *E. coli* LE392 indicate all the bands which reacted with convalescent-phase sera. Solid and dotted triangles represent antigens recognized in extracts of the plasmid-containing or -cured organisms, respectively, grown at 37°C. The striped and stippled triangles represent antigens recognized by the antisera in extracts of the plasmid-containing or -cured organisms, respectively, grown at 28°C.

enterocolitica 8081c; and 68, 64, 52, 28, and 16.5 kDa in the *E. coli* LE392(pYV019::Tn*5*) strain, as well as in the *E. coli* LE392 control strain lacking the plasmid. In addition, the postvaccination sera bound to the 22-kDa plasmid-encoded protein in *Y. enterocolitica* 8081 (Fig. 6). As expected, the sera with the highest PHA titers, i.e., 32, gave the most intense bands.

Murine immune response to *Yersinia* species. Pooled sera from mice immunized with *Y. pestis* EV76 reacted with seven *Y. pestis* antigens ranging from 14 to 62 kDa, and sera from mice immunized with *Y. pestis* 195-P1 or 195-P2 reacted with the same antigens. The immunoblot is shown in Fig. 7, and the reaction data are summarized in Fig. 8. The mouse sera recognized 12 antigenic components of between 14 and 68 kDa in *Y. enterocolitica*, including the 22-kDa plasmid-specified antigen present only in *Y. enterocolitica* 8081 grown at 37°C. The components which the pooled sera recognized in *Y. pestis* and *Y. enterocolitica* grown at 37 and 28°C were otherwise identical. Six of the same antigens were recognized in *E. coli* LE392 irrespective of whether the virulence-associated plasmid was present.

Rabbit immune response to yersiniae. The rabbit sera reacted with seven antigens (of 52, 48, 42, 38, 33, 20, and 18 kDa) in *Y. pestis* strains and nine antigens (of 72, 52, 51, 48, 42, 38, 34, 16, and 14 kDa) in *Y. enterocolitica* 8081c (Fig. 9). The reactive antigens in plasmid-carrying strain 8081 were identical, with the addition of the 22-kDa protein. The antigenic reactions for the organisms grown at 28 and 37°C were very similar, except that the 22-kDa antigen was not expressed in the 28°C culture of *Y. enterocolitica* 8081. Rabbit immune sera reacted with the five major components (of 68, 64, 52, 28, and 16.5 kDa) previously described in *E. coli* LE392(pYV019::Tn*5*) and found as well in the *E. coli* LE392 control strain which lacked the virulence plasmid.

DISCUSSION

The immune responses of humans and mice to plague infection and of rabbits to *Y. enterocolitica*, as studied by immunoblotting, were highly similar. Within the 1- to 2-kDa uncertainty inherent in the molecular sizes measured from the SDS gels, it is tempting to conclude that most of the immunogenic *Y. pestis* components were antigenically identical to those present in the *Y. enterocolitica* strains shown in Fig. 2, 7, and 9. At least five major common antigens not

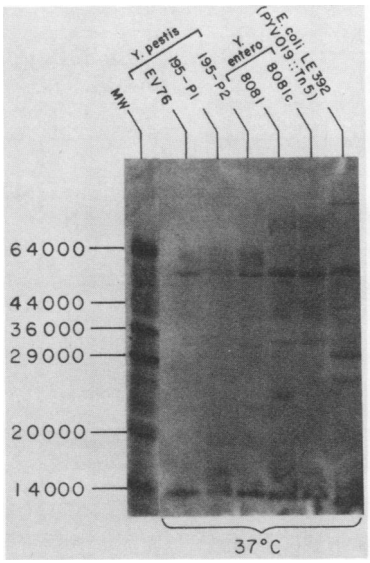

FIG. 6. Immunoblot of *Y. pestis*, *Y. enterocolitica*, and *E. coli* strains reacted with serum having a PHA titer of 32 from one subject immunized with the plague vaccine.

specified by the virulence plasmid were recognized. In addition, most of the sera reacted with two outer membrane proteins of 31 and 22 kDa expressed by *Y. enterocolitica* strains harboring the 40-MDa plasmid.

Studies of the *Yersinia* virulence proteins have been complicated by small differences in molecular weights and the different nomenclature used by various laboratories. In the new terminology of H. Wolf-Watz et al., the 140-kDa protein termed protein 1 by Bölin et al. (1) is YOP 1. YOP 1 and YOP 3, which, according to Wolf-Watz et al., is about 40 kDa, were not observed by Portnoy et al. (17). YOPs 2, 4, and 5 are proteins of 44, 36, and 24 kDa, which correspond to proteins 1 (45 kDa), 2 (38 kDa), and 3 (25 kDa) of Portnoy et al., respectively (17).

The central questions raised by this study are whether the antigenic polypeptides designated I and II in *Y. enterocolitica* (Fig. 3) are in fact the two lower-molecular-weight YOPs 4 and 5 and whether *Y. pestis* produces any of these polypeptides in vivo. The experiments of Fig. 2 and 3 are consistent with identity between polypeptide I and YOP 4 and between polypeptide II and YOP 5 by the following three criteria: (i) antigens I and II were expressed only in the virulent strains; (ii) the antigens were observed in the purified outer membrane protein preparations as well as in the whole-cell extracts; and (iii) the molecular weights of proteins I and II were identical to those of YOP 4 and 5, respectively, within measurement error. The fact that these two antigens were visualized equally well by reaction with human plague convalescent-phase serum, sera from mice infected with *Y. pestis*, and sera from *Y. enterocolitica*-infected rabbits further suggests that the 22- and 31-kDa antigenic proteins expressed by *Y. pestis* in vivo are the equivalent of YOPs 4 and 5 in *Y. enterocolitica* 8081. A more definitive proof of identity is not possible without applying criteria which are more rigorous than those used to originally define the YOPs. Interpretation of these experiments is complicated by the fact that we did not observe plasmid-specified proteins corresponding to YOPs 1, 2, and 3.

The finding that the sera from plague patients, immunized laboratory personnel, mice, and rabbits recognized five antigens common to *E. coli* as well as both *Yersinia* species was expected, since a taxonomic relationship between *Yersinia* spp. and other *Enterobacteriaceae* was established some time ago. This relationship was based on the common sensitivities of these strains to bacteriophages (11, 14, 20, and 21), the 22% hybridization homology between *E. coli* and *Y. pestis* DNA (19), and immunodiffusion experiments which demonstrated that *E. coli* shares at least three antigens with *Y. pseudotuberculosis* (13). In our experiments, *E. coli* LE392 with the pYV019::Tn5 virulence plasmid did not express any of the plasmid-associated proteins, and it exhibited the same antigenic profile as *E. coli* LE392 without the plasmid. One possible explanation for this is that expression of pYV019 genes may involve products specified by the chromosome or one or both of the other plasmids present in *Y. pestis* or all of these components. This hypothesis is further supported by the observation that *E. coli* LE392 carrying pYV019 was not virulent in mice, showing that the plasmid does not confer virulence upon a normally avirulent microbial species (15).

Two questions raised by our data are (i) why *Y. pestis* appeared not to express proteins 2 and 3 (YOPs 4 and 5) in vitro and (ii) why we did not observe other plasmid-specified antigens such as YOPs 1 and 2. Under the growth conditions used in this study, we did not detect the expression of any plasmid-associated polypeptides by *Y. pestis* in vitro. This finding was consistent with reports from several laboratories indicating that although the *Y. pestis* plasmid has sufficient capacity to encode several polypeptides, none are expressed in vitro (16–18). If other plasmid-specified outer membrane proteins had been expressed by *Y. pestis* in vivo and if they were immunogenic, then antibodies to them should have been seen in the experiments of Fig. 2 and 3, in which immunoblots of extracts of *Y. enterocolitica* cultures grown in vitro were challenged with sera from convalescent plague patients.

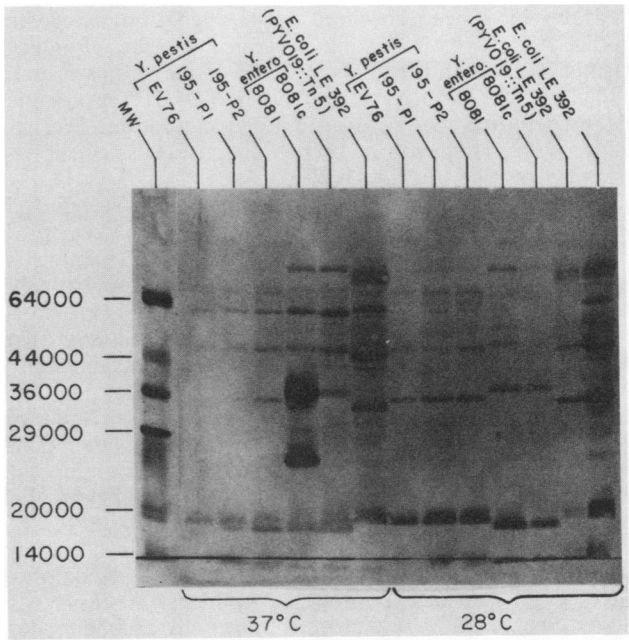

FIG. 7. Immunoblots of *Y. pestis*, *Y. enterocolitica*, and *E. coli* strains grown at the temperatures indicated and reacted with mouse antiserum to *Y. pestis* EV76.

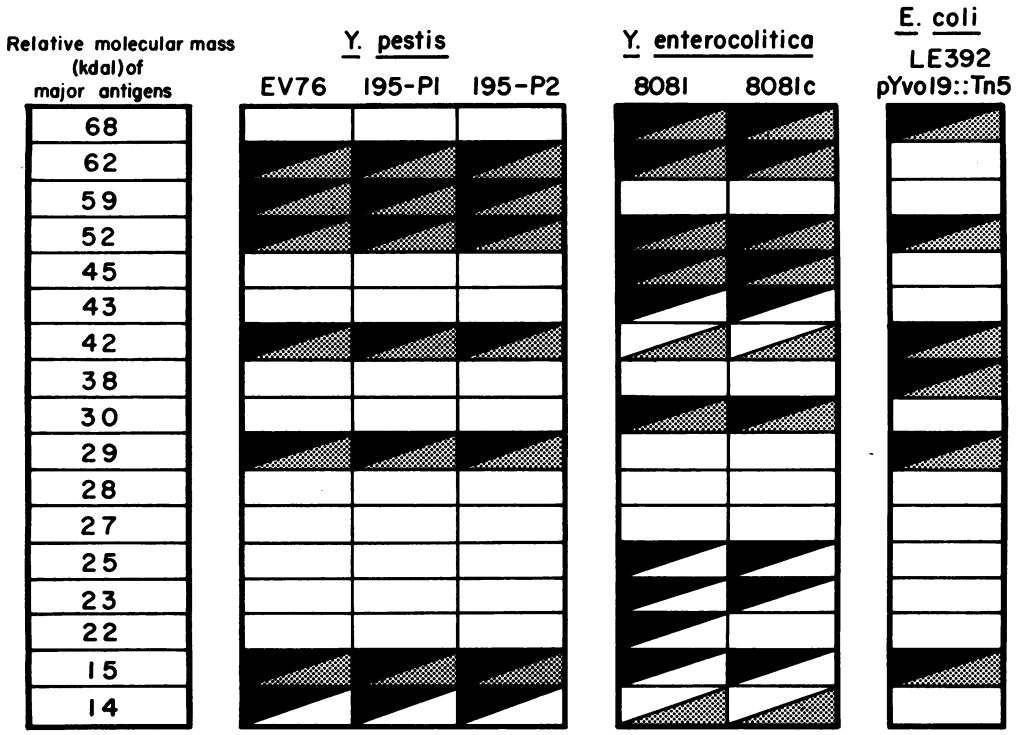

FIG. 8. Immunoblot patterns of *Y. pestis* EV76, 195-P1, 195-P2; *Y. enterocolitica* 8081 and 8081c; and *E coli* challenged with a pooled mouse antiserum to *Y. pestis* EV76. Antigens recognized in the extracts of cultures grown at 37 or 28°C are represented by solid or dotted triangles, respectively.

In these experiments, neither the silver stain nor the immunoblots gave any indication of the 140-kDa plasmid-encoded protein termed YOP 1 (1) or of the 45-kDa YOP 2. Recent studies indicate that YOP 1 may be composed of subunit polypeptides of about 51.5 kDa (H. Wolf-Watz, personal communication). If this is the case, the subunit band may have been obscured by the highly immunogenic 52-kDa protein which is not encoded by the virulence plasmid, but which is prominent in all of the strains tested. In the case of YOP 2, as well as that of YOP 1, it is possible that the epitope was destroyed by denaturation. Further studies with nondenaturing or two-dimensional electrophoresis may resolve this question.

The observation that the two acute-phase sera recognized the temperature-dependent, plasmid-encoded 22-kDa protein in *Y. enterocolitica* suggests that this polypeptide is highly immunogenic early in infection. It is also noteworthy that the immunoblots revealed antibodies to *Yersinia* antigens, including the 22-kDa plasmid-encoded protein in two convalescent-phase sera which did not have detectable PHA titers. PHA detects antibodies only to *Y. pestis* fraction 1, and patients with this fraction appear to have raised antibodies to noncapsular components of the bacteria other than fraction 1. Thus, the data suggest that the immunoblot technique is more sensitive than PHA in detecting the presence of particular antibodies.

That fewer antigenic components were recognized by sera from individuals immunized with the plague vaccine was expected. The vaccine is prepared from in vivo-passaged, fully virulent *Y. pestis* organisms treated with formaldehyde, which modifies the cell surface by denaturing or cross-linking surface components. Consequently, individuals immunized with these Formalin-killed organisms may not "see" all of the antigens present on organisms in a natural infection. However, the 22-kDa polypeptide of *Y. enterocolitica* 8081 was recognized by the sera, which indicates expression of the plasmid-encoded proteins and may be due to the in

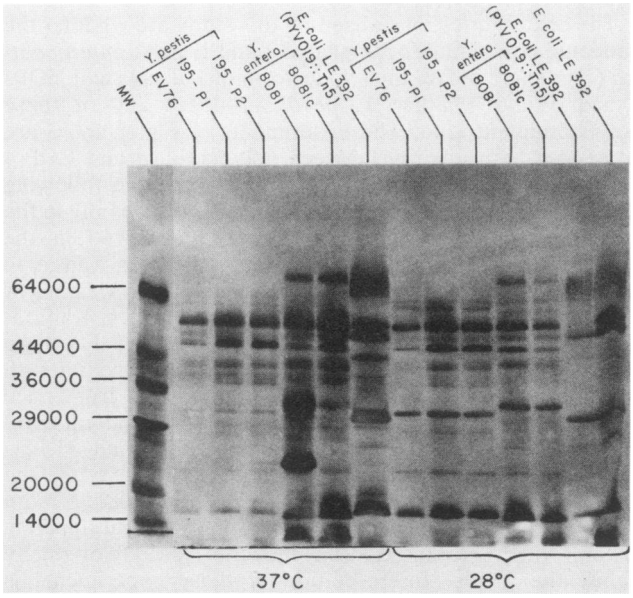

FIG. 9. Immunoblot of *Y. pestis*, *Y. enterocolitica*, and *E. coli* components challenged with rabbit antiserum to *Y. enterocolitica* 9576. The strains were grown at 28 or 37°C and subjected to electrophoresis in a 10 to 15% SDS-polyacrylamide gel.

vivo passaging of the organisms. (It is difficult to explain why sera from patients immunized with the vaccine should have recognized YOP 5, despite the fact that the organisms used to make the vaccine had been passaged for several generations in vitro and presumably would have ceased to express this YOP. We can only speculate that the ability to express YOPs was retained as a consequence of the repeated passaging of the strain in guinea pigs and mice, which is part of Cutter Laboratory's procedure for making the vaccine. Whether some antigens were lost due to the subsequent in vitro passaging or Formalin treatment of the organisms is presently being tested.)

In conclusion, the components of *Y. pestis*, including at least two plasmid-associated YOPs, i.e., YOPs 4 and 5, elicited an immune response in humans and the other mammals tested. Since humans and mice responded to many of the same antigens, mouse monoclonal antibodies to *Y. pestis* should be useful in characterizing the antigens observed in the human immune response and in following the expression of the plasmid-associated proteins in *Y. pestis* during the course of plague infection.

ACKNOWLEDGMENTS

We are grateful to Hans Wolf-Watz for valuable discussions and the generous gift of *Y. pseudotuberculosis* outer membrane preparations. We also thank Renee Sugasawara for advice throughout the course of this work and Hank Blank for technical assistance. Portions of this study appeared in a thesis for the Master of Science degree submitted by G.M. to the University of California, Berkeley School of Public Health.

This research was supported by Food and Drug Administration agreement 224-73-1019 and contract N00014-81-C-0570 from the Office of Naval Research.

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