Yersinia pestis pH 6 antigen forms fimbriae and is induced by intracellular association with macrophages

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Summary

Ability to express pH 6 antigen (Ag) is necessary for full virulence of Yersinia pestis; however, the function of the Ag in pathogenesis remains unclear. We determined the nucleotide sequence of a 4232 bp region of Y. pestis DNA which encoded the pH 6 Ag structural gene (psaA) and accessory loci necessary for Ag synthesis. Protein sequences encoded by the Y. pestis DNA were similar to accessory proteins which function in the biosynthesis of Escherichia coli fimbriae Pap, K88, K99 and CS3 as well as the molecular chaperone for the Y. pestis capsule protein. Electron microscopy and immunogold labelling studies revealed that pH 6 Ag expressing E. coli or Yersinia produced flexible 'fibrillar' organelles composed of individual linear strands, multiple strand bundles or wiry aggregates of PsA. Y. pestis associated with the murine macrophage-like cell line, RAW264.7, expressed pH 6 Ag in an intracellular acidification-dependent manner. Together with an earlier study showing that a Y. pestis psaA mutant was reduced in virulence, these results demonstrate that the expression of fimbriae which are induced in host macrophages is involved in plague pathogenesis.

Introduction

Yersinia pestis is a facultative intracellular parasite which can survive and multiply inside macrophages (Straley and Harmon, 1984a; 1984b; Charnetzky and Shuford, 1985). The interaction of Y. pestis with host macrophages has long been thought to be important in the pathogenesis of bubonic plague (Cavanaugh and Randall, 1959), although little is known about this interaction. Many of the known virulence determinants of Y. pestis are induced when the organism is cultivated under conditions that simulate the environment encountered by the organism inside the mammalian host (Straley and Brubaker, 1981). However, only two of the Yersinia outer membrane proteins (Yops) have been reported to be expressed by bacteria associated with macrophages (Pollack et al., 1986; Straley, 1991).

Approximately 30 years ago, Ben-Efraim et al. (1961) described an antigen (Ag) that was produced only when Y. pestis was cultured at temperatures above 36°C and pH values below 6.7. This new Ag was designated pH 6 Ag. More recently, we cloned a Y. pestis Ag that was similarly regulated (Lindler et al., 1990). Although antisera from the previous study (Ben-Efraim et al., 1961) were not available, we also designated the cloned protein as pH 6 Ag because of its similar regulation and biochemical characteristics (Lindler et al., 1990). Furthermore, pH 6 Ag was found to be necessary for virulence of Y. pestis in the mouse model by the intravenous route of infection (Lindler et al., 1990).

In the previous study (Lindler et al., 1990), we isolated several Escherichia coli clones which encoded loci necessary for the synthesis of the Y. pestis pH 6 Ag. However, pH 6 Ag was not regulated by pH or temperature in these E. coli clones (Lindler et al., 1990). The structural gene designated psaA mapped within a 1.7 kb EcoRI-BamHI fragment of Y. pestis chromosomal DNA. We also isolated a single Tn10lacZ insertion 1.2 kb upstream of psaA which greatly reduced the expression of pH 6 Ag by mutant Y. pestis (Lindler et al., 1990). This transposon mutation defined an auxiliary locus designated psaE and mapped within a 0.9 kb EcoRI fragment of DNA. Gene fusions to both psaE and psaA allowed us to determine the direction of transcription for both of these loci. Further, a third locus was suggested to be involved in pH 6 Ag expression by transposon mutagenesis and deletion analysis (Lindler et al., 1990). The third locus was downstream from psaA and was located near a BamHI restriction enzyme recognition site. The expression of the precursor and processed form of pH 6 Ag was altered by a single transposon mutation within 60 bp of the BamHI site (Lindler et al., 1990). We now designate this third locus psaB.

The expression of pH 6 Ag at acidic pH and 37°C suggested that it might be synthesized in host macrophage phagolysosomes or extracellularly in abscesses such as...
buboes (Lindler et al., 1990). Here, we present results which demonstrate that Y. pestis pH 6 Ag is a fibrillar structure and is induced by Y. pestis present inside cultured macrophages.

Results

DNA sequence of the Y. pestis psaE, psaA, psaB and downstream partial open reading frame

We determined the nucleotide sequence of the region of Y. pestis chromosomal DNA encoding pH 6 Ag and the accessory loci (Fig. 1). Four significant open reading frames (ORFs) were detected which would be transcribed in the direction identified previously from m-Mu dl1734 and Tn10lacZ gene fusions (see above and Lindler et al., 1990). The first ORF began at bp 397 and ended with a TGA nonsense codon at bp 1041 (Fig. 2). This ORF includes the site of the Tn10lacZ mutation which defined psaE (Lindler et al., 1990). We determined the position of the transposon insertion in pPSN1 (psaE::Tn10lacZ) by comparing the nucleotide sequences of the mutant plasmid with the non-mutagenized DNA. The Tn10lacZ mutation occurred by insertion of the transposon 3' to the guanosine residue at bp 736 (Fig. 2). The second ORF began at bp 2056, 2071 or 2077 and terminated in a non-sense codon at bp 2547. The predicted protein sequence which was measured from bp 2071 is shown in Fig. 2 because this coding region is preceded by a weak Shine-Dalgarno sequence 9bp upstream of the ATG translation initiation codon. This second ORF is located in the region previously mapped as the psaA structural gene (Lindler et al., 1990). The 158-amino-acid PsA protein (Fig. 2) has a predicted pl of 5.8. This predicted isoelectric point is in agreement with our previous findings that the pH 6 Ag was acidic when analysed by two-dimensional protein electrophoresis (Lindler et al., 1990). A third ORF was found between bp 2674 and bp 3492 (Fig. 2). The third ORF designated psaB spanned the region previously found to be necessary for the expression of the mature 15 kDa pH 6 Ag polypeptide (Lindler et al., 1990). A partial ORF began at bp 3579 and continued past the end of the DNA for which we determined the nucleotide sequence (Fig. 2). Because of the incomplete sequence information, we designated this putative protein coding region as ORF4'.

Predicted protein sequence similarities

We analysed the EMBL and Genbank Data Libraries for similarity with our predicted PsaE, PsaA, PsaB and ORF4' sequences. No significant similarity was found with the PsaE protein. The pH 6 Ag structural gene product (PsaA) had limited similarity to the E. coli Pilus adhesin, PapG (Lund et al., 1987), and an influenza virus haemagglutinin (InfH; Air, 1981). The protein alignments are shown in Fig. 3. Considering highly conservative amino acid substitutions, these regions of PsaA (Fig. 3) were 40% similar to PapG and 54% similar to InfH. The PsaB and ORF4' predicted proteins had a high degree of similarity to several accessory proteins involved with pilus or capsule expression by enteric bacteria (Fig. 4; ORF4' data not shown). The 273-amino-acid PsaB protein was similar to the E. coli chaperone proteins for CS3 (Jalajakumari et al., 1989), Pap (Holmgren and Branden, 1989) and K88 (Bakker et al., 1991) pili as well as the Klebsiella pneumoniae pilus, Mrk (Allen et al., 1991) and the molecular chaperone for the Y. pestis capsule protein fraction 1 (F1; Galyov et al., 1991). The protein sequence alignment of these molecular chaperones is shown in Fig. 4. The identity with PsaB ranged from 41% to 22% for the F1 and K88 chaperones, respectively. Considering conservative amino acid substitutions, the similarity with PsaB ranged from 61% to 22% for the F1 and K88 chaperones, respectively. Considering conservative amino acid substitutions, the similarity with PsaB ranged from 61% to 22% for the F1 and K88 chaperones. Holmgren et al. (1992) have analysed the three-dimensional structure of PapD and compared it with several other pilin chaperones. Of the 23 amino acid residues considered by Holmgren et al. to be critical for structure and function of the pilin chaperone family, 18 of these residues are identical in the PsaB sequence (Fig. 4).
Correlation of ORFs and expressed proteins

To determine if the identified ORFs produced proteins having molecular weights similar to that predicted by DNA sequence analysis, we performed in vitro transcription-translation. Several linear DNA templates corresponding to various lengths of Y. pestis chromosome were generated by the polymerase chain reaction (PCR) and used as templates for in vitro transcription and translation (Fig. 5, top). The 5' end of the DNA fragments were kept constant by priming the reactions with a T7 promoter primer complementary to a region just outside the vector plasmid multiple cloning region. The 3' end of each PCR product was initiated with various oligonucleotide primers complementary to the Y. pestis DNA present in pDG27 as described in the Experimental procedures. The covalently closed circular pDG27 and vector pSK+ plasmid DNAs were also included as template. The results of these in vitro translation products were labelled on the left: numbers indicate positions of amino acid residues within the proteins. Identical amino acid residues are shown as double vertical lines, similar amino acid residues are shown as double dots, and less similar residues are shown as a single dot. Protein sequences were aligned with the BESTFIT Program of GCG software running on the Vax computer using the default settings for amino acid similarities.

Fig. 3. Protein sequence similarities with PsaA. Represented proteins are labelled on the left; numbers indicate positions of amino acid residues within the proteins. Identical amino acid residues are shown as double vertical lines, similar amino acid residues are shown as double dots, and less similar residues are shown as a single dot. Protein sequences were aligned with the BESTFIT Program of GCG software running on the Vax computer using the default settings for amino acid similarities.

A

| PsaA | 120 | WYINDDSPKDKDLHLYVKAG | 139 |
|------|-----|-----------------------|-----|
| PapG | 298 | WYTAGSKTVKIESLYGEEG | 317 |

B

| PsaA | 52  | APNTGEIFAGKQGZDVTMLTMTDGT | 77  |
|------|-----|-----------------------------|-----|
| InfH | 265 | APYECGLTGTGMRNLKLIKMGCCQ | 291 |
vitro transcription and translation reactions are shown in Fig. 5. No specific protein species was produced by the first 358 bp of Y. pestis DNA (Fig. 5, lane A). However, the synthesis of a specific 24 kDa polypeptide was directed for the biosynthesis of pH 6 Ag (Fig. 5, lane B). The 24 kDa size of this protein is in close agreement with the 23958 predicted molecular weight of DNA present in pDG27. The 18 kDa protein may be the product of an ORF which begins at bp 1038 and terminates at bp 1526 shown). The 18 kDa protein is in close agreement with the 23958 predicted molecular weight of DNA present in pDG27.
In vitro transcription and translation of the Y. pestis pH 6 Ag coding region.

Top. The linear map of the pH 6 Ag coding region is labelled as in Fig. 1. Thick lines below the map indicate DNA templates used in the in vitro transcription and translation reactions. Linear templates were generated by the PCR as indicated in the Experimental procedures. Bottom. Fluorograph of [35S]-methionine labelled proteins produced by in vitro labelling with various DNA templates. Labelled extracts (100 000 c.p.m. per lane) were separated and processed by SDS-PAGE on 4–20% gradient gels as described in the Experimental procedures. Lanes: A, B, C and D contain linear DNA templates generated by the PCR as indicated in the top portion of the figure; lanes pDG and V contain pDG27 and pSK+, respectively; the lane labelled 'neg' did not contain any template DNA. The positions of various transcription and translation products are indicated by arrows along with their apparent molecular weights.

the ability of a Y. pestis psaA mutant and isogenic wild-type cells to agglutinate sheep erythrocytes (SRBCs). Wild-type Y. pestis whole cells cultivated at 37°C pH 6 as well as cell-free potassium thiocyanate (KSCN) extracts derived from these cells caused aggregation of SRBCs. The agglutination observed was resistant to 25 mM methyl α-D-mannopyranoside (Firon et al., 1984). In contrast, Y. pestis KIM5-3001 (psaA') did not cause agglutination of the SRBCs. We found that E. coli HB101 containing either pDG1 or pDG27 also specifically agglutinated SRBCs when cultures were grown in Luria broth (LB) at 37°C. These results suggest that all genetic information necessary for synthesis, transport and assembly of pH 6 Ag was contained within the 6.5 kb KpnI to ClaI fragment of Y. pestis DNA. However, E. coli containing pDG9 did not cause agglutination of SRBCs. The plasmid pDG9 contains a 3.1 kb KpnI to BamHI fragment of Y. pestis DNA (Fig. 1; Lindler et al., 1990) and therefore encodes PsaE and PsaA but only the first 133 amino acids of PsaB. Accordingly, PsaB and/or downstream genetic information was necessary for functional expression of pH 6 Ag in E. coli.

Electron microscopy and immunogold labelling of pH 6 Ag

Protein sequence similarities and haemagglutination reactions suggested that pH 6 Ag was fimbrial. Accordingly, we examined Y. pestis and E. coli clones for the presence of fimbriae by electron microscopy after growth using the appropriate inducing conditions. We were unable to observe fimbriae associated with the cell surface of Y. pestis KIM5-3001. This may have been because of the presence of the F1 capsular Ag produced by Y. pestis (Brubaker, 1972). F1 is a capsular protein which covers the surface and surrounding milieu of Y. pestis with ‘granular particles’ which form an extracellular matrix (Chen and Elberg, 1977). To visualize the location of F1, we labelled Y. pestis KIM5-3001 grown under pH 6 Ag-inducing conditions with monoclonal antibody (mAb) 6H3, which recognizes F1, and examined the grids by electron microscopy. These studies revealed the presence of immunogold-labelled F1 capsule associated with the cell surface and surrounding environment (data not shown). Thus, it was possible that the presence of F1 on Y. pestis KIM5-3001 was obscuring the morphology of pH 6 Ag fimbriae. Consequently, we chose, first of all, to study pH 6 Ag produced by E. coli HB101 containing pDG1.

Uranyl-acetate-stained E. coli harbouring pDG1 produced fibrillar organelles that protruded from the cell surface (Fig. 6). Fimbriae were consistently observed on the surface of E. coli clones that had been grown overnight with aeration in broth cultures; however, we had difficulty observing fimbriae on the surface of clones that had grown in logarithmic phase or on the surface of solid agar. This latter result was in agreement with our previous observation that very little pH 6 Ag could be extracted
Yersinia pestis pH 6 Ag fimbriae

A. E. coli harbouring pDG1 negatively stained with uranyl acetate expressing the three pH 6 Ag fibrillar morphotypes.
B. Higher magnification of (A) showing the ultrastructural detail of the three morphotypes of pH 6 Ag, i.e. single strands, multistranded bundles, and large aggregates.
C. E. coli HB101 containing pDG1 labelled by the immunogold technique. Cells were incubated with a 1:20 dilution of pH 6 Ag-specific antiserum. Inset is a higher magnification of (C) (region designated by arrow) which shows more clearly the binding of immunogold particles to the aggregative fibrillar morphotype.
D. E. coli HB101 containing the pH79 cloning vector labelled by the immunogold technique using similar conditions as in (C). In these micrographs, CS designates cell surface. Unlabelled arrowheads designate the single-stranded pH 6 Ag fimbriae morphotype. Arrows labelled with 'a' designate the aggregative fibrillar morphology, while arrows labelled with 'b' designate the multistranded bundles of pH 6 Ag fimbriae. Bar markers: a, 0.1 μm; b, 0.05 μm; c, 0.25 μm, (inset bar is 0.05 μm); d, 0.5 μm.

Fig. 6. Electron photomicrographs of negatively stained and immunogold-labelled E. coli containing the cloned pH 6 Ag locus.

with KSCN from the surface of E. coli clones that had been grown in logarithmic phase (Lindler et al., 1990). The fimbriae were visualized as subtle, fine, singular strands (approximately 4 nm in diameter), as multistranded bundles of three or more fimbriae, or as large aggregates (Fig. 6, A and B). The immunogold labelling technique was useful in identifying these structures as pH 6 Ag (Fig. 6C). Anti-pH 6 Ag serum coated immunogold complexes were specifically observed bound to fibrillar structures expressed on the surface of E. coli HB101 containing pDG1 (Fig. 6C) as well as pDG5 and pDG27 (data not shown). There was no binding of pH 6 Ag serum to E. coli HB101 containing the cloning vector (Fig. 6D). These studies demonstrated that the expression of pH 6 Ag in E. coli resulted in fimbriation of the host cell.

Our studies revealed that pH 6 Ag was highly conserved between Y. pestis and Yersinia pseudotuberculosis PB1/+ at both the genetic and immunologic level (see Fig. 9 later, and below). Therefore, we chose to examine the morphology of pH 6 Ag in Y. pseudotuberculosis PB1/+ since this organism does not synthesize F1 capsule (Brubaker, 1972). All three morphotypes of pH 6 Ag
were seen protruding from the surface of uranyl-acetate-stained cells (Fig. 7A). Furthermore, we observed pH 6 Ag fibrillar bundles on the grids of Y. pestis which had been immunogold labelled with anti-F1 mAb (Fig. 7B). These observations confirmed that pH 6 Ag fimbriae were produced by both Y. pestis and Y. pseudotuberculosis. Lastly, pH 6 Ag fimbriae were identified by the immunogold labelling of structures with similar morphologies on the surface (Fig. 7C) or in crude KSCN preparations (Fig. 7D) of Y. pseudotuberculosis PB1/+ grown at 37°C and pH 6. In control experiments, when Y. pseudotuberculosis was cultured at 37°C and pH 8, we did not detect any labelling (data not shown). Thus, the specificity of the immunogold reaction in identifying structures as pH 6 Ag was confirmed.

Expression of pH 6 Ag by Y. pestis associated with macrophages

Expression of Y. pestis pH 6 Ag in vitro at 37°C and acidic pH suggested that the Ag may be synthesized inside phagocytic cells such as macrophages. To test this possibility, we infected the murine macrophage-like cell line, RAW264.7, with Y. pestis which was not expressing pH 6
Yersinia pestis pH 6 Ag fimbriae

To determine if acidification of the phagolysosome was necessary to induce the synthesis of pH 6 Ag, we treated Y. pestis-infected macrophage cultures with monensin (Fig. 8B). Monensin is a carboxylic ionophore which disrupts the acidification of intracellular compartments (Horrowitz and Maxfield, 1984; Wileman et al., 1985). Long-term (greater than 10 h) exposure of Y. pestis-infected macrophages to monensin concentrations which ranged from 5–10 μM resulted in cytotoxicity to the infected RAW264.7 cell line (data not shown). However, treatment of similar infected cultures with monensin concentrations of 0.1 and 1 μM reduced the expression of pH 6 Ag by Y. pestis after 21 h of growth in RAW264.7 macrophages (Fig. 8B). In control experiments, the addition of 5 μM monensin to broth cultures of Y. pestis growing at pH 6 and 37°C had no effect on pH 6 Ag expression. Thus, Y. pestis pH 6 Ag was expressed in association with macrophages in a manner which required acidification of the intracellular environment.

Molecular epidemiology of pH 6 Ag

To determine if pH 6 Ag gene sequences were present in other Yersinia spp., we performed Southern blot (Southern, 1975) hybridization using a psaA-specific probe. The pH 6 Ag gene probe was completely internal to the psaA locus (bp 2149 to bp 2547, Fig. 2) and was generated by the PCR. The probe hybridized with a 3.2 kb EcoRI fragment of Y. pestis DNA as well as Y. pseudotuberculosis strains 7, 43, R2, MSU-D and PB1/+ (Fig. 9). However, the psaA probe hybridized with a 9.3 kb EcoRI fragment

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**Fig. 8.** Expression of pH 6 Ag by Y. pestis inside macrophages in the presence or absence of monensin. Macrophage cell line RAW264.7 was infected with Y. pestis which was not expressing pH 6 Ag (grown at 30°C and pH 7.2). The multiplicity of infection was approximately one bacterium per macrophage. A. Macrophages infected with Y. pestis psaA' (+ lanes) or Y. pestis psaA- (− lanes) strains. Samples were removed at various times after infection of the macrophages for determination of viable bacterial counts as well as Western blot analysis. The time, in hours, is indicated above each pair of lanes. The immunoreactive protein corresponding to pH 6 Ag is indicated by the arrow. The equivalent of approximately 5 × 10^8 bacterial cfu was loaded on each lane. B. Effect of the addition of monensin on the expression of pH 6 Ag by Y. pestis inside macrophages. Monensin was added to the infected macrophages as described in the Experimental procedures. The millimolar concentration of monensin is indicated above each lane. The time, in hours, is indicated above each set of three lanes. The position of pH 6 Ag is indicated with the arrow. The equivalent of approximately 4 × 10^8 Y. pestis cfu was loaded on each lane.

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**Fig. 9.** Southern hybridization of various bacterial DNAs with the psaA-specific probe. Approximately 1 μg of bacterial total genomic DNA was digested to completion with EcoRI and separated on a 0.7% agarose gel. Fractionated DNA fragments were transferred to nitrocellulose and probed with the 398 bp Y. pestis psaA probe as described in the Experimental procedures. Lanes contain: 1. Y. pseudotuberculosis 7 (Ila); 2. Y. pseudotuberculosis 43 (III); 3. Y. pseudotuberculosis 32 (Iva); 4. Y. pseudotuberculosis R2 (Vb); 5. Y. pseudotuberculosis MSU-D; 6. Y. pseudotuberculosis PB1/+ (I); 7. Y. pestis KIM-5–3001; 8. Y. enterocolitica WA; 9. Y. enterocolitica 288; 10. Y. enterocolitica 312; 11. Salmonella typhi Ty2; 12. Shigella flexneri 5. The serotypes of the Y. pseudotuberculosis strains are indicated in parentheses.
of *Y. pseudotuberculosis* 32 genomic DNA (Fig. 9, lane 3). In contrast, no *psaA* sequences were detected in DNA derived from *Yersinia enterocolitica, Shigella flexneri* and *Salmonella typhi* (Fig. 9) even when low-stringency hybridization conditions were used (data not shown). Western blot analysis of *Y. pseudotuberculosis* PB1/4 grown at 37°C and pH 6 revealed that a protein was produced which immunologically cross-reacted with *Y. pestis* pH 6 Ag (data not shown). The anti-pH 6 Ag reactive material was not produced by *Y. pseudotuberculosis* PB1/4 which was cultured at 37°C and pH 8. However, neither *Y. pseudotuberculosis* 32 nor *Y. enterocolitica* WA cultured at 37°C and pH 6 produced a protein which immunologically cross-reacted with *Y. pestis* pH 6 Ag sera (data not shown). These results indicated that all genomic DNAs of the *Y. pseudotuberculosis* strains we examined did include sequences homologous to *Y. pestis* *psaA*. Furthermore, at least *Y. pseudotuberculosis* PB1/4 produced an acid-inducible polypeptide when cultured at 37°C which immunologically cross-reacted with *Y. pestis* pH 6 Ag.

**Discussion**

Our results demonstrate that pH 6 Ag is a fibrillar structure produced by *Y. pestis* and *Y. pseudotuberculosis*. Also, we show that pH 6 Ag is induced by *Y. pestis* inside macrophages in an acidic intracellular environment. Several lines of evidence from our studies support these conclusions. First, we observed a high degree of similarity between a pH 6 Ag accessory protein (*PsaB*) and several pilin chaperone proteins. Second, cell-free KSCN extracts of *Y. pestis* *PsaA* bacteria specifically caused the agglutination of SRBCs when compared with similar extracts prepared from the isogenic *psaA* mutant strain. Third, DNA hybridization studies and immunoblotting with *Y. pestis* pH 6 Ag-specific reagents revealed that the Ag is expressed by *Y. pseudotuberculosis*. Fourth, our electron microscopy studies revealed fimbriae on the surface of *E. coli* HB101 containing cloned pH 6 Ag as well as *Y. pseudotuberculosis* expressing the Ag. Multistranded bundles of pH 6 Ag fimbriae were also present on grids prepared from *Y. pestis*. Furthermore, these structures appeared to react with pH 6 Ag-specific antiserum. Fifth, we found that induction of *Y. pestis* pH 6 Ag inside RAW264.7 macrophages required acidification of the intracellular environment (Fig. 8).

The wavy morphology of *Yersinia* pH 6 Ag fimbriae appears to be similar to CS3, a fibrillar component of colonization factor antigen II produced by enterotoxigenic *E. coli* (Levine et al., 1984). Although the primary protein sequences of *PsaA* and the CS3 fibrillin are not similar, the chaperone proteins for these two fibrillar proteins share a high degree of identity (Fig. 4). Most of the *Yersinia* pH 6 Ag fimbriae were observed to be wavy strands which could form complex aggregates. However, these flexible organelles were also seen as single 'fibrillar' strands or as laterally associated thick multifilament bundles of three or more strands. Although lateral association of *E. coli* CS3 into multifilament bundles has not been reported, similar aggregation of other thin fimbrin into thicker structures has been observed (Olsen et al., 1989; Giron et al., 1991). Surface fimbriae produced by *Y. enterocolitica, Y. pseudotuberculosis* (Old and Adegboya, 1984; Skurnik, 1984) and *Y. pestis* (Vodopianov, 1988) have been described. Immunoblotting of pH 6 Ag clones and *Y. pestis* expressing pH 6 Ag with anti-*Y. pestis* fibrillar antiserum obtained from Russia (Vodopianov, 1988; 1990; see the Experimental procedures) indicates that the fimbriae described by Vodopianov are at least partly composed of *PsaA*. The fimbriae composed of the YopA protein in *Y. pseudotuberculosis* and *Y. enterocolitica* (Kapperud et al., 1987) are not synthesized by *Y. pestis* because of a point mutation in the coding region of the gene (Skurnik and Wolf-Watz, 1989).

Various enteric major pili subunit proteins contain little amino acid similarity (Paranchych and Frost, 1988) over their entire protein sequence. Accordingly, *PsaA* did not contain any significant amino acid sequence similarity to other fibrillin proteins. However, we found a limited region of similarity near the carboxyl terminus of *PsaA* with the *E. coli* Pap pilus adhesin, PapG. Hultgren et al. (1989) demonstrated that the region of amino acids around residues 301 to 314 of PapG is necessary for the adhesin to interact with the chaperone protein, PapD. The similarity we noted between *PsaA* and PapG encompasses this region of the *E. coli* protein. Also, the similarity noted between *PsaA* and the influenza A virus haemagglutinin (Fig. 3) suggests that amino acids 52 to 77 of the *Y. pestis* fibrillin may be involved in binding of the fimbriae to host cells. However, further structure-function studies will be necessary to determine if these regions of *PsaA* are involved in chaperone binding and haemagglutination.

The newly designated *Y. pestis psaB* locus encodes a protein which appears to be a member of a group of molecular chaperone proteins (see Fig. 4 and Results). This conclusion is supported by our previous observation that mutation of DNA in the *psaB* region resulted in decreased accumulation of the 15kDa mature form of *PsaA* (Lindler et al., 1990). Bakker et al. (1991) have shown that the *E. coli* K88ab pilus chaperone, FaeE, is responsible for protecting the pilin protein from proteolytic degradation as well as preventing premature polymerization of pilin into pilus structures on the surface of the bacterium. Thus, the decrease in the accumulation of mature *Y. pestis* *PsaA* in *psaB* mutants may be due to increased proteolysis of the pH 6 Ag fibrillin subunit.

The induction of *Y. pestis* pH 6 Ag fimbriae by growth at
Acidic pH and mammalian body temperature is unusual among bacterial fimbriae. Expression of most E. coli fimbriae occurs when the bacteria are cultured at 37°C (de Graaf, 1990). Only the Vibrio cholerae toxin-coregulated pilus (tcpA) has been shown to be induced when bacteria were grown at pH 6.5 (Taylor et al., 1987). However, unlike Y. pestis psaA expression, tcpA synthesis is maximum when cultivation of the bacteria is at 30°C. To date, only the psaE and psaB loci (see Results and Lindler et al., 1990) are known to affect the expression of Y. pestis pH 6 Ag. In Y. pestis psaE mutants, pH 6 Ag expression is regulated normally although the amount of expression is greatly reduced (Lindler et al., 1990). The lack of similarity between PsaE and other fimbrial regulatory proteins may reflect the novel regulation of expression of Y. pestis pH 6 Ag. Further studies will be required to evaluate the role of PsaE and other regulatory elements in the expression of pH 6 Ag fimbriae.

Y. pestis pH 6 Ag has been shown to be expressed in vivo (Ben-Efraim et al., 1961). These studies also showed that infection of mice with Y. pestis already expressing pH 6 Ag was more rapidly fatal to animals when compared with infection with bacteria not synthesizing the Ag. Previously (Lindler et al., 1990), we found that mutation at the psaA locus resulted in a 200-fold increase in the LD₉₀ (50% lethal dose) of the mutant bacteria compared with the wild-type parent Y. pestis when mice were challenged by the intravenous route of infection. Also, the interaction of Y. pestis with host macrophages is important for the pathogenesis of plague (Cavanaugh and Randall, 1959). Expression of pH 6 Ag inside macrophages was observed in our studies (Fig. 8) as well as by others (Vodopianov et al., 1990). Taken together, these facts may lend some insight into the function of the Ag during infection by Y. pestis. Bacterial fimbriae primarily function as mediating attachment of bacteria to host cells. Induction of expression of Y. pestis pH 6 fimbriae inside macrophages may allow the pathogen to interact with other uninjured macrophages or other host cells after the bacteria are released from the infected cell. Infections caused by Y. pseudotuberculosis are usually not systemic but rather are localized infections resulting in acute ileitis and mesenteric lymphadenitis (Butler, 1983). Accordingly, the high conservation of pH 6 Ag fimbriae at the genetic and immunologic levels we observed between Y. pestis and Y. pseudotuberculosis supports the possibility that these fimbriae facilitate the initial stage of pathogenesis. Further investigation of the role of pH 6 Ag in the pathogenesis of plague infection will require the construction of Y. pestis pigmentation-positive psaA⁻ mutants. Also, the expression of pH 6 fimbriae on the surface of bacteria could facilitate their entry into macrophages or other host cells. Experiments towards these ends are currently under way.

**Experimental procedures**

**Bacterial strains, plasmids, media and growth conditions**

Routine cultivation of Yersinia strains was in brain–heart infusion broth (BHI; Difco Laboratories) or on BHI agar. When Yersinia was to be cultured at 37°C, BHI was supplemented (SBHI) with 2.5 mM CaCl₂, 0.5% yeast extract and 0.2% xylose (Lindler et al., 1990). The pH of the medium was adjusted before sterilization to either 8 or 6 with 10 N NaOH or 12 N HCl, respectively. Y. pestis strains KIM-3001 (psaA⁻) and KIM-3001.1 (psaA⁺) have been described previously (Lindler et al., 1990). Y. pseudotuberculosis strains were kindly provided by Dr Susan C. Straley, Department of Microbiology and Immunology, University of Kentucky, Lexington, KY, USA. Y. enterocolitica WA (serotype 0:8), YE 288 (serotype 0:3) and YE 312 (serotype 0:34) were provided by Dr Peter Feng, Food and Drug Administration, Washington, D.C., USA.

E. coli was routinely cultured in LB or on LB agar plates at 37°C (Ausubel et al., 1989; Maniatis et al., 1989). The standard host for cloning experiments was E. coli HB101 obtained from Bethesda Research Laboratories (BRL). M13 phage derivatives were propagated in E. coli XL1-Blue (recA1, endA1, gyrA96, thiI, hsdR17, supE44, relA1, lac, F° proAB, lacZΔM15, Tn10) obtained from Stratagene Cloning Systems.

Plasmids pPSN1, pDG1, pDG4, pDG6 and pDG9 containing the cloned pH 6 Ag of Y. pestis KIM5 were described previously (Lindler et al., 1990). Plasmid pDG5 contains a 9 kb ClaI fragment of Y. pestis KIM-5 chromosomal DNA present in pDG4 (Lindler et al., 1990) cloned into the vector pC20R (Marsh et al., 1984). All of the genetic material shown in Fig. 1 is present in pDG5 plus 2.5 kb of DNA to the left of the KpnI site. Plasmid pDG27 contains the 6.5 kb KpnI to ClalDNA fragment from pDG6 in the pSK⁺ vector (Stratagene). The direction of transcription of the Y. pestis genes psaE, psaA, psaB and ORF4 in pDG27 is the same as that of the T7 promoter present in pSK⁺.

For selection of antibiotic-resistance phenotypes, the following antibiotic concentrations were used (µg ml⁻¹): chloramphenicol, 25; ampicillin, 100; streptomycin, 100; and tetracycline, 25.

**Recombinant DNA techniques and DNA sequencing**

Restriction endonucleases, T4 DNA ligase and frozen E. coli competent cells were purchased from BRL. Plasmid DNA was purified from E. coli hosts with the Qiagen midi-plasmid purification kit (Qiagen). Rapid screening of bacteria for plasmid DNA was as described previously (Del Sal et al., 1988).

DNA restriction fragments were separated on 0.7% agarose gels (Maniatis et al., 1989) and transferred to nitrocellulose filters as described by Southern (1975). A DNA probe specific for the Y. pestis psaA sequence was generated by the PCR as described below. The PCR reaction was initiated by oligonucleotides which were homologous to bp 2155 to 2180 and the inverse complement of bp 2524 to 2547 (Fig. 2). The DNA probe was labelled using the random primers DNA labelling system (BRL). High-stringency filter hybridization and post-hybridization washes were as described (Silhavy et al., 1984). Low stringency was achieved using similar conditions except that 25% formamide was included in the hybridization solution. Post-hybridization washes under low-stringency conditions...
were similar to those above except that the final two washes were in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at 30°C. Washed and dried filters were autoradiographed at −70°C with X-omat AR film (Eastman Kodak Co).

Initial DNA sequencing was on single-stranded templates generated from M13 derivatives (Ausubel et al., 1989). A 3.1 kb KpnI to BamHI fragment liberated by restriction digestion of pDG9 was ligated into similarly cleaved M13mp18 and M13mp19 as described elsewhere (Ausubel et al., 1989). This 3.1 kb DNA fragment included the psaE, psaA and the 5' end of the psaB loci of Y. pestis. The nucleotide sequence of the remainder of the psaB locus and downstream material was obtained from double-stranded DNA template, pDG27. The DNA sequence of both strands was determined using overlapping oligonucleotide primers. Primers were synthesized on an Applied Biosystems Incorporated (ABI) Model 380b oligonucleotide synthesizer. Nucleotide sequences were determined by the chain termination method (Sanger et al., 1977) using Sequenase version 2.0 (United States Biochemical).

DNA Sequence manipulation was with the PC/Genie software package (Intelligenetics Corp.). Protein or nucleotide database searches and alignments were with the Genetic Computer Group (GCG) sequence analysis software package for the VAX computer (Devereux et al., 1984).

**Protein gel electrophoresis, the PCR and in vitro transcription and translation**

Linear DNA templates were generated by the PCR. The PCR reactions contained, in a 100 µl volume: 10 ng of pDG27 template DNA, 50 pmol of oligonucleotide primers and Hot Tub Polymerase (Amersham Corp.) according to the manufacturer's specifications. Oligonucleotide primers used to initiate polymerization at the 3' end of the DNA fragments were as follows: fragment a, GGAGCGCTCAATAGCC; fragment b, GCTTTACATTGCTGTGTTTGC; fragment c, GCATAAGGTAAAGACACC; fragment d, CCAAGGAGCAGCTATCCCGC. The DNA primer that initiated synthesis at the 5' end of the above sequences was the T7 promoter primer, TAATACGACTCACTATAGGG. The annealing times and temperatures were maximized for each primer combination to yield the specific synthesis of the linear fragment. Linear DNA fragments were purified by the Qiagen PCR purification kit (Qiagen Inc.). *In vitro* [35S]-methionine labelling of proteins encoded by the above linear and plasmid DNA templates was with a commercially available *E. coli* S30 extract (Promega Corp.). Reactions were according to the manufacturer's directions except that they contained 50 units of T7 RNA polymerase (BRL).

*In vitro* [35S]-methionine-labelled proteins were separated on 4–20% denaturing polyacrylamide gels (SDS–PAGE) according to the method of Laemmli (1970). After electrophoresis, gels were impregnated with Enhance (New England Nuclear), dried, and fluorographed at −70°C.

**RAW264.7 infection and Western blotting**

Murine macrophage cell line RAW264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C in an atmosphere of 7.5% CO2 as described previously (Kelly et al., 1991). Before infection with *Y. pestis*, macrophages were treated with 0.05% trypsin and 0.53 mM EDTA then dislodged from the tissue culture flask. Suspended macrophages were washed three times with DMEM and resuspended in like medium to a concentration of approximately 3–5 × 10⁶ per ml. A sample of *Y. pestis* which had been grown overnight at 30°C was added to the macrophage suspension to give a multiplicity of infection (m.o.i.) of approximately one bacterium per macrophage. The infected mixture of cells was centrifuged at 500 × g for 5 min at room temperature. After centrifugation, the pelleted bacteria and macrophages were incubated at 37°C for 5 min. The supernatant was decanted and the cells were then suspended in fresh DMEM. Samples of 0.1 ml were placed into 96-well culture plates (Corning 25860, Corning Glass Works) and incubation continued at 37°C for 20 min. The medium was removed from each well and replaced with like medium containing 5 µg/ml gentamicin. In some experiments, various concentrations of monensin were added to the DMEM containing gentamicin. After 30 min further incubation at 37°C, an initial time zero sample was taken. Samples were prepared by washing groups of 10 wells three times with Hanks' balanced salt solution (HBSS). Samples for determination of viable bacterial counts were pools of five of these wells that had been overlaid with 0.1 ml of ice-cold sterile water. After complete lysis of the macrophage cells, the remaining bacteria were appropriately diluted in 0.9% NaCl and inoculated onto BHI agar plates. The cfu ml−1 sample was determined after incubation at 30°C for 48 h. Using these macrophage infection conditions, uptake of *Y. pestis* ranged from 30 to 60% of the input bacteria. Samples for Western blot analysis consisted of the contents of five wells extracted with a total of 0.1 ml SDS–PAGE sample buffer (Laemmli, 1970). The extracts were heated at 95°C for 15 min and stored at −20°C. Similar extracts were made at the times indicated. Macrophage culture medium was changed with fresh medium when the growth medium became acidic.

Proteins from the above extracts were electrophoresed and transferred to nitrocellulose (Towbin et al., 1979) then processed as described previously (Lindler et al., 1990). Primary antibody (Ab) was pH 6 Ag-specific rabbit polyclonal antiserum diluted 1:1000 (Lindler et al., 1990). To determine if pH 6 Ag was immunologically cross-reactive with the *Y. pestis* EV76 temperature- and pH-induced fimbriae previously described (Vodopianov, 1988), primary antibody was rabbit anti-*Y. pestis* fimbrial antiserum kindly provided by Boris Mishankin, Research Anti-plague Institute, Rostov-on-Don, Russia. The latter antiserum was reacted with Western blots of crude whole-cell extracts of *E. coli* pH6 Ag clones and *Y. pestis* expressing the Ag or negative controls. Secondary antibody was biotinylated donkey-anti-rabbit serum (Amersham) diluted 1:1000. Immunoreactive protein was visualized by reaction with streptavidin horseradish peroxidase (Amersham) and the 3,3',5'-tetramethylbenzidine dihydrochloride (TMB) horseradish peroxidase substrate system (Kirkegaard and Perry Laboratories).

**Haemagglutination assay**

Heparinized SRBCs were washed three times in normal saline (NS; 0.9% NaCl) and suspended in the wash solution to 0.3% (v/v). *E. coli* expressing pH 6 Ag or negative controls were grown overnight at 37°C in LB. *Y. pestis* strains were grown...
overnight in SBHI pH 6 at 37°C. E. coli and Y. pestis were aerated by agitation at 120 r.p.m. in a New Brunswick Innova Model 4300 shaking incubator (New Brunswick Scientific). For haemagglutination assay, 0.1 ml of washed SRBCs was mixed with an equal volume of NS-washed overnight bacterial culture. Bacteria were mixed with SRBCs in a 1.5ml microcentrifuge tube followed by incubation for 2h at 37°C. NS was included as a negative control. After incubation, tubes were observed macroscopically for agglutination of the SRBCs. Tubes which appeared negative by macroscopic examination were also examined microscopically.

Electron microscopy and immunogold labelling

E. coli was grown in LB overnight at 37°C. Y. pestis KIM-3001.1 (psaA) or the isogenic wild-type strain were grown at 37°C and pH 6 as described (see above and Lindler et al., 1990). Y. pseudotuberculosis PB1/+ was grown at 37°C in SBHI adjusted to either pH 8 or 6 until cultures reached mid-log phase. Bacteria were washed twice and suspended in distilled water. The bacterial suspension was placed on carbon coated 300-mesh copper grids then negatively stained with 0.5% uranyl acetate or 1% phosphotungstic acid (PTA) pH 7.2 and examined directly with a Phillips 400 HM transmission electron microscope operated at an accelerating voltage of 80 kV.

Immunogold labelling was as described elsewhere (Beesley, 1989). Briefly, bacteria suspended in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g NaH2PO4, 0.24 g KH2PO4 per litre, pH adjusted to 7.4 with HCl) were deposited on 0.25% formvar carbon coated 300-mesh copper grids and partially dried. Primary antibody (Ab) was polyclonal Y. pestis pH 6 Ag-specific sera (Lindler et al., 1990) diluted as indicated in fig. legends 6 and 7. In some experiments, Y. pestis-containing grids were reacted with mAb 6H3, which recognizes the F1 capsular Ag; 6H3 was kindly provided by Dr John Ezzet, Department of Bacteriology, United States Army Research Institute of Infectious Disease, F. Detrick, MD, USA. The secondary Ab was goat anti-rabbit or anti-mouse IgG labelled with 10 nm gold particles diluted 1:10. After final washing with distilled water, the labelled bacteria were stained with 1% PTA pH 7.2, and examined as described above.

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References

Air, G.M. (1981) Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. Proc Natl Acad Sci USA 78: 7639-7643.

Allen, B.L., Gerlach, G., and Clegg, S. (1991) Nucleotide sequence and functions of mtk determinants necessary for the expression of type 3 fimbriae in Klebsiella pneumoniae. J Bacteriol 173: 916-920.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989) Current Protocols in Molecular Biology. New York: Wiley.

Bakker, D., Vader, C.E.M., Roosendaal, B., Mooi, F.R., Oudega, B., and de Graaf, F.K. (1991) Structure and function of the periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic Escherichia coli. Mol Microbiol 5: 875-886.

Beesley, J.E. (1989) Colloidal gold: immunonegative staining method. In Colloidal Gold: Principles, Methods and Applications. Vol. 2. Hayat, M.A. (ed.). London: Academic Press, Press, pp. 243-254.

Ben-Efraim, S., Aronson, M., and Bichowsky-Slomnicki, L. (1961) New antigenic component of Pasteurella pestis formed under specific conditions of pH and temperature. J Bacteriol 81: 704-714.

Bichowsky-Slomnicki, L., and Ben-Efraim, S. (1963) Biological activities in extracts of Pasteurella pestis and their relation to the pH 6 antigen. J Bacteriol 86:101-111.

Brubaker, R.R. (1972) The genus Yersinia: biochemistry and genetics of virulence. Curr Topics Microbiol 57:111-158.

Butler, T. (1983) Plague and other Yersinia infections. In Current Topics in Infectious Disease. Greenough, W.R., and Merigan, T.C. (eds.) New York: Plenum Medical Book Company.

Cavanaugh, D.C., and Randall, R. (1959) The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague. J Immunol 83: 348-363.

Charnetzky, W.T., and Shutford, W.W. (1985) Survival and growth of Yersinia pestis within macrophages and an effect of the loss of the 47-megadalton plasmid on growth in macrophages. Infect Immun 47: 234-241.

Chen, T.H., and Elberg, S.S. (1977) Scanning electron microscopic study of virulent Yersinia pestis and Yersinia pseudotuberculosis type I. Infect Immun 15: 972-977.

de Graaf, F.K. (1990) Genetics of adhesive fimbriae of intestinal Escherichia coli. Curr Topics Microbiol Immunal 151: 29-53.

Del Sal, G., Manfioletti, G., and Schneider, C. (1988) A one-tube plasmid mini-preparation suitable for sequencing. Nucl Acids Res 16: 9878.

Devereux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 12: 387-407.

Firon, N., Olek, I., and Sharon, N. (1984) Carbohydrate-binding sites of the mannose-specific fimbrial lectins of enterobacteria. Infect Immun 43: 1086-1090.

Galyov, E.E., Karlishv, A.V., Chernovskaya, T.V., Dolgikh, D.A., Smirnov, O.Y., Volkovoy, K.I., Abramov, V.M., and Zavyalov, V.P. (1991) Expression of the envelope antigen F1 of Yersinia pestis is mediated by the product of catM gene having homology with the chaperone protein PapD of Escherichia coli. FEBS Lett 286: 79-82.

Giron, J.A., Ho, A.S.Y., and Schoolnik, G.K. (1991) An inducible bundle-forming pilus of enteropathogenic Escherichia coli. Science 254: 710-713.

Holmgren, A., and Branden, C. (1989) Crystal structure of chaperone protein PapD reveals an immunoglobulin fold. Nature 342: 248-251.

Holmgren, A., Kuehn, M., Branden, C., and Hultgren, S. (1992)
Conserved immunoglobulin-like features in a family of periplasmic pili chaperones in bacteria. *EMBO J* **11**: 1617–1622.

Horwitz, M.A., and Maxfield, F.R. (1984) *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *J Cell Biology* **99**: 1936–1942.

Hultgren, S.J., Lindberg, F., Magnusson, G., Kihlbreg, J., Tennent, J.M., and Normark, S. (1989) The PapG adhesin of uropathogenic *Escherichia coli* contains separate regions for receptor binding and for the incorporation into the pilus. *Proc Natl Acad Sci USA* **86**: 4357–4361.

Jalajakumari, M.B., Thomas, C.J., Halter, R., and Manning, P.A. (1989) Genes for biosynthesis and assembly of CS3 pilus of CFA/II enterotoxigenic *Escherichia coli*: novel regulation of pilus production by bypassing an amber codon. *Mol Microbiol* **3**: 1685–1695.

Kasperud, G., Namork, E., Skurnik, M., and Nesbakken, T. (1987) Plasmid-mediated surface fimbriae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to the outer membrane protein Yop1 and possible importance for pathogenesis. *Infect Immun* **55**: 2247–2254.

Kelly, N.M., Young, L., and Cross, A.S. (1991) Differential induction of tumor necrosis factor by bacteria expressing rough or smooth lipopolysaccharide phenotypes. *Infect Immun* **59**: 4491–4496.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Levine, M.M., Ristaino, P., Marley, G., Smyth, C., Knutton, S., Boedeker, E., Black, R., Young, C., Clements, M., Cheney, C., and Pataki, R. (1984) Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxicogenic *Escherichia coli*: morphology, purification and immune responses in humans. *Infect Immun* **44**: 409–420.

Lindler, L.E., Klempner, M.S., and Straley, S.C. (1990) *Yersinia pestis* pH 6 antigen: genetic, biochemical and virulence characterization of a protein involved in the pathogenesis of bubonic plague. *Infect Immun* **58**: 2569–2577.

Lund, B., Lindberg, F., Marklund, B., and Normark, S. (1987) The PapG protein is the \(\alpha\)D-galactopyranosyl-(1-4)-\(\beta\)D-galactopyranose-binding adhesin of uropathogenic *Escherichia coli*. *Proc Natl Acad Sci USA* **84**: 5998–5992.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Marsh, J.L., Erle, M., and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection of insertional inactivation. *Gene* **32**: 481–485.

Mooi, F.R., Claassen, I., Bakker, D., Kuipers, H., and de Graaf, F.K. (1986) Regulation and structure of an *Escherichia coli* gene coding for an outer membrane protein involved in the export of K88ab fimbrial subunits. *Nucl Acids Res* **14**: 2443–2457.

Norgren, M., Båga, M., Tennent, J.M., and Normark, S. (1987) Nucleotide sequence, regulation and functional analysis of the *papC* gene required for cell surface localization of Pap pilus of uropathogenic *Escherichia coli*. *Mol Microbiol* **1**: 169–178.

Old, D.C., and Adembola, R.A. (1984) Relationships among broad-spectrum and narrow-spectrum mannose-resistant fimbrial hemagglutinins in different *Yersinia* species. *Microbiol Immunol* **28**: 1303–1311.

Olsen, A., Jonsson, A., and Normark, S. (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* **338**: 652–655.

Paranchych, W., and Frost, L.S. (1988) The physiology and biochemistry of pili. *Adv Microb Physiol* **29**: 53–113.

Pollack, C., Straley, S.C., and Klemmep, M.S. (1986) Probing the phagolysosomal environment on human macrophages with a Ca\(^{2+}\)-responsive operon fusion in *Yersinia pestis*. *Nature* **322**: 834–836.

Roosendaal, B., and de Graaf, F.K. (1989) The nucleotide sequence of the *fanD* gene encoding the large outer membrane protein involved in the biosynthesis of K99 fimbriae. *Nucl Acids Res* **17**: 1263.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467.

Shine, J., and Dalgarno, L. (1974) The 3'-terminal sequence of the *Escherichia coli* 16S ribosomal RNA complementary to nonsense triplets and ribosome-binding sites. *Proc Natl Acad Sci USA* **71**: 1342–1346.

Silhavy, T.J., Berman, M.L., and Enquist, C.M. (1984) *Experiments with Gene Fusions*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Skurnik, M. (1984) Lack of correlation between the presence of plasmids and fimbriae in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *J Appl Bacteriol* **56**: 355–363.

Skurnik, M., and Wolf-Watz, H. (1989) Analysis of the yopA gene encoding the Yop1 virulence determinants of *Yersinia* spp. *Mol Microbiol* **3**: 517–529.

Souther, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**: 503–517.

Straley, S.C. (1991) The low-Ca\(^{2+}\) response virulence regulon of human-pathogenic yersiniae. *Microb Patho* **10**: 87–91.

Straley, S.C., and Brubaker, R.R. (1981) Cytoplasmic and membrane proteins of *yersinia* cultivated under conditions simulating mammalian intracellular environment. *Proc Natl Acad Sci USA* **78**: 1224–1228.

Straley, S.C., and Harmon, P.A. (1984a) Growth in mouse peritoneal macrophages of *Yersinia pestis* lacking established virulence determinants. *Infect Immun* **45**: 649–654.

Straley, S.C., and Harmon, P.A. (1984b) *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. *Infect Immun* **45**: 655–569.

Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987) Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* **84**: 2833–2837.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrofretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354.

Vodopianov, S.O. (1988) Double control of pili formation in *Yersinia pestis*. *Mikrobiol Zh* **50**: 40–45.

Vodopianov, S.O. (1990) The effect of a 37°C/low pH signal on cells of *Yersinia pestis*. *Mikrobiol Zh* **52**: 88–92.

Vodopianov, S.O., Popova, G.O., Vasileva, G.I., and Mischankin, B.N. (1990) The phenomenon of pilus formation in the interaction of *Yersinia pestis* with macrophages in experimental animals. *Zh Mikrobiol Epidemiol Immunobiol* **3**: 3–6.

Von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. *Nucl Acids Res* **14**: 4683–4690.

Wileman, T., Harding, C., and Stahl, P. (1985) Receptor-mediated endocytosis. *Biochemistry* **232**: 1–14.
