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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 PsaA. *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 *Eco*RI–*Bam*HI fragment of *Y. pestis* chromosomal DNA. We also isolated a single Tn*10lacZ* 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 *Eco*RI 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 *Bam*HI 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 *Bam*HI 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

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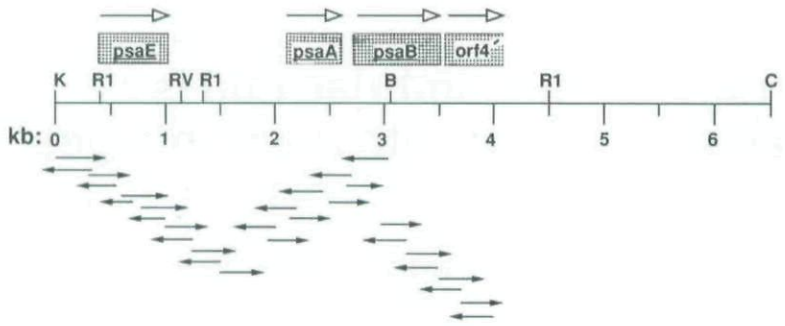

Fig. 1. Nucleotide sequencing strategy of the *Y. pestis* pH 6 Ag locus and surrounding DNA. Open arrows indicate the directions of transcription of the various loci. Shaded boxes indicate coding regions of the *Y. pestis* genes. Restriction enzyme recognition sites are as follows: B, *Bam*HI; C, *Cla*I; R1, *Eco*RI; RV, *Eco*RV; and K, *Kpn*I.

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 dI1734 and Tn*10lacZ* 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 Tn*10lacZ* mutation which defined *psaE* (Lindler *et al.*, 1990). We determined the position of the transposon insertion in pPSN1 (*psaE*::Tn*10lacZ*) by comparing the nucleotide sequences of the mutant plasmid with the non-mutagenized DNA. The Tn*10lacZ* 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 nonsense 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 (1974) sequence 9 bp 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 PsaA protein (Fig. 2) has a predicted pI 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* P 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% for F1 chaperone to 47% with the K88 chaperone. 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.

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        KpnI
1       GGTACCCTGCTCGCGGGCGGCTTGTATGCCATGCTGAAACGCCCTGACGCGATGGTGGCGAAGGTCGCGTAACGCTCAACGTGCCGGCGCGTTTCAGATC
101     GAATCATCGGCGCTTGTCAGCAGTCTGAAATGCCAGTCAGTTAATTCTGACTGGCATTTTTTATGGGTGTTATAAAATCAGTCTCATTTTAATGAAATGC
201     GAAAATAAAAGGCAGCTAATATTTTTTTGTTAAATTATTAAGATAATAATCTTAATAAGACTTAATCTTGTGCTGGGTTGACTTTTGTTTAAATATTGTT
                                                                                EcoRI
301     TAGCTTTTGTGTATAAAATTTTTCCAATTAAGACTCACTTATGGCTATTGAGCCGTCCTAATATTCTGAATTCCAGATCAGGGGAGGTGAGGGCAAATGA
                                                                                                    M  S
                                                                                                psaE
401     GTCACTGTGTTGTTTTAAATAAATTAGAAAGTGTGTTAATAATTGGTGACAGTAGATATGCTTTATCCAAGAATGAGGTGCTGCTGTTAGAGTGTCTTTA
          H  C  V  V  L  N  K  L  E  S  V  L  I  I  G  D  S  R  Y  A  L  S  K  N  E  V  L  L  L  E  C  L  Y

501     TTTACGTGCCGGTGATGTGATCTCCCATGATGAATTACTGACAACCTGTTGGCCTGATCGAGTCGTCTCCCCAACATCACTGCCGGTTGCTATTAAACAT
          L  R  A  G  D  V  I  S  H  D  E  L  L  T  T  C  W  P  D  R  V  V  S  P  T  S  L  P  V  A  I  K  H

601     ATCCGTGATGTATTCCGTAAAATAACCCGGAGTGAGGTGATAAAGACATATAAGAATGAGGGCTATAGCTATCAAAAGGATAGTGTACTAATTATTATTG
          I  R  D  V  F  R  K  I  T  R  S  E  V  I  K  T  Y  K  N  E  G  Y  S  Y  Q  K  D  S  V  L  I  I  I  D
                                          *
701     ATGATGGCAGCACCGAAAAAGAGAGTCATAGCGCAGCATATACCAGAAAGGAAAAACCCGACATACCAATAAAATTGGTTGGATTACAAATACTTTCTCA
            D  G  S  T  E  K  E  S  H  S  A  A  Y  T  R  K  E  K  P  D  I  P  I  K  L  V  G  L  Q  I  L  S  H

801     CCTGAACTCAACCTTTTTTATCGCTATTATGATGGTCATCATCATCATATTTTTCATGGTAGGAGGTAATGATATTGTCTCATTTATTGATAGTGATACC
          L  N  S  T  F  F  I  A  I  M  M  V  I  I  I  I  F  F  M  V  G  G  N  D  I  V  S  F  I  D  S  D  T

901     AACAGTGTCATTATAACGAATGTGACAACCAAGATGAATGGCCCAACGGCAGGCTTACCGAAGGTGAAGAATAGTATGATATTTAAGGATGATTTTGGTT
          N  S  V  I  I  T  N  V  T  T  K  M  N  G  P  T  A  G  L  P  K  V  K  N  S  M  I  F  K  D  D  F  G  L

1001    TAGTCATTATCTGTGATCAATCGGAATGCAAACAGCAATGAAAGCAAAATCACTTACTCTCATATCTATAACAGTAATGTTTTTCTTATTTTTAATCTAT
          V  I  I  C  D  Q  S  E  C  K  Q  Q
                                          EcoRV
1101    AGCTTTAATGACTTATTTTTTTATTCTGAAGTGAAATATGGCGATATCCATGAGCATCTTGATTTAAGAATGCAAGGGATAAGATTTAGCCTCTCACATT
1201    ATATTATTGATGATAAGTCTCAACTGGTGATTTCAGAGGGAATATATGGTATTGGTTTGAAAATGCCAACGGGGAAGTATTACCTATTCCCTCTTCATTC
                                          EcoRI
1301    ATATCAGTCATCCCCTGATAATATGGCCAGAGGATCGCTGAATTCATTGGCCTCACCGTTGTCTTTGTATGTCTATGAAATACATAATAAAAAAAATAAC
1401    GTTGTCACCTTTTTTAATGGTGACAGAGGCTTTATTGATGTCAATGGAGAAACGATCCACTTATCTTCTCTGTTTCTCGGGGTACAAGGAGAACATATCC
1501    ATACGTCCTATCATGACGTTAGTTAATCATTTTATCTATTGATTTTCTATTTTAGATGACATTTTTAAATAATAAATATGGCGTCTGTCTATATTGGTAT
1601    CTTTAGAACCGTTTTTTACTCCTTAGGTACTATGCTCATCATTATTAATGCTCTTTCATACAGATAATAATCAACTAAAGCATGAGAGTTAATGTCATGA
1701    TAGATAATGAAATAAAAGCGTCAAATAGCATTGGGTTGTTTTCCAAGATTAATCTTAACATTATTATTTAAAATTTAATCATTAATAATTTTAAACATCC
1801    AGAAGTGGGTTATATGATGACGCTGTTAGTGGTGATCGGGAGTTGCAAAAAATAAAGTTGCAGAAAACACAACCACAACAGGAACGAAACTGGAAACTGG
1901    TGTTTCGGGGAAAATAGGATTTATTTTCTCTATCTATAGGTCTGCGACTGAGTTATTAATATAAATATATTGATTTACAATGTAGTTATATTTAATTGCC
2001    CAATATCACCACTAGCATATTCTACGCTATGTCGTAATAGCAATTATTACATTTAATGGAGACTGTTCTCATGAAAATGAAATGTTTTGCGAAAAATGCG
                                                                                M  K  M  K  C  F  A  K  N  A
                                                                            psaA
2101    CTGGCGGTTACCACACTAATGATCGCTGCTTGTGGTATGGCAAACGCTTCTACTGTCATTAACTCCAAGGATGTTTCTGGTGAGGTGACTGTCAAGCAGG
        L  A  V  T  T  L  M  I  A  A  C  G  M  A  N  A  S  T  V  I  N  S  K  D  V  S  G  E  V  T  V  K  Q  G

2201    GAAACACATTCCACGTCGATTTTGCGCCTAACACAGGAGAGATTTTTGCGGGTAAACAGCCGGGTGATGTCACTATGTTTACGCTAACTATGGGTGATAC
          N  T  F  H  V  D  F  A  P  N  T  G  E  I  F  A  G  K  Q  P  G  D  V  T  M  F  T  L  T  M  G  D  T

2301    TGCACCACACGGTGGTTGGCGTTTGATTCCAACAGGGGACTCAAAAGGTGGATATATGATCAGCGCCGATGGTGACTATGTTGGTTTATACAGTTATATG
          A  P  H  G  G  W  R  L  I  P  T  G  D  S  K  G  G  Y  M  I  S  A  D  G  D  Y  V  G  L  Y  S  Y  M

2401    ATGTCATGGGTAGGTATAGATAATAACTGGTATATAAATGATGACTCTCCTAAAGATATAAAAGATCATCTGTACGTTAAGGCAGGGACTGTCCTTAAAC
        M  S  W  V  G  I  D  N  N  W  Y  I  N  D  D  S  P  K  D  I  K  D  H  L  Y  V  K  A  G  T  V  L  K  P

2501    CAACGACTTATAAATTCACGGGGCGTGTTGAAGAGTATGTATTTTAAATAACGAGTAATCATAGAACATGATGGCTTGCTGTCATGTTCTATGTATGTTT
          T  T  Y  K  F  T  G  R  V  E  E  Y  V  F

2601    TTGTTATTGATGTTTTATTGTGAGTTTTTTTAAAATTCACAATAAAATATCATGATAATGTGAGTGATTAAAAATGAAGAATCTATTTTTTTCTGCATAT
                                                                                M  K  N  L  F  F  S  A  Y
                                                                            psaB
2701    AAAAAAGTATTTTCTTATATCACATCAATAGTCATATTCATGGTGTCTTTACCTTATGCTTATTCTCAAGATGTTGTTGTCAATACGACTAAGCATCTTT
        K  K  V  F  S  Y  I  T  S  I  V  I  F  M  V  S  L  P  Y  A  Y  S  Q  D  V  V  V  N  T  T  K  H  L  F

2801    TTACTGTAAAAATAGGGACAACGCGGGTCATTTACCCTTCGTCTTCGACAAAAGGGGTATCCGTATCGGTGGCTAATCCACAGGATTATCCAATATTGGT
          T  V  K  I  G  T  T  R  V  I  Y  P  S  S  S  T  K  G  V  S  V  S  V  A  N  P  Q  D  Y  P  I  L  V

2901    ACAAACCCAAGTTAAAGATGAAGATAAGACGTCGCCCGCTCCTTTTATTGTTACCCCTCCTTTATTCAGGCTTGATGCTGGGTTACAAGGCCGTGTACGT
          Q  T  Q  V  K  D  E  D  K  T  S  P  A  P  F  I  V  T  P  P  L  F  R  L  D  A  G  L  Q  G  R  V  R
                                                                            BamHI
3001    ATTATTCGGACCGGTGGAAAATTCCCTGAAGATCGTGAAACACTGCAATGGTTGTGCCTAACGGGGATCCCACCTAAGAACGGTGATGCTTGGGGCAATA
        I  I  R  T  G  G  K  F  P  E  D  R  E  T  L  Q  W  L  C  L  T  G  I  P  P  K  N  G  D  A  W  G  N  T
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```
3101    CGCAAAATAATCCAAAAAATTCATCCCCAACTATGGATATTCAAATGTCTATCAGTACCTGCATAAAATTATTATTCAGACCCGATAAAGTTAAAGGAGA
          Q  N  N  P  K  N  S  S  P  T  M  D  I  Q  M  S  *  S  T  C  I  K  L  L  F  R  P  D  K  V  K  G  D

3201    TCCGACGGATAGTGCGGATTCATTAACTTGGAGATATAAAGGTAACTACTTGGAAGTTAATAATCCAACGCCATTCTACATGAATTTTTATTCACTCCGT
          P  T  D  S  A  D  S  L  T  W  R  Y  K  G  N  Y  L  E  V  N  N  P  T  P  F  Y  M  N  F  Y  S  L  R

3301    ATTGGTGATGAAAAAATAAATTTATCTGATTTAGGTTCAAAGGATGAAATAAAAAATGGCAGCTATGTTCCGCCATTCTCTTCTAGGGATTTTATTATTC
          I  G  D  E  K  I  N  L  S  D  L  G  S  K  D  E  I  K  N  G  S  Y  V  P  P  F  S  S  R  D  F  I  I  P

3401    CGGTAAAAAATAAAGGTAAGGCAACAGAGGTTTTTTGGCAAGTGATTAATGATAATGGTGGTGTGAGCCGGGAATTTAAAAGCACAGTCCAGTAAGTTTC
          V  K  N  K  G  K  A  T  E  V  F  W  Q  V  I  N  D  N  G  G  V  S  R  E  F  K  S  T  V  Q

3501    TATTTATATTTTTCAGAAAATACCTGGGGTATTATTTTGAAAAAATTAATAGTTCAATTTACTACTATTACTCTATTGATGAGCACTTCATTTTTAGTGG
                                                                                                  M  S  T  S  F  L  V  G
                                                                                          orf4'
3601    GTGCCCAGCGTTATTCTTTTGATCCCAATCTGCTAGTGGATGGCAATAACAACACTGATACCTCGTTATTTGAACAGGGCAATGAATTACCGGGCACCTA
          A  Q  R  Y  S  F  D  P  N  L  L  V  D  G  N  N  N  T  D  T  S  L  F  E  Q  G  N  E  L  P  G  T  Y

3701    TTTGGTGGATATTATCTTGAATGGGAATAAAGTGGACTCTACGAATGTGACATTTCATTCGGAGAAATCGCCATCAGGAGAGCCTTTCTTGCAATCTTGC
          L  V  D  I  I  L  N  G  N  K  V  D  S  T  N  V  T  F  H  S  E  K  S  P  S  G  E  P  F  L  Q  S  C

3801    TTAACCAAGGAGCAGCTATCCCGCTATGGTGTGGATGTTGATGCCTATCCCGAGTTATCTCCAGCATTAAAAAACTCACAGACAAACCCGTGTGTCAATT
          L  T  K  E  Q  L  S  R  Y  G  V  D  V  D  A  Y  P  E  L  S  P  A  L  K  N  S  Q  T  N  P  C  V  N  L

3901    TAGCCGCTATCCCTCAGGCCAGTGAAGAGTTCCAATTTTATAATATGCAGTTGGTACTGAGTATTCCACAAGCGGCTTTACGGCCTGAAGGTGAAGTGCC
          A  A  I  P  Q  A  S  E  E  F  Q  F  Y  N  M  Q  L  V  L  S  I  P  Q  A  A  L  R  P  E  G  E  V  P

4001    AATAGAACGCTGGGATGATGGTATTACGGCTTTTTTGCTGAACTACATGGAAATATCAGTGAAACCCAGTTTCGTCAAAATGGTGGATACCGGCGTTCAC
          I  E  R  W  D  D  G  I  T  A  F  L  L  N  Y  M  E  I  S  V  K  P  S  F  V  K  M  V  D  T  G  V  H

4101    AATATATCCAGTTATATCCCGGTT
          N  I  S  S  Y  I  P  V
```

Fig. 2. Nucleotide sequence of *Y. pestis* pH 6 Ag including *psaE*, *psaA*, *psaB* and ORF4'. Nucleotide residue numbers are shown on the left. Putative protein products are labelled and shown below the DNA sequence. Predicted secretion signal peptides are underlined below the amino acid sequence of the proteins (Von Heijne, 1986). Restriction enzyme recognition sites are overlined and labelled above the DNA sequence. Potential Shine–Dalgarno (Shine and Dalgarno, 1974) sequences are underlined below the DNA sequence. The asterisk above the nucleotide sequence indicates the point of insertion of Tn*10lacZ* in pPSN1 (*psaE*::Tn*10lacZ*). These sequence data appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number M86713.

4). Similar results were obtained from database searches with the ORF4' amino acid sequence (data not shown). Primary protein sequence identity with ORF4' ranged from 29% to 22% for the *E. coli* Pap (Norgren *et al.*, 1987), K88 (Mooi *et al.*, 1986), and K99 (Roosendaal and de Graaf, 1989) pilus accessory proteins PapC, FaeD and FanD, respectively. The FaeD, FanD, and PapC proteins are members of a family of proteins each having a molecular mass of approximately 85 kDa (de Graaf, 1990). The PapC family is a group of outer membrane proteins necessary for the transport and assembly of pilin subunits into mature pili in *E. coli* (de Graaf, 1990).

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*

A

```
PsaA    120 WYINDDSPKDIKDHLYVKAG 139
            ||.:... .|..:||...|
PapG    298 WYTAGSKTVKIESRLYGEEG 317
```

B

```
PsaA     52  APNTGEIFAGKQPGDVTMFTLTMGDT  77
             ||: |.::.|| .| : . .|.||:.
InfH    265  APEYGHLITGKSHGRILKNNLPMGQC  291
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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. *Y. pestis* pH 6 Ag structural gene product (PsaA) similarity to the *E. coli* PapG adhesin.
B. Similarity alignment of *Y. pestis* PsaA with the influenza A virus haemagglutinin (InfH) of strain Duck/Alberta/60/76.

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     PsaB   mknlffsayk kvFsyitsiv ifMvsLp.Ya YSqdvvvntt khlFtVkIGt 49
     F1     ...mil.... nrLstLgiit fgMlsFa..a nSaqpdikfa skeYgVtIGe 21
     Cs3    .......... .....Mtpik liFaaLsLFp CSniyAnnit tqkFeailGa 35
     PapD   .......... .....Mirkk ilMaaipLFv iSgadA.... ....aVsldr 27
     MrkB   .......... .......mkr iaLffcfiFs Faahan.... ....nIiVng 25
     FaeE   ..vskrnavt tfFtnrvtka lgMtlalMmt CqsamA.... ....slaVdq 40
  Consensus ---------- --F--M---- --M--L-LF- CS---A---- ---F-V--G-

            **              *             *
     PsaB   TRVIYPssst kGvsvsVaNp QDyPILVQtq VkdE..DkTs p...aPFIVT 94
     F1     sRIIYPlDaa .GvmvsVkNt QDyPVLIQsR IydENkEkes e...dPFVVT 67
     Cs3    TRVIYhlDg. nGeslrVkNp QisPILIQsk VmdE..gskd n...adFIVT 79
     PapD   TRaVFdGsek smTldisNdn kqlPyLaQAW IEnENqE... kiitgPvIaT 74
     MrkB   TRfIYPGnek eiTvqlsNta dr.PaLatAW lDngNaDaTp dtittPFIIT 74
     FaeE   TRyIFrGDkd alTitvtNNd kErtfggQAW VDnivekdTr pt....FVVT 86
  Consensus TRVIYPGD-- -GT---VNN- QD-PILIQAW VD-EN---T- -----PFIVT

          *             *            * *       *   * *
     PsaB   PPLFRLDAgl QgrvRIIrtg Gk..FPeDrE tLqWLcLtgI PPKngDaWgn 142
     F1     PPLFRLDAKq QnslRIaqag Gv..FPrDkE SLkWLcvkgI PPKdeDiWvD 115
     Cs3    PPLFRLDAKr etdiRIVMvn Gl..YPKDrE SLktLcvrgI PPKqgDlWan 127
     PapD   PPvqRLDpga ksmvRlsttp diskLPqDrE SLFYFnLreI PP.....RsE 119
     MrkB   PPisRvDAKs gqtlRIkLgs nag.LaKDkE tLWWLnLleI PPveasqknE 123
     FaeE   PsFFkvkpng QqtlRIIMas d..hLPKDkE SvYWLnLqdI PP......al 128
  Consensus PPLFRLDAK- Q---RIIM-- G---LPKD-E SL-WL-L--I PPK--D-W-E

                              *     *    **
     PsaB   tqNnpknssp tmd....iQm sIsTCIKLLF RPdkvKgdPt d.sADsLtWr 187
     F1     datnkqkfnp dkdvgvfvQf AInnCIKLLv RPneLKgtPi q.fAEkLsWk 164
     Cs3    nekef..... ...vgmklnv sInTCIKLiL RPhnLpkldi n.segqieWg 168
     PapD   kaN....... ......vlQi AlqTkIKLFY RPaaiKtrPn evwqDqLiln 156
     MrkB   gqN....... ......ilQl AIrsrfKFiY RPagLgnrda a..AEkLals 158
     FaeE   egs....... ......giav AlrTklKLFY RPkaLlegrk gaeegislqs 165
  Consensus --N------- --------Q- AI-TCIKL-Y RP--LK--P- ---A--L-W-

               *     *  **                                   *
     PsaB   ykGnyLeVnN PTPFYmnfys LriGdeKinl sDlgskdeik ngsyvpPfSs 237
     F1     vdGGkLiaeN PsPFYmnIge LtfGGKsi.. .......... pshyipPkSt 202
     Cs3    irdGnLvakN kTPYYfTIvn asfnGKal.. .........k tpgtLgPyeq 207
     PapD   kvsGgyrIeN PTPYYvTVig LggseKqaee gEfeT....v ...MLsPrSe 199
     MrkB   anGssLsVsN PTPFYiTVsr isrnGgKaln s..kT....v ...MFaPqSs 199
     FaeE   rpdGrtmlvN tTPYifaIgs LldGngKkia tDngTtqkll ...MFmPgde 212
  Consensus --GG-L-V-N PTP-Y-TI-- L--GGKK--- -D--T----- ---M--P-S-

                             ** *
     PsaB   rdfiiPvKnk gKateVfWqV INDnGGvsre fkstVq.... .......... 273
     F1     wafdLP.Kgl agarnVsWrI INDqGGldrl ysknVtl... .......... 238
     Cs3    klytLPsK.i svsglVkWeI IgDlGesset kkfnI..... .......... 241
     PapD   qtvksa.ny. ...ntpylsy INDYGG.... .....rpvls ficngsrcsv 235
     MrkB   qtiaLs.sav sKgetltvnn INDYGa.... .....dvavk vtvk...... 233
     FaeE   vqv....... .KgnvVkvds lNDYGelqtw tinkkkpaap eaakaekadt 254
  Consensus ----LP-K-- -K---V-W-I INDYGG---- ----V----- ----------
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Fig. 4. Protein sequence alignment of predicted PsaB amino acid residues with *E. coli*, *Klebsiella pneumoniae* fimbrial chaperone proteins and the *Y. pestis* chaperone for capsule protein F1. Predicted protein sequences for the *Y. pestis* chaperones (PsaB, F1), *E. coli* chaperones for CS3 fimbriae, Pap pilus (PapD), K88 fimbriae (FaeE) and *K. pneumoniae* mannose-resistant fimbriae (MrkB) are labelled on the left. Residue numbers are shown to the right of each protein sequence. Asterisks indicate residues conserved among the PapD family of pilus chaperone proteins (Holmgren *et al.*, 1992). Capital letters indicate similar amino acids. The consensus sequence indicates positions at which three of the six aligned proteins have similar residues.

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 by the first 1045 bp of the *Yersinia* DNA present in pDG27 (Fig. 5, lane B). The 24 kDa size of this protein is in close agreement with the 23 958 predicted molecular weight of PsaE. A slightly faster migrating species was produced by these reactions. This result suggested the removal of the protein signal sequence from PsaE that was predicted by primary protein sequence analysis (Fig. 2). Template C (Fig. 5, lane C) specifically directed the synthesis of 18, 17 and 15 kDa proteins. The 17 kDa and 15 kDa species reacted with pH 6 Ag specific antiserum when similar extracts were analysed by Western blotting (data not shown). The 18 kDa protein may be the product of an ORF which begins at bp 1038 and terminates at bp 1526 (Fig. 2). We are unsure if the 18 kDa protein is necessary for the biosynthesis of pH 6 Ag. However, the predicted translation product of this ORF (bp 1038 to bp 1526) did not have significant similarity to proteins in the current databases. A protein with a size similar to the 30 648 molecular weight predicted for the *psaB* gene product is specifically synthesized from the appropriate region of *Y. pestis* DNA (Fig. 5, lane D). An 81 kDa polypeptide was encoded by the 3 kb region of DNA downstream from *psaB* (Fig. 5, lane pDG versus to lane V). Given the protein sequence similarity of the first 182 amino acid residues predicted between ORF4 and the 85 kDa *E. coli* PapC pilin transport family (see above), it was likely that the observed 81 kDa protein (Fig. 5, lane pDG) is encoded by the complete ORF4.

Haemagglutination induced by the Y. pestis *pH 6 Ag*

Previously, pH 6 Ag was shown to agglutinate red blood cells derived from sheep, rabbits and guinea-pigs (Bichowsky-Slomnicki and Ben-Efraim, 1963). We tested

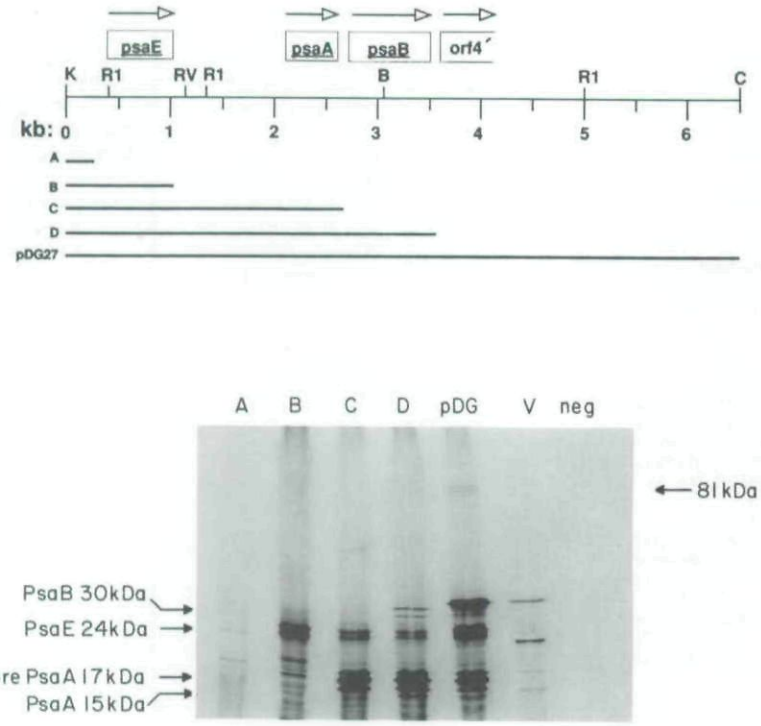

Fig. 5. *In vitro* transcription and translation of the *Y. pestis* pH 6 Ag coding region.
Top. The linear map of the pH 6 Ag coding region is labelled as in Fig. 1. Thick lines below the map indicate DNA templates used in the *in vitro* transcription and translation reactions. Linear templates were generated by the PCR as indicated in the *Experimental procedures*.
Bottom. Fluorograph of [^{35}S]-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* KIM5-3001.1 (*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 *Kpn*I to *Cla*I fragment of *Y. pestis* DNA. However, *E. coli* containing pDG9 did not cause agglutination of SRBCs. The plasmid pDG9 contains a 3.1 kb *Kpn*I to *Bam*HI 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

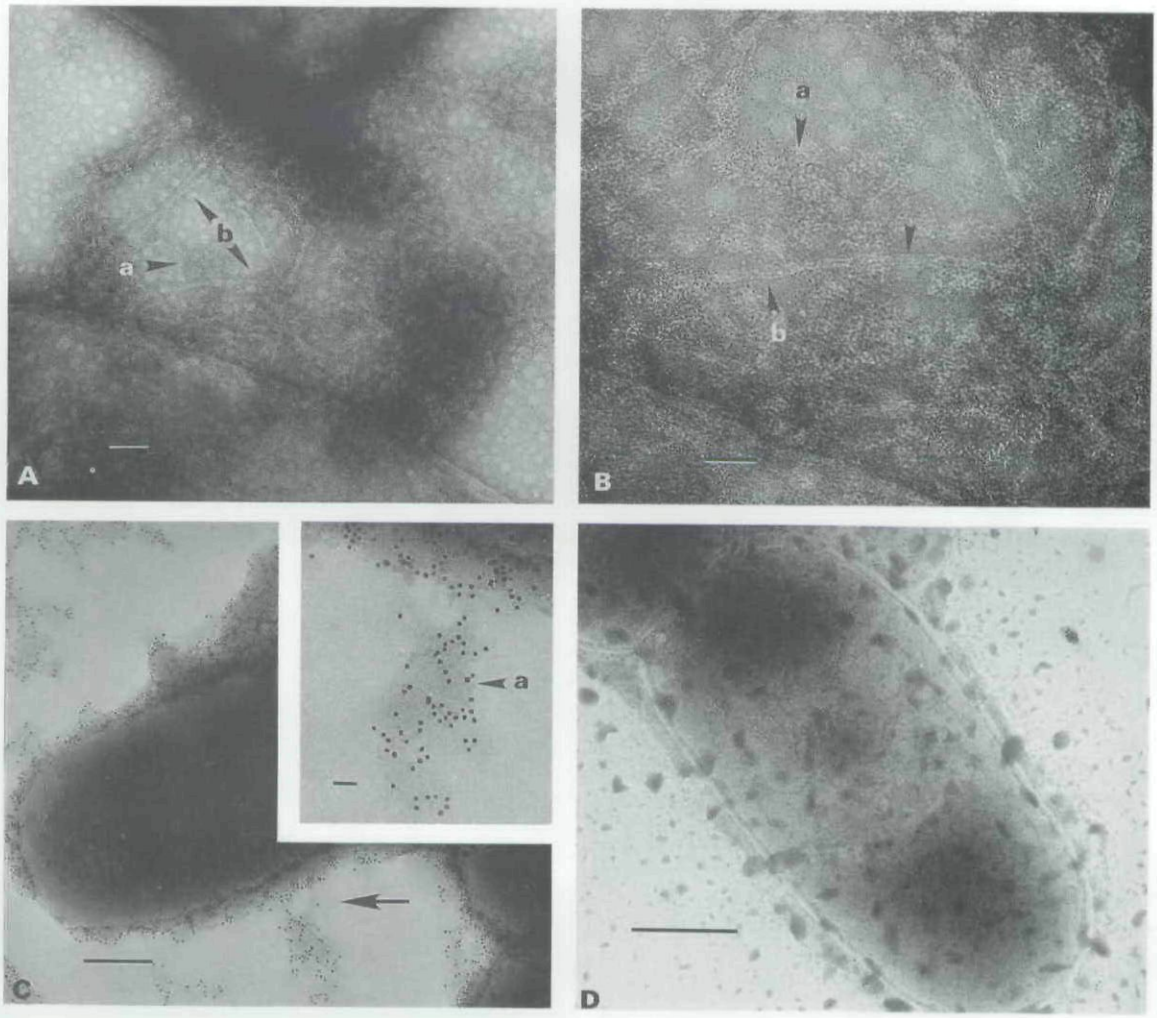

Fig. 6. Electron photomicrographs of negatively stained and immunogold-labelled *E. coli* containing the cloned pH 6 Ag locus.
A. *E. coli* harbouring pDG1 negatively stained with uranyl acetate expressing the three pH 6 Ag fibrillar morphotypes.
B. Higher magnification of (A) showing the ultrastructural 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 pHC79 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.

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

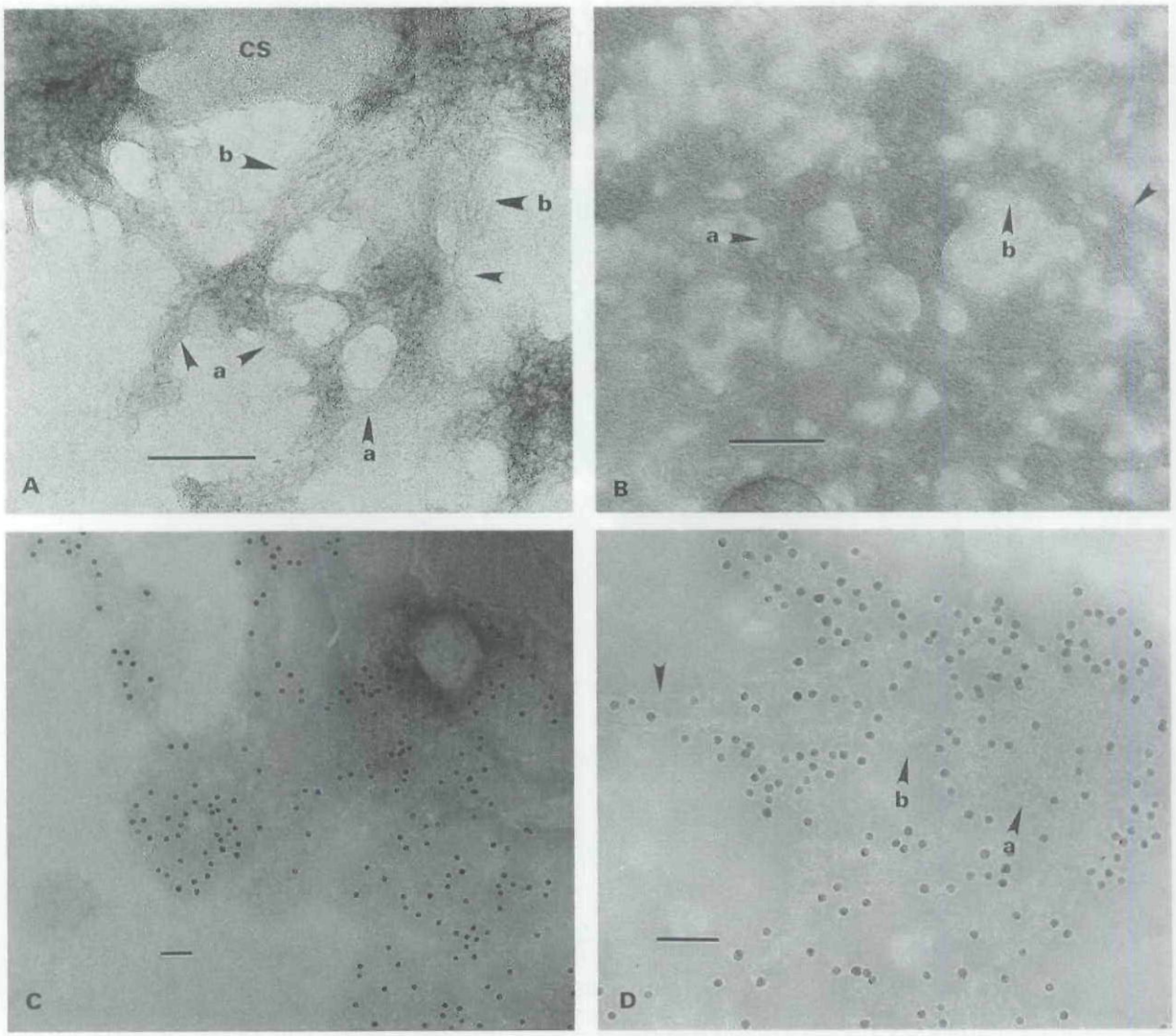

Fig. 7. Electron photomicrographs of negatively stained and immunogold-labelled *Yersinia* spp.
A. High-magnification photomicrograph of *Y. pseudotuberculosis* PB1/+ negatively stained with uranyl acetate.
B. Fibrillar pH 6 Ag on a grid after immunogold labelling of *Y. pestis* KIM 5-3001 with undiluted mAb 6H3 and negative staining with PTA.
C. *Y. pseudotuberculosis* PB1/+ labelled by the immunogold technique. Cells were incubated with a 1:1000 dilution of pH 6 Ag-specific antiserum.
D. Crude KSCN extract of *Y. pseudotuberculosis* PB1/+ grown at 37°C and pH 6. Immunogold labelling was with a 1:1000 dilution of anti-pH 6 Ag antiserum. No labelling was observed when KSCN extracts of *Y. pseudotuberculosis* PB1/+ grown at 37°C and pH 8 were treated similarly. See the Fig. 6 legend for a description of arrows and other fig. markers. Bar markers in A,B and C represent 0.1 μm and the bar marker in D represents 0.05 m.

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

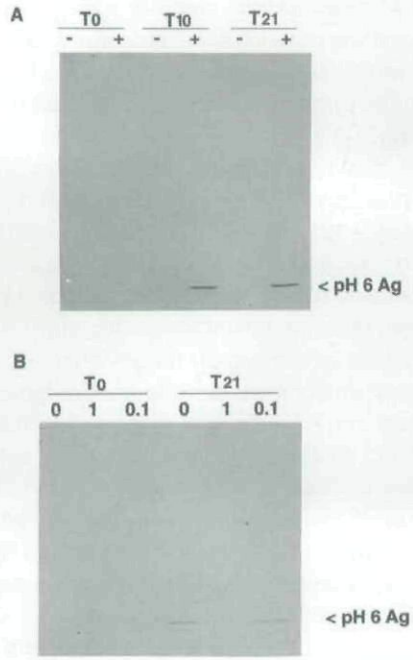

Fig. 8. Expression of pH 6 Ag by *Y. pestis* inside macrophages in the presence or absence of monensin. Macrophage cell line RAW264.7 was infected with *Y. pestis* which was not expressing pH 6 Ag (grown at 30°C and pH 7.2). The multiplicity of infection was approximately one bacterium per macrophage.
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 5×10^5 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 micromolar 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 4×10^4 *Y. pestis* cfu was loaded per lane.

Ag. We removed samples of macrophages at various times after infection with *Y. pestis* for the determination of viable colony-forming units (cfu) and Western blot analysis. We found no difference in the survival of *Y. pestis* KIM-5 3001.1 *psaA* mutant bacteria and the wild-type parent strain. Expression of pH 6 Ag was not observed immediately after infection of the macrophages (Fig. 8A). However after 10 h in the macrophage intracellular environment, pH 6 Ag was expressed by *Y. pestis psaA*$^+$ bacteria (Fig. 8A). It was not likely that the expression of pH 6 Ag observed was due to acidification of the macrophage growth medium. Two lines of evidence support this: (i) the pH of the medium removed from the infected RAW264.7 cells before sampling was approximately 7; and (ii) the inclusion of gentamicin in the macrophage growth medium to inhibit extracellular replication of the bacteria.

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 (Horwitz 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 *Eco*RI 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 *Eco*RI fragment

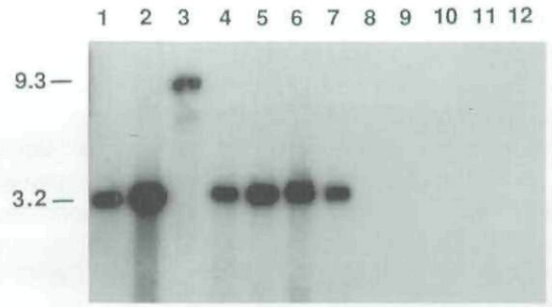

Fig. 9. Southern hybridization of various bacterial DNAs with the *psaA*-specific probe. Approximately 1 μg of bacterial total genomic DNA was digested to completion with *Eco*RI and separated on a 0.7% agarose gel. Fractionated DNA fragments were transferred to nitrocellulose and probed with the 398 bp *Y. pestis psaA* probe as described in the *Experimental procedures*. Lanes contain: 1, *Y. pseudotuberculosis* 7 (IIa); 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. pertis* KIM5–3001; 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/+ 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/+ 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/+ 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 wiry 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 wiry 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 fimbriae into thicker structures has been observed (Olsen *et al.*, 1989; Giron *et al.*, 1991). Surface fimbriae produced by *Y. enterocolitica*, *Y. pseudotuberculosis* (Old and Adegbola, 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* fimbrial 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 pilus 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 15 kDa 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 cholera* 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 LD_{50} (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 uninfected 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 $CaCl_2$, 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 KIM5-3001 (*psaA*$^+$) and KIM5-3001.1 (*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*, *thi1*, *hsdR17*, *supE44*, *relA1*, *lac*, (F' *proAB*, *lacI*^{q}ZM15, Tn*10*)) 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 *Cla*I fragment of *Y. pestis* KIM-5 chromosomal DNA present in pDG4 (Lindler *et al.*, 1990) cloned into the vector pIC20R (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 *Kpn*I site. Plasmid pDG27 contains the 6.5 kb *Kpn*I to *Cla*I DNA 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 ($\mu g\,ml^{-1}$): 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 *Kpn*I to *Bam*HI 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. DNA 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/Gene 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 pmoles 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, GGACGGCTCAATAGCC; fragment b, GCTTTCATTGCTGTTTGC; 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* [^{35}S]-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 [^{35}S]-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 En^{3}hance (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% CO_2 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 × 10^6 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^{-1} 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 salts 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* pH 6 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 3,3'5,5'-tetramethylbenzidine dihydrochloride (TMB) horseradish peroxidase substrate system (Kirkegaard and Perry Labratories).

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 1.5 ml microcentrifuge tube followed by incubation for 2 h 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 Na_2HPO_4, 0.24 g KH_2PO_4 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 Ezzel, 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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