Biol Metals (1991) 4:41–47

BIOLOGY OF METALS

Characterization of a hemin-storage locus of *Yersinia pestis*

Michael L. Pendrak and Robert D. Perry

Department of Microbiology and Immunology, Louisiana State University Medical Center Shreveport, 1501 Kings Highway, P. O. Box 33932, Shreveport, LA 71130-3932, USA

Summary. The pigmentation phenotype (Pgm^+) of *Yersinia pestis* refers to temperature-dependent storage of hemin as well as expression of a number of other physiological characteristics. Spontaneous mutation to a Pgm^- phenotype occurs via a large chromosomal deletion event and results in the inability to express the Pgm^+ characteristics. In this study, we have used transposon insertion mutants to define two regions of a hemin-storage (*hms*) locus. A clone (pHMS1) encompassing this locus reinstates expression of hemin storage (Hms^+) in *Y. pestis* spontaneous Pgm^- strains KIM and Kuma but not in *Escherichia coli*. Complementation analysis using subclones of pHMS1 in *Y. pestis* transposon mutants indicates that both regions (*hmsA* and *hmsB*),which are separated by about 4 kb of intervening DNA, are essential for expression of the Hms^+ phenotype. The 9.1-kb insert of pHMS1 contains structural genes encoding 90-kDa, 72-kDa, and 37-kDa polypeptides. Two-dimensional gel electrophoresis analysis of cells from Pgm^+, spontaneous Pgm^-, and Hms^- transposon strains, as well as a spontaneous Pgm^- strain transformed with pHMS1, indicated that two families of surface-exposed polypeptides (of about 87 and 69–73 kDa) are associated with the Hms^+ phenotype.

Key words: *Yersinia pestis* – Hemin storage – Iron – Pigmentation – Congo red

Introduction

The ability to utilize heme or hemin compounds as sources of nutritional iron is a characteristic increasingly identified in pathogenic bacteria. The yersiniae (Perry and Brubaker 1979), neisseriae (Dyer et al. 1987; West and Sparling 1985), as well as *Haemophilus influenzae* (Pidcock et al. 1988), *Klebsiella pneumoniae* (Ward et al. 1986), *Shigella flexneri* (Lawlor et al. 1987), *Vibrio cholerae* (Stoebner and Payne 1988) and *Vibrio vulnificus* (Helms et al. 1984) all utilize hemin as a sole source of iron. The ability to utilize hemin compounds may provide such pathogens with an alternative nutritional source of iron which is more abundant in mammals and unavailable via siderophore-dependent iron-acquisition systems. However, the heme moiety or inorganic iron must still be withdrawn from such host ligands as hemopexin, albumin, haptoglobin, myoglobin or hemoglobin (Griffiths 1987; Weinberg 1978). Organisms such as *H. influenzae* (Pidcock et al. 1988), *K. pneumoniae* (Ward et al. 1986), *V. vulnificus* (Helms et al. 1984) and the pathogenic neisseriae (Dyer et al. 1987) can utilize some but not all of the above heme complexes. However, *Y. pestis* effectively obtains iron from all forms of bound hemin and hemoglobin (Sikkema and Brubaker 1987; Staggs and Perry, unpublished results).

In addition to heme utilization system(s), *Y. pestis* cells also possess a hemin-storage (Hms) system. This property was originally described by Jackson and Burrows (1956a; 1956b) as the pigmentation phenotype (Pgm^+) due to the ability of wild-type cells of *Y. pestis*, grown at 26° C but not at 37° C, to absorb sufficient quantities of exogenous hemin or Congo red (Surgalla and Beesley 1969) to form dark 'pigmented' colonies on solidified media. Other physiological characteristics associated with the Pgm^+ phenotype include sensitivity to the bacteriocin pesticin (Brubaker 1969; Sikkema and Brubaker 1987), the ability to grow at 37° C in an iron-deficient medium containing iron chelated by citrate (Sikkema and Brubaker 1987) and the expression of a number of unique peptides including four iron-repressible outer-membrane peptides (Sikkema and Brubaker 1989; Straley and Brubaker 1982). Spontaneous Pgm^- mutants form white ('nonpigmented') colonies and are avirulent in mice via intraperitoneal or subcutaneous injection unless given sufficient iron to saturate transferrin (Brubaker et al. 1965; Jackson and Burrows 1956b, Une and Brubaker 1984). Although spontaneous Pgm^- organisms no longer express any of

Offprint requests to: R. D. Perry

the above physiological characteristics, they retain functional hemin-utilization systems (Perry and Brubaker 1979; Sikkema and Brubaker 1989; Staggs and Perry, unpublished results). Thus spontaneous Pgm$^-$ mutants of *Y. pestis* are defective in hemin storage but not in hemin utilization.

Recently several *hms*::mini-*kan* mutants were isolated which no longer express hemin storage at 26° C. Using DNA adjacent to these inserts as a probe of genomic DNA, it was determined that a massive chromosomal deletion event, encompassing at least 18 kb of DNA, was the mechanism of spontaneous mutation to the Pgm$^-$ phenotype. A clone containing 9.1 kb of *Y. pestis* DNA (pHMS1) restored temperature-regulated expression of hemin storage in a spontaneous Pgm$^-$ strain of *Y. pestis* KIM (Perry et al. 1990). To reflect the increased complexity of the *pgm*$^+$ genotype, the acronym Hms is used to refer only to the hemin-storage component of the Pgm$^+$ phenotype while Pgm$^+$ and Pgm$^-$ refer specifically to wild-type organisms and their spontaneous deletion mutants. The *pgm* locus (encompassing at least 18 kb) refers to all genomic DNA deleted in the spontaneous Pgm$^-$ mutants. This study identifies two regions, *hmsA* and *hmsB*, within a 9.1-kb locus essential for expression of the Hms$^+$ phenotype in *Y. pestis* as well as changes in cell-surface structures associated with this locus.

Materials and methods

Bacteria and plasmids. All *Y. pestis* strains used in this study are avirulent due to the absence of functional Lcr plasmids (named pCD1 in strain KIM) (Brubaker 1983; Perry et al. 1986; Straley and Bowmer 1986). The Pgm$^+$ determinant and the low-Ca^{2+} response (Lcr$^+$) virulence regulon are genetically and biochemically unrelated (Brubaker 1983; Perry et al. 1990). Pgm$^+$ strains are denoted by a + behind the strain designation. Strain KIM6+ contains two (pMT1 and pPCP1) of the three endogenous plasmids of wild-type *Y. pestis* strains. *Y. pestis* Kuma+ and Kuma are Pgm$^+$/Pgm$^-$ isogenic strains possessing pMT2 and pPCP2 (Perry and Brubaker 1979; Perry et al. 1990). *Y. pestis* KIM6-2008, KIM6-2009, KIM6-2011, KIM6-2012, KIM6-2024, and KIM6-2027 are Hms$^-$, Km^r mutants (Perry et al. 1990) resulting from single insertions of the transposable element mini-*kan* (Way et al. 1984) into the *hms* locus of *Y. pestis* KIM6+ (pAMH62). *E. coli* strains χ2338 (Jacobs et al. 1986), DH5α, and HB101 (Ausubel et al. 1987) served as hosts for recombinant plasmids. Recombinant plasmid pNPM1 is the cloning vector, pBGL2, with a 19.5-kb *Sal*I DNA insert (containing *hms2012*::mini-*kan*) from KIM6-2012 (Perry et al. 1990). Plasmid pBGL2 (Ap^r, 4.8 kb) was produced by excision of a 1.78-kb *Bgl*II fragment from pHC79 (Hohn and Collins 1980). Recombinant plasmid pHMS1 (Km^r, 16.4 kb) contains a 9.1-kb *Sau*3AI insert from *Y. pestis* KIM6+ ligated into the *Bam*HI site of the low-copy-number cloning vector pLG338 (Stoker et al. 1982). Deletion of *Eco*RI or *Bam*HI fragments from pHMS1 resulted in subclones pNPM7 and pNPM8 (see Fig. 1). Plasmid pHMS1.1 (expressing Cm^r) was constructed by ligation of a 9.7-kb *Hin*dIII–*Sal*I fragment from pHMS1 into pACYC184. Plasmid pHMS1.1 contains the entire insert from pHMS1 plus about 300 bp of pLG338 DNA at both ends of the insert. Deletion of a *Bam*HI fragment from pHMS1.1 resulted in pNPM9, while pNPM11 derives from elimination of a *Hin*dIII–*Sma*I fragment from pHMS1.1 (see Fig. 1).

Cultivation and labelling of bacterial cells. All bacterial strains were stored at −20° C in buffered glycerol (Beesley et al. 1967). *Y. pestis* cells were grown in liquid heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% xylose (HIBX) and plated on tryptose blood agar base (Difco) plates (TBA). *E. coli* strains DH5α and HB101 were grown in Luria broth (LB) and plated on LB solidified with agar (Difco). Growth of *E. coli* χ2338 cells in LB requires supplementation with diaminopimelic acid, thymidine, and tryptophan (Jacobs et al. 1986). The CR agar of Surgalla and Beesley (1969) was used to test for Pgm$^+$ and Hms$^+$ phenotypes. All bacteria harboring antibiotic resistances were cultivated with the appropriate antibiotic at a concentration of 50 μg/ml for kanamycin (Km) or chloramphenicol (Cm). Minicells were isolated from *E. coli* χ2338 and labelled with [^{35}S]methionine (ICN Radiochemicals, Irvine, CA) as previously described (Clark-Curtiss and Curtiss III 1983). In surface-labelling experiments, cells of *Y. pestis* were grown with aeration (200 rpm setting on a New Brunswick model G76 gyratory shaker water bath) in deferrated PMH. Hemin, $MgCl_2$, and $MnSO_4$ supplements were added after Chelex 100 (Bio-Rad Laboratories, Richmond, CA) treatment of PMH (Dyer et al. 1987). PMH is essentially the chemically defined TMH medium of Straley and Bowmer (1986) with glucose substituted for potassium gluconate. At 26° C, growth of Pgm$^+$ cells in liquid PMH occurs without the severe clumping observed in several other liquid media (Perry and Brubaker 1979; unpublished observations). Cells were surface-labelled with ^{125}I (ICN Radiochemicals) using iodobeads (Pierce, Rockford, IL) as previously described (Armstrong and Parker 1986).

Protein gel electrophoresis. Labelled minicell products were analyzed on one-dimensional sodium dodecyl sulfate (SDS) gels (Laemmli 1970) containing 12% (mass/vol.) polyacrylamide, impregnated with En^{3}Hance (New England Nuclear Research Products, Boston, MA) and dried. Extracts from ^{125}I-labelled cells were analyzed on two-dimensional gels: first by isoelectric focusing (IEF) followed by SDS/PAGE (O'Farrell and O'Farrell 1977). Molecular mass markers labelled with ^{14}C were purchased from Bethesda Research Laboratories (BRL, Gaithersburg, MD). Gels were visualized by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −70° C.

In vitro DNA manipulations. Reactions with DNA restriction endonucleases, T4 DNA ligase, DNA polymerase I, or calf intestinal alkaline phosphatase were performed according to manufacturer's specifications. Genomic DNA from *Y. pestis* was isolated using a lysozyme/SDS/proteinase K procedure (Ausubel et al. 1987) and purified by phenol and chloroform extractions. Small-scale plasmid isolations (Kado and Liu 1981; Birnboim and Doly 1979) were used for rapid plasmid screening. Highly purified plasmid DNA was isolated by differential DNA precipitation (Humphreys et al. 1975) of cleared cell lysates (Birnboim and Doly 1979). *E. coli* strains were transformed via a standard $CaCl_2$ procedure (Ausubel et al. 1987). Plasmid DNA was introduced into *Y. pestis* cells by electroporation (Dower et al. 1988). Briefly, late-log-phase cells were harvested, washed once in sterile distilled, deionized water, and once in sterile 10% (mass/vol.) poly(ethylene glycol), before resuspension to about 4×10^{10} cells/ml 10% poly(ethylene glycol); 25 μl of this suspension was mixed with about 2.5 μg plasmid DNA and incubated 20 min on ice prior to electroporation with a BRL cell-porator. A field strength of 2 kV/cm (300-V discharge with an electrode gap of 0.15 cm) with the low resistance (no parallel resistor) and 50-μF settings were used. Electroporated cells were incubated 1 h in HIBX prior to plating on the appropriate selective media.

Results and discussion

Isolation and mapping of hms::*mini*-kan *insertion sites*

Recently, Perry et al. (1990) isolated several *Y. pestis* mutants containing a mini-*kan* element inserted into an

hms locus. The mini-*kan* insertion site was precisely mapped in one of these mutants from a recombinant clone (pNPM1) containing a 19.5-kb *Sal*I fragment isolated from KIM6-2012. This fragment contains the transposon and 17.8-kb of genomic DNA that is deleted in spontaneous Pgm$^-$ strains (Perry et al. 1990). To map the mini-*kan* insertion sites of five other Hms$^-$, Km^r mutants, 15–24-kb *Sal*I genomic DNA fragments from KIM6-2008, KIM6-2009, KIM6-2011, KIM6-2024, and KIM6-2027 were separately ligated into the *Sal*I site of plasmid pBGL2, and transformed into *E. coli* HB101 or DH5α. Transformants containing a 19.5-kb *Sal*I insert encoding Km^r were obtained from each transposon mutant strain and named pNPM2–6.

Figure 1 shows the restriction enzyme and mini-*kan* insertion sites which have been mapped within the 17.8-kb *Sal*I genomic fragment. Four inserts lie between 9.6–11.5 kb on the map and two others lie ≈3.8 kb 'downstream' between 15.25 and 15.4 kb (Fig. 1). Using the *Sal*I insert from pNPM1 as a probe. Perry et al. (1990) isolated a clone, designated pHMS1, from a library of KIM6+ genomic DNA. The Hms$^+$ phenotype is restored when spontaneous Pgm$^-$ mutant KIM6 carries pHMS1. All six transposon inserts are encompassed by the cloned region of pHMS1 (Fig. 1). The insertion pattern and the complementation data below suggest at least two separate genetic units reside within the cloned locus. We broadly define these two regions as *hmsA* (≈8.7 to ≈14.1 kb) and *hmsB* (≈14.1 to ≈17.8 kb); (see Fig. 1).

Complementation analysis

In the spontaneous Pgm$^-$ strains KIM6 and Kuma, pHMS1 restored temperature-dependent expression of the Hms$^+$ phenotype. When introduced separately into KIM6, subclones pNPM7, pNPM8, and pNPM11 did not complement this strain's defect in hemin storage, indicating that both *hmsA* and *hmsB* are required for the Hms$^+$ phenotype. Since pNPM7 and pNPM8 express Km^r, these plasmids could not be used to perform complementation testing in the Km^r transposon mutants. Consequently, Cm^r subclones pNPM9 and pNPM11 were constructed. Plasmid pNPM11 restored temperature-dependent hemin storage in mutants with inserts lying within the *hmsA* region (i. e. KIM6-2012, KIM6-2027, KIM6-2009, and KIM6-2011). As expected, neither pNPM11 nor pNPM9 complemented mutations in the *hmsB* region (KIM6-2024 and KIM6-2008). Interestingly, subclone pNPM9 complemented KIM6-2012, KIM6-2027, and KIM6-2009, but not KIM6-2011 indicating that the region between 11.9–14.1 kb is necessary for complementation in this mutant. Both the mini-*kan* insertion pattern and complementation results suggest that functional *hmsA* and *hmsB* regions are essential for expression of hemin storage.

Although both regions could be transcribed onto a single mRNA of 6 kb or more, the simplest interpretation is that *hmsA* and *hmsB* encode separate transcripts. In addition, the complementation results with KIM6-2011 harboring pNPM9 suggest that the *hmsA* region contains more than one structural gene and could encode two or more separate transcripts.

Expression of pHMS1-encoded polypeptides in E. coli *minicells*

The ^{35}S-labelled peptide profiles of *E. coli* χ2338 minicells harboring either pHMS1 or the cloning vector pLG338 are compared in Fig. 2. The 9.1-kb *hms* region encoded at least three polypeptides with molecular masses of 90, 72, and 37 kDa (Fig. 2, lane 3). At 26° C, the 90 kDa peptide was not detected although both the 72-kDa and 37-kDa proteins were expressed at this temperature (Fig. 2, lane 1). Labelling at 37° C also resulted in increased expression of a 29-kDa peptide which could be encoded on the insert. Alternatively, this insert could cause over-expression of a pLG338 structural gene at 37° C. Note that the 72-kDa polypeptide corresponds in size to a family of Pgm$^+$-specific products which include iron-repressible polypeptides IrpB-E (69.1–65.1 kDa) as well as peptide F (72.8 kDa)

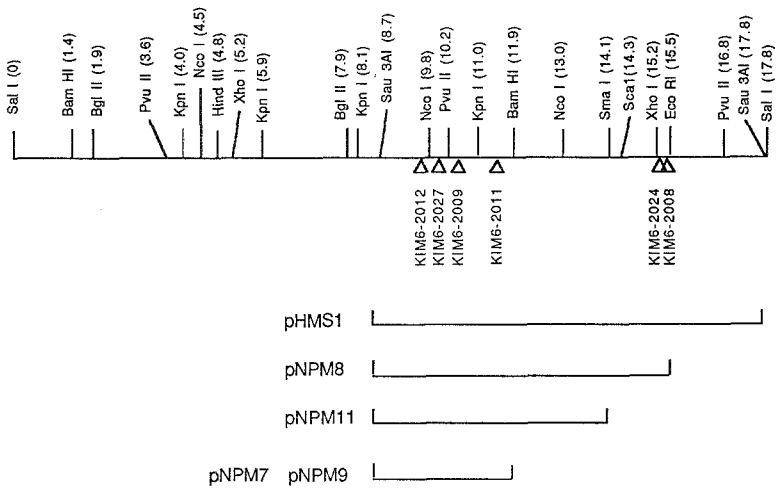

Fig. 1. Restriction map of the unique 17.8-kb *Sal*I fragment from *Yersinia pestis* KIM6+. The mini-*kan* insertion sites of six mutants are shown as triangles with strain designations listed directly below. Regions subcloned from uninterrupted *Y. pestis* KIM6+ DNA are depicted. The numbers in parentheses indicate the location of restriction sites (in kb)

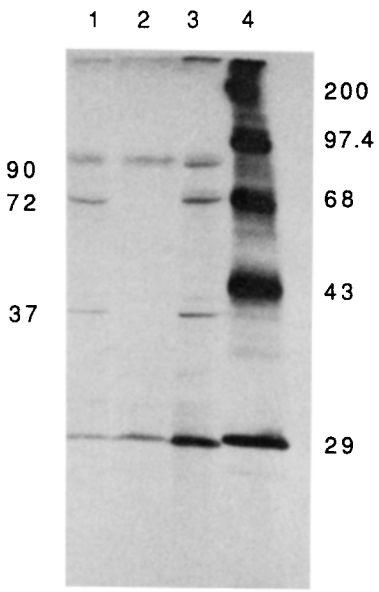

Fig. 2. Autoradiogram of plasmid-encoded ^{35}S-labelled polypeptides from *E. coli* χ2338. Minicells of χ2338 (pHMS1) were labelled at 26° C (lane 1) or 37° C (lane 3). Lane 2 shows polypeptides expressed at 37° C by pLG338. Lanes 1–3 contain 40000 cpm ^{35}S precipitable by trichloroacetic acid. Lane 4 contains BRL ^{14}C-labelled molecular mass markers with the indicated molecular masses (in kDa). The numbers on the left denote molecular masses of pHMS1-encoded polypeptides

which was described as a possible hemin-storage component (Sikkema and Brubaker 1989).

The role of these pHMS1-encoded peptides in hemin storage is unclear. In *E. coli*, none are preferentially expressed at 26° C (Fig. 2, lanes 1 and 3) and pHMS1 does not convert *E. coli* strains χ2338, HB101, or DH5α to an Hms$^+$ phenotype (data not shown). Although the reason for this is not known, it is possible that the genes encoding proteins necessary for hemin storage were not expressed in *E. coli*. However, *E. coli* HB101 harboring the intact Lcr plasmid, pCD1, does not display an Lcr$^+$ phenotype (R. D. Perry and S. C. Straley, unpublished observations) even though low-level unregulated expression of *Y. pestis* Lcr genes does occur in *E. coli* minicells (Perry et al. 1986; Price and Straley 1989). These observations suggest that strains of *E. coli* may express *Y. pestis* polypeptides without conversion to the appropriate physiological phenotype. Consequently, we attempted to identify Hms$^+$-specific surface polypeptides to compare with those expressed in minicells.

Identification of Hms$^+$-linked polypeptides

Cells of *Y. pestis* KIM6+, KIM6-2012, KIM6, and KIM6 (pHMS1) were acclimated to growth at 26° C or 37° C by serial transfer for approximately eight generations in deferrated PMH supplemented with 20μM hemin. Acclimated cells were transferred to fresh media and mid-log-phase cells were then harvested, surface-labelled with ^{125}I, and subjected to two-dimensional gel electrophoresis. KIM6+ cells grown at 26° C contained unique polypeptides in four regions (spots A–D in Fig. 3 A) that were not detected in the spontaneous Pgm$^-$ mutant KIM6 (Fig. 3D). With the exception of spot C, each region may represent three or more unique polypeptides or different isoelectric forms of the same polypeptide. Comparison of KIM6+ and the Hms$^-$ transposon mutant KIM6-2012 shows both quantitative and qualitative differences. KIM6-2012 exhibits lower expression of spot C and region D. In addition, several discrete spots in regions A, B, and D may be absent in KIM6-2012 (Fig. 3, A and C). Finally, introduction of pHMS1 into KIM6 causes over-expression in regions A, B, and C while expression of polypeptides in region D is not restored. Molecular mass measurements of these proteins from one-dimensional SDS/polyacrylamide slab gels (data not shown) indicate that polypeptides in regions A and B have a molecular mass of ≈ 87 kDa while region C and D polypeptides are ≈ 68–73 kDa. These proteins are similar in size to the minicell polypeptides (Fig. 2); however it is uncertain which, if any, of the peptides in Figs. 2 and 3 correspond to previously identified Pgm$^+$-specific peptides (Sikkema and Brubaker 1989, Straley and Brubaker 1982).

At 37° C, ^{125}I-surface-labelling of proteins in regions A, C, and D in KIM6+ is much lower than at 26° C (compare Fig. 3A with Fig. 4A). Thus loss of hemin storage at 37° C may be due to under-expression of Hms-specific surface peptides. While region B polypeptides are expressed in KIM6 cells grown at 37° C, regions A, C and D are not expressed in these cells (Fig. 4B). Both KIM6+ and KIM6 cells express peptide E at 37° C (Fig. 4); although not apparent in Fig. 3 (A and D), low-level expression of peptide E does occur at 26° C. Only KIM6-2012 cells over-express peptide E at 26° C (Fig. 3C).

The polypeptide expression patterns exhibited in Figs. 3 and 4 are too complex to identify a single polypeptide as essential for hemin storage; indeed, expression of this phenotype may require an array of outer-membrane proteins. However, the absence of region D in KIM6 (pHMS1) cells and the presence of region B in KIM6 cells grown at 37° C suggest that these polypeptides may not be involved in hemin storage. Finally, the over-expression of A and C peptides by KIM6 (pHMS1) provides the strongest evidence for their involvement in hemin storage.

Function of the Hms$^+$ phenotype

We have used the term 'hemin storage' to refer to the physiological trait of pigmented colony formation at 26° C in the presence of excess hemin or Congo red. A similar Congo-red-binding phenotype (CR$^+$) has been described in *E. coli* (Berkhoff and Vinal 1985), *Neisseria meningitidis* (Payne and Finkelstein 1977), *V. cholerae* (Payne and Finkelstein 1977), *Y. enterocolitica* (Prpic et al. 1983), *Aeromonas salmonicida* (Kay et al. 1985), and several species of *Shigella* (Daskaleros and Payne 1987;

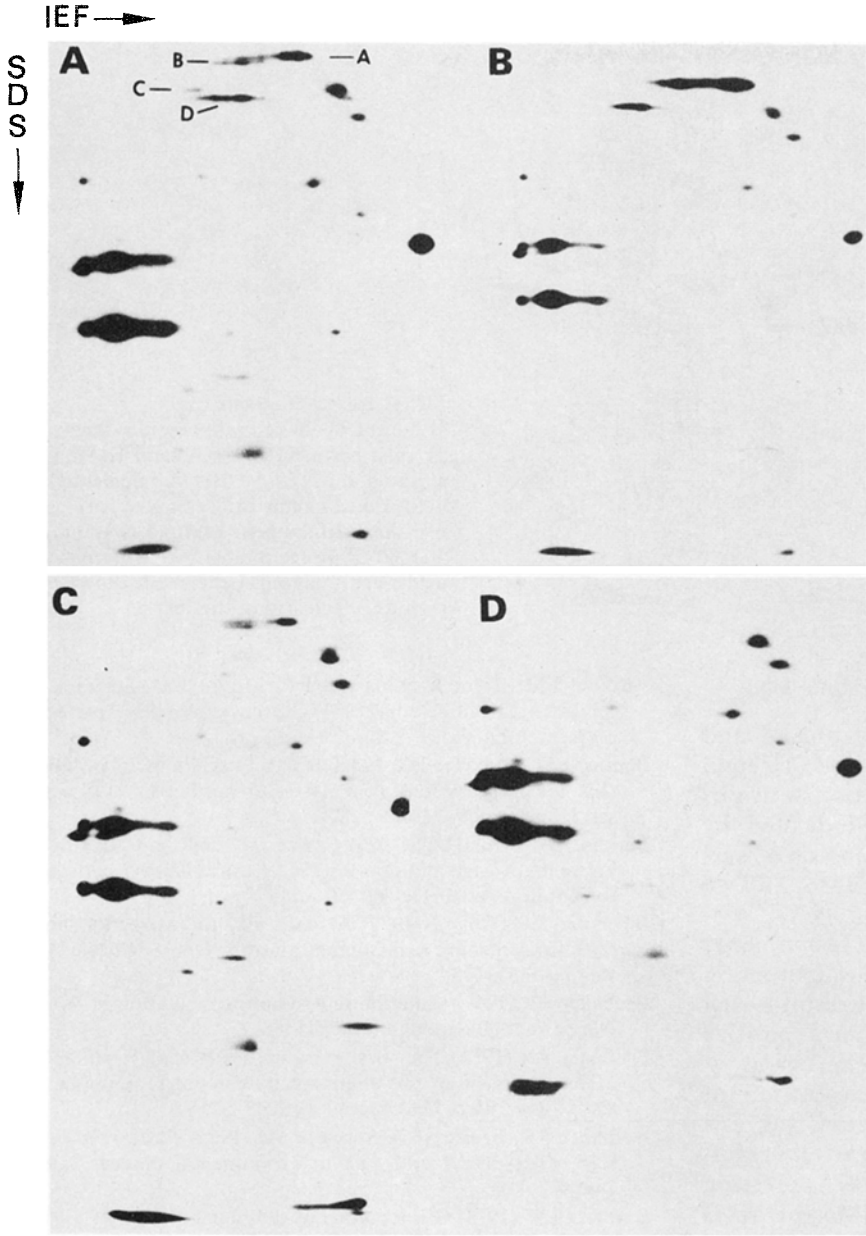

Fig. 3. Autoradiograms of cell extracts from ^{125}I-surface-labelled *Yersinia pestis* cells cultured at 26° C in PMH supplemented with 20 μM hemin and separated on two-dimensional gels. (A–D) Analysis of ^{125}I-labelled material precipitable by trichloroacetic acid (100000 cpm each) from strains KIM6+, KIM6 (pHMS1), KIM6-2012, and KIM6, respectively. Letters in **A** (A–D) indicate regions referred to in the text

Payne and Finkelstein 1977). Only *Y. pseudotuberculosis* (described as having highly variable pigmentation: Burrows 1973), *A. salmonicida* (Kay et al. 1985), *S. flexneri*, and enteroinvasive *E. coli* (Daskaleros and Payne 1987; Stugard et al. 1989) have been shown to 'bind' hemin. The physiological characteristics and regulation of CR^+ expression do not match the Pgm^+ or Hms^+ phenotype of *Y. pestis*, leaving the degree of functional and genetic similarities among these systems unresolved.

The function of the Hms^+ phenotype in *Y. pestis* is unknown. A CR^+ phenotype increases the ability of *S. flexneri* to invade and infect HeLa cells (Daskaleros and Payne 1987; Stugard et al. 1989). Thus, hemin molecules on the surface of the organism may facilitate its uptake by eucaryotic cells. Alternatively, since iron can inhibit a variety of nonspecific host defenses (van Asbeck and Verhoef 1983), an array of surface hemin molecules may serve this function. In *Y. pestis*, the expression of the Hms^+ phenotype during growth in the hemin-rich environment of the flea gut may allow the storage of hemin for later use as a nutritional source of iron in mammals.

Spontaneous Pgm^- mutants of *Y. pestis* are avirulent (Jackson and Burrows 1956b; Une and Brubaker 1984). Our studies have shown that this mutation is caused by a massive deletion of at least 18 kb of chromosomal DNA (Perry et al. 1990). Since this mutation affects the expression of the pesticin receptor (Brubaker 1969; Sikkema and Brubaker 1987), iron-repressible peptides (Sikkema and Brubaker 1989), Pgm^+-specific peptides (Sikkema and Brubaker 1989; Straley and Brubaker 1982), and Hms^+-specific peptides (this study), the role of each of these physiological traits in the virulence of plague remains to be determined.

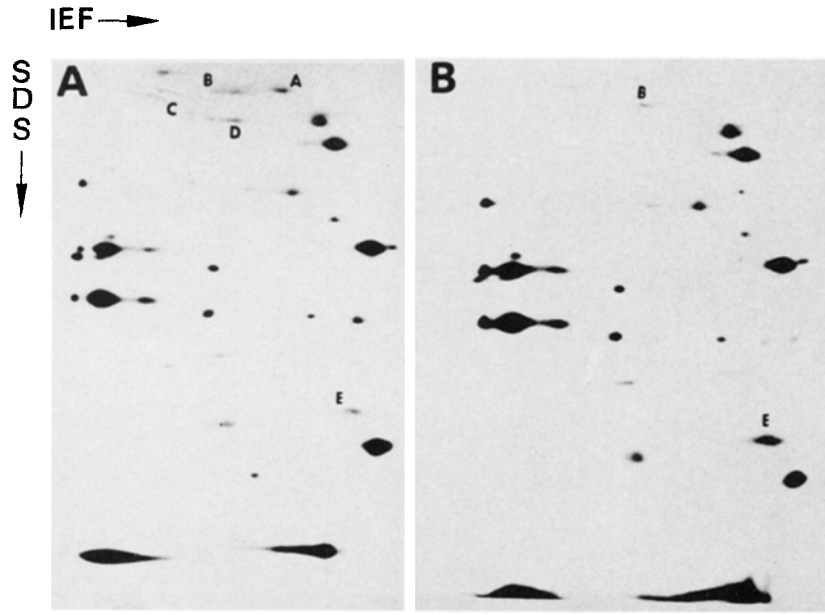

Fig. 4. Autoradiograms of ^{125}I-surface-labelled polypeptides from *Yersinia pestis* KIM6+ (**A**) and KIM6 (**B**) cultured at 37° C in PMH supplemented with 20 μM hemin and separated on two-dimensional gels. Samples containing 100000 cpm precipitable by trichloroacetic acid were analyzed. Letters A-E show regions referred to in the text

Genetic organization and regulation of the hms *locus*

We have identified two essential regions (*hmsA* and *hmsB*) in a locus necessary for the expression of hemin storage in *Y. pestis*. This *hms* locus may contain two or more separate transcriptional units as indicated by complementation studies as well as by analysis of surface proteins present in KIM6+, KIM6, KIM6 (pHMS1) and KIM6-2012.

KIM6-2012 and KIM6+ appear to differ primarily in the amount of Hms-specific polypeptides present on the cell surface. One interpretation of this result is that the KIM6-2012 min-*kan* insert lies within a positive regulatory element necessary for the expression of hemin storage. Furthermore, the over-expression of peptide E in KIM6-2012 cells grown at 26° C suggests that the putative regulatory gene may negatively regulate the structural gene for this protein. Over-expression of proteins in regions A, B, and C by KIM6 (pHMS1) suggests that this clone might contain Hms structural genes as well as the putative regulatory gene disrupted in KIM6-2012. Whether the Hms$^-$ phenotypes of the three other mutants with mini-*kan* inserts in *hmsA* are due to disruption of a regulatory or structural gene is undetermined. While these results are suggestive of regulation at the transcriptional or translational level, we have not ruled out regulation at the level of proper assembly on the cell surface.

Acknowledgments. This study was supported by Public Health Service grant AI25098 from the National Institutes of Health. We thank Paul Schuetze and Teanna Staggs for their technical assistance and thoughtful discussions. This paper is dedicated to the memory of Charles E. Lankford whose pioneering studies helped open an entirely new field of research in bacterial iron transport and metabolism.

References

Armstrong SK, Parker CD (1986) Heat-modifiable envelope proteins of *Bordetella pertussis*. Infect Immun 54:109–117

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1987) Current protocols in molecular biology. John Wiley & Sons, New York

Beesley ED, Brubaker RR, Janssen WA, Surgalla MJ (1967) Pesticins. III. Expression of coagulase and mechanism of fibrinolysis. J Bacteriol 94:19–26

Berkhoff HA, Vinal AC (1985) Congo red medium to distinguish between invasive and non-invasive *Escherichia coli* pathogenic for poultry. Avian Dis 30:117–121

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Brubaker RR (1969) Mutation rate to nonpigmentation in *Pasteurella pestis*. J Bacteriol 98:1404–1406

Brubaker RR (1983) The Vwa$^+$ virulence factor of yersiniae: the molecular basis of the attendant nutritional requirement for Ca^{2+}. Rev Infect Dis 5[Suppl 4]:S748–S758

Brubaker RR, Beesley ED, Surgalla MJ (1965) *Pasteurella pestis*: role of pesticin I and iron in experimental plague. Science 149:422–424

Burrows TW (1973) Observations of the pigmentation of *Yersinia pseudotuberculosis*. Contr Microbiol Immunol 2:184–189

Clark-Curtiss JE, Curtiss III R (1983) Analysis of recombinant DNA using *Escherichia coli* minicells. Methods Enzymol 101:347–362

Daskaleros PA, Payne SM (1987) Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. Infect Immun 55:1393–1398

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 16:6127–6145

Dyer DW, West EP, Sparling PF (1987) Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. Infect Immun 55:2171–2175

Griffiths E (1987) The iron-uptake systems of pathogenic bacteria. In: Bullen JJ, Griffiths E (eds) Iron and infection: molecular, physiological and clinical aspects. John Wiley & Sons, New York, pp 69–137

Helms SD, Oliver JD, Travis JC (1984) Role of heme compounds and haptoglobin in *Vibrio vulnificus* pathogenicity. Infect Immun 45:345–349

Hohn B, Collins J (1980) A small cosmid for efficient cloning of large DNA fragments. Gene 11:291–298

Humphreys GO, Willshaw GA, Anderson ES (1975) A simple method for the preparation of large quantities of pure plasmid DNA. Biochim Biophys Acta 383:457–463

Jackson S, Burrows TW (1956a) The pigmentation of *Pasteurella pestis* on a defined medium containing haemin. Br J Exp Pathol 37:570–576

Jackson S, Burrows TW (1956b) The virulence-enhancing effect of iron on non-pigmented mutants of virulent strains of *Pasteurella pestis*. Br J Exp Pathol 37:577–583

Jacobs WR, Barrett JF, Clark-Curtiss JE, Curtiss III R (1986) In vivo repackaging of recombinant cosmid molecules for analyses of *Salmonella typhimurium, Streptococcus mutans*, and mycobacterial genomic libraries. Infect Immun 52:101–109

Kado CI, Liu S-T (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145:1365–1373

Kay WW, Phipps BM, Ishigura EE, Trust TJ (1985) Porphyrin binding by the surface array virulence protein of *Aeromonas salmonicida*. J Bacterial 164:1332–1336

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685

Lawlor KM, Daskaleros PA, Robinson RE, Payne SM (1987) Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect Immun 55:594–599

O'Farrell PH, O'Farrell PZ (1977) Two-dimensional polyacrylamide gel electrophoretic fractionation. Methods Cell Biol 26:407–420

Payne SM, Finkelstein RA (1977) Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect Immun 18:94–98

Perry RD, Brubaker RR (1979) Accumulation of iron by yersiniae. J Bacteriol 137:1290–1298

Perry RD, Harmon PA, Bowmer WS, Straley SC (1986) A low-Ca^{2+} response operon encodes the V antigen of *Yersinia pestis*. Infect Immun 54:428–434

Perry RD, Pendrak ML, Schuetze P (1990) Identification and cloning of a hemin-storage locus involved in the pigmentation phenotype of *Yersinia pestis*. J Bacteriol 172:5929–5937

Pidcock KA, Wooten JA, Daley BA, Stull TL (1988) Iron acquisition by *Haemophilus influenzae*. Infect Immun 56:721–725

Price SB, Straley SC (1989) *lcrH*, a gene necessary for virulence of *Yersinia pestis* and for the normal response of *Y. pestis* to ATP and calcium. Infect Immun 57:1491–1498

Prpic JK, Robins-Browne RM, Davey RB (1983) Differentiation between virulent and avirulent *Yersinia enterocolitica* isolates by using Congo red agar. J Clin Microbiol 18:486–490

Sikkema DJ, Brubaker RR (1987) Resistance to pesticin, storage of iron, and invasion of HeLa cells by yersiniae. Infect Immun 55:572–578

Sikkema DJ, Brubaker RR (1989) Outer membrane peptides of *Yersinia pestis* mediating siderophore-independent assimilation of iron. Biol Metals 2:174–184

Stoebner JA, Payne SM (1988) Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect Immun 56:2891–2895

Stoker NG, Fairweather NF, Spratt BG (1982) Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. Gene 18:335–341

Straley SC, Bowmer WS (1986) Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect Immun 51:445–454

Straley SC, Brubaker RR (1982) Localization in *Yersinia pestis* of peptides associated with virulence. Infect Immun 36:129–135

Stugard CE, Daskaleros PA, Payne SM (1989) A 101-kilodalton heme-binding protein associated with Congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. Infect Immun 57:3534–3539

Surgalla MJ, Beesley ED (1969) Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. Appl Microbiol 18:834–837

Une T, Brubaker RR (1984) In vivo comparison of avirulent Vwa^- and Pgm^- or Pst^r phenotypes of yersiniae. Infect Immun 43:895–900

Van Asbeck BS, Verhoef J (1983) Iron and host defense. Eur J Clin Microbiol 2:6–10

Ward CG, Hammond JS, Bullen JJ (1986) Effect of iron compounds on antibacterial function of human polymorphs and plasma. Infect Immun 51:723–730

Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N (1984) New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene 32:369–379

Weinberg ED (1978) Iron and infection. Microbiol Rev 42:45–66

West SEH, Sparling PF (1985) Response of *Neisseria gonorrhoeae* to iron limitation: alterations in expression of membrane proteins without apparent siderophore production. Infect Immun 47:388–394