JOURNAL OF BACTERIOLOGY, Oct. 1990, p. 5929–5937
0021-9193/90/105929-09$02.00/0
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Vol. 172, No. 10

Identification and Cloning of a Hemin Storage Locus Involved in the Pigmentation Phenotype of *Yersinia pestis*†

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Received 21 March 1990/Accepted 22 July 1990

The temperature-dependent absorption of sufficient exogenous hemin or Congo red to form pigmented colonies of *Yersinia pestis* has been termed the pigmentation phenotype (Pgm$^+$). Spontaneous mutation to a Pgm$^-$ phenotype results in the loss of a number of divergent physiological characteristics, including the ability to store hemin and to bind Congo red at 26°C. In this study, we generated and isolated transposon insertion mutants that are hemin storage negative (Hms$^-$) and therefore unable to form pigmented colonies. These mutations are due to single mini-*kan* insertions within a 19.5-kilobase (kb) *SalI* fragment of chromosomal DNA. Restriction site analysis of eight mutants identified a minimum of six potentially different insertion sites spanning an ~10-kb hemin storage (*hms*) locus. The 19.5-kb *SalI* fragment (containing ~18 kb of *Y. pestis* DNA and the mini-*kan* insert) was cloned from one of these mutants, KIM6-2012. By using this cloned fragment as a DNA probe, the mechanism of spontaneous mutation to a Pgm$^-$ phenotype was identified as a massive deletion event. The deletion spans at least 18 kb of genomic DNA in spontaneous Pgm$^-$ mutants from nine separate strains of *Y. pestis*. DNA adjacent to the mini-*kan* insert was used to identify a clone containing a wild-type *hms* locus. A spontaneous Pgm$^-$ mutant of *Y. pestis* KIM containing this clone exhibits an Hms$^+$ phenotype. The *hms*::mini-*kan* mutations and cloned wild-type *hms* locus generated in this study will greatly aid in identifying the function of hemin storage in *Y. pestis*.

Pigmentation phenotype (Pgm$^+$) refers to the ability of wild-type cells of *Yersinia pestis* to absorb sufficient quantities of exogenous hemin (25, 26) or Congo red (CR) (48) to form dark pigmented colonies on solidified media. Expression of this phenotype is temperature regulated: pigmented colonies form during growth at 26°C but not at 37°C (25, 48). Spontaneous Pgm$^-$ mutants of *Y. pestis* form white, nonpigmented colonies at 26°C and are avirulent in mice via intraperitoneal or subcutaneous injection but not by intravenous injection. The 50% lethal doses of spontaneous Pgm$^-$ mutants injected intraperitoneally are restored to wild-type values when mouse serum transferrin is iron saturated (26, 49). These observations establish the Pgm$^+$ phenotype as an important plague virulence determinant that affects the iron metabolism of *Y. pestis*.

Both Pgm$^+$ and Pgm$^-$ organisms can utilize micromolar quantities of hemin as a sole iron source (32). Thus, the defect in Pgm$^-$ organisms does not reside in hemin transport but in accumulation and storage of excess hemin (25, 32). Therefore, one possible function of the Pgm$^+$ phenotype is the storage of iron, which may be abundant during growth in the flea gut.

Perry and Brubaker (32) showed that both Pgm$^+$ and Pgm$^-$ cells of *Y. pestis* can grow at 37°C in an unchelated, iron-deficient, defined medium. Growth under these conditions induces expression of a transport system, in both Pgm$^+$ and Pgm$^-$ organisms, that accumulates ferric iron (32). Later, Sikkema and Brubaker (39) noted that Pgm$^-$ cells are unable to grow at 37°C in a defined medium containing only submicromolar quantities of iron chelated by citrate. This growth defect at 37°C in an iron-chelated medium correlates with the lack of expression of four iron-repressible outer membrane peptides in spontaneous Pgm$^-$ mutants of *Y. pestis* (40). In addition to these iron-repressible peptides, several other unique peptides are associated with the Pgm$^+$ phenotype (40, 45). The functions of these peptides have not been definitively assigned.

Only Pgm$^+$ strains of *Y. pestis* lacking the plasmid pPCP1, which encodes pesticin (Pst), are sensitive to this bacteriocin (Pst^s) (3, 7, 16, 41). However, it is unlikely that the Pst receptor and the heme storage component are the same entity for two reasons. Some strains of *Yersinia pseudotuberculosis* (33), *Yersinia enterocolitica* (23, 33), and *Escherichia coli* (9, 17) are Pst^s despite being phenotypically Pgm$^-$. In addition, Sikkema and Brubaker (40) have generated a mutant of *Y. pestis* that is pesticin resistant (Pst^r) and Pgm$^+$.

Virulence-associated CR binding (CR$^+$) has been reported in a number of other organisms, including *E. coli* (4, 37), *Neisseria meningitidis* (31), *Vibrio cholerae* (31), *Y. enterocolitica* (35), and several species of *Shigella* (14, 31, 36). In some instances, CR binding is only expressed at 37°C and is associated with invasion of mammalian cells (14, 47). Hemin binding has been demonstrated only in *Shigella flexneri* (14, 47), enteroinvasive *E. coli* (47), and *Y. pseudotuberculosis* (described as highly variable pigmentation [10]). The physiological characteristics and optimal conditions for expression of these CR$^+$ phenotypes do not match the Pgm$^+$ phenotype of *Y. pestis*. Consequently the degree of functional and genetic similarities among these systems is unresolved.

As more physiological characteristics of the pigmentation phenotype are identified, our genetic understanding of the putative *pgm* locus has decreased. Spontaneous mutations to Pgm$^-$ result in loss of all of these phenotypic characteristics. The spontaneous mutation rate of 10^{-5} and the inability to obtain Pgm$^+$ revertants (7) suggested that the

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† Dedicated to the memory of Charles E. Lankford, whose pioneering studies led the way in the continually expanding research area of bacterial iron transport and utilization.

TABLE 1. *Y. pestis* strains used in this study

Strain[a]	Plasmid(s) present[b]	Relevant genotype or phenotype[c]	Reference or source
KIM6+	pMT1, pPCP1	Pgm^+ Pst^+ Lcr^- Fra^+	39
KIM6	pMT1, pPCP1	Pgm^- Pst^+ Lcr^- Fra^+	39
KIM10+	pMT1	Pgm^+ Pst^- Pst^s Lcr^-	This study
KIM10	pMT1	Pgm^- Pst^- Pst^r Lcr^-	This study
KIM10(pBGL2)	pMT1, pBGL2	Pgm^- Pst^- Pst^r Lcr^- Ap^r	This study
KIM10(pNPM1)	pMT1, pNPM1	Pgm^- Pst^- Pst^r Lcr^- Ap^r Km^r	This study
KIM6+(pAMH62)	pMT1, pPCP1, pAMH62	Pgm^+ Pst^+ Lcr^- Ap^r $LamB^+$	This study
KIM6(pHMS1)	pMT1, pPCP1, pHMS1	Pgm^- Hms^+ Pst^+ Lcr^- Fra^+	This study
KIM10(pHMS1)	pMT1, pHMS1	Pgm^- Hms^+ Pst^- Pst^r Lcr^-	This study
KIM6-2002+	pMT1, pPCP1, pAMH62	Pgm^+ Pst^+ Lcr^- Ap^r $LamB^+$ Km^r (mini-*kan*)	This study
KIM6-2003+	pMT1, pPCP1	Pgm^+ Pst^+ Lcr^- Ap^s Km^r (mini-*kan*)	This study
KIM6-2004+	pMT1, pPCP1, pAMH62	Pgm^+ Pst^+ Lcr^- Ap^r $LamB^+$ Km^r (mini-*kan*)	This study
KIM6-2005 to KIM6-2007	pMT1, pPCP1, pAMH62	Pgm^- Pst^+ Lcr^- Ap^r $LamB^+$ Km^r (mini-*kan*)	This study
KIM6-2008	pMT1, pPCP1, pAMH62	Hms^- Pst^+ Lcr^- Ap^r $LamB^+$ Km^r *hms-2008*::mini-*kan*	This study
KIM6-2009	pMT1, pPCP1	Hms^- Pst^+ Lcr^- Ap^s Km^r, *hms-2009*::mini-*kan*	This study
KIM6-2010 to KIM6-2014	pMT1, pPCP1, pAMH62	Hms^- Pst^+ Lcr^- Ap^r $LamB^+$ Km^r, *hms-2010*::mini-*kan* to *hms-2014*::mini-*kan*, respectively	This study
KIM6-2015	pMT1, pPCP1	Hms^- Pst^+ Lcr^- Ap^s Km^r *hms-2015*::mini-*kan*	This study
KIM6-2016 to KIM6-2028	pMT1, pPCP1, pAMH62	Hms^- Pst^+ Lcr^- Ap^r $LamB^+$ Km^r, *hms-2016*::mini-*kan* to *hms-2028*::mini-*kan*, respectively	This study
A1122	pEL101	Pgm^- Pst^+ Lcr^- Fra^+	16
Kuma+	pMT2, pPCP2	Pgm^+ Pst^+ Lcr^- Fra^+	32, 39
Kuma	pMT2, pPCP2	Pgm^- Pst^+ Lcr^- Fra^+	32, 39
TS+	pMT3, pPCP3	Pgm^+ Pst^+ Lcr^- Fra^+	39
TS	pMT3, pPCP3	Pgm^- Pst^+ Lcr^- Fra^+	39
Salazar+	pMT4, pPCP4	Pgm^+ Pst^+ Lcr^- Fra^+	32, 39
Salazar	pMT4, pPCP4	Pgm^- Pst^+ Lcr^- Fra^+	32, 39
K10+	pMT5, pPCP5	Pgm^+ Pst^+ Lcr^- Fra^+	39
K10	pMT5, pPCP5	Pgm^- Pst^+ Lcr^- Fra^+	39
Yokohama+	pPCP6	Pgm^+ Pst^+ Lcr^- Fra^+	32, 39
Yokohama	pPCP6	Pgm^- Pst^+ Lcr^- Fra^+	32, 39
G32+	pMT7	Pgm^+ Pst^- Lcr^- Fra^+	32, 39
G32	pMT7	Pgm^- Pst^- Lcr^- Fra^+	32, 39
A12+	pMT8	Pgm^+ Pst^- Lcr^- Fra^+	39
A12	pMT8	Pgm^- Pst^- Lcr^- Fra^+	39
Dodson+	pDMT9, pCD9	Pgm^+ Pst^- Lcr^- Fra^+	16, 39
Dodson	pDMT9, pCD9	Pgm^- Pst^- Lcr^- Fra^+	16, 39

[a] All KIM strains were derived from KIM6+; + designates pigmented (Pgm^+) strains. All mutant strains generated in this study were assigned strain numbers KIM6-2002 to KIM6-2028.

[b] The three KIM plasmids have been designated pMT1 (110 kb, no known functions), pCD1 (75 kb, Lcr genes), and pPCP1 (9.5 kb, pesticin genes, plasminogen activator-coagulase gene [Pac^+]) (41, 46). Since the plasmids in other strains are not known to be identical to the KIM plasmids, we have given previously unidentified or unnamed plasmids separate numbers. The ~60-kb pDMT9 plasmid appears to be a deleted version of the 110-kb pMT family (data not shown).

[c] Pst^+, Pesticinogenic; Pst^s, pesticin sensitive; Pst^r, pesticin resistant; Pgm^-, spontaneous mutation to nonpigmented phenotype; Hms, hemin storage; Lcr, low-Ca^{2+} response; Fra, fraction 1 antigen (16, 46).

loss of a plasmid might be the mutational mechanism. However, analysis of different strains lacking one or more of the three *Y. pestis* plasmids suggested a chromosomal location for the putative *pgm* locus (16). The purpose of this study was to begin a genetic analysis of the hemin storage (*hms*) locus of the pigmentation phenotype. We use the acronym Hms to refer only to the hemin storage component of the Pgm^+ phenotype of *Y. pestis* and retain the use of Pgm^- to refer specifically to the spontaneous mutants. Transposon insertional mutagenesis was used to generate mutants of *Y. pestis* KIM that were unable to store sufficient hemin (Hms^-) to form pigmented colonies. Several *hms*::mini-*kan* mutants have been isolated that no longer express hemin storage at 26°C. These inserts have been localized to an ~10-kilobase (kb) fragment that is deleted in spontaneous Pgm^- mutants. A 9.1-kb fragment cloned from Pgm^+ cells, which corresponds to the *hms* locus identified by the transposon insertions, complements the hemin storage defect in spontaneous Pgm^- mutants of *Y. pestis* KIM.

MATERIALS AND METHODS

Bacteria, bacteriophages, transposons, and plasmids. Characteristics of the *Y. pestis* strains used in this study are detailed in Table 1. A "+" at the end of a strain designation indicates a Pgm^+ phenotype. Strain KIM6+ contains two (pMT1 and pPCP1) of the three endogenous plasmids of wild-type *Y. pestis* strains. *Y. pestis* KIM10+ (Table 1) was cured of pPCP1 by serial cultivation at 4°C of KIM6+ and now harbors only the 110-kb cryptic plasmid pMT1. Mutant strains generated in this study were assigned numbers KIM6-2002 to KIM6-2028. All *Y. pestis* strains used in this study are avirulent due to the absence of functional Lcr plasmids (named pCD1 in strain KIM) (34, 43, 44). The low-Ca^{2+} response (Lcr^+) virulence regulon is biochemically and genetically unrelated to the Pgm^+ determinant (8). *E. coli* HB101 (6), χ2338 (27), and DH5α (1) were used as hosts for plasmids. *E. coli* NK5012 and the Tn*10* derivative mini-*kan* (on pNK862 and λ1105) were obtained from N.

Kleckner and were described by Way et al. (50). The inserts generated by this engineered transposon do not undergo secondary transpositions or Tn*10*-promoted rearrangements. The tranposase gene of mini-*kan* is controlled by a *ptac* promoter so that transposition is induced by isopropyl-β-D-thiogalactopyranoside (50). λ *vir* and λ::Tn*5* were provided by F. Grundy. *E. coli* χ1553 (18) was used as a host for bacteriophage P1L4 (12). *Salmonella typhimurium* AS80 contains pAMH62, a multicopy vector expressing the *lamB* gene product, the phage λ receptor protein (21). *E. coli* HB101(pCD1) was obtained from S. C. Straley. *E. coli* χ2338(pPCP1) was constructed by cotransformation of the temperature-sensitive cosmid pJC75-76 (13) and the yersinial plasmid pPCP1 into χ2338. *E. coli* χ2338(pPCP1, pJC75-65) cells were cured of pJC75-65 by incubation at 42°C. *E. coli* HB101(pCD1) and χ2338(pPCP1) and *Y. pestis* KIM10+ cells served as sources of plasmid DNA. The low-copy-number plasmid pLG338 (42) was used in the construction of a genomic library of *Y. pestis* DNA. Plasmid pBGL2 was constructed by excision of a 1.78-kb *Bgl*II fragment from pHC79 (22).

Cultivation of bacteria and bacteriophage. All bacterial strains were stored at −20°C in buffered glycerol (2). *Y. pestis* cells were grown in liquid heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% xylose (HIBX medium) and plated on tryptose blood agar base (TBA; Difco) plates. Cells of *E. coli* and *S. typhimurium* were grown in Luria broth (LB) and plated on LB solidified with agar (Difco). For growth of *E. coli* χ2338 cells, LB was supplemented with diaminopimelic acid, thymidine, and tryptophan (27). The CR agar of Surgalla and Beesley (48) was used to test for the Pgm^+ phenotype and to isolate spontaneous Pgm^- mutants. The Pgm^+ phenotype was also visualized by using a defined medium (PMH) supplemented with hemin (100 μM) and solidified with agar. PMH is essentially the defined TMH medium of Straley and Bowmer (43) with glucose substituted for potassium gluconate. This substitution allows 26°C growth of Pgm^+ cells in liquid PMH without the severe clumping observed in several other liquid media (32; unpublished observations). All bacteria harboring antibiotic resistances were cultivated with the appropriate antibiotic at concentrations of 50 μg/ml for ampicillin and kanamycin and 12.5 μg/ml for tetracycline. Lysates of P1L4, λ1105, λ vir, and λ::Tn*5* were generated by established procedures (1, 12). *Y. pestis* transductants were obtained as previously described (1, 18) by using stationary-phase cells, which enhances λ transduction of *Y. pestis* KIM (data not shown).

Transposon mutagenesis. Approximately 10^8 stationary-phase cells of KIM6+(pAMH62) were mixed with λ1105 (at a multiplicity of infection of 2) in a final volume of 200 μl and incubated without shaking at 37°C. After 30 min, 10 μl of 2% (wt/vol) isopropyl-β-D-thiogalactopyranoside and 0.3 ml of HIBX-ampicillin were added, and the culture was incubated at 37°C with shaking for 90 min. Samples of 100 μl were plated on CR plates containing kanamycin and incubated at 30°C for 2 days. White colonies were selected as potential transposon-induced Hms^- mutants.

In vitro DNA manipulations. DNA restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and New England BioLabs (Beverly, Mass.). T4 DNA ligase and nick translation kits were purchased from Bethesda Research Laboratories. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and Promega Corp. (Madison, Wis.). Dupont, NEN Research Products (Boston, Mass.) was the source of [^{32}P]CTP.

Bacterial genomic DNA was isolated by a lysozyme-sodium dodecyl sulfate-proteinase K method (1) and further purified by phenol and chloroform extractions. Restriction endonuclease-digested *Y. pestis* genomic DNA was fractionated on sucrose density gradients as described previously (1). *E. coli* strains were transformed by a standard $CaCl_2$ procedure (1). A modified dimethyl sulfoxide method (20) was used to transform plasmid DNA into cells of *Y. pestis*. Alternatively, plasmid DNA was introduced into *Y. pestis* cells by electroporation (15) as follows. Late-log-phase cells were harvested, washed once in sterile distilled, deionized water, once in sterile 10% (wt/vol) polyethylene glycol, and suspended in 10% polyethylene glycol at ~4×10^{10} cells per ml. Cell suspensions (25 μl) were incubated for 20 min on ice with ~2.5 μg of plasmid DNA before electroporation with a Bethesda Research Laboratories Cell-Porator. The low-ohm (no parallel resistor) and 50-μF settings were used with a field strength of 2,000 V/cm (300-V discharge with an electrode gap of 0.15 cm). Electroporated cells were incubated for 1 h in HIBX and plated on selective medium. For rapid plasmid screening, small-scale plasmid isolations were performed by the method of Kado and Liu (28) or the method of Birnboim and Doly (5). Large-scale isolation of highly purified plasmid DNA was obtained by differential DNA precipitation (24) of cleared cell lysates (5). Southern blots were probed with nick-translated probes under stringent hybridization conditions (hybridization at 42°C in the presence of 50% formamide, with a final wash at 65°C in 0.1% sodium dodecyl sulfate–0.1X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) (1). Hybridized Southern blots were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Pesticin sensitivity. A plate assay system was used to determine sensitivity or resistance to the yersinial bacteriocin pesticin. Cells of *Y. pestis* A1122 (16, 23), a pesticin overproducer, were inoculated onto a ~1-cm area of a TBA plate, incubated at 37°C for 48 h, and then killed by exposure to chloroform vapor. From overnight liquid cultures, ~5×10^5 cells of test strains were inoculated into 5 ml of molten TBA containing 2.5 mM $CaCl_2$ and 2.5 mM EDTA and overlaid onto the A1122 TBA plates (23). Plates were incubated for 18 h at 26 or 37°C.

RESULTS

The goal of this study was to use transposon mutagenesis to identify a genetic locus responsible for hemin storage in cells of *Y. pestis*. We chose to use λ1105, a λ::mini-*kan* construct which acts as a suicide delivery vehicle in nonsuppressor strains (50). The kanamycin resistance (Km^r) gene present on this transposable element could then be used as a probe to identify and isolate the responsible loci.

Construction of a λ-sensitive strain of *Y. pestis*. Cells of *Y. pestis* are normally resistant to infection by λ due to their $LamB^-$ phenotype. The LamB expression plasmid pAMH62 (21) was transduced into *Y. pestis* KIM6+ cells with a P1L4 lysate generated from *S. typhimurium* AS80. Infection of KIM6+(pAMH62) with λ *vir* did not yield plaques on plate cultures or lysis of liquid cultures. However, infection of KIM6+(pAMH62) with λ::Tn*5* did result in Km^r transductants (data not shown). These results indicate that λ transduces KIM6+(pAMH62) but does not result in a productive infection. Similar results have been previously reported for bacteriophage P1 infection of *Y. pestis* (18).

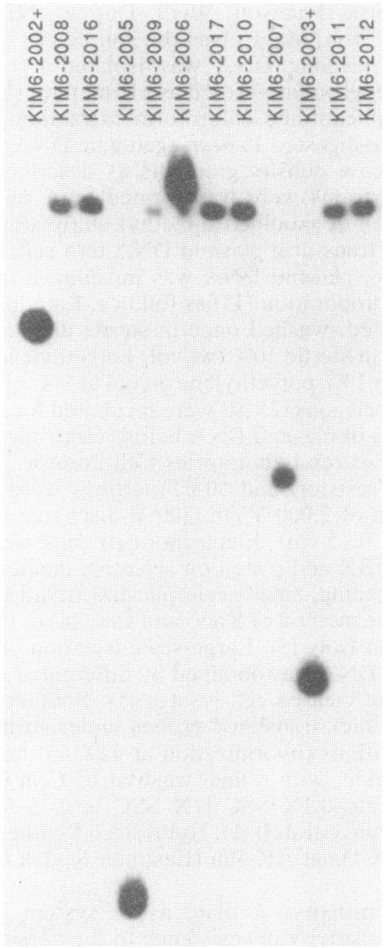

FIG. 1. Southern blot analysis of *Pst*I-digested genomic DNA from representative *Y. pestis* KIM mutants. Cells of KIM6-2002+ and KIM6-2003+ are Pgm^+ Km^r. The remaining mutants are Hms^- Km^r. The DNA probe was a *Hin*dIII fragment of the Km^r gene from mini-*kan* on plasmid pNK862.

Isolation of *hms*::mini-*kan* mutants. Twenty-four separate, replicate mutagenic procedures were performed to generate independent pools of Hms^- Km^r mutants. With a spontaneous mutation rate of 10^{-5}, a portion of the Km^r population will consist of spontaneous Pgm^- mutants with mini-*kan* insert(s) outside putative *hms* loci. Hms^- Km^r cells comprised 0.45% of the total population in cultures exposed to λ1105 for 2 h, whereas only 0.045% of the population not exposed to λ1105 was Hms^-. This 10:1 ratio indicates that a significant number of mutants should have mini-*kan* inserts within a locus involved in hemin storage.

A minimum of 5 Hms^- mutants from each independent mutagenic procedure and 10 Pgm^+ Km^r mutants were selected for Southern blot analysis. Genomic DNA was isolated from these mutants, digested with *Sal*I, and electrophoresed through a 0.7% (wt/vol) agarose gel. After transfer to nitrocellulose, the blots were hybridized, under stringent conditions, with a nick-translated *Hin*dIII fragment derived from the Km^r gene of mini-*kan*. The nonmutagenized KIM6+ parent strain showed no hybridization to the Km^r gene (data not shown). A band of ~17 kb that hybridized to the Km^r gene was present in isolates from 23 of the 24 mutagenic procedures. Twenty-four Hms^- Km^r mutants (exhibiting this ~17-kb restriction fragment) and three Pgm^+ Km^r isolates that possessed hybridizing bands of similar but not identical size (data not shown) were selected for further study.

Figure 1 shows the autoradiograph of the Southern blot of *Pst*I-digested genomic DNA from representative mutants electrophoresed through a 0.5% agarose gel to better separate large DNA fragments. Of the 24 Hms^- Km^r mutants tested, 21 contained a band of ~27 kb that hybridized to the Km^r gene. Three of the Hms^- Km^r mutants (KIM6-2005 through KIM6-2007; Fig. 1) and all of the Pgm^+ Km^r isolates that were examined (two of which are shown in Fig. 1: KIM6-2002+ and KIM6-2003+) had hybridizing bands differing in size from the ~27-kb band (Fig. 1). Similar results were obtained when a *Bgl*II digest of genomic DNA from the same three Pgm^+ Km^r mutants and eight of the Hms^- Km^r mutants (each from an independent mutagenesis) was analyzed by Southern blot hybridization (Fig. 2, lanes 10 through 20). Again, all Hms^- Km^r mutants had a common hybridizing band (~15 kb), whereas the hybridization pattern of the Pgm^+ Km^r mutants was different. These results indicate that the Hms^- Km^r phenotypes of mutants KIM6-2008 through KIM6-2015 as well as KIM6-2016 through KIM6-2028 are probably due to mini-*kan* insertions into an essential *hms* locus. Although isolates KIM6-2005 through KIM6-2007 may be spontaneous Pgm^- mutants with random mini-*kan* insertions into their genomes, it is also possible that these, as well as other transposon mutants exhibiting hybridizing bands of dissimilar sizes, could have insertions into other loci essential for the expression of hemin storage. However, we narrowed our focus to isolates KIM6-2008 through KIM6-2015 as the most likely candidates for mini-*kan* insertions into an essential *hms* locus.

Although a single hybridizing band was visualized by using three different restriction enzymes that do not cut within the transposable element, Southern blot hybridization of *Xho*I-digested genomic DNA from the eight selected mutants confirmed that these mutants contain only a single mini-*kan* insert (Fig. 2, lanes 1 through 8). The Km^r gene contains a single *Xho*I site and should therefore yield two hybridizing bands per insert. Each mutant exhibited only two bands that hybridized to the Km^r gene probe (Fig. 2, lanes 1 through 8), suggesting that each of the eight mutants analyzed has suffered a single mini-*kan* insert into its genome. From the *Xho*I hybridization patterns, there appear to be a minimum of six different insertion sites (Fig. 2, lanes 1, 2, 4, 5, 6, and 8) that affect the expression of hemin storage. Mutants KIM6-2009 and KIM6-2010 (Fig. 2, lanes 2 and 3) appear to have inserts in a common site (as do mutants KIM6-2013 and KIM6-2014; Fig. 2, lanes 6 and 7); however, the methodology employed here would not detect closely adjacent insertion sites.

Isolation of an *hms*::mini-*kan* DNA fragment. Genomic DNA from KIM6-2012 was digested with *Sal*I and size fractionated by ultracentrifugation through a sucrose density gradient. DNA fragments between 10 and 22 kb were pooled and ligated into the *Sal*I site of plasmid pBGL2 (Ap^r, 4.8 kb). Sixteen Ap^r Km^r transformants were obtained, each of which contained a 19.5-kb *Sal*I insert. One plasmid, designated pNPM1, was selected for further analysis.

Restriction enzyme sites that were mapped within the 19.5-kb *Sal*I insert of pNPM1 are depicted in Fig. 3. There are no *Xba*I or *Pst*I sites in the insert. The mini-*kan* element is bounded by the *Bam*HI sites at 9.8 and 11.2 kb on the pNPM1 map (Fig. 3). These sites and the location of the *Hin*dIII, *Pvu*I, *Sma*I, and *Xho*I sites (at 10.0, 10.4, 10.6, and

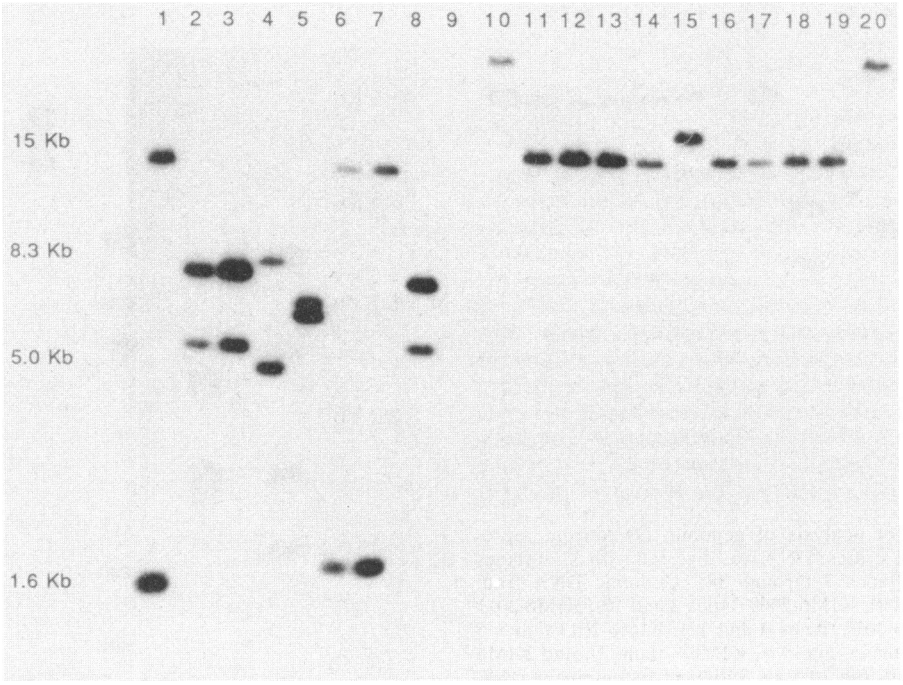

FIG. 2. Southern blot analysis of genomic DNA from representative *Y. pestis* KIM transposon mutants; DNA was digested with *Xho*I (lanes 1 through 8) or *Bgl*II (lanes 10 through 20). Lane 9 contains λ DNA molecular weight markers. Genomic DNA from KIM6-2008 (lanes 1 and 11), KIM6-2009 (lanes 2 and 12), KIM6-2010 (lanes 3 and 13), KIM6-2011 (lanes 4 and 14), KIM6-2012 (lanes 5 and 16), KIM6-2013 (lanes 6 and 17), KIM6-2014 (lanes 7 and 18), KIM6-2015 (lanes 8 and 19), KIM6-2002+ (lane 10), KIM6-2003+ (lane 15), and KIM6-2004+ (lane 20) was probed with the *Hin*dIII Km^r gene fragment.

10.8 kb, respectively) on the pNPM1 map correspond in order and spacing to sites within the known restriction map of the transposed mini-*kan* element (50).

Identification of *hms*::mini-*kan* insertion sites. To ensure that pNPM1 contains the appropriate DNA insert, digests of genomic DNA from six mutants were probed with the 19.5-kb *Sal*I insert fragment from pNPM1. The hybridization pattern obtained with this probe should contain all of the bands seen with the Km^r gene probe (Fig. 2). A single *Sal*I fragment of ~16 kb was observed when genomic DNA from each of the mutants examined was probed with the Km^r gene (data not shown) or the pNPM1 *Sal*I insert (Fig. 4, lanes 9 through 14). Likewise, Southern blots of *Xho*I-digested genomic DNA, probed with either the pNPM1 insert (Fig. 4, lanes 1 through 6) or the Km^r gene (Fig. 2), contained hybridizing fragments of similar size. With the probe from pNPM1, two additional bands, which presumably represent hybridization to the flanking *Xho*I fragments, were also observed (Fig. 4). These results indicate that the *Sal*I insert in pNPM1 corresponds to the KIM6-2012 *Sal*I genomic fragment containing the mini-*kan* insertion.

Southern blots of genomic DNA digested with *Bgl*II, *Sal*I, or *Xho*I and probed with either the Km^r gene or the pNPM1 insert allowed us to roughly map the mini-*kan* insertion sites of five transposon mutants. The pNPM1 clone allowed precise mapping of the insertion site in KIM6-2012 (Fig. 3). In mutants KIM6-2009, KIM6-2011, and KIM6-2015, the mini-*kan* insertions all appear to lie between the *Bgl*II site at 7.9 kb and the *Xho*I site at 16.9 kb (Fig. 3). The mini-*kan* insertions in both KIM6-2008 and KIM6-2013 apparently reside within the small *Xho*I to *Sal*I fragment (16.9 to 19.5 kb, Fig. 3). These data suggest that the six mutants analyzed

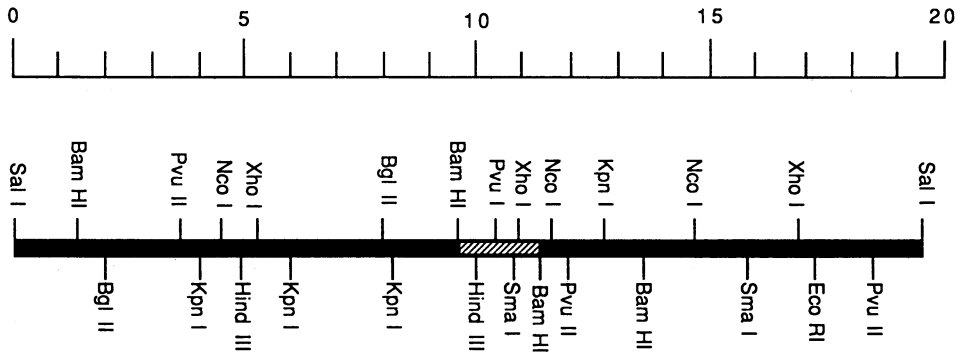

FIG. 3. Restriction endonuclease site map of the 19.5-kb *Sal*I insert in pNPM1. Identified restriction enzyme sites are shown beneath the kilobase scale. The 1.7-kb mini-*kan* insertion element (▨) and the *Y. pestis* DNA segment (■) are indicated.

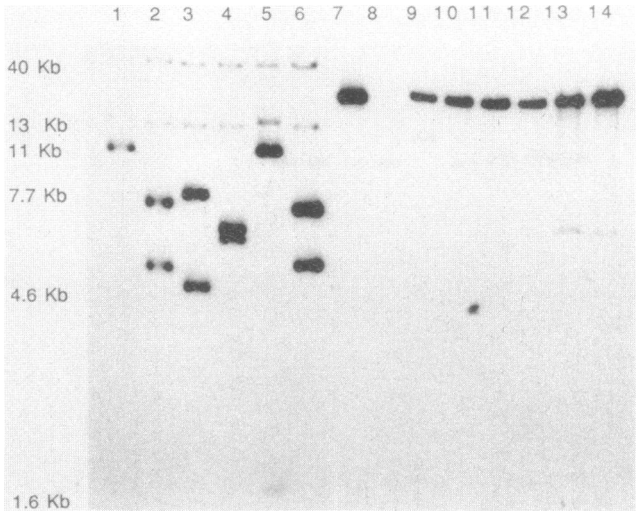

FIG. 4. Southern blot analysis of genomic DNA from representative *Y. pestis* KIM strains; DNA was digested with *Xho*I (lanes 1 through 6) or *Sal*I (lanes 7 through 14). Genomic DNA from KIM6-2008 (lanes 1 and 9), KIM6-2009 (lanes 2 and 10), KIM6-2011 (lanes 3 and 11), KIM6-2012 (lanes 4 and 12), KIM6-2013 (lanes 5 and 13), KIM6-2015 (lanes 6 and 14), KIM6+ (lane 7), and KIM6 (lane 8) was probed with the 19.5-kb *Sal*I Km^r recombinant DNA fragment from pNPM1.

probably have inserts within an ~10-kb segment (7.9 to 19.5 kb minus the 1.7-kb mini-*kan* insert; Fig. 3) that appears to define an *hms* locus in *Y. pestis*.

Analysis of the plasmids from these eight mutants did not demonstrate a mini-*kan* insert in either pMT1 or pPCP1 (data not shown). Southern blot analysis of pMT1, pCD1, and pPCP1 did not reveal any homology to the *Sal*I fragment from pNPM1 (data not shown). Thus, the mutations are due to chromosomal and not plasmid insertions.

Mechanism of spontaneous mutation to Pgm^- phenotype. If the mini-*kan* insertions were responsible for the Hms^- phenotypes of our mutants, then using the radiolabeled *Sal*I fragment of pNPM1 as a probe might identify genomic DNA alterations between Pgm^+/Pgm^- isogenic pairs of *Y. pestis* strains. Figure 4 (lanes 7 and 8) shows hybridization of this probe with *Sal*I-digested genomic DNA from KIM6+ (which is Pgm^+) and its spontaneous Pgm^- mutant, KIM6. KIM6+ possesses a band that on shorter exposures migrates slightly faster than the bands in the mini-*kan* insertion mutants. However, no hybridization to KIM6 DNA was observed (Fig. 4). Hybridization of the *Sal*I probe to KIM6+ and KIM6 genomic DNA digested with a series of restriction enzymes is shown in Fig. 5. As would be predicted from the restriction site map of pNPM1 (Fig. 3), two *Hin*dIII fragments of KIM6+ DNA hybridized with the probe (Fig. 5, lane 7). In addition, the wild-type *hms* locus contained the predicted, internal ~10-kb *Xho*I and *Bam*HI fragments (Fig. 5, lanes 9 and 11). No hybridization to KIM6 DNA was detected in any of these digests (Fig. 5, even-numbered lanes). These results suggest that a massive deletion event is responsible for the spontaneous mutation to the Pgm^- phenotype.

To determine whether this deletion mechanism is common to a variety of strains of *Y. pestis* or restricted to strain KIM, Pgm^+/Pgm^- isogenic pairs from eight non-KIM strains (Table 1) were analyzed. Genomic DNA from each organism was digested with *Hin*dIII, and the Southern blots were

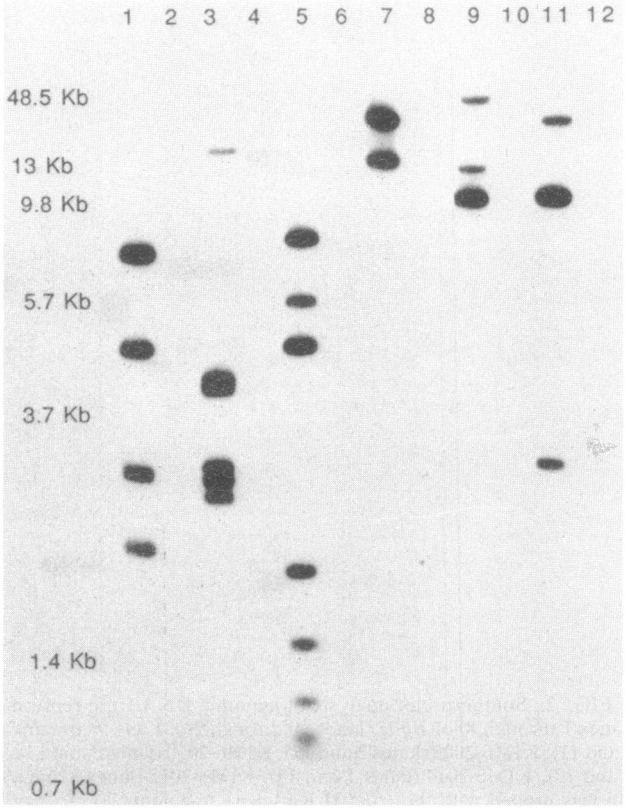

FIG. 5. Southern blot analysis of genomic DNA from KIM6+ (Pgm^+) and KIM6 (Pgm^-). Genomic DNA from KIM6+ (odd-numbered lanes) and its isogenic spontaneous Pgm^- mutant, KIM6 (even-numbered lanes), was digested with the following restriction enzymes: *Acc*I (lanes 1 and 2), *Cla*I (lanes 3 and 4), *Ava*II (lanes 5 and 6), *Hin*dIII (lanes 7 and 8), *Xho*I (lanes 9 and 10), and *Bam*HI (lanes 11 and 12). The DNA probe was the 19.5-kb *Sal*I recombinant fragment from pNPM1.

hybridized to the *Sal*I probe from pNPM1. On the basis of the hybridization results, the strains can be divided into two categories, representatives of which are shown in Fig. 6. Pgm^+ organisms of strains KIM, G32, A12, and Dodson possessed the ~19- and ~11.5-kb *Hin*dIII fragments, whereas their spontaneous Pgm^- mutants did not. In strains Kuma, TS, Salazar, Yokohama, and K10, Pgm^+ organisms exhibited three *Hin*dIII fragments (~19, ~11.5, and ~9 kb) that hybridized with the probe. Only the ~9-kb fragment was

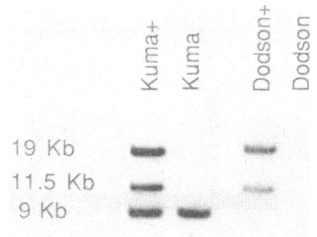

FIG. 6. Southern blot analysis of genomic DNA from *Y. pestis* Kuma and Dodson. Genomic DNA from Pgm^+ cells (Kuma+, Dodson+) and their isogenic spontaneous Pgm^- mutants (Kuma, Dodson) were digested with *Hin*dIII and probed with the 19.5-kb *Sal*I recombinant DNA fragment from pNPM1.

retained in the spontaneous Pgm$^-$ mutants. Despite these differences, the common mechanism among these strains appears to be a massive deletion that results in a Pgm$^-$ phenotype (Fig. 6).

Cloning of the wild-type *hms* locus. Genomic DNA from *Y. pestis* KIM6+ was partially digested with *Sau*3AI and size fractionated by ultracentrifugation through sucrose density gradients. DNA fragments between 7 and 23 kb were pooled, ligated into the *Bam*HI site of plasmid pLG338, and transformed into competent HB101 cells. The resulting transformants were analyzed by colony blot hybridization (1) with a 5.9-kb *Bam*HI-*Sal*I probe from pNPM1. A single clone was identified and designated pHMS1. The restriction enzyme sites in the 9.1-kb insert of plasmid pHMS1 matched those of pNPM1 (data not shown). The pHMS1 insert corresponds to the region of pNPM1 from just inside the *Sal*I site at 19.5 kb to ~1 kb past the *Nco*I site at 11.5 kb (Fig. 3).

Physiological characterization of the *hms* locus. The CR$^-$ phenotype was used as a screen for selecting Hms$^-$ Km^r mutants of KIM6+(pAMH62). In all spontaneous Pgm$^-$ mutants that have been examined, the CR$^-$ phenotype and the Hms$^-$ phenotype have been completely analogous. The eight *hms*::mini-*kan* mutants grow as white colonies on solidified PMH supplemented with hemin and therefore fulfill the original criterion of Pgm$^-$ mutants with respect to hemin storage (data not shown).

To determine whether pHMS1 is able to restore CR binding, the plasmid was electroporated into the spontaneous Pgm$^-$ mutant KIM6. Cells of KIM6(pHMS1) exhibited a CR$^+$ phenotype on CR plates and hemin storage on solidified PMH supplemented with hemin. As in Pgm$^+$ cells, the Hms$^+$ phenotype was expressed during growth at 26°C but not during growth at 37°C (data not shown). Thus, the 9.1-kb insert of pHMS1 complements the defect in hemin storage caused by the massive deletion in the spontaneous Pgm$^-$ mutant.

Although spontaneous Pgm$^-$ strains of *Y. pestis* (lacking pPCP1) are Pst^r, an Hms$^+$ Pst^r mutant has recently been isolated (40), suggesting that the structural genes for these characteristics are separate. In that case, we might expect our *hms*::mini-*kan* mutants to be Pst^s. Since these mutants carry pPCP1 and are therefore immune to pesticin, this could not be tested directly. Consequently, we transformed (or electroporated) recombinant plasmid pNPM1 or pHMS1 into *Y. pestis* KIM10 (which is Pgm$^-$ Pst$^-$ Pst^r). All strains were tested for sensitivity to pesticin by using a plate assay (see Materials and Methods). As expected, *Y. pestis* KIM10+ (which is Pgm$^+$ Pst$^-$ Pst^s) was pesticin sensitive (i.e., exhibited a zone of growth inhibition). KIM10 harboring either pNPM1 or pHMS1 remained pesticin resistant. This confirms that the pesticin receptor and hemin storage genes are separate entities. These results also suggest that a functional gene for the pesticin receptor is not present on pNPM1 or pHMS1. The intact gene could lie outside the cloned *Sal*I fragment, or it could be within the *hms* operon and downstream of the *hms*::mini-*kan* insert in KIM6-2012. Further analysis must await the construction of *hms*::mini-*kan* strains lacking pPCP1.

DISCUSSION

We generated a number of mini-*kan* insertion mutants of KIM6+(pAMH62) that no longer exhibit visible hemin storage at 26°C, the original definition of spontaneous Pgm$^-$ mutants of *Y. pestis*. In the six mutants closely analyzed, the inserts fell within an ~10-kb region of the genome. Whether these mini-*kan* inserts lie within structural or regulatory genes required for hemin storage is undetermined. A 19.5-kb *Sal*I fragment from KIM-2012, encompassing the interrupted *hms-2012*::mini-*kan* locus, was isolated and cloned. Using this fragment as a probe of genomic DNA from spontaneous Pgm$^-$ mutants from nine different strains of *Y. pestis*, we showed that a massive, spontaneous deletion event was invariably the cause of the Pgm$^-$ phenotype. This deletion includes nearly all of the *Y. pestis* DNA cloned into pNPM1 (~18 kb) and may extend well beyond this cloned segment. Finally, from a Pgm$^+$ strain (KIM6+) we cloned a 9.1-kb DNA fragment that restored temperature-regulated expression of hemin storage in Pgm$^-$ strains of *Y. pestis* KIM.

Ferber and Brubaker (16) showed that Pgm$^+$ strains of *Y. pestis* could lack Lcr and pesticin plasmids (named pCD1 and pPCP1, respectively, in KIM strains) and that spontaneous Pgm$^-$ isolates retained the cryptic 110-kb plasmid (called pMT1 in KIM strains). They consequently proposed a chromosomal location for the *pgm* locus. The possibility remained that the *pgm* locus was encoded on pMT1, which was somehow altered in Pgm$^-$ strains. However, we have shown that pMT1, pCD1, and pPCP1 do not contain sequences homologous to the ~18-kb region of DNA that is involved in the Pgm$^+$ phenotype. Thus our results confirm the chromosomal location of the *pgm* locus proposed earlier by Ferber and Brubaker (16).

A survey of Pgm$^+$/Pgm$^-$ isogenic pairs of organisms from nine different strains of *Y. pestis* identified two hybridization patterns. Pgm$^+$ organisms of strains KIM, Dodson, A12, and G32 exhibited 19- and 11.5-kb *Hin*dIII fragments that hybridized to the 19.5-kb probe from pNPM1. Spontaneous Pgm$^-$ mutants in these strains no longer possess DNA homologous to this probe. However, Pgm$^+$ isolates of strains Kuma, Salazar, Yokohama, TS, and K10 have three hybridizing *Hin*dIII fragments (19, 11.5, and 9 kb), and their spontaneous Pgm$^-$ mutants retain the 9-kb fragment. The identity of this retained fragment is uncertain. Given a spontaneous mutation rate of 10^{-5}, genomic DNA from a Pgm$^+$ population would be expected to contain a small amount of Pgm$^-$ genomic DNA. Perhaps a smaller deletion event is occurring in these strains and the 9-kb fragment is due to the presence of Pgm$^-$ DNA within our Pgm$^+$ preparation. However, if this were the case, one would expect the intensity of the 9-kb band in DNA from a Pgm$^+$ culture to be much less than was observed (Fig. 6). An alternative possibility is that a portion of the *pgm* locus is duplicated. Finally, our 19.5-kb probe may contain a repeated sequence, such as an insertion element, that is present in the genome of some but not all strains of *Y. pestis*.

The spontaneous deletion mechanism explains not only the lack of measurable reversion rates but also the simultaneous loss of physiologically distinct Pgm$^+$ characteristics that are differentially regulated in their expression. Thus, hemin storage (25, 26), pesticin sensitivity (3, 7, 16, 41), and expression of iron-repressible peptides (40), all characteristics associated with the Pgm$^+$ phenotype, may have only a genetic linkage and not a functional one. In fact, the pHMS1 clone complements the defect in hemin storage in the spontaneous Pgm$^-$ mutant but does not restore the Pst^s phenotype. This confirms previous evidence (40) that indicated that separate genes encode hemin storage functions and the pesticin receptor.

To reflect the increased complexity of the *pgm*$^+$ genotype this study has uncovered, we use the acronym Hms to refer only to the hemin storage component of the Pgm$^+$ phenotype of *Y. pestis*. The terms Pgm$^+$ and Pgm$^-$ have been retained

to 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. Although spontaneous Pgm^- mutants are avirulent (26, 49), the contribution of various, separate physiological characteristics of the Pgm^+ phenotype to the virulence of *Y. pestis* remains to be determined.

The benefit derived from using a nonreverting deletion event to prevent expression of factors necessary for survival in mammals as well as factors possibly important to survival in the flea is unclear. Species of the genus *Anabaena* undergo deletion of a 55-kb DNA segment during differentiation into a heterocyst. However, the heterocyst is a nonreplicating dead-end cell in which the deletion appears to allow expression of nitrogen-fixing activities (19). Recently, phase variants of *Bordetella bronchiseptica* were reported to occur via spontaneous deletions in the *vir* locus of that organism; however, these are small deletions of 50 to 500 base pairs (30), over 1 order of magnitude lower than the *Y. pestis* deletion. In *S. flexneri*, the CR binding determinant(s) is expressed only at 37°C and is encoded on a 230-kb virulence plasmid (14, 29, 36–38, 47). Spontaneous CR^- mutants of *S. flexneri* are due to loss of this plasmid, insertions into the plasmid, or deletions within the plasmid. Twelve classes of deletions that vary in size from ~10 kb to over ~50 kb have been identified (29, 38). These deletions cover divergent regions, and no region is common to all deletion classes (38). Finally, Carniel et al. (11) cloned a 1-kb DNA fragment from yersiniae that contains part of the coding sequence for a 190-kilodalton iron-repressible peptide. This 1-kb DNA fragment was present only in highly pathogenic *Yersinia* strains (11). It is undetermined whether this is a result of strain divergency, a separate small deletion event, or another segment of the *pgm* locus. In *Y. pestis*, the *pgm* deletion could be due to a transposition event, recombination between repetitive sequences, or the emergence of a defective, lysogenic bacteriophage. Further studies to identify the molecular mechanism of this deletion may illuminate a rationale for this high rate of nonreverting, spontaneous mutation to avirulence in an obligate parasite.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI25098 from the National Institutes of Health.

The assistance of Mounang Desai and Rachel Soloff is gratefully acknowledged. We thank Teanna Staggs and Jackie Fetherston for their thoughtful discussions and advice.

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