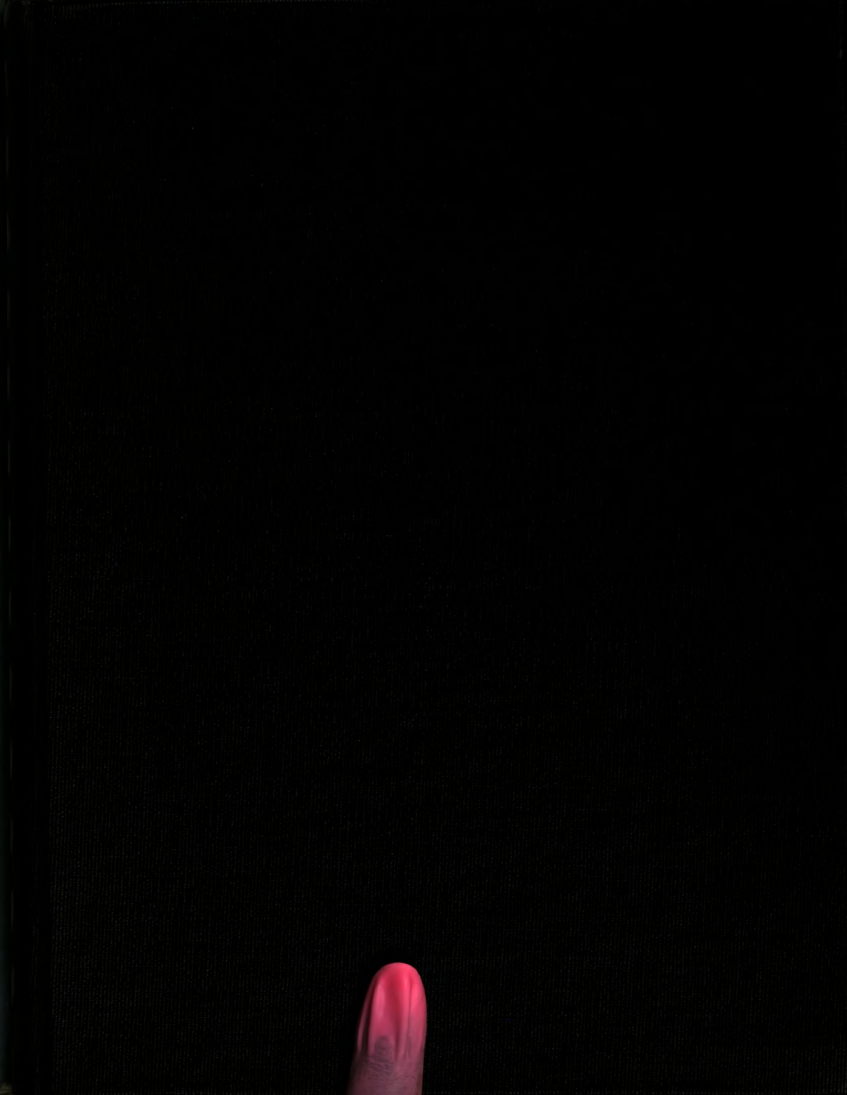

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GENETIC BASIS FOR THE HIGH-FREQUENCY MUTATION TO NONPIGMENTATION IN YERSINIA PESTIS AND ANALYSIS OF THE PIGMENTATION PHENOTYPE

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GENETIC BASIS FOR THE HIGH-FREQUENCY MUTATION TO NONPIGMENTATION IN YERSINIA PESTIS AND ANALYSIS OF THE PIGMENTATION PHENOTYPE

By

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ABSTRACT

GENETIC BASIS FOR THE HIGH-FREQUENCY MUTATION TO NONPIGMENTATION IN YERSINIA PESTIS AND ANALYSIS OF THE PIGMENTATION PHENOTYPE

By

Thomas S. Lucier

The pigmentation virulence determinant of Yersinia pestis encompasses the ability to bind hemin at 26°C, pesticin sensitivity, and a possible mechanism for iron acquisition. Their role in virulence and the genetic basis for the high-frequency mutation to nonpigmentation with concomitant loss of these traits remain obscure. We used pulsed-field gel electrophoresis (PFGE) to demonstrate that this mutation is due to a specific chromosomal deletion of approximately 100 kb. The deletion had not occurred, but was able to do so, in a rare mutant capable of hemin storage, but not Pgm associated iron transport suggesting a genetic, but not functional linkage of these traits. Total genome size was found to be 4397.9 +/- 134.6 kb, and intraspecific variation in macrorestriction patterns confirmed the existance of three distinct biotypes. Studies of the accumulation of labelled inorganic and hemin-bound

iron showed hemin binding to be mediated by the outer membrane of Pgm^+ cells at 26^oC, and to be a storage function distinct from hemin uptake. Inorganic, but not hemin-bound iron was preferentially stored on a bacterioferritin-like cytoplasmic protein. Uptake of inorganic and hemin-bound iron were non-competitive and involved different mechanisms. Gradiant plates were developed in which cells were exposed to increasing concentrations of various iron chelators to better define the Pgm asociated iron uptake system. Results indicated this was an inducible, non-specific system which mediates sensitivity to the bacteriocin pesticin in all pathogenic Yersinia species. Analysis of protein expression using SDS-PAGE and two-dimensional gel electrophoresis identified several potential components of this system. In Y. pestis, but not enteropathogenic Yersinia species the mutation to Pst^r was correlated with loss of expression of three of these peptides. Temperature profoundly influenced the ability of Y. pestis to obtain iron in a Pgm independent manner. This correlated with expression of proteins in the outer membrane and periplasm.

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LITERATURE REVIEW

The genus Yersinia (genus IX of the family Enterobacteriaceae) contains seven recognized species, three of which are important human pathogens (19,23,32). Yersinia pseudotuberculosis and Y. enterocolitica are enteropathogenic organisms which cause chronic gastrointestinal disease in human hosts. They are transmitted via contaminated food or water supplies. Y. pestis is the causative agent of bubonic and pneumonic plague, a disease transmitted among various species of rodents and occasionally from rodents to humans by the bites of certain species of fleas. This highly virulent organism can be fatal to primates and most rodents at infecting doses of 10 cells or less (30). While most commonly infecting rodents its considerable influence on human history results from periodic devastating pandemics responsible for the deaths of perhaps as many as 200 million people (23,84). Although exhibiting considerable differences in ecology and the diseases they cause, Y. pestis and Y. pseudotuberculosis chromosomal DNA is nearly indistinguishable in hybridization studies (7,14,73). Detectable genetic discrepancies are primarily in plasmid content (5,47) making their study a potentially powerful tool for understanding the genetic basis for virulence and pathogenicity. Chromosomal DNA of

Y. enterocolitica is much less closely related to that of the other two species suggesting that the divergence of Y. enterocolitica from the lineage occurred at an earlier time than did the separation of Y. pestis and Y. pseudotuberculosis (7,14,73). All three species are geographically widespread with numerous distinct strains (32). Intraspecific relationships within Y. pestis has not been extensively studied, however, a classification system which recognizes three biotypes (mediaevalis, antigua, orientalis) based on the ability to ferment glycerol and reduce nitrate has been proposed (45).

Y. pestis is acquired by its vector when it obtains a blood meal from an infected mammalian host (usually a rodent). While multiplying in the gut the bacteria produce coagulase which results in blockage of the proventriculus and prevents utilization of the blood meal by the flea. The hungry vector is induced to feed on another host where regurgitation of contaminated blood causes introduction of the bacteria into the subcutaneous tissues of the previously uninfected host. From here bacteria are transported in the dermal lymphatics to regional lymph nodes where they undergo rapid multiplication. Inflammation and necrosis at the infected nodes produces the "bubos" characteristic of bubonic plague. Spillover into the blood allows dissemination of the bacteria and establishment of foci of infection at other locations associated with the reticuloendothelial system. Establishment of foci of

infection within the lungs results in the pneumonic form of plague. Spillage of the organism into the vascular system permits ingestion by the vector and lethality (32).

Differences in pathogenicity of Y. pestis versus the enteropathogenic yersiniae may relate in part to the unique ability of the former to cause death from peripheral sites of infection (23). Wild type Y. pestis exhibited LD_{50}s in mice of 10 for intravenous, intraperitoneal, subcutaneous, and intradermal routes of injection, while the enteropathogenic yersiniae demonstrated comparable pathogenicity only after intravenous injection (23,24,30,37,38,108). Within 24 hours of intravenous injection with any of these species into mice organisms disappeared from the blood and large populations had become established in the liver, spleen and lungs. Once populations of 10^6 bacteria per gram of tissue were achieved bacteremia was again present presumably due to spillover from the heavily infected tissues (104,108,109). Invasion and multiplication of bacteria within the spleen and liver resulted in the appearance of focal necrotic lesions. A typical inflammatory response to infection failed to develop in mice injected with Y. pestis and the lesions progressively enlarged and eventually involved entire organs (104,110). This pathology of infection suggests suppression of cell mediated immunity. Although enteropathogenic yersinial infections also proved fatal, the necrotic lesions were more typical with some degree of neutrophil recruitment

(104,110). Examination of these lesions by electron microscopy showed Y. pseudotuberculosis remained extracellular (96). Given the number of in vitro studies which indicate yersiniae can survive or grow within professional phagocytes (39,41,60,83,105,106) this was a surprising observation. While there may be interactions in vivo between yersiniae and professional phagocytes which are as yet undetected, it seems probable that yersiniae exist primarily as extracellular parasites.

VIRULENCE FACTORS OF Yersinia pestis

Although effective treatments have been devised for diseases caused by the pathogenic yersiniae, their continued study is of significance since these organisms provide an important model for determining the mechanisms of bacterial virulence and pathogenicity. Methods of in vitro culture are well established, and since rodents are natural hosts a realistic in vivo system is readily available. Studies of yersiniae have provided initial observations on several mechanisms of virulence that were subsequently found to apply to other pathogenic species including i) auxotrophs blocked in purine biosynthesis are avirulent (18), ii) plasmids can mediate bacterial virulence (5,47), iii) the ability to bind hemin is associated with virulence (57,58), iv) the ability to restrict bacterial growth in vivo by

limiting iron availability acts as a host defense mechanism (59), v) hemin can serve as the sole source of iron for a prokaryote (80), vi) iron can be obtained by a non-siderophore, membrane bound specific transport system (80).

Characterization of mutants with significant increases in LD_{50} is one method of identifying virulence factors. In Y. pestis this approach has led to the recognition of several determinants of virulence. These are listed in table 1. While the factors which were the object of this study are chromosomally encoded, pathogenic yersiniae all require the 70 kb Lcr plasmid for virulence, and Y. pestis is unique in requiring three different plasmids for expression of full virulence (5,47,108). These plasmids and their products are described in table 2. In the remainder of this review I will describe these recognized virulence factors of Y. pestis.

PURINE BIOSYNTHESIS

Mutational loss of the ability to synthesize purines was first demonstrated to result in avirulence in Salmonella typhi and was hypothesized to relate to the lack of readily available purines in the tissues and serum of the host (1). This has since been shown to be an important virulence factor in a number of pathogens including Y. pestis (22). In Y. pestis it was demonstrated that if the block occurred prior to de novo synthesis of inosine monophosphate (IMP)

Table 1. Effect of loss of virulence factors in Yersinia pestis on the LD_{50} in guinea pigs and mice.

Virulence Factor[a]					LD_{50}[b]		
Lcr	Pst	Pgm	Fra	Pur	mouse	guinea pig	mouse+Fe^{3+}[c]
+	+	+	+	+	10	10	10
O	+	+	+	+	10^7	10^8	10^7
+	O	+	+	+	10^5	10^6	10
+	+	O	+	+	10^7	10^8	10
+	+	+	O	+	10	10^4	10
+	+	+	+	O[d]	10^2	10^4	10^2
+	+	+	+	O[e]	10^7	10^8	10^7

a. abbreviations for virulence factors
   - Lcr: Presence of Lcr plasmid
   - Pst: Presence of Pst plasmid
   - Pgm: absorb exogenous hemin at 26°C
   - Fra: Fraction 1 Antigen
   - Pur: ability to synthesize purines

b. cells introduced by intraperitoneal injection

c. injection of sufficient iron to saturate serum transferrin

d. mutation blocks purine synthesis prior to inosine monophosphate

e. mutation blocks purine synthesis after inosine monophosphate

TABLE 2. Plasmids in Yersinia.

Plasmid (kilobases)	Proteins Encoded	Species
9.5	Pesticin Pesticin Immunity Plasminogen Activator/ Coagulase	Y. pestis
70	V Antigen Yersinia Outer Membrane Proteins (Yops) Lcr regulatory proteins (lcr or vir genes)	Y. pestis Y. pseudotuberculosis Y. enterocolitica
100	Fraction 1 Murine Toxin	Y. pestis

there was only a relaatively small reduction in virulence, but if the block occurred at the conversion of IMP to guanosine monophosphate (GMP) the organisms were avirulent (18). The discovery that Y. pestis unable to convert IMP to GMP could not survive or reproduce in mouse peritoneal macrophages in vitro without the addition of hypoxanthine or guanosine to the culture medium supports the idea that this purine is normally unavailable to the parasite when it is within the mammalian host (105).

FRACTION 1 ANTIGEN

Fraction 1 antigen is a protein-carbohydrate complex associated with the capsule of Y. pestis (2). Complexes are composed of approximately 15 kda protein-carbohydrate (fraction 1A) and free protein (fraction 1B) subunits which will assemble into structures of up to 300 kda (6,51). Maximum expression occurs at body temperatures of mammalian hosts (6). The significance of fraction 1 antigen as a virulence factor is unclear. While Fra- strains injected intraperitoneally into guinea pigs demonstrated reduced virulence, lethality is not reduced in mice (table 1) (30).

Also encoded on the 100-kb Tox plasmid is an exotoxin which is highly lethal to mice and rats, but non-toxic in other hosts (16). This is a 120 kdal protein composed of subunits which may be 12 kDa (72), however, modified purification schemes suggest they may be larger (Lucier, unpublished data). While the role of the exotoxin in vivo

is uncertain, it may account for the more rapid death of mice following intravenous injection with Y. pestis, than with enteropathogenic species of Yersinia which do not produce exotoxin (104,108).

LCR PLASMID

Early studies of virulence in Y. pestis were hampered by a rapid shift to avirulence when cells were cultivated in vitro at 37°C but not at 26°C in a variety of commonly used media (50). Modification of media components showed this to be the result of an unexpected dependency on the presence of at least 2.5 mM Ca^{2+} for unrestricted growth of virulent cells at 37°C (56). While zinc and strontium also facilitated growth, only calcium is present in vivo in sufficient quantities to alleviate the restriction. Further studies showed restriction of growth to be Mg^{2+} dependent with lower concentrations of Ca^{2+} being permissive at magnesium concentrations below 20mM (27). A role for the Lcr plasmid in this response became apparent with the observation that calcium dependence for growth was lost in cells lacking the plasmid (5,47). Along with the restriction in vegetative growth bacteria were discovered to begin synthesizing a set of Lcr plasmid encoded putative virulence factors including Yersinia outer membrane proteins (YOPS) and V antigen (10,11,12,20,27,100,102,118). This is termed the Lcr (low calcium response) and is mediated by similar plasmids in all pathogenic species of Yersinia

(5,47,86).

The growth restriction exhibited by virulent yersiniae grown in conditions simulating the environment within host cells ($37^{o}C$, 20mM Mg^{2+}, approximately 0 mM Ca^{2+}) is the result of an ordered nutritional stepdown beginning with cessation of stable RNA synthesis and reduction of adenylate energy charge (40,119). One additional round of DNA replication and cell division occurs (109), and most protein synthesis is greatly reduced or eliminated, with the exception of most factors encoded on the Lcr plasmid and a few chromosomally encoded proteins which may be necessary for full expression of virulence (69,119). In Y. pestis growth restriction is complete, although addition of Ca^{2+} or shifting the temperature down to $26^{o}C$ restores normal growth (119). Similar responses occur in the enteropathogenic yersiniae, however, the restriction of growth is not as extreme (21,38).

Regulation of the Lcr is complex and not well understood. Structural genes for YOPS are located in several operons scattered about the plasmid and function as a coordinately controlled regulon (10,13,100). Regulatory genes designated lcr (vir in Y. enterocolitica) are encoded within an 18 kb Ca^{2+} dependence region (10,52,85,117). Five thermally inducible lcr genes have been identified in this region and transposon insertions within these loci usually result in a loss in the ability to perform the Lcr just as in cells from which the plasmid has been cured (13,52).

Spontaneous avirulent mutants which no longer expressed the Lcr were assumed to have lost the Lcr plasmid. Yet as many as 50% were found to still carry the plasmid, but with various mutations within the calcium dependence region(117). At least two other regulatory factors and the V antigen are encoded in an adjacent lcrGVH operon (88). Insertions in this region often eliminate the regulatory effects of calcium so bacteria grow and express YOPS at 37^{o}C regardless of calcium levels (117). On the opposite side of the calcium dependence region of Y. enterocolitica is the vir C operon which encodes a group of proteins necessary for the secretion of YOPs (70). This 8.5 kb region contains 13 open reading frames and is regulated in the same manner as the YOP operons. Its existence in the plasmids of the other pathogenic yersiniae has not yet been reported. While the Lcr plasmids from these three species are very similar, homology within the two regulatory regions is especially high (nearly 100%) suggesting this mechanism is conserved within the pathogenic yersiniae (88). The arrangement and nucleotide sequence of the YOP genes is more variable (10,100), yet exchanging the Lcr plasmids of Y. pestis and Y. pseudotuberculosis still results in a normal Lcr in both species (116). Thus the Lcr and its regulatory mechanisms are highly conserved and essential for Yersinia virulence.

Several comprehensive models describing regulation of the Lcr have been proposed and while they disagree in many details, there are consistent similarities (8,42,87). All

recognize two separate regulatory loops one sensitive to temperature and the other sensitive to calcium concentration. A shift to 37°C initiates production of an activator encoded by the lcr F gene (42). Additional genes in the calcium dependence region may also be involved (8). This encourages transcription of plasmid encoded genes. Counteracting the thermally induced activator system in low calcium conditions is a calcium sensitive repressor system involving at least lcr H of the lcr GVH operon (8,87,89). These systems determine the level of transcription at the YOP and lcr GVH operons. Non-polar lcr V mutants have recently been shown to lose their dependency on calcium for growth at 37°C, yet they still produce YOPs (87). Thus accumulation of V antigen in the cytoplasm may be sufficient to promote restriction of growth. Association of V antigen with a regulatory function was unexpected.

V and W antigens were initially shown to be produced by virulent yersiniae in vitro and in laboratory animals (31). V antigen is a 38 kDa monomer which may undergo aggregation, a property which may account for its initial size estimate of 90 kDa. It is found within the cytoplasm and in culture supernatants (93,102). Studies on the role of secreted V have been hampered by its autoproteolytic property (25), however, the passive protection provided by polyclonal V antiserum against infection by all pathogenic yersiniae argues for an essential role in virulence (111). Histopathology studies suggest it may suppress cell mediated

immunity and granuloma formation (109,110). Thus V may be an unusual bifunctional protein serving both a regulatory role in the cytoplasm and as a virulence factor following secretion (87).

W was originally identified as a 140 kDa lipoprotein which accumulated in the supernatant (64). Little is known of W, however, recent attempts at its isolation resulted in retrieval of a protein with characteristics of GroEL, a chaperone protein which facilitates secretion (65, Mehigh, unpublished data). W may be the GroEL - V complex.

Yops are produced by all yersinial pathogens during the Lcr, but there are species specific differences in expression. Table 3 provides a summary of identified Yops and their known characteristics. While these become major outer membrane proteins in enteropathogenic species early studies failed to discover them in the outer membrane of Y. pestis (102). This was a puzzling observation since serum from convalescent plague patients contained anti-Yop immunoglobulins (67), and when the Lcr plasmid of Y. pestis was transferred to Y. pseudotuberculosis normal outer membrane Yop expression occurred (116). With the discovery that mutants cured of the pesticin plasmid exhibited stable expression of Yops in their outer membrane (93) it became apparent that the plasminogen activator protein catalyzed the proteolysis of Yops (98). So although Yops are produced by Y. pestis they are immediately hydrolyzed upon reaching the outer membrane in pst^+ strains. This may explain the

Table 3. Properties and Distribution of Yops.

Protein	Mol Wt. (kDal)	Produced by: pestis	pseudo	entero	Essential for full virulence	Proposed Function
Yad A		O	+	+	O	Adhesion associated with expression of chronic disease, inhibit complement activation
Yop B	44	+	+	+		
Yop C	42	+	+	+		
Yop D	34	+	+	+		
Yop E	25	+	+	+	+	Stable degradation product in Y. pestis; virulence of intraperitoneally injected yopE mutant "restored" by injected iron; inhibits phagocytosis.
Yop F	76	+	+			
Yop G	58	O	+			
Yop H	45	+	+	+	+	Protein tyrosine phosphatase; inhibits phagocytosis
Yop I	43	O	+			
Yop J	31	+	+		O	
Yop K	22	+	O	O	+	Required for prompt growth in tissues
Yop L	16	+	O	O	+	Required for prompt growth in tissues
Yop M	45	+	O	O	+	Primary structure similar to platelet glycoprotein Ib; inhibit platelet aggregation and clotting
Yop N	34	+	+	+		Temperature sensor

References (3,9,13,23,49,53,62,82,91,92,100,101,104,117)

observation that a polyclonal preparation of anti-Y. pestis Yops provided complete passive protection against Y. pseudotuberculosis but was ineffective against plague (23). The continual renewal of major surface antigens may mediate the removal of bactericidal molecules involved in host defense and allow for the expression of a more acute disease.

Numerous studies have dealt with the possible roles of various Yops as virulence factors. Genetic studies involving insertional mutagenesis have helped identify Yops whose expression is necessary for full virulence (13,99,101) and a growing number of studies have addressed the specific functions of these Yops. These and other characteristics of the known Yops are summarized in table 3. Several of these have recently been the object of intensive study. Yad A is unusual in that its expresion is only temperature regulated; Ca^{2+} concentration has no effect (61). It is not expressed in Y. pestis due to a frameshift mutation and its mutational loss in Y. pseudotuberculosis enhances lethality (92). Yad A may promote chronic rather than acute disease. Both Yop E and H may inhibit the activity of professional phagocytes using different mechanisms. Yop E may interfere with microtubular function (91) while Yop H is a tyrosine phosphatase which can remove phosphate from certain host proteins and perhaps interfere with signal transduction pathways necessary for phagocytosis (9,53). Recently there has been controversy surrounding the location at which Yops

exert their effects. Since they lack signal sequences and hydrophobic regions typical of membrane spanning proteins (71) they may be released proteins which exert their effects following their release from the bacterial cell.

During the Lcr a few chromosomally encoded proteins continue to be expressed at high levels (69). The two expressed in greatest amounts are GroEL which would be necessary for transport of the large number of secreted proteins being produced and a large protein with catalase activity. Whether the latter is necessary for the expression of disease in Y. pestis remains to be resolved.

PESTICIN PLASMID

Ben-Gurion and Hertman (4) first recognized that virulent strains of Y. pestis produced a bacteriocin which they named pesticin. It is a 44 kDa protein which exhibits n-acetylglucosidase activity (46) and has a host range limited to Y. pseudotuberculosis serotype I, O8 strains of Y. enterocolitica, a few strains of Escherichia coli, and Pgm^+, Pst^- strains of Y. pestis (28). Pesticinogenic strains are protected from the effects of the bacteriocin by an immunity protein absent in non-pesticinogenic mutants (55). It seems unlikely that a bacteriocin which targets envelope associated molecules in prokaryotes could have a role in virulence, so it was surprising that Pst^- strains were no longer able to disseminate from peripheral sites of

infection in mice (24). The discovery of linked expression of a plasminogen activator protein with potent fibrinolytic activity helped explain this observation (28). It is located in the outer membrane as a transient 37 kDa monomer which autodegrades and forms a 35 kDa peptide (98). The protein was also shown to exhibit coagulase activity, however this may be an artifact restricted to rabbit blood (97), or an activity expressed only at 26^{o}C which may be significant for survival in the gut of the flea vector (67). Recent studies also demonstrated the role of this protein in the hydrolysis of Yops as previously described in this report.

The 9.5 kb pst plasmid is unique to Y. pestis and encodes pesticin, the pesticin immunity protein, and the plasminogen activator (97). Loss of the plasmid results in significantly reduced virulence by peripheral routes of infection (Table 1), presumably due to reduced ability of the bacteria to reach the lymph or blood without expression of plasminogen activator. Injection of mice with 40 ug of iron can restore virulence to Pst^{-} strains introduced intraperitoneally. Although this might imply existance of a lesion in an iron uptake mechanism associated with the pesticin plasmid, no evidence for such a mechanism has been found. This effect could also result from inhibition of the activity of host professional phagocytes by excess iron (111).

PIGMENTATION

Virulent isolates of Y. pestis can absorb exogenous hemin (57) or congo red (107) to their surface when grown on solid media at 26°C thus forming pigmented (Pgm^+) colonies. Strains which suffer mutational loss of this ability (Pgm^-) grow as white colonies and are avirulent via peripheral route of injection (58). Interestingly they remain fully virulent showing normal progression of the disease if introduced intravenously (108) indicating that the Pgm associated virulence factors are necessary for survival in or dissemination from peripheral tissues. That virulence of peripherally introduced Pgm^- strains can be restored by injecting sufficient iron (Fe^{3+}) to saturate serum transferrin (58) raised the possibility that mutation to Pgm^- resulted in the loss of an iron uptake mechanism required for full virulence.

Another association exists between pigmentation and pesticin. Pgm^+, Pst^- strains are sensitive to pesticin due to the lack of the Pst plasmid encoded immunity protein. Yet Pgm^-, Pst^- strains are resistant (17). Even in Pgm^+, Pst^- cells the effects of pesticin are inhibited by sufficient Fe^{3+} or hemin in the medium (26). It was suspected that pesticin could bind to iron repressible protein components of a high affinity iron uptake system in the cell envelope (28). Type B colicins of E. coli use this type of mechanism to attach to target cells (44). That ton B mutants of pesticin sensitive E. coli strains are also

resistant provides support for this idea (48). If this is the case, then a lesion in at least one iron uptake system would occur when cells become Pgm^-. Initial studies found no differences in growth between Pgm^+ and Pgm^- bacteria in iron deficient (less than 0.3 uM) media lacking significant iron chelating molecules (80). But in a similar medium containing citrate Pgm^- cells were unable to grow at 37°C once storage reserves were depleted (94). Both grew equally well at 26°C or at 37°C after addition of 50 uM $FeCl_3$ or hemin.

High affinity iron uptake systems of gram negative bacteria typically include outer membrane proteins expressed more strongly under low iron conditions (76). It was, therefore, considered significant that Pgm^- mutants lost the ability to express five outer membrane peptides, four of which were iron repressible (irps B-E) (95). The other peptide (peptide F) could be identical to a previously described Pgm^+ specific outer membrane peptide whose expression is temperature regulated (103), and to the 72 kDa peptide of three identified by genetic means as being required for hemin binding at 26°C (79,81). Selection for pesticin resistance in Pgm^+, Pst^- strains results in isolation of large numbers of Pgm^- mutants, however, a rare pesticin resistant mutant that retained pigmentation (Pgm^+, Pst^r) was recently obtained (95). This strain had the same growth characteristics in iron deficient media as Pgm^- cells, and failed to produce irps B-E suggesting a role for

Table 4. Phenotypic characteristics of pigmented (Pgm^+), nonpigmented(Pgm^-), and pigmented, pesticin resistant (Pgm^+, Pst^r) isolates of Yersinia pestis KIM.

	PHENOTYPE	Pgm^+	Pgm^-	Pgm^+,Pst^r
1.	Absorb hemin or congo red at 26°C.	+	-	+
2.	Virulent via peripheral route of infection.	+	-	?
3.	Virulent via intravenous route of infection.	+	+	?
4.	Pesticin sensitive. (Pst^- strains)	+	-	-
5.	Growth in iron deficient medium of Perry and Brubaker. (80)	+	+	+
6.	Growth in iron deficient Higuchi's medium.			
	a. 37°C	+	-	-
	b. 26°C	+	+	+
7.	Growth with hemin containing proteins or ferritin as the sole source of iron.	+	+	+
8.	Growth with lactoferrin or transferrin bound iron as the sole source.	-	-	-
9.	Expression of Irps B-E in iron deficient medium.	+	-	-
10.	Expression of peptide F.	+	-	+
11.	Expression of HMWPs in iron deficient media.	+	-(?)	?

one or more of these peptides in a high affinity iron uptake system and as the binding site for pesticin. These mutants still produce peptide F. Pigmentation and an iron uptake system appear to be two separate phenotypes associated with the classical Pgm virulence determinant. They employ separate proteins which are independently regulated (pigmentation by temperature and irps by iron availability). While it is not known which of these determinants is required for virulence, the inhibition of pigmentation at host temperatures makes it less likely to be significant.

Since the switch to Pgm^- did not correlate with the loss of any of the three plasmids in Y. pestis, Pgm functions were assumed to be chromosomally encoded (47). Yet the conversion to Pgm^- occurs at a frequency of 10^{-5} and is irreversible (17) implying a high frequency deletion event. Perry has recently shown this to be the case with a deletion size of at least 45 kb which contains structural genes for the peptides required for pigmentation (79,81). Irp expression is not restored by this segment of DNA so if structural genes for these peptides are also lost the deletion could be considerably larger.

IRON UPTAKE IN YERSINIAE

Iron is an essential nutrient for all cells and although it is abundant in nature it is not often available

in a readily accessible form (74). The more soluble ferrous form is quickly oxidized to ferric iron in the presence of oxygen, and forms hydroxides with a solubility constant of 10^{-38} at physiological pH values. In response to this prokaryotes have evolved special mechanisms for iron acquisition (63,74). Obtaining iron in mammalian hosts is also difficult since iron privation in extracellular fluids serves as an important defense against bacterial growth (29,113,114). Although total iron content of host tissues is far in excess of that needed by bacteria for growth, it is present as components of organic molecules such as hemin, or chelated to high affinity proteins reducing its availability to potential parasites. Important chelators include transferrin, haptoglobin and hemopexin in serum, lymph and tissue fluids, and lactoferrin in external secretions. Because of their high affinity the concentration of free iron is estimated to be 10^{-18}M; far below the 1 - 3 uM needed by most bacteria for survival without expression of special mechanisms for its acquisition (75). Removal of iron from transferrin and its sequestration in cells of the reticuloendothelial system during infection further lowers potentially available iron in the blood and tissue fluids (114). Virulence will in part depend on the ability of bacteria to obtain sufficient iron in this environment.

A widespread mechanism of iron uptake is the production and secretion of low molecular weight, high affinity iron

chelators called siderophores, and cell bound proteins for the uptake and processing of ferrisiderophores (75,76). While this is an important virulence factor in some pathogens (43,78) a growing number of pathogenic bacteria, primarily facultative intracellular parasites, have been found to rely instead on membrane bound high affinity iron binding proteins (77,80,90,95,115). The mechanisms of iron uptake in yeriniae remain poorly defined. Although some studies indicated the possible production of siderophores by Y. pestis (112) and enteropathogenic yersiniae (54), an extensive study by Perry and Brubaker found no evidence of siderophore production by any pathogenic yersiniae (80). However, enteropathogenic yersiniae, but not Y. pestis could use exogenously supplied siderophores (15,80). Pathogenic yersiniae have cell bound systems which allow uptake of iron from ferritin, hemin, hemopexin, haptoglobin, and hemoglobin (80,94,95). The mechanism is unknown, but since both Pgm^+ and Pgm^- strains can use hemin as their sole source of iron it would appear to be separate from the uptake system lost in the mutation to Pgm^- (80). Iron chelated to transferrin or lactoferrin cannot be used in vitro (95). Irps B-E may be components of a high affinity iron uptake system, however, the source from which it obtains iron in the host is unknown (95). There is also evidence that temperature may influence expression of iron uptake systems. Pgm^+ cells can grow at 37^oC in an iron deficient medium containing citrate and Pgm^- cells cannot. But at 26^oC in the same

medium they grow equally well (94). The physiological explanation for this difference is unknown.

Iron also regulates the expression of two iron repressible high molecular weight proteins (HMWP) of 190 and 240 kDa in all pathogenic yersiniae (33,35,36). They are not expressed in several avirulent strains or in the non-pathogenic species. In Y. pestis the structural genes for these proteins appear to be lost as part of the deletion event when cells become Pgm^- (34). Although originally described as being located in the outer membrane, Sikkema and Brubaker (95) failed to see their expression in two-dimensional outer membrane gels of Y. pestis grown in iron deficient medium. The reason for this discrepancy was unclear. Iron deficiency can act as a signal to regulate expression of virulence factors not involved in iron uptake (78) so the role of irps and HMWPs is not definitively known. The possibility of them serving as part of a membrane bound system for acquisition of iron in peripheral tissues which is required for virulence needs to be explored.

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CHAPTER I

(ARTICLE)

Determination of genome size, macrorestriction pattern polymorphism, and nonpigmentation-specific deletion in *Yersinia pestis* by pulsed-field gel electrophoresis.

by

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ABSTRACT

Of 16 restriction endonucleases known to hydrolyze rare 6- or 8-base recognition sequences that were tested, only SpeI, NotI, AscI, and SfiI generated fragments of chromosomal DNA from Yersinia pestis, the causative agent of bubonic plague, of sufficient length to permit physical analysis by pulsed-field gel electrophoresis (PFGE). Of the individual bands detected after single-dimensional PFGE of these digests, the largest sum was obtained with SpeI (3575.6 + or - 114.6 kb). Of these 41 bands, 3 were found to contain comigrating fragments, as judged by the results of two-dimensional SpeI-ApaI PFGE; addition of these fragments and the three plasmids of the species yielded a refined estimate of 4397.9 + or - 134.6 kb for the genome. This size was similar for eight strains of diverse geographical origin that exhibited distinct DNA macrorestriction patterns closely related to their biotypes. The high-frequency chromosomal deletion known to exist in nonpigmented mutants (unable to assimilate Fe^{3+} at $37^{o}C$ or store hemin at 26^{oC}) was shown by two-dimensional PFGE analysis with SpeI and ApaI or with SfiI and SpeI to be 92.5 and 106 kb in size respectively. The endpoints of this deletion were precise, and its size was more than sufficient to encode the eight known peptides reported to be absent in

nonpigmented mutants. This deletion had not occurred (but was able to do so) in a rare mutant capable of hemin storage but not iron transport.

INTRODUCTION

The majority of virulence factors required for the expression of bubonic plague by Yersinia pestis are encoded by the 9.5-kb pesticin (Pst), 70-kb low-calcium response (Lcr), and 110-kb capsule/exotoxin (Tox) plasmids. These plasmids or their gene products may interact with each other, the bacterial chromosome, or chromosomally encoded proteins. This phenomenon is pronounced with the Pst plasmid, known to encode the bacteriocin-pesticin, its immunity protein, and a plasminogen activator (Pst^+). The plasminogen activator promotes posttranslational degradation of certain Lcr plasmid-encoded gene products, whereas pesticin itself interacts with the product of one of the few known chromosomally encoded virulence genes of the species (7). The latter is an outer membrane protein involved in the expression of a complex phenotype first defined (24) as the ability of yersiniae to absorb exogenous chromatophores (e.g. hemin, crystal violet, and the dye Congo red (24,51)) from solid medium at room temperature and thus grow as dark or pigmented colonies (Pgm^+). Such colonies frequently contained white sectors composed of spontaneously occurring Pgm^- mutants (24,51) that, if also Pst^-, were resistant to the antibacterial activity of pesticin (9). In contrast,

Pgm^+ Pst^- isolates were killed by the bacteriocin (21). This relationship permitted the determination of a mutation rate from Pgm^+ to Pgm^- of 10^{-5} (9) and suggested that this event results in the loss of a pesticin receptor.

Pgm^- mutants failed to grow in an iron-deficient medium adequate for Pgm^+ organisms (45) and lacked detectable levels of six iron-repressible peptides expressed by the Pgm^+ parent. These consist of two high-molecular weight proteins of 190 and 240 kDa (10-12) and four smaller outer membrane peptides termed IrpB (69 kDa), IrpC (67 kDa), IrpD (69 kDa), and IrpE (65 kDa) (44). Expression of iron-repressible proteins in bacteria is commonly controlled by the Fur regulatory system (53), and this mechanism was shown to also exist in yersiniae (49). Furthermore, Pgm^- mutants failed to produce an additional outer membrane protein, termed peptide F (73 kDa (44)), that is not regulated by iron but rather undergoes repression at host but not room temperature (50). Peptide F may be identical to one of two independently discovered proteins required for binding of chromatophores (39). The diversity of function among these peptides was underscored by isolation of a rare Pgm^+ Pst^- but pesticin-resistant mutant that produced high-molecular weight proteins and peptide F but failed to express IrpB, IrpC, IrpD, and IrpE and to grow in iron-deficient medium (44, 45). Typical Pgm^- mutants were avirulent in mice by intraperitoneal or other peripheral routes of infection (50% lethal dose, 10^7 bacteria (25, 52)). However, concomitant

injection of sufficient Fe^{3+} to saturate serum transferrin reduced the 50% lethal dose to ca. 10 bacteria, a value corresponding to that determined for Pgm^+ organisms in normal mice (25). Curiously Pgm^- mutants also exhibited full virulence in normal mice when administered via the intravenous route (50% lethal dose, ca. 10 bacteria (52)).

It seems unlikely that the high-frequency mutation to Pgm^- represents inactivation of flanking fur sequences or loss of Fur repressor protein because these events promote constitutive expression of iron-repressible peptides (53). The mutation may reflect the loss of some additional unknown regulatory function or, more likely, be the consequence of a reported greater than 18-kb chromosomal deletion unique to Pgm mutants (41). However, structural genes for known missing Pgm^+-specific functions have not yet been detected within this segment of DNA, indicating that the actual size of the deletion may be larger.

The technique of pulsed-field gel electrophoresis (PFGE) was used in the present study to characterize the genome of Pgm^+ and Pgm^- cells of Y. pestis. This procedure facilitates the separation of large DNA fragments generated upon digestion of the chromosome via the action of those restriction endonucleases that cleave at rare sequences (46). For a growing number of bacterial species (28), the method has enabled accurate estimation of genome sizes, construction of physical chromosome maps, and detection of gross chromosome alteration (e.g., large deletions,

additions, inversions, and transpositions). It has also proven to be an effective method for qualitatively evaluating intraspecific genetic variation, permitting identification of individual isolates of a given species by comparison of macrorestriction patterns (1, 2, 6, 31). Although PFGE has been used extensively to define the genome of Escherichia coli (15, 47), comparable studies with other members of the Enterobacteriaceae have been limited; only one such report concerning yersiniae has appeared (furnishing an estimate of 2400 kb for the genome of Y. pestis (23).

The purpose of this study was to provide a refined estimate of the size of the Y. pestis genome and determine the size of the high frequency Pgm^- specific deletion. In addition , we present information regarding the efficacy of restriction endonucleases capable of cleaving rare sequences within the genomes of selected isolates. The resulting restriction patterns correlated with classical biotypes (16).

MATERIALS AND METHODS

Bacteria. Characterizations and derivations of the strains of Y. pestis used in this study are shown in Table 1. All isolates used in experimental work are avirulent due to the absence of the Lcr plasmid (17). Pgm^- mutants were obtained from Pgm^+ parents by cultivation on Congo red medium after incubation at 26°C (51). The isolation and characterization of Pgm^+ Pst^- (pesticin-resistant) mutant Kl15-B was reported previously (45).

Preparation of genomic DNA. DNA was prepared in agarose plugs by a modification of the methods of Lee and Smith (32). Cells were grown to late logarithmic growth phase in chemically defined medium (54) at 26°C. Chloramphenicol was then added to a final concentration of 20 mg/ml, and the cultures were incubated for an additional 1 h to permit completion of a final round of chromosome replication. Cells were then harvested by centrifugation (9000 x g for 8 minutes), washed in a buffered salt solution (20 mM Tris, 0.5 M NaCl (pH 7.6)), and, following centrifugation, resuspended in buffered salt solution to an OD_{620} of 2.2 to 2.5. This suspension was heated to 37°C and mixed with an equal volume of melted 1%

TABLE 1. Characterization and derivation or geographical origin of strains of *Yersinia pestis* used in this investigation

Strain	Plasmids			Pgm	Sensitivity to pesticin	Biotype[a]	Source or geographical origin
	Pst	Lcr	Tox				
KIM	+	+	+	+	0	M	Iran
KIM(D1)	+	0	+	+	0	M	KIM
KIM(D142)	0	0	+	+	+	M	KIM(D1)
KIM(D35)	0	0	+	0	0	M	KIM(D142)
KIM(P15)[b]	0	0	0	+	+	M	KIM(D142)
K115	0	0	+	+	+	M	[c]
K115-A	0	0	+	0	0	M	K115
K115-B	0	0	+	+	0	M	K115
K115-C	0	0	+	0	0	M	K115-B
Kuma	+	0	+	+	0	A	Manchuria
Yokohoma	+	0	+	+	0	A	Japan
Salazar	+	0	+	+	0	O	Bolivia
EV76	+	0	+	0	0	O	Madagascar
TS	+	0	+	+	0	O	Java
A12	0	0	+	+	+	O	U.S.A.
Dodson	0	0	+	+	+	O	U.S.A.

[a]M = var. *medievalis* (glycerol +, nitrate reductase -), A = var. *antiqua* (glycerol +, nitrate reductase +), and O = var. *orientalis* (glycerol -, nitrate reductase +) (16).

[b]Obtained from R. D. Perry.

[c]Strain K115 and derivatives are *leu pro* auxotrophs of strain KIM that were cured of the Pst and Lcr plasmids.

low-melting-temperature agarose (Sea-Plaque GTG; FMC BioProducts, Rockland, Maine) containing 40 ug of DNase-free RNase A per ml. The mixture was pipetted into 250-ul molds (CHEF system; Bio-Rad, Melville, N.Y.) and allowed to solidify at 4°C. Blocks were removed from the mold and incubated overnight in 15 to 20 volumes of a solution containing 10 mM Tris, 50 mM EDTA, 1 mg of lysozyme per ml, 20 ug of RNase A per ml, 0.5% Nonidet P-40, and 0.5% Triton X-100 (pH 7.5) at 37°C with gentle agitation. The blocks were then incubated for 2 days at 50°C in 15 to 20 volumes of lysis buffer (10 mM Tris, 50 mM EDTA, 1.0% Nonidet P-40, and 1 mg of proteinase K per ml (pH 7.5)) with gentle agitation. Thereafter, the blocks were transferred to 20 volumes of a solution containing 10 mM Tris, 0.1 M EDTA, and 1 mM phenylmethysulfonyl fluoride (pH 7.5) and gently agitated at room temperature for 10 h. After three washes in the same solution without phenylmethylsulfonyl fluoride, the blocks were stored at 4°C in 10 mM Tris, 50 mM EDTA (pH 8.0). No detectable degradation was observed in these stored samples over a period of 8 months.

Restriction digests and electrophoresis. Restriction enzyme digestion of DNA embedded in agarose blocks was undertaken by a modification of procedures described by Smith et al. (48). Restriction endonucleases were obtained from Promega (Madison, Wis.), New England BioLabs (Beverly, Mass.), or Boehringer Mannheim, Inc. (Indianapolis, Ind.).

Slices of blocks containing chromosomal DNA (20 to 50 ul) were washed twice for 30 minutes each at room temperature in 5 ml of a solution containing 10 mM Tris and 0.1 mM EDTA (pH 8.0). The inserts were then transferred to sterile Eppendorf tubes and gently agitated for at least 2 h at room temperature in 1.0 ml of the appropriate restriction buffer (without enzyme), as recommended by the manufacturer, containing 0.1 mg of acetylated bovine serum albumin per ml. This solution was then replaced with 0.2 ml of the same buffer containing 20 U of restriction endonuclease. Digestion was undertaken with gentle agitation for 6 to 12 h at temperatures recommended by the manufacturer. Afterwards, the inserts were first incubated for 1 h at 50°C in 1 ml of a solution of 0.1 M EDTA and 1% sodium lauryl sarcosine (pH 8.0) and then for at least 2 h at 50°C in 0.25 ml of the same solution containing 1 mg of proteinase K per ml. Inserts were then washed twice for 30 min each at room temperature in 1 ml of a solution containing 10 mM Tris and 50 mM EDTA (pH 8.0) to remove the detergent.

PFGE was performed at 15°C with a CHEF system (Bio-Rad, Inc.) in TBE buffer (45 mM Tris, 45 mM boric acid, and 2 mM EDTA (pH 7.6)) (35). Gels contained either SeaKem GTG or Sea Plaque GTG agarose (FMC BioProducts). Pulsed-field gels were run at various pulse ramps for between 2 and 30 s and 1 and 6 s in order to best resolve restriction fragments in different size ranges. To determine the smallest fragments, samples were prepared as described above

but run by conventional electrophoresis in 0.7% SeaKem agarose. For this purpose, inserts were prepared in which cells were resuspended to an OD_{620} of ten so that enough DNA would be present for visualization of small fragments. Gels were stained after electrophoresis with 0.5% aqueous ethidium bromide for 1 h and then destained with TBE buffer or distilled water. Sizes of restriction fragments were determined by comparison with standards consisting of lambda DNA concatemers (Bio-Rad, Inc.), with fragments ranging from 48.5 to 1000 kb, and HindIII lambda DNA fragments (Bio-Rad, Inc.). Restriction fragment sizes were determined by graphical analysis, or by the method of Schaffer and Sederoff (43).

Two-dimensional PFGE. Initial digestion with SpeI of SfiI was performed as described previously except that an insert sufficiently large to fill an entire well was initially used to provide as much DNA as possible for later preparation of the second dimension. Electrophoresis was performed with a 1% SeaPlaque agarose gel run in TBE buffer lacking the EDTA in order to avoid potential inhibition of digestion by the second restriction enzyme. Following electrophoresis in the first dimension, a 2-mm-wide band comprising the lane was cut out and incubated at 4°C in a 30-mm-diameter plastic centrifuge tube containing 40 ml of appropriate restriction enzyme buffer as recommended by the manufacturer (New England BioLabs, Inc.). After 6 to 12 h,

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the buffer was removed and replaced; after three such incubations, the gel strip was transferred to a digestion chamber constructed from a 5-mm (inner diameter) glass tube having Eppendorf tubes (with their tips removed) affixed to each end, where they served as plugs. After the gel strip was inserted, the chamber was filled with ca. 2.5 ml of appropriate restriction endonuclease buffer containing bovine serum albumin (0.1 mg/ml) and held at 4°C for 4 h. The buffer then received 150 U of restriction endonuclease, and the chamber was incubated for 6 h at 4°C (to permit diffusion of the enzyme into the gel strip), after which it was placed into a plastic bag and suspended in a water bath with gentle agitation for 24 h at the recommended temperature. This solution was then replaced with 2.5 ml of a solution of 0.1 M EDTA in 10 mM Tris (pH 8.0) to stop the reaction. The chamber was incubated overnight at 4°C before the strip was loaded onto 1.2% SeaKem GTG agarose gels. For the second dimension, the gels were run in TBE buffer for 27 h at 15°C and 180 V with a 2-to-12-s pulse ramp. Gels were stained and analyzed after electrophoresis as described for single dimensional gels.

Restriction enzyme digestion of the Tox plasmid. The Tox plasmid was isolated from Y. pestis KIM(D142), which lacked the Lcr and Pst plasmids, by the method of Casse et al. (13). To test for the presence of restriction sites, homogeneous plasmid was embedded in 0.5% SeaPlaque agarose

to yield a concentration of 0.5 ug of DNA per 50 ul block. These blocks were then exposed to restriction endonucleases following the procedure described above. PFGE was performed in 1% SeaKem agarose for 26 h at 15°C and 180 V with a 2 to 30 s pulse ramp. For normal agarose gel electrophoresis, 0.5 ug of purified plasmid was digested with 10 to 20 U of the desired restriction endonuclease under standard conditions (35). Electrophoresis was performed in 0.7% agarose gels run overnight at 2.5 V/cm. For both types of gels an undigested plasmid and a sample digested with BamHI were used as negative and positive controls, respectively.

RESULTS

Total DNA from cells of *Y. pestis* KIM(D142) (lacking the Pst and Tox plasmids) was subjected to PFGE after digestion with restriction endonucleases possessing 8-base recognition sequences (*NotI*, *SfiI*, *SgrAI*, *PacI*, and *AscI*) or 6-base sequences that may be rare in bacterial genomes having GC contents of 45 to 50%, as does *Y. pestis* (36) (*AvrII*, *SpeI*, *BssHII*, *SmaI*, *NheI*, *XbaI*, *DraI*, *KspI*, *SfuI*, *SspI*, and *ApaI*) (37). Enzymes providing digests suitable for single-dimensional estimation of the sizes of the genome and the Pgm^--specific deletion were *SpeI*, *NotI*, *AscI*, and *SfiI*. The remainder generated large numbers of small fragments (50 kb or less) that could not be resolved in a single dimension, although *ApaI* proved useful for PFGE in the second dimension. PFGE of undigested DNA failed to yield detectable bands (not illustrated), indicating the absence of linear extrachromosomal elements in the genome.

Genome size. Digests prepared with the four restriction enzymes potentially useful in a single dimension were analyzed by PFGE at different agarose concentrations and pulse ramps to facilitate maximum resolution of fragments in different size ranges. Conditions that

maximized the separation of lower-molecular-weight fragments were nevertheless insufficient to resolve the small bands generated by AscI and especially SfiI, although, as noted below, these enzymes distinguished between the DNA of Pgm^+ and Pgm^- organisms. The sizes and nomenclature of fragments identified by single-dimensional PFGE with SpeI, NotI, SfiI, and AscI are listed in Table 2. Many of these bands were unusually broad or intense, indicating that they represented the occurrence of multiple overlapping fragments (Fig. 1). Single-dimensional PFGE after digestion with SpeI generated 41 distinct bands, of which only 3 (J, P, and T) appeared to be possible comigrating fragments (Table 2).

Two-dimensional gels performed with SpeI and then ApaI permitted the resolution of 101 detectable fragments, exhibiting a total length of 3853.8 kb (Fig. 2). This gel was used to compare the sizes of individual bands generated by SpeI alone (Table 2) with the sum of their fragments after further digestion with ApaI followed by PFGE in the second dimension. This sum was greatly in excess for bands J, P, and T. The actual number of fragments present in each of these three bands was thus taken to be the closest next highest multiple of the sizes determined by single-dimensional PFGE. Accordingly, bands J and P occurred as triplets and band T was a doublet. The sum of all fragments detected after digestion with SpeI was therefore 4208.4 kb (Table 2). This value was significantly higher than those determined with SfiI or AscI (Table 2),

Figure 1. Pulsed-field gels of chromosomal DNA from Pgm^+ cells of *Y. pestis* KIM(D142), showing the longest (A, 2 to 30 s) and shortest (B, 1 to 6 s) pulse ramps used to resolve restriction fragments generated by *SpeI* (lanes 2 and 7), *NotI* (lanes 3 and 8), *SfiI* (lanes 4 and 9), and *AscI* (lane 5). Concatemeric lambda DNA molecular size markers are shown in lanes 1 and 10, and lane 6 contains lambda *HindIII* molecular size markers. Numbers indicate the lengths (in kilobases) of the molecular size markers. Conditions were 180 V and 15°C for 27 h in 1% SeaKem GTG agarose.

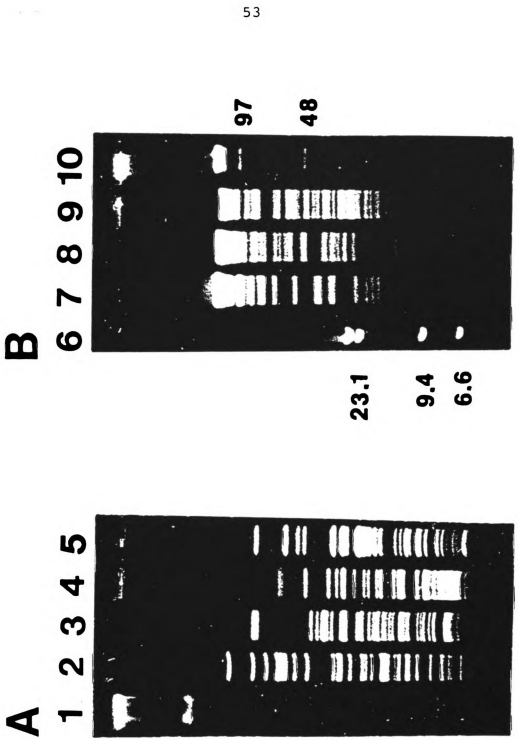

Figure 1

Table 2. Sizes of DNA fragments detected by PFGE of the genome of Y. pestis KIM(D142) after digestion with selected restriction endonucleases.[a]

Band	*Spe*I		*Not*I		*Sfi*I		*Asc*I
	Size (kb)	Error[b] (kb)	Size (kb)	Error (kb)	Size (kb)	Error (kb)	Size (kb)
A	312.6	3.6	264.9	12.5	220.7	1.8	245.0
B	263.2	7.3	258.2	11.2	182.8	1.0	201.7
C	239.3	5.4	176.5	6.0	149.2	1.3	184.0
D	222.3	5.5	168.4	6.4	141.5	1.3	175.4[c]
E	216.3	5.8	161.1 (2)[c]	4.7	133.6 (3)	0.7	139.7[c]
F	209.8	4.6	157.0	4.5	122.3	1.8	131.5
G	195.7	8.0	150.5	5.5	111.8	0.7	126.0
H	183.0	6.4	137.2 (3)	7.0	106.0	2.3	113.6[c]
I	148.2	2.9	124.3	7.3	99.3	5.5	107.5[c]
J	142.2 (3)	3.7	119.0 (2)	6.5	92.2 (2)	4.7	104.0
K	130.8	3.1	110.3	6.8	84.2 (2)	6.3	97.0[c]
L	121.0	3.9	105.5	5.1	70.8 (3)	4.7	90.0
M	116.0	5.5	100.5 (2)	5.0	56.8	5.7	72.5
N	109.2	3.8	94.8 (2)	3.8	<50[d]		65.0[c]
O	99.7	4.0	90.7	4.1			58.0
P	95.9 (3)	3.8	87.6	5.0			54.8[c]
Q	91.9	4.5	81.5	3.2			45.5
R	85.9	3.4	78.4	4.4			39.5
S	82.4	3.9	73.8 (2)	3.8			32.3
T	74.3 (2)	5.0	66.2	6.0			29.7
U	65.0	3.7	62.4	3.6			27.2
V	52.3	2.9	58.6	4.1			23.2
W	40.5	1.0	52.8	3.8			21.8
X	38.9	1.2	47.5 (3)	3.5			20.8
Y	34.4	1.2	35.8	2.4			19.1
Z	31.4	2.7	33.1 (2)	1.9			17.5
AA	24.6	1.2	32.0	1.7			16.5
BB	21.4	1.0	29.4 (2)	1.7			13.7[c]
CC	20.0	1.3	23.8 (2)	1.8			13.0
DD	18.3	0.8	20.1	1.2			
EE	16.6	0.7	13.8	1.1			
FF	14.4	0.4	12.5	1.1			
GG	13.1	0.4	10.7	0.9			
HH	12.3	0.4	9.2	0.2			
II	8.8	0.4	8.5	0.3			
JJ	7.3	0.5	6.1	0.6			
KK	6.1	0.2	2.7	0.2			
LL	3.6	0.1					
MM	3.2	0.2					
NN	2.1	0.1					
OO	1.6	0.1					
Total	4,208.4		3,996.5		>2,156.4		>2,475.0

[a] Number of determinations: *Spe*I, 6; *Not*I, 6; *Sfi*I, 4; and *Asc*I, 1.
[b] Error, largest deviation (either plus or minus) from the average among the individual values.
[c] Probable multiple fragments.
[d] Presence of numerous multiple fragments of 50 kb or less.
[e] Number of fragments per band as determined by two-dimensional PFGE.

Figure 2. Two-dimensional pulsed-field gel of chromosomal DNA from Pgm^+ cells of *Y. pestis* KIM(D142) after digestion with *SpeI* (first dimension) and then *ApaI* (second dimension) under the conditions defined in the text. The lanes across the top of both figures represent a single dimensional gel of the *SpeI* digest shown at the same scale as the two-dimensional gel illustrated directly beneath. (A) Photograph of the two-dimensional gel and (B) interpretive diagram showing the locations of fragments in the photograph; the three fragments drawn as open circles are those absent in similar gels of chromosomal DNA from isogenic Pgm^- organisms (see Fig. 5). Numbers in the margin indicate the location of lambda *HindIII* and concatemeric lambda DNA molecular size markers (in kilobases).

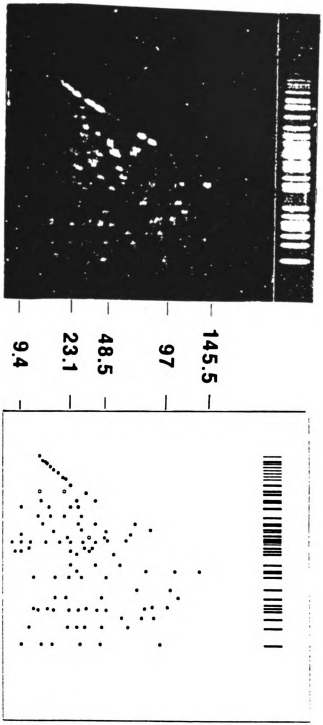

Figure 2

with which the generation of small fragments prohibited performance of PFGE in a second dimension. It also exceeded the estimate of 3853.8 kb made by summation of individual fragments detected in the two-dimensional gel owing to ApaI-mediated loss of small undetectable pieces of DNA, as discussed below.

NotI gave sufficient fragment resolution for a second determination of genome size; however, the bands were closely spaced within the 20 to 150 kb size range, making the analysis more difficult than that performed with SpeI. Single-dimensional pulsed-field and conventional gels indicated the presence of 37 bands with an apparent total length of 2751.6 kb. However, the width and intensity of several bands indicated the existence of comigrating fragments (Fig. 1). Two-dimensional gels performed with NotI and then ApaI resulted in the appearance of 92 fragments with a total length of 3330.8 kb. Analysis as described for SpeI-ApaI two-dimensional gels permitted the identification of 10 bands composed of multiple fragments, which are indicated in Table 2. Correcting for this comigration of the single-dimensional gels resulted in a chromosome size estimate of 3996.5 kb, which is similar to that described above for the analysis of SpeI digests.

The Tox plasmid was isolated and subjected to hydrolysis by SpeI, NotI, AscI, and SfiI. Attempts to detect the products of these digestions following PFGE of normal agarose electrophoresis were not successful,

indicating the absence of corresponding recognition sites. Similarly, PFGE of purified but undigested Tox plasmid failed to form a visible band, indicating that the plasmid in circular form is unable to emerge from the well (33), and two-dimensional PFGE of an isolate lacking all three plasmids of the species (Y. pestis KIM(Pl5)) yielded results identical to those obtained with strains possessing the Tox plasmid (Not illustrated). Accordingly, all of the fragments detected by PFGE were of chromosomal origin. Addition of the known sizes of plasmid DNA (189.5 kb) to that determined with SpeI for the chromosome (4208.4 kb) thus provides an estimate of 4397.9 kb for the genome of wild-type Y. pestis. Adding plasmids to the NotI estimate (3996.5 kb) results in a comparable total genome size of 4186.0 kb.

Intraspecific variation. The macrorestriction patterns of a few additional strains of Y. pestis, selected on the basis of diverse geographical ofigin and biotype, were compared with that of strain KIM (Fig. 3). Although each strain exhibited a unique pattern of fragments, their number and range of sizes were similar, suggesting that there were no major variations in genome size. The patterns corresponded well with the ability to ferment glycerol, a primary determinant in assigning biotype (16). For example, the patterns of glycerol-negative strains (Y. pestis subsp. orientalis) were significantly more similar to each other

Figure 3. Pulsed-field gels of genomic DNA from selected strains of *Y. pestis* after digestion with *SpeI* on (left) a 1% SeaKem GTG agarose gel run under the conditions defined in the legend to Fig. 1 (favoring resolution of fragments of greater than 100 kb) and (right) a 1.2% SeaKem GTG agarose gel run under conditions of 180 V at 15°C with a 2- to 12-s pulse ramp for 27 h (favoring resolution of fragments of less than 100 kb). Lanes: 1, lambda DNA concatemeric molecular size markers (shown in kilobases in both left margins); 2, strain KIM(D1); 3, strain Kuma; 4, strain Yokohama; 5, strain Salazar; 6, strain EV76; 7, strain TS; 8, strain Dodson; 9; strain A12; 10, lambda *HindIII* size markers (shown in kilobases in the far right margin).

Figure 3

TABLE 3. Pairwise comparisons of SpeI-generated total genomic DNA fragments from selected strains of *Yersinia pestis* after single-dimensional pulsed-field gel electrophoresis

	Strain						
Strain	Kuma	Yokohoma	Salazar	EV76	TS	Dodson	A12
KIM	46/70[a]	42/66	28/64	26/64	24/64	20/64	28/66
	65.7[b]	63.6	43.8	40.6	37.5	31.3	42.4
Kuma		64/70	22/68	24/68	20/68	20/68	24/70
		91.4	32.4	35.3	29.4	29.4	34.3
Yokohoma			22/64	24/64	18/64	18/64	22/66
			34.4	37.5	28.1	28.1	33.3
Salazar				54/62	48/62	42/62	44/64
				87.1	77.4	67.7	68.8
EV76					48/62	42/62	44/64
					77.4	67.7	68.8
TS						54/62	52/64
						87.1	81.3
Dodson							56/64
							87.5

[a]No. of fragments scored as identical/sum of fragments detected in both strains.

[b]Ratio x 100.

than they were to those of any of the glycerol-positive isolates (Table 3). The members of Y. pestis subsp. orientalis tested all exhibited fragments of 232.4, 213.0, 169.7, 60.5, 54.6, and 51.2 kb plus those between 40 and 30 kb. Within this variety, the patterns of the two isolates from North America (strains A12 and Dodson) showed greater similarity to that of the TS strain from Java than to those of strains from South America (Salazar) and Madagascar (EV76). The patterns of the two glycerol-positive strains of Y. pestis subsp. antiqua (Kuma and Yokohama) were highly similar to each other and more closely resembled that of strain KIM (Y. pestis subsp. mediaevalis) than those of Y. pestis subsp. orientalis. Digests of the three glycerol-positive isolates tested exhibited bands with sizes corresponding to those of fragments B, C, G, H, X, and Y listed in Table 2.

The Pgm^--specific deletion. Total DNA from Pgm^+ (substrain D142) and isogenic Pgm^- (substrain D35) cells of Y. pestis KIM was digested with SpeI, NotI, AscI, or SfiI and compared after single-dimensional PFGE. At least one fragment was always missing in the digests of Pgm^- mutants (Fig. 4). These missing fragments were the 182.8 kb band B for SfiI, and 30.6 kb band Y for SpeI, and the 72.5 kb band M for AscI. In digests prepared with NotI, the 78.4 kb band Q became narrower and less intense owing to probable loss of a multiple comigrating fragment. This consistent

Figure 4. Pulsed-field gels of chromosomal DNA from Pgm^+ (strain KIM(D142), lanes 1,3,5,and 7) and Pgm^- (strain KIM(D35) lanes 2,4,6, and 8) derivatives of *Y. pestis* KIM digested with SpeI (lanes 1 and 2), NotI (lanes 3 and 4), AscI (lanes 5 and 6), and SfiI (lanes 7 and 8). Electrophoresis was performed in 1% SeaKem GTG agarose under the conditions described in the legend to Fig. 1. Positions of lambda concatemeric molecular size markers are indicated (in kilobases). Solid arrowheads indicate the positions of Pgm^+-specific bands, and the open arrow designates the location of the 77-kb junctional fragment in DNA from Pgm^- mutants digested with SfiI.

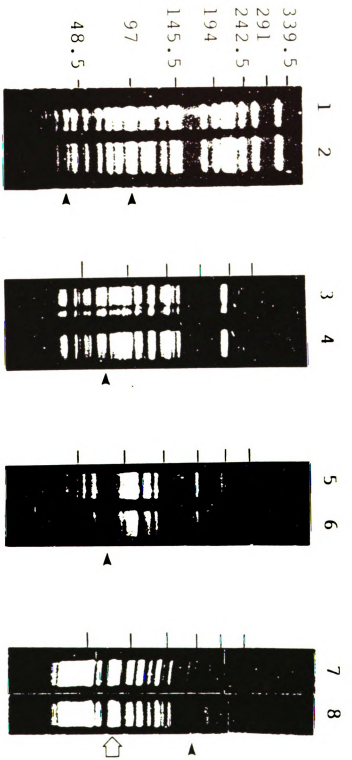

Figure 4

disappearance of fragments in digests of DNA afrom Pgm$^-$ organisms is in accord with the concept that the mutation reflects the occurrence of a large deletion. Examination of DNA from six Pgm$^-$ mutant clones of Y. pestis KIM(D142) revealed identical macrorestriction patterns for SfiI, NotI, and SpeI (only one isolate was analyzed with AscI), suggesting the occurrence of a deletion with precise or nearly precise endpoints.

Analysis of the deletion by single-dimensional PFGE after digestion with SpeI, NotI, or AscI failed to reveal the existence of new junctional fragments in digests of Pgm$^-$ mutants. Accordingly, two-dimensional PFGE was used to provide an estimate of the size of the deletion. Analysis of Pgm$^-$ mutants by digestion with SpeI and then ApaI showed the disappearance of the 31.4 kb band Z (shown as two smaller fragments after cleavage with ApaI in Fig. 2) and of an additional fragment of about 40 kb that constitutes part of one of the three 95.9 kb fragments composing band P (Fig. 5). A Pgm$^-$-specific fragment is visible in a location indicating that it is slightly larger than band Y (Fig. 5), with an estimated size of approximately 34.8 kb. The loss of two fragments totaling 127.3 kb and the appearance of a 34.8 kb fragment suggest a deletion of 92.5 kb.

Digests of Pgm$^-$ mutants prepared with SfiI lacked the 182.8 kb band B. If most or all of the deleted Pgm$^+$-specific sequence is contained within this large fragment, then one or possibly two junctional fragments should be

Figure 5. Two-dimensional pulsed-field gel of chromosomal DNA from Pgm^- cells of *Y. pestis* KIM(D35) after digestion with *SpeI* (first dimension) and then *ApaI* (second dimension) under the conditions defined in the text. The lane across the top of the figure represents a single-dimensional gel of the *SpeI* digest shown at the same scale across as the two-dimensional gel illustrated directly beneath. An interpretive diagram showing the location of missing fragments in comparison to a similar gel of chromosomal DNA from the parental Pgm^+ strain is given in Fig 2B. The white arrow within the field indicates the location of the Pgm^--specific junctional fragment. Solid arrowheads show the location of missing Pgm^+-specific fragments, and the open arrowhead indicates the position of the junctional fragment on the single-dimensional gel. Numbers indicate the location of lambda *HindIII* and concatemeric lambda DNA molecular size markers (in kilobases).

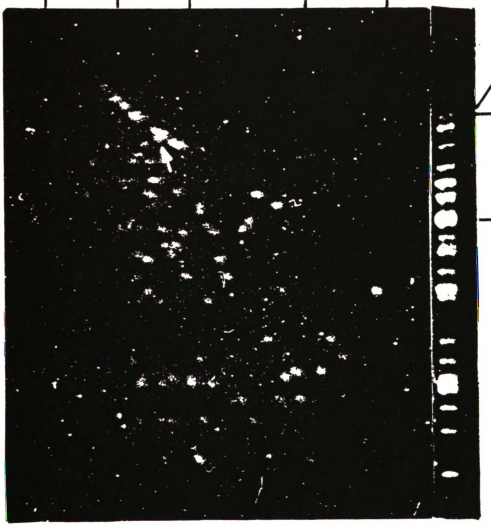

Figure 5

detected in digests of Pgm$^-$ mutants following two-dimensional PFGE. Furthermore, use of SpeI for the second dimension of SfiI-generated digests of Pgm$^+$ organisms should result in the appearance of the 95.9 kb and 31.4 kb fragments noted above in the column previously occupied in the first dimension by SfiI band B. Both of these assumptions were correct. Fragments of 95.9 and 31.4 kb were detected when SfiI band B was digested with SpeI and then subjected to second-dimensional PFGE (Fig. 6A). DNA from Pgm$^-$ mutants, or course, lacked all of the SpeI-generated fragments originating at the location of SfiI band B (Fig. 6B). However, two new fragments of 38 and 23 kb were observed at a location that corresponded to the position of the 77 kb junctional fragment first detected in single-dimensional gels of DNA from Pgm$^-$ mutants digested with SfiI (Fig. 4). No further differences were evident in single-dimensional (SfiI) or two-dimensional (SfiI and SpeI) gels, an observation consistent with occurrence of most or all of the Pgm$^-$-specific deletion within SfiI band B. The 77 kb junctional fragment would thus arise following deletion of a 105.5 kb segment of DNA from SfiI band B. This value corresponds closely to the independent 92.5 kb estimate determined by two-dimensional PFGE with SpeI and ApaI.

Mutation to pesticin resistance. DNA was prepared from the rare Pgm$^+$ Pst$^-$ (pesticin-resistant) mutant Y. pestis

Figure 6. Two-dimensional pulsed-field gels of chromosomal DNA from (A) Pgm^+ strain KIM(D142) and (B) Pgm^- strain KIM(D35) cells of Y. pestis after digestion with SfiI (first dimension) and SpeI (second dimension). Lanes across the top represent single-dimensional gels of SfiI digests at the same scale as the two-dimensional gels shown directly beneath. The white arrowheads in the field of panel B indicate the locations of Pgm^--specific junctional fragments. The solid arrowhead shows the location of the missing 183-kb SfiI fragment, and the open arrow indicates the position of the 77-kb junctional fragment on the single-dimensional gel of DNA from Pgm^- cells. Numbers indicate the locations of lambda HindIII and concatemeric lambda DNA molecular size markers (in kilobases).

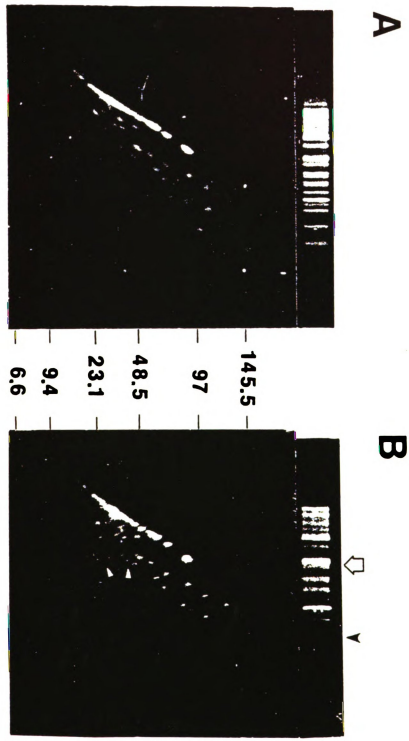

Figure 6

KIM(K115-B) and subjected to digestion by appropriate restriction endonucleases. DNA composing the Pgm^+-specific 31.4 kb SpeI band Z and 182.8 kb SfiI band B was retained in this isolate (Fig. 7). However, DNA from an isogenic Pgm^- (*Y. pestis* KIM(115-C)) mutant isolated on Congo red agar, like that of typical Pgm^- mutants (e.g., *Y. pestis* KIM(D35)), lacked both of these Pgm^+-specific fractions. These results would be expected if the event accounting for the loss of IrpB, IrpC, IrpD, and IrpE in the rare mutant was a mutation undetectable by PFGE in the Irp operon contained within the 93 to 106 kb deletable sequence of typical Pgm^+ yersiniae. This could be a small deletion or insertion or a polar mutation (introduction of a nonsense codon or frameshift).

Figure 7. Pulsed-field gels of chromosomal DNA from Pgm^+ strain KIM(D142) (lanes 1 and 6), Pgm^- strain KIM(D35) (lanes 2 and 7), Pgm^+ (pesticin-resistant) strain Kl15-B (lanes 3 and 8), and Pgm^- (pesticin-resistant) strain Kl15-C (lanes 4 and 9) derivatives of Y. pestis KIM digested with SpeI (lanes 1 to 4) or SfiI (lanes 6 to 9). Conditions were as described in the legend to Fig. 1A. Positions of lambda concatemeric molecular size markers (lane 5) are given in kilobases. Arrows indicate the location of Pgm^+-specific fragments.

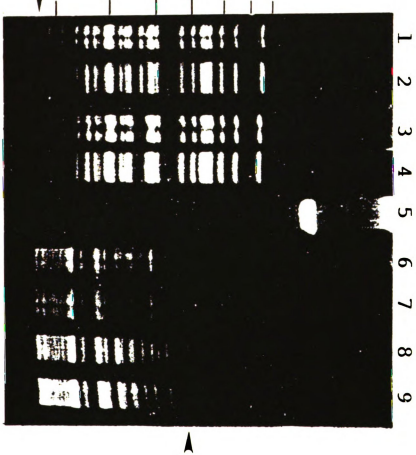

Figure 7

DISCUSSION

Y. pestis is related to *E. coli*, as judged by significant DNA homology and the existence of numerous shared physiological determinants (7,8). Smith et al. (47) used PFGE of *NotI* restriction digests to estimate a genome size of 4550 kb for *E. coli* K-12 that was later refined to 4700 kb (15). The size of 4208.4 kb reported here for the chromosome of *Y. pestis*, an obligate intracellular parasite restricted to fixed niches (7), is thus 89.5% of that determined for the more metabolically active *E. coli*. This smaller size may reflect the absence in *Y. pestis* of catabolic and regulatory functions (7) utilized by *E. coli* for existence as a commensal organism or for survival in soil and water. In this context, it is of interest that a value of 5900 kb was estimated for the genome of *Pseudomonas aeruginosa* (42), a robust saprophyte capable of growth in numerous natural environments.

Genomic analysis of *Y. pestis* was difficult due to a lack of restriction endonucleases that cut at only a few sites on the chromosome. No enzyme-generated fragments larger than the 312.6 kb band A were detected after digestion with *SpeI*. This observation contrasts with the case of *E. coli*, for which *NotI* digests contained fragments

of up to 1000 kb in size (47). Although NotI has been used for analysis of the genome of Y. pestis as well (23), the closely packed bands and larger number of comigrating fragments observed in NotI digests suggested that SpeI might be a better choice for use in PFGE. Our difficulty in finding restriction endonucleases that produced few eneough fragments to permit detailed analysis was probably due in part to the 45.6% GC content (36) of the yersinial chromosome and to its relatively large size. It thus became necessary to find enzymes that recognized specific rare sequences rather than relying on those distinguishing 6- or 8-base sequences containing either all C/G or A/T. Indeed, all of the enzymes tested that recognized 6-base sequences containing CTAG (SpeI, XbaI, NheI, and AvrII) produced digests with comparatively few fragments, although only those prepared with SpeI could be accurately analyzed. This finding with Y. pestis is in accord with the observation that CTAG is a rare sequence in bacteria with GC contents of greater than 45% (37).

Our results also emphasized the need for careful evaluation of single-dimensional pulsed-field gels when using this method for determining the size of a bacterial genome. Digests prepared with AscI initially seemed adequate, but the small sum of the visible fragments suggested considerable comigration, resulting in a low estimate of total size. Similar situations have been described for other species, including P. aeruginosa, for

which initial determinations of genome size by single-dimensional PFGE were 2700 kb (19). Later work involving two-dimensional PFGE (3) and additional restriction enzymes (42) resulted in higher estimates of 5,300 and 5,900 kb, respectively.

Two-dimensional PFGE was useful in this study for confirming the genome size estimated from single-dimensional gels and for identifying comigrating fragments. Nevertheless, the sum of fragments located within a given column of their progenitor as determined by single-dimensional PFGE. This difference was caused by both generation in the second dimension of fragments of 10 kb or less that were too faint for reliable resolution and to a larger error in measuring fragments in two-dimensional gels as they approached sizes of 150 kb. These errors probably account for the discrepancy of 311 kb observed between the values obtained by single-dimensional PFGE after correction for comigrating fragments (4,208.4 kb) and by summing the sizes of visible fragments after two-dimensional PFGE (3,853.8 kb). The smaller value obtained from the two-dimensional gel makes it unlikely that the length determined by single-dimensional PFGE is a significant underestimate of the correct genome size.

The usefulness of macrorestriction pattern analysis in identification of unknown strains of a given species and in determining intraspecific relationships and genetic history has been established (1-3, 6, 31). For example,

macrorestriction patterns determined by PFGE for both Lactococcus lactis and Streptomyces ambofaciens exhibited 70 to 80% band identity, as opposed to 23 to 30% band identity for distantly related strains (6, 31). These values are very similar to those determined in this study for selected isolates of Y. pestis. Our results are in accord with the accepted division of the species into glycerol-negative (Y. pestis subsp. orientalis) and glycerol-positive strains and suggests that division of the latter into Y. pestis subsp. antiqua and Y. pestis subsp. mediaevalis (16) is equally legitimate. These determinations, however, were primarily undertaken to define the limits of diversity within the species. Further work will be required to fully exploit the potential of macrorestriction pattern analysis to help trace the historical spread of plague. In this context, it is possibly significant that strain TS of Y. pestis subsp. orientalis, isolated in Java, exhibited significant band identity with the two North American isolates tested, Dodson (87.1%) and A12 (81.3%), and that the latter two were equally similar to each other (87.5%). This relationship favors the hypothesis that plague has not always been present in the New World but rather was introduced from Indochina at the start of the present century (18, 22, 34).

Although detection of a large deletion in Pgm^- organisms was anticipated (41, 44-46), its extent was difficult to determine by PFGE because the size of missing

fragments depended upon which restriction endonuclease was used. New junctional fragments in digests of Pgm$^-$ mutants were not always evident after single-dimensional PFGE, and their detection usually required migration in a second dimension. Our determination of the size of the deletion is not precise because fragments of 10 kb or less could not be accurately resolved in two-dimensional gels. The estimate of 92.5 to 105.5 kb for the deletion may thus be a significant underestimate, although the concurrence of the two independent analyses suggests that this value is reasonably accurate. The deletion is certainly large enough to include all of those structural genes encoding the peptides required for pigmentation (11, 39, 41, 44) and assimilation of iron (11, 44) not expressed in Pgm$^-$ mutants.

Larger high-frequency deletions (250 to 2,100 kb) occur at specific regions of the chromosome in species of Streptomyces, although the exact positions of their endpoints and their extents appear to be variable. Amplification of DNA sequences associated with the deletion may also occur in these organisms (4, 5, 30). We found no evidence in this study for similar augmentation or for occurrence of variation in either the size or location of the deletion. These observations suggest that the Pgm$^-$ specific deletion involves a DNA sequence found as a single copy on the chromosome and that its endpoints are highly specific. Chromosomally encoded virulence determinants similarly vulnerable to high-frequency deletion have been

described for other pathogenic bacteria. These factors include two two groups of hemolysin and fimbrial genes of uropathogenic E. coli that are lost as part of exact deletions of 75 and 100 kb, respectively (20, 26, 27). The precision of these events reflects the occurrence of short direct repeats at each end of the DNA segment that evidently mediate site-specific recombination. Similarly, genes encoding the synthesis and retrieval of the siderophore aerobactin occur in a cluster found either in the chromosome or on the ColV plasmid of several enteric species (14). Flanking ISl elements give this segment the appearance of a composite transposon (29, 38, 40). Although they no longer function in transposition, these elements also mediate nearly precise deletions of the aerobactin genes through site-specific recombination.

The high frequency and apparent precision of the Pgm^- specific deletion in Y. pestis may also occur as a consequence of site-specific recombination. Further study of the deletable segment may show it to be an iron uptake and storage gene cluster that allows the bacterium to colonize its host. Its current instability in the chromosome may reflect an ability in the past to invade bacterial genomes.

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CHAPTER II

(ARTICLE)

Iron and Hemin Storage in *Yersinia pestis*

by

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(Manuscript to be submitted for publication)

ABSTRACT

The pigmentation (Pgm^+) phenotype,a required virulence determinant of Yersinia pestis, encompasses a number of characteristics that now appear to be genetically but not necessarily physiologically linked. We now use hemin storage (Hms^+) phenotype to define a Pgm^+ trait involved in the temperature-dependent storage of exogenous hemin. In this study, we have identified the outer membrane as the site of iron storage which occurs only at 26°C in Pgm^+ cells of Y. pestis grown with hemin as the iron source. Outer membrane storage of hemin does not occur in spontaneous Pgm^- cells nor in Pgm^+ cells cultured at 37°C. We also identified a soluble inorganic iron storage pool in both Pgm^+ and Pgm^- cells. Expression of inorganic iron storage is temperature-independent and is associated with a bacterioferritin-like protein. At 37°C, these iron and hemin storage pools are relatively independent of each other. While the function(s) of these storage pools is undetermined, they may play important roles in the pathogenesis of the plague bacillus.

INTRODUCTION

Although procaryotes must generally survive and grow in iron-deficient environments, pathogens living within host tissues may encounter potentially iron-rich conditions. However, this iron will be bound to host molecules including high affinity ligands such as transferrin and lactoferrin or hemin (from hemoglobin, hemopexin, and haptoglobin) and ferritin (7,24,49). One possible iron rich environment is the flea gut (7) which is the site of multiplication for part of the life cycle of Yersinia pestis, the etiologic agent of bubonic and pneumonic plague (8). Hemolyzed blood would provide an abundance of hemin compounds and inorganic iron. When grown in laboratory conditions simulating this environment (26^{o}C) virulent cells of Y. pestis accumulate sufficient levels of exogenous hemin (20,21) or Congo red (44) from solidified media to form colored or "pigmented" (Pgm^{+}) colonies. Spontaneous Pgm^{-} mutants fail to accumulate these compounds, however, they still utilize all tested hemin compounds as nutritional sources of iron (33,36,37). Thus one defect in spontaneous Pgm^{-} organisms lies in hemin storage but not in hemin utilization.

While hemin storage appears to be rare in prokaryotes, inorganic iron storage on the cytoplasmic protein bacterioferritin may be widespread (45). This iron-storage

protein has been detected in six diverse genera: Azotobacter (6,11,39), Escherichia (1,50), Pseudomonas (29), Rhodopseudomonas (28), Rhodospirillum (2), and Streptomyces (15). The bacterioferritins from the first three genera have been more extensively characterized and show striking similarities to eucaryotic ferritins in subunit structure, consisting of multiple copies of a 15-18 kDa polypeptide, and in their nonhemin iron storage properties (1,27,39). Unlike eucaryotic ferritins, bacterioferritins contain one hemin moiety for every 2-5 subunit polypeptides. Although no function for bacterioferritins has been proven, iron storage could prevent formation of toxic radicals and provide iron for growth under subsequent iron starvation conditions (1).

In this study we examined hemin and inorganic iron storage properties in isogenic Pgm^+ and Pgm^- cells of Y. pestis KIM. Hemin storage occurred primarily in the outer membranes of Pgm^+ cells while storage of excess inorganic iron occurred in a soluble fraction of both Pgm^+ and Pgm^- cells. Peptides associated with the inorganic iron storage pool may represent a Y. pestis bacterioferritin. Under iron excess conditions incorporation of inorganic iron and iron from hemin appeared to be independent processes and incorporation from one source did not greatly repress incorporation from the other.

MATERIALS AND METHODS

Bacteria. An isogenic Pgm^+ and Pgm^- pair of Y. pestis KIM derivatives were used in this study. The Pgm^- mutation results from the spontaneous deletion of approximately 102 kb of chromosomal DNA (16,26). Both derivatives possess endogenous toxin (pmTl) and pesticin plasmids, but are avirulent due to the absence of the low-calcium response (Lcr) plasmid (pCDl) (5,36,46). The Pgm^+ determinant and the Lcr virulence regulon are genetically and biochemically unrelated (5,34).

Cultivation and labelling of bacterial cells. Bacterial strains were stored at $-20^{o}C$ in buffered glycerol (3). The Congo red (CR) agar of Surgalla and Beesley (44) was used to test for the Pgm^+ phenotype. Cells of Y. pestis were grown with aeration (200 rpm setting on a New Brunswick Model G76 gyratory shaker water-bath) in the synthetic medium of Higuchi et al (18) as modified by Zahorchak and Brubaker (51). In this study $FeSO_4$ was omitted from the medium which was further deferrated by 8-hydroxyquinoline extraction (48) (hereafter called modified Higuchi's medium). Hemin and $FeCl_3$ supplements were added after deferration. Glycerol stocked cells were inoculated onto tryptose blood agar (Difco, Detroit, MI) slants and

incubated for 48 h at 26°C. Slant cultures were suspended in 33 mM phosphate buffer (pH 7.0) and used to inoculate modified Higuchi's medium (supplemented with 100 uM $FeCl_3$ or 50-87 uM hemin) to an optical density (OD) of 0.1 at 620 nm. Cells were acclimated to the medium by growth at 26°C or 37°C by serial transfer for approximately eight generations (33). Acclimated cells were transferred to fresh modified Higuchi's medium and mid-log phase cells harvested for analysis. Growth of cultures was determined by OD_{620} measurements.

Cells were labelled to a constant specific activity with either $^{55}FeCl_3$ (New England Nuclear Research Products (NEN), Boston, MA) or (^{55}Fe)hemin by addition of the isotope to modified Higuchi's medium. The radioactive medium was sterilized by filtration and used for cell cultivation as described above. The final concentration and specific activities in radioactive media were 100 uM with 13,000 cpm ^{55}Fe per nmol inorganic Fe or 87 uM hemin with 1,700 cpm (^{55}Fe)hemin per nmol. Cell-associated radioactivity was quantitiated by filtration (0.22 um pore size, Millipore Corp., Bedford, MA) of approximately 10^9 CFU's followed by membrane washing with ice-cold nonradioactive medium and scintillation counting.

Cell fractionation. Mid-log phase cells were fractionated into periplasmic, cytoplasmic, outer membrane, and inner membrane components as previously described

(41,42). Briefly, lysozyme-EDTA treatment and centrifugation separated spheroplasts from periplasm. Spheroplasts were lysed by sonication followed by successive isopycnic sucrose gradient centrifugations to separate cytoplasm, outer membranes and inner membranes. Although the sucrose density outer membrane banding pattern was significantly lower in all cells grown at 26°C, SDS-PAGE polypeptide profiles showed these fractions were authentic, isolated outer membranes (data not shown). Radioactivity associated with each cell fraction was determined by scintillation spectroscopy of appropriate aliquots.

To analyze soluble cell components, mid-log phase cells were harvested by centrifugation, washed with 33 mM phosphate buffer (pH 7.0), and resuspended in 50 mM N-2-hydroxyethylpiperazine- N'-2'ethanesulfonic acid (HEPES) - 1.0 mM sodium citrate (pH 7.8, hereafter called column buffer). Cell suspensions were sonicated on ice in 15 second bursts for 1 minute. Cellular debris and particulate membrane fragments were removed by centrifugation (10,000 x g, 15 minutes, 4°C). Protein concentrations were determined by the method of Lowry et al (25) and all samples diluted to 10 mg protein/ml. One ml samples were applied to Bio-Gel A-1.5m (100-200 mesh, Bio-Rad Laboratories, Richmond, CA) for molecular sieving. Samples were held at 4°C and eluted with column buffer at a flow rate of 0.15 ml/min. Protein concentrations of column fractions (3 ml volumes) were estimated by OD_{280} measurements. Radioactivity of column

fractions was determined by drying 1.0 ml aliquots in scintillation vials overnight at 80°C, adding 10 ml Complete Counting Cocktail (Research Products International Corp., Mount Prospect, IL), and quantitating cpm on a LS7500 Scintillation System (Beckman Instruments, Inc. Fullerton, CA).

Identification of the soluble iron storage peptide. Fractions eluting from the Bio-Gel A-1.5m column at an elution volume of approximately 270 ml displayed the highest radioactive peak under most conditions (Fig. 1). These fractions from Pgm^- cells grown at 37°C with $FeCl_3$ were pooled for further analysis. Pooled fractions were dialyzed overnight with three changes of buffer against 50 mM Tris-HCl pH 7.8 and then loaded onto a DEAE cellulose column (Whatman Biosystems, Ltd., Maidstone, England) at room temperature. Proteins were eluted from the column using a 0 to 0.5 M NaCl gradiant in 50 mM Tris-HCl (pH 7.8) over a period of two hours with a flow rate of 1.0 ml per minute. Five ml fractions were collected and their optical density and radioactivity were measured as described above for the A-1.5m column. Proteins in each fraction were identified by combining 25 ul from each fraction with an equal volume of SDS-PAGE sample buffer. These were used for SDS-PAGE and subsequent silver staining (23,30).

(^{55}Fe)Hemin synthesis. (^{55}Fe)Hemin was synthesized

from $^{55}FeSO_4$ (NEN) and protoporphyrin IX (Sigma Chemical Co., St. Louis, MO) essentially by the methoed of Chang et al. (10). Briefly, iron was incorporated into the prophyrin ring by incubation in a nitrogen atmosphere at 80°C in pyridine-glacial acetic acid solvent. (^{55}Fe)Hemin was separated from reactants by chloroform extractions and acid precipitation (10). Analysis of authentic hemin (Sigma Chemical Co.) and our (^{55}Fe)hemin product revealed virtually identical visible absorption spectra (data not shown). Hemin concentrations were determined spectrophotometrically at 580 nm against a standard curve constructed with authentic hemin. The term hemin is used throughout this report as a generic term for iron-containing protoporphyrin IX without regard to oxidation of salt states.

RESULTS

Subcellular localization of hemin and iron pools. The amount of label removed from the culture medium during growth was influenced by temperature, bacterial strain, and the form in which the label was present. All cells grown in the presence of inorganic iron removed less than 5% of the label from the medium. Cells grown with labelled hemin removed 19-25% with the exception of Pgm^+ cells at 26^oC which removed approximately 85% of the hemin.

Table 1 reports the storage of inorganic or hemin-associated iron within subcellular fractions in specific activities (nmoles of iron/mg protein). Although several differences are notable, Pgm+ cells of Y. pestis KIM grown at 26^oC with hemin clearly stored excess iron in their outer membranes. Under these conditions outer membranes contained 51-fold more iron than Pgm^- cells and 317-fold more iron than Pgm^+ cells grown at 37^oC. Inner membranes and periplasms of Pgm^+ cells grown at 26^oC contained considerably more iron than these fractions from Pgm^- cells grown under the same conditions. The large amount of hemin accumulated on the outer membrane could result in more passing into the periplasm and inner membrane, or it could represent low level contamination of these samples with outer membranes during the fractionation procedure.

TABLE 1. Specific Activities of Hemin and Inorganic Iron in Subcellular Fractions from Pgm^+ and Pgm^- cells of *Yersinia pestis* KIM[a]

Subcellular Fractions	Specific Activities[b]							
	Pgm^+ Cells				Pgm^- Cells			
	26°C Growth		37°C Growth		26°C Growth		37°C Growth	
	Fe^{3+}	Hemin	Fe^{3+}	Hemin	Fe^{3+}	Hemin	Fe^{3+}	Hemin
Outer Membranes	40.8	697.4	1.6	2.2	8.0	13.7	15.8	3.4
Inner Membranes	8.8	17.4	5.9	9.7	3.3	3.4	5.2	6.7
Periplasm	12.2	20.4	14.6	21.2	3.1	5.4	4.4	18.7
Cytoplasm	5.1	3.7	11.3	3.0	2.8	1.5	6.3	2.3

[a] Cells were cultured at 26°C or 37°C to a constant specific activity in a chemically defined medium containing either 100 μM $^{55}Fe^{3+}$ or 87 μM [^{55}Fe]-hemin.

[b] nmoles of iron / mg protein

Distribution of iron did not differ significantly in Pgm^+ and Pgm^- cells grown at 37°C with hemin. There is no evidence of significantly more uptake of hemin bound iron into the cytoplasm at 26°C by Pgm^+ cells than by Pgm^- cells supporting the assumption that the function of pigmentation is outer membrane adsorption of hemin and is not directly involved in iron uptake.

Table 1 identifies several differences in the distribution of inorganic iron label between Pgm^+ and Pgm^- cells. At 26°C the pattern was very similar to that seen in cells grown with hemin, although the 5-fold difference between Pgm^+ and Pgm^- cells in outer membrane specific activities was substantially less. Inorganic iron may have a low affinity for the proteins involved in hemin storage. Alternatively, a distinct iron acquisition system expressed in Pgm^+ cells may be responsible for the observed increase in iron absorption. When grown at 37°C, Pgm^- outer membranes have an approximately 10-fold higher iron content than analogous Pgm^+ membranes. In addition, the iron contents of both cytoplasmic and periplasmic fractions are significantly lower in Pgm^- cells than in Pgm^+. It is possible that inorganic iron gets held up at the outer membrane of Pgm^- mutants, while Pgm^+ cells at 37°C more efficiently transported iron across their outer membranes.

Protein associated with inorganic iron storage. To analyze differences between soluble fractions of Pgm^+ and

Pgm^- cells of Y. pestis KIM, labeled cells were sonicated and soluble fractions separated from particulate and membrane fractions by centrifugation. Soluble cell extracts were subjected to column chromatography molecular seiving through Bio-Gel A-1.5m. The resulting radioactive and OD_{280} profiles of Pgm^+ and Pgm^- soluble cell extracts are shown, respectively, in Figs. 1 and 2. The most notable feature was a radioactive peak at 270 ml elution volume which was present in both Pgm^+ and Pgm^- cells grown under all conditions. Comparison to standards of known size indicated a native molecular weight of approximately 620 kDa for proteins eluting at this volume. This fraction appeared to contain an inorganic iron storage molecule since growth with hemin significantly reduced the specific activity of the fractions. Of the total labelled iron in the cytoplasm 53% - 76% was found in this peak for cells grown with $^{55}FeCl_3$ and 16% - 39% for cells grown with ^{55}Fe-hemin. Although inorganic iron appeared to be stored preferentially in this fraction, we have not determined whether inorganic iron is the stored form in hemin grown cells. The smaller soluble storage component observed in cells grown to a constant specific activity with hemin (Table 1 and Figs. 1 and 2) suggests that inorganic iron is the primary form stored in this component. The labelled iron seen here in hemin grown cells may represent secondary hemin storage in this component or inorganic iron removed from the porphyrin ring.

The 9 fractions which eluted from 262-285 ml and

Figure 1. Bio-Gel A-1.5m column chromatography profiles of soluble extracts from Pgm^+ cells of Yersinia pestis cultured at 37^oC (panels A and B) or 26^oC (panels C and D). Cells were labeled to constant specific activity with either $^{55}FeCl_3$ (panels A and C) or ^{55}Fe-hemin (panels B and D). Closed circles represent optical densities of column fractions while open circles are nmoles of iron/ml of eluant.

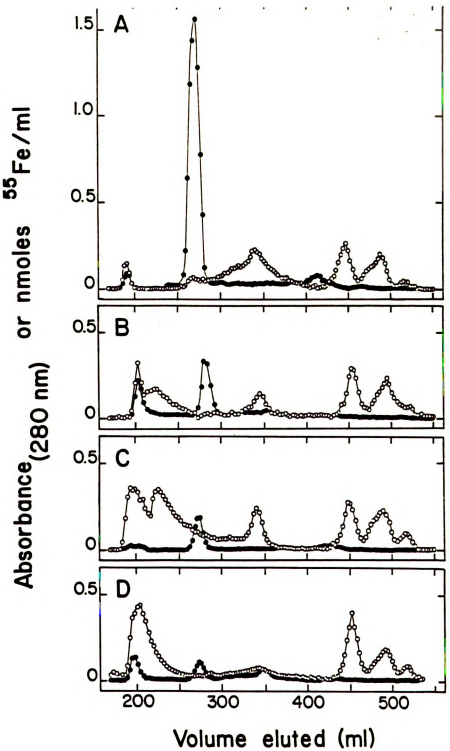
A
B
C
D
1.5
1.0
0.5
0
0.5
0
0.5
0
0.5
0
200
300
400
500
Volume eluted (ml)
Absorbance (280 nm) or nmoles ^{55}Fe/ml

Figure 1

Figure 2. Bio-Gel A-1.5m column chromatography profiles of soluble extracts from Pgm^- cells of Yersinia pestis cultured at 37^oC (panels A and B) or 26^oC (panels C and D). Cells were labelled to constant specific activity with either $^{55}FeCl_3$ (panels A and C) or ^{55}Fe-hemin (panels B and D). Closed circles represent optical densities of column fractions while open circles are nmoles of iron/ml of eluant.

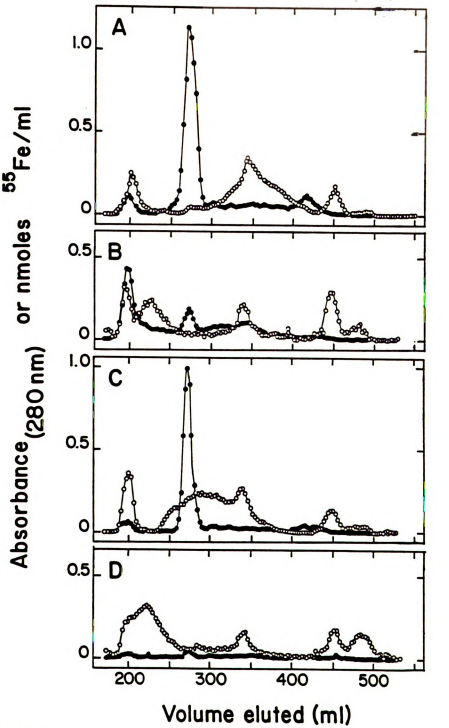
A
B
C
D
1.0
0.5
0
200
300
400
500
Absorbance (280 nm) or nmoles ^{55}Fe/ml
Volume eluted (ml)

Figure 2

comprised the radioactive peak of Pgm- cells grown at 37°C with $FeCl_3$ were pooled, dialyzed against 50 mM Tris-HCl pH 7.8, and passed over a DEAE column. There was a single radioactive peak containing 90% of the ^{55}Fe loaded onto the column which eluted at 0.35 M NaCl (Table 2). SDS-PAGE analysis (Fig. 3) demonstrated that radioactivity was associated with a 620 kDa protein which was composed of multiple 19 kDa subunits. Following DEAE there is a new 16 kDa peptide which may be a degradation product of the 19 kDa subunits. Thus cytoplasmic iron storage appears to involve a molecule with the characteristics of bacterioferritin.

A smaller radioactive peak (exception: Pgm^+ grown with hemin at 37°C) eluted from the A1.5m columns at approximately 200 ml. This peak was larger in 37°C samples, especially those grown with hemin. Mehigh and Brubaker (manuscript in preparation) have shown that a very large multimeric, hemin containing protein with moderate catalase activity elutes at this location, and is expressed at higher levels at 37°C. The higher specific activity of this peak in cells grown with (^{55}Fe)hemin compared to those grown with $^{55}FeCl_3$ suggests direct incorporation of exogenously supplied hemin into this protein, and implies the existence of a mechanism for transport of intact hemin molecules into the cytoplasm.

Interaction between hemin and iron storage pools. To determine if iron from hemin and inorganic iron become part of a common pool in Y. pestis KIM cells grown under iron-

Table 2. Isolation of bacterioferritin from soluble cell extract of Pgm^- cells grown with $^{55}FeCl_3$ at 37°C.

Preparation	Volume (ml)	Protein (mg/ml)	Total (mg)	^{55}Fe (nMol/ml)	Total (nMol)	Spec. Activ. (nMol/mg)	% Recovery[a]
Crude	3	10.27	30.80	64.7	194.1	6.3	100
A-1.5m	51	0.03	1.53	3.2	163.2	106.7	84
DEAE	25	0.05	1.25	5.9	147.5	118.0	76

a. Percentage of counts in the crude soluble cell extract still present in the sample.

Figure 3. Silver-stained 12.5% SDS-PAGE gel of pooled column chromatography fractions containing the soluble inorganic iron pool. Analysis of Pgm^- cells grown with $^{55}FeCl_3$ at 37°C showing crude soluble cell extract (lane A), radioactive peak from Bio-Gel A-1.5m column at about 270 ml elution volume (lane B), and radioactive peak from DEAE cellulose column eluting at 0.35 M NaCl (lane C). Numbers on the left are the positions of molecular weight standards in kDa. The arrows indicates the locations of the presumptive bacterioferritin subunits.

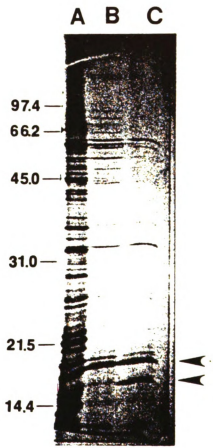

Figure 3

excess conditions, Pgm^+ and Pgm^- cells were labelled to a constant specific activity by growth for approximately eight generations at 37°C in modified Higuchi medium with $^{55}FeCl_3$ or (^{55}Fe)hemin. These cells were used to inoculate two separate cultures containing the same radiolabelled iron source with or without the other iron source, unlabelled and in excess. If there is an interaction between the two forms of iron pools, accumulation of radioactive label in cultures containing both iron sources should diverge from those with a single iron source. For both Pgm^+ and Pgm^- cells grown with $^{55}FeCl_3$, the addition of 5-fold excess unlabelled hemin did not inhibit the continued accumulation of inorganic iron (Fig. 4). However, for cells grown with (^{55}Fe)hemin, addition of 5-fold excess unlabelled inorganic iron resulted in an initial release of some accumulated hemin without significantly affecting subsequent hemin acquisition. This caused a lower specific activity in both Pgm+ and Pgm- cells exposed to both iron sources (Fig. 5). Thus inorganic iron may have an initial slight inhibitory affect on hemin accumulation without significantly affecting the continued rate of acquisition.

Figure 4. 37°C growth and $^{55}FeCl_3$ accumulation by Pgm^+ (panels A and B) and Pgm^- (panels C and D) cells of Y. pestis. Cells were grown to constant specific activity prior to transfer to medium containing 50 uM $^{55}FeCl_3$ alone (panels A and C) or with 250 uM unlabelled hemin (panels B and D). Open circles are cell growth measured by optical density at 620 nm. Closed circles are nmoles of accumulated iron per ml of culture.

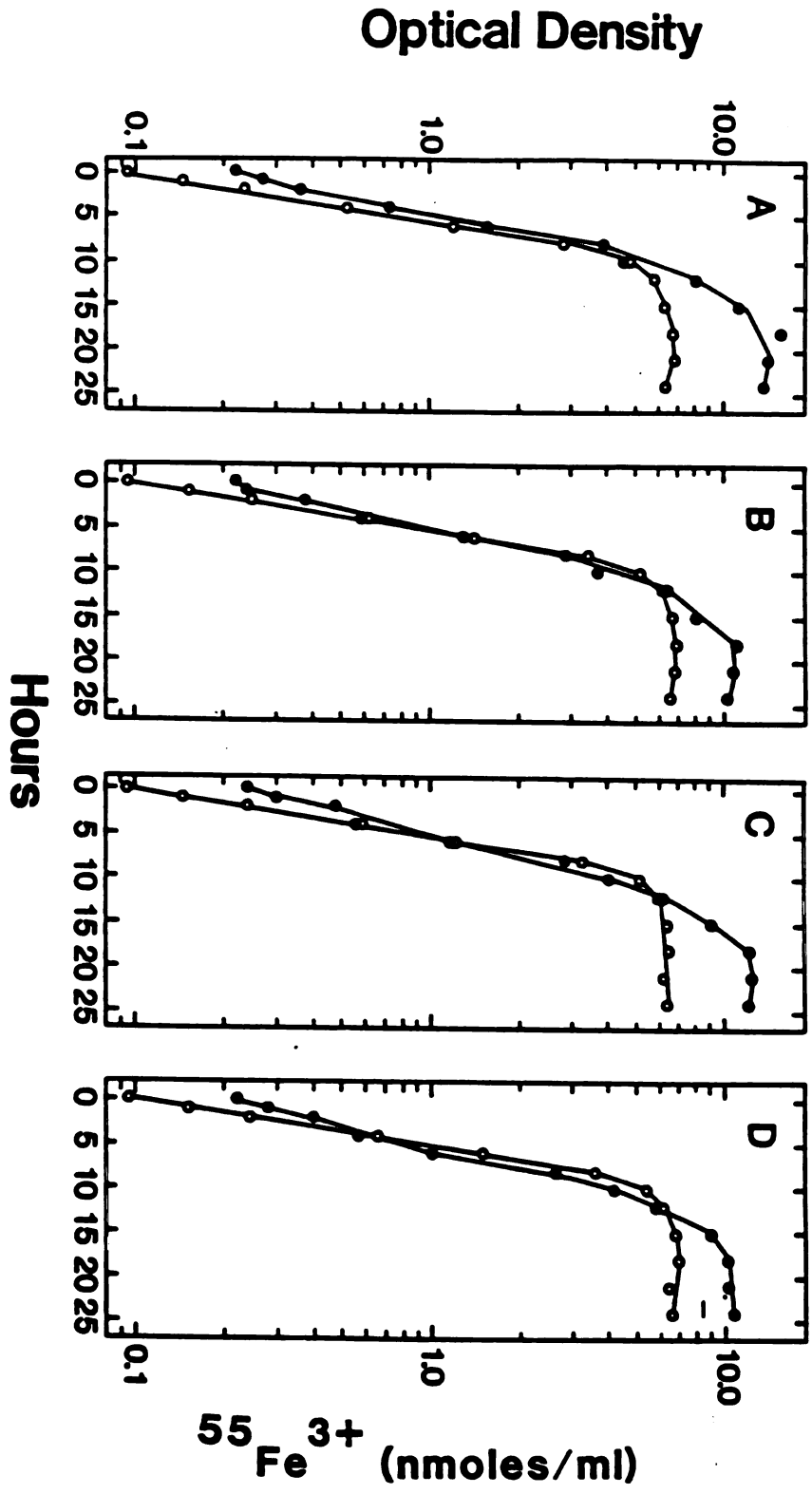
Optical Density
0.1
1.0
10.0
A
B
C
D
0 5 10 15 20 25
Hours
55Fe3+ (nmoles/ml)

Figure 4

Figure 5. $37^{o}C$ growth and (^{55})hemin accumulation by Pgm^{+} (panels A and B) and Pgm^{-} (panels C and D) cells of Y. pestis. Cells were grown to constant specific activity prior to transfer to medium containing 20 uM (^{55})hemin alone (panels A and C) or with 1 mM unlabelled $FeCl_3$ (panels B and D). Open circles are cell growth measured by optical density at 620 nm. Closed circles are nmoles of accumulated iron per ml of culture.

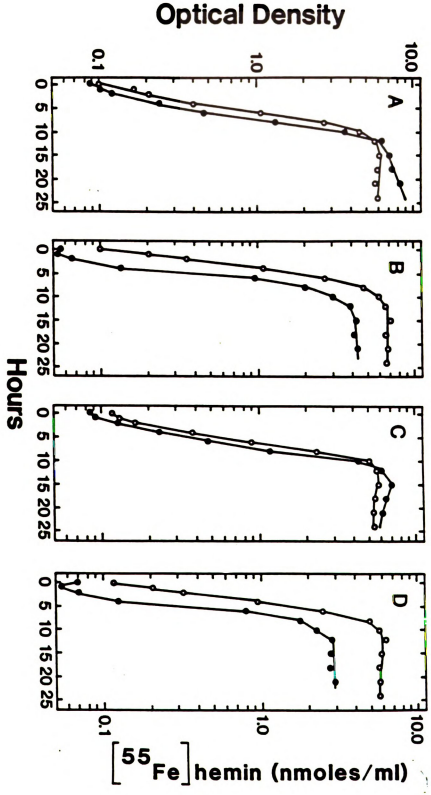
Optical Density
0.1
1.0
10.0
A
B
C
D
0 5 10 15 20 25
Hours
[55Fe]hemin (nmoles/ml)

Figure 5

DISCUSSION

Jackson and Burrows (20,21) first described the pigmentation phenotype as a required virulence determinant identified by the accumulation of sufficient exogenous hemin to form pigmented colonies at 26°C but not at 37°C. The characteristics linked to the Pgm^+ phenotype now include sensitivity to the bacteriocin pesticin, (4) growth at 37°C in an iron-chelated medium, (35) expression of unique iron-regulated outer membrane polypeptides (30), and expression of unique Hms-specific peptides (32,36,42). Spontaneous Pgm^- cells lose all of the above characteristics due to a 102 kb chromosomal deletion (16,26). It is now apparent that the Pgm^+ phenotype of Y. pestis consists of numerous, separable, and independent traits and we use the term hemin storage (hms) to specifically refer to the physiological trait of pigmented colony formation at 26°C in the presence of exogenous hemin or Congo red. The original observation by Jackson and Burrows (20) demonstrated that hemin molecules were retained for storage without apparent removal of inorganic iron. We have localized the site of hemin storage in Pgm^+ cells to the outer membrane and shown that it is restricted to cells grown at 26°C presented with hemin as the iron source. A number of Hms-specific outer membrane polypeptides have now been identified that may be

involved in hemin binding (32,36,42).

The function of the Hms^+ phenotype in Y. pestis is unknown. The extent to which hemin is removed from the medium by Pgm^+ cells at 26°C shows that hemin storage is enormous and raises several possible roles for the expression of such a system. The thermal regulation of this system suggests the flea gut as the environment where this phenotype would be highly expressed. Although several Hms-specific proteins are expressed at low levels at 37°C (32), the time period that stored hemin remains absorbed following shift up to 37°C upon entry into the mammalian host is unknown. However, any putative function in the mammalian environment would likely occur soon after entry. This is also suggested by the finding that Pgm^- cells are only avirulent via peripheral (subcutaneous or intraperitoneal) routes of injection and retain full virulence if introduced intravenously (46). Iron stored during growth in the hemin-rich environment of the flea gut may be used to allow rapid initial multiplication prior to induction of systems capable of obtaining inorganic iron or hemin from mammalian sources. Storage may simply prevent free hemin from participating in generation of damaging oxygen radicals in either the flea or the mammalian host. A recent study suggests that the hemin storage aspect (32,34) of the Pgm^+ phenotype may be necessary for long-term survival in the flea and for blockage of the flea proventriculus (22). Alternatively, hemin molecules on the surface of Y. pestis

may facilitate its uptake by eucaryotic cells. Such a function has been proposed for Shigella flexneri where the CR^+ phenotype correlates with increased ability to invade and infect HeLa cells (13,43) but not with hemin transport and nutritional utilization (31). However, the physiological characteristics and regulation of Cr^+ phenotype of S. flexneri is expressed at 37°C, leaving the degree of functional and genetic similarities between these systems unresolved. A final putative function for hemin storage in Y. pestis is the inhibition of a variety of nonspecific host defenses (47) by an array of surface hemin molecules. Although none of the above putative functions are mutually exclusive, evidence for the validity of any of these functions awaits future experimentation.

We speculate that the inorganic iron storage pool may represent a Y. pestis bacterioferritin whose expression is independent of growth temperature and pigmentation phenotype. Native size, subunit molecular weight, behavior on DEAE and the relative amount of cytoplasmic iron associated with the molecule in cells grown with $FeCl_3$ all closely match results for bacterioferritins from other species (1,2,11,27,28,29,39,45). Bacterioferritins are often difficult to purify by traditional means and some minor contaminants remain in our preparations. Future work should result in highly purified preparations which will be used to analyze hemin content, iron binding capacity and absorption spectra data to help confirm our tentative

identification of this molecule as bacterioferritin. Although the purpose of bacterioferritins is unproven, stored iron in Y. pestis may allow rapid multiplication after injection into the mammalian host prior to expression of iron acquisition systems which can extract iron or hemin from host ligands. However, the relatively low amount of storage on this component when cells were grown with hemin at 26°C seems inconsistent with this idea. Alternatively, this pool may simply prevent excess free iron from damaging the bacterial cell through generation of oxygen radicals (45).

While cells of Y. pestis can utilize either inorganic iron or hemin as sole sources of nutritional iron (33,36,37), our results indicate that accumulation of one form does not have a significant inhibitory effect on the accumulation of the other. Utilization of either inorganic iron or hemin compounds suggests that Y. pestis cells should possess enzymatic activities for inorganic iron insertion into and removal from porphyrin rings. Such activities would lead to a functional linkage between the hemin and inorganic iron pools. If such a linkage exists, it was not evident from the hemin and inorganic iron competition experiments performed here under iron and hemin surplus conditions. These results also suggest separate uptake mechanisms for hemin bound iron and inorganic iron. Results of the studies of the interaction of the iron pools, the preferential accumulation of hemin iron in fractions from

the Al.5m columns containing an enormous hemin binding protein at 37°C, and the lower specific activities of the presumptive bacterioferritin fractions from the Al.5m columns of cells grown with hemin all indicate a mechanism for uptake of exogenous hemin without prior removal of iron, and possibly delayed removal of iron from the hemin even after internalization. Other bacteria have been shown to employ hemin specific uptake systems which allow them to use hemin as an iron source (12,14,16,17,33,40). This would account for the inability of inorganic and hemin-iron sources to interfere with each other's uptake into Y. pestis cells, however, the apparent continued separation of these pools following uptake may occur only under iron-surplus conditions. The significance of these observations and their relevance to growth in vivo is uncertain. Since cells were grown to constant specific activity intracellular iron availability would be high and it is unlikely that high affinity transport mechanisms which are probably expressed in the mammalian host would be operational (10,36,37,38). Under conditions of iron deprivation encountered in the host there may be greater unification of the iron pools following uptake.

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CHAPTER III

Association of a system for iron acquisition with sensitivity to pesticin in pathogenic yersiniae, and the effect of iron deficiency on protein expression.

by

Thomas S. Lucier and Robert R. Brubaker

ABSTRACT

Differential growth of pathogenic species of Yersinia on plates containing increasing concentration of iron chelating molecules indicated the presence of a non-specific iron uptake system associated with pesticin sensitivity. Loss of pesticin sensitivity due to the 100 kb Pgm-specific deletion in Y. pestis, or undefined rare mutations in all three species correlated with lack of functioning of this system as indicated by reduced growth in the presence of moderate strength ferric (Y. pestis) or strong ferrous (all species) chelators. In Y. pestis, but not enteropathogenic species, this correlated with loss of expression of several previously identified iron-regulated outer membrane peptides (Irps B-D). Y. pestis showed dramatically improved growth in the presence of ferric chelators at 26°C versus 37°C which correlated with temperature specific expression of iron-regulated peptides of 34 and 28.5 kDa in the outer membrane and 33.5 and 30 kDa in the periplasm. Growth of enteropathogenic species was unrestricted by ferric chelators at both temperatures, and the relationship of this to expression of iron regulated outer membrane proteins was evaluated. Growth of Y. pestis in iron deficient medium resulted in considerable changes in protein expression in outer membrane, periplasmic and cytoplasmic membrane

fractions. Newly described iron-regulated peptides of 113 kDa in the outer membrane and 19 kDa in the periplasm along with the envelope spanning HMWPs are associated with the Pgm virulence determinant. The possible role of these peptides and the previously described Irps B-E in a Pst^S/iron uptake phenotypic component of the classical Pgm virulence factor is considered.

INTRODUCTION

Pathogenic bacteria encounter iron-deficient growth conditions due to mammalian iron and heme binding compounds such as transferrin, lactoferrin, ferritin, hemopexin, albumin, hemoglobin, and haptoglobin (20,53). To obtain iron necessary for growth in mammalian hosts bacteria have developed disparate and elaborate iron acquisition systems. Numerous siderophore-mediated iron transport systems for acquiring chelated inorganic iron have been described (13, 35). However, a number of organisms utilize siderophore-independent mechanisms to obtain iron from transferrin, lactoferrin, ferritin, and/or heme compounds (20, 30,35,36,42,44). These systems typically require outer membrane receptors along with periplasmic and cytoplasmic membrane proteins for internalization of iron. Expression of these proteins by pathogenic bacteria is typically regulated by iron availability such that iron transport genes are highly expressed only during iron starvation (33, 35). A number of these membrane proteins are also utilized by bacteriocins (31,33,35) to gain access to the bacterial cell. In enteric organisms expression of these membrane peptides and other components of iron transport systems are typically regulated by the ferric uptake regulation (Fur) protein which acts as both the iron-status

sensor and a negative regulator of transcription (35,52).

The pigmentation phenotype (Pgm^+) of Yersinia pestis confers exogenous hemin binding at 26°C (22) mediated by the expression of several non-iron-regulated outer membrane proteins (38,44,47), virulence in the mouse model (48), ability to grow in iron chelated medium at 37°C (43) and expression of four iron-regulated outer membrane peptides of 65.1 - 69.1 kDa (Irps B-E) (44). In addition, Pgm^+ cells which lack the 9.5 kb Pst plasmid are sensitive to the bacteriocin pesticin (4,15). Pgm^- mutants result from a high frequency (10^{-5}) (4) 100 kb chromosomal deletion (29). They fail to express these characteristics and are resistant to pesticin. A rare Pgm^+, Pst^r mutant which had not experienced this deletion failed to express Irps B-E and was unable to grow in iron-chelated medium (44) suggesting a role for one or more of these peptides in an iron acquisition system which also serves as a binding site for pesticin. Functional separation of this system and hemin binding was indicated by the isolation of the Pgm^+, Pst^r mutant, and the ability of a cloned hemin storage locus to restore hemin binding but not pesticin sensitivity to a Pgm^- mutant (39). Conjugational transfer of DNA which restores pesticin sensitivity but not pigmentation to Pgm^- cells also restores virulence in the mouse model by peripheral routes of injection (25). Therefore, the iron uptake system associated with the Pgm^+ phenotype and not hemin binding may be critical for virulence in the mammalian host.

The physiological nature of this system for iron acquisition remains obscure. Both Pgm^+ and Pgm^- cells can utilize ferritin and various heme containing proteins (hemoglobin, haptoglobin, hemopexin) as their sole source of iron (36,44), and neither can remove iron from transferrin or lactoferrin (44). Earlier studies implied a possible role for siderophores in iron acquisition by Y. pestis, however, subsequent intensive work failed to confirm this and indicated the presence of inducible, membrane associated systems (36,44). Proteins involved in this system should include one or more of Irps B-E, and perhaps the iron-regulated 190 kDa and 240 kDa high molecular weight proteins (HMWPs) required for virulence in all pathogenic species of Yersinia (6,8,9) which may be encoded in the 100 kb Pgm^+-specific segment of DNA in Y. pestis (7). Since citrate was the chelator inhibiting growth of Pgm^- and Pgm^+, Pst^r cells in iron deficient medium (43), these peptides could mediate a ferric dicitrate specific uptake system as seen in Escherichia coli (18,35) or a non-specific system for acquisition in iron deficient conditions. Certain strains of Y. pseudotuberculosis and Y. enterocolitica are also sensitive to pesticin (5,37). In these species pesticin resistant mutants are rare and it is not known if this mutation results in changes in expression of iron-regulated proteins, however Pst^s and Pst^r cells of both species showed equivalent growth in iron deficient media containing citrate (43). The reason for this discrepancy

between Y. pestis and the enteropathogenic species is unknown.

In this study we present evidence for the existence of an inducible, chelator non-specific system for iron acquisition correlated with pesticin sensitivity in all three pathogenic species of Yersinia, and consider the potential role of Irps B-E in this system. We further demonstrate that both Pgm^+ and Pgm^- Y. pestis acquire iron much more readily from certain ferric chelators at 26°C than at 37°C, and this ability is correlated with the expression of four previously undescribed iron-regulated peptides in the outer membrane and periplasm. In vitro growth of Y. pestis in iron deficient media results in significant changes in proteins associated with the cytoplasmic membrane, periplasm and outer membrane, including expression of previously unrecognized Pgm^+ specific peptides in the latter two locations. We confirm that expression of HMWPs in Y. pestis is Pgm^+ specific and rather than being restricted to the outer membrane they may extend from the outer membrane to the cytoplasm.

MATERIALS AND METHODS

Bacteria. Yersinia pestis KIM was used in all experiments. All strains contained the Tox plasmid, but lacked the Pst and Lcr plasmids. The Pgm^- and Pgm^+, Pst^r mutants were isolated from Pgm^+ strain KIM as previously described (4,44). Pesticin resistant Y. pseudotuberculosis PB1 serotype I and Y. enterocolitica WA serotype O8 were derived from parent strains which lacked the Lcr plasmid (43).

Media and cultivation. Bacteria were routinely grown in the media of Perry and Brubaker,(36) or in the media of Higuchi et al (21) as modified by Zahorchak and Brubaker (55). These media were made iron deficient by omitting $FeSO_4$ during their preparation, and by 8-hydroxyquinoline extraction prior to addition of magnesium salts (51). The resulting media contained 0.3 - 1.0 uM iron as determined by atomic absorption spectrophotometry (SpectrAA Zeeman atomic absorption spectrophotometer; Varian Techtron Pty, Ltd. Mulgrave, Australia). They were made iron replete by subsequent addition of $FeCl_2$ dissolved in 0.01N HCl to a final concentration of 50 uM.

Procedures for growth of bacteria for gradiant plate analysis, iron uptake tests, and cell fractionation studies

were modifications of those previously described (43). Following retrieval of bacteria from stock cultures cells were grown overnight in iron replete liquid medium at 26°C and aerated at 200 rpm on a model G76 gyratory water bath shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Bacteria were harvested by centrifugation (10,000 g for 8 minutes), washed twice with iron deficient medium, resuspended to an optical density at 620 nm of at least 1.0 and used to inoculate iron replete or iron deficient media to an optical density of 0.1 (approximately 10^8 cells per ml). Cells in this first transfer were allowed to grow at either 26°C or 37°C to an optical density of 1.0. These cultures were used to inoculate fresh cultures to an optical density of 0.1. The second transfer was allowed to grow to late log phase before being harvested for use in various tests. Cells were grown in the medium of Perry and Brubaker for most procedures. Radiolabelling was performed by growing cells in modified Higuchi's medium supplemented with 20 uCi/ml L-(^{35}S) methionine as described in Sikkema and Brubaker (44).

Gradient plates. Gradient plates were prepared in 100mm wide square or 100mm diameter circular petri dishes. Shape of the dish had no noticable effect on results. Iron deficient media of Perry and Brubaker was solidified with 1% Noble Agar (Difco Laboratories, Detroit, Mi). This medium was used since it lacks molecules which could compete for

iron with the chelators being tested. After autoclaving and cooling to 50°C the chelator was added from sterile stock solution to the desired concentration. Stock solutions were prepared in distilled, deionized water (milli-Q; Millipore Corp. Bedford, MA.) at a concentration 100 times that needed in the plates and were filter sterilized (0.45 um Acrodisc, Gelman Sciences, Ann Arbor, Mi). EDDA (ethylenediamine-N,N'-diacetic acid) was deferrated according to standard techniques (40). Stock solutions of sodium citrate and sodium pyrophosphate were extracted with 8-hydroxyquinoline to remove contaminating iron (51). Twenty ml of media was poured into each plate and allowed to solidify overnight while inclined so that the media just reached the base on the raised side of the plate. More of this media was then prepared without the iron chelator. Twenty ml of this top layer was poured onto each plate and allowed to solidify on a level surface. This resulted in an increasing concentration of the chelator across the plate. Control plates had a basal layer which lacked iron chelators.

To inoculate the plates cells were grown to an optical density of 1.0 during the second passage through iron deficient medium as described above. They were then diluted 100:1 in fresh iron deficient media and 20 ul (2×10^5 CFU/ml) was streaked across the gradiant. Plates were incubated at either 26°C or 37°C and the distance along the gradient to which cells in the streak had grown was recorded

each day until no further growth was observed. This required four days for all chelators with Yersinia pestis and anywhere from two to five days with enteropathogenic Yersinia species depending upon the chelator being tested.

Iron uptake assay. Pgm^+ or Pgm^- cells were grown in iron deficient medium to an optical density of 1.0 in the second passage as previously described. Subsequent uptake assays were adapted from previously described methods (11,36). Briefly, cells were harvested by centrifugation (10,000 g for 10 minutes), washed twice and resuspended to an optical density of 7.0 in iron deficient uptake buffer (25mM HEPES pH 7.4, 2.5 mM K_2HPO_4, 20 mM $MgCl_2$, 10 mM glucose extracted with 8-hydroxyquinoline to remove iron). Iron content of uptake buffer was determined to be 0.5 - 1.0 uM by atomic absorption spectrophotometry. Cells were kept on ice in this buffer for no more than 40 minutes prior to their use. To perform an assay 1.0 ml of cells was added to 8.9 ml of fresh uptake buffer and allowed to acclimate for 10 minutes at either 26°C or 37°C. To begin the test 0.1 ml of uptake buffer containing 5.0 uCi/ml $^{55}FeCl_3$ (NEN Products, Boston, MA.) was added and timing begun. The activity of the uptake buffer was 0.05 uCi/ml for a concentration of 30 picomolar ^{55}Fe. Samples of 0.5 ml were withdrawn at intervals, filtered (0.22 um pore size, Millipore Corp. Bedford, MA.) washed once with 5.0 ml of the same buffer less glucose and plus 0.2 mM sodium

nitrilotriacetate to remove unabsorbed iron, and counted in a Packard Tri-Carb scintillation spectrometer.

Cell fractionation. Fractionation of cells was performed using the methods described by Straley and Brubaker (46). Briefly, cells were harvested by centrifugation and resuspended in a 0.75M sucrose buffer. Conversion to spheroplasts followed treatment with lysozyme and rapid dilution with low molarity EDTA. Spheroplasts were harvested by centrifugation. The supernatants were filtered (0.45 um Acrodisc, Gelman Sciences, Ann Arbor, Mi) and saved as the periplasm fraction. Spheroplasts were disrupted by sonication, intact cells were removed by centrifugation, and the supernatant was separated into cytoplasmic and membrane fractions by centrifugation in 0.25M sucrose buffer at 240,000 g for 3 hours). The resulting pellet (membrane fractions) was resuspended and separated into inner and outer membranes by isopycnic sucrose-density gradiant centrifugation. Inner membrane contamination of outer membrane preparations using this procedure was approximately 3% (46).

Electrophoresis. Two-dimensional protein gels were performed as described by O'Farrell (34). Details of the procedure for preparation of outer membrane samples of Yersinia pestis are well established and were followed in this study (44,46,47). The same procedure was used for two

dimensional analysis of outer membranes of Y. pseudotuberculosis and Y. enterocolitica.

SDS-PAGE lane gels were performed using standard methods (26). Periplasm samples were concentrated by precipitation in cold 10% TCA and then prepared for SDS-PAGE. Inner membranes and outer membranes were concentrated by centrifugation for 4 h at 190,000 g in wash buffer (50 mM Tris-HCl pH 6.8, 10 mM $MgCl_2$, 0.2 mM dithiothreitol) followed by preparation for SDS-PAGE. Cytoplasmic fractions did not require concentration prior to electrophoretic analysis. Proteins were visualized following two dimensional electrophoresis and lane gels by silver staining (32) or autoradiography.

Materials. All reagents were of the highest quality available and obtained from Sigma Chemical Co. (St. Louis, MO.) or Bio-Rad Laboratories (Richmond, CA.). Glassware was soaked overnight in saturated chromic acid followed by copious rinsing in distilled water and distilled deionized water prior to use. Aqueous solutions were prepared in distilled deionized water.

Protein determination. Concentration of protein in samples was determined by the method of Lowry (28).

RESULTS

Gradient plates. In order to further define the apparent lesion in the ability of Y. pestis to acquire iron at 37°C after mutation to Pgm^- or Pst^r (43,44) gradiant plates were developed in which cells on the surface of the agar were exposed to progressively higher concentrations of iron chelators along the length of the streaks. Typical results are shown in Figure 1 and Figure 3A summarizes the results of all tests. All species and strains exhibited rapid growth on control plates lacking chelators. In the presence of any of the tested chelators full, rapid growth across the gradient was restored by inclusion of 50 uM hemin or 100 uM $FeCl_2$ in the overlayer confirming that inhibition of growth was due to iron deprivation rather than toxic effects of the chelators. As reported by Sikkema and Brubaker (43,44) Pgm^+ cells showed greater growth then Pgm^- and Pgm^+, Pst^r strains in the presence of citrate (Fig. 1, 3A), but this was not caused by a ferric-dicitrate specific system since growth of Pgm^+ cells was inhibited at relatively low concentrations, and similar results were obtained with other moderate strength ferric chelators (NTA, sodium pyrophosphate) (Fig. 1,3A). Interestingly the greatest difference was with the relatively strong synthetic ferric chelator EDDA demonstrating that growth inhibition is

Figure 1. Gradient plates showing growth of Yersinia pestis KIM. On each plate the top streak is Pgm^-, middle streak is Pgm^+ and bottom streak is Pgm^+, Pst^r cells. (A) gradients of 0-1 mM sodium citrate and (B) 0-1 mM EDDA. In each picture the upper plate was incubated at 37°C and the lower plate at 26°C.

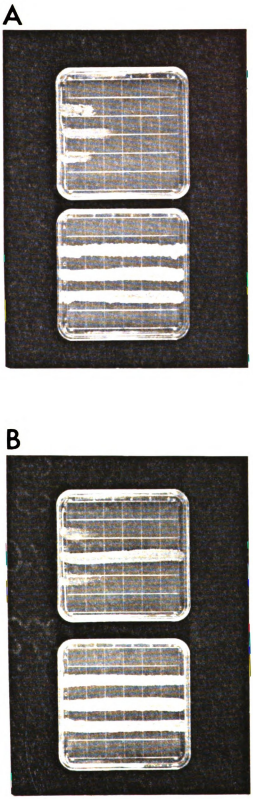


Figure 1

Figure 2. Gradient plates showing growth of (A) Yersinia pseudotuberculosis PB1 at 37°C and (B) 26°C, and (C) Yersinia enterocolitica WA at 37°C and (D) 26°C. The top streak on each plate is Pst^s bacteria and the bottom streak is Pst^r cells. All gradients are 0 - 200 uM 2,2 dipyridyl.

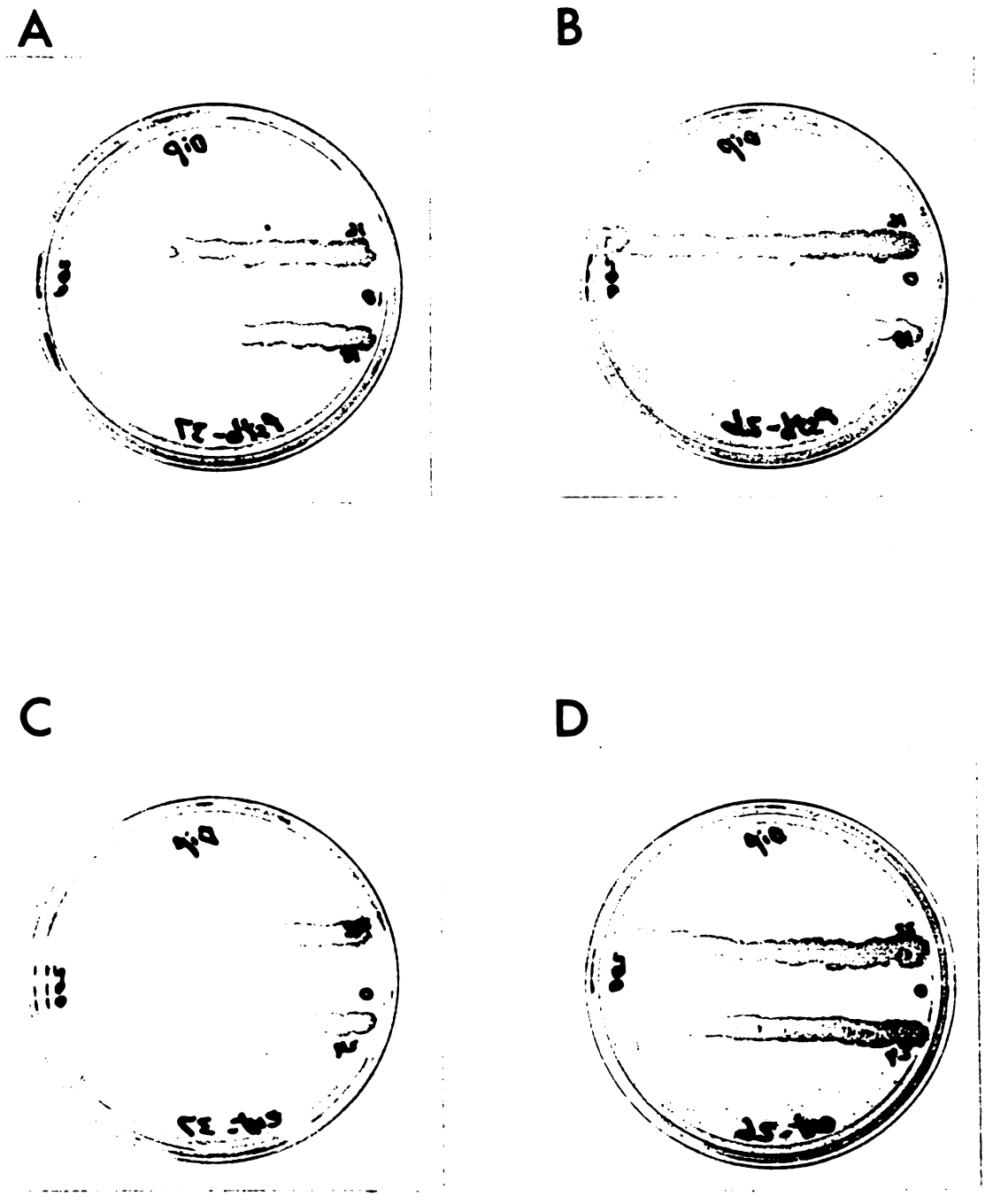


Figure 2

Figure 3. Extent of growth by Yersinia on gradient plates containing various iron chelators. Rectangles show the distance each streak grew across the plates from 0 (no growth) to 100% (complete growth across the plate). DIP = 2,2 dipyridyl (0-200 uM), BPS = bathyphenanthroline disulfonic acid (0-100 uM), CON = conalbumen (0-20 uM), DES = desferrioxamine mesylate (0-200 uM), EDDA = ethylenediamine-N,N'-diacetic acid (0-1 mM), CIT = sodium citrate (0-1 mM), PYR = sodium pyrophosphate (0-1 mM), NTA = sodium nitrilotriacetate (0-1 mM). (A) Growth of Yersinia pestis KIM Pgm^- (top rectangle for each chelator), Pgm^+ (middle rectangle), Pgm^+,Pst^r (bottom rectangle). (B) Growth of Yersinia pseudotuberculosis PBl Pst^s (top rectangle) and Pst^r (bottom rectangle). (C) Growth of Yersinia enterocolitica WA Pst^s (top rectangle) and Pst^r (bottom rectangle).

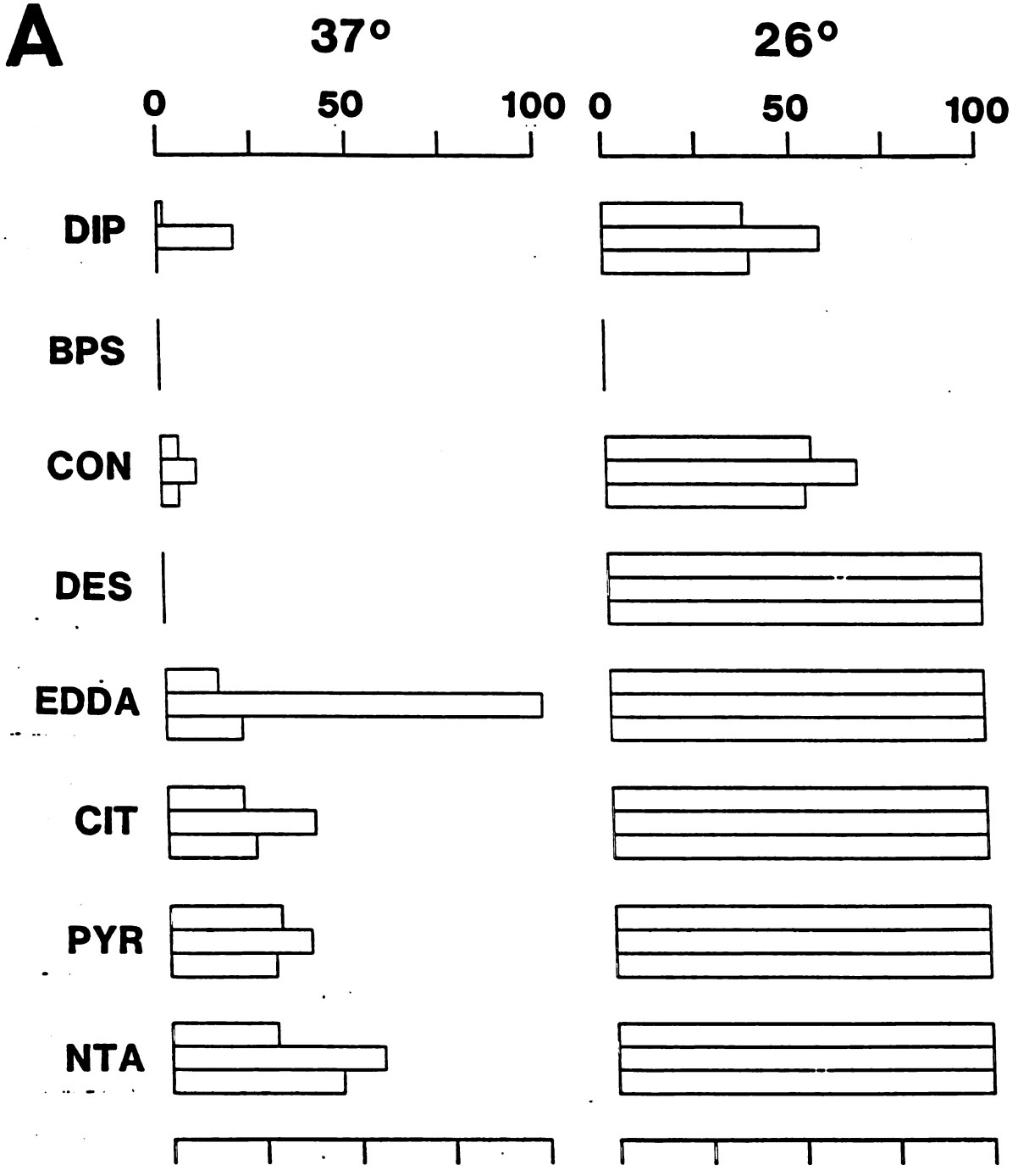
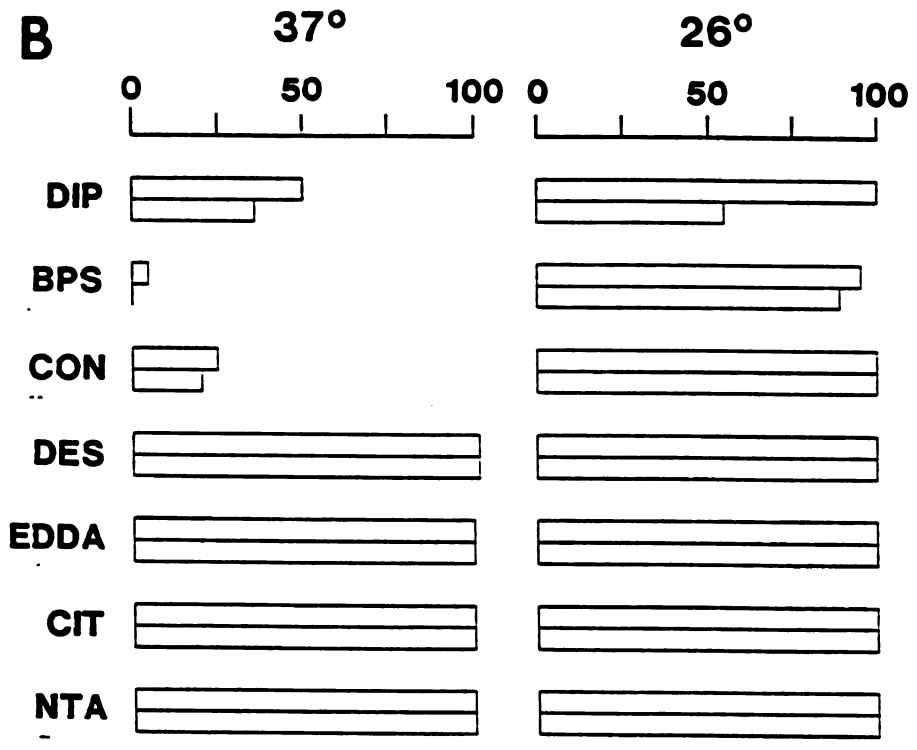


Figure 3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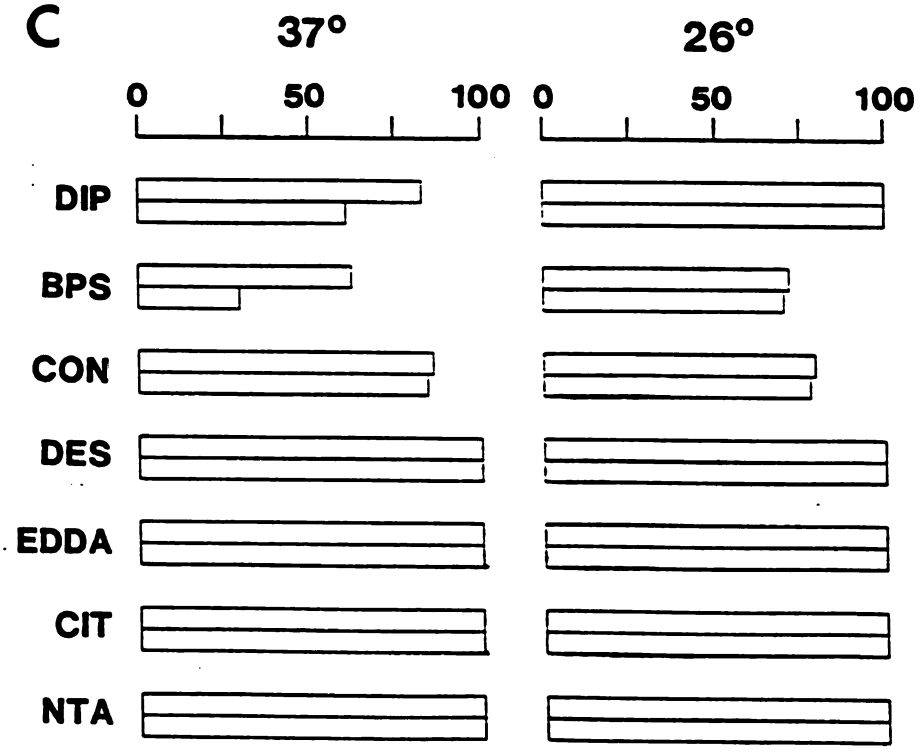
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not entirely attributable to the relative affinity of the different chelators. We confirmed previous reports that Y. pestis is unable to utilize iron bound to the hydroxamate siderophore desferrioxamine mesylate (ferrioxamine) at 37°C or to conalbumen (36). The latter was included as a negative control and while growth is indicated (Fig. 3) the streaks were faint and should be considered residual for all three species at both temperatures. The same consideration applies to results for the ferrous chelator BPS.

Temperature has a dramatic effect on the ability of Y. pestis to acquire iron from ferric chelators. All strains show unrestricted growth in the presence of ferric chelators (exception; conalbumen) at 26°C. The difference was most extreme for ferrioxamine going from no detectable growth at 37°C to complete growth across the gradiant at 26°C (Fig. 1 and 3A).

The two ferrous chelators tested (2,2 dipyridyl and BPS) were very effective at limiting growth of all three species at 26°C and 37°C (exception; Y. enterocolitica with dipyridyl at 26°C) (Fig. 2 and 3). As with ferric chelators Pgm^+ Y. pestis exhibited growth to higher concentrations of dipyridyl at 37°C than did Pgm^- and Pgm^+, Pst^r cells, however, only with this chelator was the difference maintained at 26°C. This was the only case in which Pst^r mutants of Y. pseudotuberculosis and Y. enterocolitica showed a reduced ability to acquire iron relative to Pst^s parent strains (Fig. 2 and 3), and was characteristic of

both at 37°C and of Y. pseudotuberculosis at 26°C. The similar results seen with BPS may not be significant since growth with this chelator was residual.

Enteropathogenic species were much better able to grow at 37°C in the presence of ferric chelators other than conalbumen than was Y. pestis (Fig. 2,3). With citrate and ferrioxamine both species grew substantially faster than in control plates which lacked chelators, and for the latter probably reflects the presence of a ferrioxamine-specific system (3). Since growth on BPS and conalbumen plates was residual the enhanced growth seen at 26°C with Y. pseudotuberculosis is not conclusive, although in the presence of 2,2 dipyridyl growth was greater at 26°C.

^{55}Fe uptake assays. Differences in the abilties of Pgm^+ and Pgm^- Y. pestis to grow in media containing various chelators could reflect either the loss of a non-specific system for iron acquisition or a greater iron requirement for growth of Pgm^- cells. To help resolve this studies of labelled iron uptake were initiated. These studies indicated distinct strain and temperature dependent differences in kinetics of iron uptake which correlated with the growth data obtained from gradiant plates.

Expression of inducible uptake systems occurred in low iron conditions by both Pgm^+ and Pgm^- cells at 37°C, however Pgm^+ cells acquired the nutrient more than twice as fast (Fig. 4). Thus differences in growth on gradiant plates

Figure 4. Acquisition of ^{55}Fe from uptake buffer by Yersinia pestis KIM grown at 37°C. Open circles; Pgm^+ cells grown in iron deficient medium. Closed circles; Pgm^- cells grown in iron deficient medium. Triangles; Pgm^+ cells grown in iron replete medium.

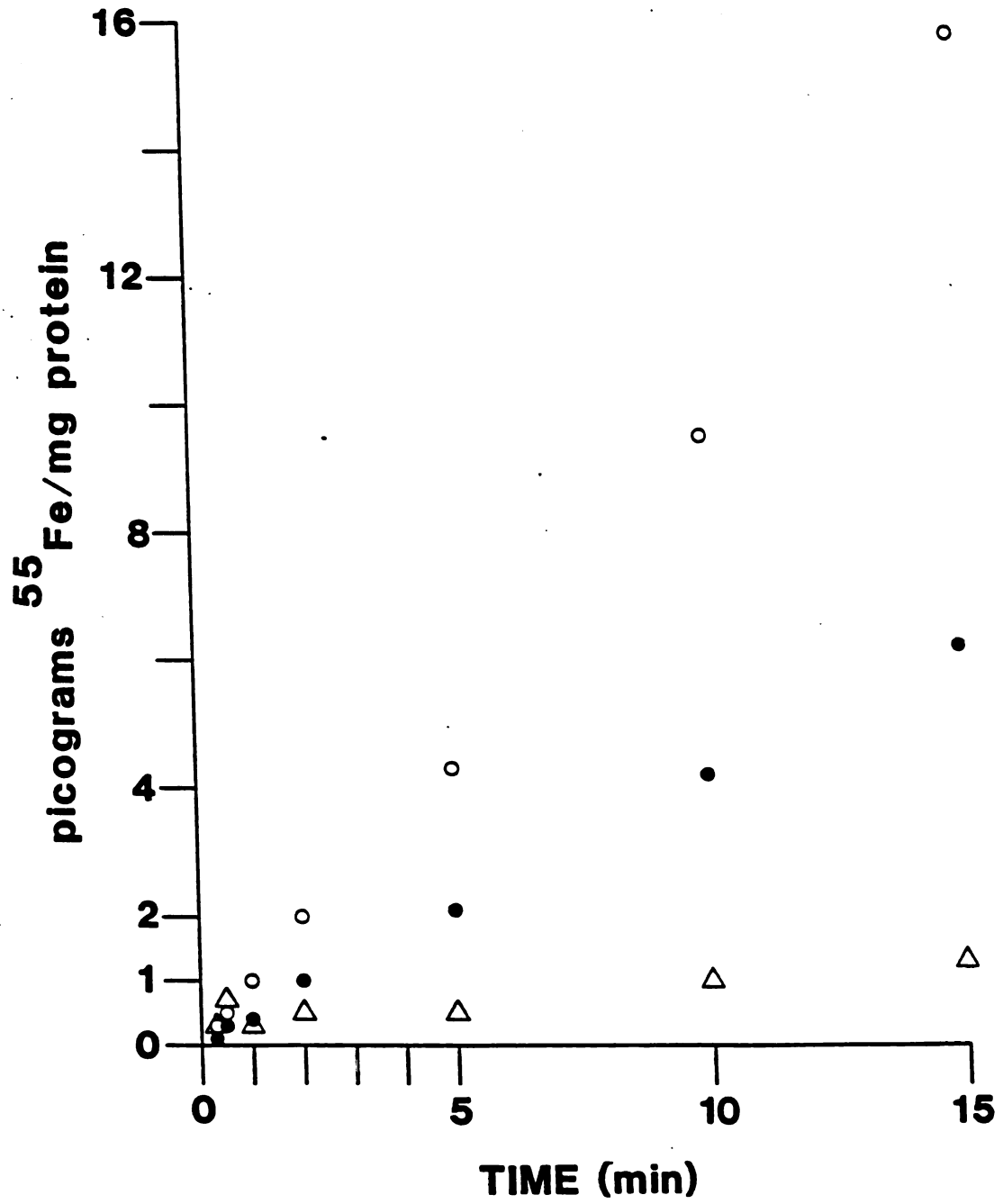
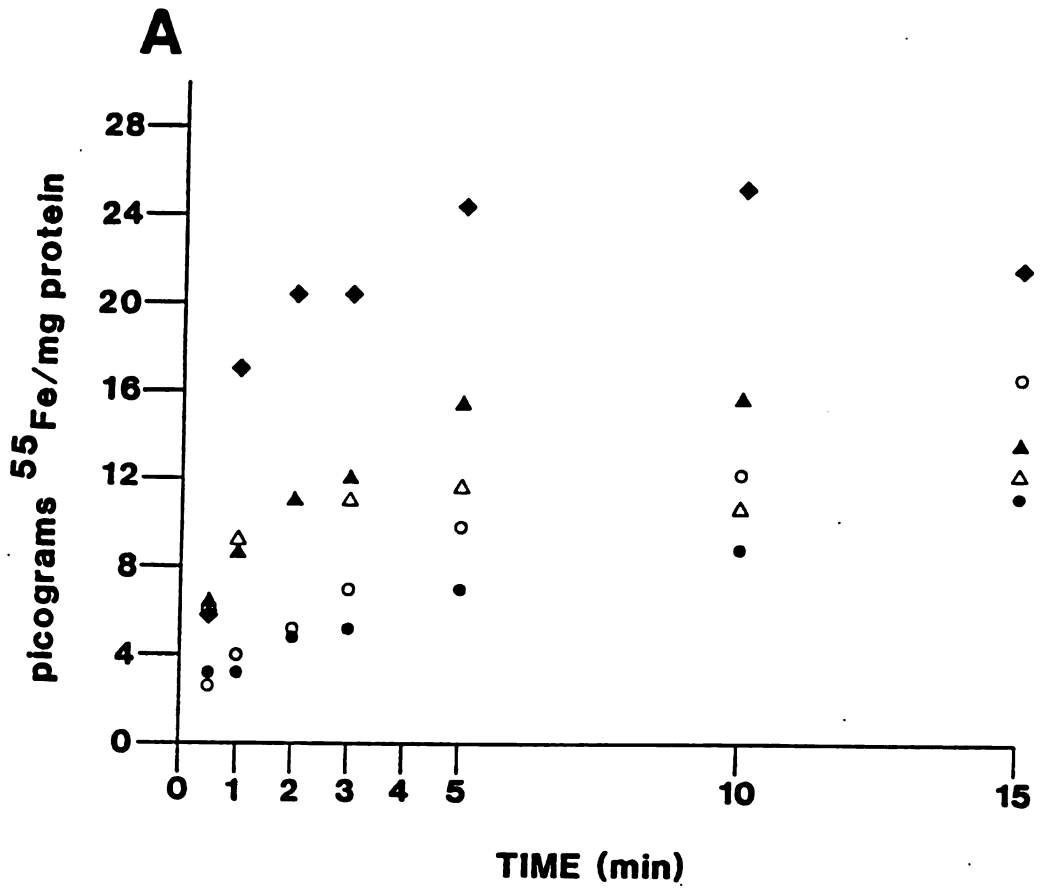

16
12
8
4
2
1
0
picograms ^{55}Fe/mg protein
0
5
10
15
TIME (min)

Figure 5. Effect of acclimation temperature on uptake of ^{55}Fe by Yersinia pestis KIM. (A) Uptake buffer lacking strong iron chelators. All were grown in iron deficient medium. Open circles; Pgm^+ cells grown and tested at 37^oC. Closed circles; Pgm^+ cells grown at 37^oC and tested at 26^oC. Open triangles; Pgm^+ cells grown at 26^oC and tested at 37^oC. Closed triangles; Pgm^+ cells grown and tested at 26^oC. Diamonds; Pgm^- cells grown and tested at 26^oC. (B) Uptake buffer containing lmM sodium citrate. Open circles; cells grown and tested at 37^oC. Closed circles; cells grown and tested at 26^oC.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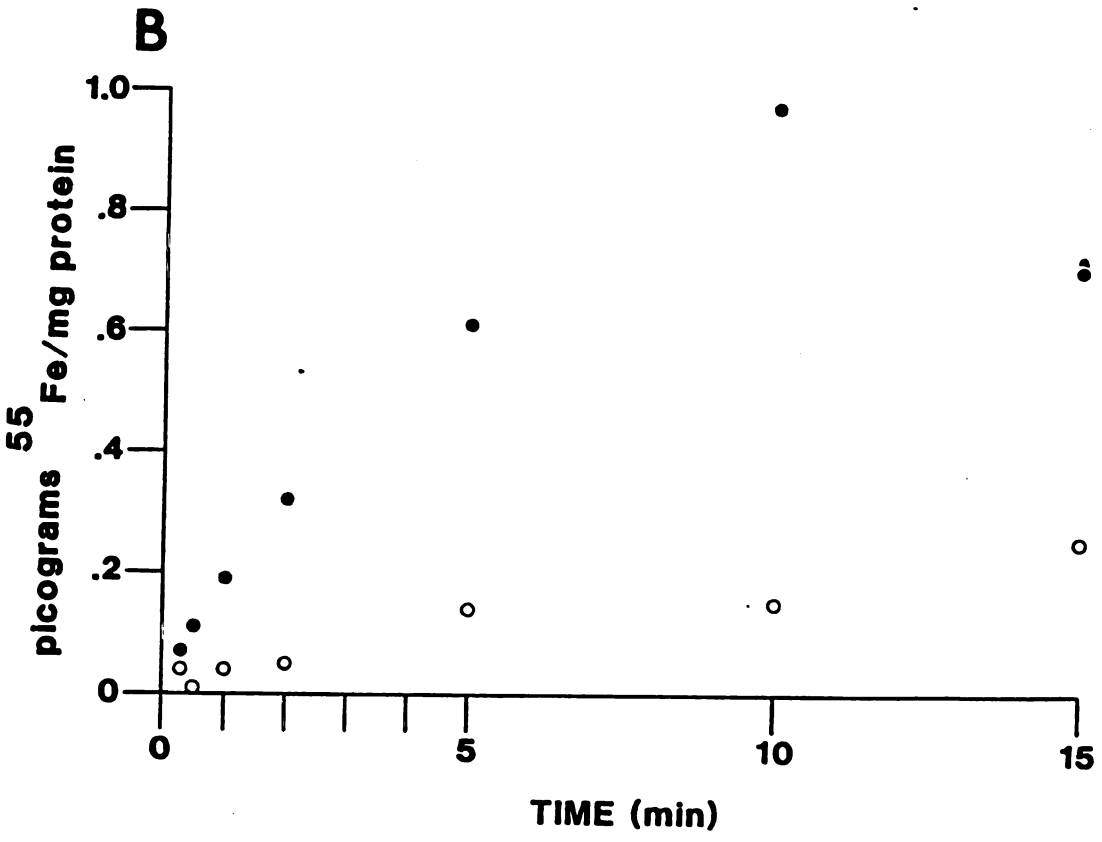
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could be due to more efficient iron uptake by Pgm^+ cells. Pgm^+ cells grown in iron replete medium acquired much less iron over the course of the experiment. The more rapid accumulation by these cells during the first 30 seconds may result from non-specific binding to the surface of the cell.

The effect of temperature on growth seen with gradiant plates was also reflected in studies of labelled iron acquisition. Pgm^- cells acclimated to iron deficient conditions at 26°C actually took in more iron than Pgm^+ bacteria acclimated at either temperature (Fig. 5A). Results for Pgm^+ cells indicate that the temperature at which the cells were grown determines the kinetics of iron uptake, and not the temperature at which the assay was performed. Initial uptake by Pgm^+ cells was significantly greater if they had been grown at 26°C when assayed at either temperature. After five minutes net uptake for 26°C grown cells had leveled off and been equalled by 37°C grown cells after 10 - 15 minutes. The same pattern was apparent with 26°C grown Pgm^- cells.

Temperature dependent differences in growth of Y. pestis on gradient plates were only apparent in the presence of chelators so we assumed that their inclusion in uptake buffer would magnify the temperature dependent difference in rate of uptake. When 1 mM citrate was included in the uptake buffer the rate of accumulation by Pgm^+ cells was reduced nearly 30-fold (Fig. 5B) as previously observed by Perry and Brubaker (36). However, the relative difference

in acquisition was substantially greater. Cells grown at 26°C continued rapid accumulation for 10 minutes and remained considerably higher than 37°C grown cells even after a significant decline at 15 minutes. Thus the temperature effect seen for growth in iron deficient media in the presence of chelators is reflected in the results of labelled iron acquisition studies.

Iron regulated protein expression in Y. pestis KIM. Protein expression was examined in Pgm^+, Pgm^-, and Pgm^+, Pst^r Y. pestis KIM grown in iron replete and iron deficient media at 26° and 37°C. Differences in protein expression which correlate with the results from growth and uptake studies could provide clues to the biochemical basis for the observed deficiency in iron acquisition, and more fully characterize phenotypic changes associated with the mutation to Pgm^-. SDS-PAGE analysis of cell fractions revealed considerable changes in protein expression in the outer membrane, periplasm and inner membrane. Peptides expressed at substantially higher levels in iron deficient conditions are listed in table 1.

Outer membrane Irps A-E and the HMWP's have been previously described (7,8,44). Pgm^- cells failed to produce any of these except Irp A. We showed that Irps A-D were expressed at both 26° and 37°C by Pgm^+ cells (Table 1, Fig. 6,7,11), and identified a previously undescribed 113 kDa Pgm^+-specific peptide (Irp F) which was only visible on lane

Table 1. Iron repressible peptides of Yersinia pestis KIM.

Designation	Location	Size (kDa)	Temperature (°C)
Irp A (44)	OM	80.1	Both
*Irp B (44)	OM	68.8	Both
*Irp C (44)	OM	67.4	Both
*Irp D (44)	OM	69.1	Both
*Irp E (44)	OM	65.1	Both
*Irp F	OM	113.0	Both
Irp G	OM	36.0	Both
Irp H	OM	34.0	26
Irp I	OM	28.5	26
Irp J	OM	22.5	37
Irp K	OM	19.5	37
Irp L	OM	13.8	Both
Irp M	OM	80-85	Both
**HMWP1 (8)	OM,IM,PP,C	240.0	Both
**HMWP2 (8)	OM,IM,PP,C	190.0	Both
Pirp A	PP	75.0	Both
Pirp B	PP	39.5	Both
Pirp C	PP	38.5	Both
Pirp D	PP	37.7	Both
Pirp E	PP	37.0	Both
Pirp F	PP	36.5	Both
Pirp G	PP	33.7	26
Pirp H	PP	31.5	Both
Pirp I	PP	31.0	Both
Pirp J	PP	30.0	26
Pirp K	PP	25.0	Both
**Pirp L	PP	19.0	Both
Mirp A	IM	127.0	Both
Mirp B	IM	115.0	Both
Mirp C	IM	94.0	Both
Mirp D	IM	55.5	Both
Mirp E	IM	36.0	Both
Mirp F	IM	35.0	Both
Mirp G	IM	33.0	Both
Mirp H	IM	29.8	Both

OM = outer membrane
IM = inner membrane
PP = periplasm
C = cytoplasm
* = absent in Pgm^- and Pgm^+, Pst^r cells
** = absent in Pgm^- but present in Pgm^+, Pst^r cells

Figure 6. Two-dimensional stained gels of outer membrane proteins from Y. pestis KIM grown at 26°C. (A) Pgm^+ cells grown in iron replete medium, (B) Pgm^+ cells grown in iron deficient medium, (C) Pgm^- cells grown in iron deficient medium. Letters indicate iron repressible peptides (Irps) designated in table 1.

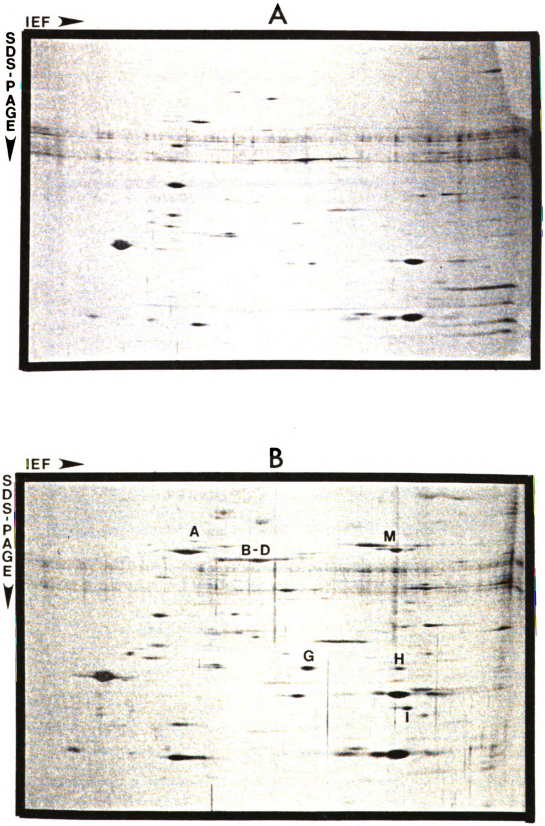

Figure 6

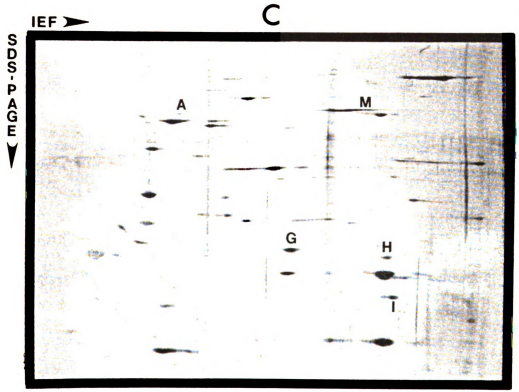

Figure 6

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Figure 7. Comparison of peptides present in a stained gel (12% SDS-PAGE) of purified outer membrane proteins of *Y. pestis* KIM grown at 37°C (lanes A-D) and 26°C (lanes E-H). Pgm^+ grown in iron replete medium (lanes A and E), Pgm^+ cells grown in iron deficient medium (lanes B and F), Pgm^+,Pst^r cells grown in iron deficient medium (lanes C and G), and Pgm^- cells grown in iron deficient medium (lanes D and H). Molecular weight markers are in the unlabelled middle lane and their sizes in kilodaltons are indicated along the left edge of the gel. Positions of detectable Irps (Table 1) are indicated along the right edge. The open arrow indicates expression only at 26°C, and asterisks show peptides not expressed by Pgm^- cells.

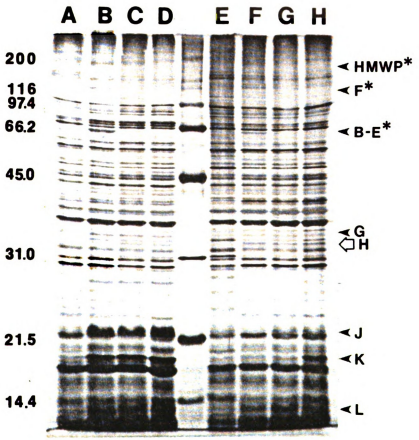

Figure 7

gels (Fig. 7,14). We were unable to confirm the presence of Irp E (65 kDa, isoelectric point = 5.98). At least eight more irps unrelated to pigmentation were identified (Fig. 6,7,11). The 36, 34 and 28.5 kDa peptides (Irps G, H, and I) were best seen in two-dimensional gels (Fig. 6,11), and the latter two (Irps H,I) were expressed only at 26°C. The Irp M group included two or three peptides only visible in two-dimensional gels (isoelectric point = 6.2 - 6.6, Fig. 6,11).

We confirmed previous observations that the HMWP's are visible in lane gels of outer membrane samples, but are not seen in two-dimensional gels (7,8,44). This would occur if the isoelectric points of these peptides were outside the 4.5 - 7.0 range of our analysis. Our results indicated that HMWP's are not restricted to the outer membrane as was previously suggested (6). HMWPs were seen in all fractions from Pgm^+ and Pgm^+, Pst^r cells at both temperatures. Since most peptides could be clearly assigned to a particular location it is unlikely to reflect poor separation of fractions during sample preparation.

Twelve iron repressible peptides including the two HMWP's were found in periplasm samples (Table 1, Fig. 8). Several were among the most strongly expressed proteins in this compartment. Pirps G and J were only expressed at 26°C. A new Pgm^+ specific peptide was identified at 19 kDa (Pirp L). It was clearly visible at 26°C, however, at 37°C there was a slightly larger peptide expressed in enormous

Figure 8. Comparison of peptides present in a stained gel (12% SDS-PAGE) of purified periplasmic proteins of *Y. pestis* KIM grown at 37°C (lanes A-D) and 26°C (lanes E-H). Pgm^+ cells grown in iron replete medium (lanes A and E), Pgm^+ cells grown in iron deficient medium (lanes B and F), Pgm^+,Pst^r cells grown in iron deficient medium (lanes C and G), and Pgm^- cells grown in iron deficient medium (lanes D and H). Positions of molecular weight standards in kilodaltons are between the gels. Positions of detectable Pirps (Table 1) are indicated along the right edge. Open arrows indicate expression only at 26°C, and asterisks show peptides not expressed by Pgm^- cells.

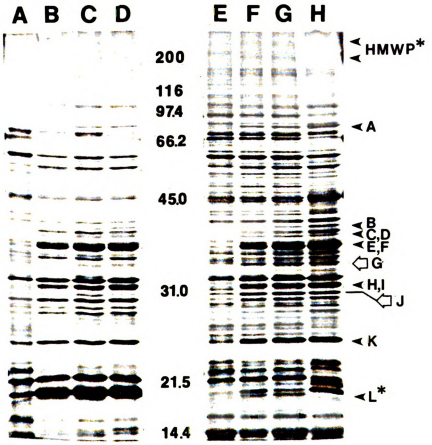

Figure 8

quantities, and it was usually detectable on the underside of this band. Although absent in Pgm^- cells, it was produced normally by the Pgm^+, Pst^r mutant.

Early attempts to isolate inner membranes were often unsuccessful due to contamination with outer membrane material. Straley and Brubaker (46) found contamination as high as 38% in their samples. By eliminating the washing of cells with phosphate buffer after harvest we reduced contamination as judged by SDS-PAGE profiles. While some contamination may be present, most identified iron regulated proteins appeared unique to these samples. No temperature dependent differences in any inner membrane proteins were observed so only the 26°C results are illustrated (Table 1, Fig. 9). They show ten irps including the HMWP's. With the exception of HMWP's none of these were Pgm^+ specific.

Cytoplasmic samples also showed no temperature or strain related differences in protein expression other than the HMWP's which were strongly expressed in this cellular compartment (Fig. 10). No other irps were visible in these samples.

Iron regulated expression of outer membrane proteins in the enteropathogenic yersiniae. Gradiant plate results indicated that Pst^r mutants of all three pathogenic species showed a similar deficiency in iron uptake in the presence of 2,2-dipyridyl. We examined outer membranes of Pst^s and Pst^r strains of Y. pseudotuberculosis PBl and Y.

Figure 9. Comparison of peptides present in a stained gel (12% SDS-PAGE) of purified cytoplasmic membranes of *Y. pestis* KIM grown at 26°C. Pgm^+ cells grown in iron replete medium (lanes A), Pgm^+ cells grown in iron deficient medium (lanes B), Pgm^+,Pst^r cells grown in iron deficient medium (lanes C), and Pgm^- cells grown in iron deficient medium (lanes D). Molecular weight standards are in the unlabelled lane and their sizes, in kilodaltons are along the left edge of the gel. Positions of detectable Mirps (Table 1) are indicated along the right edge of the gel. Asterisks show the location of proteins not expressed by Pgm^- cells.

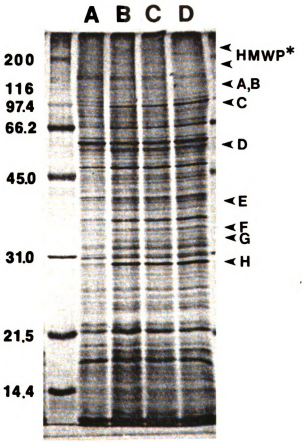

Figure 9

Figure 10. Comparison of peptides present in a stained gel (12% SDS-PAGE) of purified cytoplasmic proteins of Y. pestis KIM grown at 37°C (lanes A-D) and 26°C (lanes E-H). Pgm^+ cells grown in iron replete medium (lanes A and E), Pgm^+ cells grown in iron deficient medium (lanes B and F), Pgm^+,Pst^r cells grown in iron deficient medium (lanes C and G), and Pgm^- cells grown in iron deficient medium (lanes D and H). Molecular weight standards are in the unlabelled middle lane and their sizes, in kilodaltons are along the left edge of the gel. Positions of detectable HMWPs (Table 1) are indicated along the right edge.

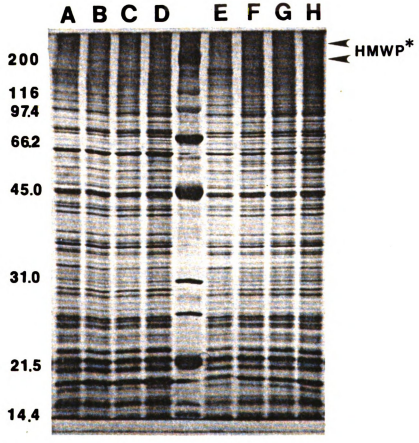

Figure 10

enterocolitica WA and compared them to those of Y. pestis KIM to search for similar changes in protein expression. We also attempted to discover if the superior ability of enteropathogenic strains to grow in the presence of ferric chelators is reflected in the expression of protein constituents of the outer membrane.

Outer membrane profiles from Y. pestis KIM and Y. pseudotuberculosis PB1 were nearly indistinguishable (Fig. 11, 12). The latter expressed peptides assumed to be homologous with Irps B-D, G, and the M group. Irp A was present and expressed at much higher levels than in Y. pestis (Fig. 12,14), but Irp F was absent (Fig. 14). Y. enterocolitica was more unique. Although it expressed peptides apparently homologous to Irps B-D, the peptide which could be Irp A had a slightly lower isoelectric point (Fig. 13). No peptides corresponding to Irp G or the M group were detectable, and unique Irps of 85 kDa and 67 kDa were expressed (Fig. 13,14). Carniel et al (8) described Irps of 240, 190, 89, 81, 79, 70, 68, and 27.5 kDa in outer membranes of Y. enterocolitica serovar O:8. We identified peptides which probably corresponded to all of these except those at 89 and 27.5 kDa. Differences in cultivation (they harvested cells well into the stationary phase), sample preparation, or strain of cells being analyzed could be responsible.

No definite differences were observed between Pst^S and Pst^r strains of enteropathogenic species (Fig. 12,13, 14).

Figure 11. Autoradiograms of two dimensional gels of purified outer membranes from Y. pestis KIM grown at 37°C. (A) Pgm^+ cells grown in iron replete medium, (B) Pgm^+ cells grown in iron deficient medium, (C) Pgm^- cells grown in iron deficient medium. Horizontal dimension is IEF (pH) and vertical dimension is SDS-PAGE (molecular weight in kilodaltons). Letters refer to Irps (Table 1).

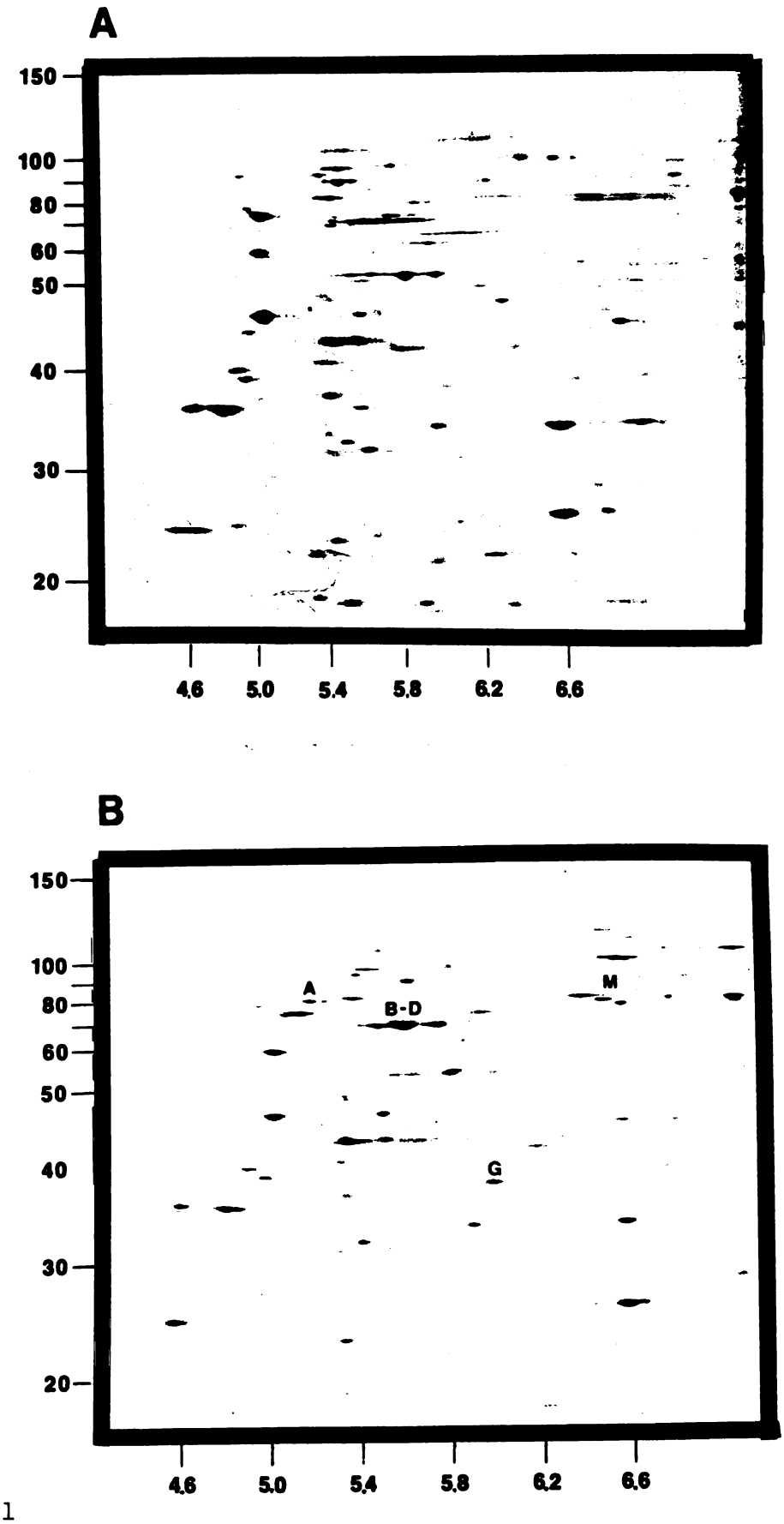
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Figure 11

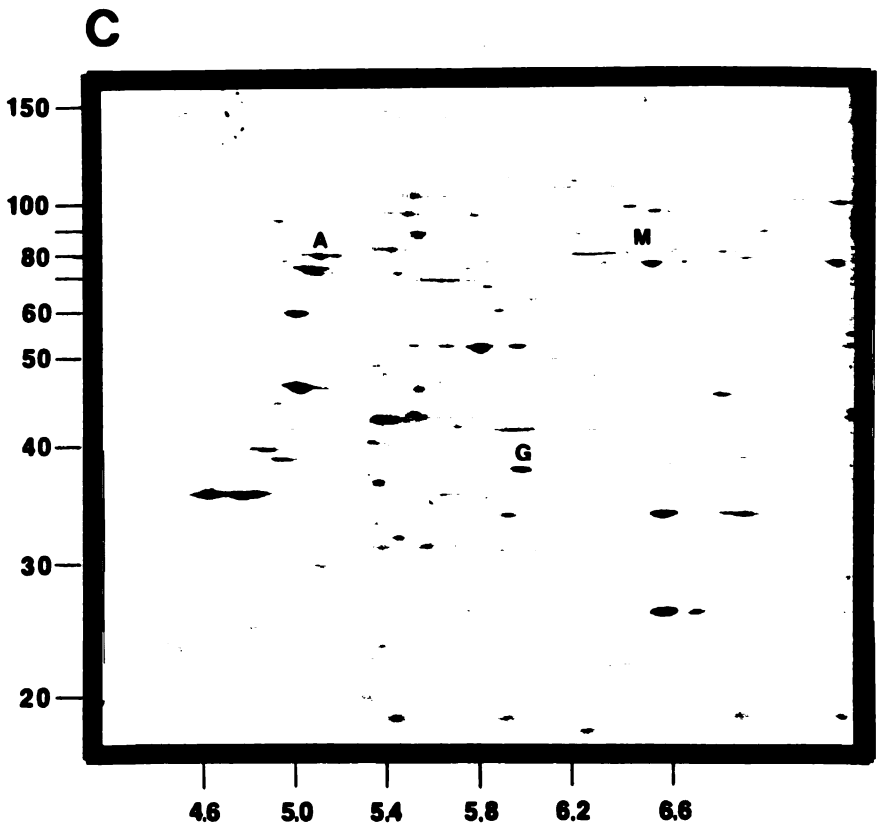

Figure 11

Figure 12. Autoradiograms of two dimensional gels of purified outer membranes from Y. psuedotuberculosis grown at 37°C. (A) Pst^s cells grown in iron replete medium, (B) Pst^s cells grown in iron deficient medium, (C) Pst^r cells grown in iron deficient medium. Horizontal dimension is IEF (pH) and vertical dimension is SDS-PAGE (molecular weight in kilodaltons). Letters refer to Irps (Table 1).

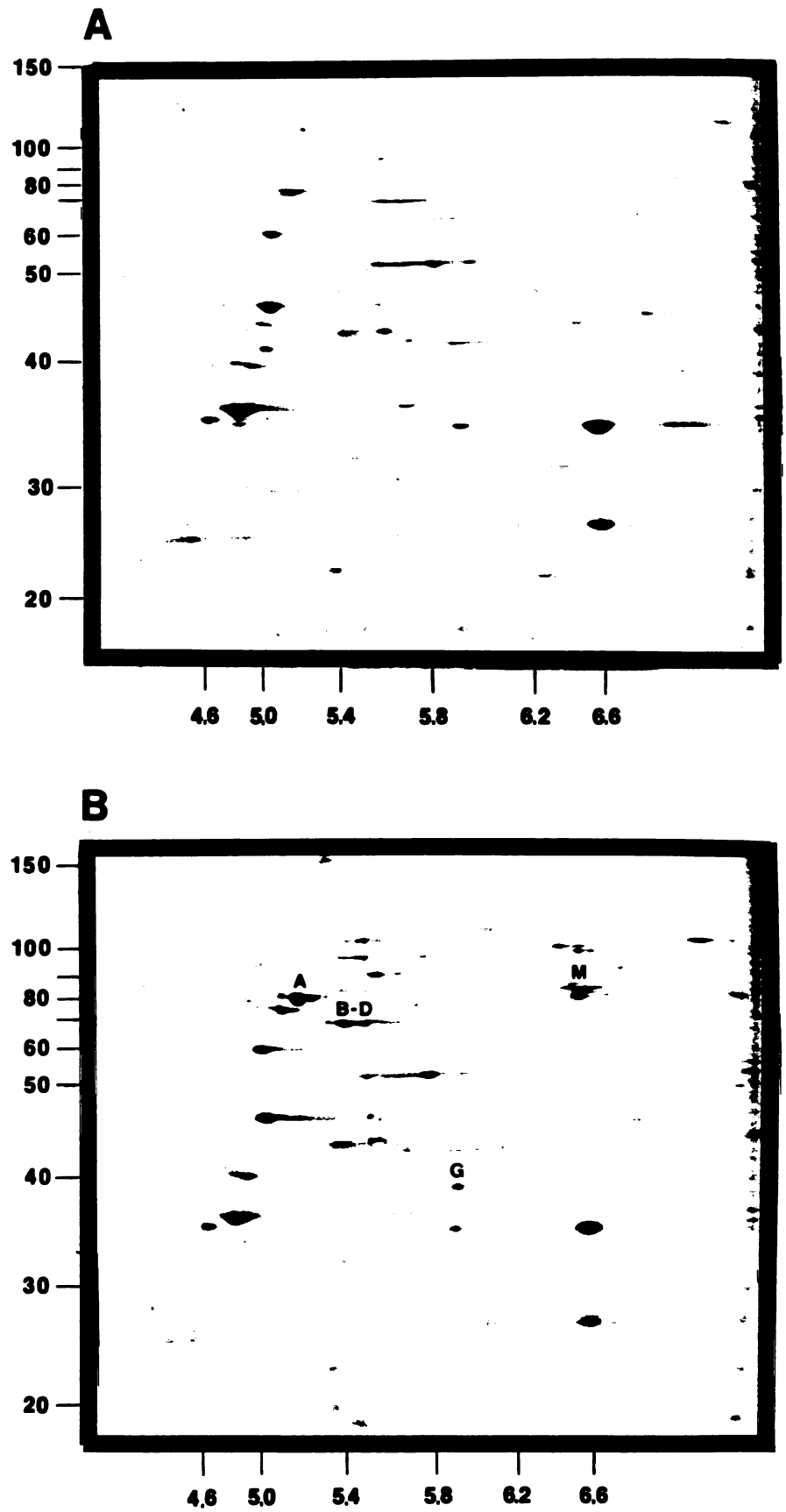
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Figure 12

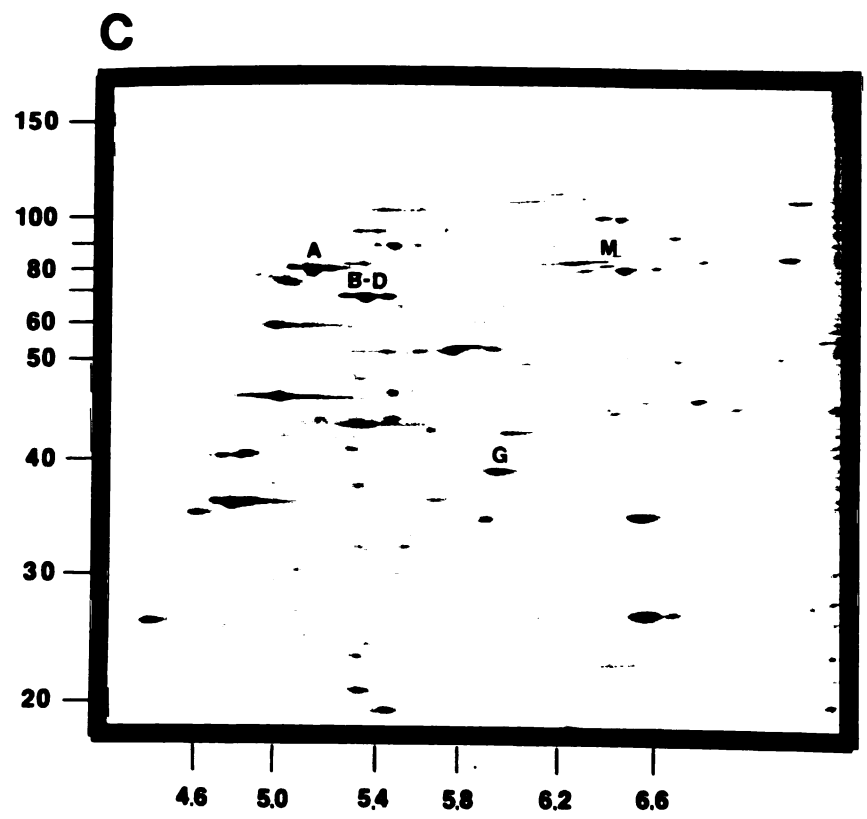

Figure 12

Figure 13. Autoradiograms of two dimensional gels of purified outer membranes from Y. enterocolitica WA grown at 37°C. (A) Pst^S cells grown in iron replete medium, (B) Pst^S cells grown in iron deficient medium, (C) Pst^r cells grown in iron deficient medium. Horizontal dimension is IEF (pH) and vertical dimension is SDS-PAGE (molecular weight in kilodaltons). Letters refer to Irps (Table 1) which may be homologous to those in other pathogenic Yersinia and arrowheads indicate Irps unique to this species.

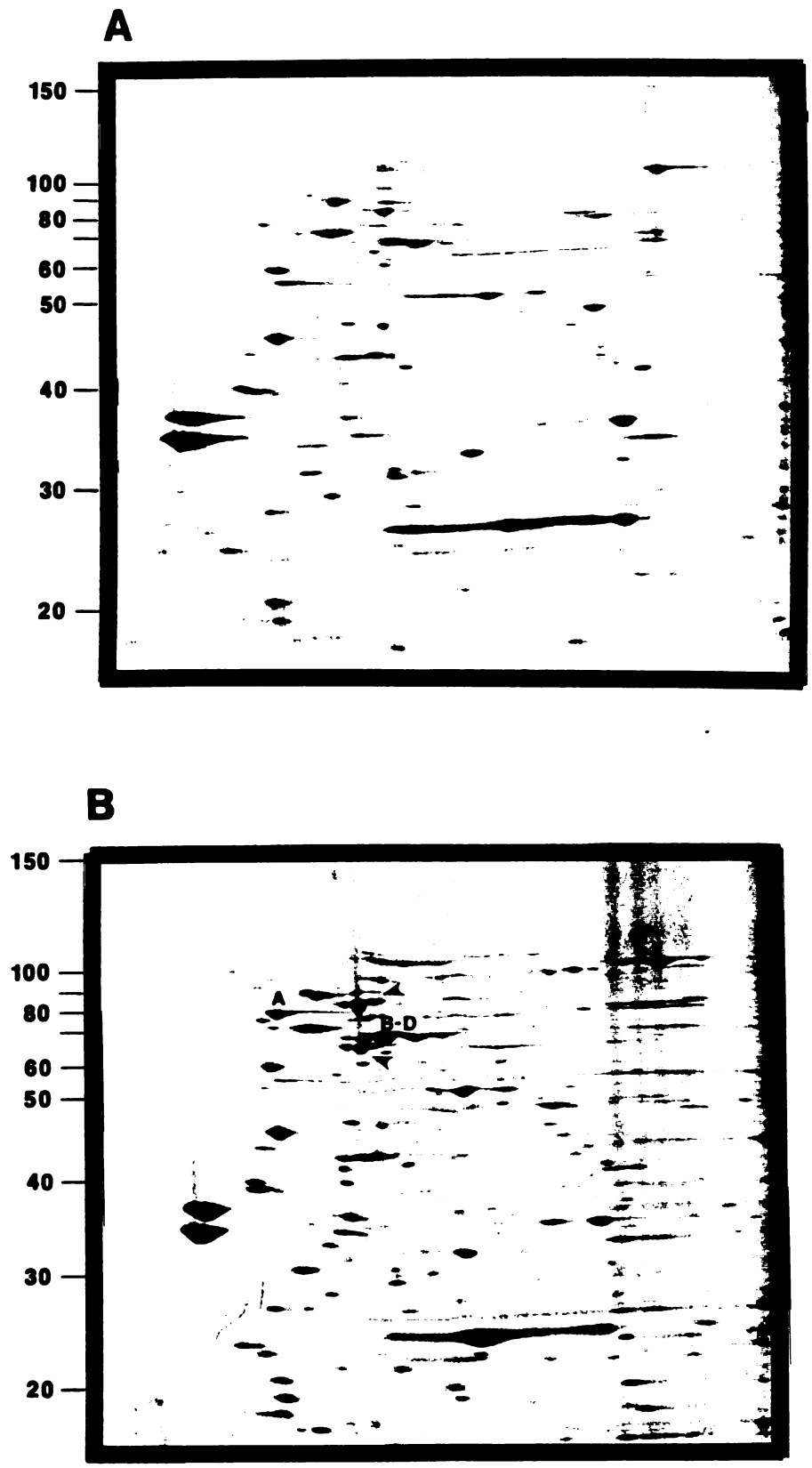

Figure 13

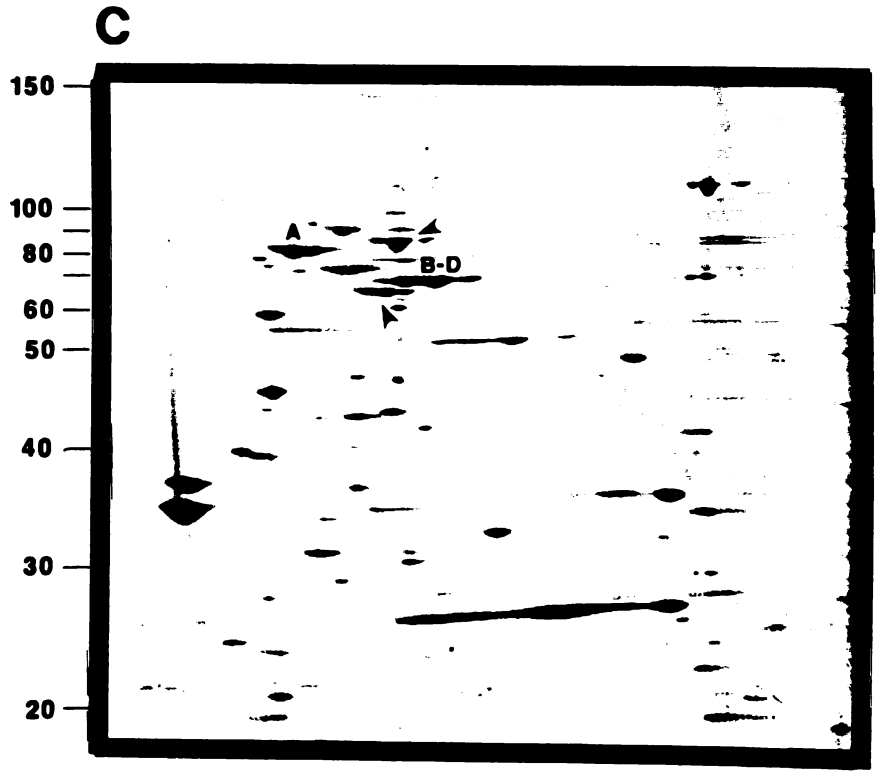

Figure 13

Figure 14. Autoradiogram of SDS-PAGE gel of purified outer membranes from Y. pestis KIM (lanes A-C), Y. pseudotuberculosis PB1 (lanes D-F), and Y. enterocolitica WA (lanes G-I). Pgm^+ (lane A) or Pst^S (lanes D and G) cells grown in iron replete medium. Pgm^+ (lane B) or Pst^S (lanes E and H) cells grown in iron deficient medium. Pgm^- (lane C) or Pst^r (lanes F and I) cells grown in iron deficient medium. All cells were grown at 37^oC. Position of molucular weight markers (kilodaltons) are indicated on the left. Labelled arrowheads indicate the positions of Irps identified in Y. pestis (Table 1). Unlabelled arrowheads show the position of detectable Irps unique to Y. enterocolitica.

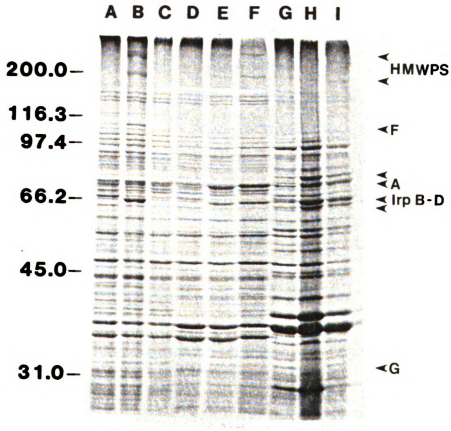

Figure 14

Since loss of Irps B-D has been linked to Pst^r and the associated lesion in iron uptake in Y. pestis the presence of these peptides in Pst^r enteropathogenic strains was unexpected. On two-dimensional, but not lane gels, enteropathogenic species exhibited a pair of 100 kDa peptides with isoelectric points of 6.4 - 6.6 in iron deficient medium. They are not visible in gels of Pst^r Y. enterocolitica. Since this change is not observed in the other species and it involves minor peptides its significance is unknown. Apparently the most likely mutational event causing loss of pesticin sensitivity in enteropathogenic species is not analogous to that in Y. pestis although the simultaneous lesion in iron uptake does occur.

DISCUSSION

Sikkema and Brubaker (43) demonstrated that Pgm^+, but not Pgm^- or Pgm^+, Pst^r Y. pestis were able to grow in iron deficient medium containing the ferric chelator citrate at 37°C. This could result from impaired ability of mutants to obtain iron through a lesion in a transport system, or a metabolic change could cause a greater iron requirement in Pgm^- cells which cannot be met under these conditions. Although we cannot discount the latter possibility, labelled iron uptake results are consistant with a lesion in iron transport. Observable changes in protein expression correlated with the mutation to nonpigmentation or pesticin resistance involve only envelope associated proteins which would be expected in a mutation affecting transport.

Although these studies failed to clarify the nature of the Pgm^+-specific transport system, similar results for citrate, pyrophosphate, EDDA, and NTA suggest it does not target a specific chelator. Why the difference in growth was greatest with EDDA is unclear, but may reflect some degree of specificity by this system for iron chelated in a particular manner, or it may relate to differences in the abilities of chelators to bind Fe^{3+} under conditions present in the agar. Metabolic activity of the cells could decrease the pH and oxygen levels in the agar and allow reduction of

some Fe^{3+} to Fe^{2+} as would the inclusion of low levels of dithiothreitol. Ferrous iron would be less tightly bound by most ferric chelators. However, very high affinity ferric chelators (desferrioxamine mesylate and conalbumin) made iron unavailable to Y. pestis at 37°C.

We clearly demonstrated the correlation between pesticin sensitivity and a system for iron acquisition in pathogenic Yersinia. Pgm^+, Pst^r showed growth essentially identical to that of Pgm^- Y. pestis on gradient plates indicating the functional separation of hemin binding and iron uptake/pesticin sensitivity described in other studies (25,39,44). In previous experiments enteropathogenic species of Yersinia failed to exhibit evidence for the association of pesticin sensitivity and ability to acquire iron which was demonstrated by Yersinia pestis (43,44). Use of citrate to chelate iron in these studies obscured the lesion since citrate actually enhances growth of enteropathogenic Yersinia in iron deficient medium. By using ferrous chelators we demonstrated a similar system associated with pesticin sensitivity in all three species.

The effectiveness with which ferrous chelators retard growth in iron deficient media suggests a possible role for ferrous iron uptake. Reduction of ferric to ferrous iron is required in several siderophore and non-siderophore based acquisition systems for release of iron from the chelator and transport across the cytoplasmic membrane (14,17). Non-specific reductase systems are believed to play a role in

iron acquisition by several pathogenic bacteria (12,14,23). Growth and iron uptake by cells utilizing these mechanisms are effectively blocked by ferrous chelators such as ferrozine and 2,2 dipyridyl (14,27). Pst^S cells may express a ferrous uptake system which can better compete with these chelators for iron than can Pgm^- (Pst^r) cells. It is also possible that the inclusion of these chelators reduces ferric iron concentrations to a point where Pst^S cells would have a significant advantage in obtaining iron due to the presence of an additional non-specific, high affinity ferric transport system. Additional studies of growth and uptake, and tests of Pst^S and Pst^r strains for ferric iron reductase activity should help clarify this.

Sikkema and Brubaker (44) speculated on a role for one or more of Irps B-E of Y. pestis in the Pst^S associated iron uptake system since their loss correlated with Pst^r and inability to grow in iron deficient media containing citrate. This is supported by our finding that iron regulated peptides corresponding to Irps B-D were expressed by enteropathogenic Yersinia. However, it was surprising that no differences in outer membrane protein expression were evident following mutation to pesticin resistance. Since these species do not experience the high frequency deletion event seen in Y. pestis loss of pesticin sensitivity may most commonly result from mutations only affecting as yet unidentified periplasmic or cytoplasmic membrane proteins needed for internalization of iron and/or

pesticin. Since these cells still readily acquire iron from most available sources , it is unlikely to be analagous to tonB mutations which result in tolerance of Pst^S strains of E. coli (16). There might also be mutational "hotspots" causing loss of function of critical peptides without concomitant loss of expression. Isolation and study of additional Pst^r mutants is ongoing to resolve this.

Unrestricted growth of enteropathogenic species in the presence of Fe^{3+} chelators at 37^oC (exception: conalbumin) suggest the operation of uptake systems not found in Y. pestis. Expression of several unique iron regulated outer membrane proteins by Y. enterocolitica may reflect this, however, little difference was apparent between Y. pseudotuberculosis and Y. pestis. Greater Irp A expression in enteropathogenic species may be involved, although there is no evidence as to its function. Both enteropathogenic species can obtain iron from ferrienterobactin and ferrioxamine (3,36), and specific receptors of 81.0 kDa (Fep A) (41) and 13.7 kDa (1) respectively have been identified in the outer membrane of Y. enterocolitica. The latter is to small to detect in our gels, but the former is similar in size to Irp A. However, since Y. pestis is unable to utilize this siderophore and it expresses Irp A in reduced, but significant amounts, it is unlikely to be the Fep A homolog.

The dramatic effect of temperature on growth of Y. pestis in the presence of various chelators is

unprecedented. Earlier studies demonstrated reduced expression of systems for iron acquisition at higher temperature in E. coli and Salmonella typhimurium, however, these effects occurred primarily at febrile rather than normal host temperatures (19,24,54). Whether this reflects temperature regulation of expression or inability of the involved proteins to function at 37°C is unresolved, however, the observation that the temperature at which cells are grown in iron deficient medium rather than the temperature at which the test is performed has a greater influence on kinetics of labelled iron uptake and the presence of outer membrane (Irps H and I) and periplasmic (Pirp G and J) iron regulated proteins expressed only at 26°C favors the former hypothesis. If this is the case, then it may be possible to obtain mutants which constitutively express 26° systems at 37°C. Attempts to isolate mutants capable of growth in the presence of ferrioxamine at 37°C have so far been unsuccessful and leave the issue unresolved.

Proteins comprising a particular system for iron acquisition are often coordinately regulated and encoded on a single operon (13,35). Thus identification of other iron repressible proteins associated with the Pgm phenotype might help define the components of the Pst^S linked uptake system. We confirmed the suspected relationship between HMWP's and pigmentation, but could not characterize them as essentially outer membrane in location as previously described (6).

Their large size and significant expression in all compartments suggests they span the envelope, extending from the cytoplasm to the outer membrane. Since iodination studies indicate they are not exposed on the surface of the cell they may only be associated with the inner leaflet of the outer membrane (6, Perry, pers. comm.). The reason for our discrepancy with Carnial et al (6) probably relates to differences in fractionation procedure. Their use of Triton X-100 to dissolve cytoplasmic membranes would destroy any association with this structure leaving only the attachment to and apparent preferential expression in the outer membrane. The newly identified 113 kDa outer membrane protein (Irp F) and 19.0 kDa periplasmic protein (Pirp L) were the only additional Pgm-specific iron repressible peptides identified. While the absence of Irp F in the Pgm^+, Pst^r mutant suggests possible close linkage with genes for Irps BE in Y. pestis, its absence in enteropathogenic species argues against it having a critical role in pesticin sensitivity or its associated iron uptake system. This system could utilize Irps B-D and possibly E as the outer membrane receptor for initial binding of iron or pesticin. If so this would be the first identified non-specific receptor for iron acquisition. Pirp L and HMWP's could facilitate transport across the periplasm and cytoplasmic membrane. This hypothesis is highly speculative since there is abundant room in the 100 kb Pgm^+-specific DNA segment for these genes to be separately encoded, and serve different

functions while being coordinately regulated. Genetic studies are underway to address this.

Iron is known to serve as a regulatory signal influencing the expression of a variety of proteins. In pathogens the low iron levels encountered in the host would cause expression of genes necessary for survival and pathogenicity (35). In Y. pestis some of the numerous iron-regulated envelope proteins are probably involved in Pgm-independent systems for iron acquisition such as utilization of iron from ferritin or hemin. Others may serve additional functions. Transcription of virulence factors including certain toxins (2,10) and hemolysins (49) are iron regulated in certain bacteria. However, none of these Pgm-independent iron regulated proteins correspond to known virulnce factors in Yersinia. Since expression of many of these peptides is subject to Fur regulation (53) it should be possible to identify their genes and study them in more detail.

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