Biol Metals (1989) 2:174-184

BIOLOGY OF METALS

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Outer membrane peptides of *Yersinia pestis* mediating siderophore-independent assimilation of iron

Daniel J. Sikkema and Robert R. Brubaker

Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824, USA

Summary. It is established that wild-type cells of *Yersinia pestis* absorb exogenous hemin or Congo red and thus grow as pigmented colonies at 26° C on media containing these chromatophores (Pgm^+). Pgm^+ isolates are known to possess a siderophore-independent mechanism of iron-transport (required for growth in iron-deficient medium) which is absent in avirulent Pgm^- mutants. Production of the bacteriocin pesticin and linked invasins (Pst^+) is an additional defined virulence factor of yersiniae; mutation of Pgm^+,Pst^- organisms to pesticin-resistance (Pst^r) results in concomitant conversion to Pgm^-. In this study, autoradiograms of two-dimensional gels of [^{35}S]methionine-labeled outer membranes from Pgm^- mutants were compared to those of the Pgm^+,Pst^+ or Pgm^+,Pst^- parent. An apparently single predominant peptide present in these preparations (> 10% of total membrane protein) existed as a family of iron-modifiable 17.9-kDa molecules focusing down to isoelectric points of about 4.6 and up to 5.89. Expression of eight detectable Pst^+-specific peptides was not significantly influenced by exogenous iron. Pgm^+ yersiniae constitutively produced pigmentation-specific peptide F and five iron-repressible peptides termed IrpA to IrpE. Typical spontaneous mutation to Pgm^- resulted in loss of peptide F and IrpB-E. A rare Pgm^+,Pst^r mutant, selected on Congo red agar containing pesticin, also lost IrpB-E but retained peptide F. This isolate, like Pgm^- mutants, failed to grow in iron-deficient medium. Regardless of phenotype, all yersiniae utilized hemin, hemopexin, myoglobin, hemoglobin, and ferritin, but not transferrin or lactoferrin, as sole sources of iron.

Key words: Iron storage — Iron-repressible peptides — *Yersinia pestis*

Introduction

Wild-type cells of *Yersinia pestis*, the causative agent of bubonic plague, are highly infectious in mice ($LD_{50} < 10$) by both intravenous and peripheral routes of injection (Burrows 1963; Brubaker 1972). The ability of these organisms to grow as pigmented colonies at 26° C but not 37° C on solid medium by absorbing certain exogenous chromatophores (e.g. hemin, crystal violet, and Congo red) serves as an important determinant of virulence (Pgm^+). Pgm^- mutants form colorless colonies on such media (Jackson and Burrows 1956a; Surgalla and Beesley 1969), are avirulent by peripheral routes of injection ($LD^{50} > 10^7$; Jackson and Burrows 1956b) but not via intravenous injection ($LD_{50} \sim 10$; Une and Brubaker 1984), and incapable of sustained growth at 37° C in iron-deficient medium (Sikkema and Brubaker 1987). An additional virulence factor is ability to produce the bacteriocin pesticin (Ben-Gurion and Hertman 1958; Hu et al. 1972; Hu and Brubaker 1974) and linked plasminogen/prothrombin activator (PAC), an invasin accounting for the ability of wild-type yersiniae to disseminate from peripheral sites of infection (Pst^+) (Brubaker et al. 1965). Mutation to Pst^- typically results in reduced invasiveness reflecting loss of the 9.5-kb Pst plasmid encoding maintenance functions, pesticin, its immunity protein, and PAC (Ferber et al.

Offprint requests to: R. R. Brubaker, Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824, USA

This is journal article no. 13025 of the Michigan Agricultural Experiment Station.

1981; Ben-Gurion and Shafferman 1981; Sodeinde and Goguen 1988). Such Pst$^-$ mutants were sensitive to pesticin (Hertman and Ben-Gurion 1959) provided that they were Pgm$^+$; pesticin-resistant (Pst^r) mutants of the latter arose at the high rate of 10^{-5} and were Pgm$^-$ (Brubaker 1970).

Mammalian extracellular fluids and many natural environments are typically deficient in available iron (Weinberg 1974). Procaryotes indigenous to these niches often excrete small molecules, termed siderophores, that serve to sequester and return the cation from otherwise unusable sources (Neilands 1972; Lankford 1973). It is established that synthesis of siderophores and their outer membrane receptors is induced in iron-deficient media (Neilands 1972). Contrary to prior findings (Wake et al. 1975), Perry and Brubaker (1979) failed to detect production of typical siderophores by Pgm$^+$ or Pgm$^-$ isolates of yersiniae grown in this environment. Nevertheless, iron-deficient medium promoted induction of iron-stress peptides in *Y. pestis* (Carniel et al. 1987; Carniel et al. 1989a; Carniel et al. 1989b). The function of these structures is unknown; they may mediate a previously described cell-bound saturable high-affinity inducible process of iron transport (Perry and Brubaker 1979).

Outer membrane iron-stress siderophore receptors of enteric bacteria can promote binding of certain bacteriocins, especially group B colicins. Mutations conferring resistance to these colicins thus cause loss of their corrsponding receptors which may be necessary for growth in iron-deficient medium (Davies and Reeves 1975). This event is distinct from mutations to tolerance such as *tonB*, an inner membrane function required for absorption of colicins to outer membrane iron-stress peptides and for occurrence of all high-affinity processes of iron-transport (Davies and Reeves 1975; Wookey 1982). The pesticin receptor has not been identified and the nature of the high-frequency pleiotropic mutation to Pgm$^-$ in *Y. pestis* is unresolved. Pesticin may, however, resemble group B colicins in that expression of sensitivity is repressed by iron (Brubaker and Surgalla 1961). Furthermore, *tonB* mutants of a pesticin-sensitive strain of *Escherichia coli* were also tolerant to pesticin (Ferber et al. 1981). The major purpose of this report is to provide a definitive two-dimensional map of outer membrane peptides of *Y. pestis*. Information obtained from this map demonstrated that mutation to Pgm$^-$ resulted in loss of four out of five major peptides found to be regulated by iron and a distinct Pgm$^+$-specific structure. Production of at least one of the missing iron-stress peptides was associated with expression of prolonged growth in iron-deficient medium but not required for utilization of various sources of organic iron.

Materials and methods

Bacteria. Y. pestis KIM (variety *mediaevalis;* Devignet 1951) described previously (Brubaker 1970) was used in all experiments. This Pgm$^+$,Pst$^+$ isolate and its isogenic Pgm$^-$,Pst$^-$, and Pst^r mutants were avirulent due to absence of the 70-kb Lcr plasmid known to mediate the low calcium response (Goguen et al. 1984). Pgm$^-$ mutants were isolated on hemin agar (Jackson and Burrows 1956a) and Pst$^-$ mutants were recovered after prolonged cultivation at 5° C, a temperature that favors loss of the Pst plasmid (Sample et al. 1987). Pst^r mutants that retained the Pgm$^+$ phenotype were obtained by selecting rare colored colonies of surviving Pgm$^+$,Pst$^-$ cells plated on Congo red agar (Surgalla and Beesley 1969) containing pesticin (10^5 units/ml) purified by the method of Hu and Brubaker (1974). Petri dishes were incubated overnight at 37° C to favor killing and then at 26° C for 2 days where expression of the pigmentation reaction by survivors is optimal.

Cultivation. Yersiniae were grown in the synthetic medium of Higuchi et al. (1959) as modified by Zahorchak and Brubaker (1982). $FeSO_4$ was omitted if the medium was intended for use in determination of iron-stress functions. In this case, further extraction was performed with 8-hydroxyquinoline (Waring and Werkman 1942) leaving a level of <0.3 μM iron as determined by flame absorption spectroscopy. Yersiniae were retrieved from stock cultures, inoculated, and subcultured at 26° C as previously described (Sikkema and Brubaker 1987). Medium used for growth of organisms to be fractionated into outer membranes contained 0.25 mM [^{35}S]methionine (20 μCi/ml) during all transfers; cells of the final transfer were incubated at 37° C and harvested during the late logarithmic growth phase. Optical density of cultures was determined at 620 nm.

Preparation of outer membranes. Established procedures for fractionation of enteric bacteria (Osborn et al. 1972) were modified for use with yersiniae as previously described in detail (Straley and Brubaker 1981). The method involved conversion of organisms to spheroplasts with lysozyme and EDTA followed by disruption via sonication. Particulate material was collected by sedimentation and then separated into inner and outer membranes by isopycnic sucrose-gradient centrifugation. Contamination of outer membrane preparations by inner membranes after use of this method is <3% (Straley and Brubaker 1981).

High-resolution two-dimensional gel electrophoresis. Standardized high-resolution two-dimensional gels were run and computer-analyzed using the PDQUEST™ system by Protein Databases, Inc. (Huntington Station, NY). To determine if samples were suitable for high-resolution analysis, electropherograms were first prepared by the method of O'Farrell (1975); results of this process as used with outer membranes of *Y. pestis* grown with excess iron have been reported (Straley and Brubaker 1981; Straley and Brubaker 1982). If found appropriate for commercial analysis, samples were prepared after the methods of Garrels (1979; 1983) as described by Blose (1986). Briefly, proteins were harvested in sample buffer

(~0.3% SDS, 5% 2-mercaptoethanol, Tris pH 8.0) and placed in a boiling water bath for 2 min. Samples were then cooled on ice, treated with DNase and RNase to reduce viscosity, snap-frozen in liquid nitrogen, packed on solid CO_2, and shipped to Protein Databases, Inc. for further processing. Samples were then counted for radioactivity and assayed for protein, volumes were noted and, after lyophilization, portions were dissolved in isoelectric focusing buffer (9.5 M urea, 2% NP-40, 100 mM dithiothreitol, and 2% basic ampholines) at 37° C for 30 min.

Two-dimensional electrophoresis was performed after the method of Garrels (1979; 1983) and, to achieve reproducible standardized high-resolution gels, all procedures and operations including sample counting, electrophoresis, exposure calculation, staining, and sample inventory were controlled and monitored by computer. Approximately 10–20 µl of sample containing 250000–400000 dpm and 20–30 µg protein were loaded onto a narrow-bore isoelectric focusing tube (0.8 mm diameter; ≈20 cm long). The latter contained 2.9% acrylamide, 2% NP-40, 9.5 M urea, and 2% pH 5–7 ampholines. Peptides were focused at 19 kV/h overnight. Second-dimension SDS-gel chromatography was carried out after the methods of Garrels (1979; 1983). The SDS-equilibrated isoelectric focusing gel was mounted on the second-dimension gel having the geometry of 24 cm × 24 cm × 1 mm (thick) and an acrylamide concentration of 10%. The second dimension was electrophoresed at a constant 60 W. Resulting gels, with their corresponding radioactive calibration strips containing known amounts of radioactive protein (Garrels 1979; 1983; Garrels et al. 1984), were then processed for fluorography and multiple autoradiographic exposures of each gel were made after the method of Garrels (1979; 1983) and Garrels et al. (1984). Gel films were scanned with an Eikonix 78/99 camera system (photodiode array camera) at a resolution of about 200 µm (1120 × 1120 pixels). They were then processed using the PDQUEST™ computer analysis programs based on the initial design and appropriate algorithms of the program of Garrels et al. (1984). After the films were scanned, merged, and spot detected, the experiment was assembled into a standard reference image and an individual sample image. For analysis, the majority of well resolved spots of individual gels were matched to the standard. For statistical analysis the correlation coefficient value (r), used to quantify the relatedness of two compared samples in scatter plots, was calculated according to Sokal and Rohlf (1969). The Mann-Whitney rank-sum (U-test), a non-parametric rank-sum test for two independent samples, was based on the ranking of the data and was calculated using the Wilcoxon method described by Sokal and Rohlf (1969) and Rohlf and Sokal (1969). The t-test, a parametric test, based on the differences between sample means assuming normal distribution of sample populations, was calculated by the methods of Spurr and Bonini (1973).

Miscellaneous. Protein in samples used for electrophoresis was determined by the method of Lowry et al. (1951). Pesticin was assayed against cells of *Yersinia pseudotuberculosis* PB1 on solid medium containing EDTA in excess Ca^{2+} (Brubaker and Surgalla 1962).

Results

Pgm^+, Pst^+ yersiniae were grown to constant specific activity with [^{35}S]methionine in either iron-deficient medium (<0.3 µM) or medium supplemented with sufficient $FeCl_3$ (100 µM) to

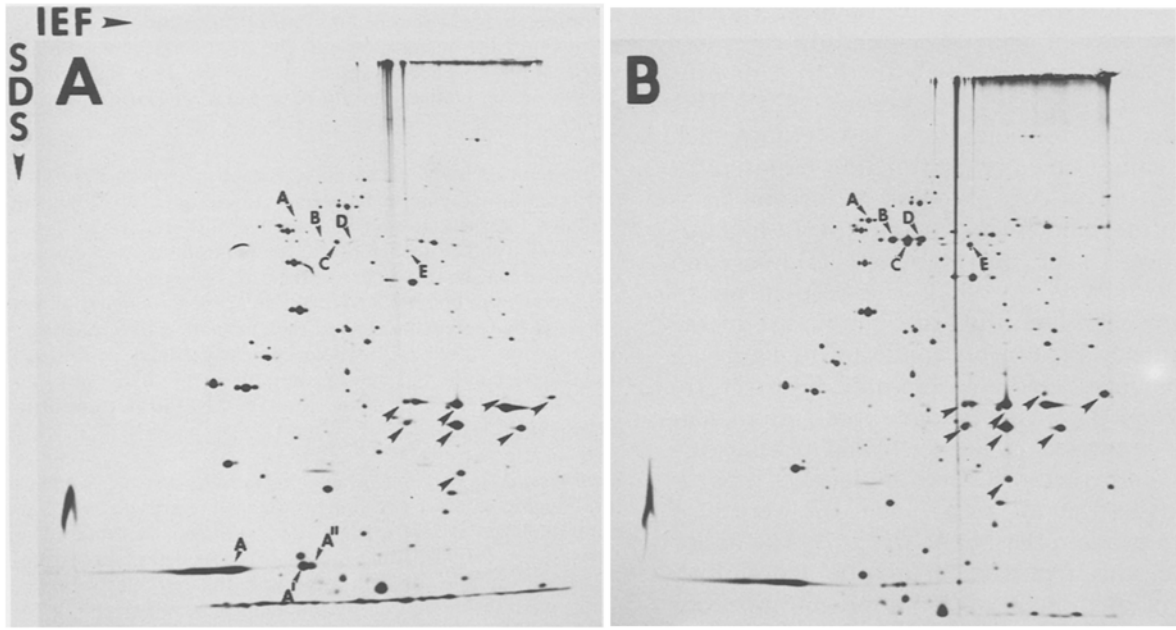

Fig. 1. Autoradiograms of two-dimensional gels of purified outer membranes of Pgm^+,Pst^+ cells of *Yersinia pestis* KIM grown at 37° C in **(A)** iron-enriched (100 µM Fe^{3+}) and **(B)** iron-deficient (<0.3 µM Fe^{3+}) media. Location of iron-repressible peptides, pesticin plasmid-dependent peptides, and iron-modifiable peptides are shown by letters at top, large *arrowheads*, and *A*, *A'*, and *A''* at bottom, respectively. IEF refers to initial migration via isoelectric focusing (acidic peptides appear at the left and basic peptides migrate to the right) and SDS refers to second direction of migration by sizing in sodium dodecyl sulfate

repress iron-stress outer membrane peptides. The organisms were then used to prepare outer membranes; autoradiograms of two-dimensional gels of the latter are shown in Fig. 1.

Iron-modifiable peptides

A major iron-modifiable peptide of 17.9 kDa comprised over 10% of the total protein within the outer membrane of Pgm^+,Pst^+ yersiniae grown with added $FeCl_3$. This component (peptide A of Fig. 1A) was resolved as a broad streak containing overlapping isoelectric points of about ~4.6 and 4.67. Molecules of identical molecular mass possessing higher isoelectric points (peptides A′ and A″ of Fig. 1A) were also expressed during growth with exogenous Fe^{3+}. This family of small constant-molecular-mass peptides focused at isoelectric points up to 5.89 when the organisms were grown without exogenous Fe^{3+}; intermediate values were observed for Pgm^- bacteria (Fig. 2B, Table 1) known to possess a lesion in assimilation of iron. Isoelectric points and concentrations of these iron-modifiable peptides are shown in Table 1.

Iron-repressible peptides

Comparison of peptides composing outer membranes of Pgm^+,Pst^+ yersiniae grown in iron-supplemented (Fig. 1A) and iron-deficient (Fig. 1B) media revealed five major iron-repressible peptides termed IrpA–IrpE. Environmental and genetic factors influencing expression of these five iron-stress functions and their physical properties are shown in Table 2.

Pst^+-specific peptides

Outer membranes were similarly prepared from Pgm^+,Pst^- yersiniae cultivated in iron-deficient medium. Comparison of the autoradiogram from the two-dimensional gel of this sample (Fig. 2A) with those of the Pgm^+,Pst^+ parent grown with (Fig. 1A) and without exogenous iron (Fig. 1B) demonstrated the presence of eight major Pst^+-specific peptides. Concentration of these structures was not significantly influenced by their expression within a Pgm^+ or Pgm^- background or by the presence of exogenous iron (Table 3).

Pgm^+-specific peptides

An autoradiogram of a two-dimensional gel prepared from outer membranes of Pgm^-,Pst^- organisms grown in iron-deficient medium (Fig. 2B) was contrasted to that of the Pgm^+,Pst^- parent (Fig. 2A). The latter possessed the five IrpA–E already defined in Pgm^+,Pst^+ yersiniae (Fig. 1).

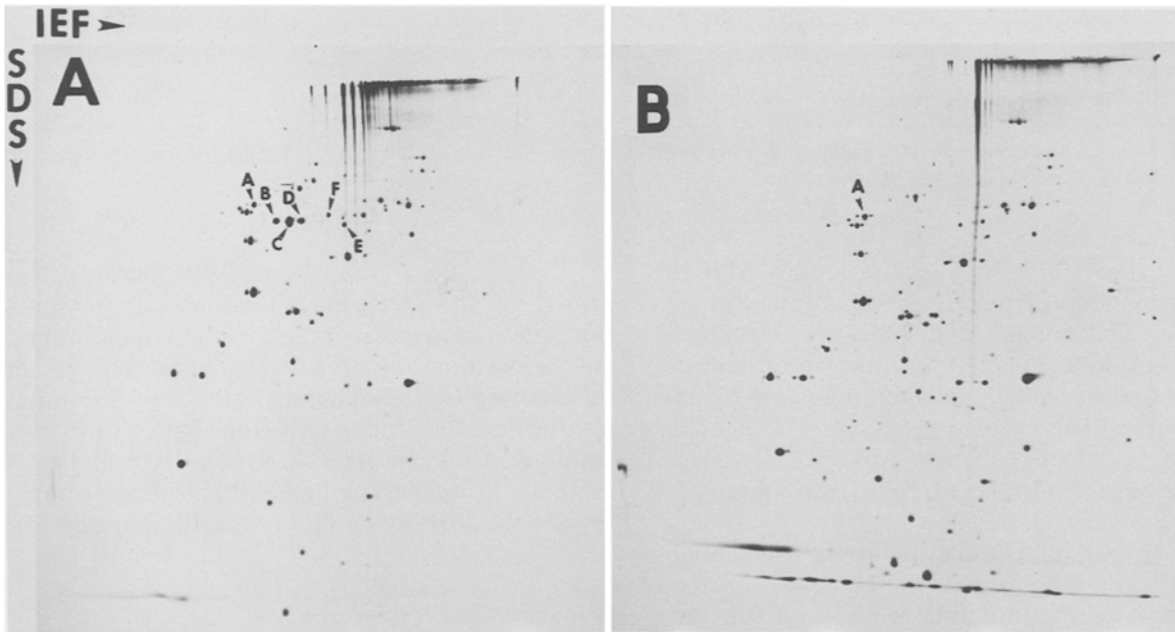

Fig. 2. Autoradiograms of two-dimensional gels of purified outer membranes of **(A)** Pgm^+,Pst^- and **(B)** Pgm^-,Pst^- cells of *Yersinia pestis* KIM grown at 37° C in iron-deficient medium (< 0.3 μM Fe^{3+}). Letters refer to iron-modifiable peptides *A, B, C, D,* and *E* and pigmentation-specific peptide *F.* IEF refers to initial migration via isoelectric focusing and SDS refers to second direction of migration by sizing in sodium dodecyl sulfate

Table 1. Distribution of 17.9-kDa iron-modifiable peptides in outer membranes of wild-type and mutant *Yersinia pestis* KIM grown at 37° C in iron-enriched and iron-deficient media

Isoelectric point	Growth with Fe^{3+}	Amount in phenotype (%)				
		Pgm^+,Pst^+	Pgm^-,Pst^+	Pgm^+,Pst^-	Pgm^-,Pst^-	Pgm^+,Pst^r
≈4.6 and 4.67	+	11.5	10.6	3.4	–	–
	0	5.8	2.3	2.5	8.2	5.3
4.96	+	0	1.6	0.30	–	–
	0	0	0.31	0.13	0.35	0
5.05	+	0	1.2	0	–	0
	0	0	1.0	0	0	0
5.21	+	1.4	0	0	–	–
	0	0	0	0	0	0
5.31	+	0	0.18	0	–	–
	0	0.18	0	0	0	0
5.41	+	0.09	0	0	–	–
	0	0.21	0	0	0	0
5.59	+	0.11	0	0	–	–
	0	0.12	0	0	0	0
5.71	+	0	0	1.3	–	–
	0	0	0	0	0	0
5.89	+	0	0	0	–	–
	0	0.36	0	0.24	0	0

Values refer to the percentage of total outer membrane protein, grown with added 100 μM $FeCl_3$ (+); or extracted medium containing <0.03 μM Fe^{3+} (0); – = not determined

However, the Pgm^-,Pst^- isolate lacked four of these components (IrpB–E) and also failed to express an additional Pgm^+-specific structure, termed peptide F (Fig. 2A). Production of peptide F, unlike that of IrpA–E, was not regulated by exogenous iron (Table 2).

Outer membrane peptides of Pgm^+,Pst^r mutants

Pgm^+,Pst^- yersiniae were incubated on Congo red agar containing added pesticin. As expected, the majority of Pst^r mutants selected on this medium arose as typical Pgm^- colonies. However, about 1 of 10000 such isolates retained the ability to absorb Congo red and were thus termed Pgm^+,Pst^r.

One of these rare Pgm^+,Pst^r mutants was grown in iron-deficient medium with [^{35}S]methionine and used to prepare outer membranes. An autoradiogram of a two-dimensional gel of this sample (Fig. 3) demonstrated that the mutation to Pgm^+,Pst^r, like that from Pgm^+ to Pgm^-, resulted in loss of IrpB–E. Unlike typical Pgm^- mutants, however, the Pgm^+,Pst^r isolate retained the ability to produce Pgm^+-specific peptide F.

Nutritional sources of iron

Ability of selected compounds to serve as sole sources of iron was determined by observing if they supported growth on conalbumin agar. As

Table 2. Properties and concentrations of iron-repressible peptides (IrpA–E) and pigmentation-specific peptide F in outer membranes of wild-type and mutant *Yersinia pestis* KIM grown at 37° C in iron-enriched and iron-deficient media

Peptide	Molecular mass (kDa)	Isoelectric point	Growth with Fe^{3+}	Amount in phenotype				
				Pgm^+,Pst^+	Pgm^-,Pst^+	Pgm^+,Pst^-	Pgm^-,Pst^-	Pgm^-,Pst^r
IrpA	80.1	5.21	+	0.04	0.09	0.03	–	–
			0	0.78	0.96	1.1	0.90	1.2
IrpB	68.8	5.37	+	0.05	0	0.07	–	–
			0	1.5	0	1.7	0	0
IrpC	67.4	5.48	+	0.03	0	0.60	–	–
			0	8.6	0	5.6	0	0
IrpD	69.1	5.59	+	0.07	0	0.10	–	–
			0	1.6	0	3.4	0	0
IrpE	65.1	5.98	+	0.05	0	0.08	–	–
			0	0.57	0	0.88	0	0
Peptide F	72.8	5.84	+	0.40	0	0.36	–	–
			0	0.40	0	0.71	0	0.88

Details as in Table 1

shown in Fig. 4, Pgm^+,Pst^+ organisms could utilize iron of hemin, hemopexin, ferritin, myoglobin, and hemoglobin. Growth did not occur in the presence of transferrin, lactoferrin, or cytochrome *c*. Similar results were obtained with conalbumin agar seeded with Pgm^-,Pst^- mutants or Pgm^+,Pst^r mutants (not illustrated).

Growth in iron-deficient medium

Yersiniae were cultivated in iron-deficient medium in order to compare the effects of mutation from wild type to Pgm^-,Pst^- and Pst^r on assimilation of the cation. Both Pgm^+,Pst^+ (Fig. 5A) and Pgm^+,Pst^- (Fig. 5B) organisms exhibited essentially full-scale growth in iron-deficient medium comparable to that in media supplemented with Fe^{3+} or hemin. In contrast, Pgm^-,Pst^+ (Fig. 5C) or Pgm^-,Pst^- (Fig. 5D) yersiniae were unable to maintain sustained growth without exogenous iron. The Pgm^+,Pst^r mutant (Fig. 5E) similarly failed to grow in iron-deficient medium. This result is consistent with the hypothesis that neither carriage of the Pst plasmid nor expression of Pgm^+-specific peptide F is required for high-affinity uptake of iron. The strong possibility exists, however, that assimilation of iron is dependent on at least one of the four IrpB–E peptides missing in Pgm^- mutants.

Discussion

It is difficult to reconcile the findings presented here with those of Carniel et al. (1987; 1989a; 1989b) who used a total membrane fraction of evident Pgm^- mutants of *Y. pestis* grown to stationary phase at 26° C. After single-dimensional electrophoresis, these workers observed two iron-stress peptides considerably larger than those reported here (i.e. 190 and 240 Da). Further study may demonstrate that these structures represent unprocessed precursors of the smaller Irp peptides described in this report. Attributes of the two-dimensional gel system used in this study were its sensitivity, reproducibility, and ability to define peptides at extremes of molecular mass and isoelectric point. For example, over 600 strutures present in the sample of outer membranes prepared for analysis were identified and quantified. The majority of these peptides were not regu-

Table 3. Properties and concentrations of pesticin plasmid-dependent peptides in outer membranes of wild-type and mutant *Yersinia pestis* KIM grown at 37° C in iron-enriched and iron-deficient media

Molecular mass (kDa)	Iso-electric point	Growth with Fe^{3+}	Amount in phenotype				
			Pgm^+,Pst^+	Pgm^-,Pst^+	Pgm^+,Pst^-	Pgm^-,Pst^-	Pgm^+,Pst^r
32.8[a]	6.29[a]	+	7.9	5.1	0	–	–
		0	10.9	4.4	0	0	0
30.3	6.30	+	9.8	10.1	0	–	–
		0	9.0	12.0	0	0	0
25.4	6.31	+	1.4	0.40	0	–	–
		0	0.80	0.66	0	0	0
32.8	5.93	+	0.43	1.1	0	–	–
		0	0.37	0.90	0	0	0
30.4	5.93	+	2.6	1.2	0	–	–
		0	2.7	3.0	0	0	0
34.1	>7.0	+	0.51	0.68	0	–	–
		0	1.2	0.43	0	0	0
34.0	6.62	+	0.08	0.17	0	–	–
		0	0.25	0.06	0	0	0

Details as in Table 1

[a] Plasminogen activator after initial processing during insertion in the outer membrane (Sodiende and Goguen 1988; Mehigh and Brubaker 1989)

lated or modified by iron and were present at low concentrations (<0.001% of total protein) consistent with possible contamination by inner membrane or cytoplasm. To avoid misinterpretation of results due to the presence of these minor components, only the first 50 predominant peptides were subjected to ranking and statistical analysis. This group contained five Irp peptides, eight Pst^+-specific peptides, a single Pgm^+-specific peptide, and a family of 17.9-kDa iron-modifiable components.

The latter, although present at high concentrations, were too small for resolution via the system of O'Farrell (1975) as performed previously (Straley and Brubaker 1982). Analogous structures have not yet been described in other microorganisms; thus they may comprise a new class of peptides involved in the assimilation or storage of iron. Their appearance at a fixed molecular mass but a widely divergent isoelectric points is consistent with post-translational modification of a single peptide rather than expression of distinct gene products, although the latter possibility has not yet been eliminated. Further study will be required to determine the nature of this divergence and why growth with excess Fe^{3+} (100 μM) or expression in a Pgm^+ genetic background (required for growth in iron-deficient medium; Sikkema and Brubaker 1987) favors focusing at acidic isoelectric points. Although bound iron *per se* might promote discrete reductions of the isoelectric point, the electrophoretic process used in this study would be expected to cause dissociation of the cation. Accordingly, these outer membrane iron-modifiable peptides may be distinct from procaryote ferritin-like proteins which are cytoplasmic and lose bound Fe^{3+} upon denaturation. Nevertheless, the subunit mass of these proteins is similar to that of the iron-modifiable components described here (Theil 1987). The possibility that these peptides represent an outer membrane iron-storage function similar to that of cytoplasmic ferritin is presently under study.

Pgm^+ organisms were found to produce five

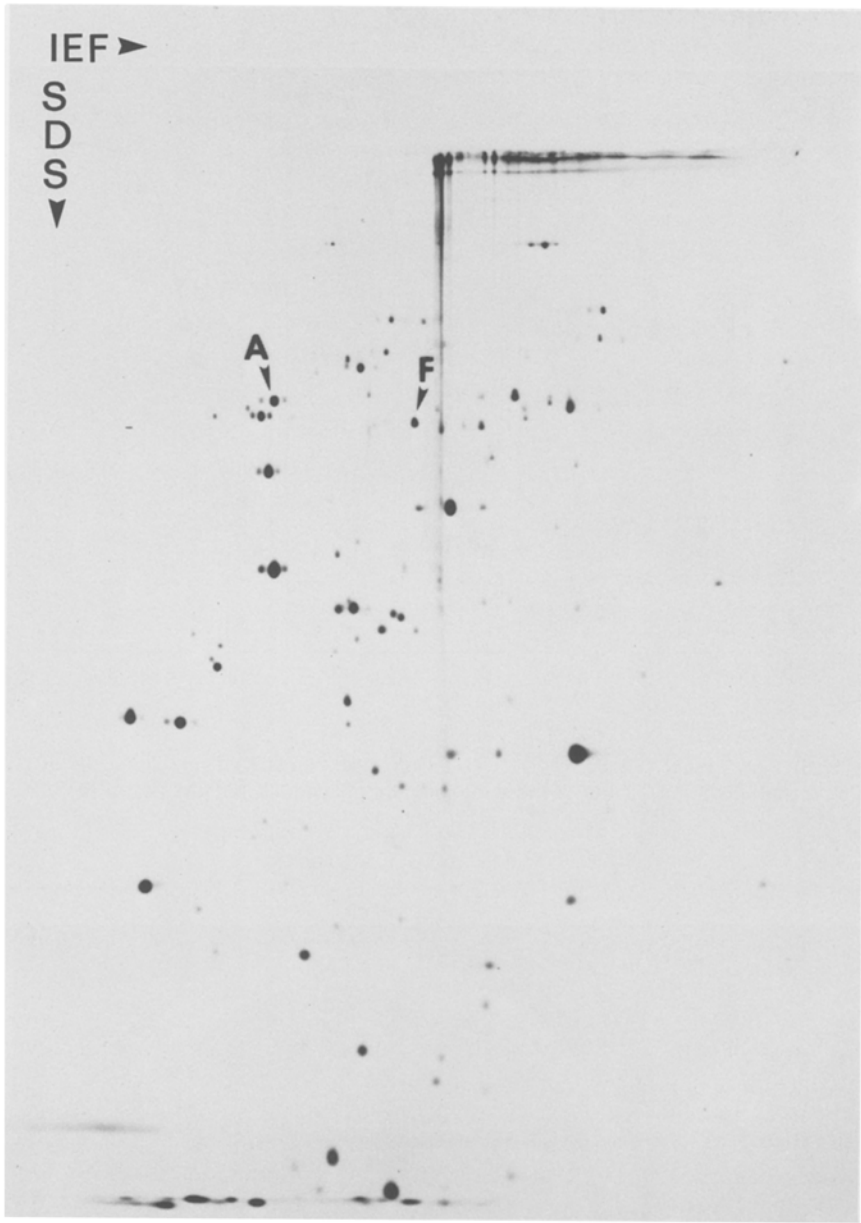

Fig. 3. Autoradiogram of two-dimensional gel of purified outer membrane of rare Pgm^+,Pst^r mutant of *Yersinia pestis* KIM grown at 37° C in iron-deficient medium (<0.3 μM Fe^{3+}). Letters refer to iron-modifiable peptide A and pigmentation-specific peptide F. IEF refers to initial migration via isoelectric focusing and SDS refers to second direction of migration by sizing in sodium dodecyl sulfate

Irp peptides during growth at 37° C in iron-deficient medium. Isogenic Pgm^- mutants, incapable of sustained multiplication in this environment (Sikkema and Brubaker 1987), shared only one of these iron-stress functions (IrpA). However, this mutation also resulted in loss of Pgm^+-specific peptide F which was not regulated by exogenous iron. To show that accumulation of the cation in this environment required full expression of Irp peptides rather than peptide F, we isolated and tested a mutant lacking IrpB–E which retained peptide F. This isolate, selected for resistance to pesticin with retention of ability to absorb Congo red, also failed to grow in iron-deficient medium suggesting that at least one of the missing Irp peptides is required for high-affinity uptake of iron.

In order to obtain this rare Pgm^+,Pst^r mutant, it was first necessary to cure the Pgm^+,Pst^+ parent of the Pst plasmid, thereby ensuring loss of immunity to pesticin (Hertman and Ben-Gurion 1959; Brubaker 1970). The resulting Pgm^+,Pst^- isolate, but not its Pgm^-,Pst^- mutant, exhibited full-scale growth in iron-deficient medium demonstrating that the Pst plasmid does not encode functions required for active transport of iron. The sensitive analytical system used in this study revealed the presence of eight Pst^+-specific peptides in outer membranes whereas only two such

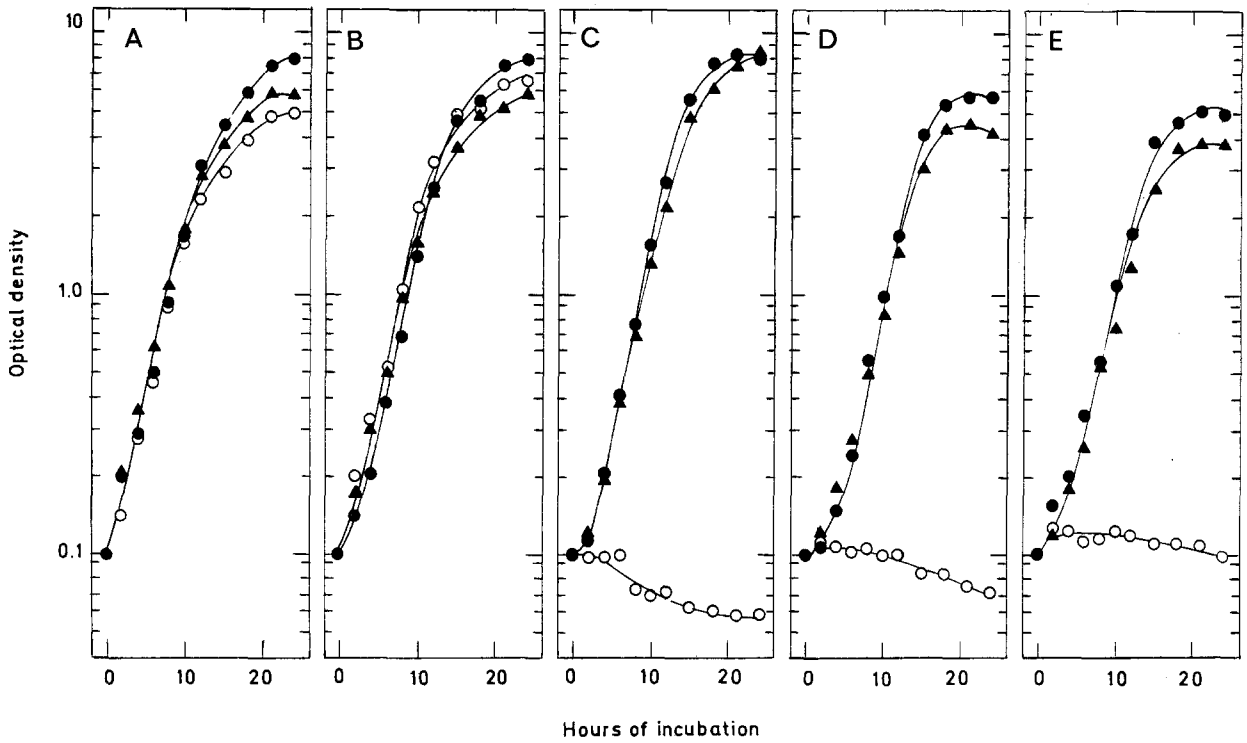

Fig. 4. Growth at 37° C of **(A)** Pgm^+,Pst^+, **(B)** Pgm^+,Pst^-, **(C)** Pgm^-,Pst^+, **(D)** Pgm^-,Pst^- and **(E)** Pgm^+,Pst^r cells of *Yersinia pestis* KIM in 8-hydroxyquinoline-extracted medium (○) and in the same medium plus added 100 μM $FeCl_3$ (●) or 50 mM hemin (▲)

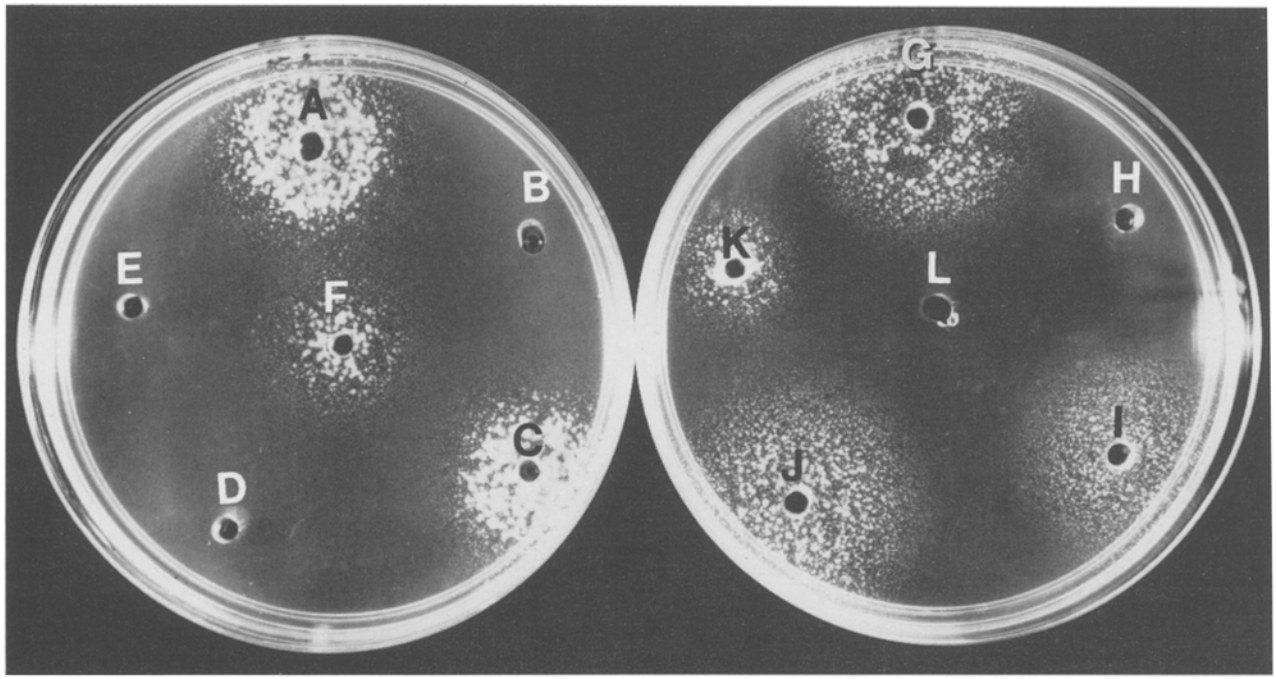

Fig. 5. Growth of Pgm^+,Pst^+ cells of *Yersinia pestis* KIM at 37° C on conalbumin agar with wells (2.5 mm diameter) containing solutions (≈0.025 ml) of *(A)* $FeCl_3$ plus protoporphyrin IX (1 μmol), *(B)* protoporphyrin IX alone, *(C)* $FeCl_3$, *(D)* transferrin (95% saturated), *(E)* lactoferrin (95% saturated), *(F)* hemin, *(G)* ferritin, *(H)* cytochrome *c*, *(I)* myoglobin, *(J)* hemoglobin, *(K)* hemopexin, and *(L)* distilled water. Concentrations of iron-containing compounds was adjusted to provide 1 nmol iron in each well

structures had previously been described (Straley and Brubaker 1982). These components were assumed to comprise PAC and its major degradation product (Sodeinde and Goguen 1988; Mehigh and Brubaker 1989). Results of further study may demonstrate that the extra Pst^+-specific structures defined here represent additional PAC degradation products and possibly the pesticin immunity protein.

Of interest was the finding that the mutation to Pgm^- involves loss of four detectable Irp peptides and a distinct Pgm^+-specific peptide not regulated by iron. Concomitant high-frequency loss of ability to express these products could reflect loss of a single regulatory gene or occurrence of a large deletion encompassing the corresponding structural genes. The former seems unlikely because yersiniae possess typical *fur* sequences (Staggs and Perry 1989) so that loss of these operators or of the complimentary Fur protein (a classical repressor) would promote constitutive production of Irp peptides rather than superrepression (Bagg and Neilands 1987; Wee et al. 1988). However, precedent exists for the occurrence of large deletions at high frequency in *Y. pestis* (Portnoy et al. 1983); this event also accounts for high-frequency loss of ability to accumulate iron in *E. coli* (e.g. *tonB;* Conkell and Yanofsky 1971). The nature of the mutation to Pgm^- is presently under investigation.

These studies have provided important insights into the significance of the Pgm^+ determinant as a virulence factor. It was correctly assumed at the outset (Jackson and Burrows 1956b) that avirulence of Pgm^- isolates resulted from inability to assimilate iron in vivo. Evidence supporting this hypothesis was the observation that injected iron phenotypically repressed the lesion in Pgm^- organisms accounting for avirulence. However, it is now established that injection of iron into the mammalian host can also enhance bacterial virulence by inhibiting a variety of nonspecific mechanisms of host defense (van Asbeck and Verhoef 1983). This effect probably accounts for the ability of the cation to increase virulence of Pst^- mutants (Brubaker et al. 1965) because, as shown, here, these isolates accomplished full-scale growth in iron-deficient medium. However, rare Pgm^+,Pst^r mutants failed to grow in this environment, indicating that the ability to express the pigmentation reaction *per se* is insufficient for active transport of iron. Accordingly, avirulence of Pgm^- mutants may reflect loss of Irp peptides rather than peptide F. This peptide possesses physical properties similar to those of the major outer membrane Pgm^+-specific peptide described previously in yersiniae grown at 26° C (Straley and Brubaker 1982) and may therefore be identical to this component.

Yersiniae were capable of utilizing a variety of sources of iron including the major intracellular reservoirs ferritin and myoglobin. Ability to grow with hemoglobin, hemopexin, and hemin as sole sources of iron suggest that these extracellular components might provide the cation during residence in vascular fluid. These molecules provide alternatives to lactoferrin and transferrin as sources of intracellular and extracellular iron, respectively, and thus could negate the established antibacterial activity of these molecules (Weinberg 1974). Both Pgm^+ and Pgm^- yersiniae used the same sources of organic iron when present in excess with equal facility as judged by growth on conalbumin agar. Accordingly, neither IrpB–E nor peptide F appear to be required for retrieval of the cation from these molecules. More likely, the Irp peptides function to assimilate low levels of Fe^{3+} and peptide F serves to promote storage of hemin. These processes are presently under investigation.

Acknowledgements. Highly purified hemopexin was generously provided as a gift by Dr Daryl Dwyer. The excellent technical assistance of Janet M. Fowler is gratefully acknowledged. This work was supported by Public Health Service grant AI13590 from the National Institute of Allergy and Infectious Diseases.

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Received May 1, 1989