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Storage Reservoirs of Hemin and Inorganic Iron in *Yersinia pestis*

ROBERT D. PERRY,[1] THOMAS S. LUCIER,[2]† DANIEL J. SIKKEMA,[2]‡ AND ROBERT R. BRUBAKER[2]*

Department of Microbiology and Immunology, University of Kentucky, Lexington, Kentucky 40536-0084,[1] and Department of Microbiology, Michigan State University, East Lansing, Michigan 48824[2]

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It is established that a high-frequency chromosomal deletion of ca. 100 kb accounts for the loss of properties making up the pigmented phenotype (Pgm^+) of wild-type *Yersinia pestis*. These determinants are known to include virulence by peripheral routes of injection, sensitivity to the bacteriocin pesticin, adsorption of exogenous hemin or Congo red at 26°C, and growth in iron-sequestered medium at 37°C. We have now identified the outer membrane as the primary site of exogenous hemin storage in Pgm^+ cells grown at 26°C. Significant outer membrane storage of hemin did not occur in Pgm^- mutants or in Pgm^+ cells cultivated at 37°C. However, both Pgm^+ and Pgm^- organisms grown at 37°C contained a periplasmic reservoir of hemin, which may be associated with a temperature-dependent ca. 70-kDa peptide recently equated with antigen 5. At 37°C, Pgm^+ and Pgm^- yersiniae also utilized a cytoplasmic ca. 19-kDa bacterioferritin-like peptide for deposition of inorganic iron. Incorporation of [^{55}Fe]hemin into pools at 37°C was not significantly inhibited by competition with excess unlabeled Fe^{3+}. However, excess unlabeled hemin modestly competed with incorporation of label from $^{55}FeCl_3$. This relative independence of storage pools observed at 37°C is consistent with physiological linkage to in vivo acquisition and transport of Fe^{3+} from ferritin and of hemin from hemoglobin, myoglobin, or hemopexin.

Although saprophytic procaryotes typically compete in iron-deficient natural environments, pathogenic species present in host tissue may encounter potentially iron-rich conditions. However, this iron is often bound to sequestered reservoirs, e.g., ferritin, or contained within high-affinity ligands, e.g., transferrin and lactoferrin. Considerable iron also exists in vivo as hemin, which is present in hemoglobin, myoglobin, hemopexin, and cytochromes (8, 27, 59). Wild-type cells of *Yersinia pestis*, the causative agent of bubonic plague, can proliferate within the iron-sequestered environments of the host as well as the hemin-rich flea gut, which is known to contain hemolyzed erythrocytes (8, 9). When grown in vitro at 26°C to simulate this unique niche, the bacteria absorb sufficient exogenous hemin (22) or its analog (24) Congo red (54) from solid media to form pigmented (Pgm^+) colonies. Spontaneous Pgm^- mutants, known to have undergone a consistent ca. 100-kb chromosomal deletion (18, 29) at high frequency (5), fail to accumulate these compounds at 26°C and thus form colorless colonies (22, 54). Pgm^- mutants are also unable to colonize the gut of the flea vector (25). However, both Pgm^+ and Pgm^- yersiniae can utilize hemoglobin, myoglobin, hemopexin, or free hemin as the sole source of iron (40, 45, 46). Thus, one defect in Pgm^- mutants lies in hemin storage but not in its utilization as a nutrient.

The occurrence of a phenotypically similar but probably physiologically distinct "pigmentation" reaction has been found in a few other pathogenic procaryotes (4, 24, 38, 42). The surface deposits collected by these species are also dissimilar to a soluble ca. 70-kDa hemin-rich peptide synthesized by *Y. pestis* at 37°C but not 26°C (31, 32, 43); this unique reservoir has been equated (31, 32) with classical antigen 5 (14). Although hemin storage thus appears to be rare in pathogenic procaryotes, inorganic iron storage on the cytoplasmic protein bacterioferritin may be widespread (55). This iron storage protein has been detected in six diverse genera: *Azotobacter* (7, 12, 49), *Escherichia* (1, 60), *Pseudomonas* (34), *Rhodopseudomonas* (33), *Rhodospirillum* (2), and *Streptomyces* (21). 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- to 18-kDa polypeptide) and in their nonhemin iron storage properties (1, 30, 49). Unlike eucaryotic ferritins, bacterioferritins contain one hemin moiety for every two to five subunit polypeptides. Although no function for bacterioferritins has been proven, iron storage probably provides the cation for growth under subsequent conditions of privation and may prevent formation of toxic oxygen radicals (1).

In this study, we examined hemin and inorganic iron storage properties in isogenic Pgm^+ and Pgm^- cells of *Y. pestis* KIM. Massive accumulation of hemin occurred at the outer membrane of Pgm^+ cells grown at 26°C but not 37°C, whereas more limited storage took place in the periplasm of both Pgm^+ and Pgm^- yersiniae. Accumulation of iron supplied as Fe^{3+} occurred, especially at 37°C, on a soluble bacterioferritin-like molecule also shared by Pgm^+ and Pgm^- cells. Under the conditions of iron excess used in these experiments, incorporation of the cation into these pools occurred by essentially independent processes as judged by minimal competition between Fe^{3+} and hemin.

* Corresponding author.
† Present address: Department of Pediatrics, University of North Carolina, Chapel Hill, NC 27599.
‡ Present address: Buffalo Veteran's Administration Medical Center, Medical Research 151, Buffalo, NY 14215.

MATERIALS AND METHODS

Bacteria. An isogenic Pgm^+ and Pgm^- pair of *Y. pestis* KIM derivatives was used in this study. Both isolates possess the species-specific ca. 100-kb exotoxin/fraction 1 (Tox) plasmid (pMT1) and ca. 10-kb pesticin (Pst) plasmid (pPCP1) but are avirulent because of the absence of the shared ca. 70-kb low-calcium response (Lcr) plasmid (pCD1) (6, 45, 56). The Pgm^+ determinant and the Lcr virulence regulon are genetically and biochemically unrelated (6, 41).

Cultivation and labeling of bacteria. Organisms were stored at −20°C in buffered glycerol (3). Congo red agar (54) was used to determine the Pgm^+ phenotype. Bacteria were grown with aeration (200 rpm) on a gyratory water bath shaker (model G76; New Brunswick Scientific Co., Inc., Edison, N.J.) in the synthetic medium of Higuchi et al. (20) as modified by Zahorchak and Brubaker (61). In this study, $FeSO_4$ was omitted from the medium, which was further deferrated by 8-hydroxyquinoline extraction (58) and is referred to below as extracted Higuchi's medium. Supplements of hemin and $FeCl_3$ were added to extracted Higuchi's medium before use in experiments. Glycerol-stored cells were inoculated onto slopes of tryptose blood agar (Difco Laboratories, Detroit, Mich.) and incubated for 48 h at 26°C. The resulting bacteria were suspended in 0.033 M potassium phosphate buffer (pH 7.0) (phosphate buffer) and used to inoculate extracted Higuchi's medium (supplemented with 50 to 100 μM radioactive $FeCl_3$ or 20 to 87 μM radioactive hemin) to an optical density of 0.1 at 620 nm. The organisms were acclimated to the medium by growth at 26 or 37°C by serial transfer for at least eight generations (40), which ensured labeling to constant specific activity. Acclimated cells were transferred to fresh extracted and supplemented Higuchi's medium, and mid-log-phase cells were harvested for preparation of subcellular fractions.

Bacteria were labeled to constant specific activities with either $^{55}FeCl_3$ (New England Nuclear Research Products, Boston, Mass.) or [^{55}Fe]hemin by addition of the carrier-free isotope to extracted and supplemented Higuchi's medium. For cell fractionation studies, the final concentrations and specific activities in radioactive medium after sterilization by filtration were 100 μM $^{55}FeCl_3$ (13,000 cpm/nmol of ^{55}Fe) or 87 μM [^{55}Fe]hemin (1,700 cpm/nmol of ^{55}Fe). Cell-associated radioactivity was quantitated by filtration (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) of approximately 10^9 bacteria followed by membrane washing with ice-cold unlabeled medium and scintillation counting.

Cell fractionation. Mid-log-phase cells were fractionated into periplasmic, cytoplasmic, outer membrane, and inner membrane components as previously described (36, 51, 52). Briefly, the method involved treatment with lysozyme-EDTA to form spheroplasts and then centrifugation to separate the spheroplasts from released periplasm. Sedimented spheroplasts were suspended and lysed by sonication and then subjected to successive isopycnic sucrose gradient centrifugations to separate cytoplasm, outer membranes, and inner membranes (36). This process, as performed previously with *Y. pestis*, resulted in ca. 3% contamination of outer with inner membranes and 5 to 38% contamination of inner with outer membranes (51). Although the sucrose density outer membrane banding pattern was significantly lower in all cells grown with hemin at 26°C, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) peptide profiles showed these fractions to be authentic isolated outer membranes (not illustrated). Radioactivity associated with these subcellular fractions was determined by scintillation spectroscopy of appropriate aliquots.

To analyze soluble iron pools, mid-log-phase cells grown to constant specific activity (100 μM $^{55}Fe^{3+}$, 4,850 cpm/nmol of ^{55}Fe; 50 μM [^{55}Fe]hemin, 635 cpm/nmol of ^{55}Fe) were harvested by centrifugation (10,000 × *g* for 30 min), washed with phosphate buffer, and suspended in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–1.0 mM sodium citrate buffer (pH 7.8) (hereafter termed column buffer). Cell suspensions were sonicated on ice in 15-s bursts for 1 min (Aminco no. FA-078; SLM Instruments, Inc., Urbana, Ill.), and the resulting cellular debris and particulate matter were removed by centrifugation (10,000 × *g* for 15 min). The resulting cell extracts were sterilized by brief exposure to $CHCl_3$ followed by gentle aeration. Protein concentrations were determined by the method of Lowry et al. (28), and all samples were diluted to 10 mg of protein per ml of column buffer. Samples of 1.0 ml were then applied to a column (2.5 by 100 cm) of Bio-Gel A-1.5m (100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) for molecular sieving. The column was maintained at 4°C, and radioactivity was eluted with column buffer at a flow rate of 0.15 ml/min. The optical density of eluted material in samples (2.7 to 3.3 ml) was estimated at 280 nm. Radioactivity was determined by drying 1.0-ml aliquots of each sample in scintillation vials overnight at 80°C, adding 10 ml of Complete Counting Cocktail (Research Products International Corp., Mount Prospect, Ill.), and quantitating the counts per minute on an LS7500 Scintillation System (Beckman Instruments, Inc., Fullerton, Calif.).

Identification of bacterioferritin-like activity. Samples eluting from the Bio-Gel A-1.5m column at a volume of ca. 260 to 280 ml displayed the highest radioactive peak in extracts of cells grown under most conditions (see Fig. 1 and 2). These fractions from Pgm^- cells grown at 37°C with $^{55}FeCl_3$ were pooled for further analysis. This preparation was dialyzed overnight against three changes of 0.05 M Tris · HCl buffer (pH 7.8) and then applied to a DEAE-cellulose (Whatman Biosystems, Ltd., Maidstone, England) column (2.5 by 46 cm) equilibrated in the same buffer at room temperature. Radioactivity was eluted from the column by using a 0 to 0.5 M NaCl gradient in this buffer at a flow rate of 1.0 ml/min. Samples of 5 ml were collected, and their optical density and radioactivity were measured as described above. Aliquots (25 μl) of each sample were also mixed with an equal volume of SDS-PAGE buffer and subjected to electrophoresis, and the resulting separated proteins were visualized by silver staining (26, 35).

Synthesis of [^{55}Fe]hemin. Protoporphyrin IX (Sigma Chemical Co., St. Louis, Mo.) and $^{55}FeSO_4$ (New England Nuclear) were used to chemically synthesize [^{55}Fe]hemin by the method of Chang et al. (11). This procedure involved incorporation of iron into the porphyrin ring by incubation in a nitrogen atmosphere at 80°C in pyridine-glacial acetic acid solvent. [^{55}Fe]hemin was then separated from reactants by $CHCl_3$ extraction and acid precipitation (11). Analysis of authentic hemin (Sigma) and our [^{55}Fe]hemin product revealed virtually identical visible absorption spectra (not illustrated). Hemin concentrations in filtered media were determined spectrophotometrically at 580 nm against a standard curve constructed by using authentic hemin. The term hemin is used throughout this report as a generic name for iron-containing protoporphyrin IX without regard to oxidation or salt states.

TABLE 1. Specific activities of stored iron in subcellular fractions of Pgm$^+$ and Pgm$^-$ cells of *Y. pestis* KIM after growth to constant specific activity

Subcellular fraction	Sp act of stored iron (nmol/mg of protein) in:							
	Pgm$^+$ cells				Pgm$^-$ cells			
	Growth at 26°C		Growth at 37°C		Growth at 26°C		Growth at 37°C	
	Fe^{3+}	Hemin	Fe^{3+}	Hemin	Fe^{3+}	Hemin	Fe^{3+}	Hemin
Outer membrane	40.8	697.4	1.6	2.2	8.0	13.7	15.8	3.4
Periplasm	12.2	20.4	14.6	21.2	3.1	5.4	4.4	18.7
Inner membrane	8.8	17.4	5.9	9.7	3.3	3.4	5.2	6.7
Cytoplasm	5.1	3.7	11.3	3.0	2.8	1.5	6.3	2.3

RESULTS

Preliminary experiments were undertaken to determine the capacity of yersiniae to remove inorganic iron and hemin from solution during logarithmic growth and entry into the stationary phase. The percentage of total label removed by bacteria from the culture medium was influenced by temperature, phenotype, and the source of iron provided. All cells grown with added $^{55}FeCl_3$ (100 μM) removed less than 5% of the label from the medium. Organisms grown with [^{55}Fe]hemin (87 μM) removed 19 to 25%, with the exception of Pgm$^+$ cells at 26°C, which removed approximately 85%.

Subcellular localization of hemin and iron pools. Accumulation within subcellular fractions of radioactivity supplied as inorganic or hemin-bound ^{55}Fe is reported in Table 1 in terms of specific activity (nanomoles of iron per milligram of protein). Although several differences are notable, Pgm$^+$ cells provided with hemin at 26°C clearly bound excess iron at their outer membranes. After growth in this environment, outer membranes of Pgm$^+$ organisms contained 51 times more iron than did outer membranes of Pgm$^-$ cells grown at 26°C and 317 times more iron than did outer membranes of Pgm$^+$ cells grown at 37°C. Inner membranes and periplasm of Pgm$^+$ cells grown at 26°C with labeled hemin also contained somewhat more iron than the corresponding fractions from Pgm$^-$ organisms did. This observation may reflect release or diffusion of hemin from the large outer membrane pool of Pgm$^+$ cells or low-level contamination of inner membranes and periplasm during the fractionation procedure. There was no indication of significantly more accumulation at 26°C of hemin-bound iron in cytoplasm of Pgm$^+$ cells than in cytoplasm of Pgm$^-$ cells. This observation supports the notion that the function of pigmentation is outer membrane adsorption of hemin rather than its transport into the cytoplasm. Distribution of iron did not differ significantly in subcellular fractions of Pgm$^+$ and Pgm$^-$ cells grown at 37°C with radioactive hemin. However, growth at this temperature resulted in the periplasm, known to be rich in hemin-binding antigen 5 (31; not illustrated), serving as a storage reservoir for cells of both phenotypes.

Also identified in Table 1 are several differences between Pgm$^+$ and Pgm$^-$ cells concerning the distribution of radioactivity provided as $^{55}FeCl_3$. The pattern at 26°C was very similar to that seen in cells grown with hemin, although the fivefold difference between outer membranes of Pgm$^+$ and Pgm$^-$ cells is substantially smaller. This difference may represent a low affinity of inorganic iron for the structure involved in hemin storage or a distinct Pgm$^+$-specific system for acquisition of iron. When grown at 37°C, outer membranes of Pgm$^-$ cells contained about 10 times more iron than did Pgm$^+$ outer membranes. In addition, the concentration of iron in both the cytoplasmic and periplasmic fractions of Pgm$^-$ cells was significantly lower than that in the same fractions of Pgm$^+$ cells. This difference may represent holdup of inorganic iron at the outer membrane of Pgm$^-$ cells as a consequence of the temperature-dependent lesion in assimilation known to exist in these mutants (40, 44, 45).

Soluble iron storage pools. To define iron storage pools and analyze their size during cultivation in different environments, yersiniae were grown to constant specific activity with labeled inorganic iron or hemin and sonicated, and soluble fractions consisting of periplasm plus cytoplasm were separated from particulate fractions by centrifugation. These preparations were subjected to molecular sieving through Bio-Gel A-1.5m; profiles of eluted radioactivity and absorbance are shown for soluble fractions of Pgm$^+$ and Pgm$^-$ cells in Fig. 1 and 2, respectively. The most notable feature of these determinations was the appearance of a radioactive peak at an elution volume of about 270 ml that was present to some extent in both Pgm$^+$ and Pgm$^-$ cells grown under all tested conditions. Comparison with standards of known sizes demonstrated that a native molecular mass of approximately 620 kDa would be anticipated for proteins eluting at this volume. This fraction appeared to contain an inorganic iron storage molecule since growth with hemin significantly reduced its specific activity. Cells grown with $^{55}FeCl_3$ contained 53 to 76% of the total soluble radioactivity in this peak as opposed to 16 to 39% for cells grown with [^{55}Fe]hemin. Although these findings illustrate that inorganic iron is preferentially incorporated into this fraction, it is not established that inorganic iron is the stored form in cells grown with hemin. Nevertheless, the lower concentration of label present in this reservoir from cells brought to constant specific activity with hemin suggests that the primary form of storage is as inorganic iron removed from the porphyrin ring. These results are also consistent with the alternative possibility of secondary storage as hemin per se.

A second, typically smaller radioactive peak was eluted upon molecular sieving at a volume of about 200 ml. This position almost exceeded the exclusion limit; it corresponds to hemin-rich antigen 5 (31). The higher specific activity of this fraction in cells grown with [^{55}Fe]hemin than in those grown with $^{55}FeCl_3$ suggests direct incorporation of exogenously supplied hemin into this host temperature-dependent storage pool.

Bacterioferritin-like material. The entire fraction containing the major radioactive peak of Pgm$^-$ cells grown at 37°C with $^{55}FeCl_3$ (Fig. 2) was chromatographed on DEAE-cellulose as described above. A single radioactive fraction containing 90% of the applied label was recovered when the concentration of NaCl in the gradient was 0.35 M (Table 2).

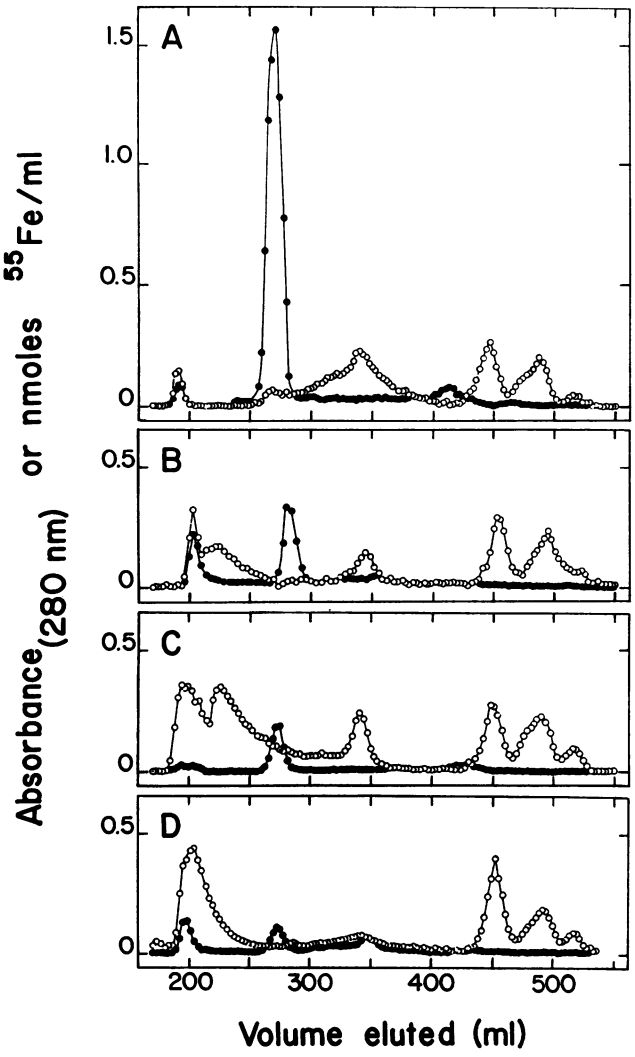

FIG. 1. Profiles of soluble extracts of Pgm$^+$ cells of *Y. pestis* eluted on chromatography with Bio-Gel A-1.5m. Growth at 37°C with $^{55}Fe^{3+}$ (A), 37°C with [^{55}Fe]hemin (B), 26°C with $^{55}Fe^{3+}$ (C), and 26°C with [^{55}Fe]hemin (D) is shown. The A_{280} (○) and recovered radioactivity in terms of nanomoles of iron per milliliter (●) are illustrated for antigen 5 (elution volume, ca. 200 ml) and bacterioferritin-like peptide (elution volume, ca. 270 ml).

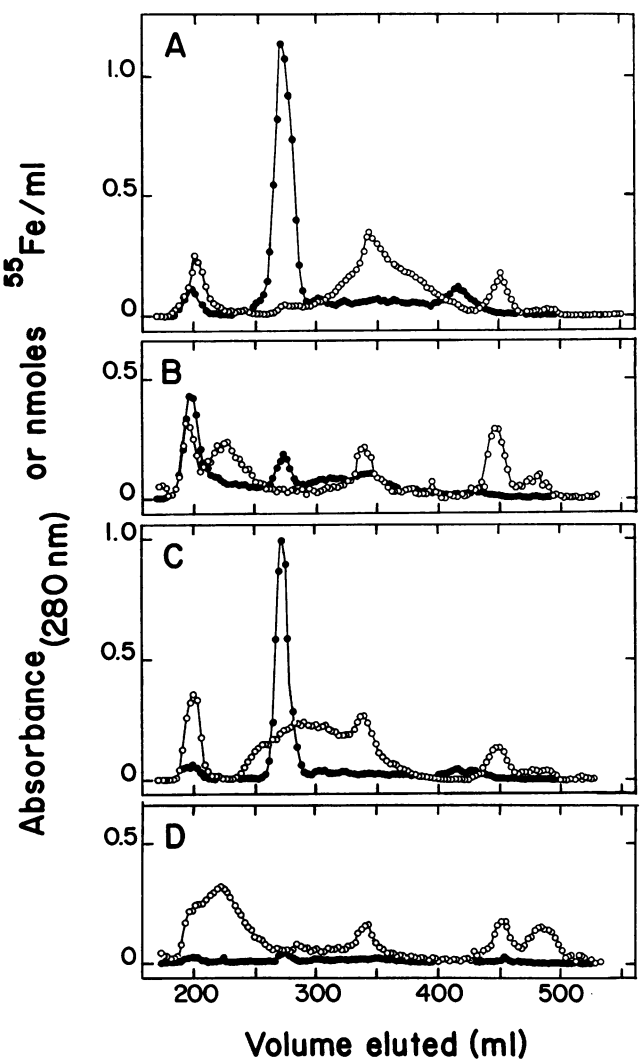

FIG. 2. Profiles of soluble extracts of Pgm$^-$ cells of *Y. pestis* eluted upon chromatography with Bio-Gel A-1.5m. Growth at 37°C with $^{55}Fe^{3+}$ (A), 37°C with [^{55}Fe]hemin (B), 26°C with $^{55}Fe^{3+}$ (C), and 26°C with [^{55}Fe]hemin (D) is shown. A_{280} (○) and recovered radioactivity in terms of nanomoles of iron per milliliter (●) are illustrated for antigen 5 (elution volume, ca. 200 ml) and bacterioferritin-like peptide (elution volume, ca. 270 ml).

Analysis of this fraction by SDS-PAGE (Fig. 3) revealed the presence of a major eluted peptide of 19 kDa plus a 16-kDa peptide that may have arisen as a degradation product of the evident 19-kDa bacterioferritin subunit. Soluble inorganic iron storage thus appears to involve a molecule with the characteristics of bacterioferritin. These findings indicate the occurrence of three distinct iron-rich depots in cells of *Y. pestis* KIM. Salient properties of these pools are compared in Table 3.

Interaction between iron storage pools. Experiments were performed to determine whether exogenous iron supplied as hemin contributes to the inorganic iron reservoir at 37°C and vice versa. Pgm$^+$ or Pgm$^-$ cells were labeled to constant specific activity with $^{55}FeCl_3$ or [^{55}Fe]hemin and then used to inoculate two parallel cultures containing the same source and specific activity of radioactive iron. One of these cultures was incubated without further addition, whereas the other received the unlabeled second source of iron in fivefold excess. If storage of one or the other form of iron was favored, accumulation of radioactivity in cultures containing this form in unlabeled excess should be inhibited. For both Pgm$^+$ and Pgm$^-$ cells grown with [^{55}Fe]hemin, the addition of excess unlabeled inorganic iron did not inhibit maximum accumulation of radioactivity in the stationary phase by more than 10% (Fig. 4). However, for cells grown to constant specific activity with $^{55}FeCl_3$, competition by fivefold excess unlabeled hemin resulted in an initial release of some previously cell-associated radioactivity, although later accumulation of label was not significantly inhibited during log phase growth. After entry into the stationary phase, this effect resulted in approximately 50 and 67% inhibition of incorporation of $^{55}FeCl_3$ in Pgm$^+$ and Pgm$^-$ cells, respectively (Fig. 5). These results are consistent with the observation that inorganic iron and hemin-bound iron are stored in

TABLE 2. Isolation of bacterioferritin-like peptide from a cell extract of Pgm$^-$ *Y. pestis* KIM grown at 37°C to constant specific activity with 100 μM $^{55}FeCl_3$

Preparation	Total vol (ml)	Protein concn (mg/ml)	Total amt of protein (mg)	Iron concn (nmol/ml)	Total amt of iron (nmol)	Sp act (nmol/mg of protein)	Recovery (%)
Cell extract	3	10.27	30.81	64.7	194.1	6.3	100
Bio-Gel A-1.5m	51	0.03	1.53	3.2	163.2	106.7	84
DEAE cellulose	25	0.05	1.25	5.9	147.5	118.0	76

distinct reservoirs and that remarkably little exchange occurs between these pools under conditions of iron or hemin surplus.

DISCUSSION

Jackson and Burrows (22, 23) first described the Pgm$^+$ phenotype as a required virulence determinant identifiable by accumulation of sufficient exogenous hemin to form pigmented colonies at 26°C but not 37°C. Characteristics now known to be lost on occurrence of the spontaneous deletion (18, 29) causing the high-frequency (5) mutation to Pgm$^-$ include sensitivity to the bacteriocin pesticin (5), growth at 37°C in iron-chelated medium (44, 45), expression of Fur-regulated (46–48) outer membrane peptides at 37°C associated with inorganic iron transport (10, 45), and production of unique hemin storage-specific peptides primarily at 26°C (39, 44, 52). The Pgm$^+$ phenotype thus consists of numerous separable and independent traits, which all appear to be either directly or indirectly concerned with storage of hemin at 26°C or high-affinity uptake of inorganic iron at 37°C. The differential temperature regulation of these two Pgm$^+$-specific traits (39, 45) makes it tempting to assume that hemin storage accounts for the ability to colonize the flea vector (25) and that inorganic iron uptake promotes death via peripheral routes of infection in mammals (23).

Essentially nothing is known about why only Pgm$^+$ cells can multiply within the hemin-rich flea gut, although an obvious possibility is that binding at the cell surface may simply prevent free hemin from participating in generation of damaging oxygen radicals (55). Surface-bound hemin might also provide some transient advantage to the bacterium after infection via a flea bite. The original observation by Jackson and Burrows (22) demonstrated that hemin molecules were retained for storage without apparent removal of inorganic iron. We have localized the site of Pgm$^+$-specific hemin storage at 26°C to the outer membrane. The extent to which hemin was removed from the medium by Pgm$^+$ cells at 26°C showed that its storage in this environment is enormous. If a similar situation occurs in the flea gut, sufficient bound iron might be transferred during infection to permit rapid multiplication prior to induction of systems capable of acquiring iron in vivo. The transient presence of bound hemin could also promote additional invasive processes including bacterial uptake by nonprofessional phagocytes. This process might facilitate dissemination from the site of injection and has been proposed for shigellae, whose ability to bind Congo red correlates with increased penetration of HeLa cells (15, 53) but not with nutritional utilization or transport of hemin (37). Incidentally, this form of pigmentation in shigellae is expressed only at 37°C, thus leaving unresolved its possible relationship with the Pgm$^+$ phenotype of yersiniae. A transient array of hemin molecules on newly introduced yersiniae could also serve to inhibit a variety of nonspecific host defenses (57), thereby providing time for induction of 37°C-dependent virulence factors (5). Analysis of mutants lacking Pgm$^+$-specific hemin-binding capabilities but not iron transport capabilities (39, 41) will allow resolution of these and other possible roles of surface-bound hemin in initiating infection.

We propose that the inorganic iron storage pool represents a bacterioferritin-like molecule that is expressed independently of the pigmentation phenotype. Native size, subunit molecular weight, elution on DEAE-cellulose, and the relative amount of cytoplasmic iron associated with the molecule in cells grown with $FeCl_3$ all closely match results obtained for bacterioferritins isolated from other species (1, 2, 12, 30, 33, 34, 49, 55). Bacterioferritins are often difficult to purify by traditional means, and some contaminating material remained in our preparations. Further effort may result in recovery of a sample of sufficient purity to permit analyses of hemin content, iron-binding capacity, and absorption spectra, thereby resolving the tentative identification of this molecule as bacterioferritin. This reservoir was favored in vitro for storage of iron provided in inorganic form, and there is no reason to assume that the same role is not fulfilled in vivo. In this case, a probable source of Fe^{3+} would be ferritin present either within host cells or free within necrotic lesions (6). Transferrin and lactoferrin are not expected to donate to this pool in vivo because they fail to serve as nutritional sources of iron in vitro (45, 46),

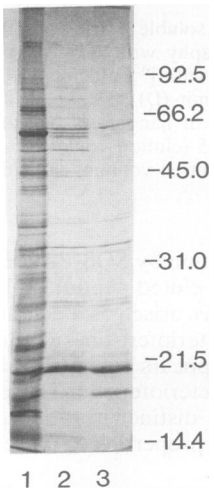

FIG. 3. Radioactive samples after SDS-PAGE (12.5% polyacrylamide) and silver staining isolated during purification of bacterioferritin-like peptide from Pgm$^-$ cells of *Y. pestis* KIM grown at 37°C to constant specific activity with 100 μM $^{55}FeCl_3$. Lanes: 1, crude cell extract; 2, radioactive fraction from Bio-Gel A-1.5m column recovered at about a 270-ml elution volume; lane 3, radioactive fraction from the DEAE-cellulose column eluting at 0.35 M NaCl. Molecular mass markers are given in kilodaltons.

TABLE 3. Some properties of three iron storage reservoirs detected in *Y. pestis* cultivated in vitro with excess inorganic iron or hemin

Anatomical location in bacterium	Temp (°C) favoring use for storage	Pgm^+ specific	Major form of stored iron	Molecular nature of storage depot
Outer membrane	26	Yes	Hemin	Unknown[a]
Soluble (probably mainly periplasmic)	37	No	Hemin	70-kDa subunit of ca. 1,000-kDa native protein
Soluble (probably mainly cytoplasmic)	37	No	Inorganic	18-kDa subunit of ca. 620-kDa native bacterioferritin-like protein

[a] Probably mediated by hemin storage-specific outer membrane peptides (39, 45, 48).

presumably because of an affinity for Fe^{3+} too high to permit its assimilation by siderophore-deficient yersiniae (40).

Additional studies are also needed to define the nature of the 37°C-specific periplasmic and probably cytoplasmic hemin storage function mediated by antigen 5. This depot is of interest because it is one of the few major stable peptides in *Y. pestis* known to be synthesized during expression of the low-calcium response (6, 31). Since antigen 5 can be recovered as a hemin-rich protein from yersiniae grown at 37°C with $FeCl_3$ as the sole source of iron (6), the activity evidently also serves as a depot for endogenously synthesized hemin. Further study is required to determine whether exogenous hemin is stored only on antigen 5 within the periplasm during growth at 37°C or whether hemin can undergo transport into the cytoplasm in intact form. In view of the large size of native antigen 5 molecules, it seems probable that significant amounts of this reservoir were lost during preparation for sizing. Nevertheless, it is evident that antigen 5 rather than the bacterioferritin-like depot served in vitro at 37°C as the major storage reservoir of iron provided as hemin. Likely sources of hemin for similar storage in vivo are hemoglobin and hemopexin in blood and myoglobin present both intracellularly and probably within necrotic lesions (6).

Although yersiniae can utilize either Fe^{3+} or hemin as the sole source of nutritional iron (40, 45, 46), our results indicate that under surplus conditions, the accumulation of one form does not have a marked inhibitory effect on the accumulation of the other. This ability to utilize either inorganic iron or hemin indicates that the organisms possess enzymatic activities for inorganic iron insertion into and removal from the porphyrin ring. Accordingly, significant exchange between the inorganic iron and hemin pools was expected. However, only modest linkage between these depots was observed, thus suggesting that the obviously distinct mechanisms of uptake were physiologically linked to separate mechanisms of storage. Other bacteria have been shown to use hemin-specific uptake systems that enable them to utilize this compound as a source of iron (13, 16, 17, 19, 50). Use of one of these processes by *Y. pestis* would account for the observed compartmentalization of iron provided as Fe^{3+} and hemin.

The significance of these observations with respect to growth in vivo is uncertain. Since the bacteria were grown to constant specific activity under conditions of great iron excess, pools would probably be saturated and the high-affinity transport mechanisms assumed to be expressed in the mammalian host should be fully repressed (40, 45–48). Perhaps under the conditions of iron deprivation encountered in vivo there would be greater unification of the iron pools following assimilation of scarce Fe^{3+} or hemin.

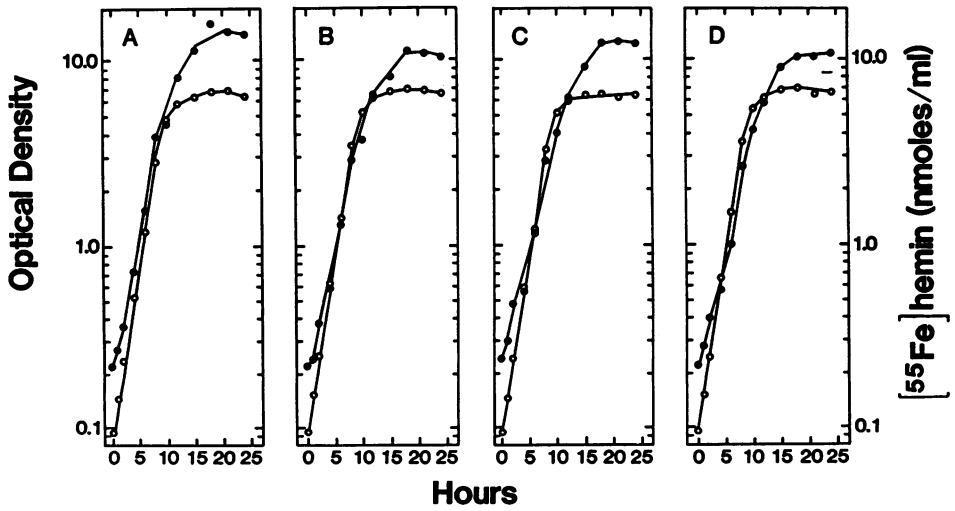

FIG. 4. Optical density at 620 nm (○) and nanomoles of cell-associated iron per milliliter of culture (●) in cultures of *Y. pestis* KIM incubated at 37°C with 20 μM [^{55}Fe]hemin. The organisms had previously been grown in this medium for at least eight generations to ensure saturation of iron storage pools at constant specific activity. (A) Pgm^+ cells without added $FeCl_3$; (B) Pgm^+ cells plus 100 μM unlabeled $FeCl_3$; (C) Pgm^- cells without added $FeCl_3$; (D) Pgm^- cells plus 100 μM unlabeled $FeCl_3$.

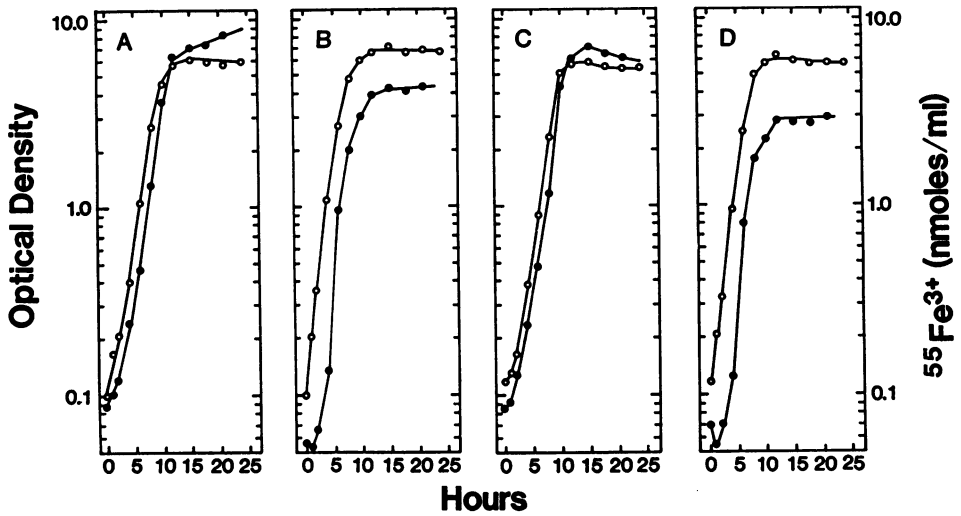

FIG. 5. Optical density at 620 nm (○) and nanomoles of cell-associated iron per milliliter of culture (●) in cultures of *Y. pestis* KIM incubated at 37°C with 50 μM $^{55}FeCl_3$. The organisms had previously been grown in this medium for at least eight generations to ensure saturation of iron storage pools at constant specific activity. (A) Pgm^+ cells without added hemin; (B) Pgm^+ cells plus 250 μM unlabeled hemin; (C) Pgm^- cells without added hemin; (D) Pgm^- cells plus 250 μM unlabeled hemin.

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