

# The haemin storage (Hms<sup>-</sup>) phenotype of *Yersinia pestis* is not essential for the pathogenesis of bubonic plague in mammals

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**The haemin storage (Hms<sup>-</sup>) phenotype of *Yersinia pestis* enables this bacillus to form greenish/brown or red colonies on haemin or Congo Red agar plates, respectively, at 26 but not 37 °C. *Escherichia coli* strains that contain mutations in genes essential for siderophore biosynthesis, porphyrin generation and/or haemin transport remain unable to utilize exogenous haemin as a nutritional iron or porphyrin source when transformed with the cloned *Y. pestis* hmsHFRS locus. Further physiological analysis of the Hms<sup>-</sup> phenotype of *Y. pestis* strain KIM6 suggests that the haemin and inorganic iron stored by the Hms system was not used nutritionally under subsequent iron-deficient conditions. *In vitro* analysis of the bactericidal effects of hydrogen peroxide, superoxide and nitric oxide showed that Hms<sup>-</sup> *Y. pestis* cells, in certain cases, were more susceptible than the Hms<sup>+</sup> parent cells to these reactive oxygen species at 26 and/or 37 °C. In adherence assays, a higher percentage of Hms<sup>-</sup> cells were associated with HeLa cells and normal human neutrophils, compared to Hms<sup>+</sup> cells. However, the Hms<sup>-</sup> phenotype did not provide any additional protection against the killing effects of neutrophils. Finally, LD<sub>50</sub> analysis in subcutaneously infected mice showed that an Hms<sup>-</sup> strain was slightly more virulent than Hms<sup>+</sup>, indicating that the Hms phenotype is not essential for the pathogenesis of bubonic plague in mammals.**

Keywords: HeLa cells, haemin binding, resistance to reactive oxygen species, neutrophils

## INTRODUCTION

Jackson & Burrows (1956a, b) defined the pigmentation phenotype (Pgm<sup>+</sup>) of *Yersinia pestis* as adsorption of haemin at 26 but not at 37 °C and iron-independent virulence from peripheral routes of infection in mice. Pgm<sup>+</sup> strains also bind Congo Red (CR) on CR agar and adsorb another flat hydrophobic molecule – guanine

(Brubaker, 1970; Surgalla & Beesley, 1969). Haemin and CR have analogous negatively charged hydrophobic groups (Kay *et al.*, 1985) and similar planar characteristics that can yield self-binding/stacking interactions (Fiel, 1989).

A 102 kb spontaneous chromosomal deletion ( $\Delta$ p<sub>gm</sub>), possibly due to a homologous recombination event between IS100 elements flanking this region, results in the Pgm<sup>-</sup> phenotype (Fetherston & Perry, 1994; Fetherston *et al.*, 1992; Lucier & Brubaker, 1992). One region of the p<sub>gm</sub> locus encodes a siderophore-dependent iron transport system (yersiniabactin) that is essential for growth in iron-chelated media at 37 °C, sensitivity to the bacteriocin pesticin (Pst<sup>S</sup>) and virulence via peripheral routes of infection (Bearden *et al.*, 1997; Brubaker *et al.*, 1965; Fetherston *et al.*, 1995; Jackson & Burrows, 1956b; Sikkema & Brubaker, 1987, 1989; Une & Brubaker, 1984).

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**Abbreviations:** ALA,  $\delta$ -aminolevulinic acid; CR, Congo Red; EDDA, ethylenediamine di(*o*-hydroxyphenylacetic acid); MEM, minimal essential medium; MEM/FCS, MEM + 5% foetal calf serum; NB, nutrient broth; NBD, NB + 0.3 mM 2,2'-dipyridyl; NOR-1, (+)-(E)-[(E)-hydroxyimino]-6-methoxy-4-methyl-5-nitro-3-hexeneamide; PMN, polymorphonuclear; SC, subcutaneous; TBA, tryptose blood agar; TGT, Tris/glucose/thymidine/leucine/proline medium

Colonization and blockage of the proventriculus of the oriental rat flea, *Xenopsylla cheopis*, lethality in fleas, and outer membrane adsorption of haemin, inorganic iron ( $\text{Fe}^{3+}$ ), and CR at 26 °C are directly associated with the haemin storage (*hms*) locus. Whether the adsorbed haemin or inorganic iron is surface-exposed or in the outer membrane has not been determined (Hinnebusch *et al.*, 1996; Jackson & Burrows, 1956a; Kutnyrev *et al.*, 1992; Perry *et al.*, 1990, 1993; Sikkema & Brubaker, 1989). Transposon insertional mutagenesis and sequence analysis of the *hms* locus elucidated the size and orientation of the four genes in the *hmsHFRS* operon (Lillard *et al.*, 1997; Pendrak & Perry, 1991, 1993). HmsH (93.4/89.5 kDa) and HmsF (74.6/72.2 kDa) are outer-membrane proteins, while the cellular location(s) of HmsR (52 kDa) and HmsS (17.5 kDa) are undetermined (Lillard *et al.*, 1997; Pendrak & Perry, 1993). Subcloning and transposon mutagenesis indicated that all four genes are needed for an Hms<sup>+</sup> phenotype and that *hmsHFRS* is the only operon within the *pgm* locus required to restore an Hms<sup>+</sup> phenotype in  $\Delta$ *pgm* strains (Lillard *et al.*, 1997; Pendrak & Perry, 1991, 1993). The Hms<sup>+</sup> phenotype results in a 5- to 26-fold increase in  $\text{Fe}^{3+}$  accretion in the outer membranes of cells grown at 26 °C, compared with Hms<sup>-</sup> strains grown at 26 °C or Hms<sup>+</sup> strains grown at 37 °C. Similarly, *hms* expression causes a 205- to 317-fold increase in haemin adsorption by Hms<sup>+</sup> cells grown at 26 °C, compared to Hms<sup>-</sup> or Hms<sup>+</sup> strains grown at 37 °C (Perry *et al.*, 1993). Curiously, a mutation in the gene encoding the iron-regulator Fur allows expression of the Hms<sup>+</sup> phenotype at 37 °C (Staggs *et al.*, 1994).

Haemin- and CR-binding phenotypes serve distinct roles in a variety of pathogens (Daskaleros & Payne, 1987; Garduno & Kay, 1992; Genco *et al.*, 1994; Kay *et al.*, 1985; Scott *et al.*, 1993); the ability to bind these substrates has been correlated with virulence, but not always with haemin utilization. A long-held hypothesis is that the Hms<sup>+</sup> phenotype allows *Y. pestis* to adsorb large quantities of inorganic iron or haemin in the flea gut, which is rich in haemin compounds from haemolysed blood, for subsequent use as an iron source in the iron-deficient environment of the mammalian host (Kluger & Bullen, 1987; Perry, 1993). Likewise, this phenotype could function as a secondary uptake system for exogenous haemin. Alternatively, the *Y. pestis* Hms<sup>+</sup> phenotype may enhance host cell adherence, invasion and/or survival in phagocytic cells as observed for haemin- or CR-binding systems in other pathogens (Daskaleros & Payne, 1987; Garduno & Kay, 1992; Stugard *et al.*, 1989). The inorganic iron and/or haemin adsorbed by Hms<sup>+</sup> strains could detoxify hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide ( $\text{O}_2^-$ ) and/or nitric oxide (NO) (Aust *et al.*, 1985; Kim *et al.*, 1994) to enhance resistance to these anti-microbial chemicals. The temperature regulation of the Hms<sup>+</sup> phenotype suggests any putative function in mammals would occur during the onset of infection.

In this study we tested the potential functions of the Hms<sup>+</sup> phenotype. The haemin and/or inorganic iron

adsorbed to the outer membranes of Hms<sup>+</sup> *Y. pestis* cells at 26 °C was not used, or efficiently mobilized, as a nutritional iron source for subsequent growth at 37 °C under iron-starvation conditions. Hms<sup>+</sup> *Y. pestis* cells, in some instances, exhibited a higher *in vitro* survival rate than Hms<sup>-</sup> cells against the killing effects of some reactive oxygen species at 26 and/or 37 °C. In addition, the Hms<sup>+</sup> phenotype enhanced adherence to HeLa and normal human polymorphonuclear (PMN) cell monolayers but did not confer protection against PMN cells. Despite these *in vitro* effects, an Hms<sup>-</sup> *Y. pestis* strain had a reduced LD<sub>50</sub> in mice challenged subcutaneously.

## METHODS

**Bacterial strains and plasmids.** The *E. coli* and *Y. pestis* strains as well as plasmids used in this investigation are described in Table 1. Except for the two strains used in virulence testing, all *Y. pestis* strains used in this study are avirulent due to the absence of pCD1 which contains the low-calcium-response stimulon genes. Plasmids were isolated by alkaline lysis and transformed into various *E. coli* strains by a standard calcium chloride transformation technique (Ausubel *et al.*, 1987) or electroporated into *Y. pestis* (Fetherston *et al.*, 1995). For biosafety considerations, we used KIM6-2053.1+ ( $\Delta$ *psa2053.1*), which lacks *psaA*, *psaB*, *psaE*, *psaF* and part of *psaC* that are essential for expression of the pH 6 antigen (Bearden *et al.*, 1997; Lindler & Tall, 1993) to generate potentially virulent *Y. pestis* strains. The *Y. pestis* DNA surrounding this deleted *psa* region was introduced into the chromosome of KIM6+ via allelic exchange (Donnenberg & Kaper, 1991; Fetherston *et al.*, 1995) using the suicide plasmid pCVDPSA1 (Bearden *et al.*, 1997). This mutation was confirmed by Southern blot analysis and by Western colony-blot analysis using polyclonal pH 6-antigen-specific antibodies (Helfman *et al.*, 1983; Lindler *et al.*, 1990).

Similarly *hmsR46*, a 6 bp deletion within the essential lysine/arginine-rich region of *hmsR* (Lillard *et al.*, 1997), was moved into KIM6+ and KIM6-2053.1+ using pKNGHM-SR46, generating KIM6-2057.1 and KIM6-2057.3 respectively. The *hmsR46* mutation in both strains was confirmed by Southern-blot and complementation analysis as well as screening on CR agar to determine the Hms phenotype. The slightly attenuated strain KIM5-2053.11+ and the potentially virulent strain KIM5-2057.31 were generated by electroporating pCD1::Mud11734-73 (Straley & Bowmer, 1986) into KIM6-2053.1+ and KIM6-2057.3 respectively.

**Bacterial media and cultivation.** All strains were stored in phosphate-buffered glycerol at -20 °C. *Y. pestis* strains were grown in heart infusion broth (Difco) for DNA isolation and on CR agar to determine Hms phenotypes (Surgalla & Beesley, 1969). For iron-deficient growth studies as well as resistance to reactive oxygen species, host cell interaction and LD<sub>50</sub> analysis, *Y. pestis* glycerol stocks were streaked onto CR plates and incubated for 48 h at 26 °C. Isolated colonies were inoculated on tryptose blood agar (TBA; Difco) slants and incubated at 26 °C for 48 h. Cells were washed off the slants with the iron-deficient defined medium PMH (Staggs & Perry, 1991) and diluted to OD<sub>620</sub> 0.1 in PMH supplemented, where appropriate, with haemin,  $\text{FeCl}_3$  and/or the iron chelator EDDA [ethylenediamine di(*o*-hydroxyphenylacetic acid); Sigma]. For KIM6+ cells, one cell in  $5 \times 10^3$  was CR<sup>-</sup>,

**Table 1.** Bacterial strains and plasmids used

Strain/plasmid	Genotype/relevant characteristics*	Source/Reference(s)
<b>Y. pestis strains</b>		
KIM6+	Hms <sup>+</sup> Lcr <sup>-</sup> Psa <sup>+</sup> Ybt <sup>+</sup> pMT1 pPCP1	Fetherston & Perry (1994), Une & Brubaker (1984)
KIM6	Hms <sup>-</sup> ( $\Delta$ pgm) Lcr <sup>-</sup> Psa <sup>+</sup> Ybt <sup>-</sup> ( $\Delta$ pgm) pMT1 pPCP1	Fetherston & Perry (1994), Une & Brubaker (1984)
KIM6-2008	Km <sup>r</sup> Hms <sup>-</sup> ( <i>hmsH-2008::mini-kan</i> ) Lcr <sup>-</sup> Psa <sup>+</sup> Ybt <sup>+</sup> pMT1 pPCP1	Pendrak & Perry (1991)
KIM6-2045.1	Hms <sup>+</sup> Lcr <sup>-</sup> Psa <sup>+</sup> Ybt <sup>-</sup> ( $\Delta$ psn2045.1) pMT1 pPCP1	Fetherston <i>et al.</i> (1995)
KIM6-2053+	Ap <sup>r</sup> Suc <sup>s</sup> Hms <sup>+</sup> Lcr <sup>-</sup> <i>psa::pCVDPSA1</i> Ybt <sup>+</sup> pMT1 pPCP1, derived from KIM6+	This study
KIM6-2053.1+	Ap <sup>r</sup> Suc <sup>r</sup> Hms <sup>+</sup> Lcr <sup>-</sup> Psa <sup>-</sup> ( $\Delta$ psa2053.1) Ybt <sup>+</sup> pMT1 pPCP1, derived from KIM6-2053+	This study
KIM5-2053.11+	Km <sup>r</sup> Hms <sup>+</sup> Lcr <sup>+</sup> Psa <sup>-</sup> ( $\Delta$ psa2053.1) Ybt <sup>+</sup> pCD1:: <i>MudI1734-73</i> ( <i>yopJ::MudI1734</i> ) pMT1 pPCP1, derived from KIM6-2053.1+	This study
KIM6-2057	Sm <sup>r</sup> Suc <sup>s</sup> <i>hmsR::pKNGHMSR46</i> Lcr <sup>-</sup> Psa <sup>+</sup> Ybt <sup>+</sup> pMT1 pPCP1, derived from KIM6+	This study
KIM6-2057.1	Sm <sup>s</sup> Suc <sup>r</sup> Hms <sup>-</sup> ( <i>hmsR46</i> ) Lcr <sup>-</sup> Psa <sup>+</sup> Ybt <sup>+</sup> pMT1 pPCP1, derived from KIM6-2057	This study
KIM6-2057.2	Sm <sup>r</sup> Suc <sup>s</sup> <i>hmsR::pKNGHMSR46</i> Lcr <sup>-</sup> Psa <sup>-</sup> ( $\Delta$ psa2053.1) Ybt <sup>+</sup> pMT1 pPCP1, derived from KIM6-2053.1+	This study
KIM6-2057.3	Sm <sup>s</sup> Suc <sup>r</sup> Hms <sup>-</sup> ( <i>hmsR46</i> ) Lcr <sup>-</sup> Psa <sup>-</sup> ( $\Delta$ psa2053.1) Ybt <sup>+</sup> pMT1 pPCP1, derived from KIM6-2057.2	This study
KIM5-2057.31	Km <sup>r</sup> Hms <sup>-</sup> ( <i>hmsR46</i> ) Lcr <sup>+</sup> Psa <sup>-</sup> ( $\Delta$ psa2053.1) Ybt <sup>+</sup> pCD1:: <i>MudI1734-73</i> ( <i>yopJ::MudI1734</i> ) pMT1 pPCP1, derived from KIM6-2057.3	This study
<b>E. coli strains</b>		
DH5 $\alpha$	Cloning strain, F <sup>-</sup> <i>endA1 hsdR17</i> ( <i>r<sub>k</sub><sup>-</sup> m<sub>k</sub><sup>+</sup></i> ) <i>supE44 thi-1 recA1 gyrA96 relA1</i> $\Delta$ ( <i>argF-lacZYA</i> ) U169 ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15)	Ausubel <i>et al.</i> (1987)
EB53	Derived from K12 AB2847, <i>aroB thi malA tsx hemA</i> , unable to transport haemin or make enterobactin and porphyrins	Eberspacher & Braun (1980)
RK4744	Derived from RK4353, <i>araD araC<sup>am</sup> hemA lacZ<sup>am</sup> mal<sup>am</sup> relA rpsL trp<sup>am</sup> trrT</i> , unable to synthesize porphyrin	Robert Kadner, University of Virginia, USA
SAB11	derived from HB101, Sm <sup>r</sup> <i>ara-14 entC galk2 hsdM hsdR lacY1 leu mtl-1 proA2 supE44 xyl-5</i> , unable to make enterobactin or transport haemin	Barghouthi <i>et al.</i> , 1991
SY327( $\lambda$ pir)	F <sup>-</sup> Nal <sup>r</sup> Sm <sup>r</sup> <i>araD</i> $\Delta$ ( <i>lac-pro</i> ) <i>argE<sup>am</sup> rif nalA recA56</i> ( $\lambda$ pirR6K)	Donnenberg & Kaper (1991)
<b>Plasmids</b>		
pACYC184	Cloning vector, Cm <sup>r</sup> Tc <sup>r</sup>	Ausubel <i>et al.</i> (1987)
pBluescript KS+	Cloning vector, Ap <sup>r</sup>	Stratagene
pBR322	Cloning vector, Ap <sup>r</sup> Tc <sup>r</sup>	Ausubel <i>et al.</i> (1987)
pCD1:: <i>MudI1734-73</i>	Km <sup>r</sup> Lcr <sup>+</sup> <i>yopJ::MudI1734</i>	Straley & Bowmer (1986)
pCVD442	Suicide vector, Ap <sup>r</sup> Suc <sup>s</sup>	Donnenberg & Kaper (1991)
pCVDPSA1	Ap <sup>r</sup> Suc <sup>s</sup> , $\Delta$ psa, 4.2 kb <i>EcoRI-SalI</i> insert from the <i>psa</i> region in pCVD442	Bearden <i>et al.</i> (1997)
pHMS1.1	Cm <sup>r</sup> , <i>hmsHFRS<sup>+</sup></i> , 9.7 kb insert from pHMS1 in pACYC184	Pendrak & Perry (1991, 1993)
pHMS1.2	Ap <sup>r</sup> , <i>hmsHFRS<sup>+</sup></i> , 9.1 kb insert from pHMS1 in pBR322	Pendrak & Perry (1993)
pKNG101	Suicide vector, Sm <sup>r</sup> Suc <sup>s</sup>	Kaniga <i>et al.</i> (1991)
pKNGHMSR46	Sm <sup>r</sup> Suc <sup>s</sup> , 1 kb <i>BstBI-SalI</i> insert from pNPM46 in pKNG101	This study
pNPM9	Cm <sup>r</sup> , <i>hmsRS<sup>+</sup></i>	Pendrak & Perry (1993)
pNPM46	Km <sup>r</sup> , <i>hmsHFS<sup>+</sup> hmsR46</i>	Lillard <i>et al.</i> (1997)
pNPM53	Ap <sup>r</sup> , <i>hmsS<sup>+</sup></i>	Lillard <i>et al.</i> (1997)

\*  $\Delta$ pgm by definition is a 102 kb deletion lacking *hmsHFRS* and the *ybt* regions. The 70 kb plasmid pCD1 is responsible for the low-calcium-reponse (Lcr<sup>+</sup>) phenotype. The 9.5 kb plasmid pPCP1 is necessary for generating pesticin, the pesticin immunity protein and plasminogen activator. The 101 kb plasmid pMT1 encodes murine toxin and capsular fraction 1 antigen. Resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline and sucrose are designated Ap<sup>r</sup>, Cm<sup>r</sup>, Km<sup>r</sup>, Sm<sup>r</sup>, Tc<sup>r</sup> and Suc<sup>r</sup>, respectively. The pH 6 antigen genes *psaA-F* are responsible for the Psa<sup>+</sup> phenotype.

*Y. pestis* strains marked with a + carry the wild-type 102 kb *pgm* locus, while those without have deletions/mutations in the *pgm* locus.

presumably due to a  $\Delta pgm$  mutation. All PMH used in this study was deferrated with Chelex-100 (Bio-Rad), which was previously shown by furnace atomic absorption spectroscopy to contain  $<0.3 \mu\text{M}$  residual iron (Staggs & Perry, 1991). *Y. pestis* cells were acclimated to iron-deficient or iron-sufficient growth conditions by two to three serial transfers in appropriately supplemented PMH. After three serial transfers of KIM6+ cells, cultures containing  $20 \mu\text{M FeCl}_3$ ,  $50 \mu\text{M}$  haemin or no additions contained one  $\text{CR}^-$  cell, in  $8 \times 10^2$ ,  $4 \times 10^3$  or  $2 \times 10^4$ , respectively. These variations in  $\Delta pgm$  deletions do not account for differences in the experiments described in this study. Similar mutations in KIM6-2008 were prevented from occurring by cultivation in PMH containing kanamycin. All cultures were of sufficient volume to maintain an approximate 10% media-to-flask-volume ratio despite repeated sampling and all experiments used early-, mid-, or late-exponential phase cells. For some experiments, cells were centrifuged and washed with PMH containing no additions to remove any exogenous haemin or  $\text{FeCl}_3$  before subsequent transfers. All glassware used for cells growing in PMH was soaked overnight ( $>12$  h) in Dichrol (Baxter Scientific Products) or chromic-sulfuric acid (46.3 g potassium dichromate per litre of  $\sim 11.5$  M sulfuric acid) and copiously rinsed with distilled and deionized water. Haemin (Sigma) was treated to remove inorganic iron as previously described (Staggs & Perry, 1991), dissolved in 10 mM NaOH and filter-sterilized. Conalbumin and  $\text{FeCl}_3$  (Sigma) were dissolved in sterile deionized water or in 10 mM HCl, respectively, and filter-sterilized. The growth of liquid cultures was measured with a Gilford 260 spectrophotometer.

*E. coli* EB53 cells were grown at 26 or 37 °C in nutrient broth with 85.6 mM NaCl (NB), NB supplemented with  $50 \mu\text{M}$  of the protoporphyrin IX precursor  $\delta$ -aminolevulinic acid (ALA) (Stojiljkovic & Hantke, 1992), or on NB containing 0.3 mM of the iron chelator 2,2'-dipyridyl (NBD) (Sigma); when appropriate, these media were solidified with 1.5% (w/v) Noble agar (Difco). *E. coli* SAB11 cells were cultivated at 26 or 37 °C in either LB broth or on solidified Tris/glucose/thymidine medium supplemented with 0.5 mM leucine and 1 mM proline (TGT) (Barghouthi *et al.*, 1991; Simon & Tessman, 1963). TGT containing either  $25 \mu\text{M}$  conalbumin (Sigma) or  $5 \mu\text{M}$  haemin was solidified with 1.4% (w/v) Noble agar. *Escherichia coli* RK4744 (Table 1) cells were grown at 37 °C in LB containing no additions,  $50 \mu\text{M}$  ALA or  $50 \mu\text{M}$  haemin.

When appropriate, antibiotics were added to the media as follows: ampicillin ( $100 \mu\text{g ml}^{-1}$ ), chloramphenicol ( $30 \mu\text{g ml}^{-1}$ ), kanamycin ( $50 \mu\text{g ml}^{-1}$ ), streptomycin ( $100 \mu\text{g ml}^{-1}$ ) and tetracycline ( $6.25 \mu\text{g ml}^{-1}$ ).

**Resistance to reactive oxygen species.** *Y. pestis* strains were grown at 26 °C through three serial transfers in PMH containing  $75 \mu\text{M}$  haemin or  $20 \mu\text{M FeCl}_3$ . For analysis of  $\text{H}_2\text{O}_2$  sensitivity, exponentially growing cells were collected, pelleted, washed and resuspended in PBS at  $10^8$  cells  $\text{ml}^{-1}$ . Cell suspensions were adjusted to 0.0, 0.5, 1.0, 2.0 or 2.5 mM  $\text{H}_2\text{O}_2$  and incubated with agitation for 30 min at 37 °C. Surviving cells were quantified by plating on TBA. The percentage survival at each  $\text{H}_2\text{O}_2$  concentration was calculated as the c.f.u.  $\text{ml}^{-1}$  of the suspension incubated with  $\text{H}_2\text{O}_2$ , divided by the c.f.u.  $\text{ml}^{-1}$  of the suspension with no additions, multiplied by 100. For a similar assay at 26 °C, concentrations of 0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 mM  $\text{H}_2\text{O}_2$  were used. Although a modest degree of autoagglutination or clumping occurs in PMH cultures of  $\text{Hms}^+$  cells at 26 °C, this did not affect the determination of c.f.u.  $\text{ml}^{-1}$  in control experiments (data not shown).

For determination of  $\text{O}_2^-$  susceptibility, *Y. pestis* cells were grown at 26 °C in PMH containing  $75 \mu\text{M}$  haemin or  $20 \mu\text{M FeCl}_3$ , as described above, transferred to unsupplemented PMH containing 0.0, 0.01, 0.1 or 1.0 mM of the  $\text{O}_2^-$  generator paraquat (Sigma) and grown with agitation at 37 °C. A similar experiment was performed at 26 °C in PMH containing 0.0, 0.1, 1.0 or 10 mM paraquat. Proliferation at 26 or 37 °C was monitored every 1.5 h. The percentage survival was calculated as the  $\text{OD}_{620}$  of cells incubated in paraquat divided by the  $\text{OD}_{620}$  of cells incubated without paraquat, multiplied by 100.

To quantify resistance to killing by NO, *Y. pestis* cells were grown at 26 °C in PMH containing no additions,  $20 \mu\text{M FeCl}_3$  or  $75 \mu\text{M}$  haemin, and cell suspensions ( $10^8$  cells  $\text{ml}^{-1}$  in PBS) of each culture were prepared as in the  $\text{H}_2\text{O}_2$ -survival assay. Cell suspensions were adjusted to 0.0, 0.52, 1.04, 2.08 or 4.16 mM of the NO generator (+)-(E)-[(E)-hydroxyimino]-6-methoxy-4-methyl-5-nitro-3-hexeneamide (NOR-1; CalBiochem). The NO from NOR-1 is rapidly released (i.e. 1.5 min half-life) at 37 °C in pH 7.4 PBS (Fukuyama *et al.*, 1995; Kita *et al.*, 1994). Cells were incubated with agitation for 30 min at 37 °C. TBA plate counts were used to determine the percentage survival. The generation of NO from NOR-1 at 26 °C is much slower, making studies at this temperature impractical (manufacturer's recommendation). Results reported for  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$  and NO are from duplicate samples of duplicate experiments.

***Y. pestis* interactions with HeLa and PMN cells.** HeLa cells grown in Eagle's minimal essential medium (MEM; Fisher) with 5% foetal calf serum (Hyclone) (MEM/FCS) were used to examine the interaction of *Y. pestis* with eukaryotic cells. Briefly, HeLa cells were cultivated in flasks containing MEM/FCS plus  $75 \mu\text{g}$  each streptomycin, gentamicin and penicillin  $\text{ml}^{-1}$  at 37 °C at 5%  $\text{CO}_2$ . After growth to a confluent monolayer, cells were removed with 2.5% trypsin in modified Hank's balanced salt solution and centrifuged. Harvested HeLa cells were washed three times in MEM to remove antibiotics and adjusted to  $10^5$  cells  $\text{ml}^{-1}$  MEM/FCS. Approximately  $4 \times 10^4$  HeLa cells were dispensed into each well of a 24-well tissue culture microplate and incubated overnight at 37 °C in a 5%  $\text{CO}_2$  atmosphere (Devenish & Schiemann, 1981; Jakoby & Pastan, 1979). Where appropriate, HeLa cell-populated microplate wells received an m.o.i. of 10 *Y. pestis* cells that were previously grown in supplemented or unsupplemented PMH through two serial transfers and washed with MEM. Microplates were centrifuged in a free swinging microplate carrier at 600 g in a Sorvall RT6000B centrifuge for 10 min at room temperature to facilitate the association of the non-motile *Y. pestis* cells with the HeLa cell monolayer (Devenish & Schiemann, 1981). The monolayer was rinsed twice with MEM/FCS to remove non-adherent extracellular bacteria 15 min after infection. Cell-associated bacteria were released from HeLa cells by cold water disruption and c.f.u.  $\text{ml}^{-1}$  were determined by plating on TBA. The percentage of cell-associated *Y. pestis* was calculated as the c.f.u.  $\text{ml}^{-1}$  of adherent and intracellular bacterial cells divided by the total number of cells added  $\text{ml}^{-1}$ , multiplied by 100. For invasion analyses, extracellular bacteria were killed by a 1 h incubation in the presence of  $75 \mu\text{g}$  gentamicin  $\text{ml}^{-1}$  (Devenish & Schiemann, 1981). Viable intracellular bacteria were quantified as above.

For neutrophil survival studies, blood was drawn, following procedures approved by the University of Kentucky Internal Review Board, from healthy human subjects who had given their informed consent. Anti-coagulated whole blood samples were layered, without mixing, over an equal volume of PMN cell isolation solution (Robbins Scientific) and centrifuged at 500 g for 35 min. The middle band consisting of PMN cells

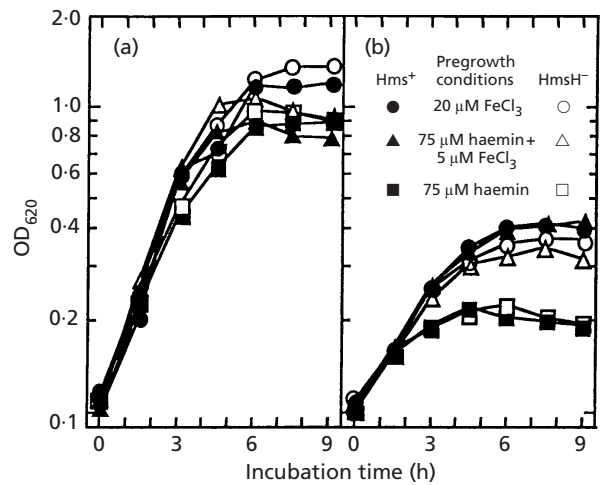
was harvested, rinsed and diluted to  $10^6$  PMN cells  $\text{ml}^{-1}$  with RPMI 1640 (Whitaker MA Bioproducts) containing 10% foetal calf serum and 1 mM glutamine (complete RPMI). Cell viability was determined by trypan blue dye exclusion. PMN cell purity was evaluated using the Diff-Quik Differential Staining system (Baxter Scientific Products) (Thompson, 1966). PMN cells ( $10^5$ ) were added to each well of a 96-well plastic microplate and incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 30 min prior to addition of bacteria. PMN cell monolayers were infected with *Y. pestis* cells resuspended in complete RPMI at an m.o.i. of 10. The percentage of cell-associated *Y. pestis* was calculated as described in the HeLa cell experiments. *Y. pestis* cells surviving exposure to PMN cells were quantified as the bacterial c.f.u.  $\text{ml}^{-1}$  present in PMN cell cultures from 0.5–4 h post infection divided by the c.f.u.  $\text{ml}^{-1}$  of cell-associated *Y. pestis* at time 0, multiplied by 100. Control experiments indicated that MEM/FCS and complete RPMI supported the growth of *Y. pestis* cells during the experimental time courses in the absence of HeLa or PMN cells (data not shown).

**Virulence testing.** *Y. pestis* cells were grown at  $26^\circ\text{C}$  in PMH containing  $50\ \mu\text{M}$  haemin, washed and diluted to the appropriate infectious dose in mouse-isotonic PBS. Four groups of five female NIH/Swiss Webster mice (5–7 weeks old) were infected subcutaneously (SC) with  $100\ \mu\text{l}$  10-fold serial dilutions of *Y. pestis* cell suspensions. The mice were observed daily for 21 d. SC LD<sub>50</sub>s were calculated according to Reed & Muench (1938). All procedures involving animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

## RESULTS

### Neither haemin nor ferric chloride adsorbed by the Hms system serve as nutritional sources of iron

To determine whether the Hms<sup>+</sup> phenotype functions to store organic or inorganic iron at  $26^\circ\text{C}$  (i.e. in the flea) for use as a nutritional source of iron during iron-restricted growth at  $37^\circ\text{C}$  (i.e. in mammals), we grew *Y. pestis* KIM6+ and KIM6-2008 (*hmsH2008::mini-kan*) cells in PMH in the presence of  $\text{FeCl}_3$ , haemin or both at  $26^\circ\text{C}$  through two serial transfers (8 generations). After pelleting and washing to remove any residual iron and/or haemin, cells were inoculated to OD<sub>620</sub> 0.1 incubated at  $37^\circ\text{C}$  in PMH with no additions and serially transferred up to five times, to test their ability to use adsorbed inorganic iron and/or haemin as a nutritional source. Transfers were performed when cultures attained OD<sub>620</sub> 0.7 for the first two transfers, 0.6 for the third transfer and 0.5 for the fourth transfer. If the Hms<sup>+</sup> strain were able to use haemin and/or iron adsorbed by the Hms system, Hms<sup>+</sup> cultures would have faster replication times and higher final yields than Hms<sup>-</sup> cultures during these serial transfers. However, there was no significant difference in the growth rate or final yield of Hms<sup>+</sup> cells compared to Hms<sup>-</sup> cells previously grown with either  $\text{FeCl}_3$  or  $\text{FeCl}_3$  and haemin (data not shown). Interestingly, Hms<sup>+</sup> cells previously grown with haemin alone did not grow as well as their Hms<sup>-</sup> counterparts. Indeed, by the third serial transfer, this culture never achieved the designated OD required for a fourth serial transfer. The growth rates of all cultures slowed and the final yields decreased with

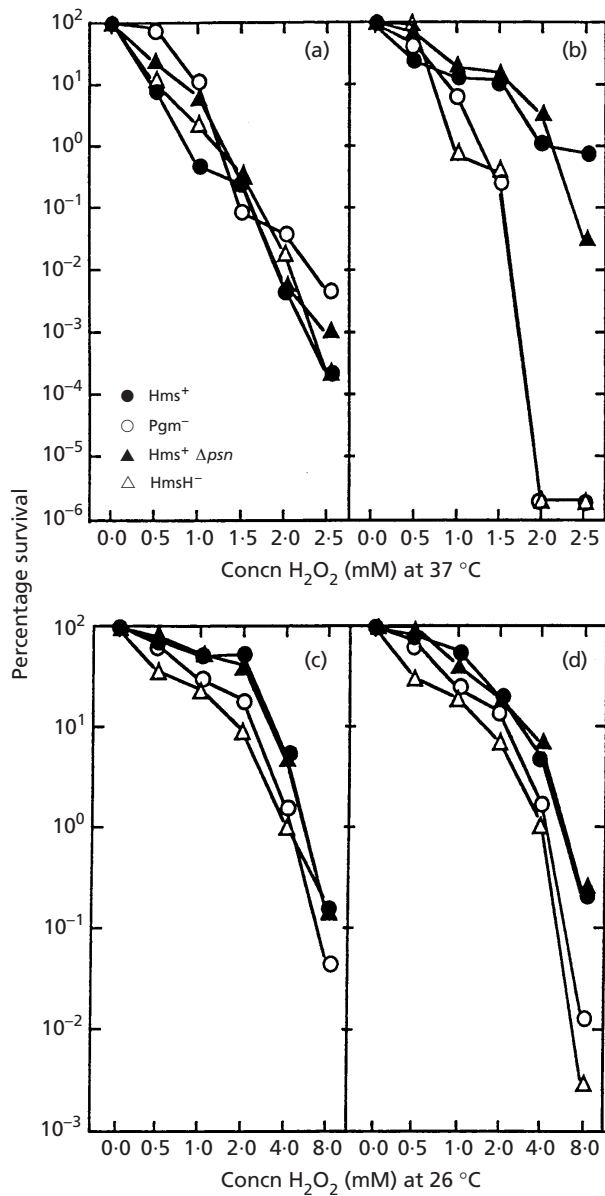


**Fig. 1.** Role of haemin and/or inorganic iron adsorption by the Hms system in iron-chelated growth at  $37^\circ\text{C}$ . Hms<sup>+</sup> (KIM6+) or Hms<sup>-</sup> (KIM6-2008) strains were grown at  $26^\circ\text{C}$  through two transfers in PMH supplemented with iron and/or haemin. Cultures were diluted to OD<sub>620</sub> 0.1, incubated at  $37^\circ\text{C}$  in PMH containing  $20\ \mu\text{M}$  EDDA and serially transferred (first and second transfers shown in panels a and b, respectively) when OD<sub>620</sub> reached 0.6. Results shown are from one of two replicate experiments.

progressive transfers suggesting that intracellular iron stores were being depleted (data not shown).

To further exacerbate iron starvation, we performed a similar experiment where cultures previously grown at  $26^\circ\text{C}$  were cultured at  $37^\circ\text{C}$  through two serial transfers into PMH containing the iron chelator EDDA (Fig. 1). Again, there was no significant difference in the growth rate of the Hms<sup>+</sup> compared to Hms<sup>-</sup> cultures. As in the previous experiment, growth of both strains was much poorer after growth with haemin at  $26^\circ\text{C}$  compared to previous growth with  $\text{FeCl}_3$  at  $26^\circ\text{C}$  (Fig. 1). However, collectively, these data show that neither  $\text{FeCl}_3$  nor haemin stored at the outer membranes by the Hms system were mobilized efficiently under the iron-starvation conditions we tested *in vitro*.

To determine if the Hms system can supply cells with either iron or porphyrin, we transformed various *E. coli* strains with defects in iron uptake and/or haem biosynthesis with recombinant plasmids encoding the entire *hmsHFRS* locus or vector plasmids. *E. coli* EB53 carries mutations in *hemA* and *aroB* (Eberspacher & Braun, 1980; Table 1), and consequently requires porphyrin precursors and added iron for growth. Approximately  $10^6$  cells of *E. coli* EB53(pHMS1.2) or EB53(pBR322) were seeded onto NB or iron-chelated NBD agar plates with equally spaced discs containing  $20\ \mu\text{l}$   $10\ \text{mM}$   $\text{FeCl}_3$ ,  $4.36\ \text{mM}$  haemin or  $10\ \text{mM}$  ALA. EB53(pHMS1.2) and EB53(pBR322) grew between the ALA and iron discs on NBD plates or around ALA discs (alone) on NB plates (data not shown). If the *hmsHFRS* locus encoded a haemin utilization system, EB53(pHMS1.2) would also have grown around the haemin disc on NBD plates.



**Fig. 2.** Role of haemin and/or inorganic iron adsorption by the Hms system in protection from  $\text{H}_2\text{O}_2$ .  $\text{Hms}^+$  [KIM6+ and KIM6-2045.1 ( $\Delta psn2045.1$ )] or  $\text{Hms}^-$  [KIM6 ( $\Delta pgm$ ) and KIM6-2008] cells were grown in PMH containing  $75 \mu\text{M}$  haemin (a, c) or  $20 \mu\text{M}$   $\text{FeCl}_3$  (b, d). After cultures reached  $\text{OD}_{620}$  0.5,  $10^8$  cells were exposed for 30 min to increasing concentrations of  $\text{H}_2\text{O}_2$  at  $37^\circ\text{C}$  (a, b) or  $26^\circ\text{C}$  (c, d).

Similar experiments using cloned genes of an authentic *Y. pestis* haem utilization system (Hmu) allowed EB53 cells to use exogenous haemin as an iron and porphyrin source (Hornung *et al.*, 1996). We also analysed the growth of *E. coli* RK4744 cells (which are unable to synthesize or transport haemin) containing pHMS1.1 or pACYC184 in LB containing ALA, haemin or no additions. For all cells, significant growth occurred only in LB supplemented with ALA. Thus, the *hms* locus did not allow transport of haemin across the *E. coli* outer

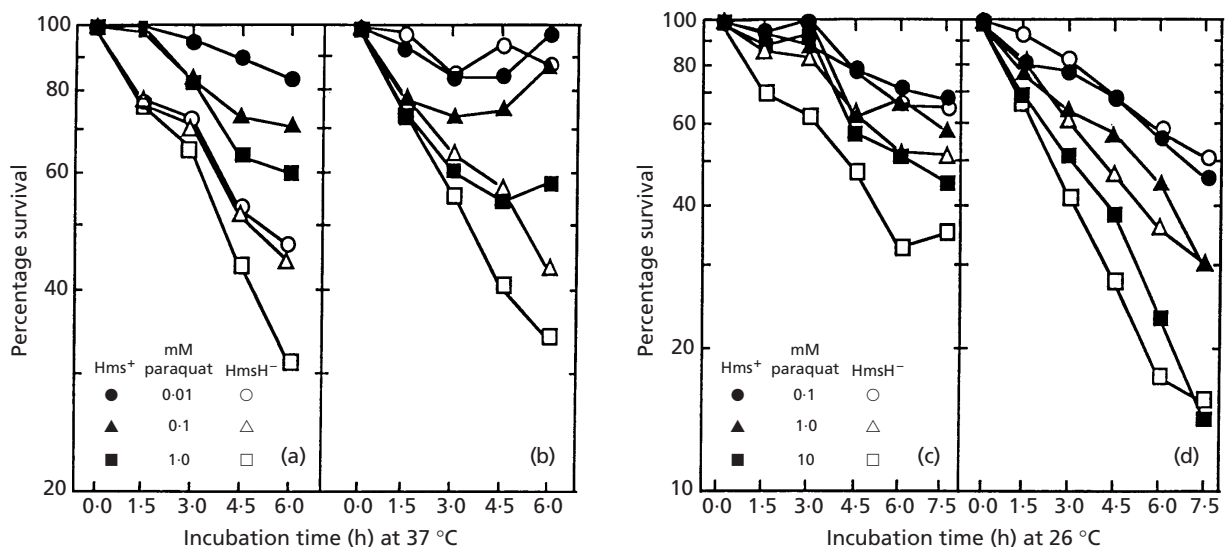
membrane. Finally, we tested *E. coli* SAB11, an *entC* mutant that is unable to synthesize enterobactin and, as a result, cannot grow in an iron-chelated medium without an iron source but can produce porphyrins (Barghouthi *et al.*, 1991). SAB11(pHMS1.1) and SAB11(pACYC184) cells were incapable of growth on TGT plates containing  $25 \mu\text{M}$  conalbumin and  $5 \mu\text{M}$  haemin. Taken together, these results suggest that the Hms system of *Y. pestis* is not involved in haemin utilization as either an iron or porphyrin source, at least in *E. coli*. Thus this system does not appear to play any role in iron acquisition for nutritional use.

### Resistance to the bactericidal effects of $\text{H}_2\text{O}_2$

Another possible function of the organic and inorganic iron adsorbed due to the  $\text{Hms}^+$  phenotype of *Y. pestis* would be to detoxify  $\text{H}_2\text{O}_2$  released by effector cells of the immune system (Aust *et al.*, 1985; Kim *et al.*, 1994). To test this, we grew two  $\text{Hms}^+$  [KIM6+ and KIM6-2045.1 ( $\Delta psn2045.1$ )] and two  $\text{Hms}^-$  [KIM6 ( $\Delta pgm$ ) and KIM6-2008 (*hmsH2008::mini-kan*)] strains of *Y. pestis* (Table 1), in PMH with haemin or  $\text{FeCl}_3$ . KIM6-2045.1 and KIM6 were used to determine whether the yersiniabactin iron transport system of *Y. pestis* would play a role in protection against the killing effects of  $\text{H}_2\text{O}_2$ . Exponentially growing cells in the third serial transfer were pelleted, washed and resuspended in PBS. Fig. 2 depicts the percentage survival of cells incubated for 30 min at either 26 or  $37^\circ\text{C}$  with increasing concentrations of  $\text{H}_2\text{O}_2$ . Higher  $\text{H}_2\text{O}_2$  concentrations were used for assays performed at  $26^\circ\text{C}$  since a  $9^\circ\text{C}$  decrease in temperature correlates with a 10-fold decrease in the bactericidal effect of  $\text{H}_2\text{O}_2$  (Dempfle *et al.*, 1983). There were no significant differences in resistance to  $\text{H}_2\text{O}_2$  between  $\text{Hms}^+$  or  $\text{Hms}^-$  strains that were grown with haemin (Fig. 2a, c). However,  $\text{Hms}^-$  strains grown in media containing added  $\text{FeCl}_3$  were strikingly more susceptible than the  $\text{Hms}^+$  strains to higher concentrations of  $\text{H}_2\text{O}_2$  (Fig. 2b, d). In assays performed at  $37^\circ\text{C}$ , there was 5 orders of magnitude difference between survival of  $\text{Hms}^+$  and  $\text{Hms}^-$  cells incubated with  $2.0 \text{ mM}$   $\text{H}_2\text{O}_2$  (Fig. 2b). At  $26^\circ\text{C}$ ,  $\text{Hms}^-$  cells were 10- to 100-fold more sensitive than  $\text{Hms}^+$  cells to  $8.0 \text{ mM}$   $\text{H}_2\text{O}_2$  (Fig. 2d). These findings show that the haemin adsorbed by the Hms system does not protect *Y. pestis* from the killing effects of  $\text{H}_2\text{O}_2$ . However, adsorption of inorganic iron by the Hms system, but not inorganic iron accumulated by the yersiniabactin system, significantly protected  $\text{Hms}^+$  cells from the lethal effects of  $\text{H}_2\text{O}_2$  *in vitro*.

### The $\text{O}_2$ generated by paraquat inhibits the growth of $\text{Hms}^-$ strains at $37^\circ\text{C}$ to a greater degree than $\text{Hms}^+$ strains

In addition to  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$  is another potent bactericidal host defence molecule that is generated by a number of immune cells (Yost & Fridovich, 1974). Resistance to  $\text{O}_2^-$  correlates with virulence and persistence of some pathogens in their respective hosts (Jackett *et al.*, 1978).

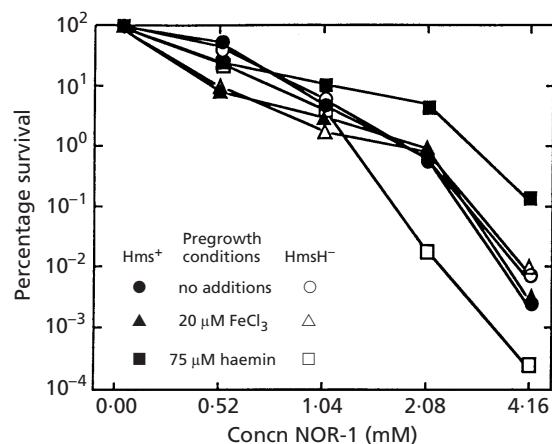


**Fig. 3.** Role of haemin and/or inorganic iron adsorption by the Hms system in protection against  $O_2^-$ . Hms<sup>+</sup> (KIM6+) or Hms<sup>-</sup> (KIM6-2008) strains were grown in PMH containing 75  $\mu$ M haemin or 20  $\mu$ M  $FeCl_3$  at 26 °C through two transfers. Following 8 h growth during the second transfer, haemin-supplemented (a, c) or iron-supplemented (b, d) cells were transferred into unsupplemented PMH containing increasing concentrations of the  $O_2^-$  generator paraquat at 37 °C (a, b) or 26 °C (c, d). The standard deviation for each condition was < 1%.

To determine if the haemin or inorganic iron adsorbed by the Hms system protects *Y. pestis* against the killing effects of  $O_2^-$ , exponentially growing KIM6+ and KIM6-2008 cells from the third PMH transfer were exposed to increasing concentrations of paraquat (methyl viologen), a quinone that catalyses the breakdown of oxygen to superoxide (Trudinger, 1970). Like  $H_2O_2$ , the killing effects of  $O_2^-$  are diminished at 26 °C compared to 37 °C, so the paraquat concentrations used at 26 °C were increased 10-fold over those at 37 °C. Cultures were grown in  $FeCl_3$  or haemin, incubated with paraquat at 26 or 37 °C and monitored for 6–7.5 hours (Fig. 3). At 37 °C, Hms<sup>+</sup> cells grown in the presence of haemin exhibited a 30–40% lower sensitivity to  $O_2^-$  compared to Hms<sup>-</sup> cells at all concentrations of paraquat (Fig. 3a). Similar results were obtained for cells grown in  $FeCl_3$  with the exception of Hms<sup>-</sup> cells exposed to the lowest concentration of paraquat; at this concentration Hms<sup>-</sup> cells survived as well as their Hms<sup>+</sup> counterparts (Fig. 3b). In the 26 °C assay, there was essentially no difference in survival of the Hms<sup>+</sup> and Hms<sup>-</sup> cells grown in haemin or  $FeCl_3$  (Fig. 3c, d). These results suggest that haemin and  $FeCl_3$  adsorbed to the outer membranes of Hms<sup>+</sup> cells provided modest *in vitro* protection against the bactericidal effects of  $O_2^-$  at 37 °C but not at 26 °C.

#### NO, produced by NOR-1, has a greater toxic effect on Hms<sup>+</sup> cells grown in haemin than on Hms<sup>-</sup> cells

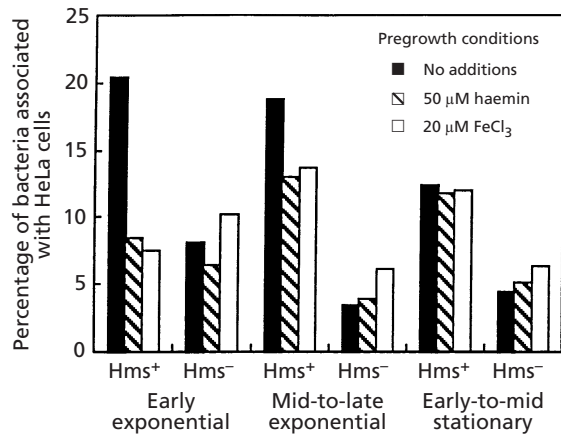
Several Gram-negative pathogens are cleared from their respective hosts by NO (De Groote & Fang, 1995; Fang, 1997). NO is a reactive oxygen species with an affinity for haem and iron-sulfur proteins (Morris *et al.*, 1995).



**Fig. 4.** Role of haemin and/or inorganic iron adsorption by the Hms system in protection against NO. Hms<sup>+</sup> (KIM6+) or Hms<sup>-</sup> cells were grown at 26 °C in PMH containing no additions, 20  $\mu$ M  $FeCl_3$  or 75  $\mu$ M haemin for two transfers. After second transfer cultures reached the middle of their exponential growth phase,  $10^8$  cells were added to increasing concentrations of the NO donor NOR-1 and incubated at 37 °C. The standard deviation for each condition was < 1%.

To test whether the haemin or inorganic iron adsorbed in the outer membranes of Hms<sup>+</sup> cells can protect against NO, we incubated *Y. pestis* cells, previously grown under three different iron conditions, with increasing concentrations of the NO generator NOR-1.

There were essentially no differences in resistance to NO between the Hms<sup>+</sup> or Hms<sup>-</sup> cells grown in PMH with no additions or  $FeCl_3$  (Fig. 4). However, the Hms<sup>+</sup> cells



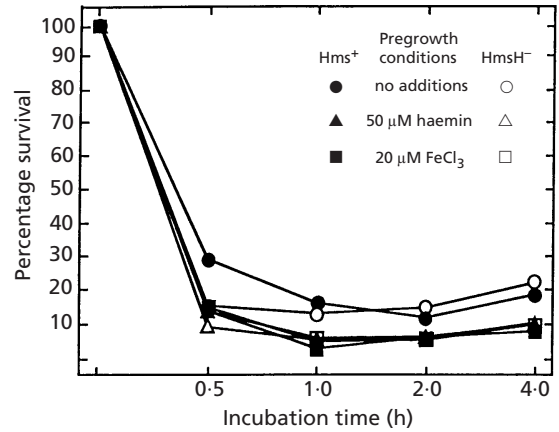
**Fig. 5.** Role of haemin and/or inorganic iron adsorption by the Hms system in association with HeLa cells. Hms<sup>+</sup> (KIM6+) or Hms<sup>-</sup> (KIM6-2008) cells were grown at 26 °C in PMH with no additions, 20 μM FeCl<sub>3</sub> or 75 μM haemin for two transfers. After second transfer cultures reached early exponential, mid-to-late exponential, or early-to-mid stationary phase, cells were added to HeLa cell monolayers at an m.o.i. of 10. Results shown are the means of duplicate samples from two independent experiments. The standard deviation for each condition was <1.5%.

grown with haemin had a 10-fold higher survival rate than cells grown in iron-replete or iron-deficient conditions and a 1000-fold higher survival rate at 2.08 and 4.16 mM NOR-1, respectively, than similarly cultured Hms<sup>-</sup> cells (Fig. 4), suggesting that absorbed haemin, but not FeCl<sub>3</sub>, can protect Hms<sup>+</sup> cells from nitric oxide.

**Y. pestis interactions with HeLa cells**

To determine if the Hms<sup>+</sup> phenotype is involved in the interaction of *Y. pestis* with eukaryotic cells, KIM6+ and KIM6-2008 cells grown at 26 °C in PMH with 20 μM FeCl<sub>3</sub>, 50 μM haemin or no additions were isolated during early exponential, mid-to-late exponential, or early-to-mid stationary phase, washed and allowed to adhere to a HeLa cell monolayer. After 4 h exposure, <0.1% of *Y. pestis* cells reached the intracellular space of the HeLa cell monolayer (data not shown) suggesting that the Hms phenotype has no effect on cell invasion.

However, under most growth conditions, Hms<sup>+</sup> cells adhered to HeLa cells significantly better than Hms<sup>-</sup> cells. After iron-deficient growth, there was 2.5-, 5.4- and 2.8-fold more association with HeLa cells by early exponential, mid-to-late exponential and early-to-mid stationary phase Hms<sup>+</sup> cells, respectively, than Hms<sup>-</sup> cells (Fig. 5). There was essentially no difference in adherence between early exponential phase Hms<sup>+</sup> and Hms<sup>-</sup> cells grown in PMH plus haemin or FeCl<sub>3</sub>. However, there was 2.8- and 2.1-fold higher association with HeLa cells by mid-to-late exponential and early-to-mid stationary phase Hms<sup>+</sup> cells, respectively, compared



**Fig. 6.** Role of haemin and/or inorganic iron adsorption by the Hms system in protection against PMN cells. Hms<sup>+</sup> (KIM6+) or Hms<sup>-</sup> (KIM6-2008) cells were grown at 26 °C in PMH with no additions, 20 μM FeCl<sub>3</sub> or 50 μM haemin for two transfers. After second transfer cultures reached mid-to-late exponential phase, cells were added to PMN cell monolayers at an m.o.i. of 10. Percentage survival of *Y. pestis* cells was determined at 0.5, 1.0, 2.0 or 4.0 h exposure to PMN cells. Results are from one of two independent experiments with PMN cells isolated from different donors. The standard deviation for each condition was <0.75%.

to Hms<sup>-</sup> cells grown in PMH plus haemin or FeCl<sub>3</sub>. In general, the largest differences in HeLa cell association between Hms<sup>+</sup> and Hms<sup>-</sup> cells occurred in mid-to-late exponential phase cells grown under any of the iron conditions tested (Fig. 5).

**Y. pestis interactions with PMN cells**

We also tested whether haemin or inorganic iron adsorbed by the outer membranes of Hms<sup>+</sup> cells could protect against the bactericidal effect of PMN cells. KIM6+ and KIM6-2008 *Y. pestis* cells were grown and isolated during mid-to-late exponential growth, as in the HeLa cell interaction experiments. *Y. pestis* cells were washed and allowed to adhere to a PMN cell monolayer. For all pregrowth conditions, there was 1.7-fold more adherence to PMN cells by Hms<sup>+</sup> than Hms<sup>-</sup> cells (data not shown). However, there was essentially no difference between the survival rate of Hms<sup>+</sup> and Hms<sup>-</sup> *Y. pestis* cells (Fig. 6). After 0.5 h incubation with PMN cells, 28% of adherent Hms<sup>+</sup> cells grown under iron deficient conditions versus 10–15% of the other cultures survived the antibacterial actions of the PMN cells. The reduction in percentage survival continued up to 2 h, after which, either the killing actions of the PMN cells in the monolayer diminished or the proliferation of *Y. pestis* cells for all pregrowth conditions increased. While there was consistently a slightly higher (5–10%) survival of *Y. pestis* cells grown under iron-deficient conditions than cells pregrown in iron-replete PMH, this effect was independent of the Hms phenotype and negligible. Thus neither the Hms<sup>+</sup> phenotype nor the presence of haemin

or inorganic iron protects *Y. pestis* from the bactericidal actions of PMN cells.

### LD<sub>50</sub> analysis of mice infected SC with Hms<sup>+</sup> and Hms<sup>-</sup> *Y. pestis* cells

To determine whether the Hms<sup>+</sup> phenotype affects the virulence of *Y. pestis* for mammals, we tested Hms<sup>+</sup> KIM5-2053.11+ and Hms<sup>-</sup> KIM5-2057.31(*hmsR46*) cells for virulence (LD<sub>50</sub>) in mice via an SC route. KIM5-2053.11+ had an LD<sub>50</sub> of 131 while the LD<sub>50</sub> for KIM5-2057.31 was <8. This difference corresponds to a >16-fold increase in virulence of the Hms mutant compared to the Hms<sup>+</sup> strain in this model of bubonic plague.

## DISCUSSION

Although CR- and/or haemin-binding phenotypes have been observed in a number of pathogenic organisms, including *A. salmonicida* (Kay *et al.*, 1985), enteroinvasive *E. coli* (Stugard *et al.*, 1989), *Neisseria meningitidis* (Payne & Finkelstein, 1977), *Porphyromonas gingivalis* (Genco *et al.*, 1994), *Shigella flexneri* (Daskaleros & Payne, 1987; Stugard *et al.*, 1989), *Treponema* species (Scott *et al.*, 1993), *Vibrio cholerae* (Payne & Finkelstein, 1977), *Yersinia enterocolitica* (Prpic *et al.*, 1983) and highly variable pigmented *Yersinia pseudotuberculosis* (Burrows, 1973), none of these phenotypes appears to be analogous to the Hms phenotype of *Y. pestis*. In some of the better defined CR-binding systems, like the S-layer proteins of *A. salmonicida* or the *crb* system of *S. flexneri*, CR adsorption has been associated with virulence, host cell attachment, protection from complement, proteolytic activity, host cell invasion and/or haemin binding (Daskaleros & Payne, 1987; Garduno & Kay, 1992).

In *Y. pestis*, the Hms<sup>+</sup> phenotype is responsible for the adsorption of haemin, inorganic iron and CR (Perry *et al.*, 1990, 1993), and is required for blockage of the proventriculus and virulence in fleas (Hinnebusch *et al.*, 1996; Kutyrev *et al.*, 1992). However, the role of the Hms<sup>+</sup> phenotype in mammalian virulence is unclear. Although *in vitro* temperature regulation suggests that the Hms phenotype would be expressed only in the early stages of mammalian infection, possible Hms functions affecting virulence in mammals include use of haemin adsorbed during growth in the flea as a nutritional source of iron during mammalian infection, transport of haemin, adherence and/or invasion of host cells, and resistance to killing by reactive oxygen species (Perry, 1993). We tested each of these potential roles of the Hms system in this study.

*Y. pestis* appears to possess a bacterioferritin iron-storage system (Lucier *et al.*, 1996); use of this intracellular store of iron probably accounts for the increasing generation times and decreasing final yields that occur with continued growth in iron-deficient PMH. If the additional haemin or inorganic iron adsorbed by the Hms system can subsequently be

mobilized to serve as a nutritional iron source during iron-starvation conditions, then Hms<sup>+</sup> cells should have a faster growth rate and/or higher yield than Hms<sup>-</sup> cells in an iron-restricted medium. However, neither haemin nor inorganic iron adsorbed by the Hms system was effectively used as nutritional iron sources during subsequent growth in deferrated PMH. Since the yersiniabactin iron transport system allows continued growth in this medium (data not shown), we also tested this hypothesis under conditions that used EDDA to completely restrict iron acquisition; again no significant differences between Hms<sup>+</sup> and Hms<sup>-</sup> cultures were observed (Fig. 1). Other differences in growth rate or yield were due to previous growth conditions and not the Hms phenotype. For instance, haemin-loaded Hms<sup>+</sup> and Hms<sup>-</sup> cells grew more poorly in an iron-chelated environment than iron-loaded cells (Fig. 1). This suggests that haemin is a poorer iron source or is not stored as efficiently as inorganic iron. In addition, Hms<sup>+</sup> cells previously grown with haemin exhibited poorer growth compared to similarly treated Hms<sup>-</sup> cells and were unable to sustain growth past the third serial transfer in iron-deficient PMH (data not shown). This suggests that the Hms<sup>+</sup> phenotype may sequester haemin and prevent it from entering intracellular storage pools. This Hms adsorption effect has also been observed with guanine. At 26 °C, an Hms<sup>+</sup> *Y. pestis* purine auxotroph adsorbs exogenous guanine, which prevents its nutritional use (Brubaker, 1970). Our results clearly show that haemin or iron adsorbed by the Hms system was not available for nutritional use.

We also investigated the possibility that the *hmsHFRS* operon encodes a haemin or inorganic iron transport system. However, none of the *E. coli* iron and haemin metabolic mutants that contained the *hmsHFRS* genes were able to use haemin as an iron or porphyrin source. While *E. coli* may not contain all the genes necessary for the *hmsHFRS* operon to function as a transport system, the *Y. pestis* haemin transport system (Hmu) does function in *E. coli*. In addition, an Hms<sup>+</sup> Hmu<sup>-</sup> *Y. pestis* mutant is unable to utilize haemin; this indicates that the Hms<sup>+</sup> phenotype cannot functionally replace the Hmu haemin transport system (Hornung *et al.*, 1996). These results suggest that the *hmsHFRS* locus of *Y. pestis* does not encode a haemin uptake system nor mobilize adsorbed iron/haemin for nutritional use. Therefore, haemin adsorbed by the Hms system is not used nutritionally.

Another possibility is that organic and inorganic iron adsorbed by the Hms system protects *Y. pestis* from the bactericidal effects of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and/or NO. While iron can catalyse production of reactive oxygen species that cause extensive damage to biological macromolecules (Morris *et al.*, 1995), it can also decompose O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and OH<sup>•</sup> (Griffiths, 1987). Similarly, the toxic effect of NO is mediated by its affinity for haem and iron-sulfur binding proteins (Fang, 1997; Morris *et al.*, 1995). Thus, these reactive oxygen species may be inactivated by the haemin or iron adsorbed by the Hms system. Our results suggest that, under certain conditions, the Hms system

provides varying degrees of protection against different reactive oxygen species.

While adsorbed haemin did not protect cells against  $H_2O_2$ ,  $Hms^+$  cells loaded with  $FeCl_3$  were 5 orders of magnitude less sensitive than  $Hms^-$  cells to the higher concentrations of  $H_2O_2$ , although the overall survival of these cells was quite low (Fig. 2).  $Hms^+$  *Y. pestis* cells grown at 26 °C in PMH with  $FeCl_3$  exhibit a small degree of clumping (data not shown). While this autoagglutination might provide some protection against  $H_2O_2$  for internal cells in the clump, cultures grown in PMH without additions also autoagglutinate and did not survive the lowest concentration of  $H_2O_2$  used (data not shown). Hence, the protective effect was not due to autoagglutination caused by the  $Hms^+$  phenotype.

Exposure to  $O_2^-$  at 37 °C, resulted in a 20–40% higher survival rate for  $Hms^+$  cells previously grown in haemin than for similarly grown  $Hms^-$  cells (Fig. 3a); except for the highest paraquat concentration, survival at 26 °C did not correspond to the  $Hms^+$  phenotype (Fig. 3c). For cells loaded with  $FeCl_3$  (Fig. 3b, d) differences in resistance to  $O_2^-$  were observed only at 37 °C at paraquat concentrations of 0.1 and 1.0 mM. These results suggest that haemin and, to a lesser degree, inorganic iron adsorption provided modest protection against *in vitro* bactericidal effects of  $O_2^-$ .

In addition to  $O_2^-$  and  $H_2O_2$ , murine and human immune cells use NO to clear infectious microbes (De Groote & Fang, 1995; Fang, 1997). Pathogens possessing a defence against NO would have a distinct advantage over microbes that did not. The lipoarabinomannan acids from *M. tuberculosis* and the iron-dependent superoxide dismutase from *S. typhimurium* have been shown to indirectly inhibit the effects of NO (Anthony *et al.*, 1994; Fang, 1997). We tested whether the  $Hms^+$  phenotype could protect *Y. pestis* from NO. There was essentially no variance in vulnerability to NO between cells grown in PMH with no additions (iron-starved) or  $FeCl_3$ . However at the highest concentration of NOR-1,  $Hms^+$  cells grown in haemin had a >10-fold higher survival rate than cells grown in  $FeCl_3$  or no additions and a 1000-fold higher survival rate than  $Hms^-$  cells grown with haemin (Fig. 4). NO has a high affinity for haem and non-haem iron in prosthetic groups of proteins and must be internalized to carry out its deleterious effects (De Groote & Fang, 1995; Fang, 1997; Morris *et al.*, 1995). Both  $Hms^+$  and  $Hms^-$  cells have a functional haemin transport system (Hornung *et al.*, 1996). However, in the  $Hms^+$  cells grown with haemin, NO could interact with the haemin bound to the outer membrane. In addition,  $Hms^-$  cells may internalize more haemin via the Hmu transport system, increasing their sensitivity. Thus, outer-membrane adsorption of haemin may be responsible for protecting  $Hms^+$  cells from NO.

While our results suggest the Hms system can protect cells against reactive oxygen species generated *in vitro*, the real question is whether the Hms system enhances

survival of *Y. pestis in vivo*. Since PMN cells are among the first immune cells to arrive at an inflammatory focus (i.e. flea bite), resistance to, or evasion of, PMN cells would be a distinct survival advantage. Early studies demonstrated that *Y. pestis* cells are readily phagocytized and destroyed by PMN cells (Cavanaugh & Randall, 1959). However, these studies were performed with  $Hms^+$  cells grown in the absence of haemin. Therefore we tested whether haemin adsorbed by the Hms system would play a part in interactions with PMN cells.  $Hms^+$  cells adhered to PMN cells at slightly higher levels than  $Hms^-$  cells (21% versus 13%; data not shown). Despite this, there was no major difference between the survival of  $Hms^+$  and  $Hms^-$  *Y. pestis* cells incubated with PMN cells, whether adsorbed haemin or inorganic iron was present or absent. These results suggest that while the  $Hms^+$  phenotype enhanced adherence to PMN cells, it is not involved in protecting *Y. pestis* from the anti-microbial effects of these host cells.

In *A. salmonicida*, haemin-binding by the A-layer increases the association of the bacteria with macrophages (Garduno & Kay, 1992). Similarly, binding of haemin to *S. flexneri* enhances their uptake into HeLa cells and correlates with host cell invasion (Daskaleros & Payne, 1987; Stugard *et al.*, 1989). Both  $Hms^+$  and  $Hms^-$  cells of *Y. pestis* are readily taken up by macrophages (Charnetzky & Shuford, 1985; Straley & Harmon, 1984). However, previous studies showed that *Y. pestis* cells grown at 37 °C did not efficiently invade HeLa cells (Sikkema & Brubaker, 1987). Since the  $Hms^+$  phenotype is maximally expressed at 26 °C (Pendrak & Perry, 1991), we tested whether the  $Hms^+$  phenotype of *Y. pestis* could affect invasion of, or adherence to, HeLa cells. We found that, in agreement with the observations of Sikkema & Brubaker (1987), *Y. pestis* invaded HeLa cells at an insignificant rate (<0.1%), indicating that the Hms system is not involved in invasion. However, adherence of *Y. pestis* cultures to HeLa cells showed some interesting differences. The highest level of attachment to HeLa cells, which also correlates with Hms-induced autoagglutination, was achieved by mid-to-late exponential phase  $Hms^+$  cells under all growth conditions (Fig. 5). Up to 5-fold more mid-to-late exponential phase  $Hms^+$  cells adhered to a HeLa cell monolayer than  $Hms^-$  cells. Interestingly, the Hms system appears to enhance association of *Y. pestis* cells with the proventriculus of the flea (Hinnebusch *et al.*, 1996) just as it consistently increased association with HeLa cells (Fig. 5).

To definitively determine whether the  $Hms^+$  phenotype has a vital role in mammalian virulence, we conducted LD<sub>50</sub> testing in mice.  $Hms^+$  and  $Hms^-$  cells were grown at 26 °C with haemin and injected SC to simulate a flea bite route of infection. The *hmsR46* mutant showed a >16-fold increase in virulence compared to the  $Hms^+$  strain. Thus, expression of the Hms system is detrimental to pathogenesis in mammals. However, this modest adverse effect is probably negligible in nature given the extremely low LD<sub>50</sub> of the  $Hms^+$  strain.

The results of this study failed to provide evidence that supported any of the postulated functions of the *Y. pestis* Hms system in mammalian virulence. However, we did observe increased adherence of Hms<sup>+</sup> cells to PMN cells (2-fold) and HeLa cells (5-fold). Since more Hms<sup>+</sup> than Hms<sup>-</sup> cells adhered to PMN cells without diminishing the killing potential of these host cells, the Hms system may account for the moderately increased virulence of Hms<sup>-</sup> cells we observed in mice. Consequently, the only demonstrated physiological role of the *Y. pestis* Hms<sup>+</sup> phenotype remains virulence in fleas and blockage of the flea proventriculus that probably increases transmission of plague from fleas to mammals (Hinnebusch *et al.*, 1996; Kuttyrev *et al.*, 1992). This may also correlate with the enhanced adherence to mammalian cells and autoagglutination reactions that we observed. The mechanism for these features may involve glycosyl transferase activity. HmsR shows 52.2% amino acid similarity to IcaA of *Staphylococcus epidermidis*. The IcaABC system appears to be necessary for expression and secretion of a polysaccharide intercellular adhesin that promotes cell aggregation (Heilmann *et al.*, 1996). However, additional experiments must be performed to determine if a similar mechanism is involved in the Hms-dependent adherence we observed and whether the Hms<sup>+</sup> specific effects of resistance to reactive oxygen species and/or adherence play a role in the flea.

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