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HmsT, a protein essential for expression of the haemin storage (Hms$^+$) phenotype of *Yersinia pestis*

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The haemin storage (Hms) phenotype of *Yersinia pestis* has been shown to be involved in the blockage of fleas that is required for the transmission of plague from fleas to mammals. Previously, an operon encoding four genes, *hmsHFRS*, that are essential for the temperature-regulated Hms$^+$ phenotype has been characterized. Here the isolation and characterization of a fifth gene, *hmsT*, that is essential for this phenotype is described. Conceptual translation of *hmsT* suggests it encodes a 44·8 kDa protein with a pI of 7·75. The gene for HmsT is located outside of the ~102 kb *pgm* locus of *Y. pestis* that contains the *hmsHFRS* operon. Hybridization studies indicate that *Yersinia pseudotuberculosis* but not *Yersinia enterocolitica* or *Escherichia coli* possesses a highly homologous gene. HmsT belongs to a family of PleD-related proteins with four highly conserved regions of homology. Although PleD is a regulator, the functions of the other members of this family have not been experimentally determined. The iron-responsive regulator, Fur, has previously been implicated in temperature regulation of the Hms phenotype. A good potential Fur-binding site (FBS) is located upstream of *hmsT*. *Y. pestis* M23 and two of five *Y. pseudotuberculosis* strains, which all exhibit a temperature-constitutive Hms phenotype, contain a 6 bp insertion in the putative FBS. *E. coli* MG1655 contains homologues of *hmsHFRST* (*ycdSRQPT*) but has an Hms$^-$ phenotype. Only *ycdQ* and *ycdP* complement mutations in their respective homologues, *hmsR* and *hmsS*, in *Y. pestis*.

Keywords: haemin binding, plague, pigmentation phenotype, temperature regulation

INTRODUCTION

The pigmentation (Pgm$^+$) phenotype of *Yersinia pestis* was first described as the ability of cells grown on haemin agar to form greenish brown colonies at 26 °C (but not at 37 °C) and correlated with iron-independent virulence in mice (Jackson & Burrows, 1956a, b). Other characteristics subsequently associated with the Pgm$^+$ phenotype include sensitivity to pesticin (Brubaker, 1969; Une & Brubaker, 1984), production of several iron-regulated proteins (Fetherston *et al*., 1995; Fetherston & Perry, 1994; Sikkema & Brubaker, 1989) and growth in iron-chelated media at 37 °C (Fetherston *et al*., 1995; Sikkema & Brubaker, 1987, 1989). These characteristics are genetically linked on a 102 kb region of the *Y. pestis* chromosome termed the *pgm* locus. Spontaneous deletion of the *pgm* locus (Pgm$^-$ phenotype), probably mediated by the IS100 elements that flank this region, causes loss of all of the above characteristics (Fetherston & Perry, 1994; Fetherston *et al*., 1992; Lucier & Brubaker, 1992). Except for haemin adsorption, all Pgm characteristics have been linked to the yersiniabactin region, which encodes a siderophore-dependent iron transport system (Bearden *et al*., 1997; Gehring *et al*., 1998; Perry & Fetherston, 1997). *Y. pestis* and highly virulent strains of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* all possess the

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Abbreviations: CR, Congo red; FBS, Fur-binding site; Hms, haemin storage; OM, outer membrane; Pgm, pigmentation.

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yersiniabactin iron transport system but Y. *pestis* also encodes a haemin adsorption system, termed haemin storage (Hms; Carniel *et al*., 1989; Lillard *et al*., 1997; Perry & Fetherston, 1997; Perry *et al*., 1990; Schubert *et al*., 1998).

The *hms* locus, within the *pgm* locus, encodes four *hms* genes, *hmsH*, *hmsF*, *hmsR* and *hmsS*, that are necessary for the Hms^+ phenotype in Y. *pestis* (Fetherston *et al*., 1992; Lillard *et al*., 1997; Pendrak & Perry, 1991, 1993; Perry *et al*., 1990). While HmsH and HmsF appear to be outer-membrane (OM) proteins (Lillard *et al*., 1997; Pendrak & Perry, 1991), the cellular location(s) of HmsR and HmsS are undetermined. Although sequence analysis (Lillard *et al*., 1997) did not reveal a Fur-binding site (FBS) in the promoter region of *hmsHFRS*, a Y. *pestis fur* mutant exhibits an Hms phenotype that is constitutive (Hms^c) with respect to temperature (Hms^+ at both 26 and 37 °C) (Staggs *et al*., 1994).

Hinnebusch *et al*. (1996) discovered that Y. *pestis* strains expressing the Hms^+ phenotype colonize and eventually block the proventriculus of the Oriental rat flea, *Xenopsylla cheopis*; Hms^- strains are able to establish an infection within the flea midgut but do not colonize and block the proventriculus. Blockage of the flea foregut results in repeated attempts to feed and is required for effective transmission of the plague bacillus to the mammal (Bacot, 1915; Bacot & Martin, 1914; Pollitzer, 1954). An investigation examining possible roles for the Hms^+ phenotype in the early stages of mammalian infections failed to demonstrate a role for this system in mammalian plague (Lillard *et al*., 1999).

Temperature appears to regulate the Hms^+ phenotype in Y. *pestis*. Both Pgm^+ KIM6+ and Pgm^- KIM6 (Δ*pgm*) cells carrying pHMS1, a low-copy-number plasmid encoding HmsHFRS, retain a temperature-regulated Hms^+ phenotype. However, studies with Pgm^- Y. *pestis* M23 derivatives containing pHMS1 exhibit an Hms^c phenotype (Lillard *et al*., 1997; Pendrak & Perry, 1991, 1993; Perry *et al*., 1990). This suggests that additional factor(s) encoded outside the *pgm* locus may be required for regulating or establishing an Hms^+ phenotype (Lillard *et al*., 1997). Here we report on the cloning and characterization of a gene, *hmsT*, found outside the *pgm* locus that is required for expression of the Hms^+ phenotype in Y. *pestis*.

METHODS

Bacterial strains, media and plasmids. The plasmids and bacterial strains used in these experiments are listed in Table 1. All Y. *pestis* strains are completely avirulent since they lack the low-calcium-response virulence plasmid pCD1 (Perry & Fetherston, 1997). All Y. *pestis*, Y. *pseudotuberculosis* and Y. *enterocolitica* strains were grown in heart infusion broth or on Tryptose Blood Agar Base and all *Escherichia coli* strains were grown in either Luria broth or Terrific broth and on Luria broth agar plates. Testing of various *E. coli*, Y. *pestis*, Y. *pseudotuberculosis* and Y. *enterocolitica* strains for the Hms phenotype was performed on Congo red (CR) agar (Surgalla & Beesley, 1969). When required, media contained ampicillin at 100 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, chloramphenicol at 30 μg ml^{-1} or tetracycline at 12·5 μg ml^{-1}.

Recombinant DNA techniques. Plasmids were isolated from either *E. coli* or *Yersinia* strains by alkaline lysis (Birnboim & Doly, 1979) and, when necessary, further purified by polyethylene glycol precipitation (Humphreys *et al*., 1975). Plasmids were transformed into *E. coli* by a standard $CaCl_2$ procedure (Sambrook *et al*., 1989) or into *Yersinia* strains by electroporation (Fetherston *et al*., 1995). Genomic DNA was isolated by a modified lysozyme/SDS/proteinase K procedure (Fetherston *et al*., 1992). Experiments using restriction endonucleases or DNA-modifying enzymes were performed according to the manufacturer's specifications. Labelling of DNA fragments for Southern blot and colony blot analyses was achieved by random priming using [^{32}P]dCTP (New England Nuclear) and a Rediprime labelling kit (Amersham), as per the manufacturer's instructions. The internal *Hin*dIII fragment from *hmsT* (Fig. 1), the *Bam*HI fragment of the *Tn*10 derivative mini-*kan* (Perry *et al*., 1990), or the ~4 kb *Bam*HI fragment adjacent to the mini-*kan* insert in pAMHS4 (Fig. 1; Table 1) were used as probes in Southern blot and colony blot analyses.

Sequencing reactions were performed via the dideoxynucleotide chain termination method (Sanger *et al*., 1977) using [^{35}S]dATP (Amersham), Sequenase version 2.0 (Amersham/USB) and 7-deaza-dGTP. Samples were electrophoresed through a 6% polyacrylamide gel containing 8·3 M urea (Sigma) cast in Tris/borate/EDTA buffer (Sambrook *et al*., 1989). Dried gels were exposed at room temperature to Kodak Biomax MR film. Homology searches of DNA and protein databases were performed using BLAST (Altschul *et al*., 1990, 1997; Gish & States, 1993). Based on the sequence of the ~800 bp *Hin*dIII fragment containing a portion of *hmsT*, synthetic oligonucleotide primers were designed to extend and complete sequencing of both DNA strands. Sequence analyses and manipulations were done using the Intelligenetics software suite and protein analysis software at the ExPASy site (http://expasy.hcuge.ch/www/tools.html). Alignments were performed using CLUSTAL W (Thompson *et al*., 1994).

Clones containing the region around *hmsT* were identified from a *Bam*HI genomic library of Y. *pestis* KIM6+ DNA (Fetherston *et al*., 1992). The mutated *hmsT8* gene was cloned from Y. *pestis* strain M23 by isolation of 3·0–3·5 kb DNA fragments digested with *Eco*RI–*Pst*I; these fragments were ligated into pBR322. Authentic clones were identified by Southern and colony blot analyses. The *hmsT* promoter region from Y. *pseudotuberculosis* strains was amplified by PCR using the following oligonucleotides: 5′-CTCCTGGATCCCGTGAGGTATTTATTCCG-3′ and 5′-TTCCCGTTAACTATCTACCCAGCCCAGTA-3′. Reactions containing *Pfu* DNA polymerase, 0·2 mM dNTPs and 0·2 μM primers consisted of 30 s at 48 °C, 30 s at 72 °C and 30 s at 94 °C for 30 cycles. PCR products were cloned into pBluescript II KS+ for sequencing. To obtain the *E. coli ycdSRQPT* region, MG1655 genomic DNA was digested with *Acc*III and 6–12 kb fragments were isolated and ligated into the *Xma*I site of pBluescript II KS+. Clones containing the ~8·4 kb *ycdSRQPT* region were identified by PCR using the following oligonucleotides: 5′-TTTGACCAGCCCATCACCATC-3′ and 5′-AATTTGCCTGGCTGCGCGAGAA-3′. Reactions containing *Taq* DNA polymerase, 0·2 mM dNTPs and 0·2 μM primers consisted of 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C for 30 cycles. All oligonucleotides were purchased from Integrated DNA Technologies.

Protein analysis. *In vitro* transcription/translation using an

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics*	Source or reference
Y. pestis		
KIM6+	Hms$^+$	Fetherston *et al.* (1992)
KIM6	Hms$^-$ (Δ*pgm*)	Fetherston *et al.* (1992)
KIM6-2050	Hms$^-$ (*hmsT2050*::mini-*kan*)	This study
KIM6-2051	Hms$^-$ (*hmsT2051*::mini-*kan*)	This study
KIM6-2052	Hms$^-$ (*hmsT2052*::mini-*kan*)	This study
KIM6-2057.1	Hms$^-$ (Δ*hmsR*46, in-frame deletion)	Lillard *et al.* (1999)
M23+	Hms$^+$	Fetherston *et al.* (1992)
M23	Hms$^-$ (*hmsR8*)	Fetherston *et al.* (1992); Lillard *et al.* (1997)
M23-2	Hms$^-$ (Δ*pgm*)	Fetherston *et al.* (1992)
Y. enterocolitica		
WA-LOX	Serotype O:8; Pst^s	Perry & Brubaker (1983)
Y. pseudotuberculosis		
PB1/0	Serotype I; Pst^s	Perry & Brubaker (1983)
Neilson	Serotype I; Pst^r	R. R. Brubaker
EP2	Serotype II	R. R. Brubaker
43	Serotype III	R. R. Brubaker
YPIII	Serotype III; Pst^r	R. R. Brubaker
E. coli		
DH5α	Cloning strain	Ausubel *et al.* (1987)
HB101	Cloning strain	Ausubel *et al.* (1987)
MG1655	K-12 strain used in genome sequencing project	Blattner *et al.* (1997)
Plasmids		
pAHMS3	19 kb, Ap^r Tc^r, *hmsT2050*::mini-*kan*, 14·2 kb *Bgl*II *kan* fragment from KIM6-2050 ligated into pBGL2	This study
pAHMS4	19 kb, Ap^r Tc^r, *hmsT2051*::mini-*kan*, 14·2 kb *Bgl*II *kan* fragment from KIM6-2051 ligated into pBGL2	This study
pAHMS5	19 kb, Ap^r Tc^r, *hmsT2052*::mini-*kan*, 14·2 kb *Bgl*II *kan* fragment from KIM6-2052 ligated into pBGL2	This study
pAHMS8	10·5 kb, Cm^r, *hmsT*$^+$, 6·3 kb *Bam*HI fragment from pAHMS496 ligated into pACYC184	This study
pAHMS10	8·0 kb, Cm^r, 4·2 kb *Bam*HI–*Hin*dIII fragment from pAHMS8 ligated into pACYC184	This study
pAHMS10.1	6·8 kb, Ap^r, 4·2 kb *Bam*HI–*Hin*dIII fragment from pAHMS10 ligated into pUC18	This study
pAHMS11	5·2 kb, Cm^r, 1·6 kb *Bam*HI–*Hin*dIII fragment from pAHMS8 ligated into pACYC184	This study
pAHMS14	6·9 kb, Tc^r, *hmsT*$^+$, 3·3 kb *Pst*I–*Eco*RI fragment from pAHMS8 ligated into pBR322	This study
pAHMS14.1	6·9 kb, Tc^r, *hmsT8*, 3·3 kb *Pst*I–*Eco*RI fragment from chromosome of M23-2 ligated into pBR322	This study
pAHMS16	4·4 kb, Ap^r, *hmsT*$^+$, 1·9 *Kpn*I–*Fsp*I fragment from pAHMS14 ligated into *Kpn*I–*Hin*cII site of pUC18	This study
pAHMS16.1	5·6 kb, Tc^r, *hmsT*$^+$, 1·9 kb *Kpn*I–*Fsp*I fragment from pAHMS16 ligated into pBR322	This study
pAHMS16.3	10·1 kb, Ap^r, *hmsT*$^+$, *Eco*RI linkers added to 1·9 kb *Kpn*I–*Fsp*I fragment from pAHMS16 and ligated into *Eco*RI site of pLC8.2	This study
pAHMS17	6·9 kb, Tc^r, *hmsT8* promoter with *hmsT*$^+$ coding region, 2·5 kb *Pst*I–*Nde*I fragment from pAHMS14 + 0·7 kb *Nde*I–*Eco*RI fragment from pAHMS41.1 ligated into *Pst*I–*Eco*RI site of pBR322	This study
pAHMS17.2	11·4 kb, Ap^r, *hmsT8* promoter with *hmsT*$^+$ coding region, *Eco*RI linkers added to 3·3 kb *Pst*I–*Eco*RI fragment from pAHMS17 and ligated into *Eco*RI site of pLC8.2	This study

Table 1 (*cont.*)

Strain or plasmid	Relevant characteristics*	Source or reference
pAHMS68	~35·5 kb, Ap^r, *hmsT*$^+$, ~29·1 kb *Bam*HI insert ligated into pHC79	Fetherston *et al.* (1992); this study
pAHMS131	~45·7 kb, Ap^r, *hmsT*$^+$, ~39·3 kb *Bam*HI insert ligated into pHC79	Fetherston *et al.* (1992); this study
pAHMS496	~27·6 kb, Ap^r, *hmsT*$^+$, ~21·2 kb *Bam*HI insert ligated into pHC79	Fetherston *et al.* (1992); this study
pAHMS497	~27·6 kb, Ap^r, *hmsT*$^+$, ~21·2 kb *Bam*HI insert ligated into pHC79	Fetherston *et al.* (1992); this study
pEYCD1	11·4 kb, Ap^r, *E. coli ycdSRQPT*$^+$, ~8·4 kb *Acc*III fragment from MG1655 ligated into *Xma*I site of pBluescript II KS+	This study
pEYCD1.1	14·4 kb, Km^r, *E. coli ycdSRQPT*$^+$, ~8·4 *Bam*HI–*Asp*718 fragment from pECYD1 ligated into *Bam*HI–*Kpn*I sites of pLG338	This study
pHMS1	16·4 kb, Km^r, *hmsHFRS*$^+$, 9·1 kb *Sau*3AI insert ligated into *Bam*HI site of pLG338	Lillard *et al.* (1997); Perry *et al.* (1990)
pHMS1.1	13·9 kb, Cm^r, *hmsHFRS*$^+$, 9·7 kb *Sal*I–*Hin*dIII fragment from pHMS1 ligated into pACYC184	Lillard *et al.* (1997); Pendrak & Perry (1993)
pHMS1.2	13·4 kb, Ap^r, *hmsHFRS*$^+$, 9·7 kb *Sal*I–*Hin*dIII fragment from pHMS1 ligated into pBR322	Pendrak & Perry (1993)
pNPM9	7·4 kb, Cm^r, *hmsRS*$^+$, 3·2 kb *Sau*3AI–*Bam*HI fragment from pHMS1 ligated into pACYC184	Lillard *et al.* (1997); Pendrak & Perry (1991)
pNPM11	9·3 kb, Cm^r, *hmsFRS*$^+$, 5·4 kb *Sau*3AI–*Sma*I fragment from pHMS1 ligated into pACYC184	Lillard *et al.* (1997); Pendrak & Perry (1991)
pNPM22	9·9 kb, Cm^r, *hmsH*$^+$ *hms*F′, 5·9 kb *Bam*HI–*Sal*I fragment from pHMS1 ligated into pACYC184	Lillard *et al.* (1997); Pendrak & Perry (1993)
pNPM29	23 kb, Km^r Cm^r, *hmsH*$^+$, *hmsF*::Mud*II*1734-29, Mud*II*1734 insert in pHMS1.1	Lillard *et al.* (1997); Pendrak & Perry (1993)
pNPM38	23 kb, Km^r Cm^r, *hmsHFR*$^+$, *hmsS*::Mud*II*1734-38, Mud*II*1734 insert in pHMS1.1	Lillard *et al.* (1997); Pendrak & Perry (1993)
pACYC184	4·2 kb, Cm^r Tc^r, moderate-copy-number cloning vector	Ausubel *et al.* (1987)
pBGL2	4·8 kb, Ap^r Tc^r, cloning vector	Perry *et al.* (1990)
pBluescript II KS+	3·0 kb, Ap^r, high-copy-number cloning vector	Stratagene
pBR322	4·4 kb, Ap^r Tc^r, moderate-copy-number cloning vector	Ausubel *et al.* (1987)
pLC682	11·2 kb, Ap^r Sp^r, single-copy-number cloning vector	Liu *et al.* (1983)
pLC8.2	8·2 kb, Ap^r, single-copy-number cloning vector constructed by removing ~3·0 kb *Hin*dIII fragment from pLC682	This study
pLG338	7·3 kb, Km^r Tc^r, low-copy-number cloning vector	Stoker *et al.* (1982)
pUC18	2·7 kb, Ap^r, high-copy-number cloning vector	Sambrook *et al.* (1989)

* Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline.

E. coli S30-based extract system (Promega) was performed on purified plasmids according to the manufacturer's instructions. The proteins were acetone-precipitated and equal amounts of radiolabel were separated by 12% SDS-PAGE (Sambrook *et al.*, 1989). Gels were dried and exposed to Kodak Biomax MR film.

RESULTS

The Hms$^+$ phenotype requires *hmsHFRS* and an additional locus

Previously we demonstrated that the *hmsHFRS* operon encoded on pHMS1 restored a temperature-regulated Hms$^+$ phenotype when transformed into *Y. pestis* KIM6 (Δ*pgm*) (Lillard *et al.*, 1997; Perry *et al.*, 1990). *Y. enterocolitica* WA-LOX, *Y. pseudotuberculosis* PB1/0, and *E. coli* HB101 and DH5α were phenotypically Hms$^-$ (Table 2). Southern blot analysis indicated that *Y. pseudotuberculosis* PB1/0 possesses an *hmsHFRS* operon but the other three strains showed no significant hybridization to this region (data not shown). Complementation analysis showed that a recombinant plasmid encoding *hmsFRS* (pNPM11) but not one encoding only *hmsRS* (pNPM9) yielded an Hms$^+$ phenotype in *Y. pseudotuberculosis* (Table 2). While this suggests that strain PB1/0 has a mutation in *hmsF*, we have not determined whether functional products from the *Y. pseudotuberculosis hmsR* and *hmsS* genes are expressed. *Y. enterocolitica* WA-LOX, *E. coli* DH5α or *E. coli* HB101 cells carrying pHMS1 were all Hms$^-$ when grown at 30 or 37 °C on CR plates. In contrast, *Y. pseudotuberculosis* PB1/0(pHMS1) cells were Hms^c (red colonies form on CR plates at 26–30 and 37 °C; Table 2). These results suggest that one or more genes in addition to *hmsHFRS* are essential for an Hms$^+$ phenotype and that these gene(s) are defective or absent in *Y. enterocolitica* and *E. coli* but present and functional in *Y. pseudotuberculosis*. Since *Y. pestis* KIM6 has deleted the entire 102 kb *pgm* region and a plasmid encoding *hmsHFRS* can restore an Hms$^+$ phenotype to

Table 2. Hms phenotype of yersiniae and *E. coli* strains

Strain	Relevant genes (chromosome/plasmid)*	CR phenotype†	
		30 °C	37 °C
Y. pestis			
KIM6+	*pgm*$^+$ (*hmsHFRST*$^+$)	+	−
KIM6	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$	−	−
KIM6(pHMS1)	*hmsT*$^+$ Δ*pgm*/*hmsHFRS*$^+$	+	−
KIM6(pHMS1)+	*hmsHFRST*$^+$/*hmsHFRS*$^+$ (lcn)	+	−
KIM6(pHMS1.1)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsHFRS*$^+$ (mcn)	+	+
KIM6(pHMS1.2)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsHFRS*$^+$ (mcn)	+	+
KIM6(pEYCD1)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*ycdSRQPT*$^+$ (hcn)	−	−
KIM6(pEYCD1.1)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*ycdSRQPT*$^+$ (lcn)	−	−
KIM6(pEYCD1)(pNPM11)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsFRS*$^+$(mcn) *ycdSRQPT*$^+$ (hcn)	−	−
KIM6(pEYCD1)(pNPM22)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsH*$^+$ (mcn) *ycdSRQPT*$^+$ (hcn)	−	−
KIM6(pEYCD1)(pNPM29)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsH*$^+$ *hmsF*::Mu*dII1734-29* (mcn) *ycdSRQPT*$^+$ (hcn)	−	−
KIM6(pEYCD1)(pNPM38)	Δ*pgm* Δ*hmsHFRS*) *hmsT*$^+$/*hmsHFR*+ *hmsS*::*Mud*II1734-38 (mcn) *ycdSRQPT*$^+$ (hcn)	+	−
KIM6-2050	*hmsT*::mini-*kan2050 hmsHFRS*$^+$	−	ND
KIM6-2051	*hmsT*::mini-*kan2051 hmsHFRS*$^+$	−	ND
KIM6-2052	*hmsT*::mini-*kan2052 hmsHFRS*$^+$	−	ND
KIM6-2050(pAHMS16.1)	*hmsHFRS*$^+$ *hmsT*::mini-*kan2050*/*hmsT*$^+$ (mcn)	+	−
KIM6-2051(pAHMS16.1)	*hmsHFRS*$^+$ *hmsT*::mini-*kan2051*/*hmsT*$^+$ (mcn)	+	−
KIM6-2051(pAHMS17.2)	*hmsHFRS*$^+$ *hmsT*::mini-*kan2051*/*hmsT8* (scn)	+	−
KIM6-2051(pEYCD1)	*hmsHFRS*$^+$ *hmsT*::mini-*kan2051*/*ycdSRQPT*$^+$ (hcn)	−	−
KIM6-2057.1	*hmsHFS*$^+$ *hmsT*$^+$ Δ*hmsR*46	−	−
KIM6-2057.1(pEYCD1)	*hmsHFS*$^+$ *hmsT*$^+$Δ*hmsR*46/*ycdSRQPT*$^+$ (hcn)	+	−
KIM6(pAHMS14.1) +	*pgm*$^+$ (*hmsHFRS*$^+$) *hmsT*$^+$/*hmsT8* (mcn)	+	−
KIM6(pHMS1)(pAHMS16.3)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsHFRS*$^+$(lcn) *hmsT*$^+$ (scn)	+	+
KIM6(pHMS1)(pAHMS17.2)	Δ*pgm* (Δ*hmsHFRS*) *hmsT*$^+$/*hmsHFRS*$^+$ (lcn) *hmsT8* (scn)	+	+
M23+	*pgm*$^+$ (*hmsHFRS*$^+$) *hmsT8*?	+	−
M23	*hmsHFS*$^+$ *hmsR8hmsT8*	−	−
M23-2	Δ*pgm* (Δ*hmsHFRS*) *hmsT8*	−	−
M23(pHMS1)	*hmsHFS*$^+$ *hmsT8 hmsR8*/*hmsHFRS*$^+$ (lcn)	+	+
M23-2(pHMS1)	*hmsT8* Δ*pgm*/*hmsHFRS*$^+$ (lcn)	+	+
Y. pseudotuberculosis			
PB1/0	*hmsH*$^+$ *hmsF*$^-$ *hmsR*? *hmsS*? *hmsT8*	−	−
PB1/0(pNPM9)	*hmsH*$^+$ *hmsF*$^-$ *hmsR*? *hmsS*? *hmsT8*/*hmsRS*$^+$ (mcn)	−	−
PB1/0(pNPM11)	*hmsH*$^+$ *hmsF*$^-$ *hmsR*? *hmsS*? *hmsT8*/*hmsFRS*$^+$ (mcn)	+	−
PB1/0(pHMS1)	*hmsH*$^+$ *hmsF*$^-$ *hmsR*? *hmsS*? *hmsT8*/*hmsHFRS*$^+$ (lcn)	+	+
Neilson(pHMS1)	*hmsHFRST*?/*hmsHFRS*$^+$ (lcn)	+	+
EP2(pHMS1)	*hmsHFRST*?/*hmsHFRS*$^+$ (lcn)	+	+
43(pHMS1)	*hmsHFRST*?/*hmsHFRS*$^+$ (lcn)	+	+
YPIII(pHMS1)	*hmsHFRST*?/*hmsHFRS*$^+$ (lcn)	+	+
Y. enterocolitica			
WA-LOX		−	−
WA-LOX(pHMS1)	*hmsHFRS*$^+$ (lcn)	−	−
WA-LOX(pHMS1)(pAHMS14)	*hmsHFRS*$^+$ (lcn) *hmsT*$^+$ (mcn)	+	+
E. coli			
HB101		−	−
HB101(pHMS1)	*hmsHFRS*$^+$ (lcn)	−	−

Table 2 (*cont.*)

Strain	Relevant genes (chromosome/plasmid)*	CR phenotype†	
		30 °C	37 °C
DH5α		−	−
DH5α (pHMS1)	/*hmsHFRS*$^+$ (lcn)	−	−
DH5α (pAHMS14)	/*hmsT*$^+$ (mcn)	−	−
DH5α (pHMS1)(pAHMS14)	/*hmsHFRS*$^+$ (lcn) *hmsT*$^+$ (mcn)	+	+
MG1655	/*ycdSRQPT*$^+$	−	−

* *pgm*$^+$, non-mutated 102 kb *pgm* locus; Δ*pgm*, entire *pgm* locus deleted; scn, lcn, mcn and hcn, single-, low- (< 10 copies per cell), moderate- (< 100 copies per cell) and high-copy-number (> 100 copies per cell) number plasmids, respectively.

† Hms phenotypes were determined on CR agar. Results with Y. *pestis* M23 derivatives, previously reported by Lillard *et al*. (1997), are shown here for comparison.

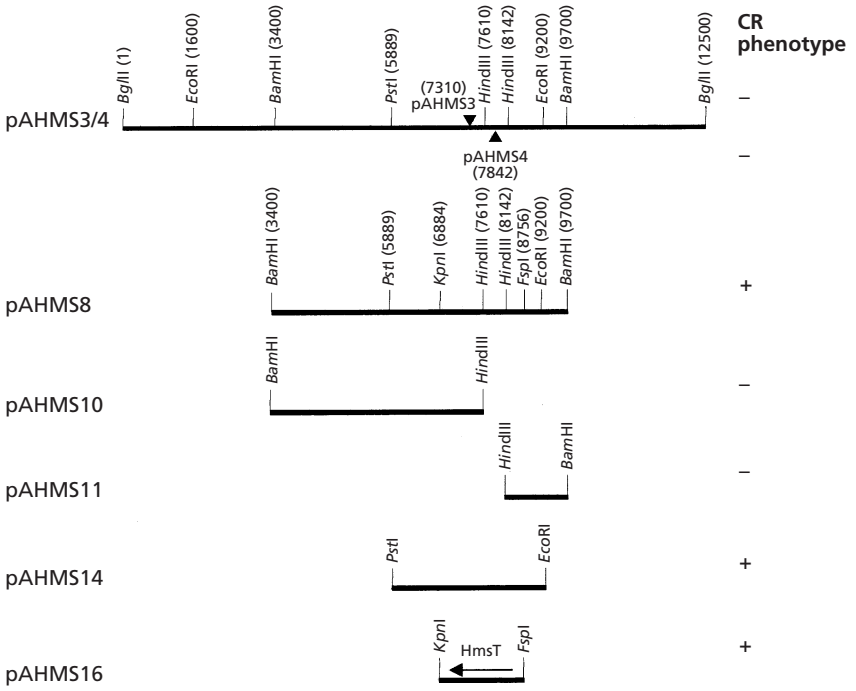

Fig. 1. Restriction site maps of recombinant plasmids of the *Y. pestis hmsT* region. Positions of restriction sites are in parentheses. The mini-*kan* insertion sites in pAHMS3 and pAHMS4 are indicated by triangles. The Hms (CR) phenotype with KIM6-2051 carrying each plasmid is shown. The *hmsT* coding region is depicted as an arrow in pAHMS16.

this strain (Fetherston & Perry, 1994; Fetherston *et al*., 1992; Lucier & Brubaker, 1992), the additional *hms* gene(s) must lie outside the *pgm* region.

Identifying the *hmsT* locus

We used mini-*kan* mutagenesis of Y. *pestis* and identified common-sized DNA fragments containing this transposon from Hms$^-$ mutants to isolate and clone the *hmsHFRS* locus (Perry *et al*., 1990). To identify possible mini-*kan* inserts into additional essential *hms* gene(s), we re-examined the original Southern blots of these mini-*kan* mutants and identified a second, common-sized DNA fragment present in several independent mutants. Of seven selected candidate mutants, three had an ~13 kb *Bgl*II fragment that hybridized to the mini-*kan* probe (data not shown). These fragments were cloned into pBGL2 and designated pAHMS3-5 (Table 1). pAHMS3-5 contained cross-hybridizing inserts and restriction endonuclease mapping of these plasmids suggested that the mini-*kan* inserts in pAHMS4 (from Y. *pestis* KIM6-2051) and pAHMS5 (from Y. *pestis* KIM6-2052) were in nearly identical locations, ~300 bp from the *Hin*dIII site at bp 8142 (Fig. 1). The mini-*kan* insertion in pAHMS3 (from Y. *pestis* KIM6-2050) is ~300 bp to the left of the *Hin*dIII site at bp 7610 (Fig. 1). Using the ~4kb *Bam*HI fragment adjacent to the mini-*kan* insert in pAHMS4 (Fig. 1) as a probe for Southern blot analysis indicated the presence of this DNA in Y. *pestis* KIM6 and Y. *pseudotuberculosis* PB1/0. Under these conditions, no hybridization to DNA from Y. *enterocolitica* WA-LOX was noted (data not shown).

To clone the wild-type locus from Y. *pestis*, the *Bam*HI genomic library from KIM6+ (Fetherston *et al*., 1992)

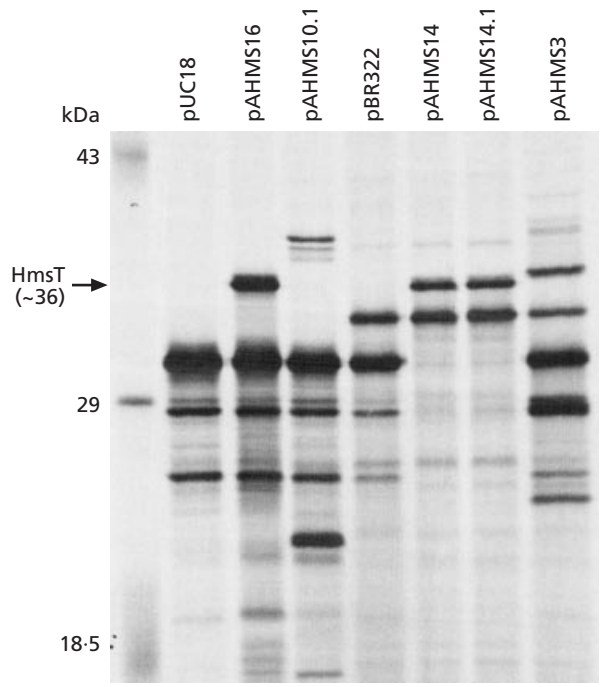

Fig. 2. Autoradiogram of plasmid-encoded proteins labelled with ^{35}S-labelled amino acids by *in vitro* transcription/translation. Sizes of molecular mass markers (unmarked lane) are indicated. pAHMS16 and pAHMS14 are HmsT$^+$; pAHMS14.1 encodes HmsT8; pAHMS10.1 and pAHMS3 (*hmsT*::mini-*kan2050*) are HmsT$^-$.

was screened by colony blot hybridization using the pAHMS4 *Bam*HI fragment as a probe. Fourteen potential positive clones were electroporated into KIM6-2050 and KIM6-2051. Four of the 14 recombinant plasmids (pAHMS131, pAHMS497, pAHMS496 and pAHMS68) restored an Hms$^+$ phenotype on CR agar to both KIM6-2050 and KIM6-2051. Plasmid DNA from these four clones and KIM6+ genomic DNA was digested with *Bam*HI and probed with pAHMS4. Two of the clones, pAHMS496 and pAHMS497, showed an identical hybridization pattern to that of DNA from KIM6+. We used pAHMS496 to subclone a 6·3 kb *Bam*HI fragment into pACYC184 to yield pAHMS8. This plasmid restored an Hms$^+$ phenotype to both KIM6-2050 and KIM6-2051 (Fig. 1). Other subclones that lacked the ~500 bp *Hin*dIII fragment did not complement either *Y. pestis* mutant. The 1·9 kb *Fsp*I–*Kpn*I insert in pAHMS16 was the smallest subclone that complemented both strains containing the mini-*kan* insertions. Only one intact ORF is encoded within this subclone (Fig. 1; see below).

Characterization of HmsT

DNA sequencing and analysis identified a possible 1170 bp ORF that we have designated *hmsT*. There are several possible methionine and leucine starts; since none of these has strong ribosome-binding sites, we have used the first methionine as the putative start of HmsT. The predicted amino acid sequence suggests that HmsT has a molecular mass of 44·8 kDa and a pI of 7·75. *In vitro* transcription/translation of plasmids containing *hmsT* and separation of polypeptides by SDS-PAGE yielded a molecular mass estimate of ~36 kDa (Fig. 2). This indicates that HmsT either migrates aberrantly during SDS-PAGE or one of the alternative start sites is used. A putative promoter region, 108 bp upstream from the first methionine (Fig. 3) was identified as well as a stem–loop structure ($\Delta G = -24$ kcal mol^{-1}) within the *hmsT* ORF, ~100 bp from its end. A similarly located stem–loop structure has been identified within the coding region of *hmsF*; however, its function, if any, has not been determined (Lillard *et al.*, 1997). Although other potential −10 and −35 regions

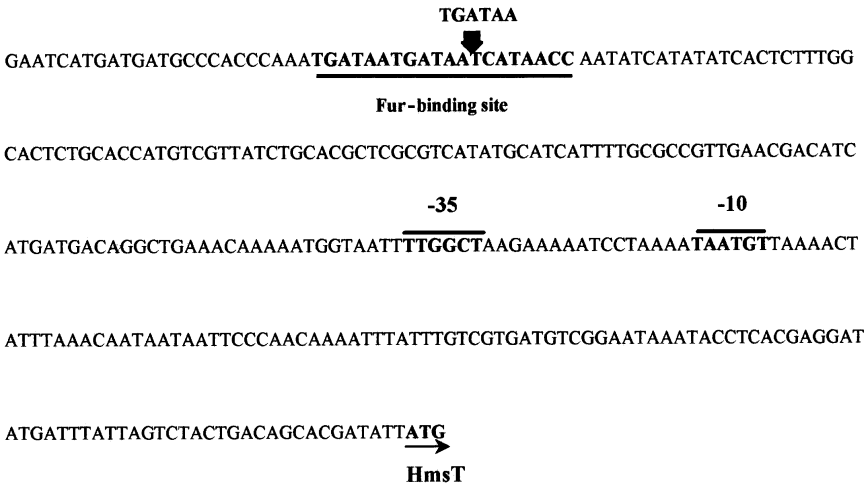

Fig. 3. Nucleotide sequence of the *hmsT* and *hmsT8* promoter regions of *Y. pestis* KIM6+ and M23, respectively. Putative −10 and −35 regions are overlined and labelled. The potential FBS is underlined and labelled. The first possible methionine start for *hmsT* is shown. The *hmsT8* promoter region contains a third TGATAA repeat (shown above *hmsT* sequence) that is absent in the *hmsT* promoter region.

```
                          Box I                  Box II                                  Box III            Box IV
Y. pestis HmsT        DPLTSLYNRR--22aa--DIDHFKAYNDNYGHTMGDQAL--14aa--D--3aa--RYGGEEF--60aa--ADEALYRAKRNGRN
S. sp. PleD           DPLTKLFNRR--25aa--DVDHFKRFNDSLGHDAGDRIL--14aa--D--3aa--RYGGEEI--60aa--ADKALYDAKQAGRN
C. crescentus PleD    DQLTGLHNRR--25aa--DIDFFKKINDTFGHDIGDEVL--14aa--D--3aa--RYGGEEF--61aa--ADEGVYQAKASGRN
V. anguillarum Orf3   DPLTDLYNRR--23aa--DVDYFKKINDQYGHEAGDEVL--14aa--D--3aa--RWGGEEF--61aa--ADQALYQAKSEGRN
E. coli Orf460        DVLTKLLNRR--25aa--DVDKFKEINDTWGHNTGDEIL--14aa--D--3aa--RYGGDEF--59aa--ADEALYIAKRRGRN
B. subtilis Orf359    DFLTGVYNRR--26aa--DIDHFKTINDQYGHHEGDQVL--14aa--D--3aa--RIGGEEF--60aa--ADQMLYKAKETGRN
E. coli YcdT          DPLTNIFNRN--23aa--DIDHFKKVNDTWGHPVGDQVI--14aa--D--3aa--RVGGEEF--61aa--ADNALYEAKETGRN
S. sp. Orf315         DQLTQLANRR--25aa--DIDYFKNYNDHYGHQGGDDCL--15aa--D--3aa--RYGGEEF--62aa--ADQYLYEAKAQGRN
E. coli Orf371        DGMTGVYNRR--25aa--DIDHFKSINDTWGHDVGDEAI--14aa--D--3aa--RFGGDEF--58aa--ADLALYKAKKAGRN
S. sp. PleD           DSLTQVSNRR--25aa--DIDHFKQFNDFYGHLSGDDCL--15aa--D--3aa--RYGGEEF--62aa--ADENLYKAKRQGRN
T. pallidum Orf387    DMMTQMKLKH--24aa--DIDFFKQINDTHGHLCGDLVL--14aa--D--3aa--RYGGEEF--59aa--ADSALYQAKQNGRN
A. aeolicus Orf431    DPLTGLYNRR--25aa--DIDNFKKINDTYGHDVGDLVL--14aa--D--3aa--RFGGEEF--59aa--ADQALYEAKRKGKN
Consensus             D lT l nrr        DiD FK iND yGH  GD vl        D       RyGGeEf        AD alY AK  GrN
                                         v                 i                                    v
```

Fig. 4. CLUSTAL W amino acid sequence alignments of *Y. pestis* HmsT with selected homologous proteins. Similarities outside Boxes I–IV were not significant and are not shown. Spacing between Boxes I–IV and a conserved aspartic acid residue (D) are shown. On the consensus line, identical residues are indicated by upper case letters, while lower case letters denote the predominant amino acids at those sites. Aligned proteins are *Synechocystis* (*S.*) sp. PleD (768 aa; D64005), *C. crescentus* PleD (454 aa; L42554), *Vibrio anguillarum* Orf3 (206 aa; U17054), *E. coli* Orf460 (D00790), *Bacillus subtilis* Orf359 (P54595), *E. coli* YcdT (AE000204), *Synechocystis* (*S.*) sp. Orf315 (D90903), *E. coli* Orf371 (P21830), *Synechocystis* (*S.*) sp. PleD (275 aa; D90917), *Treponema pallidum* Orf387 (AE001265) and *Aquifex aeolicus* Orf431 (AE000670). For identification in this figure, unnamed ORFs were given numbers to match their amino acid lengths. *C. crescentus* PleD is a demonstrated response regulator (Hecht & Newton, 1995). *Synechocystis* sp. Orf315 and *T. pallidum* Orf387 are described as hypothetical regulatory components.

are present in the promoter region, none of these had sequence matches and/or spacing of the putative −10 and −35 regions as good as the one identified in Fig. 3. The proximity of the putative promoter region to *hmsT* and the oppositely oriented ORFs on either side of *hmsT* suggest that *hmsT* lies within a monocistronic operon.

One intriguing feature of the *hmsT* promoter region is the location of a putative FBS ~250 bp upstream from the first potential methionine start of HmsT (Fig. 3). This putative FBS matches the *E. coli* FBS consensus (Stojiljkovic *et al*., 1994) at 17 out of 19 residues. A *Y. pestis fur* mutant has an Hms^c phenotype (Staggs *et al*., 1994), suggesting that Fur is involved in temperature regulation of the Hms phenotype. The *hmsHFRS* promoter does not have an obvious FBS. Although the *hmsHFRS* promoter region does show ~50% nucleotide identity to the *hmsT* promoter region, there is no clear indication of shared regulatory motifs between the *hmsT* and *hmsHFRS* promoter regions.

Since carriage of *hmsHFRS* alone did not confer an Hms$^+$ phenotype to *E. coli* and *Y. enterocolitica*, we transformed pAHMS14 (*hmsT*$^+$) into these strains. Both *E. coli* DH5α and *Y. enterocolitica* WA-LOX carrying all five *hms* genes were Hms^c (Table 2). This suggests that *Y. pestis hmsHFRS* and *hmsT* are all required for an Hms$^+$ phenotype in these organisms. If any additional genes are involved in the Hms$^+$ phenotype, they would be present in the yersiniae and *E. coli*. In addition, multiple copies of all five *hms* genes caused loss of temperature regulation of the phenotype in *Y. pestis*, *Y. enterocolitica* and *E. coli* (Table 2).

HmsT and HmsHFRS homologies

A BLAST search of the databases identified similarities between HmsT and the C terminus of the *pleD* gene from *Caulobacter crescentus* and *Synecocystis* spp., as well as genes from several other organisms (Fig. 4). Hecht & Newton (1995) identified four consensus regions within PleD (Boxes I–IV). The highest degree of similarity among the HmsT-related proteins we selected lie in or nearby these boxes. An alignment of the increased number of available sequences revealed an additional highly conserved arginine in Box I and glycine-arginine-asparagine residues in Box IV. Furthermore, an invariant aspartic acid residue between Boxes II and III was found (Fig. 4).

The *E. coli* YcdT homologue to HmsT is encoded adjacent to and transcribed divergently from a putative operon that encodes *ycdSRQP*; previously, homology was noted between HmsHFRS and the products of *ycdSRQP* which have the same gene order as *hmsHFRS*, (AE000204; Blattner *et al*., 1997; Lillard *et al*., 1997). Table 3 shows the features of these proteins and the percentage similarities and identities of the homologues. The highest degree of similarity (83%) was between HmsR and YcdQ. HmsT had the lowest similarity (33·8% similarity) to its homologue, YcdT (Table 3). YcdT contains the four PleD-related boxes (Fig. 4) and 60 additional amino acids at the N terminus that are not present in HmsT. A comparison of the regions outside Boxes I–IV of YcdT from *E. coli* strain MG1655 with HmsT shows a modest level similarity throughout the remainder of the protein. Nevertheless, the level of similarity was higher than that detected for other PleD-related proteins (Table 3 and data not shown). While the *ycdT* coding region contains a stem–loop structure ($\Delta G = -16{\cdot}84$ kcal mol^{-1}), the nucleotide sequence and location does not match the stem–loop in *hmsT*. The *ycdSRQP* and *ycdT* divergent promoter region showed no significant similarity to the promoter regions for *hmsHFRS* and *hmsT*. In addition, the prominent stem–loop structure within the *hmsF* coding region (Lillard *et al*., 1997) is not present in the *E. coli* homologue, *ycdR*.

Table 3. Comparison of *Y. pestis* HmsHFRST to *E. coli* YcdSRQPT

Signal peptide processing sites were identified using Signal P (Nielsen *et al*., 1997); pI values were calculated using the Intelligenetics software suite. For HmsH, HmsF, YcdS and YcdR only the pIs for the processed proteins are reported. The pIs for proteins HmsH, HmsR and HmsS differ slightly from previously reported values obtained by using PC GENE (Lillard *et al*., 1997).

Y. pestis **proteins (predicted no. aa/pI)**	***E. coli*** **ORFs (predicted no. aa/pI)**	**Percentage identity**	**Percentage similarity**
HmsH (822/4·99)	YcdS (807/5·67)	41·1	58·2
HmsF* (673/5·24)	YcdR (672/5·56)	48·3	60·8
HmsR (457/10·83)	YcdQ (441/9·05)	66·2	83·0
HmsS (155/6·21)	YcdP (137/10·91)	28·4	50·0
HmsT (390/7·75)	YcdT (452/6·73)	22·5	33·8

*HmsF values reported here reflect a correction in the previously reported sequence (Lillard *et al*., 1997). The calculated unprocessed and processed molecular mass of HmsF is 76·8 and 74·3 kDa, respectively. The GenBank entry for *hmsHFRS* (U22837) has been corrected.

The similarities between the coding regions of these genes prompted us to test the *E. coli* genes for their ability to complement *hms* mutations in *Y. pestis*. *E. coli* strain MG1655 had an Hms$^-$ phenotype on CR plates at 30 and 37 °C. *Y. pestis* KIM6 (Δ*pgm*) cells carrying either pEYCD1 or pEYCD1.1 (high-copy-number and low-copy-number plasmids, respectively, containing *ycdSRQPT*), had an Hms$^-$ phenotype on CR plates. However, pEYCD1 restored an Hms$^+$ phenotype to *Y. pestis* KIM6-2057.1 (in-frame Δ*hmsR*) and KIM6(pNPM38) (*hmsHFR*$^+$ *hmsS*::Mud*II*1734–38) (Table 2). In contrast, *Y. pestis* strains KIM6-2051 (*hmsT2051*::mini-*kan*; this study), KIM6(pNPM11) (*hmsH*$^-$ *hmsFRS*$^+$), KIM6(pNPM22) (*hmsH*$^+$) and KIM6(pNPM29) (*hmsH*$^+$ *hmsF*::Mud*II*1734–29) (Lillard *et al*., 1997) were not complemented by pEYCD1 (Table 2). Thus, *ycdQ* and *ycdP* can replace *hmsR* and *hmsS*, respectively. However, *ycdS*, *ycdR* and *ycdT* cannot replace their respective homologues *hmsH*, *hmsF* and *hmsT*.

Temperature regulation of the Hms$^+$ phenotype

Temperature regulation of the Hms$^+$ phenotype was noted with its discovery. Although originally defined as on at 26 °C and off at 37 °C (Jackson & Burrows, 1956a), we have found that formation of red colonies on CR agar occurs at 30 °C (unpublished observations). To more precisely delineate the transition temperature, we incubated *Y. pestis* KIM6+ cells on CR agar at temperatures of 30, 31, 32, 33, 34, 35, 36 and 37 °C for 48 h. Coloured colony formation occurred up to 34 °C but not at 35 °C and above. However, the transition was not abrupt; at temperatures of 32–34 °C, colonies were less intensely red. At 35 °C, colonies were faintly pink compared to 37 °C (data not shown).

Multiple copies of both *hms* operons (*hmsHFRS* and *hmsT*) resulted in a temperature-independent Hms^c phenotype in *E. coli*, *Y. enterocolitica* and *Y. pestis*, while multiple copies of one operon or the other retained temperature regulation in *Y. pestis* KIM6 (Table 2). In contrast, a low-copy-number plasmid (pHMS1) containing intact *hmsHFRS* transformed into *Y. pestis* strain M23 resulted in a Hms^c phenotype. The mutation responsible for this constitutive phenotype was neither in the plasmid nor within the *pgm* locus (Lillard *et al*., 1997). To discover if the mutation was in *hmsT* we cloned the 3·3 kb *Pst*I–*Eco*RI fragment containing *hmsT* (Fig. 1) from M23(pHMS1). Sequencing of the promoter region and entire *hmsT* ORF identified one mutation – a 6 bp insert, TGATAA, within a putative FBS. This mutation, designated *hmsT8*, introduces a third copy of the TGATAA sequence found in the FBS located upstream of *hmsT* (Fig. 3).

To determine if this mutation would elicit a Hms^c phenotype in *Y. pestis*, we constructed the single-copy-number plasmid, pAHMS17.2, which contains the *hmsT8* promoter region cloned in front of the wild-type *hmsT* coding region. Introduction of this plasmid into KIM6-2050 and KIM6-2051 gave a temperature-regulated Hms$^+$ phenotype; however, this plasmid, as well as a single-copy-number plasmid (pAHMS16.3) encoding wild-type *hmsT*, caused an Hms^c phenotype in KIM6(pHMS1) (Table 2). Our observations suggest that the pLC682-derived plasmids are low-copy-number and not single-copy-number, at least in *Y. pestis* (data not shown). This again indicates that multiple copies of both *hmsT* and *hmsHFRS* results in an Hms^c phenotype.

Since gene copy number disrupts Hms temperature regulation, we analysed a number of *hmsT* genes as an alternative approach to evaluating the role of this gene in temperature regulation. Four different strains of *Y. pseudotuberculosis* display an Hms^c phenotype when transformed with pHMS1 (*hmsHFRS*) (Table 2). We used PCR to amplify the *hmsT* promoter region of each strain and sequenced the cloned PCR products. The *hmsT* gene from *Y. pseudotuberculosis* strains 43 and YPIII had the TGATAA insertion in the possible FBS while strains PB1/0, Neilson and EP2 did not. Thus we cannot conclude that *hmsT* or the putative FBS as-

sociated with this gene are involved in temperature regulation of the Hms phenotype.

DISCUSSION

In *Y. pestis*, the Hms$^+$ phenotype causes adsorption of haemin, inorganic iron, CR and guanine to the OM (Brubaker, 1970; Jackson & Burrows, 1956a; Perry *et al*., 1993; Perry *et al*., 1990; Surgalla & Beesley, 1969). It functions to block the proventricular valve and ultimately causes death of the flea (Hinnebusch *et al*., 1996; Kutyrev *et al*., 1992). This blockage is essential for effective transmission of the plague bacillus from fleas to mammals and thus is critical to the survival of *Y. pestis* in nature (Bacot, 1915; Bacot & Martin, 1914; Pollitzer, 1954). The Hms$^+$ phenotype is regulated by temperature as determined by coloured colony formation on haemin agar and CR agar – on at 26 °C and off at 37 °C (Jackson & Burrows, 1956a; Surgalla & Beesley, 1969). In this study we determined that the transition temperature is 34–35 °C. However, the transition is not abrupt; colonies at 34 °C were less intensely positive than those at 26 or 30 °C and colonies at 35 °C had a faint pinkish hue compared to white colonies at 37 °C.

We initiated our search for a fifth essential gene since *Y. pestis hmsHFRS* genes failed to convert *E. coli* and *Y. enterocolitica* to an Hms$^+$ phenotype. Re-examination of *Y. pestis* Hms$^-$ mini-*kan* mutants led to the isolation of *hmsT*. *hmsT*, located outside of the *pgm* locus, potentially encodes a 44·8 kDa protein with a pI of 7·75. *E. coli* and *Y. enterocolitica* cells carrying plasmids encoding *hmsT* and *hmsHFRS* form red colonies on CR agar. Thus these five genes are necessary for this phenotype. Any other putative genes contributing to the Hms$^+$ phenotype would be common to *E. coli* and the yersiniae.

HmsT shows similarities to a number of protein sequences from disparate prokaryotes in the database; most of these proteins have no experimentally determined function. One exception is PleD of *C. crescentus*. PleD is a response regulator required for the loss of motility and stalk formation in the transition from a motile swarmer cell to a stalk cell (Hecht & Newton, 1995). However, the similarities between PleD and HmsT do not reside within the response regulator domains but in the C terminus and HmsT does not possess any currently recognized DNA-binding motifs. Sequence alignments by Hecht & Newton (1995) identified four conserved regions (Boxes I–IV) in the C terminus and these regions are highly conserved in HmsT and other proteins added to the database since 1995 (Fig. 4). Hecht & Newton (1995) designated proteins with these motifs as members of a GGDEF (Box III sequence) family; GGEEF now seems to be the more conserved Box III sequence (Fig. 4). A comparative analysis of selected PleD-related proteins in the database revealed a slight increase in the size of Box I and Box III (Fig. 4). Furthermore, an invariant aspartic acid residue is present between Box II and Box III in the 12 sequences we have illustrated (Fig. 4). Except for YcdT, there is very little similarity of these proteins to HmsT outside of Boxes I–IV. YcdT and HmsT had significant regions of similarity outside the four boxes, indicating a closer lineage between these two proteins than to other members of the family. However, these homologies do not clearly suggest a functional role for HmsT.

In addition to *hmsT*, which we describe in this study, a four gene operon, *hmsHFRS*, is essential for the Hms$^+$ phenotype (Lillard *et al*., 1997; Pendrak & Perry, 1991, 1993; Perry *et al*., 1990). The *hmsHFRS* operon is located within a deletable region of the *Y. pestis* chromosome, the 102 kb *pgm* locus, and lies >15 kb from one of the two IS100 elements that delineate the ends of the *pgm* locus (Fetherston & Perry, 1994; Fetherston *et al*., 1992; Lucier & Brubaker, 1992). Precursors of OM proteins HmsH and HmsF contain cleavable signal peptide export signals; the locations of HmsR and HmsS are undetermined (Lillard *et al*., 1997; Pendrak & Perry, 1991, 1993). We have recently corrected an error in reporting the sequence of *hmsF* (Lillard *et al*., 1997; U22837) that omitted 19 codons of the ORF. Although the *Y. pestis* Hms$^+$ phenotype would appear to serve a unique function, *E. coli* strain MG1655, which displays an Hms$^-$ phenotype, possesses homologues of the *hms* genes. The *E. coli ycdSRQP* operon has the same gene order as *hmsHFRS* ((Blattner *et al*., 1997); AE000204). Despite the high degree of similarity of several *ycd* and *hms* gene products (Table 3), *E. coli* MG1655 and *Y. pestis* KIM6 carrying recombinant *ycdSRQPT* genes were Hms$^-$ as determined by failure to form red colonies on CR agar. Complementation analysis of *hms* mutations with *ycdSRQPT* genes indicated that only YcdQ and YcdP functionally replace their homologues HmsR and HmsS, respectively. The inability of YcdS and YcdR to replace HmsH and HmsF, respectively, is perplexing. Pairwise comparisons indicate that these homologues have the second and third highest degrees of identity and similarity of the five Hms homologues. In addition, alignments of HmsH with YcdS and HmsF to YcdR did not show any significant non-conserved regions, rather the similarities are evenly distributed throughout these proteins.

In contrast to *hmsT*, which is encoded outside the *pgm* locus of *Y. pestis* KIM6+ and therefore at least 15 kb distant from *hmsHFRS*, the *E. coli* homologue (*ycdT*) is adjacent to *ycdSRQP* (Blattner *et al*., 1997; Fetherston & Perry, 1994; Lillard *et al*., 1997). However, the '102 kb *pgm* locus' may not be identical for all *Y. pestis* strains. In strain S55-797, a mutation causing an Hms$^-$ phenotype lies within the *pgm* region but outside the *hmsHFRS* operon (Buchrieser *et al*., 1998). If this mutation resides within *hmsT*, the S55-797 strain may have an *hms* gene organization similar to the *E. coli* MG1655 operons, *ycdSRQP* and *ycdT*. Alternatively, the mutation may have occurred in an additional, essential *hms* gene. In either case, a fifth essential *hms* gene appears to be encoded within the *pgm* region of *Y. pestis* S55-797 (Buchrieser *et al*., 1998) but is absent from the *pgm* loci of *Y. pestis* KIM6, Kuma and M23-2 since *hmsHFRS* are the only genes from the *pgm* locus needed

to restore an Hms$^+$ phenotype to these Δ*pgm* strains (Lillard *et al*., 1997; Perry *et al*., 1990; unpublished observations).

Previously, and during the course of this study, we noted several instances in which temperature regulation of the Hms phenotype was disrupted. Except for a *fur* mutation (Staggs *et al*., 1994), these examples require multiple copies of one or both *hms* operons. Hms temperature regulation has been observed at two levels; coloured colony formation on CR and haemin agars (Jackson & Burrows, 1956a; Surgalla & Beesley, 1969) and the level of iodination of HmsH and HmsF (Pendrak & Perry, 1991, 1993). Here we relied on CR agar phenotypes to determine that cells of *Y. pestis*, *Y. enterocolitica* and *E. coli* were Hms^c when harbouring multiple copies of both operons.

In addition, an *hmsT8* mutation may cause loss of temperature regulation in M23(pHMS1) and M23-2(pHMS1) (Lillard *et al*., 1997; this study). Given the Hms^c phenotype of the *Y. pestis fur* mutant, it is intriguing that the *hmsT8* mutation is a 6 bp insert into a putative FBS site upstream of the M23 *hmsT* gene; this is the only difference in or nearby the M23 *hmsT8* gene in comparison to the KIM6+ *hmsT* gene. However, this putative FBS is widely separated (Fig. 3) from its usual location around the −10 to −35 region of Fur- and iron-regulated promoters (Braun *et al*., 1990). Although Fur regulation of non-iron-regulated genes has been noted (Crosa, 1997; Guerinot, 1994), a role for Fur in temperature regulation has not been identified in any system other than the *Y. pestis* Hms system (Staggs *et al*., 1994). How insertion of a third TGATAA repeat within the putative *hmsT*-associated FBS would disrupt Fur binding or how Fur achieves temperature regulation is undetermined. The higher mutation rate in M23 derivatives (Lillard *et al*., 1997) will require transfer of the *hmsT8* mutation into a more stable strain before its role in disruption of temperature regulation is conclusive.

Of four *Y. pseudotuberculosis* strains carrying pHMS1 and exhibiting an Hms^c phenotype, only two strains contained the *hmsT8* mutation in the putative FBS. Since only the *hmsT* promoter region was sequenced in these strains, the mutation affecting temperature regulation could reside in the *hmsT* ORF or in the *hmsHFRS* operon. Alternatively, the copy number of pHMS1 may be higher in *Y. pseudotuberculosis* than in *Y. pestis*. While *Y. pestis* KIM6(pHMS1) cells retain a temperature-dependent Hms phenotype, higher copy number plasmids (pBR322 and pACYC184) carrying only the *hmsHFRS* operon also yielded an Hms^c phenotype in KIM6 (Table 2).

At this time the mechanisms of temperature regulation of and haemin binding by the Hms system are undetermined. While HmsT may play a regulatory role, the evidence is not conclusive and regulatory roles for other Hms proteins, especially HmsR and HmsS, are feasible. Alternatively, all the Hms proteins may be required to form a complex essential for the Hms phenotype. Overexpression of all five proteins from recombinant, multicopy genes could cause loss of temperature regulation. Again, evidence of complex formation by Hms proteins and haemin-binding by individual Hms proteins or a putative complex is lacking.

NOTE ADDED IN PROOF

While investigating the mechanism of the *pgm* locus deletion, J. M. Hare and K. A. McDonough (Department of Biomedical Sciences, University at Albany and David Axelrod Institute, New York, USA) have also identified and cloned the *hmsT* locus (*J Bacteriol*, in press).

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