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 hmsHFRS (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
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") 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. Blockage of the flea midgut results in repeated attempts to feed and is required for effective transmission of the plague bacillus. For regulating or establishing an Hms" phenotype in *Y. pestis*, the low-calcium-response virulence plasmid pCD1 (Perry & Fetherston, 1997). All *Y. pestis* 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 CaCl₂ 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 [³²P]dCTP (New England Nuclear) and a Rediprime labelling kit (Amersham), as per the manufacturer's instructions. The internal HindIII fragment of the *hrns* derivative mini-kan (Perry *et al.*, 1990), or the ~4 kb BamHI fragment adjacent to the mini-kan insert in pAMHSA (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 [³²P]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 HindIII 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 Inteigenetics 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 BamHI genomic library of *Y. pestis* KIM6+ DNA (Fetherston *et al.*, 1992). The mutated *hmsT* gene was cloned from *Y. pestis* strain M23 by isolation of 30–35 kb DNA fragments digested with EcoRI–PstI; these fragments were ligated into pBR322. Authentic clones were identified by Southern and colony blot analyses. The BamHI promoter region from *Y. pseudotuberculosis* strains was amplified by PCR using the following oligonucleotides: 5'-CTCCTGGA-TCCCCGTGAGGTTATTTATCCG-3' and 5'-TTTCCGTGTT-AACATCTACCCAGCCCGAGTA-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 I1 KS derivative mini-kan (Perry *et al.*, 1991, 1993; Gish & States, 1993). Genomic DNA was digested with EcoRI–PstI; these fragments were ligated into pBR322. Authentic clones were identified by Southern and colony blot analyses. The BamHI promoter region from *Y. pseudotuberculosis* strains was amplified by PCR using the following oligonucleotides: 5'-TTTGGACCCAGCCCATCACCATC-3' and 5'-AATTGCTGGCTGGGCGGAGAA-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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. pestis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIM6 +</td>
<td>Hms'</td>
<td>Fetherston et al. (1992)</td>
</tr>
<tr>
<td>KIM6</td>
<td>Hms' (Δpgm)</td>
<td>Fetherston et al. (1992)</td>
</tr>
<tr>
<td>KIM6-2050</td>
<td>Hms' (hmsT2050::mini-kan)</td>
<td>This study</td>
</tr>
<tr>
<td>KIM6-2051</td>
<td>Hms' (hmsT2051::mini-kan)</td>
<td>This study</td>
</tr>
<tr>
<td>KIM6-2052</td>
<td>Hms' (hmsT2052::mini-kan)</td>
<td>This study</td>
</tr>
<tr>
<td>KIM6-2057.1</td>
<td>Hms' (ΔhmsR46, in-frame deletion)</td>
<td>Lillard et al. (1999)</td>
</tr>
<tr>
<td>M23 +</td>
<td>Hms'</td>
<td>Fetherston et al. (1992)</td>
</tr>
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<td>M23</td>
<td>Hms' (hmsR8)</td>
<td>Fetherston et al. (1992)</td>
</tr>
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<td>M23-2</td>
<td>Hms' (Δpgm)</td>
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</tr>
<tr>
<td>Y. enterocolitica</td>
<td>Serotype O:8; Pst'</td>
<td>Perry &amp; Brubaker (1983)</td>
</tr>
<tr>
<td>WA-LOX</td>
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<td></td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB1/0</td>
<td>Serotype I; Pst'</td>
<td>Perry &amp; Brubaker (1983)</td>
</tr>
<tr>
<td>Neilson</td>
<td>Serotype I; Pst'</td>
<td>R. R. Brubaker</td>
</tr>
<tr>
<td>EP2</td>
<td>Serotype II</td>
<td>R. R. Brubaker</td>
</tr>
<tr>
<td>43</td>
<td>Serotype III</td>
<td>R. R. Brubaker</td>
</tr>
<tr>
<td>YPIII</td>
<td>Serotype III; Pst'</td>
<td>R. R. Brubaker</td>
</tr>
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<td>Cloning strain</td>
<td>Ausubel et al. (1987)</td>
</tr>
<tr>
<td>DH5x</td>
<td>Cloning strain</td>
<td>Blattner et al. (1997)</td>
</tr>
<tr>
<td>HB101</td>
<td>Cloning strain</td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12 strain used in genome sequencing project</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
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<td></td>
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<tr>
<td>pAHMS3</td>
<td>19 kb, Ap' Tc', hmsT2050::mini-kan, 14.2 kb BglII kan fragment from KIM6-2050 ligated into pBGL2</td>
<td>This study</td>
</tr>
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<td>pAHMS4</td>
<td>19 kb, Ap' Tc', hmsT2051::mini-kan, 14.2 kb BglII kan fragment from KIM6-2051 ligated into pBGL2</td>
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<td>pAHMS5</td>
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<td>pAHMS8</td>
<td>10.5 kb, Cm', hmsT', 6.3 kb BamHI fragment from pAHMS496 ligated into pACYC184</td>
<td>This study</td>
</tr>
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<td>8.0 kb, Cm', 4.2 kb BamHI–HindIII fragment from pAHMS8 ligated into pACYC184</td>
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</tr>
<tr>
<td>pAHMS10.1</td>
<td>6.8 kb, Ap', 4.2 kb BamHI–HindIII fragment from pAHMS10 ligated into pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS11</td>
<td>5.2 kb, Cm', 1.6 kb BamHI–HindIII fragment from pAHMS8 ligated into pACYC184</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS14</td>
<td>6.9 kb, Tc', hmsT', 3.3 kb PstI–EcoRI fragment from pAHMS8 ligated into pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS14.1</td>
<td>6.9 kb, Tc', hmsT8, 3.3 kb PstI–EcoRI fragment from chromosome of M23-2 ligated into pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS16</td>
<td>4.4 kb, Ap', hmsT', 1.9 KpnI–FspI fragment from pAHMS14 ligated into KpnI–HincII site of pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS16.1</td>
<td>5.6 kb, Tc', hmsT', 1.9 kb KpnI–FspI fragment from pAHMS16 ligated into pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS16.3</td>
<td>10.1 kb, Ap', hmsT', EcoRI linkers added to 1.9 kb KpnI–FspI fragment from pAHMS16 and ligated into EcoRI site of pLC8.2</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS17</td>
<td>6.9 kb, Tc', hmsT8 promoter with hmsT' coding region, 2.5 kb PstI–Ndel fragment from pAHMS14 + 0.7 kb Ndel–EcoRI fragment from pAHMS41.1 ligated into PstI–EcoRI site of pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pAHMS17.2</td>
<td>11.4 kb, Ap', hmsT8 promoter with hmsT' coding region, EcoRI linkers added to 3.3 kb PstI–EcoRI fragment from pAHMS17 and ligated into EcoRI site of pLC8.2</td>
<td>This study</td>
</tr>
</tbody>
</table>
The Hms+ phenotype requires H. A. JONFS, J. W. LILLARD, JR and R. D. PERRY encoded on pHMS 1 restored a temperature-regulated E. coli

additional locus

Table 1 (cont.)

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAHMS68</td>
<td>~ 35.5 kb, Ap’, hmsT’, ~ 29.1 kb BamHI insert ligated into pHCS9</td>
<td>Fetherston et al. (1992); this study</td>
</tr>
<tr>
<td>pAHMS131</td>
<td>~ 45.7 kb, Ap’, hmsT’, ~ 39.3 kb BamHI insert ligated into pHCS9</td>
<td>Fetherston et al. (1992); this study</td>
</tr>
<tr>
<td>pAHMS496</td>
<td>~ 27.6 kb, Ap’, hmsT’, ~ 21.2 kb BamHI insert ligated into pHCS9</td>
<td>Fetherston et al. (1992); this study</td>
</tr>
<tr>
<td>pAHMS497</td>
<td>~ 27.6 kb, Ap’, hmsT’, ~ 21.2 kb BamHI insert ligated into pHCS9</td>
<td>Fetherston et al. (1992); this study</td>
</tr>
<tr>
<td>pPEYCD1</td>
<td>114 kb, Ap’, E. coli ycdSRQPT’, ~ 84 kb Accl fragment from MGI655 ligated into XmaI site of pBluescript II KS+</td>
<td>This study</td>
</tr>
<tr>
<td>pPEYCD1.1</td>
<td>144 kb, Km’, E. coli ycdSRQPT’, ~ 84 kb BamHI–Asp718 fragment from pECDY1 ligated into BamHI–KpnI sites of pLG338</td>
<td>This study</td>
</tr>
<tr>
<td>pHMS1</td>
<td>164 kb, Km’, hmsHFRS’, 91 kb Sau3AI insert ligated into BamHI site of pLG338</td>
<td>Lillard et al. (1997); Perry et al. (1990)</td>
</tr>
<tr>
<td>pHMS1.1</td>
<td>13.9 kb, Km’, hmsHFRS’, 97 kb SalI–HindIII fragment from pHMS1 ligated into pACYC184</td>
<td>Lillard et al. (1997); Pendrak &amp; Perry (1993)</td>
</tr>
<tr>
<td>pNPM9</td>
<td>7.4 kb, Km’, hmsRS’, 3.2 kb Sau3AI–BamHI fragment from pHMS1 ligated into pACYC184</td>
<td>Lillard et al. (1997); Pendrak &amp; Perry (1991)</td>
</tr>
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<td>pNPM11</td>
<td>9.3 kb, Km’, hmsFRS’, 54 kb Sau3AI–SmaI fragment from pHMS1 ligated into pACYC184</td>
<td>Lillard et al. (1997); Pendrak &amp; Perry (1991)</td>
</tr>
<tr>
<td>pNPM22</td>
<td>9.9 kb, Km’, hmsH’, hmsE’, 5.9 kb BamHI–SalI fragment from pHMS1 ligated into pACYC184</td>
<td>Lillard et al. (1997); Pendrak &amp; Perry (1993)</td>
</tr>
<tr>
<td>pNPM29</td>
<td>23 kb, Km’ Cm’, hmsH’, hmsF:: MudI1734-29, MudI1734 insert in pHMS1.1</td>
<td>Lillard et al. (1997); Pendrak &amp; Perry (1993)</td>
</tr>
<tr>
<td>pNPM38</td>
<td>23 kb, Km’ Cm’, hmsHFR’, hmsS:: MudI1734-38, MudI1734 insert in pHMS1.1</td>
<td>Lillard et al. (1997); Pendrak &amp; Perry (1993)</td>
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<td>pACYC184</td>
<td>42 kb, Cm’ Tc’, moderate-copy-number cloning vector</td>
<td>Ausubel et al. (1987)</td>
</tr>
<tr>
<td>pBGL2</td>
<td>4.8 kb, Ap’ Tc’, cloning vector</td>
<td>Perry et al. (1990)</td>
</tr>
<tr>
<td>pBluescript II KS+</td>
<td>3.0 kb, Ap’, high-copy-number cloning vector</td>
<td>Stratagene</td>
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<tr>
<td>pLC8.2</td>
<td>8.2 kb, Ap’, single-copy-number cloning vector constructed by removing ~ 30 kb HindIII fragment from pLC682</td>
<td>This study</td>
</tr>
<tr>
<td>pLG338</td>
<td>7.3 kb, Km’ Tc’, low-copy-number cloning vector</td>
<td>Stoker et al. (1982)</td>
</tr>
</tbody>
</table>

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

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 (Δpgm) (Lillard et al., 1997; Perry et al., 1990). 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+ (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

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 (Kodak Biomax MR film). Gels were dried and exposed to Kodak Biomax MR film.

Hybridization to this region (data not shown). Complementation analysis showed that a recombinant plasmid encoding hmsHFRS (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+ (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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genes (chromosome/plasmid)*</th>
<th>CR phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>30 °C</td>
</tr>
<tr>
<td><strong>Y. pestis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIM6+</td>
<td>pgm⁺ (hmsHFRST⁺)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺</td>
<td>−</td>
</tr>
<tr>
<td>KIM6(pHMS1)</td>
<td>hmsT⁺ Δpgm/hmsHFRS⁺</td>
<td>+</td>
</tr>
<tr>
<td>KIM6(pHMS1)+</td>
<td>hmsHFRST⁺/hmsHFRS⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6(pHMS1.1)</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺/hmsHFRS⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6(pHMS1.2)</td>
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<td>+</td>
</tr>
<tr>
<td>KIM6(pEYCD1)</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺/ycdSRQPT⁺ (hcn)</td>
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<tr>
<td>KIM6(pEYCD1.1)</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺/hmsHFRS⁺ (mcn) ycdSRQPT⁺ (hcn)</td>
<td>−</td>
</tr>
<tr>
<td>KIM6(pEYCD1)(pNPM51)</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺/hmsHFRS⁺ (mcn) ycdSRQPT⁺ (hcn)</td>
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<td>KIM6(pEYCD1)(pNPM22)</td>
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<td>KIM6(pEYCD1)(pNPM29)</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺/hmsHFRS⁺ (mcn) ycdSRQPT⁺ (hcn)</td>
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<td>hmsT⁺ mini-kan2051 hmsHFRS⁺</td>
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<td>KIM6-2052</td>
<td>hmsT⁺ mini-kan2052 hmsHFRS⁺</td>
<td>−</td>
</tr>
<tr>
<td>KIM6-2050(pAHMS16.1)</td>
<td>hmsHFRS⁺ hmsT⁺ mini-kan2050/hmsT⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6-2051(pAHMS16.1)</td>
<td>hmsHFRS⁺ hmsT⁺ mini-kan2051/hmsT⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6-2051(pAHMS17.2)</td>
<td>hmsHFRS⁺ hmsT⁺ mini-kan2051/hmsT⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6-2051(pEYCD1)</td>
<td>hmsHFRS⁺ hmsT⁺ mini-kan2051/ycdSRQPT⁺ (hcn)</td>
<td>−</td>
</tr>
<tr>
<td>KIM6-2057.1</td>
<td>hmsHFRS⁺ hmsT⁺ ΔhmsR46</td>
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</tr>
<tr>
<td>KIM6-2057.1(pEYCD1)</td>
<td>hmsHFRS⁺ hmsT⁺ ΔhmsR46/ycdSRQPT⁺ (hcn)</td>
<td>+</td>
</tr>
<tr>
<td>KIM6(pAHMS14.1) +</td>
<td>pgm⁺ (hmsHFRS⁺) hmsT⁺/hmsT[:,:MudII1734-29</td>
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</tr>
<tr>
<td>KIM6(pAHMS16.3)</td>
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<td>+</td>
</tr>
<tr>
<td>KIM6(pAHMS17.2)</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺/hmsHFRS⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>M23⁺</td>
<td>pgm⁺ (hmsHFRS⁺) hmsT⁺</td>
<td>+</td>
</tr>
<tr>
<td>M23</td>
<td>hmsHFRS⁺ hmsR8/hmsT⁺</td>
<td>−</td>
</tr>
<tr>
<td>M23⁻</td>
<td>Δpgm (ΔhmsHFRS) hmsT⁺</td>
<td>−</td>
</tr>
<tr>
<td>M23(pHMS1)</td>
<td>hmsHFRS⁺ hmsT⁺ hmsR8/hmsHFRS⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>M23-2(pHMS1)</td>
<td>hmsT⁺ Δpgm/hmsHFRS⁺ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB1/0</td>
<td>hmsH⁺ hmsF⁺ hmsR⁺ hmsS⁺ hmsT⁺</td>
<td>−</td>
</tr>
<tr>
<td>PB1/0(pNPM9)</td>
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<td>−</td>
</tr>
<tr>
<td>PB1/0(pNPM11)</td>
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<td>−</td>
</tr>
<tr>
<td>PB1/0(pHMS)</td>
<td>hmsH⁺ hmsF⁺ hmsR⁺ hmsS⁺ hmsT⁺/hmsFRS⁺ (mcn)</td>
<td>−</td>
</tr>
<tr>
<td>Neilson(pHMS)</td>
<td>hmsHFRST⁺/hmsHFRS⁺ (mcn)</td>
<td>−</td>
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<td>43(pHMS)</td>
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<td>−</td>
</tr>
<tr>
<td>YPHILL(pHMS)</td>
<td>hmsHFRST⁺/hmsHFRS⁺ (mcn)</td>
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</tr>
<tr>
<td><strong>Y. enterocolitica</strong></td>
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<td></td>
</tr>
<tr>
<td>WA-LOX</td>
<td>hmsHFRS⁺ (mcn)</td>
<td>−</td>
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<tr>
<td>WA-LOX(pHMS1)</td>
<td>hmsHFRS⁺ (mcn)</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>HB101</td>
<td>hmsHFRS⁺ (mcn)</td>
<td>−</td>
</tr>
<tr>
<td>HB101(pHMS1)</td>
<td>hmsHFRS⁺ (mcn)</td>
<td>−</td>
</tr>
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</table>
Table 2 (cont.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genes (chromosome/plasmid)*</th>
<th>CR phenotype†</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>30 °C</td>
</tr>
<tr>
<td>DH52</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>DH52 (pHMS1)</td>
<td>/hmsHFRS+ (lcn)</td>
<td>–</td>
</tr>
<tr>
<td>DH52 (pAHMS14)</td>
<td>/hmsT+ (mcn)</td>
<td>–</td>
</tr>
<tr>
<td>DH52 (pHMS1)(pAHMS14)</td>
<td>/hmsHFRS+ (lcn) hmsT+ (mcn)</td>
<td>+</td>
</tr>
<tr>
<td>MG1655</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

*pgm*: non-mutated 102 kb pgm locus; Δpgm, 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.

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 BglII 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 HindIII 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 HindIII site at bp 7610 (Fig. 1). Using the ~4kb BamHI 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 BamHI genomic library from KIM6+ (Fetherston et al., 1992)
HmsT of *Yersinia pestis*. 

Plasmid DNA from these four clones and KIM6+ genomic DNA was digested with BamHI 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 BamHI 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 HindIII fragment did not complement either *Y. pestis* mutant. The 1.9 kb *FspI*-KpnI 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 pl 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 (ΔG = -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 was screened by colony blot hybridization using the pAHMS4 BamHI 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 BamHI 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 BamHI 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 HindIII fragment did not complement either *Y. pestis* mutant. The 1.9 kb *FspI*-KpnI 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).

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are present in the promoter region, none of these had sequence matches and/or spacing of the putative FBS -2.50 bp upstream from the -10 and -35 regions as good as the one identified in Fig. 3. The proximity of the putative promoter region to the -10 indicates that it 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 (Stojilkovic et al., 1994) at 17 out of 19 residues. A Y. pestis fur mutant has an Hms' 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 DH5a and Y. enterocolitica WA-LOX carrying all five hms genes were Hms' (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 PldE gene from Caulobacter crescentus and Synechocystis spp., as well as genes from several other organisms (Fig. 4). Hecht & Newton (1995) identified four consensus regions within PldE (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 PldE-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 PldE-related proteins (Table 3 and data not shown). While the ycdT coding region contains a stem–loop structure (ΔG = -1684 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

<table>
<thead>
<tr>
<th><em>Y. pestis</em> proteins (predicted no. aa/pl)</th>
<th><em>E. coli</em> ORFs (predicted no. aa/pl)</th>
<th>Percentage identity</th>
<th>Percentage similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HmsH (822/4-99)</td>
<td>YcdS (607/5-67)</td>
<td>41.1</td>
<td>58.2</td>
</tr>
<tr>
<td>HmsF* (673/5-24)</td>
<td>YcdR (672/5-56)</td>
<td>48.3</td>
<td>60.8</td>
</tr>
<tr>
<td>HmsR (457/10-83)</td>
<td>YcdQ (441/9-05)</td>
<td>66-2</td>
<td>83.0</td>
</tr>
<tr>
<td>HmsS (155/6-21)</td>
<td>YcdP (137/10-91)</td>
<td>28-4</td>
<td>50.0</td>
</tr>
<tr>
<td>HmsT (390/7-75)</td>
<td>YcdT (452/6-73)</td>
<td>22-5</td>
<td>33-8</td>
</tr>
</tbody>
</table>

*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 (∆*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) (*hmsHFRS*: MuddIH1734-38) (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*: MuddIH1734-29) (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' 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' 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 *PstI*-EcoRI 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' 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' 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' 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' 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-
associated 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 pink 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* HmsΔ mini-kan mutants led to the isolation of *hmsT*. *hmsT*, located outside of the *pgm* locus, potentially encodes a 448 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; GGE EF 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); AE002004. 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 *hms*, the S55-797 strain may have an *hms* gene organization similar to the *E. coli* MG1655 operons, *ycdSRQPT* 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.
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. enterocolitica and E. coli were Hms+ 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+ 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 hmsT 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+ 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 hmsF HFRS 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 hmsF HFRS operon also yielded an Hms+ 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 hmsF locus (J Bacteriol, in press).

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