Identification of critical amino acid residues in the plague biofilm Hms proteins

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Yersinia pestis biofilm formation causes massive adsorption of haemin or Congo red *in vitro* as well as colonization and eventual blockage of the flea proventriculus *in vivo*. This blockage allows effective transmission of plague from some fleas, like the oriental rat flea, to mammals. Four Hms proteins, HmsH, HmsF, HmsR and HmsS, are essential for biofilm formation, with HmsT and HmsP acting as positive and negative regulators, respectively. HmsH has a β -barrel structure with a large periplasmic domain while HmsF possesses polysaccharide deacetylase and COG1649 domains. HmsR is a putative glycosyltransferase while HmsS has no recognized domains. In this study, specific amino acids within conserved domains or within regions of high similarity in HmsH, HmsF, HmsR and HmsS proteins were selected for site-directed mutagenesis. Some but not all of the substitutions in HmsS and within the periplasmic domain of HmsH were critical for protein function. Substitutions within the glycosyltransferase domain of HmsR and the deacetylase domain of HmsF abolished biofilm formation in *Y. pestis*. Surprisingly, substitution of highly conserved residues within COG1649 did not affect HmsF function.

INTRODUCTION

An intimate association of bacterial cells with a surface and formation of multicellular aggregates is commonly referred to as a biofilm. The organization of these bacterial communities is vastly diverse and dependent upon the production of extracellular substances constituting unique biofilm matrices. Such bacterial architectures are environmentally beneficial and can contribute to dissemination and virulence of an organism (Branda *et al.*, 2005; Davey & O'Toole, 2000).

The *Yersinia pestis* Hms⁺ phenotype is a manifestation of biofilm formation causing massive adsorption of haemin or Congo red (CR) during growth at 26–34 °C but not at 37 °C. This is required for colonization and eventual blockage of the flea proventriculus – allowing efficient transmission of plague from some fleas, like the oriental rat flea, to mammals (Bacot & Martin, 1914; Bacot, 1915; Hinnebusch *et al.*, 1996; Lillard *et al.*, 1997, 1999; Pendrak & Perry, 1991, 1993; Perry *et al.*, 1990, 1993, 2004; Perry & Fetherston, 1997; Pollitzer, 1954). Biofilm formation is not involved in mammalian virulence – an in-frame deletion in *hmsR* did not affect the LD₅₀ in a bubonic plague model (Lillard *et al.*, 1999). Recent studies have demonstrated Hms-dependent biofilm

formation *in vitro* as well as in fleas and nematodes (Darby *et al.*, 2002; Jarrett *et al.*, 2004; Jones *et al.*, 1999; Kirillina *et al.*, 2004). CR binds basic and neutral polysaccharides while wheat germ agglutinin binds to *N*-acetylglucosamine, suggesting that the Hms-dependent biofilm contains an exopolysaccharide (EPS) component. In addition, dispersin, an enzyme from *Actinobacillus actinomycetemcomitans* which hydrolyses β -1,6-*N*-acetyl-D-glucosamine, prevents plague biofilm formation but does not disrupt a preformed biofilm (Itoh *et al.*, 2005; Spiers *et al.*, 2002; Tan & Darby, 2004; Wood, 1980).

The 102 kb *pgm* locus in *Y. pestis* contains the high-pathogenicity island (Ybt) and the *hmsHFRS* operon encoding four of the six *hms* genes required for biofilm formation and its regulation. *Escherichia coli* K-12 *pgaABCD* (formerly *ycdSRQP*) encode proteins with similarities (ranging from 34 to 83 %) to Hms proteins and are required for the synthesis of an EPS containing poly- β -1,6-*N*-acetyl-D-glucosamine (Blattner *et al.*, 1997; Deng *et al.*, 2002; Fetherston *et al.*, 1992; Jones *et al.*, 1999; Lillard *et al.*, 1997; Wang *et al.*, 2004). HmsH and HmsF are outer-membrane proteins while HmsS, HmsR, and HmsT are located in the inner membrane. While HmsH is predicted to be a β -barrel protein with a large periplasmic domain, its function and that of HmsS are unknown. HmsR, a putative glycosyltransferase, and HmsF, with a polysaccharide deacetylase domain, are likely involved in the synthesis and modification of the EPS component of the biofilm. HmsF also has a

Abbreviations: CR, Congo red; CV, crystal violet; EPS, exopolysaccharide.
A table of primers is available with the online version of this paper.

conserved but uncharacterized domain, COG1649, that is present in other HmsF-like proteins in Gram-negative bacteria (Kirillina *et al.*, 2004; Pendrak & Perry, 1991; Perry *et al.*, 2004). Proteins with this domain are assigned to the TIM barrel glycosyl hydrolase superfamily by Pfam (Bateman *et al.*, 2004). Recently, *gmhA*, a gene encoding phosphoheptose isomerase, has been identified as important for biofilm formation (Darby *et al.*, 2005).

Temperature regulation of plague biofilm formation does not involve transcriptional regulation or mRNA stability. Rather, this is achieved by proteolytic degradation of HmsR, HmsH and HmsT at 37 °C. HmsT, a putative diguanylate cyclase, may be the key to this temperature regulation. It is a positive regulator of biofilm formation and is involved in the synthesis of cyclic-di-GMP (c-di-GMP). HmsP, a negative regulator of biofilm formation, is an EAL phosphodiesterase and likely degrades c-di-GMP. Like other glycosyltransferases involved in EPS or cellulose productions, the enzymic activity of HmsR may depend upon c-di-GMP (Bobrov *et al.*, 2005; Hare & McDonough, 1999; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 2004; Simm *et al.*, 2005).

Previously, we used site-directed amino acid substitutions to identify several critical residues of HmsT and HmsP (Kirillina *et al.*, 2004). In this study, specific amino acids within conserved domains or within regions of high similarity in HmsH, HmsF, HmsR and HmsS were selected for site-directed mutagenesis. Substitutions within the glycosyltransferase domain of HmsR and the deacetylase domain of HmsF abolished biofilm formation in *Y. pestis* while substitutions in HmsS and HmsH had diverse effects. Surprisingly, substitution of highly conserved residues within COG1649 did not affect HmsF function.

METHODS

Bacterial strains and cultivation. All bacterial strains used in this study are described in Table 1. *E. coli* cells were grown on Luria broth agar (LBA). Where appropriate, ampicillin (Ap; 100 µg ml⁻¹), kanamycin (Km; 50 µg ml⁻¹), streptomycin (Sm; 50 µg ml⁻¹) or chloramphenicol (Cm; 20 µg ml⁻¹) were added to cultures. Strains with arabinose-inducible expression were grown in the presence of 0.2% (w/v) arabinose. *Y. pestis* cells were streaked onto Congo red (CR) plates from buffered glycerol stocks stored at -80 °C and incubated at 26 °C for 48 h. For most experiments, individual colonies were inoculated onto Tryptose Blood Agar Base (TBA; Difco) slants and incubated at 26 °C for 24–48 h. Cells were washed off TBA slants with Heart Infusion Broth (HIB; Difco) and grown overnight with aeration in HIB at the appropriate temperature. Cultures were transferred to fresh medium the next morning. For some experiments, *Y. pestis* cells were grown in TMH medium (Straley & Bowmer, 1986). Cell growth was monitored on a Spectronic Genesys5 spectrophotometer at 620 nm. For Western blot analyses, isolated colonies from TBA plates were spread onto CR plates and allowed to form a lawn (48 h). Cells were scraped off the plate and suspended in sample buffer.

Crystal violet (CV) staining. Cells attached to borosilicate glass test tubes were detected with CV staining essentially as described by O'Toole *et al.* (1999). Briefly, cells were grown overnight on TBA

slants and used to inoculate TMH to an OD₆₂₀ of 0.1. Cultures were grown for 16–18 h in a shaking water bath at 26 °C and then exposed to 0.01% CV for 15–20 min. Cells attached to the culture tube were washed three times with distilled water and the CV staining the cells was solubilized with a mixture of 80% ethanol/20% acetone. The amount of dye solubilized, representing the mass of attached bacterial cells, was measured at 570 nm on a Spectronic Genesys5 spectrophotometer.

CR-binding assay. The original CR-binding assay (Hartzell *et al.*, 1999) was modified as previously described (Kirillina *et al.*, 2004). Briefly, *Y. pestis* cells were grown overnight (≈16 h) in HIB at 26 °C. Cells were pelleted and resuspended in HIB/CR medium [1% (w/v) HIB containing 0.2% galactose and 30 µg CR ml⁻¹] such that all cultures had an equivalent wet weight of cells per µl of medium. An ≈20 mg wet cell weight aliquot of each culture was incubated for 3 h on a rocking platform at room temperature. The amount of CR bound by the cells was determined by measuring the absorbance of cell-free supernatants at 500 nm with a Spectronic Genesys5 spectrophotometer and subtracting this value from the reading obtained with uninoculated HIB/CR medium.

Plasmids and recombinant DNA techniques. All the plasmids used in this study are listed in Table 1. Plasmids were purified from overnight cultures by alkaline lysis (Birnboim & Doly, 1979) or with a Qiaprep spin Miniprep kit (Qiagen) and further purified when necessary by polyethylene glycol precipitation (Humphreys *et al.*, 1975). A standard CaCl₂ protocol was followed to introduce plasmids into *E. coli* (Sambrook & Russell, 2001). *Y. pestis* cells were transformed by electroporation as previously described (Fetherston *et al.*, 1995). All plasmid DNA and PCR products used for construction of chromosomal deletion mutants or cloned amino acid substitution mutants were sequenced by Elim Biopharmaceuticals to ensure that the correct mutation had been introduced and no secondary mutations had occurred. In a few instances, aberrant amino acid substitution clones were identified and these were also characterized.

Construction of *hmsH*, *hmsR*, *hmsF* and *hmsS* mutants. Suicide vector pKNG101 (Kaniga *et al.*, 1991) was used to generate in-frame deletions in *hmsH* and *hmsR*. Fragments spanning the upstream and downstream regions of *hmsH* were obtained by *NsiI/XmnI* (1147 bp) and *SwaI/BamHI* (1149 bp) digestions of pNPM22 and cloned into the *PstI-BamHI* sites of pBluescript SK. The resulting construct, pBSSKΔ*hmsH*, and pKNG101 were both digested with *Sall* and *XbaI* followed by ligation of the resulting 2.4 kb fragment from pBSSKΔ*hmsH* into pKNG101, yielding pKNGΔ*hmsH* (Table 1). For the *hmsR* deletion, 1.1 kb *Sall-NcoI* and 1.1 kb *BsrBI-BamHI* fragments of the upstream and downstream region of *hmsR* in pHMS1.2 were cloned into the *Sall-BamHI* sites of pWSK29, yielding pWSK29Δ*hmsR*. This plasmid was digested with *Sall/XbaI* and the resulting 2.2 kb fragment was cloned into the same sites in pKNG101, yielding pKNGΔ*hmsR* (Table 1).

PCR and DNA sequencing (Elim Biopharmaceuticals) confirmed that the correct DNA sequences were present in pKNGΔ*hmsH* and pKNGΔ*hmsR* prior to electroporation into *Y. pestis* KIM6+. As previously described (Bearden & Perry, 1999), transformants were selected on TBA plates containing 50 µg Sm ml⁻¹ and grown overnight in HIB without antibiotics to select for sucrose-resistant isolates that had completed the allelic exchange. Strains in which the wild-type gene was replaced by the deleted version were identified by PCR. One isolate of each mutation was selected and named KIM6-2115 (in-frame Δ*hmsH*2115) and KIM6-2118 (non-polar Δ*hmsR*2118). These deletion mutations remove 569 of 822 and 327 of 457 aa in HmsH and HmsR, respectively.

Red-mediated recombination (Datsenko & Wanner, 2000) was employed to construct non-polar deletions in *hmsF* and *hmsS* in *Y.*

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics†	Source or reference
<i>Y. pestis</i> *		
KIM6+	Pgm ⁺ (Hms ⁺) Pla ⁺	Fetherston <i>et al.</i> (1992)
KIM6	Pgm [−] (Δ <i>pgm</i> ; Hms [−]) Pla ⁺	Fetherston <i>et al.</i> (1992)
KIM6-2115	Hms [−] (in-frame Δ <i>hmsH2115</i>) Pla ⁺	This study
KIM6-2116	Hms [−] (non-polar Δ <i>hmsF2116</i>) Pla ⁺	This study
KIM6-2118	Hms [−] (in-frame Δ <i>hmsR2118</i>) Pla ⁺	This study
KIM6-2119	Hms [−] (non-polar Δ <i>hmsS2119</i>) Pla ⁺	This study
<i>E. coli</i>		
DH5 α	Cloning strain	Ausubel <i>et al.</i> (1987)
DH5 α (λ <i>pir</i>)	Strain for maintenance of R6K origin suicide plasmid	S. C. Straley, University of Kentucky
Plasmids		
pBluescript SK	2.9 kb, Ap ^r , high-copy-number cloning vector	Stratagene
pBAD30	4.9 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible promoter upstream of multiple cloning site	Guzman <i>et al.</i> (1995)
pBADHmsF	6.9 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible expression of <i>hmsF</i> , 2 kb <i>SwaI</i> – <i>Tafl</i> fragment from pNPM11 ligated into the <i>SmaI</i> site of pBAD30	This study
pBADHmsF-aa substitutions	6.9 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible expression of <i>hmsF</i> with the following substitutions – K107A, D114A, D115A, DD114/5AA, R123A, W143A, R161A, W167A, Q169A, E172A, E180A, H184A, Q354A, D358A, D362A, R373A, R384A, W387A, Q388A, D466A, D467A; all derived from pBADHmsF	This study
pBADHmsR	6.3 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible expression of <i>hmsR</i> , 1.4 kb PCR fragment generated from pHMS1.2 ligated into <i>EcoRI</i> – <i>XbaI</i> sites in pBAD30	This study
pBADHmsR-aa substitutions	6.3 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible expression of <i>hmsR</i> with the following substitutions – T114A, D176A, D269A, Q305A, R308A, L324A; all derived from pBADHmsR	This study
pBADHmsS	5.4 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible expression of <i>hmsS</i> , 0.5 kb PCR fragment generated from pHMS1.2 ligated into <i>EcoRI</i> – <i>XbaI</i> sites in pBAD30	This study
pBADHmsS-aa substitutions	5.4 kb, Ap ^r , <i>araC</i> ⁺ , arabinose-inducible expression of <i>hmsS</i> with the following substitutions – N73A, W80A, Y83A, R87A; all derived from pBADHmsS	This study
pBSSK Δ <i>hmsH</i>	5.3 kb, Ap ^r , 1.15 kb <i>NsiI</i> – <i>XmnI</i> and <i>SwaI</i> – <i>BamHI</i> fragments from pNPM22 ligated into <i>PstI</i> – <i>BamHI</i> sites of pBluescript SK	This study
pHmsH-aa substitutions	9.9 kb, Cm ^r , <i>hmsH</i> ⁺ <i>hmsF</i> ' expressing <i>hmsH</i> with the following substitutions – R143A, E269A, E281A, D289A, A350V; all derived from pNPM22	This study
pKNG101	6.8 kb, Sm ^r <i>Suc</i> ^s (<i>sacB</i> ⁺), R6K origin suicide vector	Kaniga <i>et al.</i> (1991)
pKNG Δ <i>hmsH</i>	9.2 kb, Sm ^r , 2.4 kb <i>Sall</i> – <i>XbaI</i> fragment from pBSSK Δ <i>hmsH</i> ligated into <i>Sall</i> – <i>XbaI</i> sites pKNG101	This study
pKNG Δ <i>hmsR</i>	9.0 kb, Sm ^r , 2.2 kb <i>Sall</i> – <i>XbaI</i> fragment from pWSK Δ <i>hmsR</i> ligated into <i>Sall</i> – <i>XbaI</i> sites pKNG101	This study
pNPM11	9.3 kb, Cm ^r , encodes intact <i>hmsF</i> , <i>hmsR</i> and <i>hmsS</i> genes	Pendrak & Perry (1991, 1993)
pNPM22	9.9 kb, Cm ^r , <i>hmsH</i> ⁺ <i>hmsF</i> '	Lillard <i>et al.</i> (1997); Pendrak & Perry (1993)
pNPM38	23 kb, Km ^r Cm ^r , <i>hmsHFR</i> ⁺ , <i>hmsS</i> ::MudIII1734–38	Lillard <i>et al.</i> (1997); Pendrak & Perry (1993)
pWSK29	5.4 kb, Ap ^r , Km ^r , low-copy-number cloning vector	Wang & Kushner (1991)
pWSK Δ <i>hmsR</i>	7.6 kb, Ap ^r , 1.1 kb <i>Sall</i> – <i>NcoI</i> and 1.1 kb <i>BsrBI</i> – <i>BamHI</i> fragments ligated into <i>Sall</i> – <i>BamHI</i> sites of pWSK29	This study

*All *Y. pestis* strains are avirulent from the absence of the low-calcium response virulence plasmid pCD1 (Lcr[−]). *Y. pestis* strains with a plus sign possess an intact 102 kb *pgm* locus.

†Ap^r, Cm^r, Km^r and Sm^r, resistance to ampicillin, chloramphenicol, kanamycin and streptomycin, respectively.

pestis KIM6(pKD46)+. Electrocompetent cells were made as previously described (Fetherston *et al.*, 1995) from cultures grown at 26 °C in HIB to OD₆₂₀ ≈ 0.6 and then incubated for 1.5 h with 0.2 % arabinose to induce expression of the Red recombinase (Datsenko & Wanner, 2000). Gene-specific primers (see Table S1, available as supplementary material with the online version of this paper) were used to amplify the *cam* gene cassette from pKD3. Cells, transformed with 0.5–2 µg purified PCR product, were plated on TBA plates containing Cm to select for recombinant cells. To cure pKD46 from selected recombinants, colonies, grown at 37 °C on TBA without Ap, were isolated and screened for sensitivity to Ap. For selected mutants, the presence of the *cam* cassette was confirmed by PCR. Flippase-expressing pCP20 was electroporated into selected Cm^r mutants and the transformation mixture was plated, after overnight incubation at room temperature, on TBA plates containing Ap. As described above, cells sensitive to Cm and Ap (excised Cm cassette and pCP20-cured) were isolated. Mutations were confirmed by PCR and the resulting strains designated KIM6-2116 (Δ *hmsF*-2116) and KIM6-2119 (Δ *hmsS*-2119). These non-polar mutations remove all of the coding region for the HmsF protein (673 aa) and 150 of 155 aa residues in the HmsS protein. The *hmsF* deletion in KIM6-2116 overlaps with *hmsR* and potentially removes four N-terminal amino acids of HmsR. Despite this elimination of the predicted start site (Lillard *et al.*, 1997), expression of this version of HmsR is functional (see Results).

Construction of alanine substitutions in HmsH, HmsF, HmsR and HmsS. Most of the amino acid substitutions were made by a two-step PCR cloning scheme (Fig. 1) using two outside primers (1, 2) and two overlapping interior primers (3, 4) carrying the desired mutation. This method has been used to avoid the occurrence of PCR mutations while amplifying a long template even with

Fig. 1. Diagram of the two-step PCR-substitution. DNA primers 3 and 4 span the region of interest and carry an appropriate mutation ('X' represents altered sequence yielding an amino acid substitution). Oligonucleotides 1 and 2, incorporating restriction sites 1 and 2, were used to clone the mutated gene into an expression vector with an inducible or native promoter.

DNA polymerases with proof-reading activity. First, two smaller PCR products were generated (primers 1-3 and 4-2), overlapping at the region of interest by primers carrying the desired mutation (primers 3 and 4). PCR products were gel purified, with the exception of *hmsR* mutations, where a *DpnI* digestion was used to eliminate carryover template DNA. Second, the products of the first PCR (1-3, 4-2), hybridizing across the site of interest and with the introduced amino acid codon substitution (3-4), underwent a fill-in cycle to yield a full-length (1-2) double-stranded template. Third, the outside primers (1, 2) were added to the reaction to complete the second PCR and thus generate a gene fragment carrying the desired mutation. The fill-in PCR consisted of one 2 min cycle at 95 °C followed by two cycles at 95 °C for 30 s, 50 °C for 30 s and 68 °C for 4 min. After introducing the outside primers (1, 2), the PCR reaction continued with a 2 min cycle at 95 °C followed by 30 cycles at 95 °C for 30 s, 50 °C for 30 s and 68 °C for 4 min. A 4 min extension time was applied in the case of a ≈ 2 kb fragment (*hmsF*) and was modified accordingly for fragments of different sizes. The final PCR product was column purified and concentrated (Zymo Research), digested (site1, site2 in Fig. 1) and cloned into a target vector. ProofStart DNA polymerase (Qiagen) was used in most amplification reactions, unless stated otherwise. In some cases the QuickChange site-directed mutagenesis kit (Stratagene) was used, following the manufacturer's instructions. Sequences of primers specific for each amino acid substitution are given in Table S1.

Mutations in *hmsH* were constructed in pNPM22 by insertion of modified *hmsH* PCR fragments digested with *BlpI* and *XhoI* for the HmsH-R134A change and with *BlpI* and *XmnI* for HmsH changes E269A, E281A, D289A and A350V. These recombinant plasmids (e.g. pHmsH-E269A; Table 1) express *hmsH* genes and a truncated *hmsF* from the native *hmsHFRS* promoter.

Plasmid pNPM11 was digested with *SwaI*/*TatI* to subclone *hmsF* into the *KpnI*–*SmaI* sites of pBAD30. An isolated *EcoRI*–*XbaI* fragment from this plasmid containing the *hmsF* gene was treated with DNase I (long Klenow fragment) and cloned into the *SmaI* site of pBAD30, yielding pBADHmsF. All HmsF substitutions were constructed in pBAD30 by insertion of the modified *hmsF* PCR fragments generated from pBADhmsF and digested with *EcoRI* and *XbaI* (Table 1).

Plasmid pHMS1.2 was used as a template to amplify wild-type *hmsR* and *hmsS* DNA. Both genes were cloned as *EcoRI*–*XbaI* fragments into the same sites in pBAD30, generating pBADHmsR and pBADHmsS (Table 1). HmsR D176A and D269A mutations were constructed with the QuickChange site-directed mutagenesis kit (Stratagene). Platinum Pfx DNA polymerase (Invitrogen) was used in the course of *hmsR* mutagenesis. Substitutions in *hmsS* were constructed by insertion of the modified *hmsS* PCR fragments generated from pBADhmsS and digested with *EcoRI* and *XbaI* (Table 1).

Sequence analysis. Amino acid sequences presented in protein alignments (Fig. 2) were obtained from the NCBI Entrez Genome Project website. All sequence alignments were generated in T-Coffee (version 1.6; <http://www.ch.embnet.org/software/TCoffee.html/inter-ref>) (Notredame *et al.*, 2000).

Western blot analysis. Equal protein concentrations were separated on polyacrylamide gels containing SDS prior to Western blotting onto PVDF membranes (Immobilon P; Millipore). For detection of HmsR, samples were not boiled. A modified Towbin procedure (Towbin *et al.*, 1979) was used for immunodetection. Briefly, PVDF membranes were blocked with 5 % non-fat dry milk or 1.5 % BSA in 10 mM Tris/HCl (pH 7.6), 137 mM NaCl (TBS) with 0.1 % Tween 20 (TBST) and then incubated with the appropriate antibody diluted in TBST. Antibodies, raised against portions of HmsH, HmsF and HmsS proteins, were generated in rabbits by Animal Pharm Services. Antiserum against a synthetic immunogenic

Fig. 2. Alignment of the amino acid sequences in conserved regions of *Y. pestis* HmsH (A), deacetylase (B₁ and B₂) and COG1649 (B₃) domains of HmsF, HmsR (C), and HmsS (D). All alignments were generated in T-Coffee (version 1.6). The black and grey boxes represent residue identity and similarity, respectively. Residue numbering of Hms proteins begins with the putative initiating methionine. Arrows indicate residues altered to alanines in *Y. pestis* Hms proteins. The *E. coli* *pgaABCD* genes encoding proteins involved in biofilm formation (Wang *et al.*, 2004) were originally annotated as *ycdSRQP* (Blattner *et al.*, 1997). Names of organisms were abbreviated as follows: *Erwinia carotovora*, ECA; *Pseudomonas fluorescens*, PFL; *Escherichia coli*, ECO; *Xanthomonas oryzae*, XOR; *Bacillus subtilis*, BSU; *Thermoanaerobacter tengcongensis*, TBT; *Geobacter sulfurreducens*, GBS; *Leptospira interrogans*, LIN; *Ralstonia solanacearum*, RSO; *Xanthomonas axonopodis*, XAX; *Gluconobacter xylinus*, GXY; *Staphylococcus epidermidis*, SEP.

peptide of HmsR was generated by Research Genetics. Following incubation with horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech), the immunoreactive proteins were detected with the ECL enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech). Alternatively, proteins interacting with alkaline phosphatase-labelled anti-rabbit IgG molecules were detected with the chemiluminescent substrate CSPD (Roche). Immunoblot results were visualized on Kodak Biomax light film.

RESULTS

All four *hmsHFRS* operon-encoded proteins have orthologues in *E. coli*, proteins PgaABCD (formerly YcdSRQP), and other Gram-negative bacteria. The amino acid substitutions in the Hms proteins constructed and tested in this study were selected from regions of high sequence similarity comprising identified domains or regions with potential

enzymic activities (Fig. 2). To test the effect of these substitutions, in-frame deletions of individual *hms* genes in *Y. pestis* were constructed. These mutations yielded an Hms[−] phenotype at 26–30 °C: white colonies on CR plates and absence of aggregation in liquid broth cultures. The four cloned wild-type *hms* genes complemented their respective in-frame or non-polar chromosomal mutations. Variants of *hms* genes with single amino acid substitutions in pBAD30 with arabinose-inducible expression (*hmsF*, *hmsR* and *hmsS*) or in versions of pNPM22 (e.g. pHmsH-R134A) with expression of *hmsH* alleles from the native *hmsH* promoter were tested for the ability to bind CR or to attach to glass in CV-staining assays as parameters of biofilm formation. The cellular level of wild-type and variant Hms proteins was assessed by Western blot analysis (Table 2).

HmsH and HmsF amino acid substitutions

HmsH and HmsF are proven outer-membrane proteins of *Y. pestis* (Pendrak & Perry, 1991, Pendrak & Perry, 1993; Perry *et al.*, 2004) and have amino acid sequence identities/similarities of 41·1%/58·2% and 48·3%/60·8%, respectively, to *E. coli* proteins PgaA and PgaB (Fig. 2). HmsH is the largest Hms protein (822 aa) with a predicted β -barrel structure typical of a transmembrane protein in the outer membrane. We have changed charged residues R134, E269, E281 and D289 to alanines in the N-terminal portion of HmsH. These residues are within a periplasmic domain predicted by the hidden Markov model (PRED_TMBB; <http://bioinformatics.biol.uoa.gr/PRED-TMBB>). Alanine substitutions E269A and D289A in HmsH had no significant effect on the Hms phenotype. The E281A change, where the stability of the resulting HmsH-E281A protein was affected (Table 2), apparently complemented the $\Delta hmsH$ mutation on CR plates but gave significantly decreased values in our quantitative assays for CR adsorption and CV staining (Fig. 3A, B) compared to complementation with wild-type *hmsH*. The HmsH-E281A protein was defective in biofilm formation as assessed by CV staining (Fig. 3). The HmsH-R134A protein also appears defective in biofilm formation by the CV staining assay but failed to show a similar defect in quantitative CR binding. A neutral change, *hmsH*-A350V, fully complemented the $\Delta hmsH$ mutation as expected (Fig. 3).

HmsF shows highest overall similarity to a number of HmsF-like proteins of unproven function or enzymic activity (Fig. 2B₁ and 2B₃). Several of the conserved residues in this group are not highly conserved in enzymes with proven deacetylase activity from a wide variety of organisms. Comparison of the deacetylase domains of these proteins, including *Bacillus subtilis* PdaA with known crystal structure (Blair & van Aalten, 2004), to *Y. pestis* HmsF (Fig. 2B₂) indicated highly conserved residues related to enzymic activity. The highly conserved nature of all these residues (Fig. 2B_{1–3}) suggests they might be essential.

The D114A alteration in HmsF, corresponding to the D73 residue of PdaA, abolished the HmsF protein function,

Table 2. Effect of amino acid substitutions in HmsHFRS proteins on the Hms phenotype and protein functionality

Gene-aa substitution	Hms phenotype*	Protein	
		Functional	Levels†
<i>hmsH</i> -wt	Red	Yes	–
<i>hmsH</i> -R134A	Red	Yes	wt
<i>hmsH</i> -E269A	Red	Yes	wt
<i>hmsH</i> -E281A	Red	Partially	‡
<i>hmsH</i> -D289A	Red	Yes	wt
<i>hmsH</i> -A350V	Red	Yes	wt
<i>hmsF</i> -wt	Red	Yes	–
HmsF deacetylase domain			
<i>hmsF</i> -K107A	Red	Yes	wt
<i>hmsF</i> -D114A	White	No	wt
<i>hmsF</i> -D115A	White	No	wt
<i>hmsF</i> -DD114/5AA	White	No	wt
<i>hmsF</i> -R123A	Red	Yes	wt
<i>hmsF</i> -W143A	Pink	Partially	wt
<i>hmsF</i> -R161A	Red	Yes	wt
<i>hmsF</i> -W167A	Red	Yes	wt
<i>hmsF</i> -Q169A	Red	Yes	wt
<i>hmsF</i> -E172A	Red	Yes	wt
<i>hmsF</i> -E180A	Red	Yes	wt
<i>hmsF</i> -H184A	White	No	wt
HmsF COG1649 domain			
<i>hmsF</i> -Q354A	Red	Yes	wt
<i>hmsF</i> -D358A	Red	Yes	wt
<i>hmsF</i> -D362A	Red	Yes	wt
<i>hmsF</i> -R373A	Red	Yes	wt
<i>hmsF</i> -R384A	Red	Yes	wt
<i>hmsF</i> -W387A	Red	Yes	wt
<i>hmsF</i> -Q388A	Red	Yes	wt
<i>hmsF</i> -D466A	Red	Yes	wt
<i>hmsF</i> -D467A	Red	Yes	wt
<i>hmsR</i> -wt	Red	Yes	–
<i>hmsR</i> -T114A	Red	Yes	wt
<i>hmsR</i> -D176A	White	No	wt
<i>hmsR</i> -D269A	White	No	wt
<i>hmsR</i> -Q305A	White	No	wt
<i>hmsR</i> -R308A	White	No	wt
<i>hmsR</i> -L324A	Red	Yes	wt
<i>hmsS</i> -wt	Red	Yes	–
<i>hmsS</i> -N73A	Red	Yes	wt
<i>hmsS</i> -W80A	White	No	wt
<i>hmsS</i> -Y83A	White	No	‡
<i>hmsS</i> -R87A	Red	Yes	wt

*CR binding assessed as red to white colonies on CR plates incubated at 26 °C.

†wt, variant protein level same as wt protein level.

‡Variant protein not detected by Western blot analysis.

confirming the importance of this residue in the deacetylase domain. Moreover, the D115A substitution in the ‘TFD(D)G’

Fig. 3. Analysis of CV staining and CR binding of *Y. pestis* cells expressing amino acid substitutions in HmsH (A and B) and HmsF (C and D) proteins. CV staining of cells attached to borosilicate test tubes after growth at 26 °C (A and C) and quantitative CR binding by *Y. pestis* cells after 3 h incubation at 26 °C with CR (B and D). Strains: KIM6+ (*hmsHFRS*⁺), KIM6 (Δ *hmsHFRS*⁺), KIM6-2115 (Δ *hmsH2115*) and KIM6-2116 (Δ *hmsF2116*). HmsH and HmsF proteins with and without amino acid substitutions were expressed from pNPM22 derivatives and pBADHmsF plasmids, respectively. Grey and open bars indicate formation of CR⁺ and CR⁻ colonies on CR plates, respectively. The hatched bar indicates formation of an intermediate pinkish colony on CR plates. Data shown represent a mean of at least four independent experiments carried out with sample duplicates. Displayed error bars refer to standard deviation values.

conserved motif and alteration of the most conserved H184 residue in HmsF both caused biofilm-defective phenotypes (Fig. 3C, D). HmsF with a double substitution (D114A and D115A) retained the expected phenotypic loss as well. Another strongly conserved residue among proven deacetylases, K107, was not critical for HmsF function (Fig. 3C, D). However, the K107A change caused an ~50 % loss in CR binding. Among the residues conserved in HmsF-like proteins (R123, W143, R161, W167, Q169, E172 and E180), only the W143 substitution had an effect on HmsF function (Table 2). The W143A substitution (identical to W143 of *E. coli* PgaB but not present in other deacetylases) had an intermediate-pink phenotype on CR agar plates (Table 2) and an adverse effect in both quantitative assays we used to assess the Hms phenotype (Fig. 3C, D).

The C-terminal part of *Y. pestis* HmsF has a predicted domain COG1649 that is present in other HmsF-like proteins of Gram-negative bacteria (Fig. 2B₂). None of the nine individual substitutions in conserved residues in the COG1649 region of HmsF caused a biofilm-defective phenotype (Table 2). An altered HmsF protein with a large deletion (aa 374–674) in COG1649 failed to complement our Δ *hmsF* mutant. However, Western blots did not detect the truncated protein, indicating that either the stability of the protein was affected or our polyclonal antibody against HmsF does not detect the truncated protein (data not shown).

HmsR and HmsS amino acid substitutions

The similarities of the putative glycosyltransferase HmsR from *Y. pestis* to those of *Staphylococcus epidermidis* – IcaA

and *E. coli* – PgaC (formerly YcdQ) and to the cellulose synthetase BscA from *Salmonella typhimurium* have been described previously (Jones *et al.*, 1999; Kirillina *et al.*, 2004; Lillard *et al.*, 1997; Wang *et al.*, 2004). The functionality of these proteins is critical for biofilm formation in all these bacteria (Heilmann *et al.*, 1996; Lillard *et al.*, 1997; Pendrak & Perry, 1993; Wang *et al.*, 2004; Zogaj *et al.*, 2001). Amino acid sequence comparison of the cellulose synthase BscA from *Gluconobacter xylinus* with many other putative glycosyltransferases, including HmsR, IcaA and PgaC, led to the identification of a conserved 'D, D, D35QxxRW' motif (Saxena & Brown, 1997) (Fig. 2C). We therefore tested alanine substitutions at positions D176, D269, Q305 and R308 as well as in non-conserved residues T114 and L324. Expression of HmsR-T114A and HmsR-L324A complemented the $\Delta hmsR$ mutation as well as the wild-type *hmsR* gene in forming red colonies on CR agar and

restored high levels of CR binding and CV staining (Fig. 4). However, substitutions D176A, D269A, Q305A or R308A in HmsR did not complement the $\Delta hmsR$ mutant, giving rise to white colonies on CR plates and only low levels of CV staining. For all HmsR variants tested, the results of the quantitative CR-binding assays correlated with CV-staining assays (Fig. 4A, B). Hence, four conserved residues from the glycosyltransferase motif 'D, D, D35QxxRW' in HmsR, D176, D269, R305, R308, are critical for production of the biofilm extracellular matrix.

HmsS (17.5 kDa) is the smallest Hms protein in *Y. pestis*, with two predicted transmembrane domains but no other identified domains. A 'NA(V)xLIxW(A)x(Y)x(Q)x(R)' sequence is highly conserved in *E. coli* PgaD, *Erwinia carotovora* HmsS and *Pseudomonas fluorescens* HmsS (Fig. 2D) and was selected for mutagenesis. Expression of

Fig. 4. Analysis of CV staining and CR binding of *Y. pestis* cells expressing amino acid substitutions in HmsR (A and B) and HmsS (C and D) proteins. CV staining of cells attached to borosilicate test tubes after growth at 26 °C (A and C) and quantitative CR binding by *Y. pestis* cells after 3 h incubation at 26 °C with CR (B and D). Strains: KIM6+ (*hmsHFRS*⁺), KIM6 ($\Delta hmsHFRS$ ⁺), KIM6-2118 ($\Delta hmsR2118$) and KIM6-2119 ($\Delta hmsS2119$). HmsR and HmsS proteins with and without amino acid substitutions were expressed from pBADHmsR and pBADHmsS plasmids, respectively. Grey and open bars indicate formation of CR⁺ and CR⁻ colonies on CR plates, respectively. Data shown represent a mean of at least two (HmsS: three) independent experiments carried out with sample duplicates. Displayed error bars refer to standard deviation values.

HmsS-N73A or HmsS-R87A in KIM6-2119 ($\Delta hmsS$) restored CR binding and biofilm formation. On the other hand, substitutions W80A and Y83A gave a strong biofilm-defective phenotype equal to that of KIM6 ($\Delta hmsHFRS$), with little CV staining or CR binding. Thus, the W80A and Y83A, but not the N73A and R87A, residues are critical to the function of HmsS.

DISCUSSION

Previously, we used transposon insertion mutants and complementation to conclude that all four genes in the *hmsHFRS* operon are essential for CR binding and thus biofilm formation (Lillard *et al.*, 1997; Pendrak & Perry, 1993). In this study, we constructed non-polar deletion mutations in each gene of the operon and demonstrated loss of biofilm formation in each mutant via quantitative CR-binding and CV-staining assays. Moreover, biofilm formation was restored by complementation with the corresponding gene (Figs 3 and 4). This definitively confirms our previous conclusions that HmsH, HmsF, HmsR and HmsS are each essential for biofilm formation.

Genes encoding orthologues of each of these *Y. pestis* proteins have been identified in a number of diverse organisms (Bell *et al.*, 2004; da Silva *et al.*, 2002; Jones *et al.*, 1999; Lee *et al.*, 2005; Paulsen *et al.*, 2005; Salanoubat *et al.*, 2002; Wang *et al.*, 2004). Of these, the *E. coli* PgaABCD system is involved in formation of an EPS containing a β -1,6-*N*-acetyl-D-glucosamine polymer, and an EPS with a similar component is also part of the *Y. pestis* biofilm (Itoh *et al.*, 2005; Wang *et al.*, 2004). Several other proteins related to HmsH, HmsF, HmsR and HmsS are from genome sequence projects (Bao *et al.*, 2002; Methé *et al.*, 2003; Nascimento *et al.*, 2004; Ren *et al.*, 2003). We have used these in amino acid sequence alignments with the Hms proteins to identify highly conserved residues that might be critical to the function of these four Hms proteins. Alteration of selected conserved residues to alanine identified critical residues, residues with a moderate effect, and highly conserved residues that are not important for forming a functional Hms protein.

HmsH is predicted to form a β -barrel protein with a large N-terminal periplasmic domain (Bagos *et al.*, 2004; Kirillina *et al.*, 2004). Our mutagenesis study concentrated on this periplasmic domain to avoid a loss of phenotype due to disruption of the β -barrel structure. Two of four residues (E269 and D289) had no effect on biofilm formation while the other two (R134 and E281) had only a moderate effect on the composition of the usual *Y. pestis* KIM6+ biofilm. Complementation with both mutated HmsH proteins yielded CR⁺ colonies on CR plates, indicating no change in a long-term phenotype (CR binding over >48 h). However, both HmsH-E281A and HmsH-R134A had a significant reduction in CV staining compared to HmsH, indicating an alteration in biomass bound to glass over a period of ~16 h. HmsH-E281A also caused a reduction in

CR binding in liquid assay over a 3 h period. Thus, R134 or E281 of HmsH are not absolutely crucial to the function of HmsH but may moderately reduce its efficacy.

HmsF belongs to a large family of deacetylases that possess strong sequence similarities across a wide range of bacteria, with some variability due to the distinct carbohydrate substrates used. The role of *B. subtilis* PdaA in spore germination has been described in detail and *in vitro* enzymic studies have demonstrated PdaA deacetylase activity with an *N*-acetylmuramic acid substrate (Fukushima *et al.*, 2002, 2005; Gilmore *et al.*, 2004). PdaB deacetylase is involved in assembly of peptidoglycan during the course of *B. subtilis* sporulation (Fukushima *et al.*, 2004). Within the HmsF deacetylase domain, we selected 11 highly conserved residues to mutate. Four residues are highly conserved in proven deacetylases (Fig. 2B₂) while the other seven residues are highly conserved in HmsF-like proteins (Fig. 2B₁) but not in proven deacetylases (Fig. 2B₂). Alteration of the HmsF residues D114 and D115, corresponding to D73 and N74 of PdaA, completely prevented biofilm formation, possibly by affecting the putative deacetylase activity of HmsF. The H184A substitution (corresponding to H124 in PdaA) also abolished CR binding and CV staining. However, the HmsF containing the K107A alteration appears to produce another attenuated CR-binding mutant with reduced short-term binding at 3 h that does not affect the formation of CR colonies by 48 h of growth (Fig. 3, Table 2). The W143A substitution caused negligible CR binding and CV straining that led to an intermediate-pink colony formation on CR plates, suggesting this residue is not essential but does affect protein function – possibly enzymic activity. W143 was the only substitution in HmsF-like conserved residues that had any effect on the functionality of HmsF.

A second region of *Y. pestis* HmsF with high similarity to other proteins (Fig. 2B₂) is COG1649; proteins with this domain of unknown function are assigned to the TIM barrel glycosyl hydrolase superfamily by Pfam (Bateman *et al.*, 2004). Nine alanine substitutions of highly conserved residues within the COG1649 domain of HmsF did not affect HmsF – all altered proteins were able to fully restore an Hms⁺ phenotype (Table 2). However, expression of a truncated HmsF lacking the COG1649 domain failed to complement the $\Delta hmsF$ mutant. Truncated HmsF protein was not detected by HmsF antiserum used in immunodetection of full-length HmsF proteins. The recombinant protein used to produce HmsF antiserum lacked the first 87 aa but contained both the deacetylase and COG1649 domains (Kirillina *et al.*, 2004; Pendrak & Perry, 1991; Perry *et al.*, 2004). This may indicate protein instability or lack of antigenic domains recognized by our HmsF antibody. Further studies will be required to determine whether the COG1649 domain is important to the function of HmsF.

There are several lines of evidence indicating that HmsR is a likely β -1,6-*N*-acetyl-D-glucosamine transferase. First, HmsR has a high degree of similarity to IcaA and PgaC,

which are both required for the synthesis β -1,6-*N*-acetyl-D-glucosamine (Heilmann *et al.*, 1996; Jones *et al.*, 1999; Wang *et al.*, 2004). Second, PgaC was able to restore the CR-binding phenotype of an *hmsR* mutant when expressed from a high-copy-number plasmid (Jones *et al.*, 1999). Third, DspB, a β -hexosaminidase and biofilm-dispersing enzyme of *A. actinomycetemcomitans* (Kaplan *et al.*, 2003), shown to specifically hydrolyse the glycosidic linkages of poly- β -*N*-acetyl-D-glucosamine, caused complete inhibition of biofilm formation by *Y. pestis* KIM6+ (Itoh *et al.*, 2005). Our results further support this conclusion. A non-polar *hmsR* deletion abrogated synthesis of an extracellular matrix which binds CR and expression of HmsR proteins with substitutions in conserved residues of the glycosyltransferase motif 'D, D, D35QxxRW', i.e. D176A, D269A, R305A and R308A, failed to restore CR binding and CV staining to this mutant (Fig. 2; Table 2). The substitutions D236Y, D333R, Q369M and R372A within this motif in the AcsAB cellulose synthase of *G. xylinus* impaired the enzymic activity *in vitro* (Saxena & Brown, 1997; Saxena *et al.*, 2001). Consequently, we propose that the substitutions in HmsR at similar positions may abolish its enzymic activity in poly-*N*-acetylglucosamine production.

The essential HmsS protein, which is associated with the inner membrane like HmsR and HmsT (Lillard *et al.*, 1997; Perry *et al.*, 2004), has two predicted transmembrane regions but no other currently recognized domains. Alignment of HmsS-like proteins identified a conserved region ['NA(V)xLlxW(A)x(Y)x(Q)x(R)'; Fig. 2D]. N73A and R87A changes did not affect HmsS protein function. In contrast, a dramatic loss of HmsS function was related to W80A and Y83A alterations (Fig. 4A, B). Both W80 and Y83 are within a predicted cytoplasmic region of HmsS.

These site-directed mutagenesis studies have identified critical residues within predicted enzymic domains in HmsF and HmsR and in a conserved region of HmsS. Two residue changes in HmsH and two in HmsF (HmsH-R134A, HmsH-E281, HmsF-K107A, HmsF-W134A) had differential effects in our biofilm assays. The reason for this differential effect is obscure. The ability of cells expressing HmsF-K107A to achieve normal attachment levels while displaying an EPS-defective phenotype suggests that this mutant produces an EPS form that is not conducive to CR binding. In addition, these and some of the other amino acid residue substitution mutations may affect protein-protein interactions that may be important for biofilm formation.

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