Role of the $hprT$–$ftsH$ locus in *Staphylococcus aureus*

James K. Lithgow,1 Eileen Ingham2 and Simon J. Foster1

1Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, UK
2Department of Microbiology, University of Leeds, Leeds LS2 9JT, UK

The roles of two adjacent genes in the *Staphylococcus aureus* chromosome with functions in starvation survival and the response to stressful conditions have been characterized. One of these, *hprT*, encoding a hypoxanthine–guanine phosphoribosyltransferase homologue, was initially identified in a transposon mutagenesis screen. Mutation of *hprT* affects starvation survival in amino-acid-limiting conditions and the ability of *S. aureus* to grow in high-salt concentrations. Downstream of *hprT* is *ftsH*, which encodes a membrane-bound, ATP- and Zn$^{2+}$-dependent ‘AAA’-type protease. Mutation of *ftsH* in *S. aureus* leads to pleiotropic defects including slower growth, sensitivity to salt, acid, methyl viologen and potassium tellurite stresses, and reduced survival in amino-acid- or phosphate-limiting conditions. Both *hprT*–*lacZ* and *ftsH*–*lacZ* gene fusions are expressed maximally in the post-exponential phase of growth. Although secretion of exoproteins is not affected, an *ftsH* mutant is attenuated in a murine skin lesion model of pathogenicity.

INTRODUCTION

*Staphylococcus aureus* is a medically important human pathogen that is capable of causing a variety of infections, ranging from minor skin and wound infections to life-threatening diseases (Lowy, 1998). This capability is due to the repertoire of toxins, exoenzymes, adhesins and immune-modulating proteins that *S. aureus* produces. As the bacterium cycles from its host to the external environment, it must survive a range of stresses and nutrient-limiting conditions, and it is the adaptability of this organism to survive in changing environments that allows it to be successful as an opportunistic pathogen.

We have characterized the starvation survival and stress responses of *S. aureus* (Clements & Foster, 1999; Watson et al., 1998b). A number of transposon insertion mutants defective in starvation survival have been isolated (Watson et al., 1998a). By this approach, genes involved in oxidative stress resistance, DNA-repair mechanisms and cytochrome biosynthesis have been shown to be involved in starvation survival (Clements et al., 1999a, b; Watson et al., 1998a). One such mutant with a defect in starvation recovery is SPW20, in which a transposon is inserted in a gene putatively encoding a hypoxanthine–guanine phosphoribosyltransferase (HprT) homologue. HPRTs (EC 2.4.2.8) are enzymes involved in the conversion of purine bases into nucleotides. These purine bases can be recovered from degraded nucleic acids rather than from de novo synthesis, and thus the activity is known as purine salvage or recycling. Most bacteria do possess de novo purine synthesis pathways; however, *hprT* was recently shown to be required for virulence in *Listeria monocytogenes* and is essential for growth of *Bacillus subtilis* (Kobayashi et al., 2003; Taylor et al., 2002).

Downstream of the *hprT* gene in the published genome of *S. aureus* (Kuroda et al., 2001) is another open reading frame (ORF) encoding a homologue of the ATP- and Zn$^{2+}$-dependent protease FtsH. FtsH belongs to the ‘AAA’ family of proteins (ATPases Associated with diverse Cellular Activities), and *ftsH* homologues are ubiquitous in eubacteria and eukaryotic organelles such as mitochondria and chloroplasts (Langer, 2000; Ogura & Wilkinson, 2001). FtsH metalloproteases are anchored to the cytoplasmic membrane via two transmembrane segments, with the short N- and long C-terminal parts facing the cytoplasm. These proteases catalyse the degradation of denatured or damaged proteins, and are also thought to assist in refolding of proteins, or ‘chaperone’ activity, which helps maintain a quality control of proteins in the membrane and cytoplasm. The *ftsH* gene is essential in *Escherichia coli*, *Lactococcus lactis* and *Helicobacter pylori* (Ge & Taylor, 1996; Nilsson et al., 1994; Ogura et al., 1991) and, although not essential in *B. subtilis* or *Caulobacter crescentus*, *ftsH* mutants exhibit a pleiotropic phenotype with defects in salt and heat tolerance, cell growth and starvation survival (Deuerling et al., 1997; Fischer et al., 2002). A recent study showed that a clone expressing antisense *ftsH* RNA prevented the growth of *S. aureus*, suggesting essentiality in this organism (Forsyth et al., 2002).

In this study, we examine the role of the *hprT* and *ftsH*
genes in *S. aureus*. Mutation of *hprT* has little effect on growth and has only a minor effect on starvation survival and osmotic tolerance. In contrast, we show that although the *ftsH* gene is not essential, a *ftsH* mutant has multiple defects including significantly slower growth, reduced viability in starvation conditions, sensitivity to multiple stresses, including salt, acid, methyl viologen and tellurite, and is significantly attenuated in a murine skin lesion model of pathogenicity.

**METHODS**

**Media and growth conditions.** *S. aureus* and *E. coli* strains and plasmids are listed in Table 1. *E. coli* was grown in Luria–Bertani medium at 37°C. *S. aureus* was grown at 25 or 37°C with shaking at 250 r.p.m. in brain–heart infusion (BHI) (Oxoid) or chemically defined medium (CDM) (Watson *et al.*, 1998b). For CDM growth experiments, cultures were inoculated from overnight CDM cultures to an OD₆₀₀ value of 0-005. For growth on solid media, 1% (w/v) agar was added. When included, antibiotics were added at the following concentrations: ampicillin, 100 mg l⁻¹; chloramphenicol (cat), 5 mg l⁻¹; erythromycin (ery), 5 mg l⁻¹; lincomycin (lin), 5 mg l⁻¹; tetracycline (tet), 5 mg l⁻¹.

**Construction of strains and plasmids.** Mapping of the *hprT–ftsH* locus and design of oligonucleotide primers were done using the *S. aureus* 8325 genomic DNA sequence (http://www.genome.ou.edu/staph.html), and protein homologies were investigated using the NCBI-BLAST homology search program (http://www.ncbi.nlm.nih.gov/blast/). DNA manipulations and gel electrophoresis were carried out according to methods described by Sambrook *et al.* (1989). SPW20 was isolated and characterized by Watson *et al.* (1998a).

<table>
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<th>Strains</th>
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<tr>
<td>8325–4</td>
<td>Wild-type strain cured of prophages</td>
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<tr>
<td>RN4220</td>
<td>Restriction deficient transformation recipient</td>
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<td>Functional <em>rshU</em> derivative of RN4220</td>
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*Restriction sites are underlined.
Stress resistance and starvation survival assays. To measure growth in the presence of high-salt concentrations, cultures were inoculated from overnight BHI cultures to an OD₆₀₀ value of 0.08 into either BHI or BHI containing 2 M NaCl at 37 °C. Hydrogen peroxide resistance assays were carried out as described previously (Watson et al., 1998a) with the following modifications. Cells were grown in amino-acid-limiting CDM (1% w/v, glucose) to exponential phase (OD₆₀₀ 0.1). Following the addition of H₂O₂ to a final concentration of 10 mM and incubation, cells were serially diluted in PBS containing catalase at 10 mg ml⁻¹, and viability was assessed by overnight growth on BHI agar. Tellurite resistance assays were performed in the same way except using potassium tellurite (K₂TeO₃) at a final concentration of 200 mM instead of H₂O₂ and dilution with PBS after treatment. To determine the MIC of tellurite, S. aureus strains were grown overnight in BHI, and approximately 10³ cells were spotted onto BHI agar plates containing varying concentrations of K₂TeO₃. The MIC of tellurite was determined as the lowest concentration at which there was no bacterial growth. Acid-resistance assays were performed by growing cells to exponential phase in BHI, followed by acidification of the medium to pH 2 with hydrochloric acid. Cells were serially diluted in 4× PBS and viability was determined after growth on BHI agar. Disc diffusion assays were performed as follows. Five millilitres of BHI top agar (0.7%, w/v) was seeded with 5 μl of an exponential-phase S. aureus BHI culture (OD₆₀₀ 0.2) and used as an overlay on a BHI agar plate. Sterile 13 mm antibiotic discs were placed on top of the overlay, and 20 μl of 500 mM diamide, 35 μl of 2 M methyl viologen or 20 μl of 1 M K₂TeO₃ was added to the disc. Zones of growth inhibition were measured after 24 h incubation at 37 °C. Starvation survival experiments were performed in amino-acid-limiting, glucose-limiting or phosphate-limiting CDM (Watson et al., 1998b). Cultures of 50 ml were grown for 24 h with shaking at 37 °C, then kept static at 25 °C. Samples were serially diluted and viability assessed by growth on BHI agar. The results presented here are representative of three independent experiments that showed less than 10% variability.

β-Galactosidase assays. Expression of hprT-lacZ and ftsH-lacZ in S. aureus was measured in BHI cultures of J35 or J37 shaking at 37 °C. Cultures were inoculated to an OD₆₀₀ value of 0.01 from exponential-phase BHI cultures. To test for induction, subinhibitory concentrations of diamide (200 μM), methyl viologen (25 μM) or K₂TeO₃ (200 μM) were added after 2 h growth. Levels of β-galactosidase activity were measured as described previously (Horsburgh et al., 2002b) using 4-methylumbelliferyl β-D-galactoside as substrate. Assays were performed in duplicate and the values were averaged. The results presented here are representative of two independent experiments that showed less than 10% variability.

Virulence testing of strains in a murine skin lesion model. Pathogenicity was tested as described previously (Chan et al., 1998). Statistical significance was evaluated on the recovery of strains using the Student’s t-test with a 5% confidence limit.
RESULTS

Identification and mutagenesis of hprT and ftsH

The Tn917 insertion mutant SPW20 was isolated in a previous screen for starvation survival mutants of *S. aureus* (Watson *et al*., 1998a). In this strain, the transposon is inserted in an ORF of 185 aa that shares 59% identity with the putative hypoxanthine–guanine phosphoribosyltransferase (Hprt) enzyme from *B. subtilis*. The genetic background of the SPW20 mutant is *S. aureus* 8325–4, which has an 11 bp deletion in the *rsbU* gene that is required for full expression of the accessory sigma factor σ^B^ (Horsburgh *et al*., 2002a). This sigma factor is known to modulate virulence-determinant expression and stress resistance. It was important to test the role of *hprT* in a strain with a functional *rsbU* and σ^B^, and therefore the *hprT::* Tn917 mutation was transduced from its original 8325–4 background into the *rsbU* strain SH1000 (Horsburgh *et al*., 2002a), creating strain J45.

Two hundred and seventeen nucleotides downstream from the *hprT* stop codon is another ORF encoding a protein of 697 aa that shares 66% identity with FtsH from *B. subtilis*. The possibility existed that the starvation survival defect in SPW20 was due to a polar effect on *ftsH*. The *hprT–ftsH* gene arrangement (Fig. 1) is similar in *S. aureus*, *B. subtilis* and *Listeria monocytogenes*, but not in all bacteria (Schumann, 1999). Considering the multiple functions and essentiality of *ftsH* in many bacteria, it was of interest to determine the role of this gene in *S. aureus*. A strain carrying an *ftsH::tet* knockout mutation, J27, was constructed in an *S. aureus* SH1000 background. This strain was viable and able to grow in rich and minimal medium. Using these strains we investigated the contribution of *hprT* and *ftsH* to the physiology of *S. aureus*.

Role of *hprT* and *ftsH* in starvation survival

The starvation survival ability of the *hprT* and *ftsH* mutants in the SH1000 background was tested (Fig. 2). In amino-acid-limiting CDM, J45 (*hprT*) showed a 25-fold reduction in viability compared to SH1000 after 27 days prolonged incubation at 25 °C. J27 (*ftsH*) lost viability much quicker, and after 27 days its viability was 10^4^ fold less than SH1000 (Fig. 2a). In phosphate-limiting CDM, J45 (*hprT*) was no less viable than SH1000, but J27 (*ftsH*) showed a drastic reduction in viability, approximately 10^7^ fold lower than SH1000 after 23 days incubation (Fig. 2b). Neither mutant was different from the wild-type in glucose-limiting CDM (data not shown).

Mutation of *hprT* affects growth in high-salt medium and mutation of *ftsH* has pleiotropic defects in growth and stress resistance

In amino-acid-limiting CDM, the growth of J45 (*hprT*) was not significantly different from that of SH1000. However, J27 (*ftsH*) exhibited a significant lag in growth (Fig. 3a).
containing 2 M NaCl, both mutants have a significant growth lag compared to SH1000 (Fig. 4a). S. aureus strains have a naturally high level of resistance to the rarely occurring oxyanion tellurite (TeO$_2^-$), which exerts toxic effects on cells (Taylor, 1999). When exponential-phase cultures were challenged with 200 mM K$_2$TeO$_3$, the J27 (ftsH) mutant cells demonstrated 10$^6$-fold less viability after 6 h compared to both SH1000 and J45 (hprT), with addition of chloramphenicol to maintain plasmids in (b).

**Fig. 3.** Growth in amino-acid-limiting CDM. (a) ■, S. aureus SH1000; ▲, J27 (ftsH); ○, J45 (hprT). (b) Complementation of J27 (ftsH) growth lag with pJIM78 ftsH plasmid. □, J126 [SH1000(pCU1)]; △, J109 [J27(pJIM78) (ftsH)]; ▲, J111 [J27(pCU1) (ftsH)]. Cultures were grown with shaking at 37 °C, with addition of chloramphenicol to maintain plasmids in (b).

viable after 5 h (Fig. 4c). J27 (ftsH) was also more sensitive to both 1 M K$_2$TeO$_3$ and 2 M methyl viologen in disc diffusion assays. This defect could be complemented using the ftsH plasmid pJIM78, but not by introduction of the control vector pCU1 (Fig. 4d). Strain J45 (hprT) was no more sensitive than SH1000 to tellurite or methyl violagen discs, and neither J27 (ftsH) nor J45 (hprT) showed increased sensitivity using disc diffusion assays with the thiol oxidant diamide. Resistance to H$_2$O$_2$ or heat shock was not affected in either mutant (data not shown).

**hprT–lacZ and ftsH–lacZ fusions are expressed in late-exponential phase of growth**

Strains J35 (hprT–lacZ) and J37 (ftsH–lacZ) were constructed to measure the expression of the hprT and ftsH genes. Both genes reach a maximum level of expression in the late-exponential/early-stationary phase of growth (Fig. 5). Since the ftsH mutant had shown sensitivity to methyl violagen and K$_2$TeO$_3$, these compounds were added to the medium at subinhibitory concentrations (25 and 200 μM, respectively) in the early stages of growth, but neither compound appeared to induce a change in the expression pattern of ftsH–lacZ (data not shown).

**Mutation of ftsH affects pathogenicity**

*S. aureus* infection and dissemination through tissues is dependent on its ability to produce and secrete a number of virulence factors such as haemolysins. Neither J27 (ftsH) nor J45 (hprT) was defective in haemolytic activity on rabbit or sheep blood agar plates. In addition, no differences could be seen in the profile of exoproteins produced from culture supernatant extracts on SDS-PAGE gels, nor in extracts of cytoplasmic proteins (data not shown). In a mouse lesion model of infection, a significantly lower percentage of recovery was observed for J27 (ftsH) compared to SH1000 (Fig. 6). The mean percentages of recovery and Student’s *t*-test *P* values were as follows: SH1000, 343 %; J27 (ftsH), 73 %, *P* < 0.011.

**DISCUSSION**

**Role of hprT in S. aureus**

Many bacteria are able to produce nucleotides by de novo synthesis pathways. In the purine nucleotide synthesis pathway, inosine monophosphate (IMP), a molecule produced from reactions originating in sugar and amino acid metabolism, is converted into either ATP or GTP. The external environment of bacteria often contains nucleosides (e.g. guanosine, xanthosine) and nucleobases (e.g. guanine, xanthine) that have been excreted by living cells or arise from degradation of dead cells. Intracellular breakdown of nucleic acids may also be a source of nucleosides and nucleobases. To prevent the loss of valuable precursors, bacteria have evolved salvage pathways that enable recovery of purine and pyrimidine bases and nucleosides, which can then be recycled into nucleotides or used as a source of
energy, carbon or nitrogen (Nygaard, 1993). HprT catalyses the addition of a ribose-phosphate moiety to either guanine or hypoxanthine, using phosphoribosyl pyrophosphate (PRPP). Thus, guanine or hypoxanthine is converted into the nucleotides GMP or IMP, respectively.

The hprT gene is clearly not essential for the growth of S. aureus, although it has a minor role in the ability to grow in high-salt and survive in starvation conditions due to likely defects in nucleotide recycling. The expression of hprT–lacZ in rich medium is maximal in post-exponential phase, which matches the role of hprT in recycling as the culture becomes nutrient-limited. In the presence of high-salt concentrations, the defect in nucleotide recycling results in a decreased growth rate which implies an important role for nucleotide recycling under these stressful environmental conditions. The hprT gene was recently shown to be essential in B. subtilis (Kobayashi et al., 2003). This result is perhaps surprising since the viability of mutants in the study was examined using a rich medium, in which de novo nucleotide synthesis pathways would be expected to function normally. The authors suggest that the hprT gene product may have a second, unsuspected role in B. subtilis. A recent study showed that a Listeria monocytogenes hprT mutant defective in surface-attached growth and virulence was unable to accumulate (p)ppGpp in response to nutrient starvation, and thus was unable to mount a stringent response. It was suggested that HprT is needed to maintain intracellular GDP and GTP at levels sufficient for the activity of the RelA (p)ppGpp synthetase (Taylor et al., 2002). The stringent response is important in S. aureus, and in this organism the relA gene is essential (Gentry et al., 2000). S. aureus 8325-4 has been previously shown by radioactive labelling/TLC to produce ppGpp and pppGpp (Cassels et al., 1995).
Using these methods we found that both S. aureus 8325-4 and SPW20 (hprT) were able to synthesize ppGpp and pppGpp molecules (data not shown), indicating that the S. aureus hprT gene is not required for (p)pGpp synthesis, and that the starvation survival defect is not due to an absence of (p)pGpp. From these results it is clear that mutation of hprT does not have the major effects it has in B. subtilis and Listeria monocytogenes. It is possible that S. aureus has other activities that compensate for the inactivation of HprT. Interestingly, the 5' end of the HprT ORF overlaps the 3' end of the preceding gene which encodes the putative protein YacA; thus the yacA and hprT genes may be transcriptionally and translationally coupled. The function of YacA is unknown, although it has been proposed to be essential based on antisense RNA studies (Forsyth et al., 2002). The role of YacA and its possible interaction with HprT are currently being investigated.

**Role of ftsH in S. aureus**

In E. coli, FtsH is involved in protein assembly into and through the cytoplasmic membrane (Akiyama et al., 1994). The FtsH protein has been shown to degrade membrane proteins such as the secretory machinery subunit SecY (Akiyama et al., 1996a; Kihara et al., 1995) and subunit a of the F1F0 ATPase complex (Akiyama et al., 1996b). This activity is thought to prevent the potentially harmful accumulation of free subunits of membrane-embedded complexes. There is also evidence that FtsH has a chaperone-like function, independent of its proteolytic activity, since certain defects in growth and protein translocation could be partially suppressed by overproduction of the molecular chaperones GroEL/ES or HtpG (Shirai et al., 1996). In addition, FtsH also degrades cytoplasmic regulatory proteins such as the transcription factor σ32 (Herman et al., 1995; Tomoyasu et al., 1995). The ftsH gene is essential in E. coli, Lactococcus lactis and H. pylori and the basis of essentiality in E. coli has been attributed to the role of FtsH in balancing phospholipid and lipopolysaccharide synthesis (Ogura et al., 1991). ftsH was recently suggested to be essential in S. aureus RN450, a strain closely related to SH1000 (Forsyth et al., 2002). The study identified a single antisense RNA clone corresponding to ftsH that prevented growth of S. aureus, compared to the multiple independent clones found for a number of other essential genes, including yacA described above. Our evidence suggests that ftsH is not essential in S. aureus; however, as has been shown for B. subtilis and C. crescentus ftsH mutants, the S. aureus ftsH mutant has a pleiotropic phenotype. In S. aureus, mutation of ftsH results in defects in growth, stress resistance, starvation survival and pathogenicity. S. aureus J27 (ftsH) has a significant growth lag in CDM which can be complemented by a plasmid carrying...
only ftsH, suggesting that the growth defect is not due to polar effects downstream of the ftsH::tet insertion. Also, the hprT mutation does not result in the same CDM growth defect and is unlikely to be polar on ftsH, and thus they probably represent independent transcriptional units.

The S. aureus ftsH mutant showed sensitivity to 2 M NaCl, acid stress, methyl viologen and K2TeO3, implying a role for FtsH in the stress response of S. aureus. Methyl viologen induces the production of internal superoxide (O2−), which can lead to the production of more toxic reactive oxygen species such as H2O2, the hydroxyl radical (OH−) or peroxynitrite (OONO−), all of which can damage macromolecules (Clements & Foster, 1999). The exact mechanism of toxicity of the tellurite ion (TeO32−) is unknown, but is thought to be due to its strong oxidizing ability (Taylor, 1999). Tellurite can be reduced by glutathione and/or other reduced thiols, leading again to O2− production. The increased sensitivity of the S. aureus ftsH mutant to acid, methyl viologen and tellurite may be explained by an inability to degrade and turn over proteins that have been oxidatively damaged by O2− or denatured by acid during acid stress.

S. aureus J27 (ftsH) has a more pronounced loss of viability in starvation conditions, as has been shown for C. crescentus ftsH mutants (Fischer et al., 2002). The degradation of existing proteins may be a major source of amino acids during starvation and, in addition, the pH of amino-acid-limiting medium has been shown to fall during prolonged starvation (Watson et al., 1998b), which would also increase the acid stress on cells. Unable to utilize a source of amino acids and sensitive to acid stress, ftsH mutants are therefore at an obvious disadvantage in starvation conditions, which may explain their reduced viability in amino-acid- or phosphate-limiting medium.

Since mutation of ftsH does not affect haemolysin production and overall exoprotein secretion in S. aureus, the attenuation in a mouse subcutaneous abscess model of pathogenicity is more likely to be caused by a growth or stress response defect, rather than an inability to produce virulence factors. How FtsH exerts its effects at the molecular level is still largely unknown in any organism. Mutation of ftsH has been shown to cause changes in gene expression in E. coli and B. subtilis (Tomoyasu et al., 1993; Zellmeier et al., 2003) and FtsH is involved in the degradation of regulatory components in E. coli (Herman et al., 1995; Tomoyasu et al., 1995). Another function of FtsH may be to prevent the accumulation of damaged proteins, which would otherwise lead to defects under stressful conditions. It is likely that such pleiotropic defects are due to the multiple targets for such an important cellular component.

ACKNOWLEDGEMENTS

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REFERENCES


