Characterization of the ftsH gene of *Bacillus subtilis*

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INTRODUCTION

The ftsH gene of *Bacillus subtilis* has recently been genetically identified (Geisler & Schumann, 1993) and has been sequenced in its entirety as part of the *Bacillus* genome sequencing project (Ogasawara et al., 1994). It is important since it encodes a prokaryotic member of the AAA-protein family of ATPases (ATPases associated with diverse cellular activities: Confalonieri & Duguet, 1995; Kunau et al., 1993). Over 100 members of this protein family are found and are found not only in bacteria but also in eukaryotes, including *Saccharomyces cerevisiae*, *Xenopus laevis* and a number of mammals including humans. The AAA-proteins are involved in a multitude of varied activities, including protein secretion, protein assembly and proteolysis, peroxisome biogenesis, and cell cycle control. All members of this protein family have in common a large domain of about 200 amino acids containing an ATP-binding site, termed the AAA-module.

The first prokaryotic member of this family to be discovered was the FtsH protein of *Escherichia coli* (Ogura et al., 1991; Tomoyasu et al., 1993a), also referred to as hflB (Herman et al., 1993), and subsequently genes that could encode homologous proteins have been identified in *Lactobacillus lactis* (Nilsson et al., 1994), *B. subtilis* (Ogasawara et al., 1994) and more recently from the genome sequencing projects for *Haemophilus influenzae* and *Mycoplasma genitalium*. The homology shared between the five prokaryotic FtsH proteins is substantial and extends beyond the AAA-module. First, all contain two hydrophobic segments at the N-terminus. In the case of the *E. coli* and *L. lactis* proteins these segments have been shown to anchor FtsH to the phospholipid membrane by two transmembrane loops exposing the C-terminal AAA-module to the cytoplasm (Akiyama et al., 1994a; Nilsson et al., 1994; Tomoyasu et al., 1993b). Second, all contain a conserved C-terminal domain which is similar to the active site motif of zinc-metalloproteases. Interestingly, three membrane-bound mitochondrial proteins from *S. cerevisiae* (Yta10p, Yme1p/Yta11p and Yta12p) show remarkable similarity to the bacterial FtsH proteins, including the transmembrane domains and zinc metalloprotease active site (Tauer et al., 1994; Thorsness et al., 1993).

ftsH mutations are highly pleiotropic and affect cell growth, viability (Ogura et al., 1991) and a number of protein–membrane-associated events such as the secretion and assembly of polypeptides into membranes (Akiyama et al., 1994a, b; Tomoyasu et al., 1993a, b). The pleiotropy associated with ftsH mutants has led to the suggestion that FtsH could function in some way as a chaperone, facilitating protein secretion and proteolysis. In *B. subtilis* and *L. lactis* ftsH mutants have been identified which are salt-sensitive, suggesting that FtsH may also be involved in the cell’s response to stress (Deuerling et al., 1995; Geisler & Schumann, 1993; Nilsson et al., 1994). Recently, the *E. coli* FtsH protein...
has been shown to be directly involved in the proteolytic degradation of both the \( \sigma^{22} \) protein and the heat-shock transcription factor \( \sigma^{32} \) (Herman et al., 1995; Tomoyasu et al., 1995). FtsH has also been shown to be involved in the degradation of uncomplexed SecY, an essential component of the protein translocation machinery of \( E. \ coli \), which may partially explain its pleiotropic effects on protein assembly and secretion (Kihara et al., 1995). The putative active-site motif for this proteolytic activity has been demonstrated to be essential for FtsH function (Qu et al., 1996).

In this work we have characterized the \( ftsH \) gene of \( B. \ subtilis \). We show that under certain conditions (e.g. in minimal medium) FtsH is required for cell growth and is in addition required for the cell's response to osmotic stress and spore formation.

METHODS

Bacterial strains, general methods and sporulation experiments. All strains used in this work were congenic derivatives of the wild-type (Spo'), prototrophic, strain PY79. Routine cloning methods, PCRs, etc., were as described by Sambrook et al. (1989). \( B. \ subtilis \) methods (preparation of chromosomal DNA, transformations, etc.) were as described by Harwood & Cutting (1990). Spectinomycin was used for growth and selection at a concentration of 100 \( \mu \)g ml\(^{-1}\). The 'exhaustion' method was used to induce sporulation (Nicholson & Setlow, 1990). For measurement of \( \beta \)-galactosidase activity, samples (10 ml) were removed, cells pelleted, and frozen at \(-70^\circ\)C. The method of Miller (1972) was used to determine enzyme activity using the \( \beta \)-galactosidase substrate ONPG and specific activity was expressed as \( \Delta A_{420} \) per min per ml culture per OD\(_{660}\) unit \( \times 1000 \).

Construction of \( ftsH::spc \). Two oligonucleotide primers FTSS (5'-CGGGATCCGGATAAAACCGAGCGGAAGA-3') and FTSSR (5'-GCTCTAAGGTGCCCGAATCGATA-3') that annealed to sequences (underlined) upstream (FTSF) and downstream (FTSRT) of the \( ftsH \) locus were used to amplify, by PCR, a 2.5 kb product containing the entire \( ftsH \) gene (see Fig. 1). This DNA was digested with \( SacI \) and \( EcoRI \), which cut within the \( ftsH \) ORF and ligated to a 1.1 kb \( SacI \)-\( EcoRI \) spectinomycin-resistance (Sp\(^{R} \)) cassette (from pJR74 (LeDeaux & Grossman, 1995). The ligated mixture was introduced directly into competent cells of \( B. \ subtilis \) strain PY79 with selection for Sp\(^{R} \) at 30 \( ^\circ \)C. Approximately 100 Sp\(^{R} \) transformants were isolated by this procedure. Transformants should arise if the Sp\(^{R} \) cassette had inserted by a marker replacement between the \( SacI \) and \( EcoRI \) sites of the \( ftsH \) ORF to create a deletion (~1 kb) and insertion mutation. A double crossover recombination would occur if the 5' and 3' ends of the Sp\(^{R} \) cassette are flanked by the corresponding upstream and downstream fragments of the \( ftsH \)-amplified DNA. Several putative \( ftsH \) mutants were isolated and checked as follows. First, linkage of Sp\(^{R} \) to a chloramphenicol resistance marker adjacent to the \( spoIE \) locus (5 kb upstream of \( ftsH \))

![Fig. 1. Physical map of the \( ftsH \) locus. The top of the figure shows a schematic representation of the FtsH protein showing the AAA-module and Zn\(^{2+}\)-binding domain. Shown below are constructions described in the text. (a) The 2.5 kb DNA fragment amplified by PCR and used for construction of the \( ftsH::spc \) mutant is indicated. (b) Physical map of the \( ftsH \) locus and neighbouring \( hprT \) gene (encoding hypoxanthine guanine phosphoribosyltransferase). (c) Structure of the chromosome anticipated in our mutant construction. (d) Structure of the \( ftsH::spc \) mutant chromosome as determined by Southern hybridization analysis. The 1 kb \( SacI \)-\( EcoRI \) fragment of \( ftsH \) that was duplicated and cloned, adjacent to Sp\(^{R} \) at the \( EcoRI \) site is shaded. (e) Structure of the \( ftsH-lacZ \) fusion. A 713 bp 5' fragment of \( ftsH \) was amplified by PCR and fused to \( lacZ \) in the vector pTKlac. Sites: H, HindIII; E, EcoRI; S, SacI.](image-url)
was confirmed by DNA-mediated transformation. Second, we used Southern hybridization analysis to examine the ftsH locus of the mutant chromosome. Analysis (not shown) of all clones revealed that insertion had not occurred between the SacI and EcoRI sites as predicted but rather at the EcoRI site alone. This insertion consisted of the Sp4 cassette followed by a copy of the 1 kb SacI-EcoRI ftsH fragment fused to Sp4 (shown in Fig. 1). This construction separates the terminal 123 codons of the ftsH ORF and leaves intact a functional ORF of 505 residues. It is unclear why our experimental approach failed to generate the anticipated ftsH null mutation; most probably, though, such an allele of ftsH would be lethal, severely affecting cell growth, as is the case with E. coli. One mutant, EL727 (ftsH::spc), was used for further characterization.

**Immunological detection of FtsH.** Membrane proteins (50 μg) purified from vegetatively growing cells (see below) were fractionated on 12% SDS-polyacrylamide gels. Proteins were transferred to PVDF membrane (Millipore) and incubated with anti-E. coli FtsH serum (1:5000). FtsH was detected using the ECL Western blotting detection kit (Amersham).

**Construction of ftsH-lacZ.** Two oligonucleotide primers, FTSF (see above) and OL61 (5'-CCCAAGCTTCTCTGTA-TACTGCTTCGC-3') were used to amplify, by PCR, a 713 bp DNA product from the wild-type chromosome containing the 5' region of ftsH (see Fig. 1). FTSF anneals to sequences within the hprT gene upstream of ftsH and OL61 anneals to sequences within the ftsH ORF. The amplified product was digested with BamHI and HindIII, which cut the 5' and 3' ends of the PCR product, and ligated to the vector pTKlac cleaved with BamHI and HindIII. pTKlac contains a multiple cloning site upstream of the E. coli lacZ gene, facilitating the construction of transcriptional lacZ fusions (Kenney & Moran, 1987). One plasmid was isolated in E. coli that contained the ftsH 5' region fused upstream and in the same direction as lacZ in pTKlac (see Fig. 1c). This plasmid, pEL8, was introduced into B. subtilis PY79 by DNA-mediated transformation with selection for CmR. CmR transformants arose due to a single, Campbell-type, integration of pEL8 into the ftsH locus since pTKlac cannot replicate autonomously in B. subtilis. Chromosomal DNA prepared from one CmR transformant was used as a source of ftsH-lacZ and introduced into subsequent strains by DNA-mediated transformation.

**Purification of membrane proteins.** Cultures (250 ml) of cells growing exponentially (OD650 ~ 3.0) in LB medium were harvested and washed once with ice-cold buffer (50 mM Tris/HCl, pH 7.5, 1 mM MgCl2, 1 M KCl, 0.1 mM PMSF) and frozen rapidly at −70°C. To break the cells, the pellet was thawed and suspended in ice-cold buffer A (100 mM Tris/HCl, pH 8.0, 1 mM MgCl2, 1 mM β-mercaptoethanol, 0.1 mM PMSF). Approximately 0.2 ml glass beads was added and the mixture subjected to repeated cycles of sonication (5 × 30 s) until more than 50% cell lysis had occurred. The broken cells were then diluted with 8 ml buffer A and the mixture centrifuged (8000 g, 10 min, 4°C). The supernatant was removed and centrifuged (48000 g, 60 min, 4°C) and the pellet resuspended in 5 ml buffer B (50 mM Tris/HCl, pH 8.0, 1 mM β-mercaptoethanol, 0.1 mM PMSF) and centrifuged again (48000 g, 60 min, 4°C). The ‘membrane’ pellet was suspended in 0.2 ml buffer B and frozen in a dry-ice/ethanol bath for storage.

**Detection of penicillin-binding proteins in vegetative membranes.** A reaction mixture (final volume 50 μl) was prepared containing 50 μg membrane proteins (see above), 50 mM Tris/HCl (pH 8.0), 1 mM MgCl2 and 40 μM [3H]benzylpenicillin (Amersham) and incubated for 14 min at room temperature. The labelling reaction mixtures were then immediately mixed with gel loading dye and fractionated on SDS-polyacrylamide gels. Gels were treated with Enhance (NEN, DuPont), dried and exposed for 7 d at −70°C.

**RESULTS**

The ftsH::spc mutant encodes a truncated protein, FtsH'

We constructed an insertional ftsH::spc mutant which disrupted translation of 123 residues of the C-terminus of ftsH and left intact 505 codons of the ftsH ORF. We prepared and purified membrane proteins from vegetatively growing wild-type and mutant cells and size-fractionated polypeptides by SDS-PAGE. Using an anti-FtsH serum prepared to the E. coli FtsH protein we visualized FtsH protein in wild-type and ftsH::spc membranes by Western blotting (Fig. 2). In wild-type membrane extracts we observed a band migrating at approximately 70 kDa, absent in the ftsH::spc mutant, and which corresponded to the molecular mass of the full-length FtsH polypeptide. The mutant, however, showed the presence of a band of approximately 57 kDa.

**Fig. 2.** Immunological detection of FtsH and FtsH' in membrane fractions. Membrane proteins were purified from vegetatively growing cultures (OD650 ~ 3.0) of a wild-type strain (PY79; lanes 1) and an ftsH::spc mutant (EL727; lanes 2). (a) Coomassie-blue-stained proteins after fractionation on 12% SDS-polyacrylamide gels. The position of the 70 kDa FtsH polypeptide is indicated. (b) Western blotting of the same gel fractionation with anti-E. coli FtsH serum. Indicated on the figure are the 70 kDa FtsH band present only in wild-type membranes and the 57 kDa band found in ftsH::spc membranes and which represents a truncated form of FtsH (FtsH').
which was absent in wild-type membranes. This band is most likely the 57 kDa polypeptide synthesized from the truncated ORF present in the \textit{ftsH}:\textit{spc} mutant, and we term this species \textit{FtsH}'. However, the abundance of this polypeptide relative to wild-type \textit{FtsH} was significantly reduced. Since \textit{FtsH'} was present in the cytoplasmic membranes this polypeptide must presumably contain the N-terminal transmembrane anchors present in \textit{FtsH}. It should also be noted that \textit{FtsH'} would contain all of the proposed functional domains required for activity of the \textit{E. coli} \textit{FtsH} protease. Thus, it is reasonable to assume that \textit{FtsH'} has at least partial activity and that the \textit{ftsH}:\textit{spc} mutant cannot be considered a null.

Our Western analysis also showed the presence of a 42 kDa band present only in wild-type membranes. Incubation (5–20 min) of membrane extracts at 30 °C and 37 °C prior to SDS-PAGE analysis caused a marked increase in the intensity of this band and we infer that it is a degradation product of \textit{FtsH}. \textit{FtsH} (and, to a lesser degree, \textit{FtsH'}) appeared to exist as two distinct bands. On higher dilutions of antiserum we could not separate these bands although the upper, slower-migrating, band always produced a stronger signal (data not shown).

**\textbf{FtsH is required for cell growth in minimal medium and for viability during stationary phase}**

When grown on LB agar plates containing spectinomycin the \textit{ftsH}:\textit{spc} mutant appeared to grow normally and was essentially indistinguishable from the congenic wild-type strain (PY79). Growth occurred at 30 °C, 37 °C and 42 °C and we observed no filamentation of cells during growth. However, \textit{ftsH}:\textit{spc} mutant cells were about twice the length of wild-type cells at equivalent stages of cell growth. Cells in LB medium showed a normal growth rate but failed to reach the same density as a wild-type culture at stationary phase (Fig. 3a), while in glucose minimal medium the mutant was unable to grow at all (Fig. 3c). In DSM sporulation medium (Fig. 3b), the mutant grew essentially at the same rate as the wild-type, but at stationary phase there was a marked decline in cell density as the culture started to lyse. After 20 h incubation, up to 50% of the culture had lysed although continued lysis did not occur beyond this point. The increase in OD$_{595}$ seen in wild-type cells beginning at hour 10 is due to the formation of phase-bright refractile spores which superficially increase the cell density measurements.

**\textbf{FtsH is required under conditions of osmotic stress}**

In other work a salt-sensitive mutation in \textit{B. subtilis} \textit{ftsH} has been described (Deuerling \textit{et al.}, 1995; Geisler \& Schumann, 1993) and a similar phenotype reported for a mutation in \textit{L. lactis} \textit{ftsH} (Nilsson \textit{et al.}, 1994). To determine whether our \textit{ftsH}:\textit{spc} mutant exhibited a salt-sensitive phenotype we grew the mutant in LB medium and challenged exponentially growing cells with 7% NaCl. Under such conditions growth of the \textit{ftsH}:\textit{spc} mutant was immediately arrested, whereas wild-type cultures experienced a lag followed by a resumption of normal growth (Fig. 4). On LB agar plates...
supplemented with 7% NaCl the ftsH:spc mutant was unable to form colonies at 30 °C, 37 °C or 42 °C.

**The ftsH:spc mutant affects the amounts of active PBP3s in the cytoplasmic membrane**

In *E. coli* FtsH is thought to be involved in the membrane assembly of PBP3 (Begg *et al.*, 1992). The functional homologue of PBP3 in *B. subtilis* is PBP2B, which, like its *E. coli* counterpart, is believed to be essential for cell septum formation and thus cell division (Yanouri *et al.*, 1993). We examined penicillin-binding proteins in vegetative membranes prepared from wild-type and mutant cells (Fig. 5). Following SDS-PAGE of ftsH:spc membrane proteins we could detect a reduction in the amounts of both PBP2B and PBP4. In contrast though, we observed a marked accumulation of PBP2A. We have repeated these experiments with more than one preparation of membrane proteins and although some of the differences were small (e.g. PBP2B) they were also reproducible.

**FtsH is essential for spore formation**

On DSM sporulation agar plates the ftsH:spc mutant formed colonies that after 24 h incubation underwent extensive cell lysis. By phase-contrast microscopy we could observe the presence of a very low number of phase-bright refractile spores. It should be noted that under conditions of cell lysis visual examination of sporulation is misleading due to the enhancement of the relative amount of spores (which do not lyse). When sporulation was induced in DSM medium by the exhaustion method cells were unable to initiate spore formation at 30 °C, 37 °C or 42 °C. Ten hours after the onset of sporulation less than 0.01% of the viable culture formed heat-resistant spores compared to almost 70% in wild-type cells.

To determine whether the cascade of sporulation-specific gene expression occurred in the ftsH:spc mutant we introduced lacZ transcriptional fusions to different sporulation genes into the ftsH:spc mutant and examined whether expression of these developmental genes was initiated in the mutant. With all genes tested (*spoIIE*, *spoIID*, *spoIVF*, *ssPE* and *gerE*) we observed no developmental gene expression. In particular, expression of *spoIIE-lacZ* (Fig. 6) was prevented in the ftsH:spc mutant. *spoIIE* expression commences some 30 min after the initiation of sporulation, prior to formation of the two cell-types (Beall & Lutkenhaus, 1991) and is controlled by the vegetative transcription factor σ^V^ and the sporulation-specific DNA-binding protein Spo0A.

**ftsH expression**

To examine expression of ftsH we constructed an ftsH-lacZ transcriptional fusion and measured ftsH-directed β-galactosidase synthesis in a spo^+^ strain. In DSM medium, at the end of stationary phase, cells enter
the sporulation life cycle so both vegetative and developmental gene expression can be monitored. Fig. 7 shows that ftsH expression was maintained during vegetative growth but steadily declined as the cells entered the stationary phase of growth and continued to do so as the cells initiated spore formation.

Although ftsH–lacZ expression declined as the cells initiated spore formation there were still significant levels of ftsH-directed β-galactosidase activity present 4–5 h after the commencement of spore development, suggesting that ftsH expression might be developmentally controlled. The early stages of sporulation-specific gene expression are controlled by RNA polymerase associated with σH (EoH; Errington, 1993). We introduced ftsH–lacZ into a mutant (RL56; spo0HΔHindIII) unable to synthesize σH and measured ftsH expression. Our results (Fig. 7) showed that there was no substantial reduction in ftsH-directed β-galactosidase synthesis in the absence of EoH and the pattern of ftsH–lacZ expression was essentially identical to that of spo0⁺ cells. In mutants unable to synthesize the sporulation-specific transcription factors σF, σE, σO or σK, and in a strain unable to synthesize the stationary-phase transcription factor ρH, ftsH expression was similarly unaffected (not shown).

As shown above, the ftsH mutant is unable to respond to conditions of osmotic stress and in order to recover from a hypersaline environment FtsH protein may be synthesized in increased amounts. To investigate this we measured ftsH–lacZ expression under conditions of salt stress (Fig. 8). Immediately following exposure to NaCl there was a twofold increase in ftsH expression. As cells adjusted to the high osmotic environment and finally resumed growth so ftsH–lacZ expression declined to its normal level.

**DISCUSSION**

The ftsH: ∶spc mutant constructed in this work encoded a truncated protein which contained the principal domains required for its proteolytic activity and membrane assembly. Most probably, this mutant protein retains some activity, yet our results show clearly that the protein accumulated in the membranes in reduced amounts. Modified activity or these reduced levels were, however, sufficient to produce significant cell growth defects, these being an inability to attain the normal cell density at stationary phase, failure to grow in minimal media, defective incorporation of some PBPs into the cytoplasmic membrane and failure to differentiate under conditions of nutrient depletion.

The relative abundance of three PBPs was altered in the ftsH mutant: there was an accumulation of PBP2A and a reduction in the amounts of PBP2B and PBP4. The function of PBP4 is least understood in B. subtilis but PBP2A has been implicated in the synthesis of the cell’s side walls (Sowell & Buchanan, 1983) and the presence of this protein in membranes declines rapidly as cells enter sporulation and when there is no requirement for cell elongation (thus PBP2A might be homologous to PBP2 of E. coli). In contrast, PBP2B is required for formation of the septum and indeed this protein is considered essential for cell growth (Yanouri et al., 1993). Furthermore PBP2B is also required for sporulation, where it is used for construction of the asym-
metric spore septum, and synthesis of this polypeptide increases dramatically during development. PBP2B is homologous to E. coli PBP3 and both proteins play similar functional roles in septum formation and cell division (Yanouri et al., 1993). As in B. subtilis, membrane levels of E. coli PBP3 are substantially reduced in an ftsH mutant (Begg et al., 1992).

One explanation for the defective PBP assembly is that FtsH is involved in the membrane assembly of these proteins, perhaps in the role of a chaperone. Alternatively, FtsH could play a more direct role in the translocation of the PBPs into the plasma membrane. In E. coli SecY is an important component of the protein translocation machinery and normally exists in the membrane complexed with SecE. In the absence of FtsH, uncomplexed SecY accumulates to levels where it is deleterious to protein export, cell growth and viability (Kihara et al., 1995). Thus, FtsH can play a direct role in protein assembly and translocation by eliminating abnormally assembled SecY by proteolysis. In B. subtilis, FtsH could have an analogous role in controlling protein–membrane interactions such as the assembly of the PBPs.

In addition to causing a defect in cell growth the ftsH mutation affected two other of the cell’s responses to environmental stress, namely recovery from osmotic shock and spore formation. Under conditions of high osmolarity the ftsH mutant was unable to recover and maintain cell growth. This phenotype has been observed previously with other mutations in the B. subtilis and L. lactis FtsH proteins (Deuerling et al., 1995; Nilsson et al., 1994) and suggests that FtsH plays an important role in the cell’s response to osmotic stress. Under such conditions we have shown that transcription of the ftsH gene is induced twofold and continues to be overexpressed until the cell has recovered from the hypersaline environment and resumed cell growth. Enhanced expression of ftsH under stress is reminiscent of the prokaryotic heat-shock response, and a simple explanation for the increased levels of FtsH would be that it may be required for the degradation of a regulatory factor required for the cell’s survival in a hypersaline environment, in a manner analogous to the FtsH-mediated degradation of the heat-shock transcription factor, σ32 (Herman et al., 1995; Tomoyasu et al., 1995). Intriguingly, FtsH is anchored to the membrane by two transmembrane domains which would expose a stretch of over 80 amino acids on the outer face of the cytoplasmic membrane beneath the peptidoglycan cell wall. Conceivably, this domain might be required as a sensory domain for responding to stress conditions. In other experiments (E. Lysenko & S. Cutting, unpublished) we have found that in a sigB mutant unable to synthesize the transcription factor σB, ftsH was induced normally following exposure to salt. Thus, σB is not required for the salt-induced expression of ftsH.

We have also shown that the ftsH mutant is unable to enter the developmental life cycle under starvation conditions. Using an ftsH–lacZ fusion we have demonstrated that expression of ftsH actually declines as cells differentiate, so the involvement of FtsH here must be different from that in the osmotic stress response. Interestingly, spoIIE was not expressed in the ftsH mutant. spoIIE transcription is controlled by σA and Spo0A. Spo0A is activated by an elaborate phosphorelay signal transduction pathway requiring a number of cellular inputs (cell cycle, nutritional and extracellular signalling: Burblys et al., 1991; Ireton & Grossman, 1994). Failure to elicit spoIIE–lacZ expression suggests that Spo0A may be inactive in the ftsH mutant. Although only a hypothesis, this could explain the block in development under conditions of nutrient depletion.

In summary, our work shows that FtsH is most likely a general factor involved in cell growth, response to stress conditions and in spore formation. It should be noted that most of these events are related to membrane functions.

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