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Escherichia coli Requires the Protease Activity of FtsH for Growth

Maithri M. K. Jayasekera,* Susan K. Foltin,* Eric R. Olson,† and Tod P. Holler*,[1]

**Department of Biochemistry and †Department of Infectious Diseases, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105*

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FtsH protease, the product of the essential *ftsH* gene, is a membrane-bound ATP-dependent metalloprotease of *Escherichia coli* that has been shown to be involved in the rapid turnover of key proteins, secretion of proteins into and through the membrane, and mRNA decay. The pleiotropic effects of *ftsH* mutants have led to the suggestion that FtsH possesses an ATP-dependent chaperone function that is independent of its protease function. When considering FtsH as a target for novel antibacterials, it is necessary to determine which of these functions is critical for the growth and survival of bacteria. To address this, we constructed the FtsH mutants E418Q, which retains significant ATPase activity but lacks protease activity, and K201N, which lacks both protease and ATPase activities. These mutants were introduced into an *E. coli ftsH* knockout strain which has wild-type FtsH supplied from a plasmid under control of the inducible *araBAD* promoter. Since neither mutant would complement the *ftsH* defect produced in the absence of arabinose, we conclude that the protease function of FtsH is required for bacterial growth. © 2000 Academic Press

Key Words: **FtsH; protease; ATPase.**

ATP-dependent proteases are responsible for both the rapid turnover of specific regulatory proteins and the degradation of misfolded or abnormal proteins. *Escherichia coli* has several different ATP-dependent proteases, including Lon (also called La), the Clp proteases, and FtsH protease (for a review, see Ref. 8). In contrast to Lon and the Clp complexes, which are cytosolic proteases that have somewhat overlapping specificities, FtsH is inserted in the inner membrane of the bacterial cell with two membrane-spanning regions (23) and processes a distinct set of substrates (e.g., 3, 11, 15, 19). Its ATPase domain is also distinct, placing FtsH in the AAA family of ATPases (for a review, see Ref. 4). Members of the AAA protein family are present in a variety of organisms, and are involved in a variety of cellular processes, including cell cycle regulation, gene expression, peroxisome formation, 26S-proteosome formation and nuclear division. FtsH and its homologs are unique in this family by virtue of their protease domain.

In addition to its role in the degradation of specific bacterial proteins, the *ftsH* gene of *E. coli* has been identified in diverse genetic screens. It has been shown to be important, for example, in the lysis/lysogeny decision in lambda phage infection of *E. coli* (12), in the processing of protein into and through the inner bacterial membrane (1, 2), and in RNA stability (9). This diversity of effects caused by a single protein has led to the suggestion that the product of the *ftsH* gene performs a chaperone function within the cell that is separate from its ATP-dependent protease function (23). Similar arguments have been used to suggest that the cytoplasmic ATP-dependent proteases of *E. coli,* the Lon and Clp proteases, also have separate ATP-dependent chaperone functions in addition to their ATP-dependent protease functions (23). These two separate functions are diagrammed in Fig. 1.

FtsH protease is the only ATP-dependent protease of *E. coli* that has been shown to be essential for bacterial growth (24). The *ftsH* homolog in *Helicobacter pylori* has also been shown to be essential (7), and while its homolog in the Gram-positive *Bacillus subtilis* is not essential, cells lacking *ftsH* grow poorly (6). When considering FtsH protease as a potential target for the discovery of novel antibacterials, we addressed the question of whether selective elimination of the protease function would be sufficient to stop bacterial

[1] To whom correspondence should be addressed. Fax (734) 622-1355. E-mail: tod.holler@wl.com.

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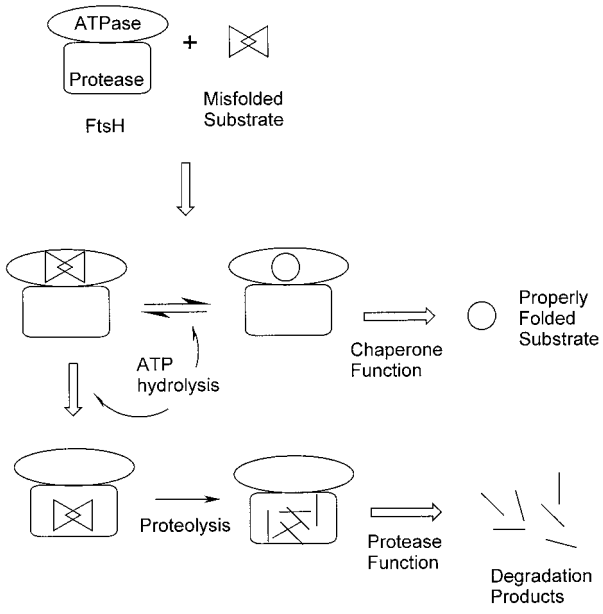

FIG. 1. The potential chaperone and protease activities of FtsH protease.

growth. We constructed site-directed mutants of FtsH that lack protease function then tested the ability of these mutants to complement an *ftsH* defect in *E. coli*.

CLONING AND EXPRESSION OF FtsH AND SUBSTRATE CII

The FtsH protease used for *in vitro* activity assays was expressed from a partially synthetic gene prepared in two steps. First, a ca. 200-bp synthetic cassette encoding the amino terminus of FtsH (27) was synthesized from three oligonucleotides of 80mers and one 60mer (sequence not shown) by the primer extension–amplification method (13) and cloned between the *Nde*I and *Bam*HI sites of pUC19. Next, the *Bam*HI fragment from Kohara phage 520 (16) that contains the rest of the *ftsH* gene and about 200 bp of downstream sequence was moved into the *Bam*HI site of the pUC construct to create pUC19-ftsH. To create the high-level protein expression plasmid pET21a-ftsH-his6, the assembled *ftsH* gene was subcloned from pUC19-ftsH into pET21a (Novagen) and then modified to fuse a thrombin cleavage site and hexa-histidine tag to the C-terminus of the expressed protein. The entire modified *ftsH* gene was sequenced at this stage. When introduced into HMS174(DE3) and induced with IPTG,[2] a protein of the appropriate size (70.9 kDa) was observed on SDS–PAGE gels.

[2] Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol.

The gene encoding substrate λ-CII was obtained from Kohara phage (16) by PCR and inserted between the *Nde*I and *Bam*HI sites of pET15b (Novagen) to create pET15b-cII. This construct fused pET15b sequences encoding an N-terminal hexa-histidine tag to the *λ-cII* gene. The entire tagged gene was sequenced and then pET15b-cII was introduced into BL21(DE3). When induced with IPTG, a protein of appropriate molecular weight (11 kDa) was observed on SDS–PAGE gels. Cells containing the CII were lysed by French press, and the supernatant clarified by centrifugation. After a preliminary ammonium sulfate precipitation at 30% saturation, the soluble his-tagged λ-CII was precipitated with ammonium sulfate at 55% saturation. Sequential chromatography on Ni-nitriloacetate resin (Qiagen) and heparin-Sepharose (Pharmicia) provided essentially pure protein, as judged by SDS–PAGE Coomassie blue staining.

Attempts to purify active FtsH protease by detergent extraction and chromatography, as described (25), were routinely without success. We were, however, able to demonstrate that membrane fractions isolated from a sucrose gradient from FtsH-C-his overexpressing cells were able to cleave recombinant, N-terminally his-tagged λ-CII protein in an ATP-dependent reaction that was sensitive to *o*-phenanthroline (data not shown).

PREPARATION AND CHARACTERIZATION OF FtsH MUTANTS IN VITRO

To selectively remove the protease activity of FtsH, we chose to mutate the glutamate of the signature zinc-binding motif HEAGH to glutamine (E418Q). This mutation was expected to eliminate proteolysis with a minimum of structural perturbation since, in matrix metalloproteases, this residue typically participates in the hydrolysis of amide bonds by activating a water molecule, rather than chelating the active site zinc (22). An equivalent mutation in matrilysin decreased k_{cat}/K_m by 600-fold (5) and an identical mutation in the yeast FtsH homolog YmeI has been reported to eliminate the protease activity (28). A previously characterized FtsH mutation tolZ21 (20) also has a mutation in the signature zinc-binding motif, but this mutation

TABLE I

Activities of FtsH Proteins *in Vitro*

FtsH variant	ATPase activity (μmol Pi/h/mg)	Protease activity (As shown in Fig. 2 by SDS–PAGE)
Wild type	105 ± 3.0	+
E418Q	80 ± 0.8	–
K201N	10 ± 1.0	–

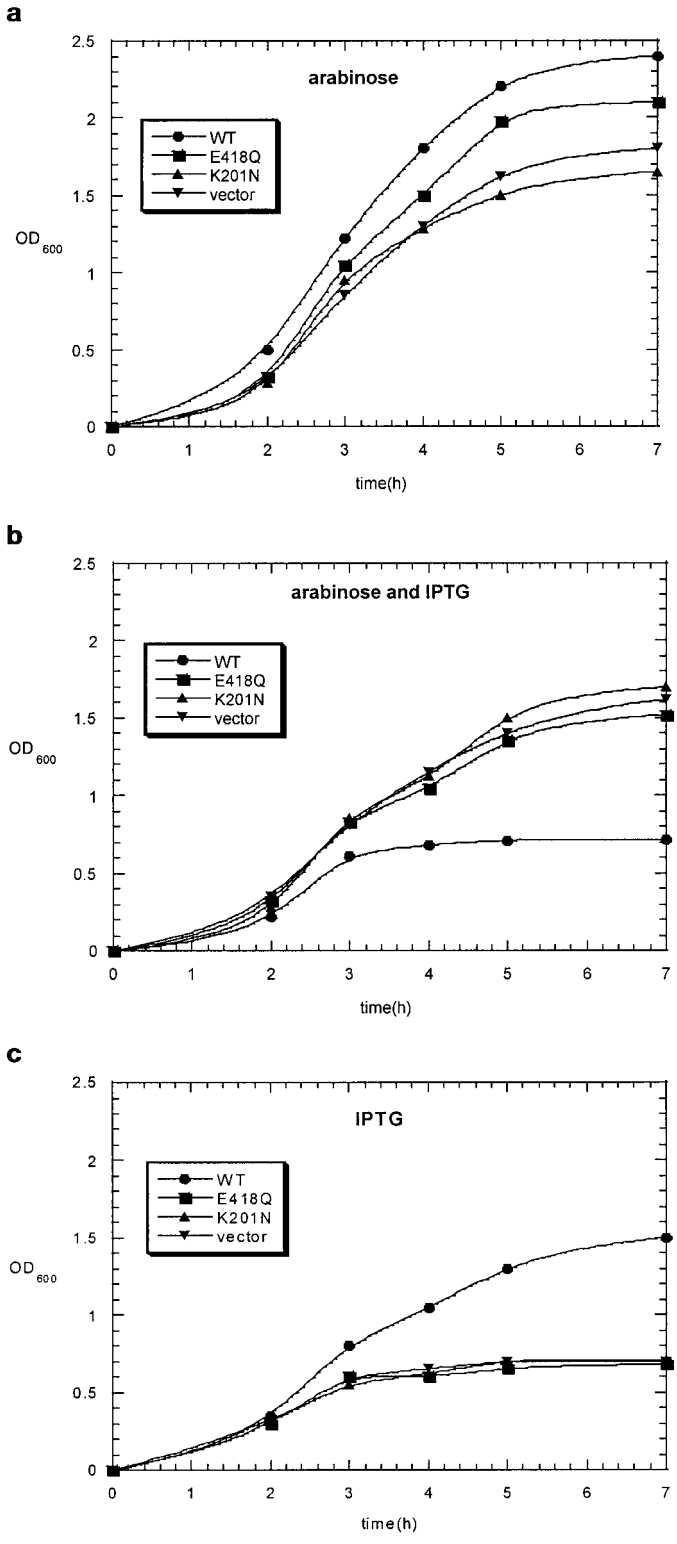

FIG. 2. Complementation of FtsH depletion by wild-type FtsH and site-directed mutants. (a) Growth of strain W3110-MJ1 containing pSR-plac (vector), pSR-plac containing wild-type *ftsH* (native), pSR-plac containing *ftsh* mutant E418Q (418Q), or pSR-plac containing *ftsH* mutant K201N (201N) in LB media containing kanamycin (10 μg/mL), chloramphenicol (5 μg/mL), ampicillin (20 μg/mL), and 0.01% arabinose. (b) Growth of strains in (a) after shift to LB media containing kanamycin (10 μg/mL) chloramphenicol (5 μg/mL), ampicillin (20 μg/mL), and 01.0% arabinose plus 0.5 mM IPTG. (c) Growth of strains in (a) after shift to LB media containing containing kanamycin (10 μg/mL), chloramphenicol (5 μg/mL), ampicillin (20 μg/mL), and 0.5 mM IPTG. All cultures were grown at 37°C.

(H421Y) is expected to cause loss of the zinc atom and therefore significant structural perturbation. The possibility that this mutation also causes significant perturbation of the potential chaperone function suggests that this mutation is not appropriate for studying the isolated effects of the protease function. To remove the ATPase activity, and therefore the protease activity as well, we chose to replace lysine residue 201 with asparagine. This mutation removes the positive charge from the "P-loop" and typically abrogates binding of ATP to this motif (for a review, see Ref. 21). This residue has previously been shown to be required for ATPase activities of both FtsH (27) and YmeI (28). Both of these mutants were introduced by site-directed mutagenesis of the C-terminally his-tagged *ftsH* gene in pET21a, and expressed in *E. coli* HMS-174(DE3). The level of overexpression in each case was indistinguishable from wild-type.

Membrane preparations from these overexpressing cells were assayed for protease activity by 37°C incubation of 0.06 mg/mL membrane proteins with 0.02 mg/mL N-terminally his-tagged λ-CII in buffer containing 50 mM Tris–HCl, pH 8.0, 500 μM ATP, 5 mM $MgCl_2$, 1 μM $ZnCl_2$, and 5 mM DTT, followed by SDS–PAGE. This method demonstrated that although the wild-type protein was able to completely digest all of the CII in 30 min, neither mutant was able to digest a significant amount of CII in the same amount of time (figure not shown). The ATPase activities of the three FtsH variants were then assayed under the same conditions, using the malachite green assay for phosphate (17). The results are in Table I. While this work was in progress, Karata *et al.* (14) published a study that included ATPase activities for ftsH and the mutant H421Y. The ATPase-specific activity of our wild-type FtsH is approximately fourfold higher than that reported for the purified protein by them. Our specific activities were measured in the presence of protease substrate, while theirs was determined in the absence of a protease substrate. Significant stimulation of ATPase activity by protease turnover has been documented for other ATP-dependent proteases. Assuming that the ATPase measurement for K201N is background ATPase activity from the membrane, we estimate that the protease-deficient mutant E418Q retains 74% of the ATPase activity of the wild-type. In contrast, Karata *et al.* report that the H421Y mutant retains only 23% of wild-type ATPase activity, support-

ing our choice of E418Q as the appropriate mutant for this study.

COMPLEMENTATION

With the appropriate *ftsH* mutants in hand, we employed a genetic system to test their ability to complement an *ftsH* null mutant. A chromosomal *ftsh::Kan* allele prepared by Akiyama *et al.* (1) was transduced from *E. coli* strain YJ430 to *E. coli* strain W3110 carrying a plasmid (pBAD33-ftsH) that expressed wild-type FtsH under control of the arabinose promoter (10). This plasmid contains the pACYC origin of replication and a chloramphenicol resistance marker. The resulting strain, W3110-MJ1, demonstrates the expected antibiotic resistances and requires arabinose for growth. A second expression system was constructed in order to supply the knockout strain with FtsH in the absence of arabinose. The wild-type, E418Q and K201N variants of *ftsH* were inserted into plasmid pSR-plac, a derivative of pBR322 in which the *lac* promoter has been inserted between the *Eco*RI and *Bam*HI sites. These plasmids are compatible with pBAD33-ftsH and carry an ampicillin resistance marker. Vector pSR-plac and the three derivatives containing the ftsH variants were used to transform W3110-MJ1. All of the resulting strains were capable of growth on LB agar plates containing kanamycin, chloramphenicol, ampicillin, and 0.01% arabinose. When streaked on plates containing antibiotics plus IPTG instead of arabinose, only the strain containing pSR-plac-wild-type ftsH was able to form colonies.

A more precise test for growth inhibition is to shift a growing culture from permissive to nonpermissive conditions. W3110-MJ1 strains containing pSR-plac-ftsH variants were grown in LB containing antibiotics and 0.01% arabinose to an optical density of 0.4 at 600 nm. The cells were pelleted, washed twice with media lacking arabinose, and resuspended in media containing arabinose, or IPTG, or a combination of IPTG and arabinose. Growth curves determined by following the optical density at 600 nm are presented in Fig. 2. All of the strains continued to grow well in the presence of arabinose. In the presence of both arabinose and IPTG, the cells containing wild-type pSR-plac *ftsH* stopped growing after 3 to 4 h, suggesting that an overexpression of FtsH is detrimental to growth. In the presence of IPTG alone, only the cells containing wild-type pSR-plac-ftsH continued to grow. The others stopped growing after 3–4 h, demonstrating that the protease activity of FtsH is required for growth.

While this work was in progress, Ogura *et al.* (19) demonstrated that FtsH protease is a key component in the system that maintains a balanced synthesis of major membrane components in *E. coli.* They further identified LpxC (EnvA) as a key substrate for FtsH in this system. Our work is entirely consistent with their results and serves to independently confirm that the protease activity of FtsH is of primary importance for the growth of *E. coli.*

The potential chaperone activity of the ATPase domains of ATP-dependent proteases Lon (26) and YmeI (18) have been recently reported. Overexpression of the protease-deficient Lon mutant was shown to overcome a chromosomal Lon deficiency, while more normal levels of expression were unable to do so. Greater than stoichiometric quantities of the isolated ATPase domain of YmeI were shown to stabilize and reactivate urea-denatured dihydrofolate reductase. These experiments demonstrate the ability of the ATPase domains of these proteins to bind and sequester a protein substrate. The inability to demonstrate turnover, however, makes the relevance of a separate chaperone activity to the critical functions of these proteins in the cell suspect. We have shown that the protease function of FtsH is required for growth in *E. coli,* since the protease-deficient mutant, which retains significant ATPase activity, is unable to sustain growth.

REFERENCES

1. Akiyama, I., Ogura, T., and Ito, K. (1994) *J. Biol. Chem.* **269,** 5218–5224.
2. Akiyama, I., Shirai, Y., and Ito, K. (1994) *J. Biol. Chem.* **269,** 5225–5229.
3. Akiyama, Y., Kihara, A., and Ito, K. (1996) *FEBS Lett.* **399,** 26–28.
4. Beyer, A. (1997) *Protein Sci.* **6,** 2043–2058.
5. Cha, J., and Auld, D. S. (1997) *Biochemistry* **36,** 16019–16024.
6. Deuerling, E., Mogk, A., Richter, C., Purucker, M., and Schumann, W. (1997) *Mol. Microbiol.* **23,** 921–933.
7. Ge, Z., and Taylor, D. E. (1996) *J. Bacteriol.* **178,** 6151–6157.
8. Gottesman, S. (1996). *Annu. Rev. Genet.* **30,** 465–506.
9. Granger, L. L., O'hara, E. B., Wang, R., Meffen, F. V., Armstrong, K., Yancey, S. D., Babitzke, P., and Kushner, S. R. (1998) *J. Bacteriol.* **180,** 1920–1928.
10. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) *J. Bacteriol.* **177,** 4121–4130.
11. Herman, C., Thevenet, D., D'Ari, R., and Bouloc, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3516–3520.
12. Herman, C., Ogura, T., Tomoyasu, T., Hiraga, S., Akiyama, Y., Ito, K., Thomas, R., D'Ari, R., and Bouloc, P. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10861–10865.
13. Holler, T. P., Foltin, S. K., Ye, Q. Z., and Hupe, D. J. (1993) *Gene.* **136,** 323–328.
14. Karata, K., Inagawa, T., Wilkinson, A. J., Tatsuta, T., and Ogura, T. (1999) *J. Biol. Chem.* **274,** 26225–26232.
15. Kihara, A., Akiyama, Y., and Ito, K. (1995) *Proc. Natl. Acad. Sci USA* **92,** 4532–4536.
16. Kohara, Y., Akiyama, K., and Isono, K. (1987) *Cell.* **50,** 495–508.
17. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem.* **100,** 95–97.
18. Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. (1999) *Nature* **398,** 348–35.

19. Ogura, T., Inoue, K., Tatsuta, T., Suzaki, T., Karata, K., Young, K., Su, L. H., Fierke, C. A., Jackman, J. E., Raetz, C. R., Coleman, J., Tomoyasu, T., and Matsuzawa, H. (1999) *Mol. Microbiol.* **31,** 833–844.
20. Qu, J.-N., Makino, S., Adachi, H., Koyama, Y., Akiyama, Y., Ito, K., Tomoyasu, O., Ogura, T., and Matsuzawa, H. (1996) *J. Bacteriol.* **178,** 3457–3461.
21. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) *Trends Biochem. Sci.* **11,** 430–434.
22. Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis Ruth, F. X., McKay, D. B., and Bode, W. (1995) *Adv. Exp. Med. Biol.* **4,** 823–840.
23. Suzuki, C. K., Rep, M., Maarten van Dijl, J., Suda, K., Grivell, L. A., and Schatz, G. (1997) *Trends Biochem. Sci.* **22,** 118–123.
24. Tomoyasu, T., Murata, K., Suzaki, T., Bouloc, P., Kato, A., Niki, H., Hiraga, S., and Ogura, T. (1993) *J. Bacteriol.* **175,** 1352–1357.
25. Tomoyasu, T., Gamer, J., Baku, B., Kanemori, M., Mori, H., Rutman, A. J., Oppenheimer, A. B., Yura, T., Yamanaka, K., Niki, H., Hiraga, S., and Ogura, T. (1995) *EMBO J.* **14,** 2551–2560.
26. Van Melderen, L., and Gottesman, S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6064–6071.
27. Wang, R. F., O'Hara, E. B., Aldea, M., Bargmann, C. I., Gromley, H., and Kushner, S. R. (1998) *J. Bacteriol.* **180,** 1929–1938.
28. Weber, E. R., Hanekamp, T., and Thorsness, P. E. (1996) *Mol. Biol. Cell.* **7,** 307–317.