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Characterization and Expression in *Escherichi coli* of *Streptococcus pneumoniae* FtsH

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FtsH is a membrane-bound, ATP-dependent metalloprotease that is involved in a variety of cellular functions including the regulation of responses to heat and stress shock. Previously, we had cloned and sequenced pneumococcal *ftsH* gene whose deduced amino acid sequence was very similar to those of several gram-positive bacteria and *Escherichia coli*, except for the N-terminal domain that was responsible for membrane anchoring. In order to better understand the role of *Streptococcus pneumoniae* FtsH, we expressed pneumococcal *ftsH* gene in *Escherichia coli*. When it was expressed from a strong promoter, P_{tac}, a considerable amount of the recombinant FtsH was produced, although the prolonged induction resulted in not only accumulation of break-down products but also ceasing of the further growth of *E. coli* host. This indicated that the expression of the exogenous *ftsH* gene was tightly regulated since the excessive FtsH appeared detrimental to bacterial cells. In Western blotting, the pneumococcal FtsH protein, whether native or recombinant, was reactive to anti-*E. coli* FtsH serum. The observation that FtsH proteins were well conserved throughout the bacterial kingdom and its expression level was fine-tuned suggests an important role for this protein in the stress adaptation which may be related to infecting process by pneumococci.

Key Words: *Streptococcus pneumoniae, ftsH*, ATP-dependent protease, Shock response

INTRODUCTION

Despite of the extensive use of antibiotics over several decades, *Streptococcus pneumoniae* still remains as one of the serious human pathogens. It is a major cause of bacterial pneumonia, meningitis and bacteremia as well as an important cause of otitis media in children (17). In a previous study, we observed that heat-shock proteins (DnaK and DnaJ) and an ATP-dependent protease FtsH were induced when pneumococcus caused infection in mice (18).

The *ftsH* gene encodes a membrane protein with a large cytoplasmic domain significantly homologous to the members of the AAA (ATPase associated with a variety of cellular activities) family whose ATPase activity is associated with a variety of cellular functions (13, 25, 26). FtsH is involved in the assembly of proteins within the plasma membrane, protein export, and the degradation of several regulatory proteins (2-4, 27). In addition, FtsH plays a significant role in regulating the heat-shock and stress-shock responses in several bacteria including *Escherichia coli* (16), *Lactococcus lactis* (21) and *Bacillus subtilis* (15). In *E. coli*, the heat-shock transcription factor, σ^{32} is negatively

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modulated by not only the DnaK chaperone system through the inactivation and destabilization of σ^{32} (28) but also FtsH through the degradation of σ^{32} (16, 27), suggesting that FtsH is fine-tuning the heat- and stress-shock in accordance with DnaK and DnaJ. *L. lactis* (21) and *B. subtilis* (12) show heat- and salt-sensitivity without the production of functional FtsH. Moreover, studies have shown that a number of pathogens express heat-shock genes while infecting hosts (1, 5, 10, 20, 30). Therefore, the role for pneumococcal FtsH in the regulation of shock responses might be related to the infection process caused by this pathogen. As the first step to address this question, we have expressed the pneumococcal *ftsH* gene in *E. coli* and characterized.

MATERIALS AND METHODS

Strains and plasmids. *S. pneumoniae* D39 strain (7) was a gift from Dr. J. Yother (University of Alabama). Pneumococcus was grown at 37℃ in the presence of 5% CO_2 either on blood agar plates (BAP) containing 5% sheep blood or in the liquid media of Todd-Hewitt broth (Difco Laboratories, Detroit, MI) containing 0.5% yeast extract (THY) anaerobically in a GasPak system (Beckton Dickinson Microbiology Systems, Cockeysville, MD). *E. coli* strain XL1-Blue (11) was grown in LB media (24) with appropriate antibiotics. Plasmid pKK223-3 (9) was obtained from Pharmacia (Uppsala, Sweden).

Inverted PCR (iPCR) and DNA sequence analysis. The upstream region of the *ftsH* gene was amplified from pneumococcal genomic DNA (23) by performing iPCR (22) as described (19). The amplified DNA was sequenced by cyclic sequencing using sequencing primers (Table 1). DNA sequence homology search was carried out by GenBank Blast Program.

Subcloning and Expression of pneumococcal *ftsH* gene in *E. coli*. Pneumococcal genomic DNA was denatured at 94℃ for 5 minutes and amplified using PCR primers (Table 1) and Taq polymerase (Boehringer Mannheim, Germany) by running 30 cycles of denaturing at 94℃ for 1 minute, annealing at 55℃ for 1 minute and extension at 72℃ for 2 minutes. Amplified *ftsH* gene was then digested with *Sma*I and *Hind*III and ligated into pKK223-3. *E. coli* XL1-Blue was electroporated with the ligated DNA (24). Ampicillin-resistant transformants were screened by restriction analysis, and the positive recombinant was designated

Table 1. Primers for sequencing and PCR used in this study

	Sequences (5' - 3')
sequencing primers	
f-12*	CCTTAGTAACAGATGGCGTGAAAAAC
f-13	GGAGTATTGAAAGAGGAAGTGTATTCAAATTAG
f-15*	CTCCATTCTGTTCCCTTTAGCTCC
f-8	GAACAAACAGCTTATGAAATTGATGAAGAGG
PCR primers#	
s	CCTTGTTGGATAAcCCgGgAGGAC
as*	CCATTTTCTCCAagCTtTTCCCTTTAG

*antisense primers
PCR primers (dotted-underlined in Fig. 1A) contain several substituted nucleotides to introduce appropriate restriction sites (underlined) and are shown in lower case

pJJ1. The overnight culture of pJJ1 in LB media containing ampicillin (100 μg/ml) and tetracycline (10 μg/ml) was diluted 20-fold in LB media, incubated with vigorous shaking for 3 hours at 37℃ and treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 hour.

SDS-PAGE and Western blotting. *E. coli* strains XL1-blue, pKK223-3 and pJJ1 were grown and incubated with or without 1 mM IPTG. *S. pneumoniae* was grown overnight in THY media. One ml of *E. coli* or pneumococcal cells was spun down, mixed with one tenth volume of SDS loading buffer, and heat-denatured. Total proteins were size-fractionated on NuPAGE™ 4~12% Bis-Tris Gel for SDS-PAGE (NOVEX, San Diego, CA) and either stained with Coomassie blue (24) or electroblotted onto 0.45 μm nitrocellulose filter (Schleichers & Schuell, Keene, NH). The filter was treated with the rabbit anti-*E. coli* FtsH serum that was obtained from Dr. T. Ogura (Kumamoto University School of Medicine, Japan) and then with the goat anti-rabbit IgG-horse radish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Western Blotting Luminol Reagent (Santa Cruz Biotechnology) was used for chemiluminescence detection on X-ray films (Fuji Film, Japan).

RESULTS AND DISCUSSION

In a previous study, several pneumococcal genes appeared to be induced after mouse-passage and were identified to encode heat-shock proteins (DnaK and DnaJ), an ATP-dependent protease FtsH, a transposase, and some unidentified genes (18). In this study, we tried to characterize and express the recombinant FtsH in *E. coli* based on the nucleotide sequence of the pneumococcal *ftsH* gene (19).

After sequence analysis was completed, it turned out that the upstream region contained

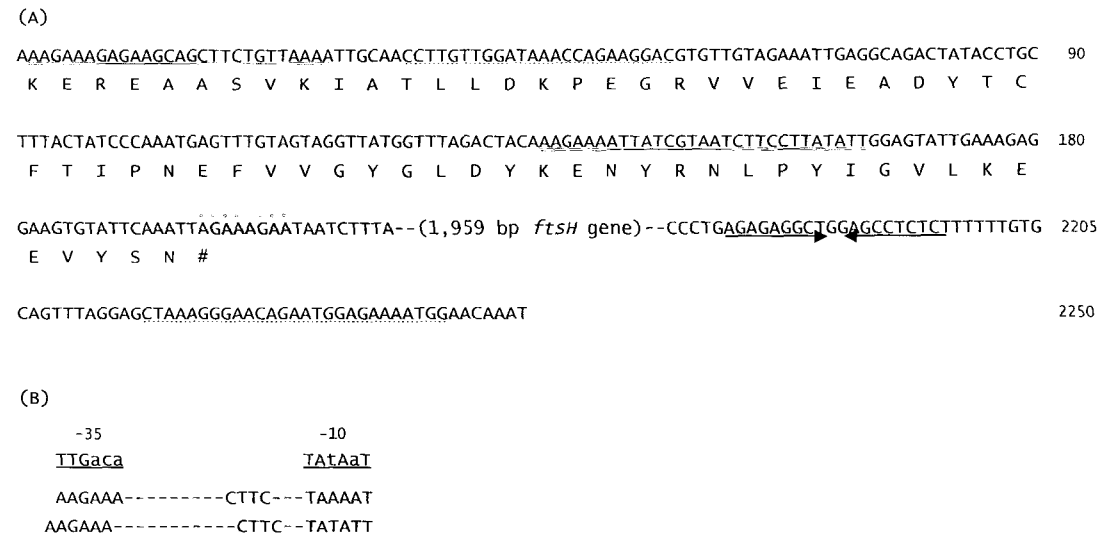

Figure 1. Flanking sequences of *S. pneumoniae ftsH* gene. **(A)** Nucleotide sequence of the upstream and downstream regions are shown. Two conserved sequences resembling the consensus for gram-positive promoters are underlined. The deduced amino acid sequence for the *hprt* gene is shown in one-letter code below the nucleotide sequence with the translation stop codon (#). The putative ribosome-binding site (bold-faced) and transcription terminator (arrowed) for the *ftsH* gene are indicated. The PCR primers used for the cloning of *ftsH* gene are dotted-underlined. **(B)** Two putative promoter sequences are aligned to the consensus promoter sequence for gram-positive bacteria.

several promoter-like AT-rich segments (Fig. 1A). At least two putative promoter sequences that resemble consensus sequences for -35 and -10 regions of the gram-positive bacteria (29) were found very similar to each other (Fig. 1B). Interestingly, an open reading frame coding for the putative hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene was located immediately upstream of the *ftsH* gene. Since the stop codon of *hprt* gene was located only 15 bp from the initiation codon of *ftsH* gene, it is uncertain at this point if the *ftsH* promoter is overlapped with the coding region of the *hprt* gene or if the *ftsH* gene is co-transcribed with the *hprt* gene from a common promoter as seen in *Helicobacter pylori ftsH* gene that is included in a stress-responsive operon (8). An attempt to recognize the rho-independent terminator for *hprt* gene within the coding sequence of the *ftsH* gene was failed. However, a putative terminator consisted of an inverted repeat followed by a stretch of T's was located immediate downstream of the *ftsH* gene (Fig. 1A).

For the expression of the recombinant FtsH protein (rFtsH) in *E. coli*, the pneumococcal genomic DNA was amplified using PCR primers that were designed to cover the 1,959-bp *ftsH* gene along with ca. 180 bp at 5'-upstream spanning several AT-rich promoter-like segments and ca. 70 bp at 3'-downstream region including a putative terminator (Fig. 1A). The 2.2-

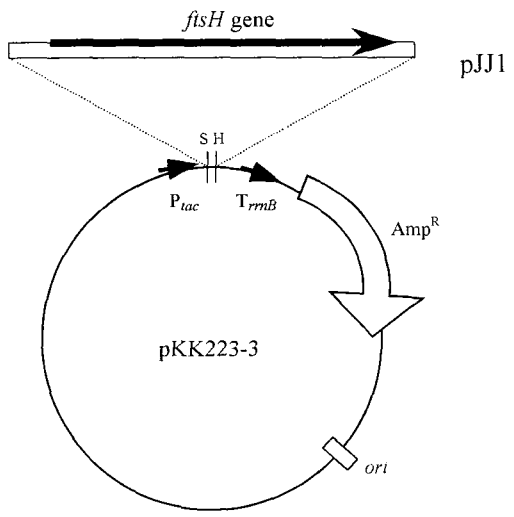

Figure 2. Cloning of pneumococcal *ftsH* gene and its flanking sequences. The 2.2-kb PCR fragment was inserted next to P_{tac} promoter in pKK223-3 to yield pJJ1. S: *Sma*I, H: *Hind*III.

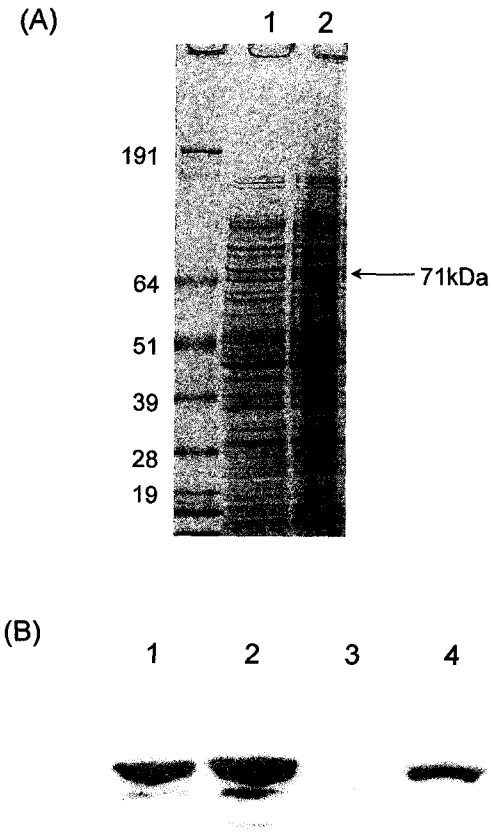

Figure 3. Expression of the pneumococcal rFtsH in *E. coli*. **(A)** SDS-PAGE of total proteins of *E. coli* (pJJ1). Overnight culture in LB medium containing ampicillin was diluted in LB, grown for 3 hours and incubated for 1 hour with IPTG. Total proteins were size-fractionated on NuPAGE™ 4~12% Bis-Tris Gel and stained with Coomassie blue. Lane 1: *E. coli* (pJJ1) before IPTG induction, lane 2: *E. coli* (pJJ1) after induction. **(B)** Western blots of the native and recombinant FtsH of *S. pneumoniae*. *E. coli* strains were grown as in (A). *S. pneumoniae* was grown overnight to a stationary phase. Total proteins were size-fractionated, electroblotted onto filters and reacted with the anti-*E. coli* FtsH serum. Lane 1: *E. coli* (pJJ1) before IPTG induction, lane 2: *E. coli* (pJJ1) after induction, lane 3: *E. coli* (pKK223-3), lane 4: *S. pneumoniae*.

kb PCR product was digested with the two restriction enzymes, whose recognition sites were introduced within the priming sites, and then inserted in front of a strong transcriptional terminator, T_{rrnB}, to stabilize the construct within an IPTG-regulated expression vector, pKK223-3 (9) (Fig. 2). The upstream region contained translation stop codons in all three different frames, indicating the rFtsH contains no extra amino acids before its own initiation codon. The positive clone, pJJ1, produced a detectable level of rFtsH after IPTG induction on Coomassie blue-stained SDS-PAGE (Fig. 3A, lane 2). In Western Blotting, both the recombinant and native pneumococcal FtsH were reactive to the anti-*E. coli* FtsH serum (Fig. 3B, lanes 2 and 4). As expected from the molecular weight calculated from the deduced amino acid sequence, the size of rFtsH (71 kDa) was same as that of native FtsH. Since the endogenous *E. coli* FtsH (71 kDa) was produced in the host strain at a very low level (6), it was not visible in Western Blotting unless loaded in an excessive amount (Fig. 3B, lane 3).

When compared with the host and vector *E. coli* strains, pJJ1 was producing a considerable amount of rFtsH even before the addition of IPTG (Fig. 3B, lane 1), suggesting the leaky expression from the P_{tac} promoter. It appeared that the presence of $lacI^{Q}$ in XL1-Blue strain was not sufficient to block the expression of the recombinant protein. In addition, the possible transcription from the promoter-like AT-rich sequences located upstream of the *ftsH* gene could not be ruled out. Although the P_{tac} promoter is regarded as a very strong promoter (9), the induced level of rFtsH was relatively moderate with only 3 to 4-fold increase (Fig. 3B, lanes 1 and 2), as seen in the induction of the recombinant mycobacterial FtsH in *E. coli* (6). Moreover, only break-down products were accumulated after 1 hr of IPTG induction, resulting in ceasing of the further growth of *E. coli* host (data not shown). Therefore, it is speculated that the expression of the exogenous *ftsH* gene was fine-tuned in a certain way that the total amount of the recombinant protein was not increased as the excessive FtsH was detrimental to bacteria. This is in accordance to the observation of a difficulty in the cloning of *ftsH* gene in a high copy-number plasmid and a moderate increase in FtsH level after heat shock treatment of *E. coli* carrying a plasmid with the additional *ftsH* gene (Dr. T. Ogura, personal communication).

FtsH plays an important role in the assembly of proteins within the plasma membrane, protein export, and the degradation of several regulatory proteins (2-4, 27). Moreover, FtsH is deeply involved in regulating bacterial stress responses by the degradation of the heat-shock transcription factor σ^{32}. Since heat shock genes from a number of human pathogens are expressed during the initiation of host infection and the survival of the organism (1, 5, 10, 20, 30), both heat shock and the infection process appear to be resulted from the similar patterns of gene regulation and expression. Although the *ftsH* genes of gram-positive *L. lactis* (21) and *B. subtilis* (12) are not essential in contrast to those of gram-negative *E. coli* (25) and *H. pylori* (14), it is assumed that pneumococcal FtsH also plays a role in stress adaptation, which may have any relation to the process of host infection. Construction of *S. pneumoniae* mutant lacking the functional *ftsH* gene is in progress to see whether this gene is essential in viability and whether the mutant is able to cause infection in hosts.

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