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The *tolZ* Gene of *Escherichia coli* Is Identified as the *ftsH* Gene

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***Escherichia coli tolZ* mutants are tolerant to colicins E2, E3, D, Ia, and Ib (Tol$^-$), can grow on glucose but not on succinate or other nonfermentable carbon sources (Nfc$^-$), and show temperature-sensitive growth (Ts). A 1.8-kb DNA fragment that complemented the *tolZ* mutation was cloned. The DNA fragment was sequenced, and one open reading frame was found. This frame was identical to a part of the *E. coli* FtsH protein, an ATP-dependent metalloprotease that binds to the cytoplasmic membrane. The *tolZ* gene was located at 69 min on the *E. coli* genetic map, and the mutation was complemented by a plasmid carrying the *ftsH* gene, indicating that the *tolZ* gene is identical to the *ftsH* gene. The mutated *tolZ21* gene was also cloned and sequenced and was found to have a single base change that caused an amino acid alteration of His-418 to Tyr in the FtsH protein. The *tolZ21* mutant showed Hfl$^-$ (high frequency of lysogenization) and Std$^-$ (stop transfer-defective) phenotypes, both of which are due to a mutation in the *ftsH* (*hflB*) gene. However, the *ftsH1*, *ftsH101*, and *hflB29* mutants did not show Tol$^-$ and Nfc$^-$ phenotypes. The *tolZ21* mutant was found to have a suppressor mutation, named *sfhC*, which allowed cells to survive. The *sfhC* mutation alone caused no Tol$^-$, Nfc$^-$, Ts, or Hfl$^-$ phenotypes in the *tolZ21* mutant.**

We previously published a report on *Escherichia coli tolZ* mutants (19) which show cross-tolerance between group A (10) colicins E2 and E3 and group B (9) colicins D, Ia, and Ib (Tol$^-$ phenotype). The *tolZ* mutants can grow on glucose as a carbon source but not on succinate or other nonfermentable carbon sources (pyruvate, malate, and lactate, etc.) (Nfc$^-$ phenotype). *tolZ21* strain UM21 shows temperature-sensitive growth (Ts) (19). The *tolZ21* mutation causes a defect in the generation of the electrochemical proton gradient (19). These findings suggest that the *tolZ* gene is involved in the formation of the active cytoplasmic membrane or alternatively that the TolZ protein may be one of the membrane proteins constituting an energy-producing system like the respiratory chain.

In this study, in order to reveal the function of the TolZ protein, we cloned a 1.8-kb *E. coli* DNA fragment complementing the *tolZ* mutation. On the basis of the nucleotide sequence of the cloned DNA fragment, the localization of the gene on the genetic map, and the results of a complementation experiment on the *tolZ* mutation, we concluded that the *tolZ* gene is identical to the *ftsH* (*hflB*) gene (13, 25, 28, 35, 36).

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All bacterial strains used were derivatives of *E. coli* K-12 and are listed in Table 1. The plasmid vectors used were pLG339 (30), pUC118, pUC119 (39), pHSG262 (5), and pHSG576 (31). λ$^+$, λ c17, and λ c17 cII were used to examine the Hfl (high frequency of lysogenization) phenotype. The P1*vir* phage was used for P1 transduction.

Media and growth conditions. Bacteria were usually cultured at 30°C or 37°C with shaking in L broth containing 0.1% glucose and 0.002% thymine. Davis medium (19) was used for genetic experiments. Media were solidified with 1.5% agar for plates and 0.5% agar for top agar. The following antibiotics were added at the given concentrations, when required: tetracycline, 10 μg/ml; chloramphenicol, 20 μg/ml; ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; and streptomycin, 25 μg/ml.

Phenotype tests. Typical phenotypes caused by the *tolZ21* mutation are Tol$^-$, Nfc$^-$, and Ts, as described above. The Tol, Nfc, and Ts phenotypes were defined as a strain's sensitivity to colicins E2 and E3, its ability to grow on a Davis plate containing 30 mM succinate at 37°C, and its ability to grow on a Davis plate containing 0.5% glucose at 42°C, respectively. The sensitivity of a strain to colicins was examined by the cross-streak method (19). Wild-type strain W2252 was used as a control.

The Hfl phenotype was examined as described previously (4), on the basis that λ c17 does not lyse Hfl$^-$ cells but lyses Hfl$^+$ cells. The Std (stop transfer-defective) phenotype was examined according to the method described previously (1).

Recombinant DNA techniques. Recombinant DNA techniques as well as the preparation of λ phage DNA were performed as described previously (26). Cosmid DNA was packaged into λ phage particles using Gigapack II Gold Packaging Extract (Stratagene). DNA sequencing was performed by the dideoxy chain termination procedure (27) using an Applied Biosystems model 370A DNA sequencer.

Genetic techniques. The mating methods, P1 lysate preparation, and P1 transduction were based on those described previously (20).

Subcellular fractionation of periplasmic, cytoplasmic, and membrane fractions and detection of FtsH protein by Western blotting (immunoblotting). Periplasmic fractions of *E. coli* cells harboring plasmids were prepared as described previously (3). The resultant cells were disrupted by sonication, and the soluble and insoluble fractions were separated by ultracentrifugation at 100,000 × *g* for 1 h and used as the cytoplasmic and membrane fractions, respectively. Protein samples were separated by sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis (18) and electrophoretically transferred onto nitrocellulose membranes. The membranes were treated with anti-FtsH serum (35), and the immunoblots were developed with an enhanced chemiluminescence Western blotting detection kit (Amersham), used according to the manufacturer's instructions. The membranes were exposed to RX film (Fuji Film).

RESULTS

Cloning of a chromosomal DNA segment complementing the *tolZ21* mutation. To clone the *tolZ* gene, we used a chromosomal DNA library (15) of the wild-type strain W3110 prepared with cosmid vector pHSG262 (kanamycin resistance) (5). After in vitro packaging of DNA into λ phage particles, *tolZ21* strain UM21 was infected by the phage particles, and kanamycin-resistant (Km^r) colonies were isolated on L broth plates containing kanamycin. The colonies were scored on Davis medium-succinate plates. Of 2,300 Km^r colonies, 12 grew on Davis medium-succinate plates, and all of them were

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TABLE 1. *E. coli* K-12 strains used in this study

Strain	Genotype	Source or reference
MV1184	*ara* Δ(*lac-proAB*) *rpsL thi* (ϕ80Δ*lacIZ*ΔM15) Δ(*srl-recA*)*306*::Tn*10*; F′ (*traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15)	39
W2252	HfrC *metB1 relA1 spoT1*	19
UM21	Like W2252 but *tolZ21 sfhC*	Reference 19 and this study
KM101	Like UM21 but *rpsL101*	19
KM103	Like KM101 but *srlC360*::Tn*10 recA56*	This study, recombinant from KM101 × JC10240
JC10240	Hfr PO45 *recA56 srlC300*::Tn*10 thr-300 ilv-318 rpsE300*	8
AR3099	Like UM21 but *tolZ*$^+$ *zha-3168*::Tn*10kan*	This study
AR719	*zgj-203*::Tn*10 thr-1 leu-6 thi-1 supE44 lacY1 tonA21*	1
CSH57B	*leu purE trp his metA/B ilvA argG thi ara lac xyl mtl gal tsx rpsL*	National Institute of Genetics, Mishima, Japan
KL14	Hfr PO68 *relA1 spoT1 thi-1*	*E. coli* Genetic Stock Center
G11	Hfr PO124 *hisA323*	*E. coli* Genetic Stock Center
AR754	*thr-1 leu-6 thi-1 supE44 lacY1 tonA21 zha-6*::Tn*10 ftsH1*(Ts)	34
ST2	Δ*lacX74 galE galK thi rpsL* Δ*phoA degP41*::*kan ftsH101*; pKY221	1
X9393	*hflB29 zgj-25*::Tn*10 araD139* Δ(*lac-pro*)	4

sensitive to colicins E2 and E3. From one of the Nfc$^+$ Tol$^+$ colonies, a 40-kb cosmid clone (Fig. 1A) complementing the *tolZ21* mutation was isolated and used for further experiments.

Subcloning of the *tolZ* gene. The DNA of the cosmid clone was digested with *Pvu*II and then ligated with a low-copy-number plasmid vector pLG339 (30) that had been digested with the same enzyme. Because the efficiency of transformation of strain UM21 with plasmids was about 10^{-3} lower than that of the wild-type strain, the ligated DNA was used to transform *E. coli* MV1184. The plasmid DNAs isolated from all of the Km^r transformants were used to transform strain UM21, and the resultant Km^r transformants were examined. Two of the 67 Km^r transformants exhibited the Nfc$^+$, Tol$^+$, and temperature-resistant (Tr) phenotypes. Two kinds of plasmid DNA obtained from the two transformants were identical 6.2-kb *Pvu*II fragments and contained cosmid vector pHSG262 (Fig. 1B) but not pLG339, indicating that the 6.2-kb *Pvu*II fragment ligated to itself with T4 ligase. The plasmid was named pCOS6.2. The position of the 6.2-kb *Pvu*II fragment on the 40-kb cosmid clone seems to be that shown in Fig. 1A.

Figure 1A shows that the 0.9-kb and 2.5-kb *Bam*HI-*Pvu*II fragments are not contiguous on the chromosome. DNA fragments derived from the two *Bam*HI-*Pvu*II fragments of pCOS6.2 (Fig. 1B) were subcloned into multiple cloning sites of pUC118 or pUC119 (39) downstream of the *lac* promoter (Fig. 1C). The results of the complementation test on *tolZ21 recA*$^+$ strain UM21 with these constructed plasmids are shown in Fig. 1C. The transformants exhibited the Nfc$^+$, Tol$^+$, and Tr phenotypes when UM21 was transformed with any of the plasmids carrying a 0.7-kb *Eco*RV-*Sph*I fragment. On the other hand, for *tolZ21 recA* strain KM103 only pJN340 carrying a 1.8-kb *Bam*HI fragment was able to complement the Tol$^-$ phenotype of the strain but not the Nfc$^-$ and Ts ones. These results suggest (i) that the 1.8-kb *Bam*HI fragment contains a part of the *tolZ* gene, (ii) that in UM21 cells recombination between plasmid DNA and chromosomal DNA occurs, and (iii) that the mutation in the *tolZ* gene may exist in the 0.7-kb *Eco*RV-*Sph*I fragment. Sequencing of the *tolZ21* gene prepared from strain UM21 proved that there was a single mutation in this fragment (see below).

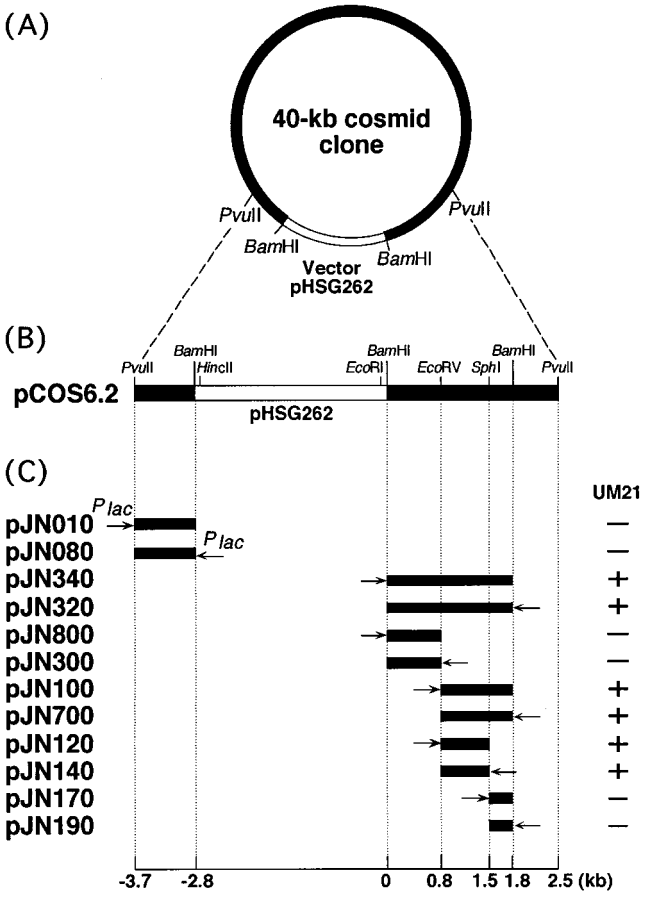

FIG. 1. Subcloning of the *tolZ* gene. (A) 40-kb cosmid clone obtained; (B) physical map of pCOS6.2; (C) plasmids carrying chromosomal DNA derived from pCOS6.2, which were constructed with pUC118 or pUC119. The results of genetic complementation of *tolZ21 recA*$^+$ strain UM21 with plasmids are also shown. Filled boxes, chromosomal DNA fragments; open box, pHSG262; +, Tol$^+$, Nfc$^+$, and Tr phenotypes; −, Tol$^-$, Nfc$^-$, and Ts phenotypes. The arrows show the direction and position of the *lac* promoter in each construct.

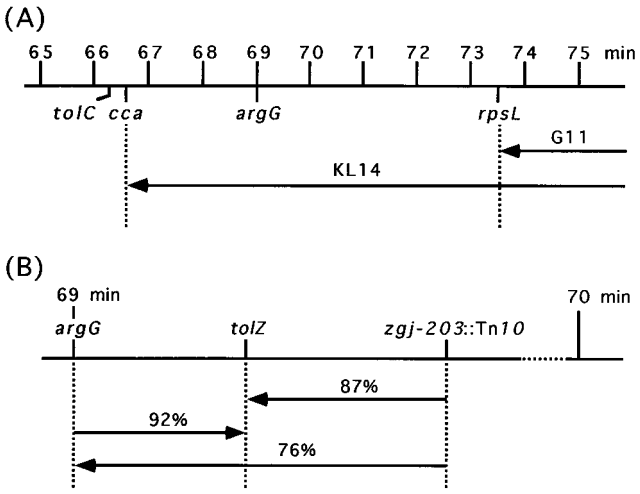

FIG. 2. Mapping of the *tolZ* gene. (A) Genetic map and origins and directions of chromosome transfer for Hfr strains KL14 and G11; (B) P1 transductional mapping of the *tolZ* gene. The approximate location of the *tolZ* gene is mapped with respect to known genetic markers. An arrow indicates the selected genetic marker used in each cross. Percentages above arrows are cotransduction frequencies.

Sequencing of the 1.8-kb *Bam*HI fragment. The cloned 1.8-kb *Bam*HI fragment was sequenced, and one open reading frame was found. On a search of a protein database, we found that this open reading frame was identical to the amino acid sequence of the *E. coli* FtsH protein (36), except that of the N-terminal 73 amino acid residues of FtsH was not included in the open reading frame. This seemed to be the reason why pJN340 carrying the *Bam*HI fragment only complemented the Tol$^-$ phenotype but not the Nfc$^-$ and Ts ones of strain KM103. The nucleotide sequence of the *Bam*HI fragment was identical to that of the part of the *ftsH* gene (data not shown).

Complementation of the *tolZ21* mutation with the *ftsH* gene. Strains UM21 (*recA*$^+$) and KM103 (*recA*) were transformed with plasmid pAR145 carrying the *ftsH* gene (36). All of the transformants obtained were able to grow on Davis medium-succinate plates, were sensitive to colicins E2 and E3, and were temperature resistant, indicating that the *ftsH* gene complemented the *tolZ21* mutation completely and that *tolZ21* is recessive to *tolZ*$^+$.

Mapping of the *tolZ* gene. The *ftsH* gene is located at 69 min on the *E. coli* genetic map (28, 36), while we previously reported that the *tolZ* gene is at 77 min (19). This is inconsistent with the results of the complementation test described above. Accordingly, we tried to map the *tolZ21* mutation again.

For mating experiments, Hfr strains KL14 and G11 (Fig. 2A) and *tolZ21 rpsL* strain KM101 were used. On 10-min mating, Nfc$^+$ recombinants of KM101 were obtained with KL14 on Davis medium-succinate plates but no Nfc$^+$ recombinants were obtained with G11. These results indicate that the *tolZ* gene is located between 67 and 73 min.

For P1 mapping, strain AR719, which carries *zgj-203*::Tn*10* at the position showing a high frequency of cotransduction with *ftsH*, was used. When strain UM21 was transduced with P1*vir* grown on AR719, 87% of the tetracycline-resistant transductants were Tol$^+$. When UM21 was transduced with P1*vir* grown on CSH57B (*argG*), 92% of the Nfc$^+$ transductants were Arg$^-$ (Fig. 2B). These results indicate that the *tolZ* gene is located at 69 min on the *E. coli* genetic map.

Mutation sequence in mutated *tolZ21* gene. The *ftsH* gene is located in a 3.05-kb *Sal*I-*Pst*I fragment (36). To clone the mutated *tolZ21* gene, the chromosomal DNA of strain UM21 was isolated and then digested with *Sal*I and *Pst*I. DNA fragments of around 3 kb were collected and purified on an agarose gel and then cloned into plasmid vector pHSG576 (chloramphenicol resistance) (31). Plasmids carrying the mutated *tolZ21* gene were obtained from four of 47 chloramphenicol-resistant colonies on the basis of restriction enzyme analysis. The cloned *tolZ21* gene was sequenced, and a single base change was found, codon CAT for His-418 being changed to TAT for Tyr. Transformants of UM21 with the plasmid carrying the cloned DNA showed the Nfc$^-$, Tol$^-$, and Ts phenotypes.

TABLE 2. Effect of *tolZ21* mutation on growth of λ phage[a]

Strain	Plaque phenotype	
	λ$^+$	λ c17
W2252 (wild type)	T	C
AR3099 (*sfhC*)	T	C
UM21 (*tolZ21 sfhC*)	VT	—
X9393 (*hflB29*)	VT	—

[a] Experiments were carried out at 30 and 37°C. λ c17 cII showed clear plaques for all strains. Abbreviations for plaque phenotypes: T, turbid; VT, very turbid; C, clear; —, no growth.

Presence of suppressor mutation in strain UM21. In the process of P1 transduction experiments at 37°C, we found that the *tolZ21* mutation could not be transduced in strain W2252, a parental strain of *tolZ21* mutant UM21, but could be transduced in UM21-derived *tolZ*$^+$ transductant AR3099. This strongly suggests that the *tolZ21* mutation is lethal for *E. coli* cells and therefore requires some other concomitant suppressor mutation(s) for its survival.

Mutations in the *fur* gene encoding the ferric uptake regulator suppress the temperature-sensitive growth of the *ftsH1*(Ts) mutant (12, 13, 25). The *fur* gene is located in the 16-min region on the *E. coli* genetic map. Genetic analyses indicated that there was no suppressor mutation in the 16-min region of strain UM21 and that the suppressor, which was tentatively named *sfhC*, was present at the 4-min region (24). Strain AR3099 carrying the *sfhC* mutation but not the *tolZ21* mutation showed Nfc$^+$, Tol$^+$, and Tr phenotypes, indicating that the phenotypes of UM21 are due to only the *tolZ21* mutation.

Hfl and Std phenotypes of the *tolZ21* mutant. Recently, it was reported that the Hfl$^-$ (13) and Std$^-$ (1) phenotypes are caused by mutations in the *ftsH* gene, *hflB29* and *ftsH101*, respectively. Table 2 shows that *tolZ21* strain UM21 exhibited the same Hfl$^-$ phenotype as the *hflB* mutant. The *sfhC* mutant strain AR3099 showed the Hfl$^+$ phenotype. Figure 3 shows that the *tolZ21* mutant also exhibited the Std$^-$ phenotype. In contrast, none of the strains AR754 (*ftsH1*), ST2 (*ftsH101*), and X9393 (*hflB29*) showed the Tol$^-$ and Nfc$^-$ phenotypes of the *tolZ21* mutant at 30 and 37°C (data not shown).

Localization of truncated FtsH protein produced by pJN340. The FtsH protein (70.7 kDa) is an integral cytoplasmic membrane protein having a large cytoplasmic C-terminal part with a functional domain. Two N-terminal hydrophobic segments span the membrane, and the 25- to 95-amino-acid region, which is flanked by the hydrophobic segments, protrudes into the periplasmic space (35). As described above, pJN340 carrying the 1.8-kb *Bam*HI fragment only complemented the Tol$^-$ phenotype of *tolZ21 recA* strain KM103. The nucleotide sequence of the *Bam*HI fragment has an open reading frame identical to that of the FtsH protein lacking the

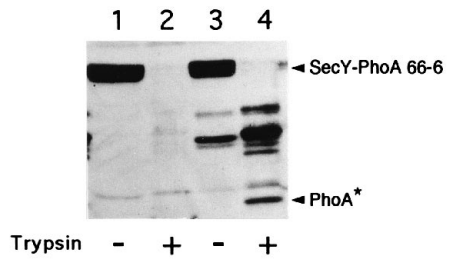

FIG. 3. Std$^-$ phenotype of *tolZ21* strain UM21. The assays were carried out as described previously (1). Cells were grown at 37°C, disrupted by treatment with lysozyme and freeze-thawing, and then treated with trypsin (50 μg/ml) as indicated. Proteins were separated by SDS–10% polyacrylamide gel electrophoresis, and this was followed by immunoblotting using anti-alkaline phosphatase (PhoA) antiserum. SecY-PhoA 66-6, the intact SecY-PhoA fusion protein produced by pKY221 (1); PhoA*, the trypsin-resistant PhoA moiety detected if it is exported in the periplasm. The extra bands between SecY-PhoA 66-6 and PhoA* for the *tolZ21* mutant represent partial degradation products of the fusion protein. Lanes 1 and 2, W2252(pKY221); lanes 3 and 4, UM21 (pKY221).

N-terminal 73 amino acid residues. When the *Bam*HI fragment was cloned into pUC118 downstream of the *lac* promoter, the resultant plasmid, pJN340, was able to carry the *lacZ-ftsH* fused gene encoding a fusion protein, which has the N-terminal 12 amino acid resides from LacZ protein plus the truncated FtsH protein. Therefore, the truncated FtsH fusion protein has only the second membrane-spanning segment, and so subcellular localization of the truncated FtsH protein was examined by immunoblotting.

Figure 4 shows that the truncated protein encoded by the fused gene on pJN340 was present in both the cytoplasmic (lane 3) and membrane (lane 4) fractions, while the wild-type FtsH protein encoded by the chromosomal *ftsH* gene was detected only in the membrane fraction (lanes 2 and 4). Most of the truncated protein in the membrane fraction was cleaved to produce a lower-molecular-weight protein (lane 4). In the periplasmic fraction, no proteins immunoreactive against anti-FtsH antiserum were detected (data not shown). These results indicate that the two membrane-spanning segments of the FtsH protein are essential for it to express the Nfc$^+$ and Tr phenotypes but not the Tol$^+$ phenotype.

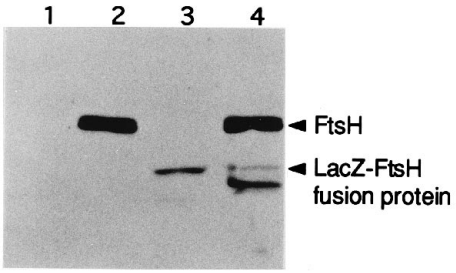

FIG. 4. Localization of the truncated FtsH fusion protein produced by pJN340. Cells were grown at 37°C and then fractionated as described in Materials and Methods. Proteins were separated by SDS–8% polyacryamide gel electrophoresis, and this was followed by immunoblotting using anti-FtsH antiserum. LacZ-FtsH fusion protein, a fusion protein which has the N-terminal 12 amino acid residues of LacZ plus the truncated FtsH protein lacking the N-terminal 73 amino acid residues. Lanes 1 and 2, cytoplasmic and membrane fractions, respectively, of MV1184(pUC118); lanes 3 and 4, cytoplasmic and membrane fractions, respectively, of MV1184(pJN340).

DISCUSSION

In this study, we identified the *E. coli tolZ* gene as *ftsH*. The FtsH protein is a membrane-bound, ATP-dependent metalloprotease which degrades the heat shock transcription factor σ^{32} (14, 34) and is required for proteolytic elimination of uncomplexed forms of the SecY protein, a component of the secretory machinery (16). FtsH belongs to a putative ATPase family (25, 36) referred to as the AAA protein family (ATPases associated with a variety of cellular activities) (17). Members of the AAA family are widely distributed among eubacteria, archaebacteria, and eukaryotes and are proposed to be associated with diverse cellular functions including cell cycle control, protein degradation, transcriptional regulation, protein secretion, and organelle biogenesis (6, 7). It is known that FtsH is involved in protein integration into membranes (1), protein export (1, 2), and λ phage development (13).

Among members of the AAA protein family, eubacterial FtsH proteins of *E. coli* (36), *Lactococcus lactis* (21), and *Bacillus subtilis* (23) and yeast *Saccharomyces cerevisiae* mitochondrial homologs Yme1p (Yta11p) (29, 33), Rca1p (Yta12p) (29, 37), and Afg3p (Yta10p) (11, 29, 32) constitute a subfamily which has an active site motif of zinc-dependent proteases, HEXXH (38), where X is a nonconserved amino acid residue. In the case of the *E. coli* FtsH protein, the active-site motif is located at positions 414 to 418, His-Glu-Ala-Gly-His (36). The *tolZ21* mutation was found to cause the alteration of His-418 to Tyr. The conserved His residues are involved in the binding of a zinc atom (38). Therefore, it might be possible that the *tolZ21* mutation caused the loss of the zinc atom of the protein, resulting in no protease activity of FtsH.

It is known that the *ftsH* gene is essential for cell viability (25). The *tolZ21* mutation was found to be in the *ftsH* gene. The *tolZ21* mutant showed the Hfl$^-$ and Std$^-$ phenotypes. However, the phenotypes of tolerance to colicins (Tol$^-$) and no growth on nonfermentable carbon sources (Nfc$^-$) were observed for the *tolZ21* mutant but not for the *ftsH1*, *ftsH101*, and *hflB29* mutants, suggesting that the two specific phenotypes of the *tolZ21* mutant may be related to the possible complete loss of FtsH protease activity. This seems to be the case. As described above, we found that the *tolZ21* mutant had a suppressor mutation which allowed cells to survive. The suppressor *sfhC* was different from the *fur* mutation and was located in the 4-min region of the *E. coli* genetic map and, more precisely, in the *hlpA-firA* or *fabZ-lpxA-lpxB* operon (24), both of which are involved in lipopolysaccharide-phospholipid biosynthesis. Recently, we also found that the null mutation of *ftsH* could be introduced into the *sfhC* mutant and that the resultant Δ*ftsH sfhC* double mutant showed Tol$^-$ and Nfc$^-$ phenotypes like the *tolZ21* mutant UM21 did (24), supporting the above-mentioned hypothesis. Further characterization of the *sfhC* suppressor is now in progress.

The *tolZ* mutants can grow on glucose but not on nonfermentable carbon sources. Several *S. cerevisiae* mutants showing phenotypes similar to those of the *tolZ* mutants have been reported. *yme1* mutants have a heat-sensitive defect in respiratory growth, being unable to grow on nonfermentable carbon sources at 37°C (33). Deletion of *YTA10* affects the ability of yeast cells to grow on nonfermentable carbon sources by impairing the activities of the respiratory complexes (NADH-cytochrome *c* oxidoreductase and ferrocytochrome *c*-oxygen oxidoreductase) in mitochondria (32). Rca1p is essential for the assembly of the mitochondrial respiratory chain and ATP synthetase complexes (37). Bcs1p, an *S. cerevisiae* protein of the AAA protein family without a zinc-dependent protease domain, is also involved in either the formation of the active

site iron-sulfur cluster or the provision of a chaperone-like function for assembly of the Rieske protein with the other subunits of the complex (22). Accordingly, *bcs1* mutants grow on glucose but cannot utilize nonfermentable carbon sources. We are now investigating the cytoplasmic membrane proteins involved in the respiration and ATP synthesis of the *tolZ* mutants.

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