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Disruption of the *hslU* Gene, Which Encodes an ATPase Subunit of the Eukaryotic 26S Proteasome Homolog in *Escherichia coli,* Suppresses the Temperature-Sensitive *dnaA46* Mutation

Tsutomu Katayama, Toshio Kubota, Makoto Takata,
Nobuyoshi Akimitsu,[1] and Kazuhisa Sekimizu[2]

Department of Microbiology, Kyushu University Faculty of Pharmaceutical Sciences, Fukuoka 812-82, Japan

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The *Escherichia coli hslVU* genes encode subunits of an ATP-dependent protease, a homolog of the eukaryotic 26S proteasome. We found that the *hslU* gene is required for the temperature (40°C)-sensitive phenotype of the *dnaA46* mutant, while other soluble ATP-dependent proteases, La (Lon) and Ti (Clp), were unrelated to this *dnaA46* phenotype. Disruption of the *hslU* gene inhibited cell growth at high temperatures. These observations suggest a specific *in vivo* role for HslVU protease in denatured proteins. As the absence of HslU produces minicells in M9 medium, the protease may be involved in the cell cycle regulation.

ATP-dependent proteases are required for various processes involved in cell regulation (1). The 26S proteasome is present in eukaryotes, and is responsible for ATP-dependent degradation of ubiquitin-conjugated proteins (1-3). Since some cyclins are included in such targets, this enzyme plays an important role for entry into M and S phases of the cell cycle (4-6). The protease activity is present in the 20S core particle of the 26S complex (7, 8).

The HslV and HslU proteins are encoded by the *hslV* and *hslU* genes, respectively, in *E. coli*, and form a multimeric complex *in vitro* which is homologous to the eukaryotic 26S proteasome (9, 10). The *in vivo* function of these genes has remained to be elucidated. Since *E. coli* proteins are not modified with ubiquitin, specific targets of the HslVU protease *in vivo* have to be identified.

The *dnaA* gene encodes an initiator protein (DnaA) that binds to the chromosomal replication origin (*oriC*), and triggers a series of initiation reactions (11). DnaA protein also plays a key role in the regulation of initiation (12-16). To search for genes responsible for regulation of DnaA protein functions, we screened *dnaA* suppressor mutants isolated by random transposon insertion (Akimitsu, N., Katayama, T., Sekimizu, K.; submitted). We now report that disruption of the *hslU* gene suppresses the *dnaA46* mutation which renders initiation temperature-sensitive. In contrast, absence of La and Ti, which are also soluble ATP-dependent proteases (17, 18), did not suppress the *dnaA46* temperature-sensitivity. These findings indicate functional differentiation of the ATP-dependent proteases. A possible role for the HslVU protease in the *E. coli* cell cycle was also given attention.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All strains were derivatives of *Escherichia coli* K12. Genotype of KH5402-1 is: *thyA thr trpE9829*(Am) *ilv tyrA*(Am) *metE deo supF6*(Ts) (19). KA456, KA459, and KA460 are derivatives of KH5402-1 carrying *clpP1*::Cm^R (20), *hslU1*::mini-*tet,* and *lon*::Tn*10,* respectively. The *lon*::Tn*10* mutation is

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[2] To whom correspondence should be addressed. Fax: +81-92-641-6648.

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derived from ME8417 (a gift from A. Nishimura). Genotype of KA413 is the same as that of KH5402-1 except for *ilv*$^+$ and *dnaA46* (21). KA737 is a temperature-resistant revertant of KA413, by transposon mutagenesis. KA456, KA461, and KA832 are derivatives of KA413 carrying *clpP1*::Cm^R, *lon*::Tn*10*, and *hslU1*::mini-*tet*, respectively. DH5α was used for cloning of plasmids (22). pKA241 bears a 6.1 kb *Hin*dIII fragment derived from chromosome of KA832 on pUC19. For transposon mutagenesis, λ1098 carrying *P*am cI857(Ts) *nin*-5 and a 'mini-*tet*' transposon was used (23).

Media. LB medium (22) was supplemented with 50 μg of thymine per ml. M9 medium (22) was supplemented with 50 μg of thymine per ml, 0.2% casamino acids, 50 μg of tryptophan per ml, 2 μg of thiamine per ml, and 0.2% glucose. Tetracycline sodium salt, ampicillin sodium salts, kanamycin sulfate, and chloramphenicol were added at 25, 50, 25, and 25 μg/ml, respectively, if required. λ broth contained 1% polypeptone and 0.25% NaCl.

Transposon mutagenesis. KA413 was grown overnight at 30°C in LB medium, diluted 10-fold in λ broth containing 0.01% yeast extract and 0.2% maltose, incubated at 30°C for 1h, and infected with λ1105, using multiplicity of infection of 1.0 in the presence of 20 mM $MgSO_4$. After 1 h incubation at 37°C, cells were collected by centrifugation, washed twice in 0.9% NaCl, and plated on LB agar plates containing tetracycline. These plates were incubated at 37°C for 2 h, and further incubated at 40°C for 36 h.

Nucleotide sequence analysis. Plasmid was purified with a QIAGEN tip, and used as a template for a cycle sequencing reaction with a fluorescent dye-conjugated primer, M13(-21), according to the manufacturer's instruction (Applied Biosystems). Products were analyzed using a DNA sequencer (Applied Biosystems).

Analysis with a flowcytometer. Cells were exponentially grown for over ten generations until the A660 reached 0.2, and incubation was continued for a further 4 h in the presence of 200 μg of rifampicin per ml. The cells were then stored in 70% ethanol at 4°C until use. Before measurement, cells were washed in buffer containing 10 mM Tris-HCl (pH 7.5) and 20 mM $MgSO_4$, and stained with 27 μg of mithramycin (Kodak) per ml and 5 μg of ethidium bromide per ml in the same buffer as above. Sell size and DNA content were simultaneously measured using a flowcytometer, BRYTE HS (BioRad) (24).

RESULTS

Isolation and characterization of a dnaA46 suppressor. To isolate suppressor mutants from a *dnaA46* mutant (KA413), we carried out transposon mutagenesis using λ1105 containing a mini-*tet* transposon. KA413 infected with this phage was incubated on LB agar plates for 36 h at 40°C, a restrictive temperature of the *dnaA46* mutant. λ1105 is unable to proliferate or lysogenize under these conditions, and the transposon randomly integrates into the chromosomal DNA (23). We isolated 150 independent colonies as candidates for suppressors of the *dnaA46* mutation.

One of those revertants, KA737, was further examined for genetic linkage between the tetracycline-resistant element (mini-*tet*) and suppressor mutation. P1 phage lysate prepared from KA737 was used for transduction of KA413. Transductants were obtained with an expected frequency on LB agar plates containing tetracycline at 30°C, and growth at 40°C was tested. Co-transduction frequency of those markers, tetracycline resistance and growth capacity at 40°C, was 100% (data not shown). This result indicates that the mini-*tet* insertion is responsible for suppression of *dnaA46* temperature-sensitivity. KA832 is representative of the *dnaA46* suppressor constructed by P1 transduction. This strain grew well at 40°C, but at 42°C growth was inhibited (Table 1).

Cloning and identification of the suppressor mutation. We cloned the *Hin*dIII fragment derived from the chromosome of KA832 on pUC19 and selected transformants for tetracycline resistance. Six independent clones were isolated, and restriction enzyme analysis revealed that all the plasmids have the same structure (data not shown).

Nucleotide sequencing of the cloned chromosomal fragment and a homology search revealed that the fragment contains a truncated form of the *glpF* gene (Fig. 1). Detailed restriction analysis indicated that the mini-*tet* transposon was inserted in the *hslU* cistron which comprises an operon with the *hslV* cistron (25). Thus, disruption of the *hslU* gene was responsible for suppression of the *dnaA46* mutation.

Growth of the dnaA46 mutant lacking La and Ti proteases. During completion of our work, HslVU proteins were reported to be the subunits of a novel ATP-dependent protease (9, 10). The HslVU proteins purified from overproducers form a multimeric complex with a considerable

TABLE 1
Suppression of a *dnaA46* Mutant

Strain	Relevant genotype	CFU/ml at 30°C	Colony-forming ability	
			40°C/30°C	42°C/30°C
KH5402-1	*dnaA*$^+$	4.8×10^9	0.67	0.63
KA413	*dnaA46*	3.2×10^9	6.9×10^{-4}	4.7×10^{-7}
KA459	*dnaA*$^+$ *hslU1*::mini-*tet*	6.1×10^9	0.90	0.89
KA832	*dnaA46 hslU1*::mini-*tet*	2.1×10^9	1.0	1.0×10^{-6}
KA456	*dnaA*$^+$ *clpP1*::Cm^R	4.0×10^9	0.45	0.70
KA457	*dnaA46 clpP1*::Cm^R	4.0×10^9	3.7×10^{-4}	7.4×10^{-7}
KA460	*dnaA*$^+$ *lon*::Tn*10*	5.3×10^9	0.83	0.85
KA461	*dnaA46 lon*::Tn*10*	4.7×10^9	3.0×10^{-4}	4.9×10^{-6}

Disruptants of the protease genes were constructed by P1 transduction using KH5402-1 and KA413 as the recipient strains. All strains were grown overnight in LB medium at 30°C and plated on LB agar plates after dilution. Colonies formed at 30, 40, and 42°C during incubation of 24 h were counted. CFU, colony-forming unit.

solubility, and degrade abnormal, hydrophobic peptides. This suggested that the HslVU protease is present in the cytosol and degrades denatured proteins.

To examine the functional specificity of soluble ATP-dependent proteases, we introduced *lon*- and *clpP*-null mutations into the *dnaA46* mutant (Table 1). The *lon* gene encodes the protease La (17), and the *clpP* gene encodes the subunit having the protease domain of the protease Ti (18). Unlike the *hslU* disruption, disruptions of the *lon* gene and the *clpP* genes did not suppress temperature sensitivity of the *dnaA46* mutation. These observations support the notion that there is functional divergence among these proteases.

Growth defect at high temperatures of the hslU-disruptant. The hslVU operon is induced by heat shock (26). The putative promoter of the *hslVU* operon has a typical sequence recognized by σ^{32} which is specific for transcription of heat shock genes (25). To search for a possible role for the HslVU protease in heat stress, we examined the colony-forming ability

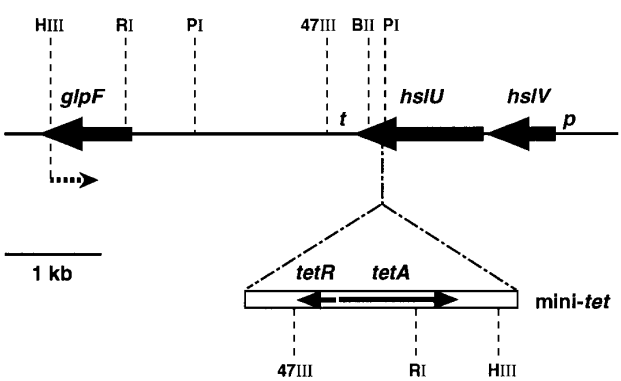

FIG. 1. The chromosomal region containing mini-*tet*. Chromosomal DNA is represented by a solid thin line. The coding regions are indicated by an arrow with a solid thick line, and the names of the genes are indicated. The direction of the arrow indicates direction of translation. The mini-*tet* transposon is shown by an open rectangle. An arrow with a broken line indicates the sequenced region and the direction of sequencing. Restriction sites are indicated: BII, *Bgl*II; HIII, *Hin*dIII; PI, *Pst*I; RI, *Eco*RI; 47III, *Eco*47III. *p* and *t* indicate the transcriptional promoter and terminator, respectively, of the *hslVU* operon. The 6.1 kb *Hin*dIII fragment containing from the truncated *glpF* gene to the *tet* gene in the mini-*tet* transposon was cloned on pUC19 (pKA241).

TABLE 2
High-Temperature Sensitivity of the *hslU*::mini-*tet* Mutant

Strain	Relevant genotype	CFU/ml 30°C	Colony-forming ability 44°C/30°C	45°C/30°C	46°C/30°C
KH5402-1	*hslU*$^+$	2.5×10^9	0.84	0.84	0.72
KA459	*hslU1*::mini-*tet*	5.0×10^9	0.88	0.84[a]	9.0×10^{-5}

Strains were grown overnight in LB medium at 30°C and plated on LB agar plates after dilution. Colonies formed at 30, 44, 45, and 46°C during incubation of 24 h were counted.
[a] Colonies are tiny pinpoints.

of the *hslU*-disruptant, at high temperatures (Table 2). Whereas both KH5402-1 (*hslU*$^+$) and KA459 (*hslU1*::mini-*tet*) grew well at 44°C, at 45°C colonies of KA459 were extremely small, and at 46°C, KA459 did not form colonies. Therefore, the HslU protein is necessary for normal growth, at high temperatures.

When the *hslU*$^+$ and *hslU*-null strains were grown at 37 and 44°C, and contents of major proteins compared using SDS-polyacrylamide gel electrophoresis, no significant change was detected (data not shown).

Cell cycle parameters in the hslU disruptant. We next examined cell cycle parameters of the *hslU* disruptant using a flowcytometer (24). When KH5402-1 and KA459 were exponentially grown at 37°C in a supplemented M9 medium, both strains grew at the same rate; a doubling time of A_{660nm} was 48 min. To inhibit initiation of new rounds of chromosome replication, rifampicin was added, and replication of whole chromosomes was completed during 4 h of further incubation. The resultant number of chromosomes per cell corresponds to number of replication origins (*oriC*) per cell (24).

Although KH5402-1 and KA459 contained almost the same level of origins (Fig. 2, A and B), cell size of KA459 was significantly smaller than that of KH5402-1 (Fig. 2, C and D). This means that the timing of initiation of chromosome replication and/or the timing of cell division during cell cycle is hastened. When cells were grown in LB medium, no significant difference in these parameters was observed (data not shown). Thus, effects of the *hslU* gene on the cell cycle may depend on conditions for growth.

DISCUSSION

Disruption of the *hslU* gene suppressed the temperature-sensitive phenotype of the *dnaA46* mutation. This suppression was seen at 40°C, but was not seen at 42°C. This suggest that a residual activity of the mutant protein is necessary for the suppression.

The mutant DnaA46 protein may be degraded by the HslVU protease; the absence of the protease should increase the amount of DnaA46 protein, which enables initiation of DNA replication at 40°C. Indeed, oversupply of DnaA46 protein suppresses the temperature-sensitivity of cell growth (27).

In addition, our study revealed that the *hslU* gene is nonessential for cell growth at 44°C or below. The disruptant was isolated by random transposon mutagenesis, and the insertional mutation was efficiently transduced to other strains.

As suggested by suppression of the *dnaA46* mutant and growth defect at high temperatures, a role of HslVU protease seems to be the degradation of denatured proteins. This is the first *in vivo* evidence to support *in vitro* observations of purified HslVU protease (9, 10).

Minicell formation of the *hslU*-null mutant implies that a positive modulator(s) for initiation of chromosome replication and/or for cell division is a proteolytic substrate of the HslVU

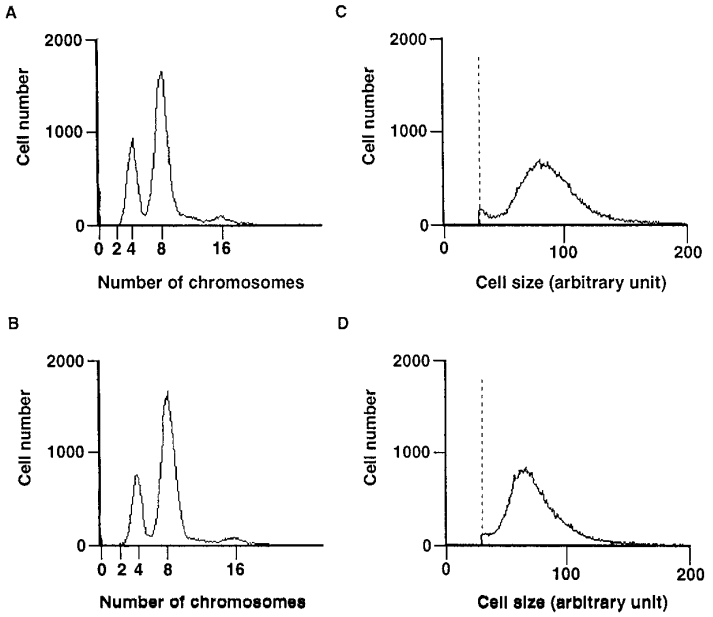

FIG. 2. Cell cycle parameters analyzed with a flowcytometer. Cells of KH5402-1 and KA459 were grown at 37°C in a supplemented M9 medium and treated in rifampicin. A, DNA content of KH5402-1; B, DNA content of KA459; C, cell size of KH5402-1; D, cell size of KA459. The dotted line on C and D indicates the threshold to eliminate noise signals.

protease. Accumulation of such stimulators in the *hslU*-null mutant should accelerate the cell cycle, the result being formation of cells of a small size. Thus, HslVU protease may degrade an intact protein for regulation of cell cycle in addition to denatured proteins. Detailed functions of HslVU protease in the cell cycle are under investigation.

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