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PROTEASE DO IS ESSENTIAL FOR SURVIVAL OF ESCHERICHIA COLI AT HIGH TEMPERATURES: ITS IDENTITY WITH THE htrA GENE PRODUCT[1]

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SUMMARY: The DNA encoding protease Do was isolated from an *E. coli* genomic DNA library in λgt11, and cloned into a Bluescript plasmid. The cells transformed with the recombinant plasmid were able to overproduce protease Do and grew normally. A mutant lacking the protease activity was also isolated by interrupting the chromosomal DNA with the *kan* gene. The mutant showed a prolonged lag period and reduced ability to degrade cell proteins as compared to its wild type. Moreover, they were unable to survive at high temperatures, similarly to the *htrA* mutants. These results suggest that protease Do may play an important role in the intracellular protein breakdown and is essential for survival at high temperatures. Identity of protease Do with the *htrA* gene product is discussed.

Soluble extracts of *E. coli* contain 9 endoproteolytic activities that appear to be distinct enzymes (1,2). Seven of these, named protease Do, Re, Mi, Fa, So, La and Ti, are serine enzymes that degrade casein and globin. Two other enzymes, protease Ci and Pi, are metalloproteases that degrade insulin and the N-terminal fragment of β-galactosidase, called auto α (3). Protease Mi and Pi are periplasmic enzymes, while all others are localized to cytoplasm (4) and therefore may play a role in the hydrolysis of intracellular proteins. However,

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physiological functions have been established for only one of these proteases thus far. Protease La, an ATP-dependent enzyme encoded by the *lon* gene, catalyzes the degradation of abnormal proteins and of certain regulatory polypeptides (5,6). This protease is also known as one of the 17 heat shock proteins that are under control of the *htpR* regulon (7,8). In addition, protease So has been implicated to play an important role in the hydrolysis of oxidatively damaged proteins (9,10). However, other proteases must also be involved in the selective breakdown of abnormal as well as normal cell proteins.

Protease Do is a multimeric enzyme with an unusual high molecular weight (11). This enzyme has recently been shown to distinctively cleave the purified Ada protein and therefore suggested to play a role in the regulation of the adaptive response to alkylating agents (12,13). As an attempt to elucidate the physiological function(s) of protease Do, we cloned the gene encoding the protease, referred to as the *ptd* gene, and isolated a mutant lacking the enzyme activity by disrupting the chromosomal *ptd* gene with the *kan* gene. The present studies demonstrated that the gene disruption results in the decrease in the overall rate of intracellular proteolysis and the loss of the cell's viability at high temperatures.

MATERIALS AND METHODS

Materials: Protease Do was purified as described previously (11). Anti-protease Do antiserum was obtained by injecting the purified enzyme to albino rabbits. A genomic library of *E. coli* DNA in λgt11 was purchased from Clontech. *E. coli* strains used were DH1 and JC7623 (*recBC, sbcB*).

Plaque Screening: The DNA clones producing protease Do were obtained by immuno-screening of a *E. coli* genomic DNA library in λgt11. Screening of the library was carried out using anti-protease Do antiserum as described by Young and Davis (14). Preparation of phage DNA and other DNA manipulations were done by following the standard procedures (15).

Cloning and Disruption of the *ptd* Gene: The 7 Kb insert DNA carrying the *ptd* gene in one of the positive phage clones was ligated into the EcoRI site of Bluscript KS$^+$. The SacI-EcoRV DNA fragment within the multicloning site of the plasmid was eliminated. The insert DNA was then trimmed to 2.2 Kb with ClaI and KpnI and ligated into the respective sites of the same plasmid. The 1.2 Kb

HaeII fragment containing the *kan* gene was isolated from pMK16 plasmid (16), and inserted into the SacI site in the recombinant plasmid by blunt-end ligation. The 3.5 Kb PvuII-KpnI fragment, in which the *ptd* gene is interrupted by the *kan* gene, was isolated and used in the linear transformation experiment for homologous recombination with the chromosomal *ptd* gene (17).

Immunoblot Analysis: Extracts were prepared from *E. coli* that had been grown in Luria broth (LB) until to A_{600} of 0.5. After the electrophoresis of the extracts in 10% polyacrylamide gels containing SDS (18), proteins in the gels were transferred to nitrocellulose membranes. The membranes were incubated with anti-protease Do antiserum and then with anti-rabbit IgG conjugated with alkaline phosphatase. Protein bands were visualized by the phosphatase reaction (19).

Measurement of Proteolysis: *E. coli* grown in M9 minimal media until to A_{600} of 0.5 were incubated with 1 μCi of [^{35}S]methionine for 5 min. The cells were washed twice and resuspended in the same media but containing 0.2 mg/ml of nonradioactive methionine. The hydrolysis of cell proteins was then estimated by following the appearance of radioactivity soluble in 10% trichloroacetic acid.

RESULTS AND DISCUSSION

To isolate DNAs encoding protease Do, we screened a *E. coli* genomic DNA library in λgt11 and obtained 5 positive clones. The 7 Kb DNA fragment from one of the clones was inserted into EcoRI site of a Bluescript plasmid and further trimmed to 2.2 Kb. *E. coli* DH1 cells transformed with the recombinant plasmid were capable of overproducing protease Do as examined by immunoblot analysis using anti-protease Do antibody (Fig. 1). Thus, it appears that the 2.2 Kb DNA contains a full length DNA sequence for the expression of the *ptd* gene.

To obtain mutants that lack the activity of protease Do, we carried out the disruption of the chromosomal *ptd* gene. The 2.2 Kb DNA was cut with SacI and ligated with the *kan* gene. The 3.5 Kb PvuII-KpnI fragment carrying the *kan* insert was then transformed to *E. coli* JC7623 for homologous recombination with the *ptd* gene in chromosome. The transformed cells lacked the band corresponding to protease Do as examined by immunoblot analysis (Fig. 1). Furthermore, no caseinolytic activity was evident in the fractions where protease Do normally eluted upon the chromatography of the cells' extract on a hydroxylapatite column (11; Fig. 2). To ascertain the lack of the protease

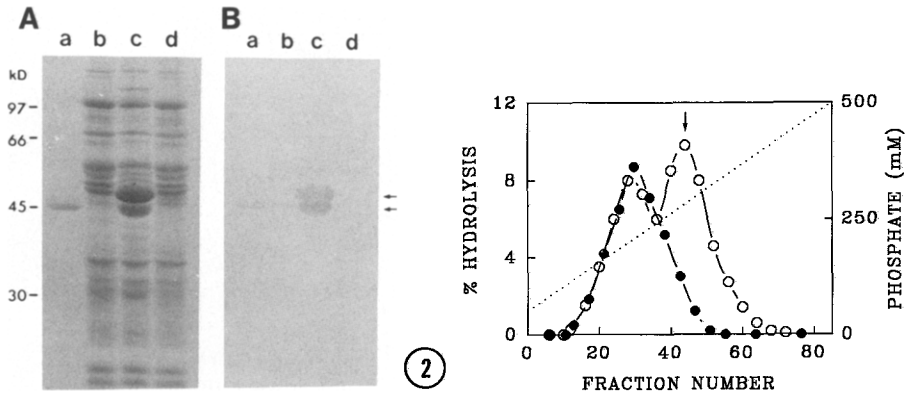

Fig. 1. Immunoblot Analysis of Protease Do in *E. coli* Cells. The cell extracts were electrophoresed and stained with Coomassie R250 (A) or immunoblotted using anti-protease Do antiserum (B). Lane a, 2 μg of the purified protease Do; b, JC7623; c, DH1 carrying the *ptd* gene in a multicopy Bluescript plasmid; d, JC7623 *ptd* mutant. The arrows indicate where protease Do migrated.

Fig. 2. Elution Profiles of Protease Do from a Hydroxylapatite Column. Extracts (40 mg) were prepared from JC7623 (○) and its *ptd* mutant (●) and loaded onto the columns (1 x 6.5 cm) equilibrated with 50 mM KH_2PO_4/K_2HPO_4 (pH 7.4). After washing the column, proteins were eluted with a linear gradient of 50-500 mM phosphate. Every second fractions were assayed for casein hydrolysis as described (1,11). The arrow indicates where protease Do elutes.

activity is indeed due to the gene disruption, the PstI-digests of *E. coli* chromosomal DNA were subjected to Southern blot analysis (20). As shown in Fig. 3, the 0.6 Kb PstI-EcoRV fragment of the *ptd* gene hybridized with a single species of chromosomal DNA fragments from both the wild type and mutant cells. On the other hand, the 0.5 Kb HindIII-XhoI fragment of the *kan* DNA hybridized only with that from the disrupted mutant. In addition, the size difference between the fragments coincided with the size of the inserted *kan*

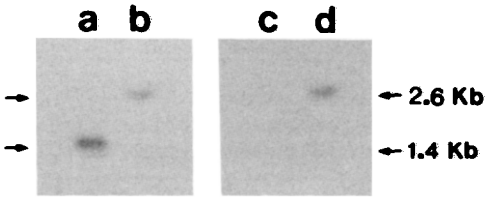

Fig. 3. Southern Blot Analysis of PstI-digests of *E. coli* Chromosomal DNA. Chromosomal DNAs were obtained from JC7623 (a and c) and its *ptd* mutant (b and d), and digested with PstI. The digests were transferred onto nitrocellulose membranes and hybridized with the 0.6 Kb fragment of the *ptd* gene (a and b) or the 0.5 Kb *kan* probe (c and d).

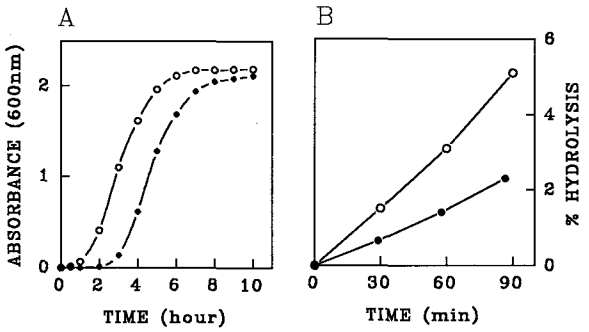

Fig. 4. Measurement of Growth Rate and Overall Rate of Proteolysis in *E. coli* Cells. The growth rates (A) and the overall rate of proteolysis (B) in JC7623 (○) were compared to those of its *ptd* mutant (●). The assays were performed as described in Materials and Methods.

DNA (1.2 Kb). These results indicate that the disruption of the chromosomal *ptd* gene is responsible for the loss of the protease activity.

To examine if the mutation induces any phenotypical change, we compared the growth rate and the overall rate of proteolysis in the mutant to those of its wild type cells. The growth rates of both cells were nearly identical although the mutant required a longer lag period (Fig. 4A). However, the breakdown of cell protein in the mutant occurred at a half-rate of that in its wild type (Fig. 4B). Thus, it appears that protease Do may play a role in the intracellular hydrolysis of normal proteins. As a preliminary experiment to investigate the role of this enzyme in the hydrolysis of denatured proteins, which may rise under heat shock, we examined the ability of the mutant in growing at high tempertures. As shown in Fig. 5, the cell density rapidly fell after shifting the

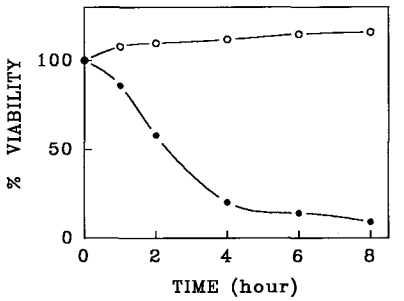

Fig. 5. Viability of *E. coli* Cells at High Temperature. *E. coli* JC7623 (○) and its *ptd* mutant (●) grown at 37℃ were shifted to 45℃ and further incubated for the indicated periods. The same aliquots of the cells were then plated on LB plates and incubated overnight at 37℃, and the number of colonies developed were counted. The cell number obtained without the temperature shift was expressed as 100% viability.

culture temperature from 37℃ to 45℃ unlike to that of its wild type. These results suggest that protease Do is essential for the cell's survival at high temperatures.

In a number of aspect, protease Do (11,13) appears to be identical to the product of the *htrA* gene (21,22), also called *degP* (23,24). [1] Both are endoproteases that use casein as substrates; [2] Both are sensitive to inhibition by serine active site-directed agents including diisopropyl fluorophosphate; [3] Both consist of subunits with similar sizes; [4] Both are processed to smaller polypeptides with similar sizes; [5] The mutations in the genes encoding the respective proteins result in the loss of the cells' viability at high temperatures; [6] Both are heat shock proteins. The protein level of protease Do increased about 2-fold upon shifting the culture temperature from 37℃ to 45℃ (unpublished observations). Perhaps only the striking difference so far been found is their subcellular localizations. The *htrA* gene product has been shown to localize primarily to the periplasmic space or inner membrane (24). On the other hand, protease Do has been reported to be a cytosolic enzyme (4,11). Determination of the DNA sequence of the *ptd* gene encoding protease Do should help in the clarification of these discrepant results and the identity of this enzyme with the *htrA* gene product, and is under vigorous investigation.

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