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# Isolation and Characterization of Mutations in the Structural Gene for Protease III (*ptr*)

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*Escherichia coli* mutants defective in protease III were isolated by enzyme assays of heavily mutagenized clones. One mutant produced thermolabile enzyme, and it is presumed to have a mutation in the structural gene of protease III. Two other mutants mapping at the same site had less than 5% of the wild-type protease III level. The genetic locus of these mutations, designated *ptr*, was located at approximately 60 min on the *E. coli* linkage map based on its high frequency (70%) of cotransduction by P1 with *argA*. Strains with less than 5% of the wild-type protease III activity grew normally and degraded nonsense fragments of β-galactosidase at wild-type rates.

There are a number of specific proteolytic processes in *Escherichia coli*. They are involved in such cellular functions as catabolism of abnormal proteins (2, 6), protein secretion (7), protein turnover during starvation (5), and inactivation of functional proteins (17). For an understanding of the mechanism of the specific proteolysis, it is necessary to evaulate the involvement of *E.* coli proteases in these processes.

Two *E. coli* proteases, protease I and protease II, have been isolated and characterized (14, 15). Studies on mutants of *Salmonella typhimurium* and *E. coli* with defective protease I and protease II indicated that these two enzymes are not involved in the degradation of aberrant proteins or in the starvation-induced proteolysis (8, 9).

We have recently purified to homogeneity a cytoplasmic endoproteolytic enzyme of *E. coli* designated protease III (4). The purified enzyme consists of a single polypeptide chain with a molecular weight of 110,000. The enzyme is most active at pH 7.4 and sensitive to metal chelating agents. It is devoid of activity towards aminopeptidase, carboxypeptidase, or esterase substrates, but rapidly degrades several small proteins. When peptides produced by autoclaving β-galactosidase are used as substrates for protease III the enzyme preferentially degrades those with molecular weights of less than 7,000. Protease III cleaves the oxidized insulin B chain at two sites, with an initial rapid cleavage at *tyr-leu* (16-17) and a second slower cut at *phe-tyr* (25-26). The biochemical features of protease III clearly indicate that it is different from protease I and protease II (4).

Here we report the isolation, mapping, and preliminary phenotypic characterization of mutations in the structural gene for protease III. A single genetic locus was found and is called *ptr*. Since we could not assume, in advance, any phenotypic characteristic for selection, the isolation of protease III mutations depended on modifying a rather complex assay for use in screening large numbers of mutagenized clones. Protease III was originally detected by its ability to degrade the small $NH_2$-terminal peptides, called "auto α" in the literature (11), can be determined in a complementation assay when they react with the products of a *lacZ* gene deletion mutation to produce active β-galactosidase. By destroying the auto α peptides, protease III lowers the amount of β-galactosidase activity formed in the complementation reaction, and this serves as the basis for its assay. To use this assay in screening for protease III mutations, we had to modify it so that all the steps, including growth of bacteria, opening of the cells, degradation of auto α, complementation, and β-galactosidase assay, could be carried out in a single tube or microtiter plate well. The details of the screening procedure are given in Materials and Methods. For this screening technique to succeed, protease III must be a major contributor to the degradation of auto α in cell-free systems. Fortunately, our studies on the purification of protease III made it clear that this was the case (4). In addition, other studies showed that the degradation of auto α in extracts was not due to protease I or protease II (unpublished data).

## MATERIALS AND METHODS

**Bacterial and phage strains.** Bacterial strains used in this study are derivatives of *E. coli* K-12 and are described in Table 1. Bacteriophage P1*kc* (ob-

TABLE 1. *Bacterial strains*

| Strain | Genotype | Derived from: | Source |
|---|---|---|---|
| 7009 | F⁻ *leu-12 met* Δ(*pro-lac*) | | Norkin (13) |
| 727 | HfrH *lacZ727 trp* | | Zipser (19) |
| 659 | F⁻, *lacZX90 trp rpsL*[a] | | Zipser (19) |
| X90F | *lacZX90 trp rpsL*/F′ *lacZX90* | 659 | Homogenotization |
| CSH74 | Hfr KL16 *thi* | | CGSC[b] |
| JB1 | Hfr P4X *relA1 metB1 uxaA1* | | CGSC |
| AT713 | F⁻ *thi argA21 cysC43 lysA22 mtl-2 xyl-7 malA1 rpsL104* | | CGSC |
| 4529X | F⁻ *thi argA21 cysC43 lysA22 mtl-2 xyl-7 malA1 rpsL104 lacZX90* | AT713 | Homogenotization |
| 7909 | F⁻ *leu-12 met* Δ(*pro-lac*) *nalA* | 7009 | Spontaneous |
| KL16N | Hfr KL16 *thi nalA* | CSH74 | P1 transduction |
| P4XS | HfrP4X *relA1 metB1 uxuA1 rpsL104* | JB1 | P1 transduction |
| 1901 | F⁻ *ptr-1 lacZ727* | 7009 | NG[c] mutation |
| 2201 | F⁻ *ptr-2 lacZ727* | 7009 | NG mutation |
| 3302 | HfrH *ptr-3 lacZ727* | 7009 | NG mutation |
| 3303 | F⁻ *ptr-3* Δ(*pro-lac*) | 3302 | 3302 × 7009 |
| 3304 | HfrH *ptr-3 lacZ727 nalA* | 3302 | P1 transduction |
| 3309 | F⁻ *ptr-3* Δ(*pro-lac*) *nalA* | 3303 | P1 transduction |
| 3311 | F⁻ *ptr-3* Δ(*pro-lac*) *rpsL104* | 3303 | P1 transduction |

[a] Formerly *strA*.
[b] *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University, New Haven, Conn.
[c] Nitrosoguanidine.

tained from A. Bukhari) was used for transductions.

**Media and bacterial growth.** Bacteria were grown in either LB solution (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of water) or on LB plates (LB with 15 g of agar per liter). For the selection of prototrophic exconjugants or transductants, M9 glucose medium supplemented with required amino acids was used. In the selection of nalidixic acid-resistant or streptomycin-resistant strains, either 40 μg of nalidixic acid per ml or 100 μg of streptomycin sulfate per ml was included in agar plates. Unless otherwise mentioned, all incubations were at 37°C.

**Genetic techniques.** The procedures used for P1 transduction and Hfr-mediated conjugation were those described by Miller (10). The *nalA* derivatives were isolated by plating $10^9$ cells on LB plates supplemented with 40 μg of nalidixic acid per ml. When *nalA* was used as a selection marker for Hfr transfers, the mating mixtures were blended vigorously with a Vortex mixer and plated with soft agar on LB plates with 100 μg of streptomycin sulfate per ml at 37°C for 90 min to express the NalA phenotype. The plates were then overlaid with soft agar containing nalidixic acid to a final concentration of 40 μg/ml. The *nalA* exconjugants were later restreaked and further tested.

**Preparation of auto α as protease III substrates.** The preparation of crude auto α was as described by Morrison and Zipser (11). The crude auto α preparation thus obtained was fractionated to get a subclass of peptides soluble in 2% acetic acid (4). The majority of the auto activity in this preparation is due to two peptides with molecular weights of 5,200 and 4,500, respectively. Both peptides can be rapidly and completely degraded by protease III (4). We found that the fractionation step using 2% acetic acid is very crucial for the preparation of auto α as protease III substrates.

**Strategy and method of protease III mutant screening.** The screening procedure for the isolation of protease III mutants was designed by using the knowledge that protease III is the major proteolytic activity against auto α activity in the crude extract of *E. coli* (4). Auto α was very useful for the mutant screening procedure because of its color formation in a previously described complementation and β-galactosidase assay coupled quantitation method (11). Since protease III is the major protease in the crude extracts that destroys auto α activity, a crude extract from a strain with defective protease III will degrade less auto α than that from a wild-type strain. As a result, more auto α molecules will be left intact after incubation with extracts of protease III mutants. The difference in the levels of remaining auto α activity can be easily visualized since, after the color-forming reactions, a preparation with high auto α activity will be deep yellow whereas a preparation with low auto α activity will be virtually colorless (11). Therefore, strains with reduced or defective protease III activity can be identified by this assay system.

Strain 7009 was grown in LB solution to a titer of 5 × $10^8$ cells per ml and then treated with 400 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine per ml at 37°C for 45 min. The treated culture was washed with LB solution and plated on LB plates at 32°C. The survivors were picked and saved on master plates. They were inoculated into LB microtiter plate wells and incubated at 32°C overnight. The microtiter plates were centrifuged at 2,000 × *g* for 20 min, and the cell pellets were treated with 50 μl of lysing buffer (100 μg of lysozyme per ml, 2 mM EDTA, 10% sucrose, and 100 mM Tris-hydrochloride, pH 8.0) at 25°C for 30 min followed by two freezings and thawings. Twenty-five microliters of 10 mM $MnCl_2$ was added into each microtiter well, and the plate was heated at 49°C for 10 min to inactivate any temperature-sensitive pro-

tease III activities. Each microtiter well then received 25 μl of auto α (at a protein concentration of 10 μg/ml), and the plates were incubated at 35°C for 3 h. At the end of the incubation, 25 μl of crude extract from XA21 in buffer I (20 mM Tris-hydrochloride, 100 mM mercaptoethanol, 5 mM EDTA, and 10 mM NaCl, pH 7.2) was added to form complemented β-galactosidase. Under the above reaction conditions, practically all the added auto α was cleaved by wild-type extracts. Thus, those wells that were yellow at the end of the screening were likely to contain mutant strains. The color difference of wild-type and protease III mutants in this test was very obvious, as shown in Fig. 1. The same screening method was used for the scoring of *ptr* recombinants after genetic crosses.

**Assay of protease III activity.** The assay of protease III activity was employed to confirm the initial screening for protease III mutants as well as for the further characterization of wild-type and mutant protease III activities. The detailed procedure for this assay was described previously (4). The assay mixture contained 0.1 ml of extract and 0.9 ml of auto α in 10 mM Tris-hydrochloride–10 mM $MgCl_2$, pH 7.2, and the rate of auto α inactivation was taken as protease activity. For the detection of temperature-sensitive protease III activities, enzyme preparations were heated at 49°C for 10 min prior to their assay.

The recovery and detection of protease III activity after native gel electrophoresis were described previously (4).

**Measurement of nonsense fragment degradation.** The procedure for the measurement of nonsense fragment degradation based on the reduction of auto α activity was described by Miller and Zipser (9).

## RESULTS

**Mutant isolation.** To carry out genetic manipulations with any mutants we obtained, we used a starting strain (7009) that can be easily converted to Hfr Hayes. The Hfr itself was not employed because experience has shown that heavy mutagenesis often destroys the Hfr character.

From about 2,500 nitrosoguanidine-mutagenized clones of 7009, three unambiguous mutants of protease III were obtained by the screening procedure described in Materials and Methods. These were crossed to HfrH, and *met*$^+$ *leu*$^+$ derivatives were selected to obtain genetically "cleaned" derivatives, some of which were Hfr's that were used for further analysis. Two of the mutants had their ability to degrade auto α reduced about 85%. One mutant required heating for 10 min at 49°C before a differential effect could be seen (Table 2).

**Characterization of the activity affected by mutation.** The lowering of degradation activity in the mutants was not due to an increased synthesis of inhibitor since the activity of mixtures of mutants and wild-type extracts gave additive results. To demonstrate that the protease III molecule had been affected by the mutation, we ran extracts from mutant strains on native polyacrylamide gels together with wild-type extracts and purified protease III. The

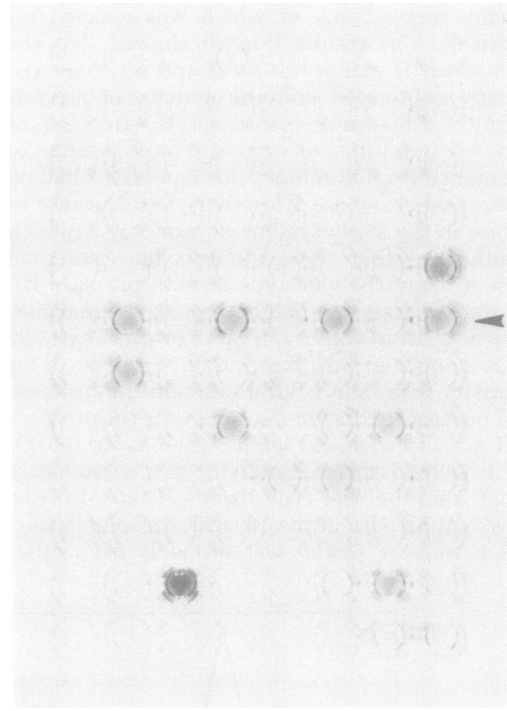

FIG. 1. *Differentiation of ptr*$^+$ *and ptr strains by protease III assay in microtiter plates. The detailed procedure for protease III mutant screening was described in Materials and Methods. The result of a genetic cross (between KL16N and 3311) involving ptr transfer is shown here as an example. The parental strains of ptr and ptr*$^+$ *are in the wells of the middle row (marked with an arrow) in alternating order. All other wells, except the darkest one (with no bacterial extract), contain nalidixic acid-resistant recombinants of this cross. The dark wells, yellow in real wells, were scored as ptr.*

TABLE 2. *Proteolytic activities of wild-type and ptr strains*[a]

| Strain | Relative specific activity (%)[b] | |
|---|---|---|
| | Untreated | Heated |
| 7009 (*ptr*$^+$) | 100 | 95 |
| 1901 (*ptr-1*) | 98 | 14 |
| 2201 (*ptr-2*) | 15 | 13 |
| 3303 (*ptr-3*) | 15 | 13 |

[a] Proteolytic activities against auto α were assayed using extracts before and after a 10-min heating treatment at 49°C in 5 mM $MgCl_2$, 5% glycerol, 10 mM Tris-hydrochloride, pH 7.2.

[b] Relative to the proteolytic activity of the untreated 7009 extract.

gels containing the extracts were sliced into many pieces, each of which was assayed for activity. The results (Fig. 2) showed that the protease III activity of *ptr-3* and *ptr-2* are virtually obliterated and the activity of *ptr-1* is highly temperature sensitive. By the use of greater quantities of extract it was possible to demonstrate that mutant *ptr-3* and *ptr-2* had no detectable protease III activity left. Because of noise in the assay system we can only say with confidence that these mutants had less than about 5% of the wild-type level of protease III. Approximately one-half of the 15% residual auto $\alpha$ inactivating activity in *ptr-2* or *ptr-3* extracts was associated with the 100,000 × *g* pellet. This activity is probably different from the protease III because, in the *ptr-1* strains, only the protease III activity was heat labile, whereas its 100,000 × *g* pellet-associated activity was as stable as that from the wild-type strains. The rest of the residual activity in the 100,000 × *g* soluble fraction from *ptr* strains was probably from other proteases, although the possibility that it was due to protease III in altered forms cannot be ruled out.

**Map position of *ptr*.** The map position of *ptr* is shown in Fig. 3, together with the sites of markers used in its mapping. Preliminary crosses with an HfrH derivative of *ptr-3* (3304) indicated that *ptr* entered later than *nalA*. A crude time of entry test with HfrP4X, using a *ptr-3* $Str^s$ $F^-$, selecting for streptomycin resistance, and then screening for protease III, showed that *ptr* enters later than streptomycin, at about 60 min. The fact that *ptr* could be transferred by HfrKL16 in short-mating-time crosses indicated that it was before 61 min. The low frequency of transfer in these crosses suggested that *ptr* was being excluded by linkage to the origin of HfrKL16. These preliminary suppositions were confirmed by P1 transduction of *ptr* with selection for *lysA* at 61 min, *argA* at 60 min, and *cysC* at 59 min. Both *ptr-3* and *ptr-1* were closely linked to *argA* (70%), somewhat more weakly linked to *lysA*

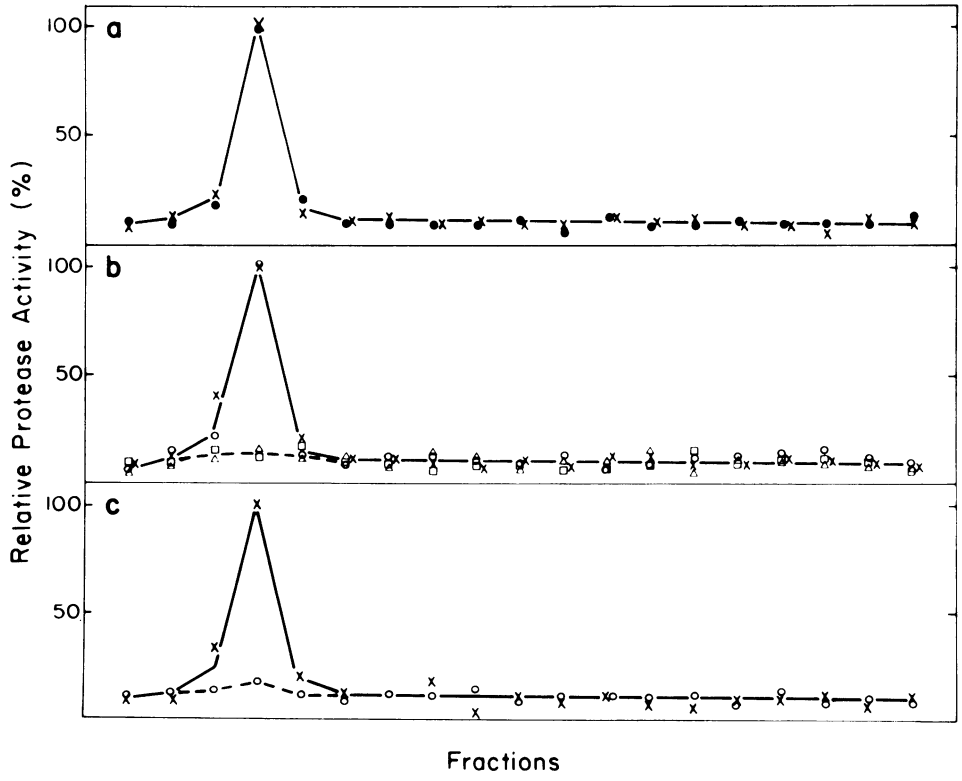


FIG. 2. *Recovery of protease III activity from native gels. Extracts from wild-type or ptr mutants at a protein concentration of 2 mg/ml were applied to polyacrylmide gels, pH 7.2 (18) and run at 4°C. At the end of the electrophoresis, the gel slabs were sliced into 3-mm pieces. They were extracted with 5 mM $MgCl_2$–10 mM Tris-hydrochloride, pH 7.2, and the extracted protease activities against auto α were measured (4). (a) Extract of 7009 (×) and purified protease III (●); (b) extracts of 7009 (×), 1901 (○), 2201 (△), and 3303 (□); (c) heated extracts (49°C, 10 min) of 7009 (×) and 1901 (○).*

(25%), and barely linked to *cysC* (see Table 3). This clearly puts *ptr* at 60 min on the recalibrated linkage map of *E. coli* K-12 (1). The fact that the protease III of *ptr-1* was heat sensitive at 49°C indicated that this locus is the structural gene for protease III.

**Phenotype of *ptr* mutants.** Many abnormal proteins are rapidly degraded in vivo in *E. coli*. Of particular interest and quite typical are the termination fragments produced by nonsense mutations. In the *Z* gene of the *lac* operon, for example, the products of virtually all nonsense mutations are rapidly degraded. To determine whether *ptr* mutants affected this degradation, we transferred several *Z* gene nonsense mutations to a *ptr-3* strain and measured the rate of nonsense fragment degradation by the auto α complementation methods described in detail elsewhere (9). The half-lives of NG521 fragment were found to be 6 min at 37°C in *ptr-3* as well as in its isogenic *ptr*$^+$ strains. Similarly, identical half-lives of NG545 fragment (1 min) were found among *ptr-3*, *ptr-2*, and their isogenic *ptr*$^+$ strains.

It had previously been shown that mutations at the *lon* locus of *E. coli* greatly slowed the rate of nonsense fragment degradation (6). We have found that *lon* mutants have wild-type levels of protease III. To see whether the residual degradation of nonsense fragments that remained in *lon* mutants was affected by the level of protease III, a strain was construced that is *lon ptr-3 lacZ545*. The rate of NG545 nonsense fragment degradation in this strain was found to be identical to an isogenic *ptr*$^+$ *lon* strain with a half-life of 7 min at 37°C.

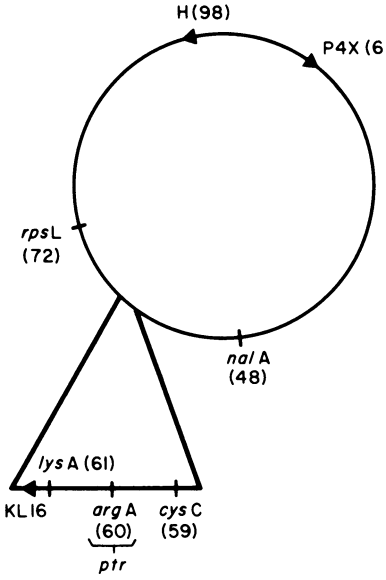


FIG. 3. *Map position of ptr.*

TABLE 3. *Cotransduction of ptr with auxotrophic markers*[a]

| Donor strain | Selected marker | No. scored | Cotransduction (%) |
|---|---|---|---|
| 3303 | *cys*$^+$ | 24 | 2 |
| | *arg*$^+$ | 96 | 72 |
| | *lys*$^+$ | 24 | 25 |
| 1901 | *cys*$^+$ | 96 | 4 |
| | *arg*$^+$ | 96 | 68 |
| | *lys*$^+$ | 96 | 23 |

[a] The recipient strain used was 4529X.

## DISCUSSION

In this manuscript, we report the isolation and characterization of *ptr* mutants using brute-force screening techniques. Three *ptr* mutants, one temperature sensitive and two with less than 5% wild-type protease III activity, have been isolated among 2,500 heavily mutagenized clones. All of the three mutations are mapped at a single site at 60 min on the recalibrated linkage map of *E. coli*. This site is almost certain to be the structural gene for protease III.

The screening procedure developed for protease III was based on the knowledge that the activity of protease III is the major enzyme in the extract of *E. coli* to degrade auto α polypeptides. Even though the auto α preparation used consists of many different polypeptides, the majority of the α-complementing activity is due to only two fragments with molecular weights of 5,200 and 4,500 (unpublished data). Both polypeptides are highly susceptible to protease III cleavage (4). Because of the rapid cleavage of auto α by protease III and the ease of detecting auto α activity, this screening procedure has been successful in isolating the *ptr* mutants.

The screening procedure used in this study is in fact an assay for protease III under fixed conditions. Modified reaction conditions can be used for the screening for other protease mutations. For example, the residual auto α inactivating activity of *ptr* extracts is quite obvious (Fig. 1, comparing the wells of *ptr* and the well with no extract). It is possible to exaggerate the residual activity in *ptr* by decreasing auto α activity added or by increasing reaction time. These altered schemes can, therefore, be used to screen for secondary mutations from a *ptr* strain. Furthermore, auto α polypeptides with molecular weights greater than 10,000 are not degradable by protease III (4). These α-polypeptides can be easily enriched by their insolubility in 2% acetic acid (4). We have found that the extracts of *E. coli* do contain activity to degrade these

α-polypeptides (unpublished data). α-Polypeptides of this class are therefore likely to be useful for biochemical as well as genetic studies for other *E. coli* proteases.

Regnier and Thang (16) reported that proteolytic activity against $^{125}$I-casein can be detected in every subcellular fraction of *E. coli*. Recently, Murakami et al. reported an ATP-stimulating proteolytic activity in the 100,000 × *g* soluble fraction of *E. coli* extracts (K. Murakami, R. Voellmy, and A. L. Goldberg, J. Biol. Chem., in press). A number of publications have indicated that an endoproteolytic activity is associated with the membrane fraction of *E. coli*, and it may be involved in the specific cleavage of signal peptides from certain precursor molecules (3, 7). Very little is now known about these enzymes. Our study using *ptr* strains indicated that the 100,000 × *g* pellet-associated auto α inactivating activity is probably due to a protease different from protease III. It is also likely that enzymes different from protease III are present in the 100,000 × *g* soluble fractions.

So far four proteases have been purified from *E. coli*. They are proteases I, II, and III and *recA* coded protease. The protease I and the protease II are most active against ester substrates but only weakly active against protein substrates (14, 15). The *recA* protease appears to be responsible for the cleavage of λ repressor (17). Our study in protease III indicated that it is undoubtedly an endoproteolytic enzyme (4). Obviously, many more proteases are present in *E. coli*. For the study of these proteolytic systems, mutants like *ptr* missing a major proteolytic activity are likely to be very useful.

We have tested the effect of *ptr* mutations on the degradation rates of two nonsense fragments, NG545 (Mr=18,000) and NG521 (Mr=73,000) (12, 19). The degradation rates of these fragments in *ptr* strains are identical to the rates in isogenic *ptr*$^+$ strains. This is quite surprising since the *ptr* strains used contain no more than 5% of the wild-type level of protease III activity. It is thus likely that other proteolytic enzymes are responsible for the degradation of these two nonsense fragments. The possibility that the *ptr* strains actually contain sufficient protease III activity in vivo, however, cannot be ruled out.

Our biochemical study on purified protease III indicated that protease III is very active for the degradation of proteins smaller than 7,000 daltons (4). It is therefore likely that protease III is responsible for the degradation of nonsense fragments subsequent to their initial cleavages by other proteolytic enzymes. Alternatively, protease III may be specific for the cleavage of some small but yet undefined substrates in vivo.

## ACKNOWLEDGMENT

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