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Degradation of *Escherichia coli* β-Galactosidase Fragments in Protease-Deficient Mutants of *Salmonella typhimurium*

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The degradation rates of several mutationally generated fragments of *Escherichia coli* β-galactosidase were determined in wild-type strains of *Salmonella typhimurium* and in mutant *Salmonella* strains lacking several proteases and peptidases. Three termination fragments (produced by *lacZ545*, *lacZ521*, and *lacZX90*) and one internal reinitiation (restart) fragment [*lacZπ(1)*] are degraded in wild-type *Salmonella* strains at the same rates observed in wild-type *Escherichia coli* strains. Mutations that lead to loss of peptidases N, A, B, P, and Q or to loss of protease I or II do not affect the decay rates of any of these fragments. In addition, all of these peptidases and proteases are present in *E. coli* mutants carrying *deg* mutations (*deg* mutations are known to stabilize β-galactosidase fragments). Apparently, none of the proteases and peptidases that are currently accessible to direct genetic analysis plays a role in the early steps of the degradation of protein fragments.

Many incomplete polypeptide chains are rapidly and specifically degraded in growing cells (16, 27). Fragments of β-galactosidase produced either by chain termination mutations or by internal reinitiation of translation can be degraded rapidly in growing *Escherichia coli* (5, 8). These unstable fragments are specifically recognized by the cell's degradation system and are degraded much more rapidly than most cellular proteins (5). Fragment degradation probably represents only one facet of a cell's ability to remove nonfunctional proteins of all types without at the same time wastefully destroying functional proteins. Degradation of several types of nonfunctional proteins has been observed in bacteria (4, 24, 25, 32) and in eucaryotic cells (27). Little is known about how such proteins are recognized or about the enzymes that degrade them.

It is known that bacteria contain a variety of intracellular peptide bond-hydrolyzing enzymes (16). Some of these enzymes can hydrolyze exogenously supplied small peptides (15, 18). The same enzymes seem to be involved in starvation-induced protein turnover (C. Yen and C. G. Miller, manuscript in preparation) and to play some role in the degradation of small peptides generated intracellularly during growth (Yen and Miller, in preparation). Two of these enzymes, peptidase N and peptidase P, have been shown to attack protein substrates (7, 30). The similarity between another of these peptidases (peptidase A, aminopeptidase I [29]) and mammalian "leucine aminopeptidase" suggests, but does not prove, that this enzyme also is capable of attacking large protein substrates. Two endoproteases (proteases I and II) that have been purified from *E. coli* (22, 23) and are also present in *Salmonella typhimurium* (14; C. Heiman and C. G. Miller, manuscript in preparation) have no known function. Neither seems to be involved in starvation-induced protein turnover (12, 17; A. L. Goldberg, personal communication). There is evidence that both of these enzymes can attack native proteins (8, 22). Both enzymes are much less active in vitro than the mammalian pancreatic proteases, and some workers have questioned the ability of protease I to attack classical protein substrates (12).

One approach to the problem of understanding the pathway of intracellular degradation of nonfunctional proteins in bacteria involves the isolation and characterization of mutants. Two general types of potentially relevant mutants have been found. One type consists of mutants that lack particular enzymes that might be involved in fragment degradation. Examples of this type are mutant strains of *Salmonella* missing peptidases (15, 18) and two different proteases (17). For these mutants, we know what enzyme is missing but not whether the missing enzyme is involved in the degradation of nonfunctional proteins. Another type of mutant has been isolated from *E. coli* by direct selection for a decreased rate of fragment deg-

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radation (2). The rates of degradation of a variety of β-galactosidase fragments are considerably slower in such *deg*⁻ strains than in the wild type. For these mutants, we know that degradation is affected, but we know nothing about the gene product altered by the *deg* mutation.

It is clearly important for understanding the degradation process to answer the following questions. (i) Can the strains missing particular proteases and peptidases degrade polypeptide fragments normally? (ii) Are the *deg* mutants lacking any of the known proteases and peptidases? The purpose of the work reported in this paper has been to answer these questions.

MATERIALS AND METHODS

Bacterial strains. The principal bacterial strains used in this work are derived from *S. typhimurium* LT2 or *E. coli* K-12 and are described in Table 1. Several of these strains have been made *pro*⁻ (to allow introduction of *E. coli* F′ *pro lac* episomes) by transduction with phage P22 (26) grown on strain JK1302 (11). JK1302 carries a deletion that includes *proAB*, *gpt* (resistance to 8-azaguanine), and *pepD* and can therefore be used to introduce a stable *pro* marker by transduction selecting for 8-azaguanine resistance. F′ *pro lac* episomes were introduced into the *pro*⁻ *Salmonella* strains by plate matings with *E. coli* F′ *pro lac* donors, selecting for Pro⁺ recombinants. (The F′ *pro lac* episomes carrying various *lacZ* mutations were constructed by homogenotization using F′ *pro*⁺ *lac*⁺ episomes and *E. coli* strains carrying the appropriate *lacZ* mutations [20] in their chromosomes.) All *Salmonella* F′ strains were tested by crossing them with a series of F⁻ *E. coli* strains carrying *lacZ545*, *lacZ521*, and *lacZX90*. Lac⁺ recombinants were observed only when the *lacZ* mutation on the episome was different from that in the chromosome of the recipient. Spontaneous Pro⁻ segregants were isolated from several of the *Salmonella* strains. As expected, these segregants had all also lost the ability to revert to Lac⁺.

Kinetics of fragment decay. Overnight cultures of the strains to be tested were diluted in fresh LB broth (19) and grown at 37°C to a density of ~5 × 10^8 cells per ml. Isopropyl-β-D-thiogalactoside (IPTG) was added (final concentration, 5 × 10^{-4} M), and incubation continued for the times indicated. Protein synthesis was stopped by adding chloramphenicol (final concentration, 200 μg/ml). Samples (7.5 to 20 ml) were pipetted onto ice immediately after the chloramphenicol addition and thereafter as appropriate. Auto-α donor activity was measured, as described previously (20), by using an extract of *lacZΔ21* as the α acceptor. (Auto-α is a small N-terminal fragment released from β-galactosidase and fragments of β-galactosidase by autoclaving [20]. It is active as an α donor in in vitro complementation.) X90 fragment decay was followed by sus-

TABLE 1. *Bacterial strains*

Parent strain	Genotype	Origin or reference	F′ *pro lac* derivatives			
			lacZ545 π(1)	*lacZ521*	*lacZX90*	*lac*⁺
Salmonella						
proB25	*proB25*	10	CM81	CM11	CM14	
TN87	*proB25 pepP1 pepQ1*	15	CM80	CM12	CM15	
CM9	*leu-485 supQ302*	See text	CM51	CM24	CM25	CM59
CM8	*leu-485 supQ302 pepN10 pepA1 pepB1*	TN215 (18)	CM50	CM22	CM23	CM58
CM18	*supQ302*	TN416 (17)	CM52	CM27	CM28	CM60
CM19	*supQ302 apeA22*[a]	TN443 (17)	CM53	CM29	CM30	CM61
CM20	*supQ302 apeA39*	TN444 (17)		CM31		
CM39	*tlp-5 supQ302*	tlp-5[b]	CM56	CM42	CM43	CM64
CM44	*supQ302 apeA22 tlp-50*	CM19	CM57	CM45	CM46	CM65
E. coli						
D2-521F	*lacZ521 deg-2 met*⁻ F′*lacZ521*	8049[c]				
D2-545πF	*lacZ545π(1) deg-2 met*⁻ F′*lacZ545π(1)*	8049[c]				
D2-X90F	*lacZX90 deg-2 met*⁻ F′*lacZX90*	8049[c]				
HR545πF	*lacZ545π(1) degR* F′*lacZ545π(1)*	1				
545πF	*lacZ545π(1) trp*⁻ F′*lacZ545π(1)*	1				

[a] *apeA* mutants lack protease I (17, 23).

[b] *tlp* mutants lack protease II (22; Heiman and Miller, in preparation).

[c] These strains were constructed from 8049, a *Δ(pro-lac) recA*⁻ *met*⁻ *deg-2* strain (2). (The *met*⁻ marker was omitted from the description of this strain given in reference 2.) Strain 8049 was crossed with an Hfr carrying *lacZ521*, and Pro⁺ Rec⁺ recombinants were isolated. Both mucoid and nonmucoid recombinants from this cross were tested for the presence of the *deg* mutation. All mucoid recombinants tested were *deg*⁻, whereas all nonmucoid strains were *deg*⁺. One of the mucoid strains (D2-521 *lacZ521 met*⁻ *deg-2*) was saved and infected with F′*lac* episomes carrying other *lacZ* nonsense mutations (20). The homogenotes (D2-521F, D2-545πF, and D2-X90F) used in this paper were isolated from these strains.

pending the pelleted samples in 0.5 ml of sodium dodecyl sulfate sample buffer (13), boiling the solution for 5 min, and subjecting it to electrophoresis on sodium dodecyl sulfate-polyacrylamide slab gels (7.5% acrylamide; 13). Gels were stained with Coomassie brilliant blue by the method of Fairbanks et al. (3). ω-Donor activity was measured as described previously (1, 28), except that an extract of strain 657 (a *lacZX90 lacZ200* double mutant) was used as the ω acceptor. For both auto-α and ω complementation, β-galactosidase activity was assayed as described previously (31).

Detection of peptidase activities after gel electrophoresis. Hydrolytic activity toward peptide substrates was determined by the procedure of Lewis and Harris (14) as described previously (18). Hydrolysis of *N*-acetyl-DL-phenylalanine β-naphthyl ester (Schwarz/Mann) was detected as described by Miller et al. (17). Protease II was detected by its ability to hydrolyze *N*-methyl-*N*-tosyl-L-lysine β-naphthyl ester (Nutritional Biochemicals Corp., Heiman and Miller, in preparation).

RESULTS

Strain construction. To study the effect of *Salmonella* peptidase mutations on the stability of various β-galactosidase fragments, *Salmonella* strains carrying F′ *pro lac* episomes were constructed. These episomes carried *lacZ* mutations leading to the production of a very long termination fragment (*lacZX90*; molecular weight, ~125,000 [20]), a termination fragment of intermediate length (*lacZ521*; molecular weight, ~73,000 [20]), and a short termination fragment (*lacZ545*; molecular weight ~18,000 [21]). The F′ *pro lacZ545* episome also carried the *lacZπ(1)* mutation, which results in production of a large restart fragment (6).

Degradation of *lacZ* termination fragments. Because of its large size, the fragment of β-galactosidase produced as a result of the *lacZX90* nonsense mutation can be easily detected on polyacrylamide gels. Loss of this X90 fragment can be followed by adding chloramphenicol (to stop protein synthesis) to an IPTG-induced culture and, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, observing the intensity of the Coomassie brilliant blue-stained X90 polypeptide. Although the Coomassie brilliant blue staining procedure is not rigorously quantitative, it is clear that in wild-type strains of both *E. coli* and *Salmonella* the X90 polypeptide is lost, with a half-life of about 10 min. Under the same conditions, no decay of wild-type β-galactosidase is seen. Stabilization of the X90 fragment by the *deg-2* mutation (in strain D2-X90F) is easily observed with this technique. This procedure is considerably faster than labeling the fragment with a radioactive amino acid and has been used to study degradation of unstable β-galactosidase proteins produced as a result of missense mutations (32).

The results of these studies (Fig. 1) are as follows. (i) Degradation of the X90 polypeptide occurs in wild-type *Salmonella* at the same rate as in *E. coli*. (Wild-type β-galactosidase is completely stable in wild-type and mutant strains [CM59 and CM61] of *Salmonella* under these experimental conditions.) (ii) The absence of peptidases N, A, B, P, and Q and proteases I

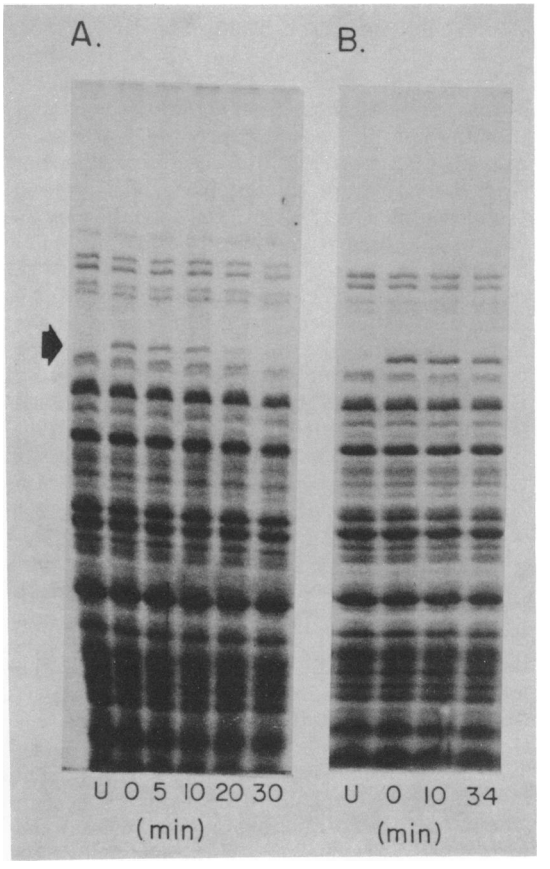

FIG. 1. *Decay of lacZX90 polypeptide and wild-type β-galactosidase. The arrow indicates the position of the X90 fragment (A) or the wild-type β-galactosidase peptide (B). Cultures of CM43 (A) or CM61 (B) growing in LB medium were induced with IPTG for 20 min before the addition of chloramphenicol. Samples were removed at the time of chloramphenicol addition (time 0) and at the later times indicated, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The first well of each series (U) contained a sample of an uninduced culture prepared in the same way except that no IPTG was added. All Salmonella strains and wild-type E. coli strains carrying lacZX90 showed results similar to those for CM43.*

and II in various combinations (Table 1; strains CM14, -15, -23, -25, -28, -30, -43, and -46 were tested) did not affect the degradation of the X90 fragment. We estimate that a difference of a factor of 2 in degradation rates would have easily been seen by these techniques.

The shorter termination fragment produced by *lacZ521* cannot be detected easily on polyacrylamide gels, because many other proteins of approximately the same size obscure the 521 peptide. The amount of the 521 peptide present can be determined, however, because of its ability to generate auto-α, an efficient α donor in in vitro complementation (2, 20). The decay kinetics of the 521 fragment in various strains were followed, therefore, by experimental procedures similar to those used for X90, except that the amount of fragment present was determined by measuring auto-α activity rather than directly from the intensity of a stained protein band. The results of these studies are as follows. (i) Loss of the auto-α activity of the 521 fragment is both qualitatively and quantitatively similar in *E. coli* and *Salmonella*. (ii) No significant differences could be detected in the initial rates of 521 auto-α decay in any of the peptidase- or protease-deficient mutants tested (Fig. 2; strains CM11, -12, -22, -24, -27, -29, -31, -42, and -45 were tested). In all strains, the half-life for this process was approximately 8 ± 2 min. In contrast, the *deg-2* mutation (in strain D2-521F) decreased the rate of decay by a factor of approximately 3 to 5.

In wild-type *E. coli*, the short *lacZ545* termination peptide decays too rapidly for accurate measurement of decay rates ($T_{1/2}$ < 1 min). Some indication of its decay rate can be obtained, however, by simply determining the level of the 545 fragment present after a short (20-min) induction. If the decay rate is slower than in the wild type, the level should increase. This is seen clearly in strains carrying *deg* mutations: the level of the 545 fragment in such strains is five- to ninefold higher than in the wild type. As in the case of the fragment 521, fragment 545 levels were determined by measuring auto-α activity (Table 2). The levels of auto-α from the 545 fragment in all of the *Salmonella* strains are extremely low (less than 1% of the level in a wild-type strain), and in some strains may approach the level of readthrough of the nonsense mutation. For this reason, the absolute values of the levels given in Table 2 are probably not extremely reliable. In several experiments, however, none of the peptidase-deficient strains showed consistently elevated 545 levels. Strains carrying *deg* mutations always contained at least fourfold-higher levels than any *deg*$^+$ strain. We believe it likely that none of the *Salmonella* peptidase or protease mutations affects 545 fragment levels. Be-

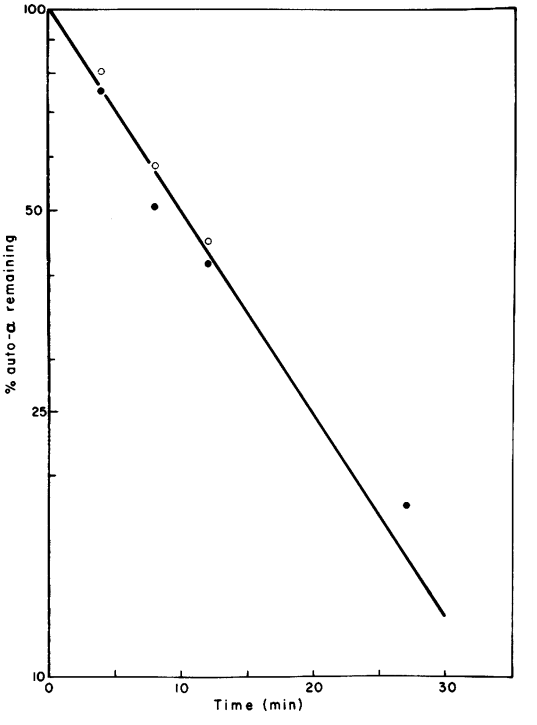

FIG. 2. *Decay of auto-α of lacZ521 in Salmonella typhimurium. Cultures were induced with IPTG for 10 min before the addition of chloramphenicol. Symbols: ●, CM27 (apeA$^+$); ○, CM31 (apeA$^-$).*

TABLE 2. *Auto-α levels in lacZ545 strains*

Strain	Relevant genotype	Auto-α level[a] (A^{420}/min per OD_{550}) × 10^3
CM50	*pepN*$^-$ *pepA*$^-$ *pepB*$^-$	8
CM51	*pep*$^+$ (parent of CM50)	7
CM52	*apeA*$^+$ (parent of CM53, CM57)	8
CM53	*apeA*$^-$	14
CM56	*tlp*$^-$	15
CM57	*apeA*$^-$ *tlp*$^-$	9
HR545πF	*degR* (*E. coli*)	130
D2-545πF	*deg-2* (*E. coli*)	79
545πF	*deg*$^+$ (*E. coli*)	15

[a] Cultures were grown and induced as described in the text. Samples (20 ml) were poured onto ice 20 min after the addition of inducer and assayed for auto-α activity. The amount of cell material present was determined by reading the optical density (OD) of the cell suspension at 550 nm. A_{420}, Absorbance at 420 nm.

cause of the experimental uncertainties in measuring these levels, small (two- to three-fold) effects cannot be excluded, however. Any differences in growth rates between the strains would lead to differences in synthesis rates of the 545 fragment. Doubling times for these strains do not differ by more than 20%, however, so no large effect of the peptidase or protease mutations could be hidden as a result of such differences. Clearly, none of the peptidase mutations has as large an effect as the *deg* mutations.

The data of Table 2 indicate that both the *degR* (1) and *deg-2* (2) mutations lead to increased levels of the 545 termination fragment. This observation was unexpected in light of previous experiments indicating that *degR* stabilized only restart fragments. We have now found that the *degR* mutation (1) not only stabilizes the $\pi(1)$ restart (see Table 3), but also both the X-90 and 521 termination fragments. In addition, the *deg-2* mutation decreases the decay rate of the $\pi(1)$ restart fragment. There is also evidence that both *degR* and *deg-2* map at *lon* (S. Gottesman and D. Zipser, manuscript in preparation).

Degradation of the π(1) restart fragment. A restart fragment produced by the *lacZ*$\pi(1)$ mutation is active as an ω donor in in vitro ω complementation. (ω complementation occurs in vitro when an ω donor, a β-galactosidase molecule or fragment containing a native C-terminal region, is mixed with an ω acceptor, a β-galactosidase molecule with a normal conformation of the N-terminal portion of the molecule but a defective C-terminal region [28]). Its decay can be followed, therefore, by measuring the loss of ω donor activity as a function of time after addition of chloramphenicol to an induced culture. The results of typical experiments are shown in Fig. 3. Decay rates for the various *Salmonella* mutants are presented in Table 3. Again, the *Salmonella* and *E. coli* strains show almost identical decay rates, and the absence of the *Salmonella* proteases does not seem to affect these rates.

TABLE 3. *Half-lives of ω-complementing activity in lacZ545$\pi(1)$ strains*[a]

Strain	Relevant genotype	$T_{1/2}$ (min)
CM51	*pep*$^+$ (parent of CM50)	13
CM50	*pepN*$^-$ *pepA*$^-$ *pepB*$^-$	11
CM52	*apeA*$^+$ (parent of CM53, CM57)	12
CM53	*apeA*$^-$	12
CM56	*tlp*$^-$	11
CM57	*apeA*$^-$ *tlp*$^-$	12
CM81	*pep*$^+$ (parent of CM80)	15
CM80	*pepP*$^-$ *pepQ*$^-$	13
HR545F	*degR*	48

[a] Cultures were grown and induced as described in the text. Samples (20 ml) were poured onto ice 30 min after the addition of inducer and assayed for ω donor activity.

Peptidases and proteases in *deg* mutants. Mutants isolated by selections requiring that the rate of fragment degradation be slowed contain mutations at a locus called *deg* (1, 2). These mutations stabilize all of the β-galactosidase fragments discussed above, as well as other fragments produced by different *lacZ* mutations. One of the simplest hypotheses explaining the phenotype of these mutants would be that they lack some protease or combination of proteases required for hydrolyzing the fragments. Therefore, we set out to determine whether the absence of any proteases or peptidases (16) could be detected in *deg* strains. Ac-

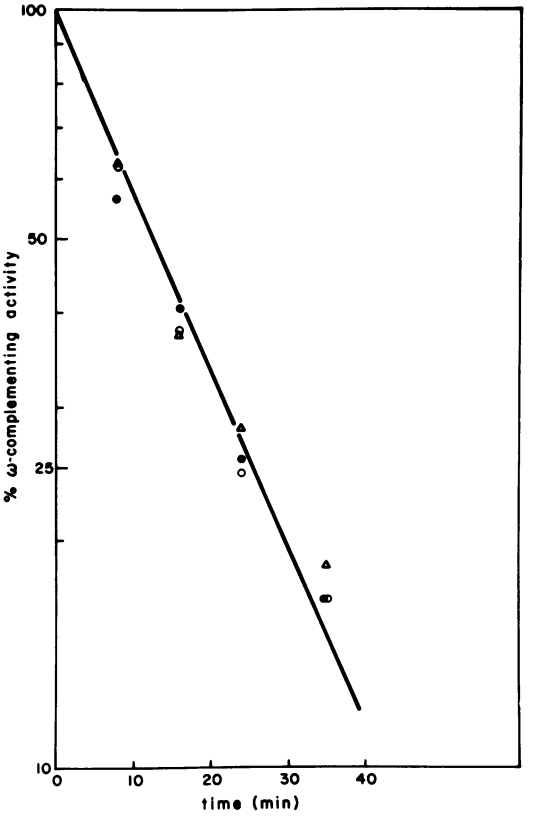

FIG. 3. *Decay of ω-complementing activity from $\pi(1)$ restart fragment. Cultures were induced with IPTG for 30 min before the addition of chloramphenicol. Symbols: ●, CM52 (wild type); ○, CM53 (apeA$^-$; protease I$^-$); △, CM57 (apeA$^-$ tlp$^-$).*

TABLE 4. *Peptidase and protease activities present in both deg mutants and the wild type*

Enzyme activity	Method of detection[a]
Peptidase	
A	Hydrolysis of Leu-Gly and Leu-Gly-Gly
B	Hydrolysis of Leu-Gly-Gly
D	Hydrolysis of Leu-Gly
N	Hydrolysis of Leu-Gly, Leu-Gly-Gly, L-alanyl-β-naphthylamide
Q	Hydrolysis of Leu-Pro
Protease	
I	Hydrolysis of *N*-acetyl-DL-phenylalanine β-naphthyl ester[b]
II	Hydrolysis of *N*-methyl-*N*-tosyl-L-lysine β-naphthyl ester

[a] See Materials and Methods.

[b] Three bands of activity toward this substrate can be detected in both the wild type and *deg* mutants (17, 23).

tivity toward appropriate peptide substrates or chromogenic protease substrates was determined after electrophoresis of crude extracts on nondenaturing polyacrylamide gels. The activities surveyed are shown in Table 4. No differences between the wild-type strain and the *deg-2* or *degR* mutants were observed.

DISCUSSION

The results presented in this paper show that none of the mutations affecting particular proteases or peptidases has any significant effect on the degradation of the β-galactosidase termination or restart fragments tested. This does not necessarily mean that none of these enzymes has any function at all in the degradation pathway. The peptidases might, for example, be required late in the degradation process to degrade small peptide intermediates to amino acids. They seem to play such a role in carbon starvation-induced protein degradation (Miller and Yen, in preparation). It is clear, however, that none of these enzymes is required for the initial inactivation of any of the fragments we have studied.

The failure to detect any qualitative differences in protease patterns between *deg* mutants and wild-type strains obviously does not rule out the possibility that *deg* directly affects some protease activity. Since neither protease I nor protease II seems to be required for growth (17; Heiman and Miller, in preparation) or for starvation-induced degradation, other proteases must be present, and these enzymes could be affected directly or indirectly by *deg* mutations. It is clear, however, that none of the well-characterized enzymes is missing in *deg* mutants.

The similarities between *Salmonella* and *E. coli* in the rates of degradation of β-galactosidase fragments are striking. Both organisms clearly possess highly efficient and apparently very similar systems for removing nonfunctional proteins. Unfortunately, the enzymes that compose these systems remain unknown.

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