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Construction and Characterization of *Escherichia coli* Strains Deficient in Multiple Secreted Proteases: Protease III Degrades High-Molecular-Weight Substrates In Vivo

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Protease III, the product of the *ptr* gene, is a 110-kDa periplasmic protease with specificity towards insulin and other low-molecular-weight substrates (<7,000 molecular weight) in vitro (Y.-S. E. Cheng and D. Zipser, J. Biol. Chem. 254:4698–4706, 1979). *Escherichia coli* strains deficient in protease III were constructed by insertional inactivation of the *ptr* gene. This mutation did not appear to affect the function of the adjoining *recB* and *recC* genes. Expression of protein A–β-lactamase, a protease-sensitive secreted polypeptide, was increased approximately twofold in *ptr* cells. A comparable increase in the half-life of protein A–β-lactamase was observed by pulse-chase experiments, suggesting that protease III is involved in the catabolism of high-molecular-weight substrates in vivo. *ptr* mutants exhibited no detectable phenotypic alterations except for a slight reduction in growth rate. When the *ptr* mutation was transferred to a strain deficient in the secreted protease DegP, a further decrease in growth rate, as well as an additive increase in the expression of the fusion protein, was observed. A *ptr degP ompT* mutant strain resulted in a further increase in expression in minimal medium but not in rich medium.

One of the major problems associated with the expression of heterologous polypeptides in *Escherichia coli* is the degradation of cloned gene products by host-specific proteases (5). At least 25 proteases and peptidases have been identified in different cellular compartments of *E. coli* (25, 28). Although most of them have been biochemically characterized, our knowledge of protein catabolism, and particularly of the physiological role of proteases, is still incomplete. In the cytoplasm, most proteins are probably degraded via an energy-dependent pathway similar to that observed in eukaryotic cells. Two ATP-dependent proteases, La and Clp, have been isolated and their role has been studied in detail (for a review, see reference 18). In addition, several proteins (e.g., oxidatively damaged glutamine synthetase) are degraded via an energy-independent process (26, 33). There is considerably less information regarding the protein turnover pathways in the cell envelope. In 1982, Talmadge and Gilbert showed that the half-life of proinsulin is increased 10 times when the protein is secreted into the periplasmic space (41). This result suggested that the rate of protein turnover in the periplasmic space is lower than in the cytoplasm. However, several recent studies have demonstrated that severe proteolysis of heterologous proteins can also occur in the periplasmic space (1, 4, 15, 19).

At present, seven proteases associated with the cell envelope of *E. coli* have been isolated. Four of these, OmpT (38, 39), DegP (36, 37), protease III (13, 14), and protease IV (21, 22), have been cloned and characterized. To elucidate the role of different cell envelope proteases in the catabolism of secreted proteins, we have investigated the degradation of a hybrid protein constructed by in-frame fusion of the genes coding for *Staphylococcus aureus* protein A and the *E. coli* enzyme TEM–β-lactamase. Although protein A–β-lactamase is fully bifunctional, indicating that both domains are folded properly, it is rapidly degraded both in vivo (3) and in vitro (16). In contrast, the native protein A and β-lactamase are stable proteins in *E. coli* and are resistant to trypsin degradation in vitro (16). We have previously reported that proteases DegP and OmpT are involved in the degradation of this model polypeptide substrate in *E. coli* (4). The fusion protein is stabilized by about fivefold in a *degP ompT* double mutant, and the effect of the two mutations on the production of protein A–β-lactamase is approximately additive (4).

E. coli protease III, the *ptr* gene product, is a monomeric 110-kDa metalloprotease which rapidly degrades oxidized insulin B-chain and preferentially cleaves autoclaved fragments of β-galactosidase of molecular weight less than 7,000 (9). However, in vitro experiments failed to detect activity against higher-molecular-weight substrates. Interestingly, the *ptr* gene is located at 61 min on the *E. coli* genetic map, flanked by the genes coding for the RecC and RecB subunits of exonuclease V (13). The protein is synthesized as a precursor containing a typical 23-amino-acid signal sequence. The 3′ end of the *ptr* gene overlaps the reading frame of *recB*, and it has been suggested that these genes may be part of an operon (14). Furthermore, a sequence that is homologous to the nitrogen-regulated promoter consensus sequence was identified downstream from the −35 and −10 regions, raising the possibility that expression of protease III is regulated by tandem promoters (10). A 50-kDa polypeptide, p50, which is probably a stable degradation product of protease III, has been detected in the periplasmic space of maxicells. p50 does not possess proteolytic activity against insulin, and the physiological role of this polypeptide has not been established (12). No detectable phenotypic alterations were identified by Dykstra and Kushner (12) in cells lacking or overexpressing *ptr*. The physiological role of protease III is still unknown.

In this article we show that protease III is involved in the in vivo degradation of at least one high-molecular-weight substrate, the protein A–β-lactamase fusion protein. The effect of mutations in *ptr* and *degP* on the expression of the fusion protein is approximately additive in both rich and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics[a]	Source or reference
E. coli		
D301	RP487 *recD1903* Δ(*lacIZYA*-U169)	C. B. Russell
JC7729	K-12 *recB21 sbcB15 his-327 leu*(Am) *trpE9829 lac str-321 thi*	E. B. Goldberg
KS272	F⁻ Δ*lacX74 galE galK thi rpsL* (*strA*) Δ*PhoA*(*Pvu*II)	36
KS474	KS272 *degP41*(Δ*Pst*I-Kan^r)	37
SF100	KS272 Δ*ompT*	4
SF101	D301 *ptr-32*::ΩCm^r	This study
SF103	KS272 *ptr-32*::ΩCm^r	This study
SF110	KS272 Δ*ompT degP41*(Δ*Pst*I-Kan^r)	4
SF115	KS272 *ptr-32*::ΩCm^r *degP41*(Δ*Pst*I-Kan^r)	This study
SF120	KS272 *ptr-32*::ΩCm^r *degP41*(Δ*Pst*I-Kan^r) Δ*ompT*	This study
Plasmids		
pACYC184	A 4.2-kbp medium-copy-number plasmid; Cm^r Tc^r	7
pCDK3	pBR325 derivative carrying a 19-kbp *Bam*HI fragment mapping (*thyA-argA*)	12
pCS1	pUC19 derivative carrying a 3.2-kbp *Pvu*I SpA-bla fragment from pFB3; Ap^r	4
pFB3	A 9.86-kbp pBR322 derivative carrying SpA-bla; Ap^r Kn^r	3
pFB5	pCS1 derivative carrying an 8-kbp *Sal*I fragment encoding *ptr*; Ap^r	This study
pFB6	pFB5 derivative (*ptr-32*::ΩCm^r); Ap^r Cm^r	This study

[a] Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance; Tc^r, tetracycline resistance; SpA-bla, protein A–β-lactamase.

minimal media. In contrast, while inactivation of *ptr*, *degP*, and *ompT* does not enhance the production of protein A–β-lactamase in LB medium, a cumulative increase in the expression of the fusion protein was observed in M9 medium.

MATERIALS AND METHODS

Strains, plasmids, bacteriophage, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteriophage T4 gene *2* mutant (amN51) was a generous gift from E. Goldberg. Cultures were grown in LB medium (Difco) supplemented with 0.2% glucose and the appropriate concentrations of antibiotics. M9 medium was supplemented with 0.2% casein amino acid hydrolysate, 0.2% glucose, and the desired antibiotics. W salts medium was described by Smith et al. (35) and consists of 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, and 0.102 g of $MgSO_4 \cdot 7H_2O$ per liter of solution containing 0.2% L-glutamine, 0.2% glucose, and 0.2% $(NH_4)_2SO_4$. Ampicillin, tetracycline, kanamycin, and chloramphenicol were added to the growth medium as required at 50, 25, 50, and 20 μg/ml (final concentrations), respectively.

Enzymes and chemicals. Restriction and modifying enzymes were purchased from Boehringer-Mannheim, New England Biolabs, and Promega. All recombinant DNA procedures were performed by the method of Maniatis et al. (27) or Ausubel et al. (2). Oxidized insulin B-chain and penicillin G were obtained from Sigma. All chemicals used were of biological grade.

Strain constructions. The strategy used to construct the *ptr* mutants is outlined in Fig. 1. Plasmid pCDK3, which contains a 19-kbp insert carrying the *thyA-argA* region of the *E. coli* genome, was digested with *Sal*I and *Bam*HI. The 8-kbp

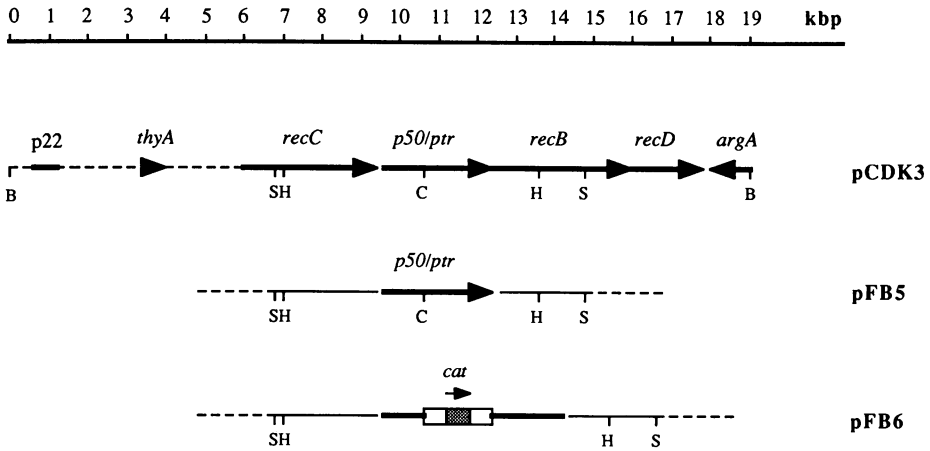

FIG. 1. Construction of the *ptr-32*::ΩCm^r mutation. An 8-kbp *Sal*I fragment from pCDK3 was cloned into the *Sal*I site of plasmid pCS1 to generate pFB5. A 1.7-kbp *Hin*cII-*Xmn*I DNA fragment from pACYC184 which contains a functional chloramphenicol gene (shaded box) was treated with Klenow fragment and ligated into the unique *Cla*I site of pFB5 to yield pFB6. The mutation was transferred to the *E. coli* genome by transforming the *recD* strain D301 with the 9.7-kbp *Sal*I fragment from pFB6 (see text). Only the relevant portions of the plasmids are shown. Restriction enzyme site abbreviations: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; S, *Sal*I.

*Sal*I fragment was isolated by using low-melting-point agarose and ligated into the unique *Sal*I site of pCS1 to yield plasmid pFB5. pFB5 contains a unique *Cla*I site, located approximately 1,000 bp downstream from the start codon of the *ptr* gene. A functional chloramphenicol acetyl transferase (*cat*) gene was obtained in a 1.7-kbp *Hin*cII-*Xmn*I fragment from plasmid pACYC184. Plasmid pFB5 was digested with *Cla*I, treated with Klenow fragment, and ligated to the 1.7-kbp fragment. Transformants were selected on chloramphenicol plates and tested for ampicillin resistance. The structure of the resulting plasmid, pFB6, was confirmed by restriction analysis.

Plasmid pFB6 was digested with *Sal*I, and the 9.7-kb fragment was isolated. Competent D301 cells were transformed with about 1.5 μg of the 9.7-kbp linearized DNA and plated on chloramphenicol plates. To ensure that no intact pFB6 had been cotransformed, the transformants were tested for ampicillin sensitivity. A Cm^r Tc^r Ap^s colony, SF101, was selected for further studies. The mutation was designated *ptr-32*::ΩCm^r. P1 transduction was used to introduce *ptr-32* into KS272, KS474, and SF110, generating SF103, SF115, and SF120, respectively. Transductants were scored for chloramphenicol resistance and tetracycline sensitivity to select for cells containing an intact *recD* gene, i.e., having exonuclease V activity. The strains carrying the *ptr* mutation grew well in rich and minimal media.

Southern blots. Southern blots were performed essentially as described by Ausubel et al. (2). Genomic DNA was isolated as described (2), separated in 0.8% agarose gels, and transferred overnight to nitrocellulose (Schleicher & Schuell BA85). DNA was cross-linked to nitrocellulose by baking for 2 h at 80°C in a vacuum oven. Nonradioactive digoxigenin-11-dUTP probes were prepared by using the Genius system (Boehringer Mannheim) according to the instructions of the manufacturer.

Pulse-chase experiments. Cells were grown in 25 ml of labeling medium supplemented with 19 amino acids but no methionine (17) to mid-exponential phase (optical density at 600 nm [OD_{600}], 0.5). [^{35}S]Met (100 μCi) was added to the growth medium. After 5 min, 1 ml of a 0.5-mg/ml solution of cold methionine was added, and a zero point was taken immediately. At specified times after initiation of the chase, 1-ml samples were transferred to microcentrifuge tubes kept on ice and containing 50 μl of a 34-mg/ml solution of chloramphenicol. The cells were centrifuged, washed with 1 ml of 10 mM Tris HCl, pH 8.0, resuspended in lysis buffer (10 mM Tris HCl [pH 8.0], 1% SDS, 1 mM EDTA), and boiled for 3 min. Immunoprecipitation was performed as described previously (4). Autoradiograms were scanned with a prototype digital Clayton video densitometer developed at The University of Texas at Austin by L. Poulson.

Penicillinase and insulin degradation assays. Samples (3 ml) from cultures grown as specified were centrifuged at 8,000 × *g* for 8 min, and the pellets were resuspended in 3 ml of 50 mM potassium phosphate, pH 6.5, and disrupted by use of a French press at 20,000 lb/in^2. The insoluble fraction was removed by centrifugation. Penicillinase activities were measured by spectrophotometry at 240 nm as previously described (3) by using a 0.5-g/liter penicillin G solution in 50 mM potassium phosphate, pH 6.5, as a substrate.

For the insulin assays, cells were grown overnight in LB medium supplemented with 0.2% glucose and the appropriate concentrations of antibiotics as required. Osmotic shock fractions were collected by the method of Nossal and Heppel (29) and concentrated by ultrafiltration with Amicon Centricon-10 microconcentrators. Periplasmic proteins (30 μg) were mixed with 100 mM ammonium bicarbonate buffer, pH 8.4, and 250 μg of oxidized insulin B-chain was added. The final volume was 500 μl. Samples were incubated for 3.5 h at 37°C. Aliquots (20 μl) (corresponding to an initial insulin concentration of 10 μg) were boiled in loading buffer to stop the reaction and loaded onto a 22% polyacrylamide gel. Purified insulin (10 μg) was used as a control.

General methods. For Western blots (immunoblots), 1-μg samples of periplasmic proteins were separated on a 15% polyacrylamide gel and transferred electrophoretically to nitrocellulose (4). Immunological detection was performed by using β-lactamase antiserum at a 1:1,250 dilution. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (24) in 15 or 22% gels. Protein concentrations were determined by a protein assay (Bio-Rad) with bovine serum albumin as a standard. Doubling times were determined by growing cells in 75 ml of LB medium containing 0.2% glucose but no antibiotics. During the exponential phase, samples were taken every 15 min and the OD_{600} was measured. Doubling times correspond to the slope of the natural logarithm of OD_{600} versus time.

RESULTS

Characterization of *ptr* mutants. Chaudhury and Smith (8) previously obtained *E. coli* mutants containing large deletions in the *ptr* gene which extended to the *recB* and *recC* genes (8). However, the absence of a functional exonuclease V activity affects cell viability and growth rates and makes *E. coli* sensitive to DNA-damaging agents (6, 8, 23). Furthermore, *recB* or *recC* mutations affect plasmid stability and consequently the synthesis rate of plasmid-encoded genes (20, 34). Preliminary studies on the effect of the *ptr* deletion on the degradation of the model substrate protein A–β-lactamase were inconclusive (data not shown). To investigate the possible degradation of the fusion protein by protease III and to compare its role with that of other cell envelope proteases, we constructed a *ptr* mutant in which the exonuclease V genes are unaffected. The *ptr-32*::ΩCm^r mutants were obtained as described in Materials and Methods, and their construction is illustrated in Fig. 1.

Southern blotting was used to demonstrate the insertion of the chloramphenicol cartridge in the *ptr* gene of strains SF103, SF115, and SF120 (Fig. 2). Briefly, a 1.7-kbp chloramphenicol fragment was recovered from pACYC184 digested with *Hin*cII and *Xmn*I and labeled with digoxigenin-11-dUTP by following the instructions of the manufacturer. Genomic DNA was digested for 7 h with *Hin*dIII and *Cla*I, separated on an 0.8% agarose gel, and transferred overnight to nitrocellulose. Figure 2 shows that the chloramphenicol probe hybridized with SF103, SF115, and SF120 DNA (lanes 2 to 4) but not with the parental KS272 DNA (lane 1). Since the *Cla*I site in the *ptr* gene is destroyed by insertion of the chloramphenicol cartridge, *Hin*dIII-plus-*Cla*I digestion of *ptr* mutant genomic DNA should yield an 8.68-kbp fragment carrying chloramphenicol resistance. As expected, the chloramphenicol probe hybridized to a fragment of approximately 8.5 kbp, suggesting correct insertion of the chloramphenicol gene. In addition, plasmid pCDK3 was digested with *Hin*dIII and *Cla*I, and the two DNA fragments of approximately 3.5 kbp were isolated, labeled with digoxigenin-11-dUTP, and hybridized to *Hin*dIII-plus-*Cla*I-digested genomic DNA. Both probes hybridized with an 8.5-kbp fragment of SF101, SF103, SF115, and SF120 DNA and a 3.5-kbp fragment of KS272 wild-type DNA (data not shown).

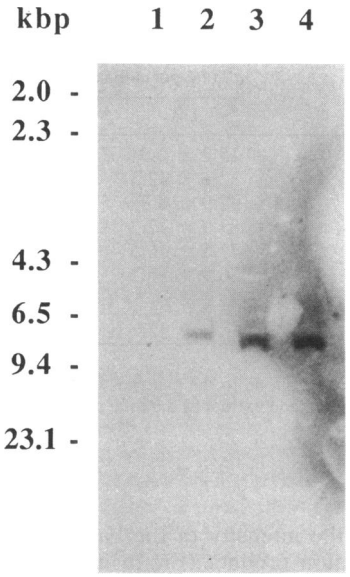

FIG. 2. Southern blot analysis of genomic DNA digested with *Hin*cII and *Cla*I. The probe was a 1.7-kbp *Hin*cII-*Xmn*I fragment from pACYC184 containing the chloramphenicol gene and labeled with digoxigenin-11-dUTP. Lanes: 1, KS272; 2, SF103; 3, SF115; 4, SF120.

Protease III is the only cell envelope protease which has been reported to rapidly degrade insulin. To delineate the role of the chloramphenicol gene insertion in the function of protease III, the following assay was developed. Strains KS272, SF103, SF110, and SF120 were grown in LB medium supplemented with glucose and the appropriate antibiotics for 24 h. The periplasmic fraction of the cells was collected by the method of Nossal and Heppel (29) and concentrated by ultrafiltration. Periplasmic proteins were incubated with oxidized insulin B-chain, and aliquots of the mixture were resolved by SDS-PAGE as described in Materials and Methods. Figure 3 shows that no appreciable degradation was observed with SF103 or SF120 (lanes 3 and 5). In contrast, no intact insulin could be detected after incubation with the

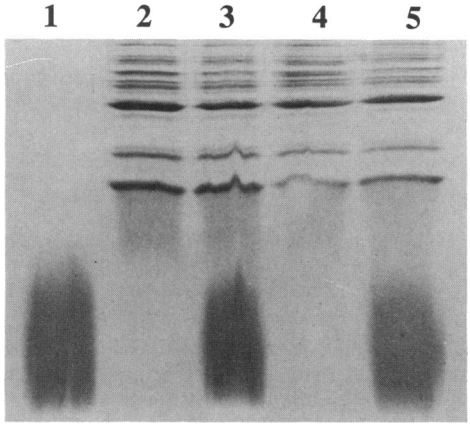

FIG. 3. Insulin degradation assays. The osmotic shock fractions of different strains were incubated with insulin as described in Materials and Methods. Aliquots corresponding to an initial insulin concentration of 10 μg were loaded on the gel. Lanes: 1, 10 μg of purified insulin control; 2, KS272; 3, SF103; 4, SF110; 5, SF120.

osmotic shock fractions of the isogenic strains KS272 and SF110 (lanes 2 and 4). Similar results were obtained with strain SF115 (data not shown). These results indicate that, even though transcription and translation of the first 1,000 bp of the *ptr* gene should be unaffected in cells carrying the chloramphenicol cartridge insertion, the resulting (putative) translation product does not possess any insulin-degrading activity.

E. coli cells containing a defective RecBCD (exonuclease V) enzyme are fully permissive for bacteriophage T4 gene *2* mutants (T4 *2*). However, T4 *2* is unable to infect cells containing an intact exonuclease V activity. Presumably, the gene *2* product prevents nucleolytic degradation of the T4 DNA by the RecBCD enzyme (30). Hence, infection with T4 *2* is an easy way to test for the RecBCD phenotype. Strains JC7729, KS272, SF103, SF115, and SF120 were infected at different multiplicities of infection with T4 *2* (amN51) as described previously (42). Although T4 *2* formed numerous plaques on the *recB* strain JC7729 (the titer of the phage was about 3×10^9 PFU/ml on JC7729), none were obtained on KS272, SF103, SF115, and SF120, indicating the presence of a fully functional exonuclease V. Furthermore, streaks of *ptr* cells exposed to 1.1 mW of UV radiation per cm^2 for up to 100 s did not display lower viability than unexposed control cells.

Degradation of protein A–β-lactamase by protease III. Protease III has been shown to preferentially degrade low-molecular-weight substrates, but its activity on large proteins has not been established (9). Plasmid pFB3 encodes a proteolytically sensitive fusion protein which is degraded by proteases DegP and OmpT (3, 4). Since degradation occurs mainly within the β-lactamase domain of the fusion protein, the total penicillinase activity is directly proportional to the amount of intact hybrid protein present in the cell (3). To examine the effect of protease III on the stability of protein A–β-lactamase, the *ptr* mutant strain SF103 was transformed with plasmid pFB3. Shake-flask cultures were grown for 24 h in LB or M9 medium as described in Materials and Methods. A twofold increase in penicillinase specific activity was observed in SF103 relative to the parental strain KS272, suggesting that protease III hydrolyzed the fusion protein (Table 2).

Plasmids pFB5 and pFB6 carry the pUC origin of replication and are maintained in high copy number in the cells. These plasmids are identical except for the presence of the chloramphenicol insert in the *ptr* gene of pFB6 (Fig. 1). When cells harboring pFB5 or pFB6 were grown in rich medium, a large fraction of the penicillinase activity was released into the extracellular fluid. Presumably, the overexpression of two secreted proteins (protein A–β-lactamase and protease III) causes alterations in the outer membrane permeability. Under these conditions, protein A–β-lactamase is no longer susceptible to the proteolytic activity of periplasmic proteases. Therefore, overnight cultures were grown in M9 medium since under these conditions release into the medium was markedly reduced. A 2.5-fold decrease in total penicillinase activity of cells overexpressing protease III was observed (Table 2), indicating that the *ptr-32*::ΩCm^r mutation can be complemented by a plasmid-encoded *ptr* gene.

The stability of protein A–β-lactamase in the *ptr* mutant SF103 and the *ptr degP* mutant SF115 was determined by pulse-chase experiments. Cultures were labeled with [^{35}S]Met for 5 min, and samples were collected for 2 h after addition of unlabeled methionine. Protein A–β-lactamase was immunoprecipitated, separated by SDS-PAGE, and

TABLE 2. Characteristics, growth rates, and total penicillinase specific activities of bacterial strains harboring different plasmids coding for the protein A–β-lactamase fusion protein

Strain (plasmid)	Strain characteristics[a]	Growth rate (per h)[b]	Total penicillinase sp act (U/mg) ± SD[c] in:	
			LB	M9
KS272(pFB3)[d]	*ompT*$^+$ *degP*$^+$ *ptr*$^+$	1.66	15.2 ± 2.9	0.6 ± 0.2
SF103(pFB3)	*ompT*$^+$ *degP*$^+$ *ptr*	1.41	32.6 ± 12.5	1.2 ± 0.1
KS474(pFB3)[d]	*ompT*$^+$ *degP ptr*$^+$	1.28	47.9 ± 8.3	2.4 ± 0.2
SF110(pFB3)[d]	*ompT degP ptr*$^+$	1.16	87.9 ± 17.2	4.8 ± 0.4
SF115(pFB3)	*ompT*$^+$ *degP ptr*	1.17	77.5 ± 16.5	3.7 ± 0.2
SF120(pFB3)	*ompT degP ptr*	1.07	86.9 ± 10.1	6.4 ± 0.8
SF103(pFB5)	*ompT*$^+$ *degP*$^+$ *ptr*			4.1 ± 0.3
SF103(pFB6)	*ompT*$^+$ *degP*$^+$ *ptr*			10.5 ± 1.1

[a] The *ptr* mutation in SF103 is complemented by plasmid pFB5.
[b] Growth rates were calculated with plasmid-free cells as described in Materials and Methods and correspond to the averages of two experiments.
[c] Total penicillinase specific activities correspond to the ratios of the sum of activities in the supernatant and extract to the total protein concentration in these fractions. The SD is expressed in units per milligram and is deduced from at least six experiments.
[d] Growth rates and penicillinase specific activities in LB medium are from Baneyx and Georgiou (4).

detected by autoradiography. The half-life of protein A–β-lactamase was about 120 min in strain SF103 compared to about 30 min in the parental strain KS272 (Fig. 4). Additional stabilization was observed in strain SF115, where the half-life of the fusion protein was approximately 180 min. Furthermore, protein A–β-lactamase was not degraded in strain SF103 or SF115 for 30 min after the initiation of the chase.

The pattern of stable degradation products in different strains was characterized by Western blotting. The cell envelope mutants KS474 (*degP*), SF100 (*ompT*), SF103 (*ptr*), SF115 (*degP ptr*), and SF120 (*degP ompT ptr*) were transformed with pFB3 and grown to early stationary phase (OD_{600}, 1.4) in LB medium. The cells were subjected to osmotic shock, and exactly 1 μg of periplasmic proteins was electrophoresed, transferred to nitrocellulose, and immunodetected with β-lactamase antiserum. While *ompT* (Fig. 5, lane 1) and *degP* (Fig. 5, lane 3) mutants exhibited similar patterns of proteolysis products, inactivation of protease III resulted in the appearance of additional bands that cross-reacted with β-lactamase antiserum (Fig. 5, lanes 2 and 4).

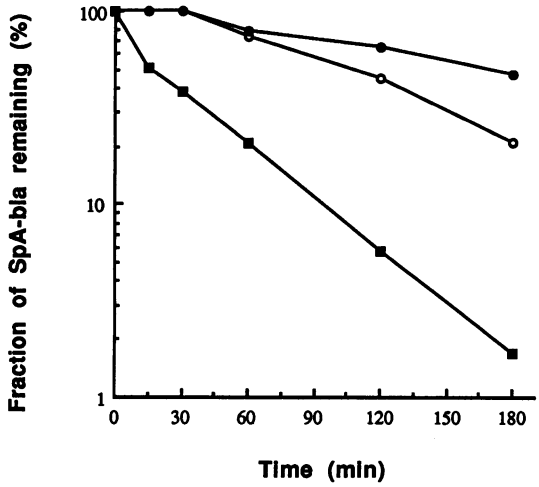

FIG. 4. Pulse-chase analysis of the stability of protein A–β-lactamase in the wild-type strain KS272 (■), the *ptr* mutant SF103 (○), and the *degP ptr* mutant SF115 (●). The fraction of protein A–β-lactamase (SpA-bla) remaining corresponds to the intensity of the band at the specified time divided by the intensity at time zero as determined by scanning of the X-ray film.

Furthermore, the intensity of the band corresponding to a major degradation product (Fig. 5, arrow 1) was lower in SF103 than in either SF100 or KS474. Finally, two low-molecular-weight degradation products (Fig. 5, arrows 2 and 3) which were evident in both *ompT* and *degP* mutants were not detected in SF103, SF115, and SF120.

To gain further insight on the role of different cell envelope proteases in the stability of protein A–β-lactamase, *ptr degP* (SF115) and *ptr degP ompT* (SF120) cells were transformed with pFB3 and grown for 24 h in LB or M9 medium. The penicillinase specific activities of the different strains are reported in Table 2. Production of protein A–β-lactamase was enhanced five- to sixfold in SF115, indicating that the effect of the *degP* and *ptr* mutations is approximately additive. Surprisingly, the penicillinase activity in the *degP ptr ompT* triple mutant grown in LB medium was comparable to that obtained with either the *degP ompT* or the *degP ptr* mutant. However, a cumulative increase in expression was observed when the cells were grown in minimal medium, suggesting that the composition of the growth medium has an important role in the turnover of secreted proteins.

The inactivation of *E. coli* proteases can affect growth rate

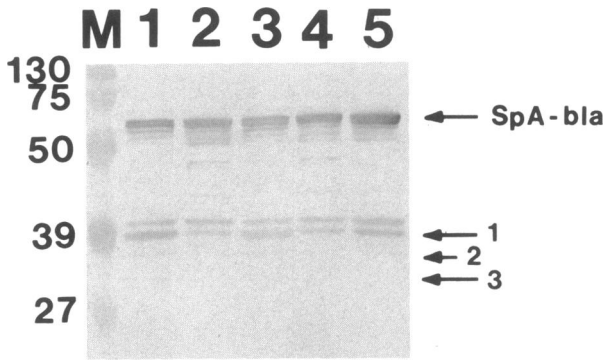

FIG. 5. Western blot analysis of protein A–β-lactamase degradation products in different cell envelope protease mutants. The position of protein A–β-lactamase (SpA-bla) is shown. One major (arrow 1) and two minor (arrows 2 and 3) carboxy-terminal degradation products whose intensities are reduced in the *ptr* mutants are shown. The N-terminal sequence of the degradation product identified by arrow 1 has been shown to correspond to the N-terminal domain of protein A (3). Lanes: M, molecular weight markers; 1, SF100; 2, SF103; 3, KS474; 4, SF115; 5, SF120.

TABLE 3. In vivo effect of divalent ions on the degradation of protein A–β-lactamase by protease III

Addition (0.1 mM)	Relative total penicillinase sp act (% ± SD)[a]	
	SF110(pFB3)	SF120(pFB3)
None	100	100
Ca^{2+}	103 ± 10	104 ± 5
Fe^{2+}	83 ± 2	76 ± 3
Zn^{2+}	146 ± 6	153 ± 9

[a] Total penicillinase activities were measured after 10 h of growth in W salts medium as described in Materials and Methods. Relative penicillinase specific activities were calculated by dividing the values obtained in the presence of 0.1 mM divalent solutions by the control values. The SD is expressed as a percentage relative to the mean.

and cell viability (4). Inactivation of protease III resulted in a 15% decrease in growth rate relative to the wild-type strain KS272 (Table 2). Furthermore, the *degP ptr* double mutant and the *degP ptr ompT* triple mutant exhibited 30 and 36% lower growth rates, respectively, than KS272.

Influence of divalent ions. Cheng and Zipser (9) have shown that protease III is sensitive to reducing agents and metal chelators, suggesting that divalent ions may be necessary for its activity. They found that the addition of 0.1 mM chloride salts of zinc, cobalt, and manganese can reactivate EDTA-inactivated protease III but has no stimulating effect on the rate of insulin degradation. In contrast, the chloride salts of magnesium, calcium, and cadmium are fairly inefficient in reactivating EDTA-inactivated protease III, even at a 2 mM concentration. To examine the in vivo effect of different divalent ions on the activity of protease III, we used the *E. coli* strains SF110 and SF120, which are deficient in DegP and OmpT. Cells transformed with pFB3 were grown in W salts medium containing 0.1% ammonium sulfate, in the presence or absence of 0.1 mM chloride salts of divalent metal ions. Cell growth was inhibited with 0.1 mM cobalt, copper, and manganese (in order of decreasing growth inhibition effect), and the role of these ions was not investigated. Table 3 shows that calcium and iron have essentially no influence on the production of the fusion protein. However, the addition of $ZnCl_2$ resulted in a 150% increase in penicillinase activity with both SF110 and SF120. It can be concluded that zinc causes some inhibition of a periplasmic proteolytic activity, which is distinct from DegP, OmpT, or protease III, or enhances the rate of protein synthesis. We favor the former hypothesis since at least one other secreted protein displays greater stability in *degP ompT* mutants grown in the presence of zinc (5a).

DISCUSSION

A rational approach for the study of protein degradation pathways and the identification of new proteolytic enzymes requires the systematic deactivation of known proteases. We have previously constructed an *E. coli* strain deficient in both DegP and OmpT (SF110) and showed that the effect of these mutations on the production of the model substrate protein A–β-lactamase is approximately additive (4). In this article, we have shown that protease III, a periplasmic protease previously thought to hydrolyze low-molecular-weight substrates, is involved in the degradation of a 63-kDa fusion protein.

In addition to protease III, a 50-kDa polypeptide known as p50 is derived from the *ptr* reading frame (12). It is possible that p50, or another polypeptide, is still synthesized in *ptr-32* strains. Nevertheless, this putative translation product does not have proteolytic activity against insulin (Fig. 3). Furthermore, it has been suggested that p50 is a stable degradation product of protease III. Inspection of the *ptr* coding sequence failed to reveal either a separate open reading frame encoding a 50-kDa protein or the existence of a Rho-independent transcription termination site (14).

The degradation of the model substrate protein A–β-lactamase by protease III was demonstrated by different criteria. First, the total penicillinase specific activity is twofold higher in cells carrying the *ptr* mutation than in the parental strain regardless of the growth medium used (Table 2). Second, this mutation can be complemented by a plasmid-encoded *ptr* gene. Third, pulse-chase experiments indicated that the half-life of the fusion protein is enhanced fourfold in the absence of protease III and that no degradation occurred in the first 30 min of the chase (Fig. 4). The fact that the half-life of the fusion protein is increased fourfold in *ptr* mutants whereas the corresponding activity after overnight growth is only twofold higher is probably due to (i) the difference in growth media used for the experiments and (ii) the fact that the protein turnover was determined in late-exponential-phase cultures whereas the activities were measured in late-stationary-phase cultures. Finally, the stable degradation products observed in early-stationary-phase *ptr* mutants are different from those in either *degP* or *ompT* single mutants (Fig. 5). It is likely that the absence of protease III confers increased stability to high-molecular-weight amino-terminal degradation intermediates of the fusion protein. This hypothesis is consistent with the fact that five Phe-Tyr (one of the two cleavage sites recognized by protease III) exist in the protein A coding sequence. In addition, the intensities of the bands corresponding to one major and two minor carboxy-terminal degradation products are markedly reduced when protease III is inactivated. Although β-lactamase does not contain the insulin cleavage sequence Phe-Tyr or Tyr-Leu, a Tyr-Ile site located close to its amino terminus may be recognized by protease III.

The above results show that, in addition to the in vitro hydrolysis of small polypeptides such as insulin, glucagon, and autoclaved fragments of β-galactosidase of molecular weight less than 7,000 protease III can degrade higher-molecular-weight substrates in vivo. A 94-kDa periplasmic metalloprotease (designated protease VII) which is activated by Ca^{2+} and degrades insulin and casein in vitro was recently described by Cook (11). Because of its striking similarity to the *ptr* gene product, this protein may be an isoenzyme or may even be identical to protease III. Like protease III, it cleaves insulin at Tyr-Leu and Phe-Tyr; in addition, it was shown to recognize three other dipeptides, namely, Asn-Gln, His-Leu, and Ala-Leu. Interestingly, the β-lactamase domain of the fusion protein contains one His-Leu and two Ala-Leu sites that may be recognized by this putative protease III activity.

We have previously shown that the inactivation of cell envelope proteases results in an increase in doubling time (4). Presumably, the accumulation of abnormal polypeptides in the periplasmic space of the cell represents a physiological burden and is detrimental to cell growth. Inactivation of *ptr* increases the doubling time by 15% relative to the wild-type strain KS272. In comparison, inactivation of *degP* caused a 25% decrease in growth rate while deletion of *ompT* had essentially no effect on growth (4). Therefore, although protease III is dispensable and its absence does not cause a detectable phenotypic alteration, a slight reduction in growth rate is observed when the gene is inactivated. As expected,

the doubling time of the cells is further increased as more secreted proteases are inactivated (Table 2).

To compare and contrast the role of protease III with that of the secreted proteases DegP and OmpT, the *ptr-32* mutation was transferred to several protease-deficient backgrounds. Table 2 shows that, on the basis of total penicillinase activity, the effect of the *degP* and *ptr* mutations is approximately additive. The *degP ompT ptr* triple mutant did not show enhanced production of the fusion protein relative to the double mutants when the cells were grown in rich medium. This phenomenon is related to the composition of the growth medium, since overnight cultures of SF120 (pFB3) in M9 salts exhibited a cumulative increase in expression relative to SF115(pFB3) cells (Table 3). At present, the reasons for the effect of the growth medium on proteolysis are not clear.

The isogenic strains SF110 and SF120 were used to examine the influence of divalent ions on proteolysis. Zinc increased the production of the fusion protein by 1.5-fold in both strains. This indicates that *E. coli* may contain at least one protease, other than OmpT, which is affected by Zn^{2+}. At present, four additional secreted proteases (IV, V, VI, and Mi) have been identified in *E. coli*. The addition of Zn^{2+} has no effect on protease VI activity (32). In contrast, protease IV, the *sppA* gene product (22), has been reported to be inhibited by $Zn(CH_3COO)_2$ (31). Degradation of protein A–β-lactamase by protease IV is unlikely, however, since (i) there is some evidence that the active site of protease IV faces the cytoplasmic side of the inner membrane (21, 40) and (ii) we did not observe a difference in penicillinase activity in *sppA* mutants transformed with pFB3 (unpublished data). To our knowledge, the influence of divalent ions on other secreted proteases such as Mi and V has not been tested.

The results discussed above show that protease III is involved in the turnover of large proteins in the periplasmic space. The protease-deficient strains developed in this study and in a previous study (4) can be employed to reduce protein turnover and may be useful for the production of proteolytically sensitive secreted proteins. These strains are isogenic and can help identify which protease(s) are involved in the degradation of a particular secreted polypeptide. They can also be used to alleviate the problem of background proteolysis that is commonly encountered in the study of protein turnover pathways.

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