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In Vivo Degradation of Secreted Fusion Proteins by the *Escherichia coli* Outer Membrane Protease OmpT

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The *Escherichia coli* outer membrane protease OmpT (protease VII) has been shown to degrade several proteins in vitro, but its function in vivo is uncertain. We demonstrate that OmpT participates in the degradation of a fusion protein secreted into the periplasmic space. A strain with mutations in *degP* (K. L. Strauch and J. Beckwith, Proc. Natl. Acad. Sci. USA 85:1576–1580, 1988) and *ompT* exhibits a cumulative decrease in protein degradation and should be useful for the expression of proteolytically sensitive secreted proteins.

Several secreted proteases are involved in the degradation of cell envelope proteins in *Escherichia coli*. One of these, OmpT (protease VII), is associated with the outer membrane. The *ompT* gene is located at approximately 12.5 min on the *E. coli* chromosome (20) and codes for a 317-amino-acid polypeptide which includes a 20-amino-acid N-terminal signal sequence (11, 26). This protein is homologous with and functionally related to the E protein of *Salmonella typhimurium* (10, 26). It was recently shown that OmpT is specific for paired basic residues and is inhibited by Zn^{2+} and Cu^{2+} ions (9, 25). In vitro, OmpT is responsible for the degradation of several proteins, including T7 RNA polymerase (9), recombinant human gamma interferon (25), the Ada protein (22), and a fusion protein between protein A and β-galactosidase (13). In addition, Hollifield et al. (14) have shown that OmpT is involved in the modification of the ferric enterobactin receptor in cell lysates. However, the role of OmpT in vivo is not clear. Grodberg and Dunn observed that T7 RNA polymerase is degraded by OmpT in intact whole cells (9, 10). This result indicates that at least the active site of this protease may be localized on the external side of the outer membrane.

Many secreted fusion proteins are highly sensitive to proteolytic degradation in the periplasmic space (1, 2, 6, 8). We have previously described the construction of a gene fusion between the protein A gene from *Staphylococcus aureus* and the *E. coli* enzyme TEM β-lactamase (2). The fusion protein is expressed constitutively from the native protein A promoter and is efficiently secreted through the cytoplasmic membrane. It exhibits β-lactam hydrolysis activity and immunoglobulin-G-binding affinity similar to those of authentic β-lactamase and protein A, respectively. Although the fusion protein is folded in a highly active conformation, it is very susceptible to proteolysis. In vitro, the half-life of the purified fusion protein in 0.05 M phosphate buffer (pH 7.0) containing 0.2 mg of trypsin per ml is approximately 7 min at 25°C. For comparison, β-lactamase is resistant to degradation for at least 1 h of incubation under identical conditions (G. Georgiou and F. Baneyx, Ann. N.Y. Acad. Sci., in press). Expression of the fusion protein in strain HB101 is accompanied by extensive degradation, resulting in the appearance of several low-molecular-weight bands that cross-react with β-lactamase-specific antiserum in immunoblots (Fig. 1, lane 1). These proteolytic products are inactive, and only the intact fusion protein exhibits enzymatic activity. The protein fragments corresponding to the two pronounced bands of approximately 39 and 37 kilodaltons in Fig. 1 have been isolated. From the N-terminal amino acid sequence it was deduced that both polypeptides contain a nearly intact protein A domain and that proteolysis occurred mainly in the β-lactamase part of the fusion protein (2). The β-lactamase domain contains three sets of paired basic residues which can serve as substrates for OmpT. To determine if OmpT is involved in the degradation of the fusion protein, strains RW193 (17) and UT4400 [RW193 Δ(*ompT-entF*)] (4) were transformed with plasmid pFB3, which codes for the protein A–β-lactamase gene fusion (2). Cultures were grown for 24 h at 37°C in shake flasks containing 25 ml of LB medium supplemented with 0.2% glucose and 50 μg of ampicillin per ml. The cells were pelleted by centrifugation at 8,000 × *g* for 8 min, and the supernatant was saved. The pellet was suspended in 50 mM potassium phosphate (pH 6.5) and disrupted in a French pressure cell, and the insoluble fraction was removed by centrifugation at 10,000 × *g* for 10 min. The clarified cell lysates were kept on ice and assayed immediately (7). The *ompT* deletion mutant exhibited a specific activity nearly threefold higher than that of its parental strain (Table 1). In addition, shake flask cultures of wild-type *ompT* strains supplemented with 0.5 mM solutions of the OmpT inhibitors $ZnCl_2$ or $CuCl_2$ had penicillinase activities approximately 70% higher than those of cultures without additives or supplemented with Mg^{2+} or Ca^{2+} ions. The increase in specific activity was proportional to the $ZnCl_2$ and $CuCl_2$ concentrations (range, 0.1 to 0.5 mM). Higher concentrations of either ion resulted in slight growth inhibition (unpublished data).

Since the deletion in strain UT4400 was large, it was possible that a protein other than OmpT was responsible for the difference in the amount of fusion protein produced. This question was addressed as follows. A 3.2-kilobase *Pvu*I fragment, which included the promoter and all the fusion protein gene 5′ to the *Pvu*I site in the *bla* part of the gene, was isolated from pFB3. This fragment was ligated to a 1.8-kilobase *Pvu*I fragment from pUC18 encoding the origin of replication and the remainder of the β-lactamase gene. The resulting plasmid, pCS1, was isolated from Amp^r transformants. Plasmid pML19 (11) encodes the complete *ompT* gene; its transformation in UT4400 resulted in the overproduction of OmpT, which does not have an adverse effect on

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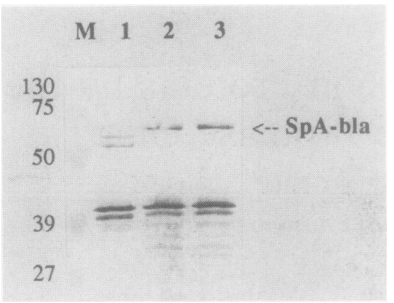

FIG. 1. Proteolytic degradation of protein A–β-lactamase in different strains. Cells were grown to an optical density at 600 nm of 1.7 as described in the text. Osmotic shock was carried out according to the method of Nossal and Heppel (19) except that a 0.5 mM solution of the OmpT inhibitor $ZnCl_2$ was added to all the working solutions to prevent possible in vitro proteolytic degradation during the incubation steps. Samples corresponding to exactly 1 μg of protein were subjected to SDS-PAGE in 15% gels, transferred to nitrocellulose filters, and analyzed immunologically with β-lactamase antiserum. Lanes: M, molecular weight markers (in thousands); 1, HB101(pFB3); 2, KS474(pFB3); 3, SF110(pFB3). The arrow indicates the position of the fusion protein.

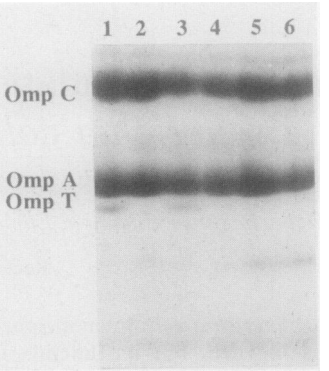

FIG. 2. SDS-PAGE of outer membrane proteins from different strains. Outer membrane fractions were prepared as described in the text. Lanes: 1, RW193; 2, UT4400; 3, KS474; 4, SF110; 5, SF100 (pFB3); 6, SF110(pFB3).

growth (9). Therefore, a *Pvu*II-*Sca*I fragment from pML19 containing the entire *ompT* sequence, along with its promoter, was inserted into the unique *Sma*I site of plasmid pCS1. In the resulting plasmid, pFB4 (7.1 kilobases), the *ompT* gene did not interfere with the regulation of the fusion protein or with the origin of replication. The specific activities of UT4400 cells transformed with pCS1 or pFB4 are shown in Table 1. As expected, cells containing the higher-copy-number plasmid pCS1 showed elevated activities compared with cells harboring pFB3, since the latter plasmid contains the pBR322 origin of replication. However, when the *ompT* gene was inserted into pCS1, a fivefold decrease in activity was observed (Table 1). This result indicates that OmpT participates in the degradation of the protein A–β-lactamase fusion protein.

Strauch and Beckwith recently identified and cloned *degP*, a gene involved in the proteolytic degradation of secreted fusion proteins (23). *degP*, which most likely codes for a protease, was also cloned independently by Lipinska et al. (15) and shown to be a heat shock protein. A *degP* mutant strain, KS474 [KS272 *degP41*(Δ*Pst*I-Kan^r)], transformed with pFB3 exhibited penicillinase-specific activity threefold higher than that of the control strain, KS272 (24). To examine the relationship and possible overlap in the functions of OmpT and DegP, we constructed a mutant deficient in both proteases. The *ompT* deletion was transferred from strain UT4400 into KS272 and KS474 by P1 transduction, generating strains SF100 and SF110, respectively. The deletion in UT4400, resulting in the loss of *ompT*, also removes several genes of the *ent* gene cluster; one consequence is resistance to colicin D. Therefore, transductants were selected for their ability to grow on colicin D plates. They were further screened for the absence of OmpT in outer membrane fractions prepared by a modification of the method described by Filip et al. (5). Cultures were grown at 37°C to mid-exponential phase, and the cells were recovered by centrifugation at 7,000 × *g*. The cells were washed, suspended in 50 mM potassium phosphate buffer (pH 6.5), and disrupted in a French press. After 8 min of centrifugation at 2,500 × *g* to remove unbroken cells, the supernatant was incubated with 0.5% *N*-lauroylsarcosine for 30 min at room temperature to solubilize inner membrane proteins. The samples were subsequently centrifuged at 100,000 × *g* for 1 h, and the pellet was suspended in 0.5 ml of 20 mM Tris hydrochloride (pH 7.5). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7% polyacrylamide gels containing 8 M urea (17). Prior to electrophoresis, the samples were boiled for 5 min in loading buffer (16). Both SF100 and SF110 lacked OmpT (Fig. 2, lanes 4 to 6). As expected, these strains did not produce a halo on enterobactin-selective plates (21).

TABLE 1. Characteristics, growth rates, and penicillinase-specific activities of bacterial strains harboring different plasmids coding for the protein A–β-lactamase fusion protein[a]

Strain (plasmid)	Strain characteristics[b]	Growth rate (per h)[c]	Total penicillinase-specific activity (U/mg) ± SD[d]
RW193(pFB3)	*ompT*$^+$ *degP*$^+$	ND	2.6 ± 0.2
UT4400(pFB3)	*ompT degP*$^+$	ND	7.2 ± 0.6
UT4400(pCS1)	*ompT degP*$^+$	ND	48.2 ± 9.2
UT4400(pFB4)	*ompT degP*$^+$	ND	8.6 ± 0.9
KS272(pFB3)	*ompT*$^+$ *degP*$^+$	1.66	15.2 ± 2.9
SF100(pFB3)	*ompT degP*$^+$	1.66	28.2 ± 7.1
KS474(pFB3)	*ompT*$^+$ *degP*	1.28	47.9 ± 8.3
SF110(pFB3)	*ompT degP*	1.16	87.9 ± 17.2

[a] Cells were grown at 37°C for 24 h in LB medium supplemented with 0.2% glucose and 50 μg of ampicillin per ml.

[b] Strain UT4400 is an *ompT* deletion mutant; however, when transformed with pFB4, which overproduces OmpT, its genotype becomes *ompT*$^+$.

[c] Growth rates were calculated from the growth curves of plasmid-free cells. ND, Not determined.

[d] Protein concentrations were evaluated by using the Bio-Rad protein assay with bovine serum albumin as a standard.

The penicillinase-specific activities of different strains transformed with plasmid pFB3 are shown in Table 1. Both SF100 and SF110 showed activities approximately twofold higher than those of the corresponding parental strains. The results in Table 1 indicate that the effects of the *degP* mutation and the *ompT* deletion on the specific activity of the fusion protein were approximately additive. To show that degradation by OmpT was occurring prior to lysis and did not result from the exposure of the fusion protein to outer membrane fragments in the cell lysate, the following experiment was carried out. KS474(pFB3) cells were harvested in 50 mM potassium phosphate buffer (pH 6.5) with or without 0.5 mM $ZnCl_2$, a concentration sufficient to completely inhibit OmpT (25). The cells were lysed in 50 mM

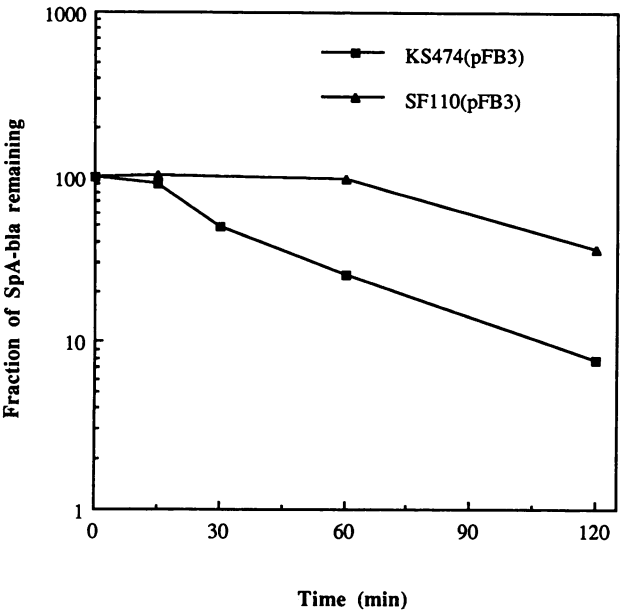

FIG. 3. Stability of protein A–β-lactamase in KS474 and the *ompT* mutant SF110. Proteins were immunoprecipitated with β-lactamase antiserum, resolved by SDS-PAGE, and subjected to autoradiography. The fraction of protein A–β-lactamase (SpA-bla) remaining corresponds to the intensity of the band at the specified time divided by the intensity at time zero, with both determined by scanning densitometry of the autoradiogram (see text).

potassium phosphate and incubated on ice, and the penicillinase activity was assayed periodically. No differences were observed for up to 2 h after lysis, indicating that the variations in specific activity did not result from degradation in vitro. Furthermore, the possibility that the outer membrane of the cells was leaky so that proteolysis was occurring in the cell growth medium was ruled out, since in KS474(pFB3) and SF110(pFB3), less than 17% of the fusion protein was found in the culture supernatant. Samples containing exactly 1 μg of protein from the osmotic shock fluid (19) of strains KS474(pFB3) and SF110(pFB3) were subjected to SDS-PAGE followed by immunoblotting with β-lactamase antiserum (7). The intensity of the band corresponding to the intact protein A–β-lactamase fusion protein was reproducibly higher in strain SF110 (Fig. 1). However, the pattern of low-molecular-weight bands cross-reacting with the β-lactamase antiserum was nearly identical in the two strains. Evidently, the fragments resulting from OmpT cleavage are unstable and are subject to further degradation by other secreted proteases. Finally, the stability of the fusion protein was also investigated by radioactive pulse-chase experiments. Cells were grown in minimal medium (7) supplemented with 50 μg of each of the 19 amino acids except methionine per ml. Cultures in mid-exponential phase were labeled with [^{35}S]Met for 2 min. The chase was initiated by adding 1 mg of unlabeled methionine per ml. The protein A–β-lactamase fusion protein was immunoprecipitated as described elsewhere (3). The immunoprecipitated proteins were resolved by SDS-PAGE in 15% polyacrylamide gels and visualized by autoradiography, and the film was scanned on a prototype Clayton densitometer (developed at The University of Texas at Austin by L. Poulson). In KS474, the half-life of the fusion protein was approximately 30 min, compared with more than 100 min for SF110 (Fig. 3). In SF110, essentially no degradation of the fusion protein was evident for the first hour following the initiation of the chase.

Mutations that decrease the rate of proteolysis also affect growth, partly because the accumulation of defective proteins is disruptive for the cell (23). The inactivation of the *degP* gene in KS272 caused a 25% decrease in the growth rate at 37°C (Table 1). In contrast, the growth of the *ompT* mutant strain SF100 was identical to that of its parental strain, KS272. The lack of a discernible effect on growth may be due to the narrow substrate specificity of OmpT. It is likely that, under normal conditions, OmpT plays a minor role in protein turnover, and therefore its absence does not affect the cell. However, strain SF110 exhibited a 10% reduction in growth rate and a longer lag relative to strain KS474 (both strains reached approximately the same optical density at 600 nm after overnight growth). Thus, the effect of the *ompT* deletion is more pronounced in a genetic background in which the proteolysis of secreted proteins has been impaired.

The results discussed above indicate that OmpT is involved in protein turnover in the periplasmic space. In agreement with our results, Hammarberg et al. (12) recently reported that the expression of a periplasmic fusion protein was significantly improved in an *ompT* mutant strain. In contrast, earlier studies have demonstrated that the OmpT-mediated degradation of T7 RNA polymerase takes place with intact *E. coli* cells (9, 10). While the latter result seems to indicate that the active site of OmpT is located on the exterior of the cell, other interpretations cannot be ruled out. For example, an outer membrane lesion may lead to the exposure of OmpT to the extracellular fluid so that it has access to T7 RNA polymerase during the assay. Besides, given the narrow substrate specificity of OmpT, it is unclear what physiological role it could play on the exterior of the cell.

Inactivation of *ompT* and *degP*, two genes involved in the degradation of secreted proteins, is not lethal for *E. coli* and results in the increased stabilization of a proteolytically unstable fusion protein. Strain SF110 may prove useful for the expression of secreted proteins in *E. coli*. Degradation of secreted proteins by OmpT seems to depend not only on the presence of paired basic residues but also on the overall conformation of the protein. We have not been able to detect any effect of the *ompT* mutation on the level of a β-lactamase–alkaline phosphatase fusion protein expressed from plasmid pB-1 (18). This protein, which is partially degraded in vivo (8; F. Baneyx, unpublished results), consists of the N-terminal sequence of TEM β-lactamase followed by the complete alkaline phosphatase and contains at least four putative OmpT cleavage sites per monomer.

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