

Identification of OmpT as the Protease That Hydrolyzes the Antimicrobial Peptide Protamine before It Enters Growing Cells of *Escherichia coli*

STEFAN STUMPE,^{1†} ROLAND SCHMID,¹ DAREN L. STEPHENS,²
 GEORGE GEORGIU,² AND EVERT P. BAKKER^{1*}

Abteilung Mikrobiologie, Universität Osnabrück, D-49069 Osnabrück, Germany,¹
 and Department of Chemical Engineering, The University of
 Texas at Austin, Austin, Texas 78712-1062²

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The influence of extracytoplasmic proteases on the resistance of *Escherichia coli* to the antimicrobial peptide protamine was investigated by testing strains with deletions in the protease genes *degP*, *ptr*, and *ompT*. Only $\Delta ompT$ strains were hypersusceptible to protamine. This effect was abolished by plasmids carrying *ompT*. Both at low and at high Mg^{2+} concentrations, *ompT*⁺ strains cleared protamine from the medium within a few minutes. By contrast, at high Mg^{2+} concentrations, protamine remained present for at least 1 h in the medium of an *ompT* strain. These data indicate that OmpT is the protease that degrades protamine and that it exerts this function at the external face of the outer membrane.

In animals, bacteria are often exposed to defensins and other cationic antimicrobial peptides produced by the host (6, 11, 12). Groisman and coworkers have introduced the strongly cationic antimicrobial peptide protamine as a model compound for the action of defensins on cells of *Salmonella typhimurium* (9, 18, 19). The site of action of protamine is the cytoplasmic membrane, where it causes membrane permeabilization, possibly by creating a large pore in the membrane (3, 10, 20). At low protamine doses, growing cells of *Escherichia coli* slowly recover from protamine treatment (20). This effect is probably due to protamine degradation by cell proteases, since the sus-

ceptibility of growing cells of *E. coli* toward protamine increases by fivefold in a strain lacking the extracytoplasmic proteases DegP, OmpT, and protease III (20). Here, we examine which of the above proteases is responsible for protamine inactivation and whether this inactivation is due to protamine degradation. For this purpose, we used strains possessing only a single extracytoplasmic protease (16) and employed high-performance liquid chromatography (HPLC) to monitor the fate of protamine in the medium of growing *E. coli* cells. We show that OmpT is the protease responsible for protamine inactivation by degradation of the toxic peptide at the external

TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid ^a	Genotype or property	Reference
KS272	F ⁻ $\Delta lacX74 galE galK thi rpsL \Delta phoA$	16
KS474	KS272 <i>degP</i>	16
SF103	KS272 <i>ptr</i>	16
SF110	KS272 <i>degP ompT</i>	16
SF115	KS272 <i>degP ptr</i>	16
SF120	KS272 <i>degP ptr ompT</i>	16
UT5600	F ⁻ <i>ara-14 leuB6 azi-6 lacY1 proC14 tsx67 $\Delta(ompT-fepC266) entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1$</i>	4
pDS319	Tet ^r ; carries an <i>ompT</i> containing 2-kb <i>E. coli</i> chromosomal <i>EcoRI-PstI</i> fragment ligated with the large <i>EcoRI-PstI</i> fragment from plasmid pBR322	
pML19	Amp ^r ; carries an <i>ompT</i> containing 2-kb <i>E. coli</i> chromosomal <i>EcoRI-PstI</i> fragment ligated with the large <i>EcoRI-PstI</i> fragment from plasmid pUC19	7, 8

^a The source for all strains and plasmids was G. Georgiou.

* Corresponding author. Mailing address: Abteilung Mikrobiologie, Universität Osnabrück, Barbarastraße 11, D-49069 Osnabrück, Germany. Phone: 49-541-9692855. Fax: 49-541-9692870. E-mail: bakker_e@sfbio1.biologie.uni-osnabrueck.de.

† Present address: Institut für Molekulare Biotechnologie, Abteilung Strukturbiologie/Kristallographie, Arbeitsgruppe Physikalische DNA-Analytik, 07745 Jena, Germany.

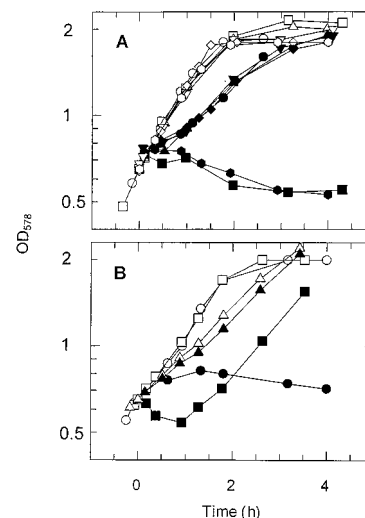


FIG. 1. Effect of *ompT* and other extracytoplasmic protease genes on protamine susceptibility of *E. coli* cells. Protamine (100 mg/liter) was added at $t = 0$ to a suspension of growing cells. Closed symbols, protamine-treated cells; open symbols, control cells. (A) Circles, strain KS272; squares, strain SF103; triangles, strain SF103; diamonds, strain KS474; inverted triangles, strain SF115; and octagons, strain SF120; (B) circles, strain UT5600; triangles, strain UT5600(pML19); and squares, strain UT5600(pDS319). The last two strains grew in the presence of 100 mg of carbenicillin and 12.5 mg of tetracycline per liter, respectively.

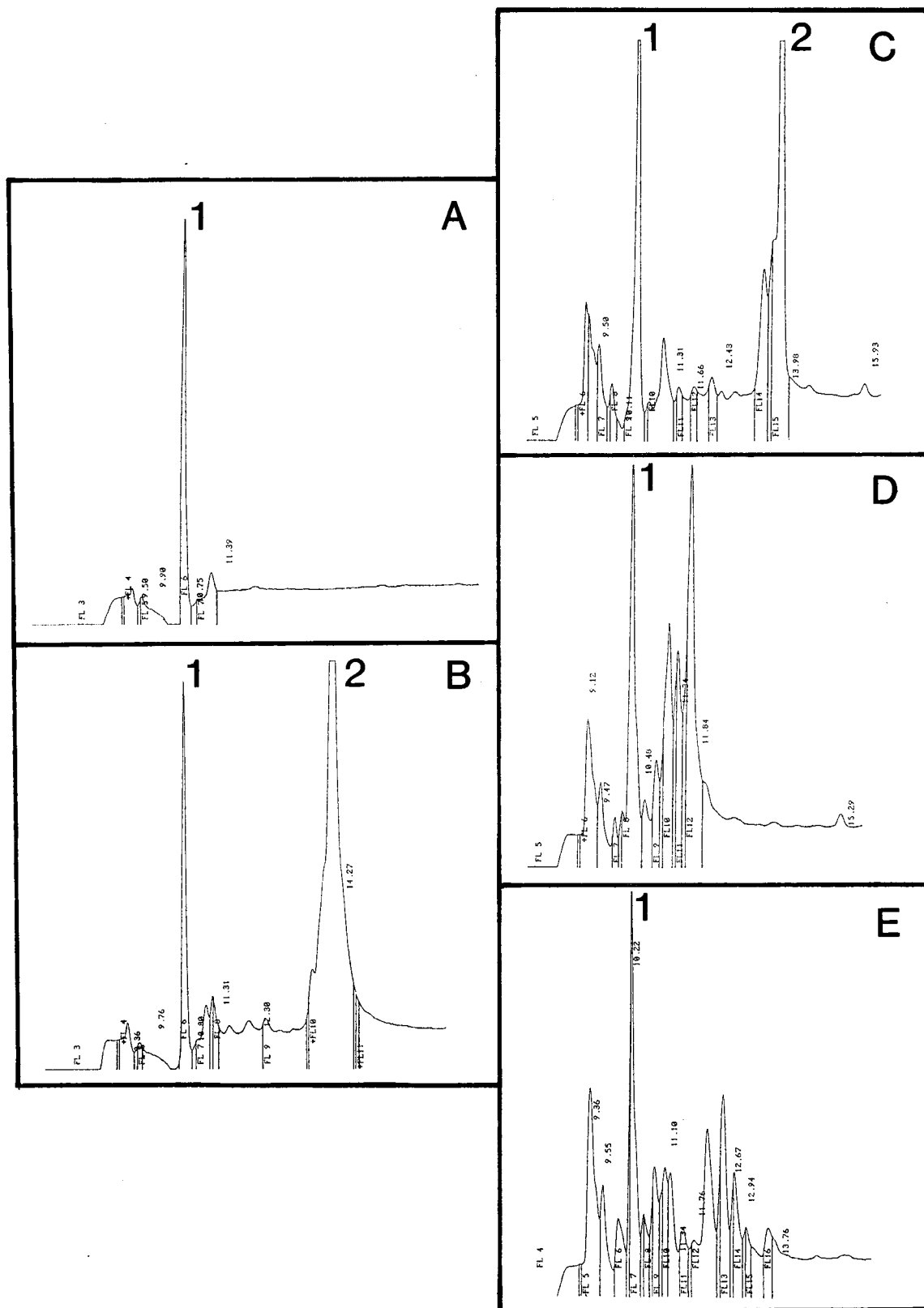


FIG. 2. Protamine vanishes rapidly from the medium of growing cells of *E. coli* KS272. The peptide content of the supernatant from centrifuged cells was analyzed by reverse-phase HPLC. (A) Growth medium; (B) growth medium plus 100 mg of protamine per liter; (C to E) chromatograms of the supernatants taken at 75 s, 7 min and 30 min and 30 min, respectively, after addition of 100 mg of protamine per liter to the cell suspension at time zero. Peaks 1 and 2 represent thiamine and protamine, respectively.

face of the cell envelope. Our data suggest that a major function of OmpT is to protect the cells against antimicrobial cationic peptides from the medium.

The strains and plasmids used in this study are listed in Table 1. Studies of protamine susceptibility of *E. coli* cells growing in shaking culture flasks were carried out as described elsewhere (20). Peptide contents and protamine concentrations in the supernatant of centrifuged cells were determined by reverse-phase HPLC separation on a C₁₈-column with an acetonitrile-H₂O gradient in a 140 apparatus from Applied Biosystems (Foster City, Calif.). The N-terminal sequences of proteins or peptides were determined by microsequencing after automatic Edman degradation in an ABI-473A Instrument (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). Protamine sulfate (from salmon milt; Calbiochem, Bad Soden, Germany) is a mixture of compounds (2). One of its major compounds, salmine AI, has the primary sequence MPRRRR SSSRPVRRRRRPRVSRRRRRRGGRRRR (1).

Protamine susceptibility of strains lacking extracytoplasmic proteases was tested in growth experiments (20). Protamine was added at a concentration of 100 mg/liter to a cell suspension with an optical density at 578 nm (OD₅₇₈) of 0.65, and the cells were grown in a minimal mineral medium containing 20 mM K⁺ ions with 10 mM glucose as the carbon source. Figure 1A shows that strain SF115 lacking protease III and DegP but not OmpT was as susceptible as the wild-type strain. These strains resumed growth at approximately 1 h after the addition of the toxic peptide. In contrast, the *ompT* strains SF110 and SF120 did not recover from protamine treatment within 6 h (Fig. 1A). Mutations in either extracytoplasmic protease gene *ptr* or *degP* in an *ompT*⁺ background exerted no effect (strains SF103, SF115, and KS474 [Fig. 1A]). This suggests that OmpT alone is sufficient for protection of cells against the toxic peptide. Further information was obtained with the nonisogenic Δ *ompT* strain UT5600 (4). As expected, this strain was hypersusceptible to protamine (Fig. 1B, closed circles). However, two types of *ompT*-containing plasmids rescued these cells. Cells harboring plasmid pML19 (an *ompT*-containing derivative of pUC19 [7, 8]) grew relatively slowly (Fig. 1B, closed triangles). Addition of protamine to these cells hardly exerted any effect on cell growth (Fig. 1B, triangles). The poor growth of UT5600(pML19) is due to the overexpression of OmpT from the high-copy-number pML19 plasmid. Therefore, we also tested the effect of *ompT* expression from the lower-copy-number plasmid pSD319, which is a pBR332 derivative. Strain UT5600(pSD319) still grew more slowly than did the control strain UT5600(pBR322) (data not shown). Moreover, addition of protamine to cells of strain UT5600(pSD319) led to considerable cell lysis, as indicated by the trough in the growth curve of this strain (Fig. 1B). This effect was only partially due to the presence of tetracycline in the growth medium of this strain and did not occur after protamine addition to cells of strain UT5600(pBR322) growing under similar conditions (data not shown). Still, the presence of *ompT* on the plasmid rescued the cells of strain UT5600(pSD319), since these cells resumed growth after approximately 2 h (Fig. 1B), whereas the control culture of strain UT5600(pBR322) did not resume growth within 5 h (data not shown). Taken together, the data from Fig. 1 show that OmpT is the extracytoplasmic protease that protects *E. coli* cells against protamine.

More information about the fate of protamine in the cell suspension came from HPLC analysis of the peptide contents of supernatants from centrifuged cells. Growth medium contained several compounds, including thiamine, with signals in the HPLC chromatogram, with a retention time of 10 to 11 min

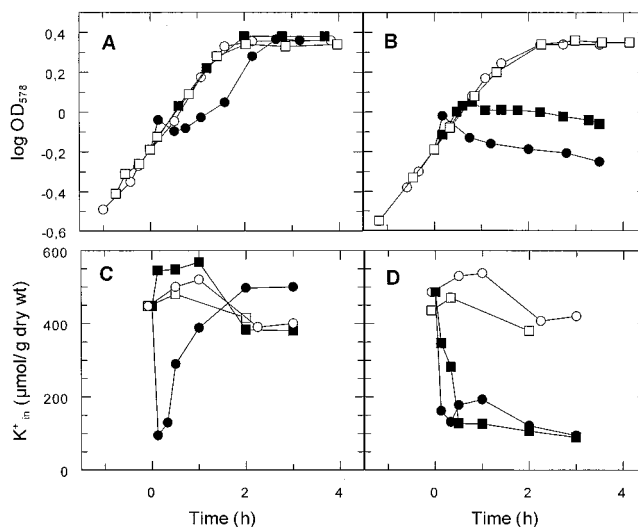


FIG. 3. High concentration of Mg²⁺ protects *ompT*⁺ cells from toxic effects of protamine. Protamine (100 mg/liter) was added at time zero to growing cells of the wild-type strain KS272 (A and C) or the *ompT* strain SF110 (B and D). (A and B) Growth curves; (C and D) K⁺ contents. Closed symbols, protamine-treated cells; open symbols, control cells; circles, cells growing at 0.4 mM Mg²⁺; and squares, cells growing at 5 mM Mg²⁺.

(Fig. 2A, peak 1). Addition of 100 mg of protamine per liter to this medium gave a major additional peak with a retention time centered at 13 to 14 min (Fig. 2B, peak 2). The form of this peak confirms that protamine consists of a mixture of compounds (2). Addition of protamine to a suspension of growing cells of the *ompT*⁺ strain KS272 led to a rapid removal of the toxic peptide from the medium (Fig. 2, traces C to E). After 75 s, the protamine peak was already much smaller (Fig. 2C), and at 450 s, it had vanished from the medium (see Fig. 2D). Several new products appeared in the medium (Fig. 2D and E). Below, we show that some of them were protamine degradation products. From these data, it is concluded that the cells removed protamine with a half-life of 1 to 2 min from the medium. However, we cannot differentiate whether this removal from the medium represents protamine adsorption to the cells (10, 23), proteolysis of the compound, or both. The Δ *ompT* strain SF110 removed protamine as rapidly from the medium as did strain KS272 (data not shown), suggesting that adsorption to the outer membrane is important in the process of protamine removal from the medium.

High Mg²⁺ concentrations prevent adsorption of protamine to the cell wall (10, 23). In order to obtain more information on the fate of protamine under these conditions, the experiments were repeated at 5 mM Mg²⁺. At this [Mg²⁺], protamine did not inhibit cell growth of the *ompT*⁺ strain KS272 (Fig. 3A). Moreover, these cells did not lose K⁺ (Fig. 3C), suggesting that under these conditions protamine did not reach the cytoplasmic membrane. By contrast, at 5 mM Mg²⁺, growth of the Δ *ompT* strain SF110 slowed down gradually and stopped completely at approximately 1 h after protamine addition (Fig. 3B). These cells lost K⁺, although much more slowly than at 0.4 mM Mg²⁺ (half-lives for K⁺ loss, 20 min and a few minutes, respectively [Fig. 3D]), suggesting that at 5 mM Mg²⁺, protamine slowly reached the cytoplasmic membrane of the *ompT* strain.

Figure 4A to C shows that at 5 mM Mg²⁺, protamine removal by strain KS272 is almost as rapid as that in the absence of Mg²⁺ (half-lives of 1 to 2 min in both cases [Fig. 4A to C and

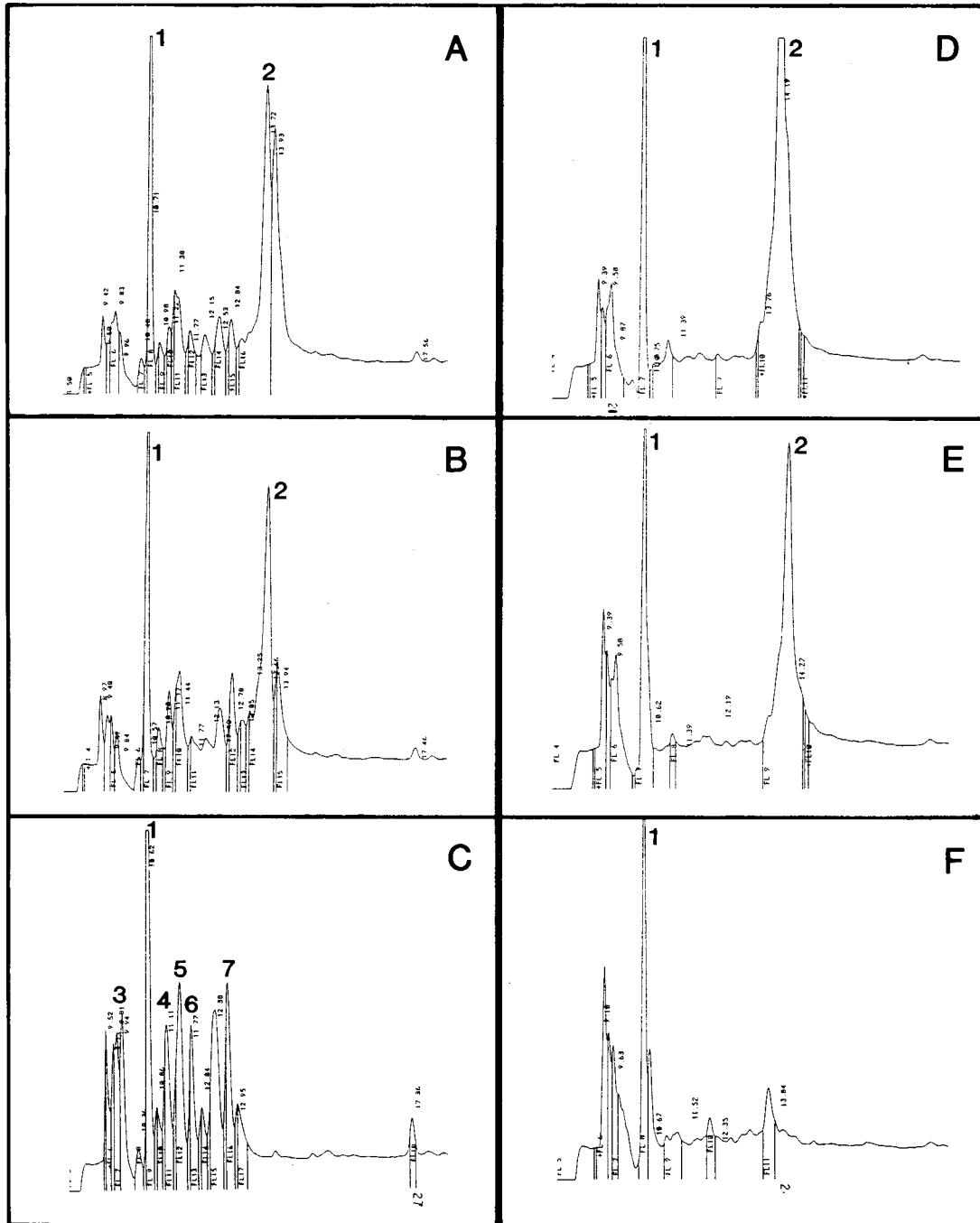


FIG. 4. Fate of protamine in the high- Mg^{2+} medium of cells of strains KS272 and SF110. Protamine (100 mg/liter) was added at time zero to the suspension of cells growing at 5 mM Mg^{2+} . (A to C) HPLC chromatograms of the supernatant of cells of strain KS272 centrifuged at 3 min, 7 min and 30 s, and 60 min, respectively, after addition of protamine to the suspension; (D to F) HPLC chromatograms of the supernatant of cells of strain SF110 centrifuged at 30, 60, and 180 min, respectively, after addition of protamine to the medium. Peaks 1 and 2 are the same as those described in the legend to Fig. 2. The N termini of peptides from peaks 3 to 7 in panel C were determined by Edman degradation (see the text for details).

Fig. 2, respectively)]. At high Mg^{2+} concentrations, several new peptides with retention times of between 9.20 and 12.67 min were formed (e.g., Fig. 4C). These compounds remained at constant concentrations for a period of 3 h in the medium (data not shown). The peptides from peaks 3 to 7 in Fig. 4C all contained several arginines at their N terminus, suggesting that they are protamine degradation products. Peptide 6 in Fig. 4C

had an N-terminal sequence, XSSRRPVRRR, indicating that it is similar to protamine 2c from rainbow trout (15). It was not yet known that salmon protamine contains this type of compound. These data suggest that at 5 mM Mg^{2+} , protamine degradation products do not adsorb to the cell wall of the *ompT*⁺ strain.

Protamine removal from the medium by the $\Delta ompT$ strain

SF110 was extremely slow (half-life of approximately 30 to 60 min [Fig. 4D to F]). Moreover, the protamine hydrolysis products observed at high Mg^{2+} concentrations with strain KS272 did not appear in the medium of strain SF110 (Fig. 4D to F). We assume that this removal of protamine from the medium represents uptake by the cells, since its time course correlated well with inhibition of growth and K^+ loss from these cells (Fig. 3B and D, respectively). It is not known whether under these conditions protamine is degraded at all and, if this is the case, in which cellular compartment proteolysis occurs.

Since protamine remained in the medium at high Mg^{2+} concentrations with the $\Delta ompT$ strain (Fig. 4D to F), we assume that under these conditions the major portion of protamine is not rapidly adsorbed by the cells. The observations that under these conditions the isogenic $ompT^+$ strain removed protamine rapidly from the medium and that its degradation products appeared in the medium (Fig. 4A to C) suggest that OmpT cleaves protamine at the external surface of the outer membrane. This idea, i.e., that the active center of OmpT is directed outward, is supported by observations that in intact *E. coli* cells OmpT degrades T7 RNA polymerase added to the medium (8) and that it activates plasminogen (13). However, the question concerning the orientation of the active site of OmpT in the outer membrane remains controversial, since other data suggest that OmpT is active in the degradation of some periplasmic proteins that come into contact with the outer membrane (17).

OmpT is known to degrade peptides between cationic residues and between cationic residues and several apolar residues (8, 14, 17, 21, 22). Therefore, it was not unexpected that OmpT is the extracytoplasmic protease that inactivates the highly cationic peptide protamine (Fig. 1, 2, and 4). We have preliminary evidence that, in addition to protamine, OmpT detoxifies the cationic peptide melittin (data not shown). Moreover, OmpT is essential for the virulence of *E. coli* strains in the human urinary tract (5). We speculate that in this situation OmpT is active in the degradation of cationic peptides (defensins) excreted by epithelial cells from the urinary tract. However, at present no data that either support or contradict this hypothesis are available.

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