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Substrate Specificity of the *Escherichia coli* Outer Membrane Protease OmpP▽

Bum-Yeol Hwang,[1,2] Navin Varadarajan,[1,3] Haixin Li,[1,5] Sarah Rodriguez,[1,3] Brent L. Iverson,[1,3] and George Georgiou[1,2,4,5]*

Institute for Cellular and Molecular Biology,[1] Departments of Chemical Engineering,[2] Chemistry and Biochemistry,[3] and Biomedical Engineering,[4] and Section of Molecular Genetics and Microbiology,[5] University of Texas, Austin, Texas 78712

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Escherichia coli **OmpP is an F episome-encoded outer membrane protease that exhibits 71% amino acid sequence identity with OmpT. These two enzymes cleave substrate polypeptides primarily between pairs of basic amino acids. We found that, like OmpT, purified OmpP is active only in the presence of lipopolysaccharide. With optimal peptide substrates, OmpP exhibits high catalytic efficiency (k_{cat}/K_m = 3.0 × 10^6 $M^{-1}s^{-1}$). Analysis of the extended amino acid specificity of OmpP by substrate phage revealed that both Arg and Lys are strongly preferred at the P1 and P1′ sites of the enzyme. In addition, Thr, Arg, or Ala is preferred at P2; Leu, Ala, or Glu is preferred at P4; and Arg is preferred at P3′. Notable differences in OmpP and OmpT specificities include the greater ability of OmpP to accept Lys at the P1 or P1′, site as well as the prominence of Ser at P3 in OmpP substrates. Likewise, the OmpP P1 site could better accommodate Ser; as a result, OmpP was able to cleave a peptide substrate between Ser-Arg about 120 times more efficiently than was OmpT. Interestingly, OmpP and OmpT cleave peptides with three consecutive Arg residues at different sites, a difference in specificity that might be important in the inactivation of cationic antimicrobial peptides. Accordingly, we show that the presence of an F′ episome results in increased resistance to the antimicrobial peptide protamine both in *ompT* mutants and in wild-type *E. coli* cells.**

The OmpP gene of F plasmids (18) encodes an outer membrane protease consisting of 292 amino acid residues. OmpP belongs to the omptin family of proteases, which includes the *Escherichia coli* OmpT, *Shigella flexneri* SopA, *Yersinia pestis* Pla, *Salmonella enterica* PgtE, and *Erwinia pyrifoliae* PlaA enzymes (13). Based on the crystal structure of OmpT (34), omptins have been classified as aspartyl proteases (family A26) and have a highly conserved active site (34). Many members of this protease family are involved in virulence by protecting cells from cationic antimicrobial peptides (32, 33), activation of the anticoagulation pathway (35), and inflammatory responses (39).

In addition, both OmpT and OmpP play a role in unwanted proteolysis of recombinant proteins expressed in *E. coli*. For example, proteins such as XynE of *Aeromonas caviae* ME-1 (16), keratinocyte growth factor-2 (14), cyclin A (38), *E. coli* H-NS (7), the omega subunit of *E. coli* RNA polymerase (6), *E. coli* initiation factor IF2 (15), and T7 RNA polymerase (8) are cleaved by OmpT or OmpP when isolated following overexpression in *E. coli*. Since OmpP and OmpT are outer membrane proteases and their active sites are positioned outside of the *E. coli* cells, cleavage of recombinant proteins most likely occurs in the purification step by membrane-bound OmpT or OmpP.

Little is known regarding the regulation, physiological role, or biochemical properties of OmpP. Early evidence suggested that its synthesis is regulated by phosphate and the growth temperature (10, 21) and that it plays a role in the catabolism of peptides under nitrogen-limited conditions (1). The enzyme was first isolated by Henning and coworkers (10), who showed that it exhibits 71% amino acid identity with OmpT, with which it also shares a strong preference for cleavage of polypeptide substrates between pairs of basic amino acids. OmpP is stable over a broad pH range (pH 6.4 to pH 9.2) and at temperatures up to 75°C but is inactivated by Mg^{2+}, Mn^{2+}, Co^{2+}, or Ca^{2+} ions (30). Striebel and Kalousek reported that OmpP is able to process eukaryotic preproteins cleaving at sites that consist of Arg ↓ Ala or Lys ↓ Gln but also at sites that contain no basic residues, such as Ala ↓ Leu or Ser ↓ Val (30).

Comprehensive information on the extended subsite specificity of a protease can be obtained by analyzing the cleavage of peptide libraries that are created either chemically or biologically (26, 29). In particular, the specificities of over 30 proteases, spanning all recognized classes, have been studied by techniques relying on phage display (5, 11, 19, 20). Substrate phage display is based on the selective cleavage of specific peptide sequences sandwiched between the gene pIII minor coat protein of fd bacteriophage and an affinity tag. The phage is immobilized on a solid support via the affinity tag, and, following treatment with a purified protease of interest, clones containing susceptible peptide sequences are cleaved, which in turn releases them from the support, allowing amplification. This process is repeated several times to reveal consensus substrate sequences.

Here, we used substrate phage display that employed a randomized octamer library to investigate the specificity of OmpP. The substrate sequences identified from this analysis revealed that the substrate specificity of OmpP is indeed very similar to its homologue, OmpT. However, some significant differences

* Corresponding author. Mailing address: Department of Chemical Engineering, University of Texas, Austin, TX 78712. Phone: (512) 471-6975. Fax: (512) 471-7963. E-mail: gg@che.utexas.edu.

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

Strain or plasmid	Genotype	Source or reference
MC1061	F$^-$ *araD139* Δ(*ara*$^-$ *leu*)*7697 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2*(r_K^- m_K^+) *mcrA mcrB1*	3
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	31
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 glnV44 relA1 lac*[F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*]	Stratagene
KS272	F$^-$ Δ*lacX74 galE galK thi rpsL* (Str^r) Δ*phoA*	2, 22
SF100	KS272 Δ*ompT*	2, 22
KS272 F′	KS272 [F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*]	This work
SF100 F′	SF100 [F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*]	This work
pOP19	pUC19 expressing *ompP* under the control of its native promoter	This study
pML19	pUC19 expressing *ompT* under the control of its native promoter	25

in the substrate preferences of the two enzymes were noted and may be relevant to the physiological role of OmpP in F pilus biogenesis and function. We further show that with optimal substrate, OmpP is a very fast enzyme, capable of cleaving peptide substrates with catalytic efficiencies, k_{cat}/K_m, in the 10^6 $M^{-1}s^{-1}$ range. As a result, the introduction of an F′ episome confers increased resistance to protamine and presumably other antimicrobial peptides.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and plasmids used in this study are listed in Table 1. The polyvalent phage display vector fUSE55, which is derived from fUSE5, was used for the construction of peptide libraries (28). Unless otherwise stated, *E. coli* cells were grown in Luria-Bertani (LB) medium (27) containing the appropriate antibiotics at 37°C in a shaking incubator.

Construction of phage libraries. First, sequences encoding the FLAG tag (DYKDDDDK) and an XhoI restriction site were inserted 5′ of gene III by ligating a synthetic DNA fragment into the SfiI site of fUSE55, giving rise to fUSE55-FLAG. Subsequently, the octamer library was constructed by PCR using fUSE55-FLAG DNA (100 pg) as the template, the 5′ primer 5′-GATAAAGG ACTCGAGGCTNNKNNKNNKNNKNNKNNKNNKGGGGCCGAAAC TGTTGAAAG-3′ (where N represents any nucleotide and K represents T/C), and the 3′ primer 5′-CAAACGAATGGATCCTCATTAAAGCCAG-3′, followed by digestion with XhoI/BamHI and ligation into fUSE55-FLAG (restriction sites are underlined). The ligated DNA (~1 μg) was used to transform electrocompetent *E. coli* MC1061 (~100 μl) via electroporation.

Substrate phage. Double-stranded phage DNA was prepared and transformed into *E. coli* BL21(DE3), which lacks the *ompT* and *ompP* genes. Immediately following electroporation, the cells were added to 1 ml of SOC medium with 0.2 μg of tetracycline/ml, allowed to grow 1 h at 37°C, and then added to 500 ml of NZY medium containing 40 μg of tetracycline/ml. The cells were grown for 20 h at 37°C and centrifuged, the supernatant was filtered, and the phage was precipitated by the addition of 0.15 volumes of 16.7% polyethylene glycol 8000 (PEG 8000) in 3.3 M NaCl solution and centrifugation at 6,200 × *g* for 40 min. The resulting pellets were resuspended in 15 ml of Tris-buffered saline (TBS). After heat treatment, the phage was precipitated with 0.15 volumes of 16.7% PEG-NaCl solution and centrifugation at 10,000 × *g* for 40 min. The resulting pellets were resuspended in 10 ml of TBS. The phage titer of the resulting peptide library was 1.5×10^{12} PFU. About 6.0×10^{11} PFU of amplified phage were bound to 0.5 g of anti-FLAG agarose beads (Sigma, St Louis, MO) at 4°C for 4 h. Unbound phages were removed by washing the beads with TBS five times. Subsequently, 0.2 μM purified OmpP was added to the beads and incubation was allowed to proceed at 37°C for 30 min. Cleaved phages were recovered from the supernatant, titered, amplified, and applied to the next round of selection. Phage that remained bound to the beads was eluted with glycine-HCl solution (pH 3.0) and titrated to assess cleavage efficiency. After the indicated rounds, phage plaques were randomly selected for sequencing.

OmpP and OmpT expression and purification. *E. coli* BL21(DE3) cells transformed with plasmid pOP19 or pML19 were grown in LB medium containing 100 μg of ampicillin/ml at 37°C for 12 h. OmpP was purified by a procedure similar to that used earlier for the purification of OmpT, with minor modifications (17, 25). Briefly, membrane proteins were solubilized in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 30 mM *n*-octyl-β-D-glucopyranoside (*n*-OG) (FisherBiotech, Fair Lawn, NJ), and 10 mM EDTA and separated by anion-exchange fast-protein liquid chromatography (Amersham Biociences, Piscataway, NJ) on a HiPrep 16/10 DEAE FF column (Amersham Biociences) with a gradient of 0 to 0.5 M NaCl in Tris-HCl buffer (pH 7.4) at a flow rate of 2 ml/min. Protein concentrations were determined by a Micro BCA protein assay (Pierce, Rockford, IL). The final purified enzymes exceeded 97% purity, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Kinetic analysis. Peptide substrates were prepared by solid-phase peptide synthesis (EZBiolab Inc., Westfield, IN) and were over 95% pure. For kinetic analysis, 0.025 nM to 10 nM concentrations of the purified enzymes were incubated with 20 μM to 500 μM concentrations of the appropriate substrate in 0.1 M MES (morpholineethanesulfonic acid) buffer (pH 6.1) containing 10 mM EDTA at 37°C for 5 to 30 min. Reactions were quenched by freezing in liquid nitrogen, and product formation was monitored by high-performance liquid chromatography (HPLC) on a Phenomenex (Torrance, CA) C_{18} reversed-phase column with 5% acetonitrile–95% H_2O for 1 min, increased to 95% acetonitrile–5% H_2O over a period of 29 min, and returned to 5% acetonitrile–95% H_2O over 5 min. The product concentration was determined at 280 nm, and the apparent rates were fitted to the Michaelis-Menten equation by nonlinear regression. The cleavage products were determined by liquid chromatography-mass spectrometry (electrospray ionization) LC-MS (ESI) on a Magic 2002 instrument (Micron Bioresources, Auburn, CA).

Site-directed mutagenesis. Mutants of OmpP were prepared with a PCR-based technique using *Pfu* polymerase, an enzyme with proofreading activity (37). PCR was performed with the primers shown in Table 2, with the expression vector of OmpP (pOP19) used as a template. The reaction was carried out with an elongation time of 12 min at 68°C to increase the yield of the PCR product with the expected size. The PCR mixtures were treated with DpnI at 37°C for 1 h and transformed into competent *E. coli* BL21(DE3) cells, and colonies resistant to ampicillin were selected.

Protamine degradation. Cells were grown in 200 μl of LB medium with protamine at the indicated concentrations in 96-well plates with shaking at 37°C. The optical density at 600 nm was monitored on a Synergy HT microplate reader (BioTek, Winooski, VT). To measure the degradation of protamine by whole cells, 450 μl of a 0.8-mg/ml solution of protamine in phosphate-buffered saline (pH 7.5) was incubated with 50 μl of cells harvested in mid-exponential phase and normalized to an optical density at 600 nm of 2. Following incubation for 30 min at 37°C, the extent of protamine cleavage was determined by HPLC on a Phenomenex C_{18} reversed-phase column under the elution conditions described in "Kinetic analysis," above.

RESULTS

Purification of OmpP. The *ompP* gene of F′ plasmid in *E. coli* XL1-Blue was cloned into pUC19 under the control of its native promoter (18), resulting in plasmid pOP19. *E. coli* BL21(DE3), a strain that lacks both the *ompP* and *ompT* genes, was used for OmpP expression. A band corresponding to the molecular weight of OmpP was readily visible in total lysates of cells transformed with pOP19 (data not shown). Given the high level of sequence identity between the two enzymes, OmpP was purified by a procedure similar to that employed for the preparation of homogeneous OmpT (12). Briefly, membrane fractions were precipitated by centrifugation and then treated with 20 mM $MgSO_4$ and the detergent

TABLE 2. Oligonucleotides used in site-directed mutagenesis of OmpP

Mutant	Primer orientation	Sequence[a]
E28L	Sense	5′-CGGGTTTATCTGCCTGAAGAAG GTGGACG-3′
	Antisense	5′-TTCTTCAGGCAGATAAACCCGT TCTTTTG-3′
L160F	Sense	5′-ACGGGAGCGTTTCCTGATAAAA TAAAAGTG-3′
	Antisense	5′-TTTATCAGGAAACGCTCCCGTT TCATTCCG-3′
K165R	Sense	5′-GATAAAATACGCGTGATTGGTT ATAAACAAC-3′
	Antisense	5′-ACCAATCACGCGTATTTTATCA GGTAACGC-3′
V211D	Sense	5′-TGAGCATTATGATAGACAAACT ACATTCCG-3′
	Antisense	5′-AGTTTGTCTATCATAATGCTCA TCATTATC-3′
R212K	Sense	5′-CATTATGTAAAACAAACTACAT TCCGAAG-3′
	Antisense	5′-TGTAGTTTGTTTTACATAATGC TCATC-3′
T214I	Sense	5′-GTAAGACAAATTACATTCCGAA GCAAAG-3′
	Antisense	5′-TCGGAATGTAATTTGTCTTACA TAATGC-3′
F216Y	Sense	5′-CAAACTACATACCGAAGCAAA GTAATAAAC-3′
	Antisense	5′-TTTGCTTCGGTATGTAGTTTGT CTTAC-3′
S264N	Sense	5′-TACGACCGTAATGATAATACTT CGGAGC-3′
	Antisense	5′-AGTATTATCATTACGGTCGTAA AGAGATG-3′

[a] Underlined portions of sequences are the mutation sites.

n-OG and extracted with *n*-OG in the presence of EDTA. Following DEAE chromatography, the pooled fractions contained OmpP at a purity of >97%, as judged by SDS-PAGE (data not shown). The purified OmpP migrated as a 33-kDa band in SDS-PAGE and was found to have a molecular mass of 33,144 Da, determined by mass spectroscopy, in good agreement with its predicted molecular mass (33,110 Da). These data indicate that the protein is not subjected to N- or C-terminal processing in the cell envelope or during purification.

Egmond and coworkers had shown that OmpT obtained after ion-exchange chromatography lacks the tightly bound lipopolysaccharide (LPS) molecule required for catalytic activity that is in contact with the polypeptide chain in the crystal structure (12). Purified OmpP was similarly inactive, but the catalytic activity could be fully restored by adding a 3 molar excess of LPS from *E. coli* (Sigma). Purified OmpP was found to be stable at 4°C and maintained over 90% of its specific activity after 2 months.

Library construction and screening. A phage library was constructed at the N terminus of the pIII protein of fd bacteriophage, which was comprised of the FLAG peptide epitope followed by a randomized sequence of eight amino acids constituting the putative protease cleavage site. The full sequence used was NH_2-DGDYKDDDDKGLEAXXXXXXXXGAETVES, where X can be any amino acid. The randomized amino acid sequence was encoded by the NNK (K = T/C) randomization scheme, which allows for codons for all possible amino acids, although a stop codon is possible at a predicted frequency of about 0.03. Following electroporation, a total of 1.1×10^8 CFU were obtained. While the number of individual clones in the library represents only a small fraction of the amino acid sequences obtainable from randomization of an octamer peptide (2.6×10^{10}), it should nonetheless be sufficient to capture the key properties of amino acids preferred by the protease (11). Sequencing of 12 randomly selected clones from this library revealed that each contained an insert with a unique octamer amino acid sequence. One out of the 12 had a stop codon in the randomized region.

Using purified OmpP, experiments were conducted to determine optimal conditions for the proteolytic release of phage bound on anti-FLAG-coated beads. A control phage, designated "RR," which encoded the known OmpP substrate sequence GHVVNHRR, was prepared. In addition, a negative-control, nonsubstrate phage designated "Non," with the sequence NHEANVHT, was prepared. The phages were prepared, purified, and incubated with 0 μM, 0.3 μM, 2 μM, or 40 μM OmpP at 37°C for 30 min. Cleavage by OmpP was detected by Western blotting using anti-FLAG and anti-pIII antisera (Fig. 1). The anti-FLAG antiserum will recognize only uncleaved pIII, while the anti-pIII antiserum will recognize both cleaved and uncleaved sequences. OmpP at a concentration of 2 μM completely cleaved the positive-control phage RR (6.0×10^{11} PFU), which therefore could no longer be recognized by anti-FLAG antibodies but was readily detected with anti-pIII antibodies. No cleavage of the same titer of the negative-control phage, Non, was detected under these conditions. At very high OmpP concentrations (40 μM), extensive nonspecific degradation of pIII was detected for both substrates in the Western blots.

Assuming that library phages containing a putative OmpP cleavage site must contain at least one basic residue and that basic residues are represented at a frequency of 0.125 with the NNK scheme, at most ~13% of the library phage could possibly be cleaved by OmpP. Consequently, if the same phage titer is employed for library screening as was used for the

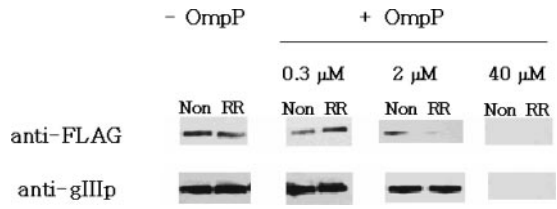


FIG. 1. Western blotting with anti-FLAG and anti-pIII of substrate phage and nonsubstrate phage after digestion by OmpP. "RR" indicates a substrate phage with an amino acid sequence of GHVVNHRR; it could be cleaved by OmpP. "Non" means a nonsubstrate phage with an amino acid sequence of NHEANVHT; it had no paired basic residues. The purified phages were digested by 0.3 μM, 2 μM, and 40 μM OmpP at 37°C for 30 min.

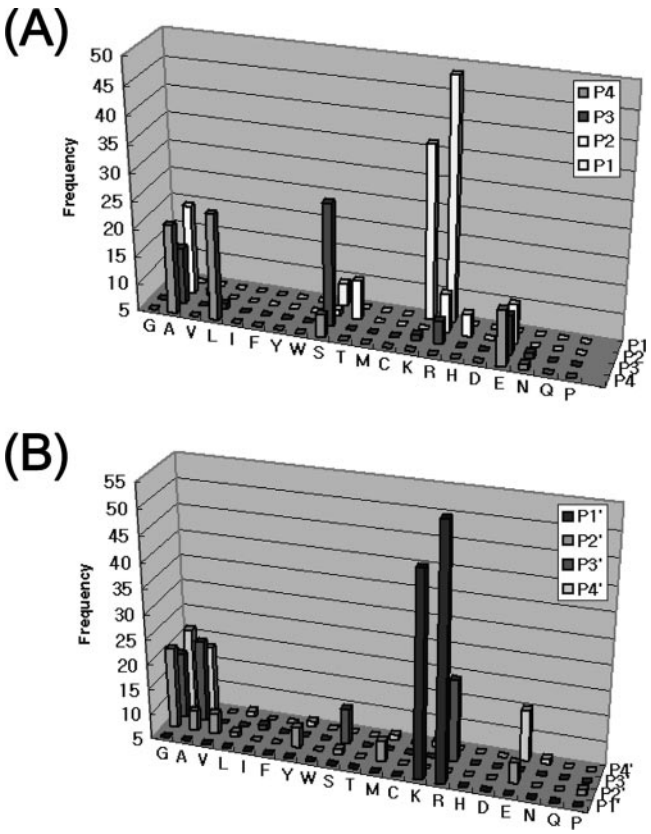


FIG. 2. Frequency of occurrence of amino acids at the P4 to P1 (A) and P1′ to P4′ (B) positions within phage from a random octamer peptide library cleaved by OmpP. These data represent the frequency of occurrence of each amino acid residue found at each of the substrate positions in 33 different phage peptides obtained from sequencing. A frequency greater than 5 is considered to be important; therefore, the *y* axis is cut off at this value.

control experiments described above, a roughly 10-fold-lower concentration of enzyme should be sufficient to give complete cleavage. Based on this rationale, library screening was carried out with a 0.2 μM concentration of enzyme.

Approximately 6.0×10^{11} PFU were bound to an excess of beads containing immobilized anti-FLAG antibodies and incubated with protease (0.2 μM) at 37°C for 30 min. In this format, incubation with protease results in the selective release of phage containing susceptible sequences from the immobilized support (19). Titering of the phage in the supernatant revealed the presence of 1.0×10^{10} PFU; thus, about 2% of the input phage were released after incubation with OmpP. For comparison, incubation under identical condition but without added protease resulted in 1.5×10^9 PFU in the supernatant.

A total of 33 clones from the fifth round of selection were sequenced to determine the amino acid compositions of their enzyme cleavage sites. No duplicate sequences were found, indicating that a large repertoire of susceptible substrates was present in the octamer randomized library. Several of these clones were amplified, and the resulting monoclonal phages were treated with OmpP and analyzed for loss of the FLAG epitope by Western blotting. As expected, all of the isolated clones exhibited partial or total cleavage of the randomized region, resulting in loss of recognition by anti-FLAG antibodies (data not shown).

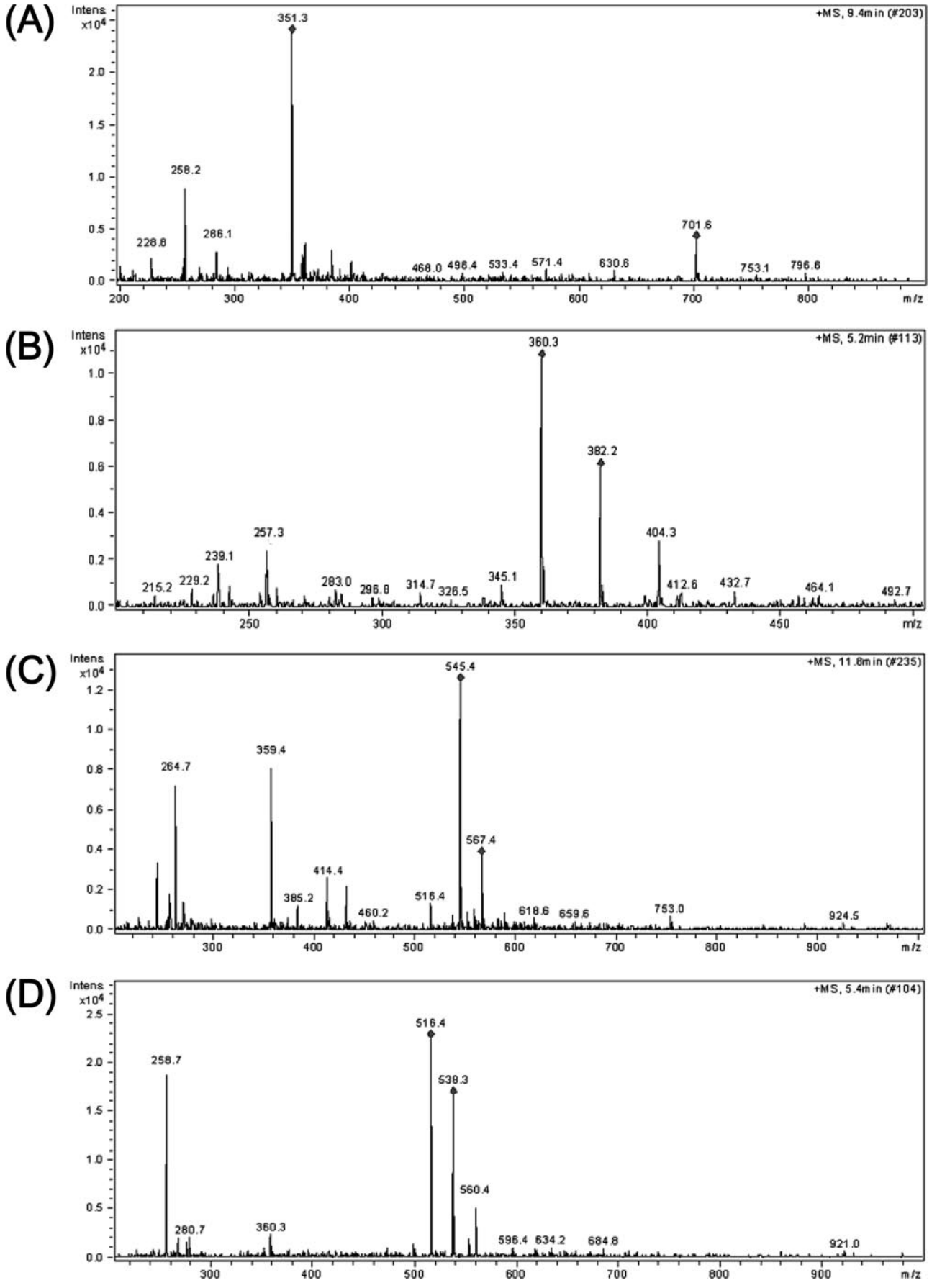
Determination of a consensus sequence. Twenty-eight of 33 fifth-round peptides contained pairs of Arg residues. Kaufmann et al. (10), who first purified OmpP, reported that it cleaves primarily between consecutive basic amino acid residues in a manner not unlike that of its homologue, OmpT. Therefore, pairs of Arg/Lys residues were assigned to the P1 and P1′ positions (Fig. 2). Five of 33 sequences contained only an Arg residue. ESI-MS analysis of the OmpP hydrolysis products from the peptide WLAASRGAG, having a sequence derived from a selected phage clone, revealed that cleavage occurred between Ser and the lone Arg; therefore, the latter residue occupies the P1′ position (see below). Ala was preferred at the P2 position (21% of clones). Thr and Arg were also found to occupy P2 with elevated frequencies (12% for both). A preference for Ser and Ala in the P3 position was also observed (27% Ser and 15% Ala). With the exception of basic residues at the P1 and P1′ cleavage sites, Ser occupation of P3 was the most highly conserved feature of selected OmpP substrates, suggesting that P3 is an important susbsite in OmpP (Fig. 2).

TABLE 3. Kinetic parameters for the OmpP- and OmpT-catalyzed hydrolysis of peptides[a]

Peptide	Amino acid sequence of peptide substrate[b]	OmpP			OmpT		
		k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
1	WLAARRGAG	13 ± 1	110 ± 20	$(1.2 \pm 0.4) \times 10^5$	8.0 ± 0.3	59 ± 10	$(1.4 \pm 0.3) \times 10^5$
2	WLAARRGRG	900 ± 150	350 ± 90	$(3 \pm 1) \times 10^6$	130 ± 40	110 ± 60	$(2 \pm 1) \times 10^6$
3	WLSARRGAG	168 ± 7	57 ± 6	$(3.0 \pm 0.2) \times 10^6$	67 ± 4	110 ± 10	$(6.1 \pm 0.3) \times 10^5$
4	WLRARRGAG	37 ± 5	32 ± 5	$(1.3 \pm 0.5) \times 10^6$	5.0 ± 0.3	12 ± 3	$(5 \pm 1) \times 10^5$
5	WLATRRGAG	9 ± 1	150 ± 40	$(7 \pm 2) \times 10^4$	5 ± 1	720 ± 220	$(8 \pm 4) \times 10^3$
6	WLARRRGAG*	2.2 ± 0.3	19 ± 4	$(1.3 \pm 0.5) \times 10^5$	1.2 ± 0.1	8 ± 3	$(1.8 \pm 0.8) \times 10^5$
7	WLSERRGAG	1.5 ± 0.1	240 ± 30	$(6 \pm 1) \times 10^3$	0.6 ± 0.1	740 ± 130	$(9 \pm 3) \times 10^2$
8	WLAASRGAG	0.17 ± 0.03	160 ± 50	$(1.2 \pm 0.5) \times 10^3$	0.03 ± 0.01	410 ± 140	$(9 \pm 5) \times 10$
9	WLAAKKGAG	13 ± 3	216 ± 84	$(8 \pm 4) \times 10^4$	1.2 ± 0.3	478 ± 183	$(3 \pm 1) \times 10^3$
10	WEEGGRRIGRGGK†	3.4 ± 0.2	23 ± 5	$(1.6 \pm 0.4) \times 10^5$	8.8 ± 0.7	55 ± 9	$(1.7 \pm 0.4) \times 10^5$

[a] Values are means ± standard deviations. We calculated the mean ratios of k_{cat}/K_m by considering the standard deviations of k_{cat} and K_m, as in the example that follows. For the cleavage of peptide 9 by OmpP, the k_{cat} is 13 ± 3 s^{-1} and the K_m is 216 ± 84 μM. The maximum k_{cat}/K_m (1.2×10^5 M^{-1} s^{-1}) was calculated from the maximum k_{cat} (16 s^{-1}) and the minimum K_m (132 μM). Similarly, the minimum k_{cat}/K_m value (3×10^4 M^{-1} s^{-1}) was calculated from the minimum k_{cat} (10 s^{-1}) and the maximum K_m (300 μM). The mean value and standard deviation of k_{cat}/K_m for peptide 9, $(8 \pm 4) \times 10^4$ M^{-1} s^{-1}, was derived from these maximum and minimum k_{cat}/K_m values.

[b] Symbols: *, the cleavage sites used by OmpP or OmpT were different; †, used as a control substrate for determination of the activity of OmpT in a previous study (36).


(A)
Intens.
x10^4
+MS, 9.4min (#203)
351.3
258.2
228.8
286.1
468.0
496.4
533.4
571.4
630.6
701.6
753.1
796.8
m/z
(B)
Intens.
x10^4
+MS, 5.2min (#113)
360.3
382.2
257.3
239.1
404.3
215.2
229.2
283.0
296.8
314.7
326.5
345.1
412.6
432.7
464.1
492.7
m/z
(C)
Intens.
x10^4
+MS, 11.6min (#235)
545.4
359.4
264.7
567.4
414.4
385.2
460.2
516.4
618.6
659.6
753.0
924.5
m/z
(D)
Intens.
x10^4
+MS, 5.4min (#104)
516.4
258.7
538.3
560.4
280.7
360.3
596.4
634.2
684.8
921.0
m/z

Overall, small aliphatic amino acids (Gly, Ala, Ser) were favored from P2′ to P4′. However, Arg and Glu were found to occupy P3′ (21% Arg) and P4′ (16% Glu) with elevated frequencies.

Hydrolysis rates of consensus-derived peptide substrates. Based on the frequency of the amino acids observed in the selected substrate phage, 10 peptides were designed and synthesized by solid-phase synthesis. Each peptide possessed an *N*-terminal Trp to facilitate detection in HPLC assays. Initial rates of hydrolysis by OmpP and OmpT were determined by measuring product formation via HPLC, and the kinetic parameters k_{cat} and K_m were evaluated by fitting the observed rate data to the Michaelis-Menten equation (Table 3). The kinetics of hydrolysis with OmpT were also measured to examine the degree of similarity in the catalytic properties of the two enzymes.

A peptide having the sequence WEEGGRRIGRGGK (peptide 10) was used as a control for OmpT activity (36). OmpP cleaved this peptide with nearly identical catalytic efficiency. Overall, OmpP is a very efficient enzyme and cleaved peptides containing an Arg-Arg sequence with k_{cat}/K_m values in the range of 6.4×10^3 $M^{-1}s^{-1}$ to 3.0×10^6 $M^{-1}s^{-1}$. Peptides 3 (WLSARRGAG) and 2 (WLAARRGRG) were the best substrate for OmpP, consistent with the finding that Ser or Ala occupation of P3 enhances enzyme activity. Occupation of P3 by Arg (peptide 4, WLRARRGAG), a residue that was represented in the round 5 sequences at a frequency slightly lower than that of Ala (3/33 and 5/33, respectively), led to a decrease in k_{cat}. However, K_m was also decreased, and thus only a modest reduction in overall catalytic efficiency was observed. Replacement of the preferred Arg in P3′ with the less-preferred Ala (peptide 1, WLAARRGAG) led to a 10-fold decrease in k_{cat}/K_m. Similarly, the presence of a Thr at the P2 position instead of the preferred Ala (peptide 5, WLATRR GAG) reduced the catalytic efficiency by 2 orders of magnitude. These amino acid substitutions had similar effects in the catalytic efficiency of cleavage by OmpT.

On the other hand, several differences in the substrate specificity of the two enzymes were also noted. Compared to OmpT, OmpP exhibited a sevenfold-higher k_{cat}/K_m value with peptide 7 (WLSERRGAG), which possessed a Glu at P2. In addition, OmpP exhibited an approximately 25-fold-higher k_{cat}/K_m value with peptide 9 (WLAAKKGAG), containing consecutive Lys residues. Peptide 8 (WLAASRGAG), containing only one Arg, was a moderate substrate for OmpP (1.2×10^3 $M^{-1}s^{-1}$) but was a poor substrate for OmpT (9.2×10 $M^{-1}s^{-1}$).

ESI-MS analysis was used to identify the positions of the OmpP and OmpT cleavage sites for each peptide. For substrates 1 to 5 and 7, cleavage occurred between two Arg-Arg residues for both enzymes. Peptide 8 was cleaved between Ser and Arg, and peptide 9 was cleaved between Lys and Lys, again by both enzymes. Interestingly, OmpP and OmpT were found to have disparate cleavage sites for peptide 6 (WLARRRG AG). OmpP cleaved between the second Arg and the third Arg, while OmpT cleaved between the first Arg and the second Arg (Fig. 3). No secondary cleavage product could be detected in either case.

Site-directed mutagenesis of OmpP. A structural model of OmpP was generated by Swiss PDB-DeepView, version 3.7 (9), with the OmpT crystal structure (PDB code, 1I78) used as a template. The generated structural model of OmpP was very similar to that of OmpT except for loop 4 (OmpT residues Asp214 to Lys217), which in OmpT was somewhat longer. Amino acids within 10 Å of four key catalytic residues (OmpT residues Asp83, Asp85, Asp210, and His212) were compared (Fig. 4). Ninety-four amino acids were included for OmpT, and 90 amino acids were included for OmpP. The two enzymes differ by a total of 16 amino acids in this region. Eight of the different residues (Glu28, Leu160, Lys165, Val211, Arg212, Thr214, Phe216, and Ser264 of OmpP) have side chains directed into the active site and are therefore potential determinants of substrate specificity. Site-directed mutagenesis was used to construct eight OmpP point mutants in which these residues had been replaced with the corresponding amino acids of OmpT (Glu28Leu, Leu160Phe, Lys165Arg, Val211Asp, Arg212Lys, Thr214Ile, Phe216Tyr, and Ser264Asn). Only variant Val211Asp showed a peptide 6 cleavage pattern different from that of wild-type OmpP. Cleavage by the Val211Asp mutant resulted in the OmpP product (cleavage at RR ↓ R) and the OmpT product (cleavage at R ↓ RR), indicating that this amino acid substitution is partly responsible for the change in cleavage specificity of the two enzymes (data not shown).

Cleavage of T7 RNA polymerase. The cleavage of an intact protein was used as a further probe of OmpP substrate specificity. Incubation of T7 RNA polymerase with purified OmpP followed by N-terminal sequencing of the two resulting proteolytic fragments (~80 kDa and ~20 kDa) suggests that cleavage occurred between Lys179 and Lys180 (-His-Val-Tyr-Lys-Lys-Ala-Phe-Met-). In contrast, a previous analysis of OmpT showed that the initial cleavage of T7 RNA polymerase occurs between Lys172 and Arg173 (-Gln-Leu-Asn-Lys-Arg-Val-Gly-His-) (17).

OmpP confers resistance to elevated concentrations of protamine. An F′ episome carrying the *ompP* gene was introduced into *E. coli* KS272 and SF100 (KS272 Δ*ompT*) by conjugation. Cell viability was examined in LB medium containing different concentrations of the antimicrobial peptide protamine, MPRRRRSSSRPVRRRRRPRVSRRRRRRG GRRRR. *E. coli* SF100 F′, which contains the episomal *ompP* gene, but not SF100, which lacks both OmpP and OmpT, was able to grow with 0.125 mg/ml protamine (data

FIG. 3. ESI-MS analysis of fragments of the substrate WLARRRGAG, cleaved by OmpP (A and B) and OmpT (C and D). The substrate WLARRRGAG (150 μM) was digested by 10 nM concentrations of either OmpP or OmpT in pH 6.1 MES buffer (50 mM) at 37°C for 30 min. (A) The ESI-MS peaks *m*/*z* 701.6 and 351.3 correspond to the masses of the protonated WLARR $[M+H]^+$ and $[M + 2H]^{2+}$, respectively. (B) The ESI-MS peaks *m*/*z* 360.3 and 382.2 correspond to the masses of the protonated RGAG $[M+H]^+$ and $[M+Na]^+$, respectively. (C) The ESI-MS peaks *m*/*z* 545.4 and 567.4 correspond to the masses of the protonated WLAR $[M+H]^+$ and $[M+Na]^+$, respectively. (D) The ESI-MS peaks *m*/*z* 516.4 and 538.3 correspond to the masses of the protonated RRGAG $[M+H]^+$ and $[M+Na]^+$, respectively.

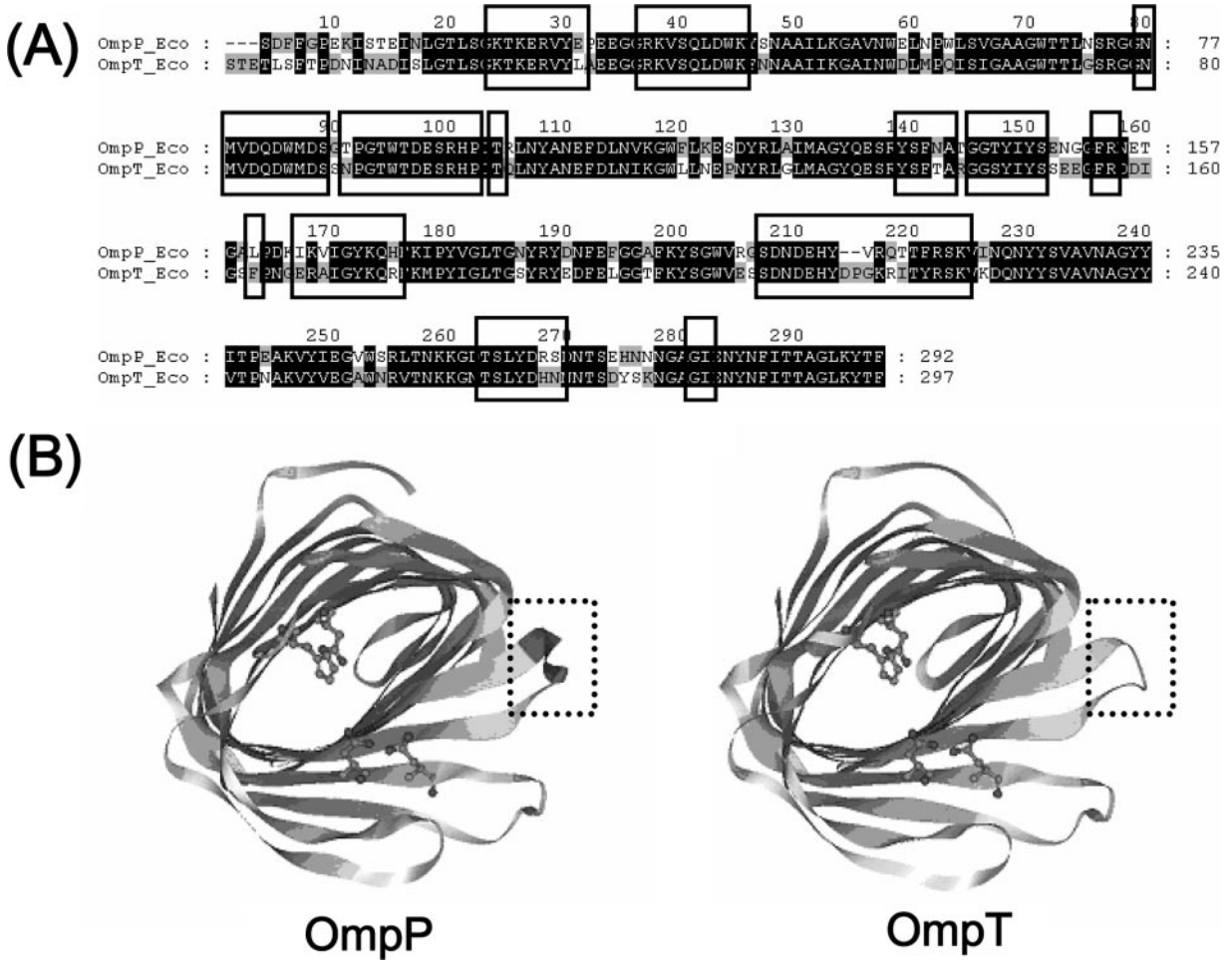


FIG. 4. Pairwise alignment of OmpP and OmpT (A) and structural models of OmpP and OmpT (B). (A) Introduced gaps are shown with hyphens. Amino acid residues within 10 Å of four key amino acids (Asp83, Asp85, Asp210, and His212) for the activity of OmpT are boxed. OmpP_Eco, CAA52338; OmpT_Eco, AAC73666. (B) The proposed catalytic residues are shown in the structural models of OmpP and OmpT. The regions corresponding to loop 4 are boxed.

not shown). KS272, the isogenic *ompT*$^+$ parent of SF100, exhibited greater resistance to protamine and was able to grow in the presence of 0.5 mg/ml of the antibiotic (Fig. 5A). However, only KS272 F′ could grow at higher protamine concentrations (Fig. 5C). We also determined the extent of degradation of protamine (0.8 mg/ml) by whole cells using HPLC analysis. After a 30-min incubation, the extent of protamine degradation in KS272 F′, KS272, SF100 F′, and SF100 was 33%, 21%, 8%, and 2%, respectively. Thus, the above data suggest that OmpP acts synergistically with OmpT to confer protection against protamine and possibly other cationic peptide antibiotics.

DISCUSSION

OmpP and OmpT are highly homologous proteins, with 71% identity and 86% similarity at the amino acid level. The level of identity increases to 78% when residues in or near the active sites are compared (i.e., within 10 Å of Asp83, Asp85, Asp210, and His212 for OmpT [34]) (Fig. 4). This high degree of active site homology might suggest that the substrate specificities of the enzymes should be similar; in fact, both proteases were found to preferentially cleave peptides between consecutive basic amino acid residues. The substrate specificity of OmpT has been investigated by several approaches (4, 20, 23, 24). McCarter et al. (20) determined the optimal subsite occupancy of OmpT by substrate phage display and showed that it has an overwhelming preference for Arg at the P1 and P1′ positions, with Lys as a less frequently observed alternative (12% at P1 and 12% at P1′, respectively).

In the present studies, we found that, like OmpT, OmpP accepts Arg at P1 and P1′ in a large fraction of the identified substrates (16/33 and 18/33, respectively). However, compared to OmpT, OmpP may have a greater ability to accept Lys, as a relatively large fraction of isolated substrate sequences contained Lys at P1 or P1′ (12/33 and 15/33, respectively). Considering differences in the codon frequency generated by the NNK scheme used to create the random oligonucleotide (1/32 for Lys and 3/32 for Arg), Lys might even be considered somewhat preferred by OmpP at both cleavage sites. Consistent with this conclusion, OmpP showed similar catalytic efficiencies for cleavage of peptide substrates containing Arg-Arg

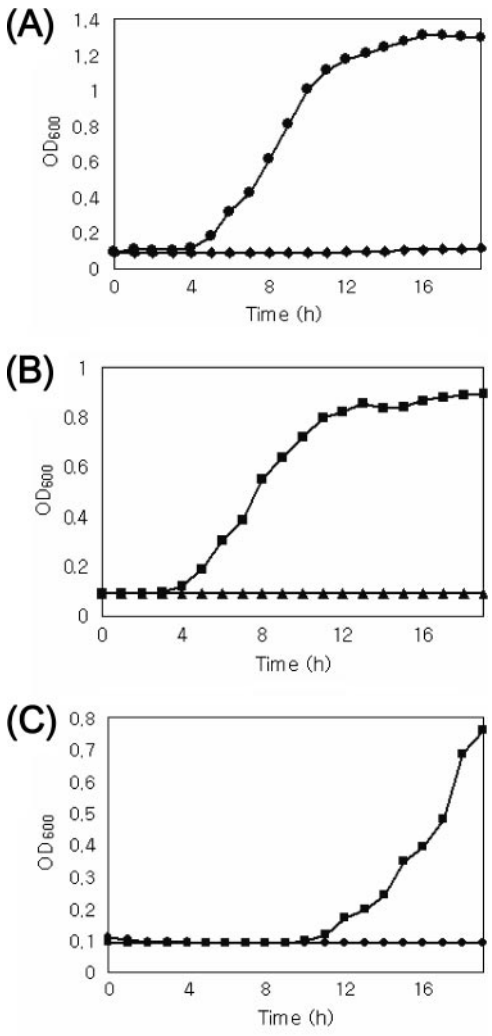


FIG. 5. Effects of *ompP* and *ompT* on protamine resistance. Protamine at 0.5 mg/ml (A and B) or 1 mg/ml (C) was added at time zero to cells growing in LB medium with shaking at 37°C in 96-well plates. The optical density at 600 nm was monitored on a Synergy HT microplate reader. The growth curves shown are as follows: ◆, SF100; ● KS272 (*ompT*$^+$); ▲, SF100 F′ carrying the episomal *ompP* gene; ■, KS272 (*ompT*$^+$) carrying the episomal *ompP* gene. The data points in the graphs are means of three independent trials.

(peptide 1) and Lys-Lys (peptide 9), and cleavage of T7 RNA polymerase occurred preferentially between two Lys residues (Lys179 and Lys180). In contrast, OmpT cleaved T7 RNA polymerase between Lys and Arg residues (Lys172 and Arg173), and it displayed a roughly 40-fold decrease in catalytic efficiency for the Lys-Lys peptide 9 substrate compared with peptide 1.

Another noticeable difference between the fine substrate specificities of OmpT and OmpP was revealed with the Arg-Arg-Arg peptide 6 (Table 3). Although both enzymes cleaved this substrate with similar overall catalytic efficiencies, product analysis revealed that they cleaved between different pairs of Arg residues. Taken together with the different propensities of the two enzymes to accept Lys at P1 or P1′, the cleavage site difference seen with an Arg-Arg-Arg sequence confirms that the two enzymes attack different cationic protein or peptide sequences with somewhat different efficiencies. This finding raises the possibility that one of the physiological roles of OmpP may be to complement the activity of OmpT in the deactivation of polycationic antimicrobial peptides. Such subtle differences may be especially important for cleavage of antimicrobial cationic peptides. Cleavage by both enzymes may result in more complete degradation of such peptides and prevent the formation of proteolytic products that still retain partial antimicrobial activity.

Previous studies indicated that OmpT favors Ala or Phe at the P2 position, while basic and hydrophilic amino acids are disfavored (4, 20). However, this P2 preference is not absolute, as the kinetic analysis in Table 3 shows that Glu (peptide 7) or Thr (peptide 5) is allowed at P2 of both OmpP and OmpT, although the k_{cat}/K_m values of the latter are almost an order of magnitude lower. Striebel and Kalousek investigated the specificity of OmpP using radioactively labeled precursor proteins (30). They reported that OmpP cleaved the *Neurospora* ubiquinol-cytochrome *c* reductase iron-sulfur subunit precursor between Arg and Ala and the rat ornithine transcarbamylase precursor between Ala and Leu. These findings disagree with earlier data (10) and with our analysis presented here. Furthermore, we could not detect any cleavage of peptides lacking a basic residue (data not shown).

In conclusion, by using substrate phage libraries, we have found that, in general, the substrate specificity of OmpP is similar to that of OmpT, with some important differences. In particular, differences in OmpP and OmpT fine specificities include the fact that OmpP appears to accept Lys at both P1 and P1′ somewhat better than does OmpT, and we observed a prominence of Ser at P3 in OmpP substrates. Likewise, OmpP showed higher activity for cleavage for a peptide possessing three consecutive Arg residues. The high activity of OmpP on peptides having consecutive Arg residues is likely to be significant for the detoxification of antimicrobial peptides. Indeed, we found that conjugative transfer of an F′ episome containing the *ompP* gene results in faster degradation of protamine, in turn allowing cell growth to proceed in the presence of higher concentrations (1 mg/ml) of this antimicrobial peptide. Wild-type *E. coli* cells expressing only OmpT do not survive under these conditions. Thus, it appears that OmpP acts synergistically with OmpT to enhance the ability of *E. coli* to resist the action of cationic antimicrobial peptides in host organisms.

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