The Type 4 Prepilin Peptidases Comprise a Novel Family of Aspartic Acid Proteases*

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Type 4 prepilins or prepilin-like-proteins are secreted by a wide range of bacterial species and are required for a variety of functions including type 4 pilus formation, toxin and other enzyme secretion, gene transfer, and biofilm formation. A distinctive feature of these proteins is the presence of a specialized leader peptide that is cleaved off by a cognate membrane-bound type 4 prepilin peptidase (TFPP) during the process of secretion. In this report we show that the TFPPs represent a novel family of bilobed aspartate proteases that is unlike any other protease. The active site pairs of aspartic acids of the two TFPPs in *Vibrio cholerae* are found at positions 125 and 189 of TcpJ and 147 and 212 of VcpD. Corresponding aspartate residues are completely conserved throughout this extensive peptidase family.

Members of the family of leader peptidases known as type 4 prepilin peptidases (TFPP) have been identified in a vast number of species of Gram-negative bacteria and an increasing number of Gram-positive bacteria. The TFPP is responsible for the cleavage and N-methylation (known collectively as processing) of the highly conserved type 4 leader peptide present at the N-terminus of the families of secreted proteins known as type 4 prepilins and type 4 prepilin-like proteins (1). Whereas the cleavage of the type 4 leader peptide is a necessary step for secretion of the mature pilin, methylation of the N-terminus of the mature pilin is not required for secretion and has no known function (2). Type 4 pilins are polymerized as the structural subunits of type 4 pili (Tfp), which are surface organelles required for diverse activities that contribute to broad attributes such as genetic transfer, virulence, and environmental persistence of a wide array of Gram-negative bacterial pathogens. Prototypical examples include microcolony formation mediated by toxin-coregulated pilus (TCP) of *Vibrio cholerae*, bacterial surface dispersal mechanisms mediated by bundle forming pilus of *Escherichia coli* (3), colonization and natural transformation mediated by PiE of *Neisseria* sp. (4), and gliding motility mediated by the Tfp of *Myxococcus* sp. (5).

The related type 4 pilin-like proteins partially comprise the main terminal branch of the general secretory pathway in Gram-negative bacteria, also known as the type 2 secretion pathway (6). The general secretory pathway is the pathway utilized by the majority of proteins that are secreted outside of Gram-negative bacteria. Such proteins include toxins such as cholera toxin of *V. cholerae* (7) and exotoxin A of *Pseudomonas aeruginosa* (8), as well as other enzymes such as pullulanase of *Klebsiella pneumoniae* (9) and hemolysin of *Aeromonas hydrophila* (10). Type 4 pilin-like proteins are also required for natural transformation of *Bacillus subtilis* (11) and *Streptococcus pneumoniae* (12).

The type 4 leader peptide is characterized by several features that are highly conserved, yet differ from those of standard leader peptides. The N-terminal leader that is cleaved from the mature protein is highly charged and immediately precedes a region of approximately 20 residues that are predominantly hydrophobic and are retained within the mature protein. The majority of type 4 prepilins or prepilin-like proteins are designated type 4a, containing leader peptides that are short (~6 residues). Type 4b preproteins contain a longer leader (~25 residues). The peptide bond on the prepeilin that is hydrolyzed by the TFPP lies between the charged and hydrophobic domains, immediately C-terminal to an invariant glycine. The new N-terminal amino acid that arises upon processing is a N-methylated phenylalanine for type 4a proteins and is typically a N-methylated methionine for type 4b proteins. The TFPPs themselves are integral cytoplasmic membrane proteins with numerous transmembrane domains. The processing reaction has been postulated to occur on the cytoplasmic side of the membrane because of the membrane orientation of the prepilin, the location of all the major nonmembrane peptidase domains, and the likelihood that the cytoplasmically located S-adenosyl methionine would provide an available source of methyl groups for the methylation step of the reaction (13).

Previous studies on the proteolytic mechanism of the TFPPs have focused on the largest cytoplasmic region of the protein designated cytoplasmic domain 1. These studies were performed on PilD, a prototypic TFPP from *P. aeruginosa*. Domain 1 of the PilD peptidase contains two pairs of cysteine residues that are conserved among many members of the TFPP family. Alteration of the 4 highly conserved cysteine residues by substitution of alanine or serine residues resulted in up to an 80–100% reduction of peptidase activity in an *in vitro* assay. However, the processing defect varied from approximately 0% to 80% for the various mutants *in vivo*, suggesting that although pilin cleavage was less efficient, it was not abolished (14). Chemical inhibitor studies supported the notion that the cysteine residues contributed to protease activity and PilD was classified as a cysteine protease, specifically as a member of the C20 family of cysteine proteases (15). The retention of partial activity by some of the *pilD* mutants as well as the subsequent discovery of TFPP’s that lack the consensue cysteine residues
have brought this mechanism of protease activity into question. For example, the XpsO TFPP of Xanthomonas campestris is characterized and shown to lack all the domain 1 cysteine residues, yet the xpsO gene fully complements a psd deletion for prepilin processing (16). Some newer members of the rapidly growing family lack the entire domain 1 region. Analysis of a current TFPP alignment also reveals a complete lack of any conserved histidine residue, a necessary component of the cysteine protease catalytic mechanism (15). These observations have led to a revised classification of PsdL and, therefore, the complete family of TFPPs, to the U12 category of proteases with unknown catalytic mechanism (17).

In the study reported here, an approach similar to the comprehensive mutational strategy employed to identify the signal peptidase I active site (18) was used to determine the active site residues of TcpJ, the TFPP responsible for processing the TcpA peptidase catalytic mechanism (15). These observations have led to a revised classification of PiiD, and therefore the complete family of TFPPs, to the U12 category of proteases with unknown catalytic mechanism (17).

For example, the XpsO TFPP of E. coli K38, which does not express TcpA, and MR90, which overexpresses TcpA prepilin at elevated temperature, 20 μl of a membrane preparation from K38 and from MR90 were subjected to electrophoresis on a 12.5% SDS-PAGE gel alongside a standard of 3 μg of trypsin, followed by staining with Coomassie Blue. The stained gel was dried using the Novex gel drying system. The dried gel was scanned by a Molecular Dynamics Personal Densitometer SI, and densitometry analysis was performed using ImageQuant version 1.2 software. The intensities (measured in pixels) of the 25-kDa trypsin band and the TcpA prepilin band were normalized to the intensity of the corresponding band in the TcpA-negative control lane (K38), and an approximately 28-kDa band from both K38 and MR90 were measured while adjusting to a single background value. The mass of the TcpA prepilin present in the MK90 lane was determined by first subtracting the intensity of the corresponding area in the K38 TcpA prepilin-lacking lane. This corrected for the staining intensity of minor protein bands that co-migrate with TcpA prepilin. By comparing the intensity values of the 28-kDa bands from the TcpA-positive and TcpA-negative lanes, a further adjustment was made to the TcpA prepilin value to account for any loading difference between the lanes. The trypsin band intensity was divided by 3 μg to determine an intensity/μg value, which was applied to the final TcpA prepilin band value to determine the mass of TcpA prepilin/μl in the membrane preparation.

The TcpA prepilin membrane preparation was diluted with 50 mM Tris, pH 8.0, buffer to a concentration of 0.2 μg/μl.

In Vitro Cleavage Assay—The in vitro TcpJ cleavage assay was designed, based on the assay developed for PsdL of P. aeruginosa (26), except that pre-TcpA was provided in a membrane preparation rather than as purified protein. TcpJ derivatives were provided in membrane preparations from the TcpJ-expressing strain JM109 (wild-type and mutant alleles). The 100-μl processing reaction was prepared by combining a 50-μl substrate fraction with a 50-μl enzyme fraction (an equivalent of 1 unit of wild-type activity) and incubated at 37 °C for 1 h. The substrate fraction was prepared by combining 5 μl of the TcpA prepilin-containing membrane preparation (1 μg of TcpA prepilin), 10 μl 0.5% (w/v) cardiolipin, 20 μl of 5× assay buffer (125 mM triethanolamine, 7.5% v/v glycerol, X-7.1001, and brought up to the total volume with H2O). The enzyme fraction was prepared by combining a standardized volume of TcpJ-containing membrane preparation and H2O to bring the volume to 50 μl. The cleavage reaction was stopped by the addition of 100 μl of 2× protein sample buffer. The volume of TcpJ preparation for all TcpJ derivatives was standardized to be equivalent to the volume containing an amount of wild-type TcpJ sufficient to cleave 50% of 1 μg of TcpA prepilin in 1 h, which is defined as 1 unit of TcpJ peptidase activity. The formation of mature TcpA from precursor was monitored by Western immunoblot analysis.

Chemical Protease Inhibitors—Chemical inhibitors were tested for their ability to prevent the cleavage of TcpA from the mature form in the in vitro cleavage assay. Various concentrations of inhibitors were added to both the enzyme (1 unit of TcpJ) and substrate fraction so that, when combined, the inhibitor concentration would remain constant. The inhibitor was incubated in the enzyme fraction at room temperature for 30 min prior to the combination of the two fractions. The 100-μl reaction was allowed to proceed for 1 h at 37 °C before 100 μl of 2× SDS protein sample buffer was added to stop the reaction. The specific concentrations of each inhibitor are listed in Table I. The 1× assay buffer, 0.001% (v/v) Triton X-100, and 1 mM EDTA (EDAC) glycine–amide inhibition protocol is a two-step chemical reaction based on the procedure developed for the selective modification of carboxyl side groups of aspartic and glutamic acids in proteins (27). Takahashi et al. (28) first suggested the use of this procedure as a method to specifically inhibit acid proteases. Membranes containing the equivalent of 6 units of TcpA activity are incubated in 80 μl of 25 mM
potassium biphthalate, pH 4.0, and 100 mM EDTA for 30 min at room temperature. 20 μl of 1× glycinamide was added to the mixture, and incubation at room temperature was continued for an additional 30 min before the reaction was stopped by the addition of 150 μl of 200 mM Tris buffer, pH 8.0. The mixture was then dialyzed against 50 mM Tris, pH 8.0, for 1 h and then centrifuged at 230,000 × g (60,000 rpm in TLA 100.3 rotor) for 15 min. The resulting membrane pellet, which contains the chemically modified TcpJ was resuspended in 60 μl of 50 mM Tris, pH 8.0. 20 μl of the membrane suspension, which contains 2 units of TcpJ, was used in the in vitro cleavage assay to determine the peptidase activity. Activity of the modified TcpJ was compared with an in vitro cleavage assay to determine the peptidase activity. Activity of the modified TcpJ was compared with an in vitro cleavage assay to determine the peptidase activity.

**Mutagenesis of tcpJ**—The TcpJ S46A, C76A, D189A, and K191A alterations were constructed in pCL10 by the Quikchange™ (Stratagene) method of mutagenesis, which utilizes inverse PCR primed by divergent overlapping primers containing the desired complementary nucleotide changes. The S46A, C51A, S65A, C73A, S81A, S65A, D125A, D125E, D125N, D125Amb, S172A, D183N, D189E, D189N, D189Amb, S191A, D212A, and D212A were constructed in pCL10, and D147A and D212A were constructed in pJM294 using other methods of inverse PCR primed by nonoverlapping divergent primers. Both primers contained an engineered common restriction site that allowed for the restriction of the PCR product by the appropriate enzyme followed by the ligation at the complementary DNA overlaps. In these cases, only one primer carried the desired mutation that was incorporated into the amplified DNA. In some cases the engineering of restriction sites into the primers was accompanied by the incorporation of silent mutations. In other primers, seamless cloning and mutagenesis was performed by incorporating the restriction enzyme site EarI or SphI at the 5’ end of each primer in the pair. These enzymes cleave just outside their recognition site, which makes possible a primer design that ensures that the recognition site is cleaved off the end of the PCR product, while complementary gene sequence overlaps remain to promote annealing prior to ligation.

**Immunoblot Analyses—**In vivo TcpA processing was monitored in *E. coli* and *V. cholerae* strains grown to mid-log phase except where indicated. 0.5 ml of cell culture was centrifuged and resuspended in 100 μl of LB. 100 μl of 2× SDS-PAGE sample buffer was added to the sample. The total sample was boiled for 5 min, and 50 μl was subjected to electrophoresis on a 12.5% SDS-PAGE gel. For both in vivo and in vitro processing, the proteins were electroblotted to nitrocellulose and probed with anti-TcpA peptide antisera. The secondary antibody, an anti-rabbit IgG conjugated to horseradish peroxidase, was used to detect TcpA by chemiluminescence using ECL reagents (Amersham Pharmacia Biotech), followed by autoradiography.

For detection of TcpJ, 20 μl of a membrane preparation from a TcpJ-expressing strain was combined with 20 μl of 2× SDS-PAGE sample buffer and incubated at 37 °C for 15 min. 20 μl of the sample was then subjected to electrophoresis on a 12.5% SDS-PAGE gel. Proteins were transferred to nitrocellulose and probed with anti-TcpJ peptide J3–1 antisera or, in the case of TcpJ (6)His and TcpJ (6)His35–81, with TetraHis Ab (Qiagen). Detection with secondary antibody was carried out as described for TcpA.
indicated in Fig. 2. 16 residues including the 7 serines, 4 cysteines, 3 aspartic acids, 1 lysine, and 1 glutamic acid indicated as shaded residues in Fig. 1 and 2 were selected for mutagenesis. 13 of the 16 potential active site residues targeted for mutagenesis are all of the residues conserved at greater than the arbitrary level of 50% in TcpJ. The three selected residues that did not meet this criterion are Ser172, Asp183, and Ser212. Ser172 of TcpJ may be functionally equivalent to residues Ser168 and Ser171 indicated as (S168) and (S171) in Fig. 1, which, in combination, are approximately 65% conserved. Asp183 and Glu183 (Fig. 1, E183) in combination are conserved in nearly 80% of the 27 TFPPs. Aspartic acid and glutamic acid can function equivalently as the active site of an acid protease (29). Mutagenesis of Ser212 was required due to the construction of the S212A/S213A double mutant, which was necessary so the analysis of the phenotype of the S213A would not be ambiguous due to the remaining serine in the adjacent position.
activity. The four mutants D125A, D125N, D189A, and D189N completely lacked all peptidase activity, as would be expected when an active site residue of a protease is converted to a nonfunctional substitute. The D125E and D189E mutants retain the carboxylic acid moiety at the R-group terminus and both retain partial peptidase activity. Processing by S212A/S213A double mutant is not shown because it was later determined that the mutant TcpJ is unstable.

In order to test the peptidase activity expressed by the tcpJ mutant constructs in V. cholerae, they were introduced into V. cholerae strain CL381, an O395 derivative with an insertional disruption in tcpJ and an in-frame deletion of vcpD, which render it completely devoid of TcpA processing activity. The resultant strains were grown under TCP-inducing conditions (LB, pH 6.5, 30 °C) and total cell protein extracts were examined by Western immunoblot analysis of TcpA (Fig. 3B). The CL381 double mutant was utilized for this analysis, because disruption of tcpJ alone does not lead to a complete loss of TcpA processing, as evidenced by the detection of some processed TcpA in the J71K-1 extract. The CL381 extract shows that the residual processing is due to VcpD, a second TFPP present in V. cholerae. Similar to the results seen in JM109, tcpJ mutants S18A, S46A, C51A, S65A, C73A, C76A, S81L, E88A, S172A, D183N, and K191A exhibited peptidase activity identical to wild-type TcpJ while C48A possessed an intermediate peptidase activity, whereas the D125E and D189E mutants restored peptidase activity, providing further support for the assignment of Asp\textsuperscript{125} and Asp\textsuperscript{189} as the active site residues.

To further extend the number of different amino acid substitutions analyzed at the 125 and 189 positions of TcpJ, amber (TAG) codons were individually engineered at each corresponding position on the pCL10 plasmid and the resultant constructs were introduced into Amber-Lys, Amber-Leu, Amber-Gln, Amber-Ser, and Amber-Tyr suppressor strains of E. coli. The strains were grown to mid-log phase, and whole cell protein extracts were examined for cleavage of TcpA by Western immunoblot analysis (Fig. 3C). No amino acid substitution at either position 125 or 189 exhibited any peptidase activity. The protein levels and membrane localization of the altered TcpJ proteins were all comparable to wild-type TcpJ.

TcpJ peptidase activity was also measured by an in vitro assay modeled after the in vitro peptidease assay devised for PilD of P. aeruginosa (26). TcpA-containing membranes from MK90 and TcpJ-containing membranes from CL318 were combined in the in vitro assay. A series of assays with an increasing volume of TcpJ-containing membranes was used to determine the volume of membrane preparation corresponding to 1 unit of TcpJ activity, which is the amount required to cleave 50% of 1 μg of TcpA prepilin into the mature form in 1 h (Fig. 4A).

The peptidase activity of proteins encoded by a subset of the tcpJ mutants was determined in the in vitro cleavage assay. TcpJ proteins with alterations S46A, C51A, S65A, C73A, and C76A were all capable of cleaving TcpA prepilin in the in vitro assay. C48A, the double mutant S212A/S213A, and the Ala, Glu, and Asn changes at positions 125 and 189 abolished all peptidase activity (Fig. 4B). These results are consistent with the in vivo analysis except for the C48A, D125E, and D189E proteins, which exhibited intermediate peptidase activity in vivo had no peptidase activity in vitro. This is not surprising since studies on PilD of P. aeruginosa have shown that the in vitro assay is a more stringent test of peptidase activity than the in vivo conditions (14). The double mutant S212A/S213A has no peptidase activity due to the fact that the enzyme is unstable as shown by the lack of any TcpJ present in TcpJ.

Asp\textsuperscript{125} and Asp\textsuperscript{189} Are the Sole Residues Required for Peptidase Activity—Alteration of the targeted residues was performed by site-directed mutagenesis of the 16 selected positions in tcpJ present on plasmid pCL10, which also carries tcpA encoding the TcpA type 4 prepilin that is the cognate substrate in vivo. Mutant and wild-type constructs were introduced into E. coli JM109, and total cell protein extracts from midlog cultures were analyzed for TcpJ protease activity by Western immunoblot detection of precursor and mature forms of TcpA (Fig. 3A). TcpJ proteins with alterations S18A, S46A, C51A, S65A, C73A, C76A, S81L, E88A, S172A, D183N, and K191A exhibited peptidase activity identical to wild-type TcpJ, whereas C48A displayed an intermediate level of peptidase activity. The four mutants D125A, D125N, D189A, and D189N completely lacked all peptidase activity, as would be expected when an active site residue of a protease is converted to a nonfunctional substitute. The D125E and D189E mutants retain the carboxylic acid moiety at the R-group terminus and both retain partial peptidase activity. Processing by S212A/S213A double mutant is not shown because it was later determined that the mutant TcpJ is unstable.

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immunoblot. All other TcpJ derivatives were detected at levels approximately equal to wild-type except for C48A and C51A, which gave faint or undetectable TcpJ signals. This is likely due to the anti-TcpJ antiserum, which is directed against a peptide that encompasses Cys^48 and Cys^51. It is likely that C48A and C51A mutants alter the epitope in TcpJ sufficiently so that the protein is not detected by the antiserum.

Cytoplasmic Domain 1 Is Not Required for TcpJ Peptidase Activity—In order to definitively determine whether the cysteine residues or any portion of cytoplasmic domain 1 is required for TFPP cleavage activity, an internal in-frame deletion corresponding to positions 35–81 of TcpJ was constructed. Since our TcpJ antibody is directed against a peptide within the region removed by the deletion, the construction was made in the 6-His epitope-tagged version of tcpJ contained on plasmid pCL11, yielding plasmid pCL11Δ35–81. TcpJ (6)His and the deletion derivative expressed from pCL11 and pCL11Δ35–81 were both able to cleave TcpA to equivalent extents in E. coli (Fig. 3A), V. cholerae (Fig. 3B), or in vitro (Fig. 4B). Interestingly, TcpJ (6)HisΔ35–81 was found to process TcpA efficiently enough to restore pilation to the tcpJ mutant strain, J71K-1 (Fig. 5).

VcpD Requires a Pair of Aspartic Acid Residues Corresponding to Those Necessary for TcpJ Activity—In order to determine the extent to which the TcpJ requirement for the aspartic acid residue pair can be generalized to other members of the TFPP family, the corresponding changes of D147A and D212A were made in VcpD. The VcpD TFPP is responsible for processing type 4 prepilins such as those involved in mannose-sensitive pilus formation and processes the extracellular protein secretion type 4 prepilin-like proteins required for the secretion of toxin and other enzymes by V. cholerae. VcpD can also cleave TcpA prepilin, although the cognate TFPP for TcpA is TcpJ (19). Wild-type, D147A, and D212A forms of VcpD expressed from plasmid pJM294 derivatives were introduced into E. coli JM109 carrying the tcpJ-expressing plasmid pRTH3G7. Strains were grown to midlog phase, whole cell protein extracts were made, and the extracts were examined by SDS-PAGE and Western immunoblot analysis with anti-TcpA antiserum (Fig. 6A). Wild-type VcpD cleaved TcpA prepilin completely under these conditions, whereas the D147A and D212A mutant derivatives were totally defective. Protein levels of VcpD in the four strains were determined by Western immunoblot analysis using the anti-TcpJ antiserum, which cross-reacts with VcpD. The D147A and D212A forms of VcpD are present at levels similar to those in wild-type VcpD. An analogous result was seen with the cleavage of the type 4 prepilin-like protein substrate of VcpD, EpsI (Fig. 6B). These experiments demonstrate that alanine substitutions at Asp^147 and Asp^212, the aspartic acid residues in VcpD that correspond to the peptidase active site Asp^125 and Asp^189 of TcpJ, completely eliminate peptidase activity for multiple substrates.

Protease Inhibitor Studies of TcpJ Activity Support the Essential Role of the Aspartic Acid Residues for Protease Activity—Utilizing the TcpJ in vitro cleavage assay, a number of chemical protease inhibitors were examined for their ability to block the peptidase activity of TcpJ (Table I). Two or more inhibitors specific to each class of protease family were tested...
for their ability to prevent TcpJ peptidase activity. The majority of the inhibitors had little or no effect on the peptidase activity, as measured by the comparison of cleaved TcpA produced in the in vitro assay in the presence or absence of inhibitor. All inhibitors were tested at the maximum suggested concentration except for the EDAC/glycinamide inhibition protocol, for which it was not required. Of all the common protease inhibitors used, only NEM inhibited cleavage greater than 50%. This agrees with the strong inhibitory effect of NEM on inhibitors used, only NEM inhibited cleavage greater than 50%. This agrees with the strong inhibitory effect of NEM on the peptidase family. For example, the typical catalytic residues of serine proteases are the three amino acids serine, aspartic acid, and histidine, but several families of serine proteases such as the signal peptidase I family utilize only serine and lysine as the catalytic residues (30). The most conserved histidine residue in the TFPP family, His\(^{189}\), is only present in 30% of the members (Fig. 1, (H30)), thus ruling out involvement of the S/D/H motif in the catalytic mechanism. The numerous highly conserved serines as well as Lys\(^{190}\), conserved in nearly 80% of the TFPP family members, raised the possibility that the proteolytic activity might involve a Ser/Lys catalytic mechanism. A further possibility was that the TFPP could be a cysteine peptidase. This potential mechanism was suggested by the pilD mutational analysis of the 4 highly conserved cysteines in the peptidase family. Mutations at the 4 cysteines failed to completely eliminate peptidase activity in vivo, but in an in vitro assay these altered proteases were nearly completely deficient of peptidase activity (14). However, since all cysteine proteases require both a cysteine and a histidine as the catalytic dyad (15), the lack of a highly conserved histidine in the TFPP family excludes any known cysteine catalytic mechanism. The possibility of an aspartic acid protease mechanism is strongly suggested for the TFPP family due to the presence of two completely conserved aspartic acid residues corresponding to TcpJ positions 125 and 189 in domains 2 and 3, respectively (Fig. 2B). However, Asp\(^{125}\) and Asp\(^{189}\) do not exist in the motif D(T/S)G, which is common to the majority of aspartic acid proteases (31). In addition, the pH optimum, which has been determined to be near neutral for TcpJ (data not shown) and PilD of P. aeruginosa (26), is in contrast to the highly acidic pH optimum of the majority of aspartic acid proteases (32). The final peptidase family comprises the metalloproteases, for which over half contain the active site motif, HEXXH (32). The TFPP family lacks any homology to the HEXXH metalloprotease motif. In light of this sequence alignment analysis, an aspartic acid protease mechanism was deemed to be the most probable correct assignment for TFPPs. However, all potential protease active site residues conserved at greater than 50% were targeted for mutagenesis in order to determine the active site with certainty.

The results from the mutational analysis of TcpJ at the 16 selected positions clearly demonstrate that only the two aspartic acids corresponding to positions 125 and 189 of TcpJ are essential for peptidase activity. Substitution of the amino acids alanine, asparagine, leucine, serine, tyrosine, glutamine, and lysine into positions 125 and 189 of TcpJ produced a stable and properly localized enzyme that lacked any protease activity. This caused for this loss of peptidase activity of the mutants at positions 125 and 189 was determined to be the loss of the carboxylic acid group of the aspartic acid. The carboxylic acid functional groups of the active site aspartic acids directly participate in the general acid-base catalysis that causes the hydrolysis (cleavage) of the peptide bond (32). When the carboxylic acid functional group was restored at positions 125 or 189 by the substitution of glutamic acid, the protease activity was also restored. The additional one carbon in length of the glutamic acid R-group was less tolerated at position 189 than 125, suggesting that the location in space of the R-group carboxylic acid at position 189 is critical to protease activity. It can be inferred from the constraint at position 189 that the carboxylic acid group of Asp\(^{189}\) directly participates in the initial chemical reduction of the pre pilin peptide bond while Asp\(^{125}\) contributes to the hydrolysis through interaction with a water molecule.
Demonstration that the complete loss of protease activity for all amino acid substitutions at positions 125 and 189 was not simply due to a conformational change was also addressed by the asparagine and serine substitutions at these positions. Asparagine and serine have small polar R-groups similar to the R-group of aspartic acid and thus would likely maintain the wild-type conformation of the enzyme.

Only one of the altered forms of TcpJ, the double mutant S212A/S213A form, was unstable and could not be assessed for protease activity. The replacement of both hydrophobic serine residues with hydrophobic alanine residues occurs at a loop region between transmembrane domains and likely prevented the proper integration of the mutant peptidase into the membrane, resulting in its degradation. In lieu of a cleavage activity result of the S212A/S213A mutant, other evidence indicates a protease active site serine does not exist at position 212 or 213.

Asparagine and serine have small polar R-groups similar to the R-group of aspartic acid and thus would likely maintain the wild-type conformation of the enzyme. Inhibition by EDAC/glycinamide strongly implicates the dependence on aspartic acids for activity of the enzyme. Inhibition by the EDAC/glycinamide protocol also suggests that the active site aspartic acids are accessible to chemical inhibitors and not shielded by the protein conformation of the cytoplasmic domains. The only protease inhibitor other than EDAC/glycinamide that inhibited at greater than 50% was NEM, which acts by modifying thiol groups of the protease Active Site of TFPP

The TFPPs differ from the majority of aspartic acid proteases in that the active site aspartic acids are not found in the D/T/S/G motif, the optimum pH for in vitro activity is near neutral as opposed to pH 2–4, and peptidase activity is not inhibited by pepstatin. Other aspartic acid proteases exist that possess these unusual traits individually. Signal peptidase II, an aspartic acid protease that cleaves prolipoproteins, does not possess the D/T/S/G motif at the active site (33). The human aspartic acid protease renin has a pH optimum range of 5.5–7.5 (34). *Pseudomonas* sp. 101 carboxyl proteinase is a bacterial aspartic acid protease that is insensitive to pepstatin (35). Aspartic acid proteases, which are insensitive to pepstatin, are often referred to as non-pepsin-like acid proteases (28). The majority of aspartic acid proteases like pepsin and human

### Table I

| Chemical(s)                  | Specificity                     | Suggested concentration | Concentration | Cleavage % |
|-----------------------------|--------------------------------|-------------------------|---------------|------------|
| No inhibitor                |                                |                         |               |            |
| K-64                        | Cysteine proteases             | (1.4–28 µM) (1–10 µM)  | 28 µM         | 96         |
| Calpain inhibitor I         | Cysteine proteases             | 45 µM                   | 45 µM         | 87         |
| NEM                         | Cysteine proteases             | 1 mM                    | 1 mM          | 15         |
| Aprotinin                    | Serine proteases               | (0.01–0.3 µM)           | 0.3 µM        | 100        |
| 3,4-DCI                     | Serine proteases               | (5–200 µM) (5–100 µM)  | 100 µM        | 65         |
| Pefabloc SC                 | Serine proteases               | (0.4–4 mM)              | 2 mM          | 62         |
| Leupeptin                   | Ser and Cys proteases          | 1 µM (1–10 µM)          | 10 µM         | 85         |
| PMSF                        | Ser and Cys proteases          | (0.1–1 mM) (3 mM)       | 1 mM          | 89         |
| Phosphoramidon              | Metallopeptidases              | (0.007–0.6 mM)          | 0.6 mM        | 89         |
| EDTA-Na                     | Metalloproteases               | (0.5–1.3 mM)            | 1.3 mM        | 100        |
| Bestatin                    | Amino peptidase(metallo-)      | 130 µM                  | 130 µM        | 81         |
| Pepstatin                   | Aspartic proteases             | 1 µM (1–5 µM)           | 1 µM          | 95         |
| EDAC/glycinamide            | Acid protease                  | 0.1 M/1.0 M             | 0.1 M/0.2 M   | 2          |

*a* Roche Molecular Biochemicals.

*b* Prolysis, a protease and protease inhibitor Web server (T. Moreau).

*c* Ref. 14.

*d* K. Takahashi, personal communication.
immunodeficiency virus type 1 protease are bilobed molecules in which the active site cleft is located between the lobes with an active site aspartic acid contributed from each lobe. In this respect, the TFPPs display a remarkable similarity. The active site can be envisioned as a cleft between two lobes corresponding to domains 2 and 3 with one active site aspartic acid residue in each lobe.

The comprehensive mutational analysis and chemical inhibitor studies that led to the discovery of the active site of TcpJ are ultimately most significant in that they allow for the prediction of the protease active site of the entire TFPP family. The identity of the active site, as well as the ability to specifically target the active site aspartic acids by a chemical protocol, provide the foundation for the development of specific protease inhibitors of all members of the TFPP family. Such inhibitors would have substantial antimicrobial effects on a wide range of pathogenic bacteria.

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REFERENCES
1. Strom, M. S., Nunn, D. N., and Lory, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2494–2498
2. Pepe, J. C., and Lory, S. (1998) J. Biol. Chem. 273, 19120–19129
3. Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R., and Chon, C. (1995) J. Biol. Chem. 270, 27349–27354
4. Tonjum, T., and Koomey, M. (1997) J. Biol. Chem. 272, 5816–5821
5. Wu, S. S., and Kaiser, D. (1995) Mol. Microbiol. 19, 1074–1078
6. Marsh, J. W., and Taylor R. K. (1998) Mol. Microbiol. 29, 1481–1492
7. Russel, M., and Model, P. (1984) J. Biol. Chem. 259, 1034–1039
8. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 32, 103–119
9. Kaufman, M. R., Seyer, J. M., and Taylor, R. K. (1991) Genes Dev. 5, 1834–1846
10. Shaw, C. E., and Taylor, R. K. (1990) Infect. Immun. 58, 3042–3049
11. Dubnau, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5261–5266
12. Pestova, E. V., and Morrison, D. A. (1998) Methods Enzymol. 244, 461–486
13. Rawlings, N. D., and Barrett, A. J. (1995) Methods Enzymol. 244, 436–457
14. Strom, M. S., Bergman, P., and Lory, S. (1993) J. Biol. Chem. 268, 15788–15794
15. Rawlings, N. D., and Barrett, A. J. (1994) Methods Enzymol. 244, 183–194
16. Hu, N. T., Lee, P. F., and Chen, C. (1995) Mol. Microbiol. 18, 769–777
17. Rawlings, N. D., and Barrett, A. J. (1999) Nucleic Acids Res. 27, 325–331
18. Tschantz, W. R., Sung, M., Delgado-Partin, V. M., and Dalbey, R. E. (1993) J. Biol. Chem. 268, 27349–27354
19. Strom, M. S., and Lory, S. (1991) J. Bacteriol. 173, 1175–1180
20. Russel, M., and Model, P. (1984) J. Biol. Chem. 259, 1034–1039
21. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 32, 103–119
22. Kaufman, M. R., Seyer, J. M., and Taylor, R. K. (1991) Genes Dev. 5, 1834–1846
23. Shaw, C. E., and Taylor, R. K. (1990) Infect. Immun. 58, 3042–3049
24. Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078
25. Pfau, J. D., and Taylor, R. K. (1998) J. Bacteriol. 180, 4724–4733
26. Nunn, D. N., and Lory, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3281–3285
27. Hoare, D. G., and Koshland, D. E., Jr. (1966) J. Am. Chem. Soc. 88, 2955–2958
28. Takahashi, K., Tanokura, M., Inoue, H., Kojima, M., Muto, Y., Yamazaki, M., Makabe, O., Kimura, T., Takizawa, T., Hamaya, T., Suzuki, E., and Miyano, H. (1991) Structure and Function of the Aspartic Proteinases, pp. 253–261, Plenum, New York
29. Takahashi, K., Kagami, N., Huang, X. P., Kojima, M., and Inoue, H. (1998) Adv. in Exp. Med. Biol. 436, 275–292
30. Rawlings, N. D., and Barrett, A. J. (1994) Methods Enzymol. 244, 19–61
31. Rawlings, N. D., and Barrett, A. J. (1995) Methods Enzymol. 248, 105–120
32. Rao, M. B., Tanksale, A. M., Ghatge, M. S., and Deshpande, V. V. (1998) Methods Enzymol. 288, 325–331
33. Sankaran, K., and Wu, H. C. (1995) Methods Enzymol. 244, 169–180
34. Inagami, T. (1981) Biochemical Regulation of Blood Pressure, pp. 39–73, John Wiley & Sons, Inc., New York
35. Reeves, P. J., Douglas, P., and Salmon, G. P. (1994) Mol. Microbiol. 12, 445–457
36. Lory, S., and Strom, M. S. (1997) Gene 192, 117–121