The Haloprotease CPI Produced by the Moderately Halophilic Bacterium *Pseudoalteromonas ruthenica* Is Secreted by the Type II Secretion Pathway[▽][†]

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The gene (*cpo*) encoding the extracellular protease CPI produced by the moderately halophilic bacterium *Pseudoalteromonas ruthenica* CP76 was cloned, and its nucleotide sequence was analyzed. The *cpo* gene encodes a 733-residue protein showing sequence similarity to metalloproteases of the M4 family. The type II secretion apparatus was shown to be responsible for secretion of the haloprotease CPI.

Moderately halophilic microorganisms include a broad variety of bacteria that are able to grow in media containing a wide range of elevated NaCl concentrations (3 to 15% NaCl) (56). Considerable attention has been focused on enzymes of moderately halophilic bacteria, since they have substantial biotechnological potential (29, 56). While several proteases from extreme halophiles, members of the halophilic archaea, have been characterized (12, 15, 19, 36, 44, 49, 50, 51, 57), fewer proteases from moderately halophilic bacteria have been purified and studied in depth (9, 14, 18, 20, 21, 33).

The moderately halophilic bacterium *Pseudoalteromonas ruthenica* CP76 was isolated from a saltern located in Isla Cristina (Huelva, Spain). This strain was selected for its ability to produce an extracellular protease, haloprotease CPI, which has a molecular mass of 38.0 kDa (46, 47). The haloprotease CPI was purified and biochemically characterized. It showed optimal activity at 55°C and pH 8.5 and tolerated a wide range of NaCl concentrations (0 to 4 M NaCl). In this work, we describe the molecular characterization of the operon encoding the haloprotease CPI and a second protease, CPII. We also report the first study of the mechanism used for the secretion of haloprotease CPI to the extracellular medium.

The N-terminal amino acid sequence of the haloprotease CPI (ADATGPGGNQKTGQYNY) (47) allowed us to identify several homologues of CPI in a BLAST search (1). We aligned (16, 53) metalloproteases from *Vibrio*, *Aeromonas*, or *Pseudomonas* species (3, 6, 23, 30). On the basis of the conserved regions, degenerate PCR primers AminoProL (GGYC CWGGYGGGYAAYCAAAAR) and ProR (CCNGNATRTC HGARAANGCTTC) were designed. We first amplified a 500-bp DNA fragment from the total DNA (27) of *P. ruthenica* CP76, which was then used in two rounds of inverse PCR (37)

to obtain a 4,680-bp fragment. Two open reading frames (ORFs) were found in this fragment (Fig. 1A). The first of them, *cpo* (2,202 bp), starts at position 148 and ends at position 2,349, encoding a polypeptide of 733 amino acids with an estimated molecular mass of 78.8 kDa. The mature secreted CPI protease corresponded to residues Ala-212 to Lys-542 of the *cpo* gene product, on the basis of its N-terminal sequence and molecular mass (38.0 kDa) (47). A BLAST analysis of the protein sequence deduced from *cpo* against the NCBI CDD database (26) revealed that the gene encodes a modular enzyme consisting of four domains: the signal sequence (28 amino acids); the N-terminal proregion (183 amino acids); the mature protease region (331 amino acids) of the M4 superfamily, with two distinct N-terminal and C-terminal domains; and the C-terminal prodomain (191 amino acids). The mature protease region of CPI showed sequence homology to metalloproteases belonging to the zincins superfamily (peptidase M4), such as EmpI from *Pseudoalteromonas* sp. strain A28 (69% identity) (25) or the metalloprotease MprI from *Alteromonas* sp. strain 0-7 (66% identity) (31). The mature protease has the highly conserved HEXXH zinc-binding motif (HEVSH) that is characteristic of this protease family (32). In the thermolysin family, another conserved amino acid sequence (the third zinc ligand motif, GXXNEXXSD) is generally found together with the HEXXH motif. The motif GGLNAFSD, comprised of residues Gly-372 to Asp-380, was also found in the protease CPI (see Fig. S1 in the supplemental material).

The *cpo* gene encodes a precursor protein with a molecular mass of 78.8 kDa. However, the haloprotease CPI has been purified as a biologically active enzyme with a molecular mass of only 38.0 kDa (47). This indicates that, upon removal of the signal peptide, the protein is proteolytically processed upstream of His-211, delineating a proregion commonly found in the microbial proteases (32). The size of the remaining polypeptide (522 amino acids) is about 55.7 kDa, indicating that the C-terminal propeptide containing the two bacterial prepeptidase C-terminal (PPC) domains is also removed by proteolytic processing. Other extracellular bacterial proteases undergo similar proteolytic processing (28); for example, it has been suggested that the zinc metalloprotease from the halophilic bacterium

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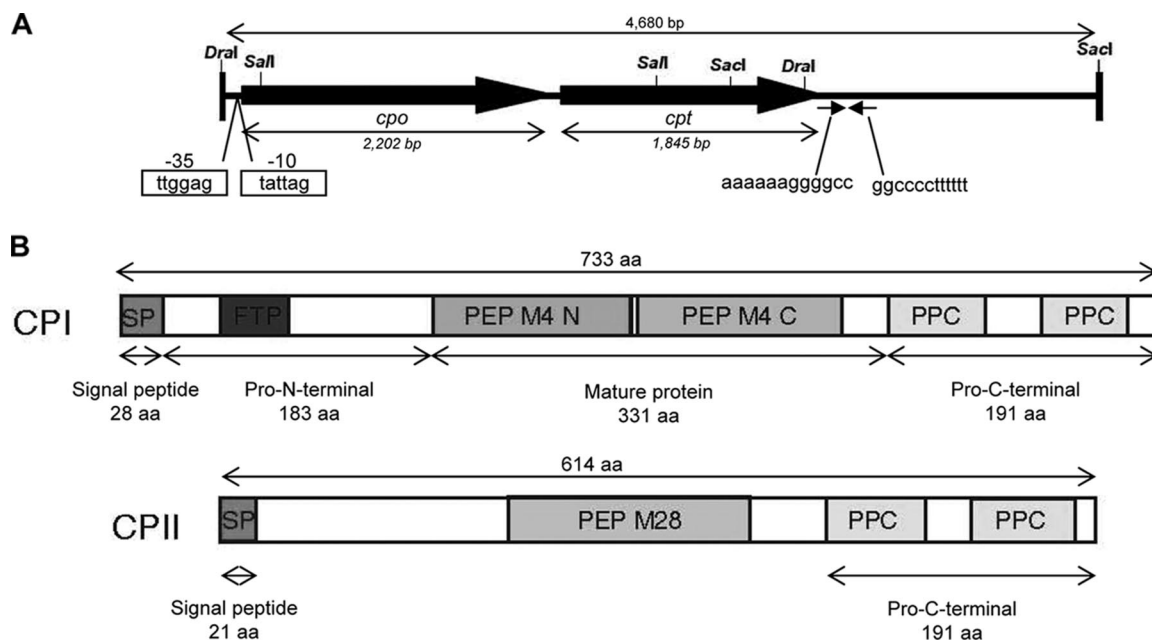
FIG. 1. (A) Restriction map of the 4,680-bp fragment. Arrows indicate the *cpo* (2,202 bp) and *cpt* (1,845 bp) ORFs and the direction of transcription. The restriction sites used in the inverse PCR strategy are indicated. The promoter region's −10 and −35 sequences are indicated in boxes. The sequences of the terminator palindromes are indicated. (B) Domain structures of CPI and CPII, determined by the conserved protein domain database (CDD) server. SP, signal peptide; FTP, fungalysin propeptide motif; PEP M4 N, thermolysin metallopeptidase N-terminal catalytic domain; PEP M4 C, thermolysin metallopeptidase C-terminal catalytic domain; PEP M28, peptidase M28 superfamily domain; aa, amino acids.

Salinivibrio sp. strain AF-2004 also undergoes both N- and C-terminal processing (21).

ORF2, designated *cpt* (positions 2409 to 4258), encodes a protein of 614 amino acids with a predicted molecular mass of 65.9 kDa. A putative Shine-Dalgarno sequence, GGGAG, was found 7 bp upstream from the start codon (ATG) of the *cpo* gene. Analysis of the sequence upstream of the *cpo* initiation codon allowed us to identify a putative promoter region, with TTGGAG for the −35 and TATTAG for the −10 sequence, respectively. A putative transcriptional terminator is found 17 bp downstream from the *cpt* stop codon. The *cpt* gene encodes a protein with high similarity to M28 metalloproteases, such as the aminopeptidase from *Vibrio cholerae* (40% identity) (13), leucyl aminopeptidase from *Vibrio proteolyticus* (41% identity) (54), and the metalloprotease MprII from *Alteromonas* O-7 (58% identity) (31). The *cpt* gene product is synthesized with a Sec-type signal peptide of 21 residues. Its catalytic domain, belonging to the aminopeptidase M28 family, is followed by the C-terminal prodomain containing two PPC domains, very similar to those found in CPI. These C-terminal polypeptides are found in various proteases produced by gram-negative bacteria. The PPC domains could be necessary for the correct folding, secretion, or maintenance of the proteases in an inactive form inside cells and are removed upon secretion (5, 22, 35).

To test whether the *cpo* and *cpt* genes constitute an operon, we carried out reverse transcription-PCR assays with primers based on their intergenic-region sequences. RNA was extracted from exponential-phase cultures of *P. ruthenica* CP76 with an RNeasy mini kit (Qiagen) and was treated with DNase I (Amersham Biosciences). Retrotranscription was performed

using a Transcriptor first strand cDNA synthesis kit with random hexamer primers (Roche). The PCRs were performed using 35 cycles of 1 min at 94°C, 30 s at 53°C, and 45 s at 72°C (45). Three pairs of primers were designed: RT-PCR1F (CC AAGCGATGACCTTCGG) and RT-PCR2R (CTTTTGTGT ATCCCAGCCGC) were designed on the basis of the region upstream of *cpo* (288 bp), RT-PCR3F (GTTCGCAATCAAC CACCAGC) and RT-PCR4R (GTGCATAAAGTCGCTTAG GCG) were designed on the basis of the region upstream of *cpo* and the region downstream of *cpt* (448 bp), and RTPCR5F (GAGGAAGTGGGACTCAGAGGC) and RTPCR6R (GA GCTCGTTATCACTCGGTGG) were designed according to the region downstream of *cpt* (444 bp). A product of the expected size, 448 bp, corresponding to the intergenic region between *cpo* and *cpt*, was amplified. These results confirmed that *cpo* and *cpt* are transcribed as a single transcriptional unit. The MprI and MprII proteases (31), homologues to CPI and CPII, respectively, are translated from an operon with the same genetic organization as that of the *cpo-cpt* operon of *P. ruthenica* CP76. This suggests that these enzymes have a common regulation mechanism, secretion pathway, and complementary function in bacterial physiology.

Little is known about protein secretion in halophilic bacteria. A wide spectrum of gram-negative bacteria utilize the type II secretion pathway for extracellular release of proteins, such as hydrolytic enzymes and toxins (39). Since the CPI precursor is synthesized with an N-terminal signal peptide, we hypothesized that the type II secretion pathway is used to secrete CPI. Some of the most highly conserved components of the type II secretion machineries are the secretins of the PulD family (2). We designed degenerate oligonucleotide primers WmpDL (G
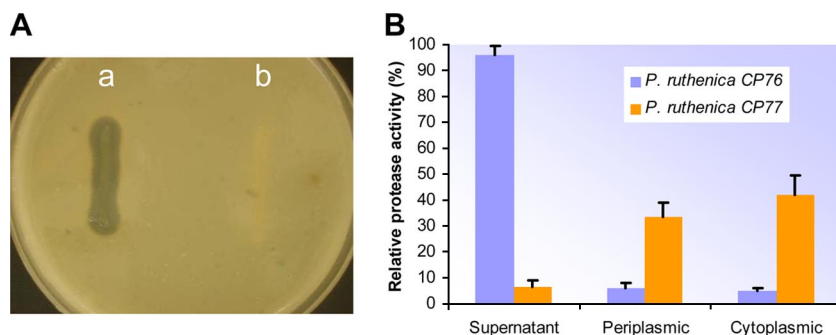
FIG. 2. Extracellular protease activity and enzymatic proteolytic assay of *P. ruthenica* CP76 and *P. ruthenica* CP77. (A) Clear zones around colonies of *P. ruthenica* CP76 show the protease activity (a); extracellular protease activity was not detected for *P. ruthenica* CP77 (b). (B) Results of enzymatic assay of proteolytic activity in the extracellular, periplasmic, and cytoplasmic fractions. Error bars show standard deviations.

TCGCTGATGAGCGTACCAAT) and WmpDR (TGATAC TTCTTGCTCAAT) on the basis of sequence alignments of PulD homologues from several gram-negative bacteria (1, 7, 10, 13, 17, 22, 42, 52). We amplified a 912-bp fragment from the *P. ruthenica* CP76 total DNA, corresponding to a fragment of a secretin gene, which we designated *wmpD*. Using an inverse PCR strategy, we amplified and cloned a 3,897-bp DNA fragment corresponding to the DNA for the PulD-type secretin from *P. ruthenica* CP76. Sequencing of this fragment revealed the presence of one complete and two partial consecutive ORFs in the same orientation. The first ORF showed homology to genes in the *pulC* family (encoding type II secretion component protein C) and was therefore designated *wmpC*. The second ORF, designated *wmpD*, encodes a 688-amino-acid protein with a molecular mass of 74.8 kDa that is highly similar to the secretin D of several type II secretion systems. The third ORF, designated *wmpE*, encodes a polypeptide with the highest identity scored for the type II secretion component PulE (40, 48).

To assess the involvement of the type II secretion pathway in the secretion of the extracellular enzymes of *P. ruthenica* CP76, the *wmpD* gene was disrupted by the insertion of an $\Omega(Sm^r)$ interposon (38) in plasmid pPD2 and subcloned into the suicide vector pJQSK200 (Gm^r) (41). The mutation was transferred by a double-recombination event to the chromosome of the wild-type strain by triparental mating using pRK600 (Cm^r) as the helper plasmid (55). The recombinant exconjugants were identified as Sm^r Rf^r Gm^s colonies on SW2 plates (47) containing 10% sucrose. The correct insertion of the $\Omega(Sm^r)$ cassette in the chromosomal *wpmD* gene was checked by PCR, and the *wmpD*::ΩSm mutant was designated *P. ruthenica* CP77. The qualitative assay, carried out by performing a modified version of the method of Kunitz (24) on SW10 skim milk plates (Fig. 2A), revealed that *P. ruthenica* CP77 is unable to secrete active CPI haloprotease. The periplasmic and cytoplasmic fractions were obtained by osmotic shock applied to the cells according to the method of Neu and Heppel (34). *P. ruthenica* CP77 showed 6% of the protease activity in the extracellular fraction seen in the parent strain. In contrast, upon cell disruption, *P. ruthenica* CP77 exhibited 34% and 42% protease activities in the periplasmic and cytoplasmic fractions, respectively, relative to the levels in the parent strain, whereas *P. ruthenica* CP76 only showed trace activities in these fractions (Fig. 2B). Thus, *P. ruthenica* CP77 does not secrete the pro-

tease and shows increased intracellular protease accumulation. This finding indicates that the type II secretion components transfer the haloprotease CPI across the outer membrane. In addition, CPI secretion by the type II pathway is required for its proteolytic activation.

Here we show that the haloprotease CPI from *P. ruthenica* CP76 is secreted by the type II secretion apparatus via its dependence on secretin D, the secretion portal of this system (4). The family of PulD proteins comprises several membrane-associated proteins that are essential for transport of macromolecules across bacterial envelope. Amino acid sequence analysis revealed a possible modular organization of proteins of this superfamily, with highly conserved C-terminal domains and dissimilar N-terminal domains. In the C-terminal domain, four highly conserved regions have been found, one of them containing a remarkable common motif: (V,I)PXL(S,G)XIPX XGXLF (11). These four regions and the highly conserved motif have been found in *P. ruthenica* CP76. In *P. ruthenica* CP76, the gene for this protein, *wmpD*, is flanked by *wmpC* and *wmpE*, as is often the case in other bacteria.

It appears, therefore, that the type II secretion system is well adapted to cope with the extreme conditions under which the bacterium *P. ruthenica* CP76 grows (concentrations of NaCl as low as 0.5% NaCl and as high as 17.5% NaCl). It would be of interest to identify specific features of the type II secretion system and its substrates that are linked to the ionic-strength conditions inside or outside the cells. The absence of extracellular protease activity in the *wmpD* mutant suggests that not only CPI but also the CPII protease may be secreted by the same pathway. This study raises a number of questions about the mechanisms of adaptation of the proteins of halophilic bacteria. While the halophilic archaea use the twin-arginine translocation (Tat) pathway for protein secretion (8, 43), the moderately halophilic gram-negative bacterium described in this work exports the haloprotease CPI to the medium using the two-step pathway. Similar to the twin-arginine pathway, the type II secretion system allows secretion of substrates that are prefolded, in this case usually in the oxidative periplasmic compartment. It would be interesting to determine if the metalloprotease produced by *Salinivibrio* sp. strain AF-2004 (21) uses the same pathway and whether the type I secretion system, which secretes protein in an unfolded state, functions in moderately halophilic bacteria.

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