Journal of Bacteriology

Autocatalytic Maturation of the Tat-Dependent Halophilic Subtilase Nep Produced by the Archaeon Natrialba magadii

Diego M. Ruiz, Roberto A. Paggi, Maria I. Giménez and Rosana E. De Castro

J. Bacteriol. **2012, 194(14):3700. DOI: 10.1128/JB.06792-11. Published Ahead of Print 11 May 2012.**

JB
Journals.ASM.org

Autocatalytic Maturation of the Tat-Dependent Halophilic Subtilase Nep Produced by the Archaeon *Natrialba magadii*

Diego M. Ruiz, Roberto A. Paggi, Maria I. Giménez, and Rosana E. De Castro

Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales Universidad Nacional de Mar del Plata–CONICET, Mar del Plata, Argentina

Halolysins are subtilisin-like extracellular proteases produced by haloarchaea that possess unique protein domains and are salt dependent for structural integrity and functionality. In contrast to bacterial subtilases, the maturation mechanism of halolysins has not been addressed. The halolysin Nep is secreted by the alkaliphilic haloarchaeon *Natrialba magadii*, and the recombinant active enzyme has been synthesized in *Haloferax volcanii*. Nep contains an N-terminal signal peptide with the typical Tat consensus motif (GRRSVL), an N-terminal propeptide, the protease domain, and a C-terminal domain. In this study, we used Nep as a model protease to examine the secretion and maturation of halolysins by using genetic and biochemical approaches. Mutant variants of Nep were constructed by site-directed mutagenesis and expressed in *H. volcanii*, which were then analyzed by protease activity and Western blotting. The Tat dependence of Nep secretion was demonstrated in Nep RR/KK variants containing double lysine (KK) in place of the twin arginines (RR), in which Nep remained cell associated and the extracellular activity was undetectable. High-molecular-mass Nep polypeptides without protease activity were detected as cell associated and extracellularly in the Nep S/A variant, in which the catalytic serine 352 had been changed by alanine, indicating that Nep protease activity was needed for precursor processing and activation. Nep NSN 1-2 containing a modification in two potential cleavage sites for signal peptidase I (ASA) was not efficiently processed and activated. This study examined for the first time the secretion and maturation of a Tat-dependent halophilic subtilase.

Subtilisin-like proteases (subtilases) are a functionally diverse superfamily of serine proteases produced by prokaryotes and eukaryotes that include both intra- and extracellular enzymes (30). These proteases serve in a number of biotechnological applications (11, 18, 24); thus, subtilases from extremophiles represent an attractive resource for both basic and applied science.

Secretory subtilases are synthesized as prepro-enzymes consisting of a signal peptide (SP) responsible for secretion, N-terminal and/or C-terminal propeptides, and the catalytic domain (S8 family). Bacterial prepro-subtilisins are processed by removal of the pre- and/or propeptides to render the active extracellular enzymes (30). Comparatively less is known regarding how extracellular proteases are transported through the archaeal membrane and processed into fully active enzymes. In this context, the maturation mechanism of the Sec-dependent subtilisin-like proteases secreted by the hyperthermophilic archaeon *Thermococcus kodaraensis* have been extensively characterized (8, 33). Many halophilic bacteria and archaea produce extracellular proteases, however, there is limited information on the pathways used by halophiles to deliver secretory proteases (27, 28), and the mechanism that leads to enzyme activation has not been examined.

Most extracellular and membrane anchored proteins are exported through the general secretory (Sec) pathway, which is conserved in all life forms (21). The Sec pathway translocates a wide variety of unfolded substrates, including proteolytic enzymes. On the contrary, the specialized Twin arginine translocation (Tat) pathway is unique in its ability to deliver fully folded proteins into and across the cytoplasmic membrane of a variety of prokaryotes and the thylakoid membrane of chloroplasts (25). The Tat secretory apparatus in *Escherichia coli* and plant thylakoids is composed of three integral membrane proteins: TatA, TatB, and TatC. TatA forms large homo-oligomeric ring-like structures that surround a central cavity which accommodates the native protein, while TatBC complexes are responsible for substrate interaction (20). In contrast, the Tat secretory apparatus of archaea and Gram-positive bacteria, except *Streptomyces* species, lacks the TatB component (23). Although TatA is strictly localized to the membrane in *E. coli*, TatA homologs have also been detected in the cytoplasm of *Bacillus subtilis*, *Streptomyces lividans*, and *Haloferax volcanii* and may be involved in substrate targeting (23).

Extreme halophilic archaea (haloarchaea) thrive in salt concentrations >2 M NaCl and, thus, they have adapted their overall physiology to optimally function under this extreme unusual condition. Genomic surveys have shown that the majority of proteins in haloarchaea are secreted through the Tat pathway and contain SP having the twin-arginine motif (RR) (13, 26). Therefore, it was proposed that the extensive use of the Tat secretory pathway in haloarchaea may ensure that secreted proteins are partially or fully folded before translocation, reflecting an evolutionary adaptation to the high salt concentrations that predominate in the environments where these organisms inhabit, as well as inside their cytoplasm (23). However, in the extreme halophilic bacteria *Salinibacter ruber* secreted proteins are predicted to rely on the Sec pathway (5), suggesting that Tat preference is a distinct adaptation of haloarchaea.

The subtilisin-like extracellular proteases produced by haloarchaea are denoted as halolysins and have been characterized from a number of species, including *Natrialba asiatica* (16), *Haloferax mediterranei* R4 (15), and *Natrinema* sp. strain J7 (28). Our labo-

Received 4 January 2012 **Accepted** 2 May 2012
Published ahead of print 11 May 2012
Address correspondence to Rosana E. De Castro, decastro@mdp.edu.ar.
Supplemental material for this article may be found at http://jb.asm.org/.

doi:10.1128/JB.06792-11

ratory has cloned and sequenced the gene encoding halolysin Nep from the haloalkaliphilic archaeon *Natrialba magadii* and, using this gene, the recombinant active enzyme was successfully synthesized in *H. volcanii* (3). The *nep* gene encodes a 541-amino-acid polypeptide with an estimated molecular mass of 56 kDa. By protein sequencing of the mature enzyme purified from *N. magadii* culture medium, the N-terminal sequence of Nep was determined and is encoded at nucleotides +364 to +406 from the translation start point (3), suggesting that Nep is synthesized with a 121-amino-acid residue propeptide (12 kDa) that is cleaved to generate the mature protease (43 to 44 kDa). Within this propeptide the Tat signal motif (G**RR**SVL) was identified, indicating that Nep is most likely secreted by the Tat pathway. In addition, Nep contains a C-terminal domain (CTD) that is common to halolysins but is not conserved in most bacterial subtilases. Recently, it was demonstrated that the CTD of the halolysin-like protease produced by *Natrinema* sp. strain J7 contributes to the stability of this protease at high salt concentrations and assists enzyme activity on protein substrates (35). Although halolysins are related to subtilases of bacterial origin, the presence of distinct protein domains (CTD) and the fact that they are structurally and functionally adapted to extreme saline conditions suggest that these enzymes may have unusual features with regard to their secretion-maturation mechanism. On the other hand, processing/activation of predicted Tat-dependent proteases has not been investigated in any organism.

In this report, by using a genetic approach we have experimentally demonstrated that halolysin Nep is secreted through the Tat system and is maturated autocatalytically. To the best of our knowledge, this is the first study which shows the (*cis*)-maturation of a subtilase *in vivo*. Evidence is provided suggesting that SP cleavage is relevant to induce protease maturation.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Fermentas (Glen Burnie, MD), T4 DNA ligase was purchased from Promega (Madison, WI), *Taq* DNA polymerase was purchased from PB-L Quilmes Argentina, and *Pfx*50 DNA polymerase was purchased from Invitrogen. Azocasein was purchased from Sigma-Aldrich (St. Louis, MO) and yeast extract from Oxoid (Remel, Lenexa, KS). Plasmid DNA was isolated by using a QIAprepSpin miniprep kit and DNA fragments were purified using a QIAquick gel extraction kit (Qiagen). All other chemicals and reagents were analytical grade and were supplied by Sigma-Aldrich.

Strains, media and growth conditions. Microorganisms used in the present study are indicated in Table 1. Cells were grown at 37 or 42°C in liquid culture (150 rpm) or on solid medium supplemented with 1.5% (wt/vol) agar. *E. coli* was grown in Luria-Bertani (LB) medium, *N. magadii* ATCC 43099 was grown in Tindall medium (1984) containing yeast extract (5 g/liter), and *H. volcanii* was grown in YPC medium (6). Media were supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or novobiocin (2 to 4 μg/ml) as needed. *E. coli* DH10β was used to establish the plasmids containing the *nep* mutant versions. *H. volcanii* spheroplasts were transformed with plasmids obtained from *E. coli* GM33 (Δ*dam*) (1). Cell growth was determined by measuring the optical density of the cultures at 600 nm (OD_{600}).

Construction of *nep* variants. Nep mutant variants were generated by QuikChange site-directed mutagenesis (Stratagene) using pET-*nep* (3) as a template and the primers listed in Table 1. Candidate mutant constructs were confirmed by sequencing the entire *nep* coding region. The *nep* variants were excised from the pET constructs with NdeI and BpU1102I, cloned into the shuttle vector pJAM, and amplified in *E. coli* GM33 to obtain unmethylated DNA. pJAM derivates were introduced in *H. volcanii* spheroplasts, plated onto selective YPC medium containing novobiocin (4 μg/ml) and 0.8% (wt/vol) skim milk, and incubated at 42°C. *H. volcanii* recombinant colonies were transferred onto YPC fresh plates containing novobiocin (2 μg/ml) and skim milk and then incubated at 37°C. Nep secretion and protease activity were evidenced by the appearance of clear halos around the colonies. The presence of plasmids containing *nep* and derivatives in the recombinant clones was confirmed by PCR.

Sample processing. *N. magadii* cultures were grown in alkaliphilic medium at 37°C for various times. *H. volcanii* cells harboring pJAM-*nep* wild type (wt) and derivatives were grown in YPC-novobiocin (2 μg/ml) at 37°C to an OD_{600} of ~1.5. The cultures were harvested by centrifugation (10,000 × *g*, 15 min, 4°C), suspended in 50 mM Tris-HCl (pH 8) containing 3 M NaCl (OD_{600} of ~20), and disrupted by ultrasonography (six times for 30 s each, 60 W). Cell debris were removed by centrifugation (10,000 × *g*, 30 min, 4°C). The supernatant (cell extracts) and cell-free culture medium were stored at 4°C until use in enzyme activity assays. To determine the subcellular localization of Nep in *N. magadii*, cell extracts were fractionated by ultracentrifugation (200,000 × *g*, 2 h, 4°C). Pellets (membrane fraction) were suspended in the same buffer and centrifuged (200,000 × *g*, 30 min, 4°C) to remove any residual contamination with the cytosolic fraction. Cytosol and membrane fractions were analyzed by SDS-PAGE and Western blotting.

Determination of protease activity. The protease activity was determined using azocasein as a substrate as previously described (10). Briefly, aliquots of the cellular or extracellular fractions were incubated at 45°C in a reaction mix containing 50 mM Tris-HCl buffer (pH 8), 1.5 M NaCl, and 0.5% (wt/vol) azocasein. Reactions were stopped by the addition of 1 volume of cold 10% (vol/vol) trichloroacetic acid, and acid-soluble products were detected by the A_{335}. One unit of protease activity was defined as the amount of enzyme producing an increase of 1 A_{335} unit per h under the assay conditions.

The protease activity in *H. volcanii* transformed with pJAM with different Nep variants was also assayed on YPC-medium containing copolymerized skim milk (0.8%) and 4 μg of novobiocin/ml. Plates were incubated at 42°C for several days in plastic bags. The appearance of clear halos surrounding the colonies were indicative of Nep protease activity.

SDS-PAGE and Western blotting. *H. volcanii* cells harboring the different Nep variants were grown to stationary phase (OD_{600} of ~1.5), and samples (1 ml) were harvested and centrifuged to separate the cells from the culture medium (10,000 × *g*, 15 min, 4°C). The cell pellets were suspended in 1× sample buffer containing 0.1% (wt/vol) SDS and 0.1 M dithiothreitol (DTT; OD_{600} of ~20). Cell-free culture media were incubated with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF; 5 mM, room temperature, 30 min), and proteins were precipitated by the addition of 1 volume of 100% acetone (4°C, 2 h). Samples were centrifuged (12,000 × *g*, 30 min, 4°C) and then washed three times with 80% (vol/vol) acetone and once with 100% acetone. The protein pellets were dried, suspended in 250 mM glycerol, and homogenized in 1× sample buffer containing 0.1% (wt/vol) SDS and 0.1 M DTT. All samples were boiled for 5 min and loaded onto 10% (vol/vol) polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes and subjected to Western blotting with 1/4,000 anti-Nep antibody diluted in blocking buffer and 1/10,000 alkaline phosphatase-conjugated secondary antibody. The molecular mass standards were the BenchMark prestained protein ladder (Invitrogen).

***In vitro trans* activation of prepro-Nep.** *E. coli* Rosetta (DE3) cells harboring pET-*nephis6* (3) were induced to synthesize the recombinant protease by incubation with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. The cells were suspended in 50 mM Tris-HCl (pH 8)–3 M NaCl, disrupted by sonication, and centrifuged to discard the cell debris. Cell lysates containing Prepro-NepHis6 were incubated in the same buffer solution for 16 h at 37°C in the presence or absence of Nep isolated from *N. magadii* culture medium (*Nm*Nep). Controls were performed with *Nm*Nep preincubated with PMSF. The samples were then analyzed by Western blotting and the azocaseinolytic activity assay.

TABLE 1 Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or primer	Phenotype, genotype, or oligonucleotide sequence (coordinates)[a]	Source or reference
Strains		
E. coli DH10β	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara-leu*)*7697 araD139 galU galK nupG rpsL* λ$^-$	Invitrogen
E. coli Rosetta (DE3)	F$^-$ *ompT* [*Ion*] $hsdS_B$ (r_B m_B) (an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
E. coli GM33	F$^-$ *dam-3 sup-85* (Am)	21
N. magadii ATCC 43099		ATCC
H. volcanii DS70	DS2 cured of pHV2	34
H. volcanii H99	DS70 Δ*pyrE2* Δ*trpA* Δ*hdrB*	2
H. volcanii KD5	H99 Δ*tatAo*::*trpA*$^+$	4
H. volcanii NH-Hv10	H99 Δ*secFD*::(*fdx-trpA*)	12
Plasmids		
pET24b(+)	Km^r; 5,309-bp expression plasmid vector	Novagen
pET-*nep*	Km^r; 1,623-bp coding region of *nep* in the NdeI-HindIII sites of pET-24b(+); *nep* expressed in *E. coli* Rosetta (DE3)	3
pET-*nep-his6*	Km^r; 1620-bp coding region of *nep* lacking the translation stop codon (TAA) in the NdeI-HindIII sites of pET-24b(+); *nep-his6* expressed in *E. coli* Rosetta (DE3)	3
pET-*nep*RR/KK	pET-*nep* modified with the primers Fw- and Rv-RR/KK	This study
pET-*nep*S/A	pET-*nep* modified with the primers Fw- and Rv-S/A	This study
pET-*nep*NSN1	pET-*nep* modified with the primers Fw- and Rv-NSN1	This study
pET-*nep*RR/KK-S/A	pET-*nep*RR/KK modified with the primers Fw- and Rv-S/A	This study
pET-*nep*NSN1-S/A	pET-*nep*NSN1 modified with the primers Fw- and Rv-S/A	This study
pET-*nep*NSN1-NSN2	pET-*nep*NSN1 modified with the primers Fw- and Rv-NSN2	This study
pJAM202	Ap^r Nv^r; 1,152-bp XbaI-to-DraIII fragment of pJAM621 blunt end ligated with a 9.9-kb BamHI-to-KpnI fragment of pBAP5010; *psmB-his6* expressed from *Hc* rRNA P2 in *H. volcanii*	14
pJAM-*nep*	NdeI-Bpu1102I fragment of pET-*nep* cloned into pJAM202	3
pJAM-*nep*RR/KK	NdeI-Bpu1102I fragment of pET-*nep*RR/KK cloned into pJAM202	This study
pJAM-*nep*S/A	NdeI-Bpu1102I fragment of pET-*nep*S/A cloned into pJAM202	This study
pJAM-*nep*NSN1	NdeI-Bpu1102I fragment of pET-*nep*NSN1 cloned into pJAM202	This study
pJAM-*nep*RR/KK-S/A	NdeI-Bpu1102I fragment of pET-*nep*RR/KK-S/A cloned into pJAM202	This study
pJAM-*nep*NSN1-S/A	NdeI-Bpu1102I fragment of pET-*nep*NSN1-S/A cloned into pJAM202	This study
pJAM-*nep*NSN1-NSN2	NdeI-Bpu1102I fragment of pET-*nep*NSN1-NSN2 cloned into pJAM202	This study
Primers		
Fw-RR/KK	GTAATGTCGGG**AAAAAG**TCAGTACTGAAAGC (31–36)	
Rv-RR/KK	GCTTTCAGTACTGA**CTTTTT**CCCGACATTAC (31–36)	
Fw-S/A	GTCGGGAACC**GCC**ATGGCATCACC (1051–1053)	
Rv-S/A	GGTGATGCCAT**GGC**GGTTCCCGAC (1053–1051)	
Fw-NSN1	CTCGGGGGAGTT**AAC**AGC**AAC**ACACCGGGACGC (91–93; 97–99)	
Rv-NSN1	GCGTCCCGGTGT**GTT**GCT**GTT**AACTCCCCCGAG (99–97; 93–91)	
Fw-NSN2	GTACTGAAAGCA**AAC**AGC**AAC**CTGGGGGCCTTC (52–54; 58–60)	
Rv-NSN2	GAAGGCCCCCAG**GTT**GCT**GTT**TGCTTTCAGTAC (60–58; 52–54)	

[a] The nucleotides changed for site-directed mutagenesis are indicated in boldface in each sequence, and the coordinates (indicated in parentheses) refer to the relative position corresponding to the first nucleotide in the ATG initiation codon of the *nep* gene (AAV66536). Nv^r, novobiocin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; *Hc*, *Halobacterium cutirubrum*.

Mass spectrometry analysis. The target bands on SDS-PAGE gels stained with Coomassie blue R-250 were excised and subjected to in-gel digestion with trypsin, followed by peptide mass fingerprinting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a MALDI-TOF-TOF spectrometer, the Ultraflex II (Bruker), in the mass spectrometry facility CEQUIBIEM, Argentina. Spectra from all experiments were converted to DTA files and merged to facilitate database searching using the Mascot search algorithm v2.1 (Matrix Science, Boston, MA) against the nonredundant protein sequences of GenBank (National Center for Biotechnology Information, Bethesda, MD) and the sequences of the full-length and mature forms of Nep deduced from the *nep* gene (AAV66536).

RESULTS

Halolysin Nep is exported by the Tat pathway. Based on genome analysis, it was observed that haloarchaea contain various numbers of putative protease genes which contain Sec or Tat signal peptides, suggesting that they probably have a transmembrane or extracellular localization (Table 2). Most of these enzymes are predicted to be transported through the Sec pathway ranging from 100% in *H. volcanii* to 61.5% in *N. magadii*. The Tat-dependent proteases are less represented except in *N. magadii* (38.5%) and *Halobacterium* sp. strain NRC-1 (36.8%).

N. magadii genome encodes six extracellular subtilisin-like proteases which probably serve a nutritional purpose (29). All of these subtilases are predicted Tat substrates including halolysin Nep.

To experimentally confirm whether transport of Nep was Tat dependent, a mutant version of this protease was constructed replacing the twin arginine residues (RR) by lysines (KK) in the Tat consensus motif (G**RR**SVL) (Fig. 1A) and expressed in *H. volcanii*

TABLE 2 Distribution of secretory proteases in haloarchaeal genomes[a]

Organism	No. (%) of secretory proteases		No. of subtilisin-like proteases
	Sec	Tat	
Halalkalicoccus jeotgali B3	7 (77.8)	2 (22.2)	0
Haloarcula marismortui ATCC 43049	15 (93.8)	1 (6.2)	0
Halobacterium sp. strain NRC-1	13 (63.2)	7 (36.8)	6
Haloferax volcanii DS2	9 (100)	0 (0)	0
Halogeometricum borinquense ATCC 700274	24 (82)	5 (18)	3
Halomicrobium mukohataei DSM 12286	20 (87)	3 (13)	0
Haloquadratum walsbyi DSM 16790	10 (83.3)	2 (16.7)	0
Halorhabdus utahensis DSM 12940	13 (72.2)	5 (27.8)	0
Halorubrum lacusprofundi ATCC 49239	14 (93.3)	1 (6.7)	2
Haloterrigena turkmenica DSM 5511	20 (71.4)	8 (28.6)	6
Natrialba magadii ATCC 43099	16 (61.5)	10 (38.5)	6
Natronomonas pharaonis DSM 2160	15 (83.3)	3 (16.7)	3

[a] The word "secretory" is used to indicate the proteases that contain a signal peptide and may be targeted to the membrane or exported to the extracellular medium. Proteases listed in the MEROPS database (http://merops.sanger.ac.uk/) were analyzed using the Signal P 4.0 (no TM) (http://www.cbs.dtu.dk/services/SignalP/) and TatFind 1.4 (http://signalfind.org/tatfind.html) programs for Sec and Tat signal peptide prediction, respectively.

DS70. Protease secretion was assessed by determination of enzyme activity on casein plates. Recombinant *H. volcanii* expressing Nep *wt* produced clear halos of proteolysis, indicating that Nep was efficiently synthesized and secreted to the extracellular medium (Fig. 1B left), whereas halos were not evident in *H. volcanii* producing Nep RR/KK, suggesting that the mutant protease was not competent for secretion (Fig. 1B middle). To further confirm this observation, *H. volcanii* cells transformed with pJAM-*nep*, pJAM-*nep*RR/KK, and pJAM without insert (control) were grown in liquid medium and harvested as the cultures reached the late exponential phase (OD_{600} ~1.5). Cell lysates and cell-free culture medium were assayed for azocaseinolytic activity and examined by Western blotting with anti-Nep antibodies to evaluate the localization of the protease. Nep polypeptides accumulated in the culture medium and a small fraction was detected associated to the cells probably due to contamination with the culture medium (Fig. 1C). Conversely, the ratio of cell-associated to secreted protease was notably higher in the Nep RR/KK variant (Fig. 1C), meaning that the mutant protease was not efficiently secreted. It should be noted that the total amount of Nep RR/KK (cell-associated plus extracellular) was lower than that of Nep, most likely as a consequence of intracellular degradation of the overexpressed mutant protease. This outcome was not surprising since it has been also observed in other Tat substrates heterologously expressed in *H. volcanii* (9). By MS analysis it was verified that the secreted polypeptide (~70 kDa) corresponded to mature Nep (see below). As previously discussed (3), the molecular size of Nep is overestimated on SDS-PAGE gels (70 kDa versus 45 kDa) due to the acidic nature of this halophilic protease. Correlating with Nep localization, high levels of extracellular azocaseinolytic activity (120 U, representing 96% of the total activity) accumulated in *H. volcanii* overexpressing Nep compared to the cells producing Nep

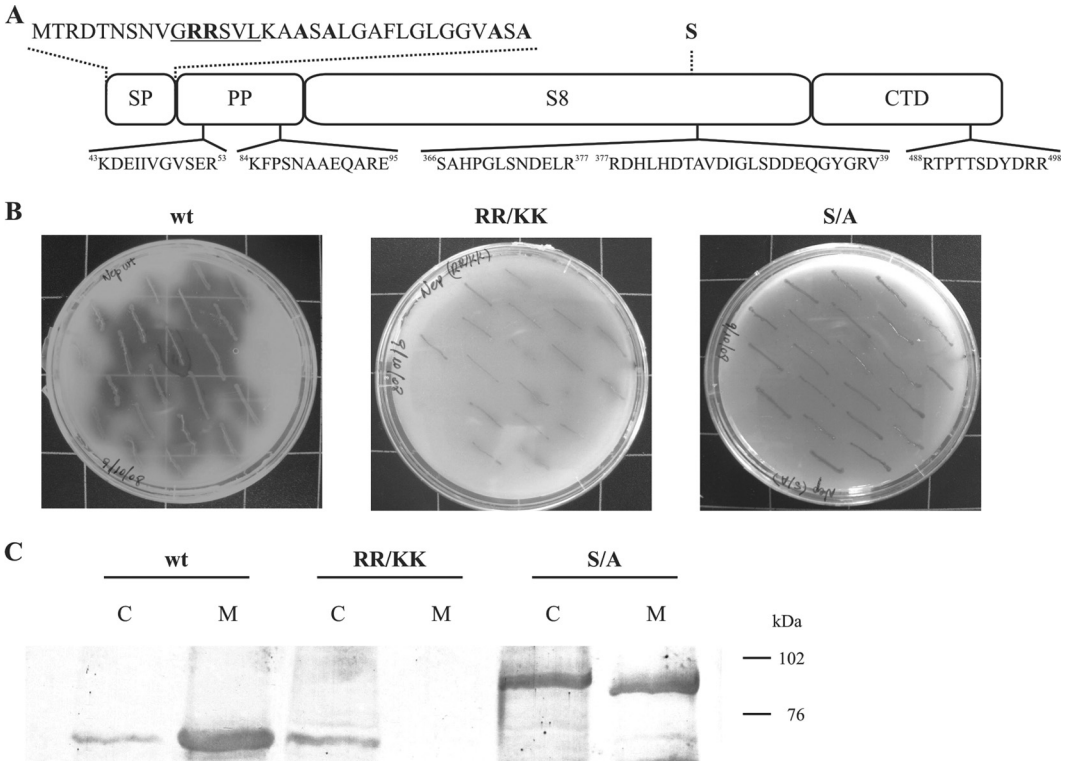

FIG 1 Secretion and processing of Nep *in vivo*. (A) Domain organization of Nep, including the signal peptide (SP), N-terminal propeptide (PP), protease domain (S8), and C-terminal domain (CTD). The amino acid residues modified by site-directed mutagenesis (indicated in boldface) in the SP ($R^{11}R^{12}$, $A^{18}SA^{20}$, $A^{31}SA^{33}$) and the catalytic serine (S^{351}) in the S8 domain are indicated above the diagram. The sequences of the Nep tryptic peptides identified by MALDI-TOF-TOF are shown below the diagram. (B) Phenotypes of recombinant *H. volcanii* DS70 cells grown in YPC-casein plates. (C) Western blot of recombinant *H. volcanii* expressing different Nep variants. Equal amounts of whole-cell lysate (lanes C) and extracellular medium (lanes M) were applied. The results are representative of at least four independent experiments.

TABLE 3 Proteolytic activity of recombinant *H. volcanii* cells expressing Nep wild type and different variants[a]

Control, wild type, or variant	Mean azocaseinolytic activity (U)[b] ± SD in:		
	Cell fraction	Culture medium fraction	Both fractions
Control	0	0	0
Wild type	4 ± 1.5 (4)	120 ± 23 (96)	124
RR/KK	19 ± 7 (100)	0 ± 0.3 (0)	20
S/A	0	0	0
RR/KK S/A	0	0	0
NSN1	3 ± 0.75 (3)	101.8 ± 16 (97)	105
NSN1-2	ND	3 ± 0.16	

[a] *H. volcanii* DS70 harboring pJAM without insert (control) or containing different modifications in the coding sequence of *nep* gene (see diagram in Fig. 1A) were grown at 37°C in YPC-novobiocin (2 μg/ml). Cells were harvested at an OD_{600} of ~1.5 and were disrupted in 50 mM Tris-HCl (pH 8) containing 3 M NaCl. The azocaseinolytic activity was determined in cell extracts and cell-free culture medium.
[b] Percentages are indicated in parentheses. These data are representative of three independent experiments. ND, not determined.

RR/KK (Table 3). The total protease activity of this variant was significantly lower (6-fold) than that observed for Nep and it remained in the cellular fraction. Altogether, these results clearly show that Nep is exported through the Tat pathway.

Validation of the Tat dependence of Nep in Tat knockout mutants (TatAt, TatCt, and TatC0 components) was not possible due to the essential nature of these proteins in *H. volcanii* (4). However, the TatA0 component is dispensable for the secretion of most *H. volcanii* Tat substrates (4). To examine whether Tat A0 was needed for Nep secretion, we expressed pJAM-*nep* in *H. volcanii* KD5, which has a Δ*tatA0* genetic background. Protease activity determination and Western blotting showed that Nep was similarly secreted and activated in the parental *H. volcanii* H99 and KD5 strains. This means that TatA0 was not needed to export this protease in *H. volcanii*. On the other hand, Nep secretion was examined in *H. volcanii* NH-Hv10 (Δ*secFD*), which has a Sec-dependent translocation defect and a severe cold sensitivity (4, 12), expecting that Nep would be efficiently secreted in this strain. Surprisingly, recombinant NH-Hv10 cells expressing Nep wt could not be obtained; however, this strain was not impaired to secrete the inactive variant Nep S/A (see below), as evidenced on Western blots. Altogether, these results support the Tat dependence of Nep and, on the other hand, suggest that Nep wt was likely secreted in the SecDF mutant, but these cells were killed due to increased protease sensitivity.

Nep maturation is autocatalytic. To examine the maturation mechanism of Nep, a modified version of this enzyme was generated changing the catalytic serine 351 by alanine (Fig. 1A). This construct was expressed in *H. volcanii*, and the mutant protease (Nep S/A) was analyzed as described for Nep RR/KK. Proteolytic activity was undetectable (Fig. 1B, right, and Table 3), confirming the key role of serine 351 in the hydrolytic catalysis of this halophilic subtilase. This phenotype correlated with the occurrence of polypeptides of higher mass than Nep (~84 kDa) in cell lysates, as well as in the extracellular medium (Fig. 1C). Nep and Nep S/A were excised from the gel, subjected to trypsin digestion, and analyzed by mass spectrometry. Peptides corresponding to the catalytic domain of Nep—S.AHPGLSNDEL.R (1295.66 Da), R.DHL HDTAVDIGLSDDEQGYGR.V (2,313.04 Da), and R.TPTTSDY DR.R (1,055.46 Da)—were identified in both Nep and Nep S/A (Fig. 1A). However, the tryptic peptides K.DEIIVGVSER.V (1,244.68 Da) and K.FPSNAAEQAR.E (1,090.53 Da), mapping within the N-terminal pro-Nep (Fig. 1A), were only detected in Nep S/A, demonstrating that this polypeptide corresponded to the unprocessed protease. The cell-associated Nep S/A had a slightly lower electrophoretic mobility than the secreted Nep S/A (Fig. 1C), suggesting that it likely corresponds to Nep precursor containing the SP (prepro-Nep). However, identification of this polypeptide by MS analysis was not successful. The triple-mutant variant Nep RR/KK-S/A remained cell associated as an inactive precursor (see Fig. S1 in the supplemental material), confirming that the occurrence of mature protease in the cellular fraction of *H. volcanii* producing Nep RR/KK was due to autoproteolysis of the accumulated precursor.

To confirm the autocatalytic nature of Nep maturation, we incubated cell lysates of recombinant *E. coli* cells overexpressing prepro-Nep in the presence or absence of Nep isolated from the culture medium of *N. magadii* (*Nm*Nep). The addition of the mature protease in *trans* triggered precursor processing and enzyme activation, as evidenced by the decrease in the molecular mass of prepro-Nep polypeptide in parallel with the increase in protease activity (Fig. 2). *In vitro trans* activation of prepro-Nep was prevented when *Nm*Nep was preincubated with the serine protease inhibitor PMSF, demonstrating that processing of the protease precursor was due to *Nm*Nep proteolytic activity (not shown). Altogether, the experimental evidence obtained *in vivo* (Fig. 1) and *in vitro* (Fig. 2) demonstrates that processing and activation of halolysin Nep is autocatalytic.

Taking into account that prepro-Nep was not capable of *cis* activation *in vitro* (Fig. 2) and that *in vivo* extracellular proteases are activated during/after translocation through the cytoplasmic membrane, we hypothesized that cleavage of the SP may be required to induce the autocatalytic removal of Nep propeptide. SPs are cleaved from secretory substrates during or after translocation

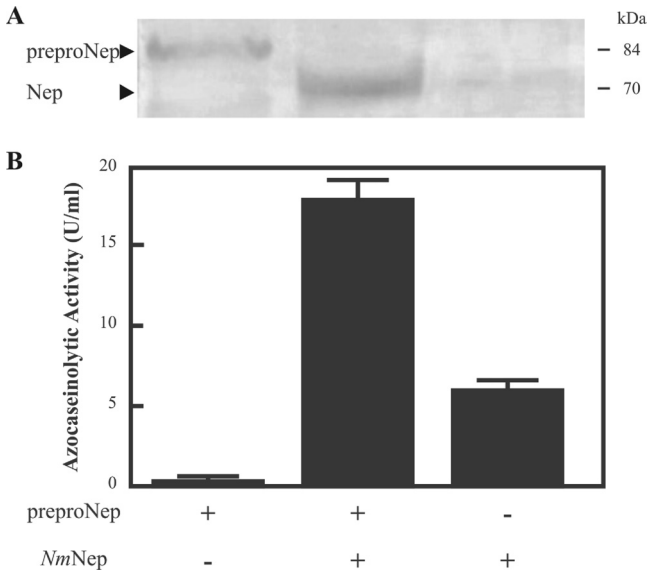

FIG 2 *trans* activation of prepro-Nep by mature Nep. Cell lysates of recombinant *E. coli* containing prepro-Nep were incubated with or without *Nm*Nep in 50 mM Tris-HCl (pH 8)–3 M NaCl at 37°C for 16 h. The samples were then analyzed by Western blotting with anti-Nep antibody (A) and azocaseinolytic activity (B).

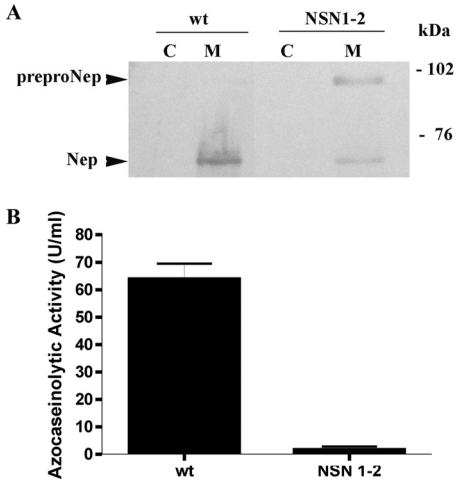

FIG 3 Protease maturation of Nep variant modified in predicted SPase I cleavage site. Western blot (A) and extracellular protease activity (B) of Nep modified in $A^{18}SA^{20}$ and $A^{31}SA^{33}$ (NSN1-2). $A^{31}SA^{33}$ was predicted as a potential SPase I recognition site. Equal amounts of whole-cell lysate (lanes C) and extracellular medium (lanes M) were applied. The results are representative of three independent experiments.

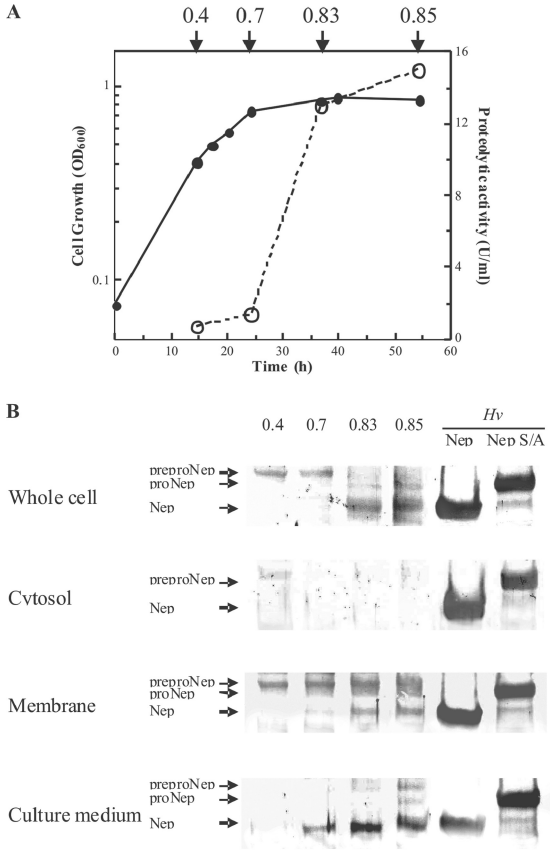

FIG 4 Synthesis and maturation of Nep in *N. magadii* during growth. (A) *N. magadii* was grown in Tindall medium at 37°C. Cell growth was monitored by changes in OD_{600} (●), and the extracellular protease activity was measured using azocasein as substrate (○). (B) Samples were withdrawn at the indicated OD_{600}, centrifuged, and processed as indicated in Materials and Methods. Extracellular (100 μl of cell-free culture medium) and subcellular (5 μg of protein) fractions were subjected to SDS-PAGE and Western blotting. The electrophoretic mobilities of mature Nep and Nep S/A (proNep) secreted into the culture medium by recombinant *H. volcanii* DS70 (Hv) are indicated on the right as a reference.

by signal peptidases type I (SPase I), which in bacteria recognize the consensus motif AXA downstream of Tat and/or Sec SP (19, 22). *H. volcanii* encodes two different SPases I, Sec11a and Sec11b, that can cleave Sec SP *in vitro* (7). The Sec11b knockout mutant is lethal, whereas the Sec11a knockout mutant is not. The prepro-Nep polypeptide contains two ASA motifs at positions 31 to 33 (ASA1) and positions 18 to 20 (ASA2) relative to the translation initiation start point (Fig. 1A). TATFIND 1.4 and Signal P 4.0 predicted that the hypothetical recognition signal for SPase I is located after A^{33} (ASA1). To investigate whether removal of the SP affected the autocatalytic processing of the Nep propeptide, a mutant version of Nep was constructed by changing A to N (ASA by NSN) in the potential SPase I recognition site, resulting in Nep NSN1. The Nep NSN1 "phenotype" was similar to that of the wt enzyme (Table 3). However, in *H. volcanii* cells expressing Nep modified in both ASA motifs (NSN1-2), the culture medium was enriched in a high-molecular-mass Nep species (probably the unprocessed precursor), which correlated with low to undetectable levels of extracellular protease activity (Fig. 3 and Table 3). If one assumes that $A^{18}SA^{20}$ could be used as an alternate SPase I cleavage site in Nep NSN1, then this result suggests that removal of the SP may be necessary to facilitate the autocatalytic maturation. Phenotypic examination of the single mutant Nep NSN2 was not possible since construction of this variant has thus far been unsuccessful.

In addition, the *H. volcanii* Δ*sec11a* deletion mutant was transformed with pJAM *nep*, and the recombinant cells were analyzed for Nep secretion by Western blotting and protease activity determination. No differential phenotype was observed compared to the wt DS70 recombinant cells transformed with the same construct (data not shown), meaning that Sec11a is not involved in Nep processing.

Synthesis, secretion, and processing of Nep in *N. magadii*. The presence of Nep precursor and mature forms as well as protease activity were examined in subcellular fractions of *N. magadii* throughout growth. At early growth stages ($OD_{600} < 0.7$), no extracellular protease activity was detectable in the medium, which was consistent with the occurrence of a high-molecular-mass polypeptide (84 kDa) in the cellular fraction (cell lysates and membranes). Toward the end of the exponential growth phase ($OD_{600} > 0.8$), the 70-kDa protein species became evident in the cellular fraction (cell lysates and membranes) and accumulated in the culture medium (Fig. 4B) in parallel with the detection of azocaseinolytic activity (Fig. 4A). Based on the similarity in the electrophoretic mobilities with Nep S/A and Nep wt and the absence or presence of protease activity, we assumed that the higher-molecular-mass species corresponded to unprocessed prepro-Nep/pro-Nep, while the lower-molecular-mass species was the mature Nep. The scarce amount of immunoreactive proteins in the cytosol suggests that the synthesis/folding and translocation of Nep may be very fast.

DISCUSSION

In this study we examined the secretion and maturation of halolysins in the model organism *H. volcanii*, based on protease Nep produced by the haloalkaliphilic archaeon *N. magadii*. Using Nep

variants modified in the RR motif typical of Tat substrates, we confirmed the Tat dependence of Nep for translocation through the archaeal cytoplasmic membrane (Fig. 1). Genome analysis indicates that *N. magadii* has the genetic potential for the Tat route since it encodes two *tatC* homologs (Nmag_2050 and Nmag_2051) and one *tatA* homolog (Nmag_3135). Thus, it is expected that Nep is secreted by the Tat pathway in the native host.

Shi et al. had suggested that the protease SptA produced by the haloarchaeon *Natrinema* sp. strain J7 was likely transported to the extracellular medium through the Tat pathway (28). Therefore, it is evident that the Tat route is conserved for secretion of these halophilic subtilases. However, the majority of the proteases encoded in haloarchaeal genomes (61.5 to 100%) are potential Sec substrates (Table 2) which represents an exception to the rule as most of the secretome in haloarchaea is predicted as Tat dependent. Although the physiological significance of this difference is not known, it is possible to speculate that since protein folding is a prerequisite for Tat-dependent protein translocation, proteases with a partial or total folded conformation may be dangerous for the host cell since they could become activated before they are exported. Since halolysins require the removal of a pro-sequence to become active enzymes, an event that takes place during or after translocation through the membrane, Tat-dependent secretion would not be a problem in these proteases.

Recently, it was reported that the haloprotease CPI from the moderately halophilic bacterium *Pseudoalternomonas ruthenica* is exported by the Sec-dependent type II secretion system (27). Thus, it seems apparent that the mechanisms used by halophilic prokaryotes to deliver extracellular proteases are not conserved.

The CTD of halolysins, including Nep, is not processed (3, 28), and its biological significance was not clearly understood. Recently, it was demonstrated that the CTD of halolysin SptA produced by *Natrinema* sp. strain J7 stabilizes this protease at high salinity and assists enzyme activity on protein substrates (35). In our study, a truncated Nep variant without the CTD was synthesized in *H. volcanii*, and this protease was detected extracellularly by Western blotting (data not shown), indicating that this tail is dispensable for Nep secretion. However, protease activity was significantly affected, suggesting that this domain is necessary to preserve Nep protease activity and/or stability, as reported for the halolysins R4 and SptA (15, 35). Although protease maturation and/or activation is well established for a number of subtilases secreted by the Sec route (32, 36), these processes have not been examined for Tat-dependent secretory proteases that are transported totally or partially folded. Our results indicate that similarly to the Sec-dependent subtilases produced by bacteria and thermophilic archaea, Nep maturation involves the autocatalytic cleavage of the N-terminal propeptide (Fig. 1 and 2). However, the precise role of Nep N-terminal pro-sequence remains to be demonstrated. Although autocatalytic activation is common in subtilisin-like proteases, most (if not all) of the experimental evidence is based on *in vitro* assays using recombinant protease variants, as is the case for Tk subtilisin (33) and aqualysin I (17). By using an *in vivo* approach (mutant protease Nep S/A expressed in *H. volcanii*), our study shows that the protease molecule itself is responsible for the initial events that lead to enzyme activation (*cis* activation). This observation is novel and has not been reported previously for any subtilase.

Based on the results obtained in the heterologous system *H. volcanii* and in the native haloarchaeon *N. magadii*, we propose a hypothetical model that relates transport and maturation of halolysin Nep (Fig. 5). Nep is synthesized as a 56-kDa precursor (prepro-Nep) consisting of the N-terminal Tat SP, an N-terminal propeptide, the protease domain and a CTD, that is exported in a (partially?) folded state through the Tat translocon. During/after transport, the Nt-Tat SP is removed by a SPase. *H. volcanii* has two different SPase type I that cleave Sec SP (7), while *N. magadii* ATCC 43099 encodes five homologs (29), but the encoded proteins have not been characterized yet. Upon reaching the extracellular medium, the Nt-propeptide is processed autoproteolytically by intramolecular cleavage (*cis* activation), yielding mature fully active halolysin Nep (45 kDa). Additional precursor molecules could be activated by either intra- or intermolecular processing (*trans* activation). Protease maturation in the extracellular milieu may result as a consequence of a conformational change in pro-Nep generated after removal of the Tat SP (Fig. 3). Alternatively, the transit through the Tat translocon and/or the different physicochemical conditions between intra and extracellular environments could position the Nep active site and the propeptide in proximity (*cis* activation). Using protein modeling and structural protein analysis, we have recently shown that activation of mature Nep during transition from a low- to a high-salt environment is due to a structural change from a more compact to a more extended conformation (31). It can be speculated that propeptide cleavage may induce similar changes in Nep. Experimental analysis of the structures of precursor and mature Nep will help to validate this hypothesis.

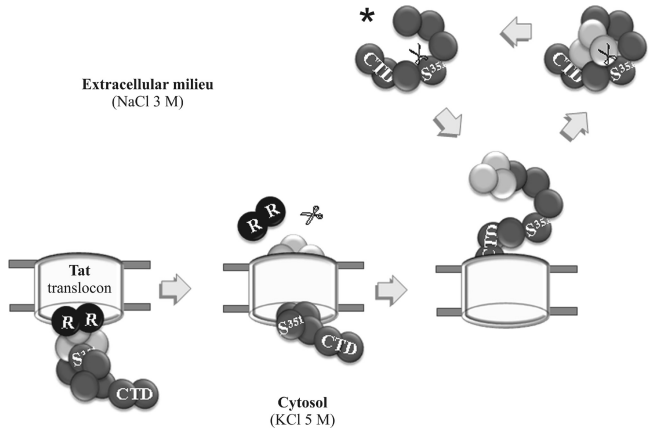

FIG 5 Proposed model for secretion and maturation of halolysin Nep. Nep is synthesized as a precursor (prepro-Nep). During/after transport across the cytoplasmic membrane, the Tat signal peptide (RR) is cleaved by SPase I (white scissors) releasing pro-Nep to the extracellular milieu. The N-terminal propeptide (light gray) is then processed autocatalytically by *cis* (right) and/or *trans* (left) protease activities (black scissors), yielding the mature fully active protease (asterisk). The C-terminal domain (CTD) is not processed. Nep catalytic serine (S^{351}) is indicated in white letters.

ACKNOWLEDGMENTS

This study was supported by research grants from CONICET (PIP-1783) and UNMdP (EXA454/09 and EXA542/11), Argentina, awarded to R.D.C.

We thank Mecky Pohlschröder (University of Pennsylvania) for the generous gifts of *H. volcanii* H99, KD5, and NH-Hv10. We thank Julie A. Maupin-Furlow (University of Florida) for plasmid pJAM202 and *H. volcanii* DS70. Finally, we thank Jerry Eichler (Ben Gurion University) for *H. volcanii* (Δ*sec11a*).

REFERENCES

1. **Allers T.** 2008. Media recipes from the Thorsten Allers Lab, p 18–20. *In* Dyall-Smith M (ed), The halohandbook: protocols for haloarchaeal genetics. http://www.haloarchaea.com/resources/halohandbook/index.html.
2. **Allers T, Ngo HP, Mevarech M, Lloyd RG.** 2004. Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. Appl. Environ. Microbiol. **70**:943–953.
3. **De Castro RE, et al.** 2008. Gene cloning and heterologous synthesis of a haloalkaliphilic extracellular protease of *Natrialba magadii* (Nep). Extremophiles **12**:677–687.
4. **Dilks K, Giménez MI, Pohlschröder M.** 2005. Genetic and biochemical analysis of the twin-arginine translocation pathway in halophilic archaea. J. Bacteriol. **187**:8104–8113.
5. **Dilks K, Giménez MI, Tripepi M, Pohlschröder M.** 2011. Protein transport into and across haloarchaeal cytoplasmic membranes, p 201–226. *In* Ventosa A (ed), Halophiles and hypersaline environments: current research and future trends. Springer, Berlin, Germany.
6. **Dyall-Smith M (ed).** 2008. The halohandbook: protocols for haloarchaeal genetics, p 144. http://www.haloarchaea.com/resources/halohandbook/index.html.
7. **Fine A, Irihimovitch V, Dahan I, Konrad Z, Eichler J.** 2006. Cloning, expression, and purification of functional Sec11a and Sec11b, type I signal peptidases of the archaeon *Haloferax volcanii*. J. Bacteriol. **188**:1911–1919.
8. **Foophow T, Tanaka S, Koga Y, Takano K, Kanaya S.** 2010. Subtilisin-like serine protease from hyperthermophilic archaeon *Thermococcus kodakaraensis* with N- and C-terminal propeptides. Protein Eng. Des. Sel. **23**:347–355.
9. **Giménez MI, Dilks K, Pohlschröder M.** 2007. *Haloferax volcanii* twin-arginine translocation substrates include secreted soluble, C-terminally anchored and lipoproteins. Mol. Microbiol. **66**:1597–1606.
10. **Giménez MI, Studdert CA, Sanchez JJ, De Castro RE.** 2000. Extracellular protease of *Natrialba magadii*: purification and biochemical characterization. Extremophiles **4**:181–188.
11. **Gupta R, Beg QK, Lorenz P.** 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. **59**: 15–32.
12. **Hand NJ, Klein R, Laskewitz A, Pohlschröder M.** 2006. Archaeal and bacterial SecD and SecF homologs exhibit striking structural and functional conservation. J. Bacteriol. **188**:1251–1259.
13. **Hutcheon G, Bolhuis A.** 2003. The archaeal twin-arginine translocation pathway. Biochem. Soc. Trans. **31**:686.
14. **Kaczowka SJ, Maupin-Furlow JA.** 2003. Subunit topology of two 20S proteasomes from *Haloferax volcanii*. J. Bacteriol. **185**:165–174.
15. **Kamekura M, Seno Y, Dyall-Smith M.** 1996. Halolysin R4, a serine proteinase from the halophilic archaeon *Haloferax mediterranei*; gene cloning, expression and structural studies. Biochim. Biophys. Acta **1294**: 159–167.
16. **Kamekura M, Seno Y, Holmes ML, Dyall-Smith ML.** 1992. Molecular cloning and sequencing of the gene for a halophilic alkaline serine protease (halolysin) from an unidentified halophilic archaea strain (172P1) and expression of the gene in *Haloferax volcanii*. J. Bacteriol. **174**:736–742.
17. **Kim DW, Matsumura H.** 2000. Requirement for the COOH-terminal pro-sequence in the translocation of aquealysin I across the cytoplasmic membrane in *Escherichia coli*. Biochem. Biophys. Res. Commun. **277**: 216–220.
18. **Kumar CG, Takagi H.** 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv. **17**:561–594.
19. **Lüke I, Handford JI, Palmer T, Sargent F.** 2009. Proteolytic processing of *Escherichia coli* twin-arginine signal peptides by LepB. Arch. Microbiol. **191**:919–925.
20. **Maldonado B, Buchanan G, Muller M, Berks BC, Palmer T.** 2011. Genetic evidence for a TatC dimer at the core of the *Escherichia coli* twin arginine (Tat) protein translocase. J. Mol. Microbiol. Biotechnol. **20**:168–175.
21. **Marinus MG, Morris NR.** 1973. Isolation of deoxyribonucleic acid methylase mutants of *Escherichia coli* K-12. J. Bacteriol. **114**:1143–1150.
22. **Ng SY, Chaban B, VanDyke DJ, Jarrell KF.** 2007. Archaeal signal peptidases. Microbiology **153**:305–314.
23. **Pohlschröder M, Hartmann E, Hand NJ, Dilks K, Haddad A.** 2005. Diversity and evolution of protein translocation. Annu. Rev. Microbiol. **59**:91–111.
24. **Rao MB, Tanksale AM, Ghatge MS, Deshpande VV.** 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. **62**:597–635.
25. **Robinson C, Bolhuis A.** 2004. Tat-dependent protein targeting in prokaryotes and chloroplasts. Biochim. Biophys. Acta **1694**:135–147.
26. **Rose RW, Bruser T, Kissinger JC, Pohlschroder M.** 2002. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol. Microbiol. **45**:943–950.
27. **Sanchez-Porro C, Mellado E, Pugsley AP, Francetic O, Ventosa A.** 2009. The haloprotease CPI produced by the moderately halophilic bacterium *Pseudoalteromonas ruthenica* is secreted by the type II secretion pathway. Appl. Environ. Microbiol. **75**:4197–4201.
28. **Shi W, et al.** 2006. An extracellular halophilic protease SptA from a halophilic archaeon *Natrinema* sp. J7: gene cloning, expression and characterization. Extremophiles **10**:599–606.
29. **Siddaramappa S, et al.** 2012. A comparative genomics perspective on the extremophilic nature of the alkaliphilic haloarchaeon *Natrialba magadii* ATCC 43099. BMC Genomics doi:10.1186/1471-2164-13-165.
30. **Siezen RJ, Leunissen JA.** 1997. Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci. **6**:501–523.
31. **Souza TACB, et al.** 2012. Correlation between catalysis and tertiary structure arrangement in an archaeal halophilic subtilase. Biochimie **94**:798–805.
32. **Takagi H, Takahashi M.** 2003. A new approach for alteration of protease functions: pro-sequence engineering. Appl. Microbiol. Biotechnol. **63**: 1–9.
33. **Tanaka S, et al.** 2007. Crystal structure of unautoprocessed precursor of subtilisin from a hyperthermophilic archaeon: evidence for Ca^{2+} induced folding. J. Biol. Chem. **282**:8246–8255.
34. **Wendoloski D, Ferrer C, Dyall-Smith ML.** 2001. A new simvastatin (mevinolin)-resistance marker from *Haloarcula hispanica* and a new *Haloferax volcanii* strain cured of plasmid pHV2. Microbiology **147**:959–964.
35. **Xu Z, et al.** 2011. Functional insight into the C-terminal extension of halolysin SptA from haloarchaeon *Natrinema* sp. J7. PLoS One **6**:e23562. doi:10.1371/journal.pone.0023562.
36. **Yabuta Y, Takagi H, Inouye M, Shinde U.** 2001. Folding pathway mediated by an intramolecular chaperone: propeptide release modulates activation precision of pro-subtilisin. J. Biol. Chem. **276**:44427–44434.