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The Lon protease from the haloalkaliphilic archaeon *Natrialba magadii* is transcriptionally linked to a cluster of putative membrane proteases and displays DNA-binding activity

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Lon protease;
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Summary

The ATP-dependent Lon protease is universally distributed in bacteria, eukaryotic organelles and archaea. In comparison with bacterial and eukaryal Lon proteases, the biology of the archaeal Lon has been studied to a limited extent. In this study, the gene encoding the Lon protease of the alkaliphilic haloarchaeon *Natrialba magadii* (*Nmlon*) was cloned and sequenced, and the genetic organization of *Nmlon* was examined at the transcriptional level. *Nmlon* encodes a 84 kDa polypeptide with a pI of 4.42 which contains the ATPase, protease and membrane targeting domains of the archaeal-type LonB proteases. *Nmlon* is part of an operon that encodes membrane proteases and it is transcribed as a polycistronic mRNA in *N. magadii* cells at different growth stages. Accordingly, *Nm*Lon was detected in cell membranes of *N. magadii* throughout growth by Western blot analysis using specific anti-*Nm*Lon antibodies. Interestingly, in electrophoretic mobility shift assays, purified *Nm*Lon bound double stranded as well as single stranded DNA in the presence of elevated salt concentrations. This finding shows that DNA-binding is conserved in the LonA and LonB subfamilies and suggests that Lon—DNA interaction may be relevant for its function in haloarchaea.
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Introduction

Energy-dependent proteolysis is not only fundamental for the degradation of defective proteins but is also a key process for the regulation of many cellular functions (Van Melderen and Aertsen 2009). ATP-dependent proteases have been classified into four major groups including: Lon, FtsH, Clp and

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the proteasome (HslUV) and they belong to the ATPases associated with diverse cellular activities (AAA^+) protein superfamily (Lupas et al. 1997). In archaea at least two ATP-dependent proteases have been identified: the soluble proteasome-PAN system and the Lon protease. Proteasomes are universal among the archaea and have been characterized from methanogens, thermophiles and extreme halophiles (Maupin-Furlow et al. 2005). Lon-type serine proteases are conserved in the three domains of life: *Bacteria*, *Archaea* and *Eukarya* (Maupin-Furlow et al. 2005). However, the overall biology of the archaeal Lon protease has been investigated to a limited extent in comparison with the bacterial and eukaryotic enzymes.

The Lon protease family can be divided into two subfamilies, LonA and LonB, based on the sources, domain structure of the proteins and differences in the characteristic sequences within the domains (Rotanova et al. 2004). The LonA subfamily consists mainly of bacterial and eukaryotic enzymes and accounts for >80% of the presently known Lon proteases. LonB are predominant in *Archaea*, however, LonB-like proteins have also been found in some bacteria. LonA is encoded by a single gene and contains an amino-terminal domain (LAN), a central ATPase (AAA^+) domain and a C-terminal protease domain (P-domain). LonB enzymes lack the LAN domain and they have a transmembrane segment that predicts a membrane-bound localization of this enzyme. The differences found between both families and the structural diversity among LonB members suggest that the archaeal-type enzyme may have conserved as well as novel biochemical and/or functional properties.

DNA-binding by ATP-dependent Lon proteases is evolutionarily conserved from bacteria to man, suggesting that it is an essential property of this protein (Lee and Suzuki 2008). It is likely that the archaeal-type LonB protease may also interact with DNA and/or RNA, however, this feature remains to be experimentally demonstrated.

Previous studies have examined the biochemical and catalytic properties of the archaeal-type LonB protease (Fukui et al. 2002; Besche et al. 2004; Im et al. 2004; Botos et al. 2005) as well the crystal structures of the P-domain and ATPase domain (Im et al. 2004; Botos et al. 2005; Young Jun et al. 2010). However, studies on the expression profile of Lon in the context of the archaeal cell have not been reported.

Haloarchaea encode Lon homologues which, to date, have not been characterized. Our laboratory has characterized extra and intracellular proteases in the alkaliphilic haloarchaeon *Natrialba magadii* (optimum growth in 3.5 M NaCl, pH 10) (De Castro et al. 2006, 2008). Studies on the *N. magadii* Lon protease will not only complement our current knowledge on the proteolytic systems of this unusual extremophile (and other haloarchaea), but will also contribute to gain insight into the overall biology of the archaeal-type LonB protease, which has been scarcely investigated.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from Fermentas (Glen Burnie, MD), and Promega (Madison, WI). Yeast extract was from Oxoid (Remel; Lenexa, KS). Plasmid DNA was isolated using QIAprep®Spin Miniprep kit, and DNA fragments were purified using the QIAquick®Gel Extraction kit (Qiagen). All other chemicals and reagents were analytical grade and were supplied by Sigma–Aldrich (St. Louis, MO).

Strains and culture conditions

Microorganisms used in this study are indicated in Table 1. Bacteria and archaea were grown at 37 °C in liquid cultures (150 rpm) or on solid medium supplemented with 1.5% (w/v) agar. Cell growth was monitored by measuring the optical density of the cultures at 600 nm (OD_{600}). *N. magadii* was grown in Tindall medium (Tindall et al. 1984) containing 20 g/L YE; *E. coli* was grown in Luria Bertani medium (LB) supplemented with the indicated antibiotics. *E. coli* DH5α was used for routine cloning and transformed by the $CaCl_2$ method (Sambrook and Russell 2001).

Cloning strategy

Genomic DNA was extracted from *N. magadii* cells as previously described (Ng et al. 1995). A DNA fragment of the *lon* gene was amplified by PCR from *N. magadii* genomic DNA using degenerate oligonucleotide primers LonF and LonR (Table 1) designed on the basis of highly conserved amino acid sequences of haloarchaeal Lon homologs. The PCR reaction contained: genomic DNA (100 ng), deoxynucleotide triphosphate (200 μM), $MgCl_2$ (1.5 mM), primers (1 μM), PCR buffer (1×) and *Taq* DNA polymerase (2 U). The reaction was incubated at: (94 °C 2 min) 1 cycle; (94 °C 30 s, 55 °C 30 s, 72 °C 30 s) 30 cycles; (72 °C 2 min) 1 cycle. The amplified DNA fragment of the expected size (~0.4 kb) was gel-purified,

Table 1. Strains, plasmids and oligonucleotide primers used in this study.

Strains and plasmids	Phenotype, genotype or oligonucleotides sequences	Source/reference
Bacterial/archaeal strains		
N. magadii ATCC 43099		ATCC
E. coli DH5α	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *supE44 relA1 lac* [F′ *proAB lacI*^{q}Z ΔM15::Tn*10*(Tet^r)]	NE BioLabs
E. coli One Shot®TOP-10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lacX74 rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-	Invitrogen
E. coli ER 1647	F⁻ *fhuA2* (*lacZ*) *r1 supE44 trp31 mcrA1272*::Tn*10*(Tet^r) *his-1 rpsL104* (Str^r)*xyl-7 mtl-2 metB1* Δ*(mcrC-mrr)*102::Tn*10*(Tet^r) *recD1040*	Novagen
E. coli BM25.8	*sup*E44, *thi* D(*lac—pro*AB) [F′ *tra*D36, *pro*AB+, *lac*I^{q}ZΔM15] *limm*434 (Kan^R)P1 (Cam^R) *hsd*R (r_{k12}^- m_{k12}^-)	Novagen
E. coli BL21 Rosetta (DE3)	F⁻ *ompT* [*lon*] hsd SB (r_B m_B) (an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
Plasmids		
pET-24b (+)	Km^r; 5309-bp expression plasmid vector	Novagen
pET-24 (+)-*Nmlon-His$_6$*	Km^r; 2334-bp coding region of *lon* lacking the translation stop codon (TAA) in the NdeI—HindIII sites of pET-24b(+); *Nm lon-his$_6$* expressed in *E. coli* Rosetta (DE3)	This work
Primers		
Lon-F (primer a)	5′-ATGAATTCGTCAACGGSCTCGCSGTSAT-3′	This work
Lon-R (primer b)	5′-TATAAGCTTGTTSGCCTYSGGGATGATGAC-3′	This work
LonR2	5′-GCGACGCGGACAAGTCCACC-3′	This work
LonR3	5′-ACCATTGCGTCCGTGCCGCG-3′	This work
LonR4	5′-GTAGACTGCGAGTTGGATGC-3′	This work
LonR5	5′-CTGCAGTTCGTAGTCCTTGC-3′	This work
LonF2	5′-CCTGCAGGATGTCTTGGTCT-3′	This work
LonF3	5′-GTCATCATTCCGAAGGCCAA-3′	This work
IPCR-R	5′-TGTGCGCGTCGATGATCTGT-3′	This work
IPCR-F	5′-GACGAGATGGTCGAGATCAT-3′	This work
Lon-*Nde*I-F	5′-ACAGATTCATATGAGCAACGATACGAACGT-3′	This work
Lon-*Hind*III-R non stop	5′-TTAAGCTTCTGTGGACTTGGGTTCGA-3′	This work
Primer e	5′-TTACTCTGCCGTCCACGTCA-3′	This work
Primer f	5′-CACCGTCGGCATTATCGTCA-3′	This work
Primer c	5′-AGAAGGACATCCACATCCAG-3′	This work
Primer d	5′-AGTCGACGCTGAACTCGATG-3′	This work

sequenced and labeled by random priming with [α-^{32}P] dCTP according to the manufacturer's instructions. The labeled fragment was used to screen a genomic library of *N. magadii* into λBlue-STAR vector (Novagen) (De Castro et al. 2008) by hybridization. Positive plaques were detected by autoradiography and purified. Cre-mediated plasmid excision was performed using *E. coli* BM 25.8. Plasmid DNA was amplified in *E. coli* DH5α, subjected to restriction enzyme mapping and sequenced using the dideoxy termination method (Sanger et al. 1997) with primers LonF2-3 and LonR2-5 (Table 1) (Macrogen and Cornell University DNA Facilities). The 5′-end of *Nmlon* was obtained by inverse PCR (I-PCR). *N. magadii* genomic DNA (1 μg) was digested with *Kpn*I and electrophoresed. DNA fragments ranging from 2 to 4 kbp were purified and subjected to self-ligation (40 ng) using T4 DNA ligase in a final volume of 100 μl at 16 °C for 20 h (Ochman et al. 1988). The ligation products were

purified and one half was used as template for I-PCR using primers IPCR-F and IPCR-R (Table 1) designed on internal nucleotide sequences of *Nmlon*. I-PCR conditions were: (94 °C 5 min) 1 cycle; (94 °C 45 s, 52 °C 45 s, 68 °C 1 min) 30 cycles; (68 °C 5 min) 1 cycle.

Bioinformatic analysis

DNA and deduced protein sequences were compared to public databases available at NCBI (www.ncbi.nlm.nih.gov) and MEROPS (www.merops.sanger.ac.uk) using BLAST. Sequence alignments were performed using CLUSTAL W2 and EMBOSS Pairwase Alignment Algorithms tools from EMBL-EBI (www.ebi.ac.uk). Transmembrane segments were predicted with Phobius (http://phobius.sbc.su.se). The genetic organization of *lon* in prokaryotes was analyzed using Microbes Online server (www.microbesonline.org).

Expression of *Nmlon* in *E. coli* and purification of the recombinant protein

For expression in *E. coli*, the *Nmlon* coding region (2334 bp) was PCR-amplified with *Pfx*50 (Invitrogen) from positive plaques using primers Lon-*Nde*I-F and Lon-*Hind*III-R-nonstop (Table 1) and the product was cloned into TOPO blunt-end vector (Invitrogen). The nucleotide sequence of the inserts was verified before subcloning. Plasmid DNA isolated from recombinant colonies was digested with *Nde*I/*Hind*III and subcloned into the expression plasmid pET-24b(+) (Novagen). The resulting construct encoded *Nm*Lon with a C-terminal polyhistidine tag *Ec-Nm*LonHis$_6$. *E. coli* Rosetta (DE3) cells harboring pET-*Nm*LonHis$_6$ was grown in LB medium containing 50 μg/ml kanamycin and 25 μg/ml chloramphenicol to an OD_{600} of 0.6—0.8. Expression was induced by addition of 0.4 mM IPTG, and samples were taken after 3 h (25 °C, 150 rpm). Cells were harvested by centrifugation at 5000 × *g* (4 °C, 10 min) and analyzed for Lon production. IPTG-induced cells were centrifuged at 10,000 × *g* (4 °C, 10 min), suspended in 0.1 M sodium phosphate buffer (pH 7.4), 10 mM $MgCl_2$ and disrupted by sonication (30 s 6, 60 W). Cell debris were removed by centrifugation at 4000 × *g* for 20 min and the supernatant (cell extract, T) was incubated in presence of 1% (v/v) Triton X-100 (10 min, on ice) and centrifuged at 12,000 × *g* (4 °C, 10 min). The supernatant was loaded onto a Ni^{2+} charged HiTrap IMAC HP column (0.7 cm × 2.5 cm) (Amersham). The column was washed with 0.1 M sodium phosphate (pH 7.4), 10 mM $MgCl_2$ and 1% (v/v) Triton X-100 with a flow rate of 0.5 ml/min. *Ec-Nm*LonHis$_6$ was eluted with the same buffer containing 0.3 M imidazole and dialyzed in 50 mM Tris—HCl (pH 8) containing 3 M NaCl. To determine the subcellular localization of *Nm*Lon, cell lysates of recombinant *E. coli* and *N. magadii* were fractionated into cytosol and membranes by centrifugation at 200,000 × *g* (2 h, 4 °C). Subcellular fractionations were analyzed by SDS-PAGE (8%) and Colloidal Coomassie Blue staining (Candiano et al. 2004) and by Western blotting. Rabbit polyclonal anti-*Nm*Lon antibodies were generated using purified *Ec-Nm*LonHis$_6$ as antigen by a specialized service (Natocor, Córdoba, Argentina). Western blots were performed by standard protocols using 1/10,000 anti-*Nm*Lon or 1/2000 anti-His$_6$ antibodies (Amersham) diluted in blocking buffer and 1/10,000 alkaline phosphatase-conjugated secondary antibody. Molecular mass standards were Full-Range Rainbow Molecular Weight Markers (Amersham).

RT-PCR

Total RNA was isolated from *N. magadii* cells at different growth stages using Trizol (Invitrogen). RNA concentration was determined spectrophotometrically at 260 nm and the integrity of RNA was evaluated electrophoretically in agarose gels. To eliminate residual contamination with genomic DNA, RNA samples were incubated with DNaseI (0.2 U/μg RNA) (Promega) at 37 °C for 2 h. The success of this treatment was checked by PCR. cDNA synthesis was performed with 2 μg of DNA free-RNA, appropriate primers (0.5 μg) (Table 1), dNTPs (0.5 mM), M-MLV-RT buffer (1×) and M-MLV reverse transcriptase (200 U) (Promega) in a final volume of 25 μl at 37 °C for 1 h. M-MLV-RT was inactivated at 70 °C for 10 min. PCR reactions were performed with 2 μl of cDNA in a final volume of 25 μl. The samples were preheated to 94 °C (5 min) followed by 35 amplification cycles consisting of denaturation (94 °C, 30 s), annealing (30 s, 50—54 °C), elongation (72 °C, 1—2 min) and a final extension cycle (72 °C, 5 min). For each primer pair, negative and positive controls were included to exclude genomic DNA contamination and to confirm primer specificity, respectively.

RT-PCR products were fractionated on 1% agarose gels and analyzed by Southern blotting (Sambrook and Russell 2001). DNA probes were amplified by PCR on *N. magadii* genomic DNA using the primers indicated in Fig. 3 and labeled with [α-^{32}P] dCTP using a random DNA labeling kit (Invitrogen). After hybridization (65 °C, overnight), the membranes were washed at high stringency (up

to 0.1× SSC-0.1% SDS at 65 °C), and incubated 10—60 min on imaging plate (Fuji) at room temperature and then visualized with a Storm analyzer (Amersham).

EMSA

*Ec-Nm*LonHis$_6$ (5—10 μg) was incubated with plasmid DNA (200 ng), genomic DNA (100 ng) or single stranded oligonucleotides (20 ng) in a solution containing 50 mM Tris—HCl (pH 8.0), 0.5, 1.5 or 3 M KCl and 10 mM $MgCl_2$ (25 μl total volume) for 30 min at 25 °C. Protein—DNA complexes were fractionated by gel electrophoresis in 0.8—1% (w/v) standard agarose (dsDNA) or 4.5% (w/v) low melting temperature agarose (ssDNA) in 1× TBE buffer. DNA bands were visualized by SYBR Safe (dsDNA) or SYBR Gold (ssDNA) staining (Invitrogen). Binding assays were performed at least three times.

Mass spectrometry

The purified *Ec-Nm*LonHis$_6$ was subjected to in gel digestion with trypsin followed by peptide mass fingerprinting by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker), in the Mass Spectrometry Facility CEQUIBIEM, Argentina. *Nm*Lon identified by mass spectrometry was deposited in the Pride database under the accession number of 10537.

Nucleotide sequence accession number

The nucleotide sequence of the *Nmlon* gene was deposited in GenBank database under the accession number of GQ118993.

Results and discussion

Cloning and sequence analysis of the gene encoding the *N. magadii* Lon protease

In this study the full-length gene encoding the *N. magadii* ATCC 43099 ATP-dependent Lon protease (*Nmlon*) was cloned by combining two different strategies: by screening a genomic library of *N. magadii* using a PCR-amplified probe specific for the Lon protease followed by I-PCR. *Nmlon* is 2334 bp long and encodes a polypeptide of 778 amino acid residues with an estimated molecular mass of 84,269 Da, which is slightly higher than that of the thermophilic Lon polypeptides *Ta*Lon (72 kDa), *Tk*Lon (70 kDa), *Af*Lon (68.2 kDa) and *Mj*Lon (71.9 kDa) (Fukui et al. 2002; Besche et al. 2004; Im et al. 2004; Botos et al. 2005). In agreement with the acidic nature of haloarchaeal proteins (Mevarech et al. 2000), the predicted *Nm*Lon polypeptide contains 20% of acidic amino acid residues and a theoretical pI of 4.42. Protein sequence alignment showed that *Nm*Lon is very similar (70—80%) to the predicted LonB proteases of haloarchaea while it is less related (30—60% similarity) to the Lon homologs of other archaea representatives.

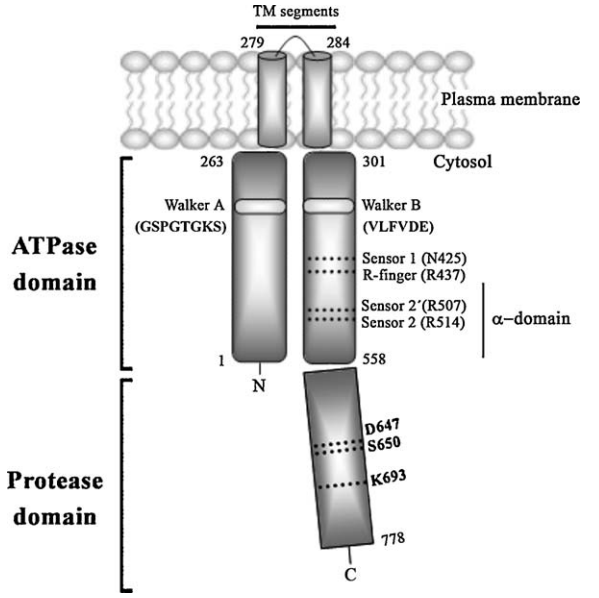

Figure 1. Schematic representation of *N. magadii* Lon protease. *Nm*Lon has an AAA$^+$ domain containing two transmembrane segments (TM) and a P-domain with a catalytic tryad (D^{647}, S^{650} and K^{693}). The positions of Walker A and B motifs, the Sensor 1, Sensor 2′, Sensor 2 and R-finger are indicated with the respective amino acid residues. Amino acid residues of the active site are indicated in bold letters.

*Nm*Lon contains the typical domains of LonB proteases: the AAA$^+$ domain at the N-terminus and the P-domain at the C-terminus of the protein (Fig. 1). The AAA$^+$ domain includes two transmembrane helices, which predict the membrane-bound localization of the haloarchaeal protease. The analysis of the primary sequence of *Nm*Lon revealed conservation of the catalytic tryad Asp647, Ser650, Lys693 (Fig. 1) critical for the protease activity of LonB proteases (Maupin-Furlow et al. 2005).

After we cloned the gene encoding *Nm*Lon, the complete genome of *N. magadii* 43099 was sequenced (CP001932), which allowed examination of the potential regulatory elements as well as the genomic organization flanking *Nmlon*. A putative TATA box (5′-TATATCAGT-3′) at −30 to −22 bp

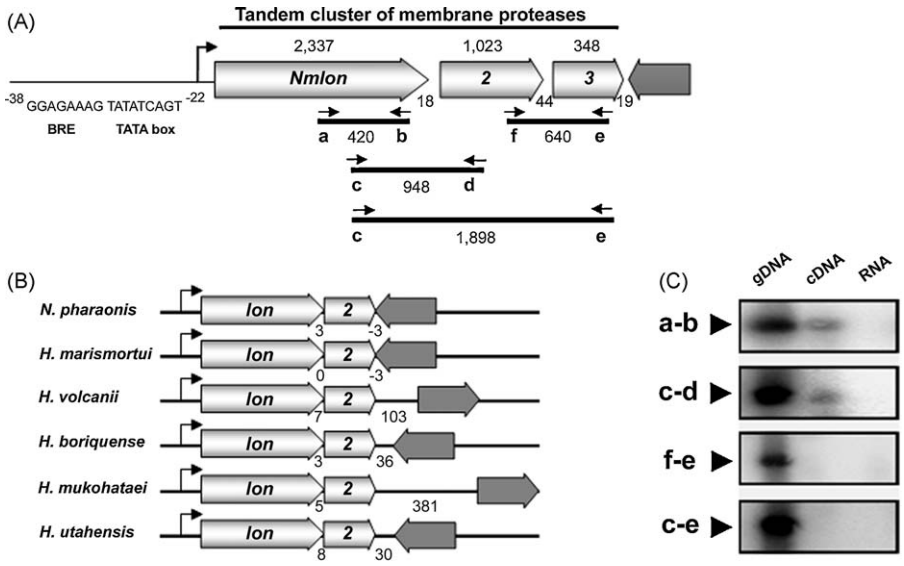

Figure 2. Genetic organization of *Nmlon*. (A) Tandem cluster of membrane proteases in *N. magadii* genome (CP001932). *Nmlon*, *orf 2*, *orf 3* and the corresponding intergenic regions are shown schematically with their respective sizes (bp). Potential TATA box and BRE-like elements of *Nmlon* promoter are indicated upstream of a putative transcription start site (arrow). (B) Genomic organization of *lon* in haloarchaea. Haloarchaeal *lon* and neighbour genes were extracted from complete genomic sequences [*Natronomonas pharaonis* DSM 2160 (CR936257), *Haloarcula marimortui* ATCC 43049 (AY596297), *Haloferax volcanii* DS2 (haloVolc1 from Genome Browser Gateway), *Halogeometricum boriquense* (ABTX00000000), *Halomicrobium mukohataei* DSM 12286 (CP001688), *Halorhabdus utahensis* DSM 12940 (CP001687). (C) RT-PCR analysis of *lon*, orf 2 and orf 3 transcripts. Total RNA from *N. magadii* was reverse-transcribed into cDNA using the reverse primers *b*, *d* or *e*. cDNAs were used as templates for PCR with primers *a*, *c* and *f* in combination with *b*, *d* and *e*, as indicated. PCR-Products were detected by Southern blotting with ^{32}P labeled-probes indicated by a bold line below the genome representation. Identical amplifications with genomic DNA (50 ng) and DNaseI-treated RNA (200 ng) were performed as positive and negative controls, respectively. PCR-Products obtained with cDNA from exponentially growing cells are shown.

and a transcription factor B recognition element (BRE) (5′-GGAGAAAG-3′) at positions −38 to −31 were identified proximal to a putative transcription start site (A) (Fig. 2A). These boxes are conserved in haloarchaeal *lon* promoters, however, their nucleotide sequences differ significantly from those of the typical promoters found in haloarchaea (5′-TTTAWAtr-3′ W = A/T, R = A/G) (Gregor and Pfeifer 2005). Further upstream (−271 to −306), three perfect 12-bp direct repeats (5′-GGTTTGGTTCGA-3′) were identified (not shown). It is possible that these elements may serve as the binding site for regulatory *trans-acting* factors as observed in other haloarchaeal promoters (Gregor and Pfeifer 2005). A potential ribosome-binding site (5′-GAACAGAG-3′) was identified at position +4 to +11.

Genetic organization of *Nmlon* in *N. magadii*

Examination of the gene organization flanking *Nmlon* revealed two open reading frames (orfs) downstream *Nmlon* (orf 1): orf 2 (1023 bp) and orf 3 (348 bp), each one flanked with their corresponding initiation and termination codons and their ribosome-binding sites at the 5′-end. The predicted gene products of orf 2 and orf 3 were annotated in *N. magadii* genome (CP001932) as ''abortive infection protein'' and ''hypothetical protein'', respectively. Based on conserved domain search and Blast search in MEROPS database, we found that the polypeptide encoded by orf 2 has six transmembrane domains, a signal peptide and contains the typical domains of membrane metallo proteases of the CAAX prenyl endopeptidase family. On the other hand, the polypeptide encoded by orf 3 contains four transmembrane domains, a signal peptide and is ∼50% similar to the C-terminal domain of an unassigned peptidase encoded by *Natronomonas pharaonis*. *Nmlon*, orf 2 and orf 3 are encoded in the same strand and are physically located next to another forming a tandem cluster (orf 2 is 18 bp apart from *Nmlon* while orf 3 is separated from orf 2 by 44 bp) (Fig. 2A) suggesting that they may be co-regulated at the level of gene expression. Adding to this observation, no promoter-like elements and transcription termination sites were evidenced in the intergenic regions, thus, we hypothesized that these genes may be functionally organized

as an operon. By inspection of the genomic organization in other haloarchaea we observed that the linkage between *lon* and orf 2 was conserved (Fig. 2B).

To examine whether *Nmlon* was co-regulated with *orf* 2 and 3, specific transcripts were analyzed in *N. magadii* cells at different growth stages by RT-PCR. PCR with primers *a* and *b* that hybridize to *Nmlon*, as well as primers *c* and *d* that hybridize to *Nmlon* and orf 2 (Fig. 2A) gave amplification products of the expected sizes (420 and 948 bp, respectively) (Fig. 2C). However, no products were amplified with primers *f* or *c* combined with primer *e* that binds *orf* 2 and 3 and *orf* 1, 2 and 3, respectively. Transcriptional continuity between *Nmlon* and (at least) orf 2 was also observed in Northern blots as evidenced by the detection of hybridization signals of similar size (>3 kb) with probes specific for *Nmlon* and orf 2—3 (data not shown). The inability to amplify a product with primers *c-e* or *f-e* might result from inefficient binding of primer *e* to the RNA template and/or premature reverse transcription termination by M-MLV-RT enzyme. On the other hand, we cannot rule out the possibility that cryptic promoters and/or terminators may be used to control gene expression in this operon in response to changing environments/conditions. Future studies will be conducted to examine *lon* mRNA expression in cells subjected to different conditions and to investigate whether *lon*, orf 2 and orf 3 are co-transcribed (or not) under such conditions.

Although the genetic organization of *lon* in archaea and bacteria predicts that this gene forms clusters with others of diverse functional categories, to the best our knowledge, the transcriptional linkage between *lon* and its neighbour genes has not been confirmed. The physiological significance of the clustering of *Nmlon* and orf 2 is not clear. Taking into account that in prokaryotes genes of related function are frequently co-transcribed as a single polycistronic mRNA, we can speculate that these proteases may be needed under similar physiological conditions. Alternatively, the physical organization of membrane proteins as tandem clusters may facilitate the mechanism of protein translocation to the cell membrane, as previously suggested (Kihara and Kanehisa 2009).

Subcellular localization and expression of Lon during growth in *N. magadii*

To validate the predicted membrane insertion of *Nm*Lon as well as to examine the cellular levels of *Nm*Lon during growth in *N. magadii*, *Nmlon* was overexpressed in *E. coli* as a fusion protein with a C-terminal His_6 tag to facilitate its purification and the generation of specific anti-*Nm*Lon antibodies.

A differential polypeptide with a relative mobility of 115 kDa was detected by colloidal Coomassie Brillant Blue staining (not shown) and Western blotting with anti-His antibody in *E. coli* cells harboring pET-*NmlonHis*$_6$ after induction with IPTG (Fig. 3). Lon polypeptide was enriched in the membrane fraction indicating that the transmembrane anchor of *Nm*Lon was functional in *E. coli*. To validate the identity of the recombinant protein, the Lon polypeptide was excised from the gel, subjected to trypsin digestion and analyzed by MALDI-TOF/TOF tandem mass spectrometry. Four peptides (SIEQQLADDYIER, GVLFVDEINTLDVR, LMTAIQEGEFSITGQSER and LVDQVIGQDEAR) were detected which corresponded to the *Nm*Lon protease (*Ec-Nm*LonHis$_6$). The molecular mass of *Ec-Nm*LonHis$_6$ polypeptide estimated by SDS-PAGE (~115 kDa) was higher than that calculated from the translated sequence of *Nmlon* (84 kDa). This fact was not surprising, as overestimation of the molecular mass by SDS-PAGE has been observed in other halophilic proteins due to the resistance of the acidic polypeptides towards SDS denaturation (Hou et al. 2000). *Ec-Nm*LonHis$_6$ was purified by Ni^{2+}-affinity chromatography to apparent homogeneity and with a high yield (20 mg protein/L cell culture). Purified *Ec-Nm*LonHis$_6$ was used to develop *Nm*Lon specific antibodies.

To assess the localization of Lon in the native microbe, cytosol and membranes isolated from *N. magadii* were probed with anti-*Nm*Lon antibodies by Western blotting. *Nm*Lon was detected associated to the membranes of *N. magadii* (Fig. 3), confirming the predicted membrane insertion of the

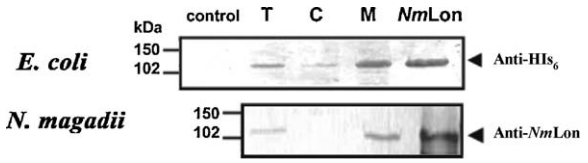

Figure 3. Heterologous synthesis and cellular localization of *Nm*Lon. Upper panel. Expression of *Nmlon* in *E. coli*. Synthesis of *Ec-Nm*LonHis$_6$ was induced by addition of 0.4 mM IPTG for 3 h. A cell lysate of *E. coli* transformed with pET24-*Nm*LonHis$_6$ was fractionated by ultracentrifugation and the recombinant protease was purified from the membrane fraction by Ni^{2+} affinity chromatography (*Nm*Lon). A cell lysate of *E. coli* transformed with empty vector was used as a negative control. *Nm*Lon was detected by Western blotting with anti-His antibody. Lower panel. Immunodetection of Lon in *N. magadii*. Cell lysate (T); cytosol (C); membranes (M).

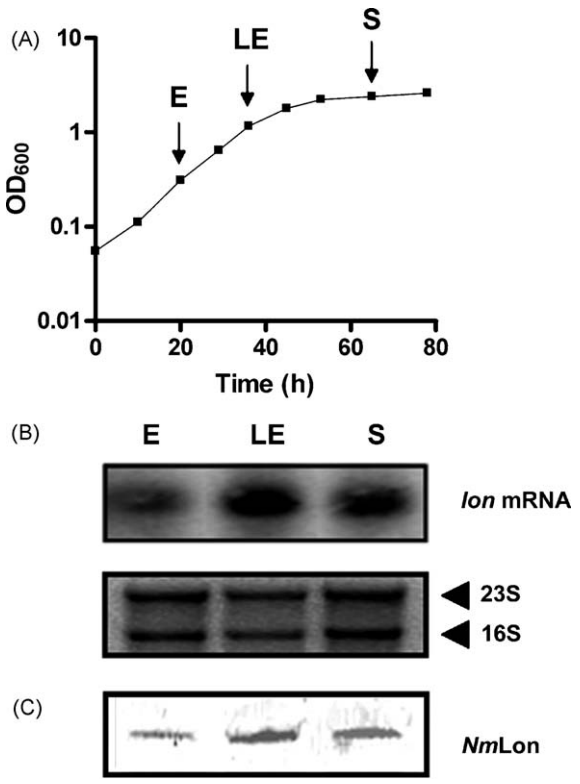

Figure 4. Detection of Lon throughout growth in *N. magadii*. (A) *N. magadii* was grown in Tindall medium containing 20 g/L YE. Samples were withdrawn in early exponential (E, OD_{600} 0.3), late exponential (LE, OD_{600} 1.2) and stationary (S, OD_{600} 2.5) growth phases (arrows). (B) Detection of Lon specific transcripts by RT-PCR. The ethidium bromide staining profile of ribosomal RNAs are shown as an indication of the quality of the RNA samples. Lon mRNA was amplified using primers a and b and detected by Southern blotting as described in Fig. 2. (C) Detection of *Nm*Lon in cell membranes (10 μg prot./lane) by Western blotting with anti-*Nm*Lon antibodies.

haloarchaeal Lon protease. This result, in agreement with those obtained for the thermophilic Lon proteases *Tk*Lon and *Ta*Lon (Fukui et al. 2002; Besche et al. 2004), clearly demonstrate that the archaeal-type LonB protease is a membrane-bound enzyme unlike the cytosolic LonA proteases of bacterial and eukaryotic origin.

The cellular mRNA and protein content of *Nm*Lon throughout growth in *N. magadii* were examined by RT-PCR and Western blotting, respectively. Lon specific transcripts were expressed in *N. magadii* cells harvested at different growth stages, correlating with the detection of an immunoreactive protein of the expected size (~115 kDa) (Fig. 4). The relative concentration of both Lon mRNA and protein seemed to increase as the culture aged. Taking into account that *in silico* analysis predicts that the Lon protease is the only identified ATP-dependent membrane-bound protease in archaea (including *N. magadii*), in parallel, we attempted to determine ATP-dependent membrane-bound caseinolytic activity. Surprisingly, although basal levels of Lon specific mRNA and protein were expressed in *N. magadii* throughout growth, protease activity was only detectable in membranes isolated from stationary phase cells (not shown), suggesting that *Nm*Lon may be activated by intracellular signals (denatured protein substrates, metabolic products) induced under specific physiological conditions. However, we cannot completely rule out the possibility that an unidentified ATP-dependent membrane-bound-casein degrading activity may be present in stationary phase cells of *N. magadii*. A quantitative analysis of the cellular content of Lon (at the mRNA, protein and activity levels) in *N. magadii* cells subjected to different conditions will provide insight into the potential physiological role of Lon in *N. magadii* and will guide future studies aimed at addressing the biological function of this protease in haloarchaea.

DNA-binding of *Nm*Lon

It is assumed that the A subfamily (bacterial and eukaryal Lon proteases) are DNA-binding proteins (Fu et al. 1997; Lee et al. 2004; Lu et al. 2007). To examine the interaction of the archaeal-type LonB protease with DNA, EMSA were performed using purified *Ec-Nm*LonHis$_6$. Considering the halophilic nature of this protein, incubations were performed in the presence of 0.5, 1.5 and 3 M KCl. As it is shown in Fig. 5A, migration of DNA was shifted upon addition of *Ec-Nm*LonHis$_6$, indicating the formation of protein—DNA complexes. Similar results were observed in the presence of 1.5 and 3 M KCl concentrations (not shown), however, migration of Lon—DNA complexes was anomalous due the high salt concentration in the samples. These results showed that *Nm*Lon can interact with DNA molecules (single and double stranded), demonstrating that DNA-binding is conserved in the LonB subfamily as well. Studies on the degree of specificity of the DNA-binding, the relevant domains of the protease involved in this interaction as well as the effect that DNA-binding exerts of the enzymatic activities of Lon are somewhat controversial (Lee and Suzuki 2008). A 35 bp element referred to as *pets* has been shown to be a target for *Ec*Lon (Fu et al. 1997). In mammalian cells, *Hs*Lon preferentially binds G-rich single stranded DNA located in the control region for mitochondrial replication and transcription (Fu et al. 1997). Based on these evidences and taking into account that both

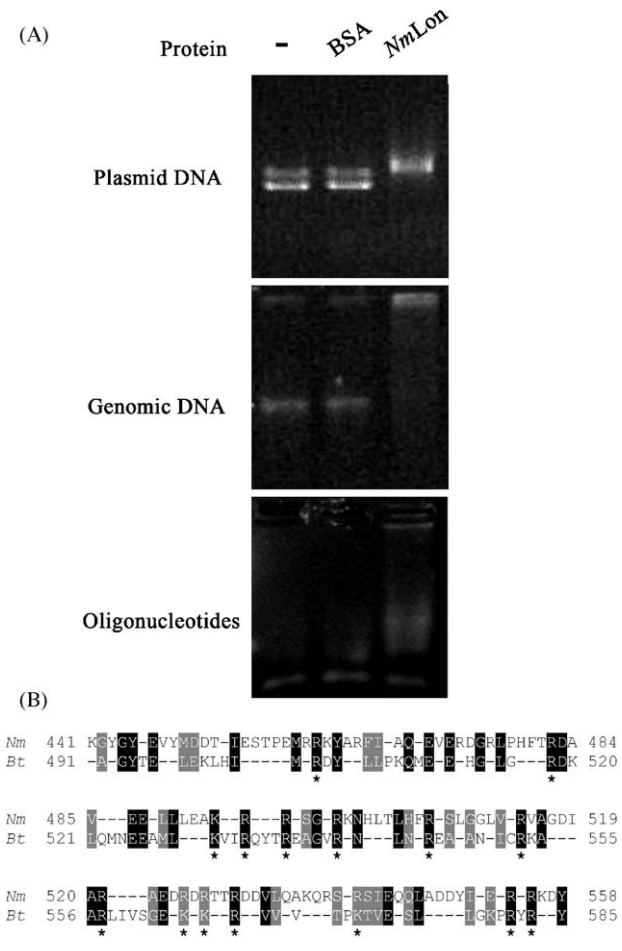

Figure 5. DNA-binding of *Nm*Lon. (A) EMSA. Binding reactions containing 5 μg of affinity-purified *Ec-Nm*LonHis$_6$ (or BSA) and DNA (200 ng plasmid, 100 ng genomic DNA or 20 ng oligonucleotides) were incubated at 25 °C for 30 min in the presence of 0.5 M KCl. Protein—DNA complexes were analyzed by agarose gel electrophoresis and stained with SYBR Safe (dsDNA) or SYBR Gold (ssDNA). (B) Comparison of the α-domains of *Nm*Lon (*Nm*) and *Bt*Lon (*Bt*). The amino acid sequence alignment was generated using EMBOSS Pairwase Alignment Algorithms. Sequences used in the analysis (GenBank accession numbers): *Nm*, residues 441—558 (ACR43883); *Bt*, residues 491—585 (AY197372). Identical residues are shown in white letters and black background; conserved residues are shown in white letters and gray background. Conserved lysines and arginines are indicated with asterisks.

the bacterial and eukaryotic Lon degrade proteins involved in transcription and/or DNA methylation, it was suggested that the DNA-binding activity of Lon may facilitate the accessibility of this protease to the specific substrates (Lee and Suzuki 2008). The α-domain of the Lon protease from *Brevibacillus thermoruber* WR-249 (*Bt*Lon) has a critical role in DNA-binding specificity and positively charged amino acids located in this domain are relevant to DNA-binding (Lin et al. 2009). To further explore on the DNA-binding activity of *Nm*Lon, the α-domain of *Nm*Lon was aligned to that from *Bt*Lon. Although the overall similarity was low (42%), about 70% of the arginine and lysine residues were conserved (Fig. 5B). The relative concentration of basic amino acids in this domain was significantly increased (*Nm*Lon α-domain R + K = 21.2%, pI = 9; *Nm*Lon full-length R + K = 9.6%, pI = 4.4) as observed in *Bt*Lon (*Bt*Lon α-domain R + K = 21%, pI = 9.9; *Bt*Lon full-length R + K = 14.5%, pI = 6.1). The importance of positive charge on DNA—protein interaction was also demonstrated for the processivity subunit of the Herpes simplex virus DNA polymerase (Komazin-Meredith et al. 2008).

Although transmembrane DNA-binding proteins are rare, there exist membrane-bound regulators in bacteria. The BcrR protein was identified as a membrane-bound sensor and DNA-binding protein, which confers high level bacitracin resistance in *Enterococcus faecalis* (Gauntlett et al. 2008). Other examples include the ToxR family in *Vibrio cholerae* and close relatives (Osorio and Klose 2000).

In summary, this study shows that haloarchaea express a membrane-bound Lon homolog that belongs to the B family and which displays DNA-binding activity *in vitro* in the presence of elevated salt concentrations, a feature that may be relevant for its physiological role. *Nmlon* is co-regulated with a membrane metalloprotease gene and is expressed at the mRNA and protein levels throughout growth in *N. magadii*. Ongoing studies are focused at elucidating the role of the Lon protease in the physiology of haloarchaea by means of biochemical and genetic studies.

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