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# Nmag\_2608, an extracellular ubiquitin-like domain-containing protein from the haloalkaliphilic archaeon *Natrialba magadii*

María Victoria Ordóñez · Débora Nercessian · Rubén Danilo Conde

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Abstract Ubiquitin-like proteins (Ubls) and ubiquitinlike domain-containing proteins (Ulds) found in both eukaryotes and prokaryotes display an ubiquitin fold. We previously characterized a 124-amino acid polypeptide (P400) from the haloalkaliphilic archaeon Natrialba magadii having structural homology with ubiquitin family proteins. The reported N. magadii's genome allowed the identification of the Nmag\_2608 gene for the protein containing P400, which belongs to specific orthologs of halophilic organisms. It was found that Nmag 2608 has an N-terminal signal peptide with a lipobox motif characteristic of bacterial lipoproteins. Also, it presents partial identity with the ubiquitin-like domain-containing proteins, soluble ligand binding  $\beta$ -grasp proteins. Western blots and heterologous expression tests in E. coli evidenced that Nmag\_2608 is processed and secreted outside the cell, where it could perform its function. The analysis of Nmag\_2608 expression in N. magadii's cells suggests a cotranscription with the adjoining Nmag\_2609 gene encoding a protein of the cyclase family. Also, the transcript level decreased in cells grown in low salinity and starved. To conclude, this work reports for the first time an extracellular archaeal protein with an ubiquitin-like domain.

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M. V. Ordóñez  $[\square] \cdot D$ . Nercessian  $\cdot R$ . D. Conde Degradación de Proteínas, Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CONICET, Funes 3250 CC 1245, 7600 Mar del Plata, Argentina e-mail: mvordone@mdp.edu.ar **Keywords** Halophilic archaea · *Natrialba magadii* · Ubiquitin-like domains · Extracellular proteins · Protein–substrate interaction

# Introduction

Ubiquitin, a small protein highly conserved in eukaryotes, acts as post-translational modifier of proteins in many cellular events (Ciechanover 1994; Ciechanover and Iwai 2004; Hochstrasser 2009). For this, ubiquitin binds to proteins in a process catalyzed by three enzymes known as E1, E2, and E3 (Ciechanover 1994; Pickart 2001). This process known as ubiquitination controls the roles of target proteins by altering their association with other macromolecules (Kerscher et al. 2006). Ubiquitin displays a stable  $\beta$ -grasp fold composed of 5  $\beta$ -sheets arranged in an antiparallel way, an  $\alpha$ -helix between the second and third  $\beta$ -sheet, and a conserved di-glycine motif in its C-terminus (Burroughs et al. 2007a, b). Because the ubiquitin protein conjugation is crucial for cellular homeostasis, the question has arisen whether prokaryotes have an equivalent mechanism.

Modern DNA sequencing techniques made possible to know the genomes of several archaeal organisms in short periods of time (Soppa 2006). This allows to reveal the sequence of many archaeal proteins but not to define their physiological role, which is often not related to any bacterial or eukaryal counterpart. Thus, assignment of roles to many new identified proteins becomes a difficult task (Makarova and Koonin 2003). Fortunately, gene context and bioinformatic sequence analysis, including structural prediction programs, became strong tools in revealing the roles of genes from a sequenced archaeal genome. These approaches have been used by several groups in the search of ubiquitin-like proteins within prokaryotes.

Several eukaryotic and prokaryotic proteins having ubiquitin fold but low amino acid sequence identity with ubiquitin have been described (Hochstrasser 2009). They are classified as ubiquitin-like (Ubls) and ubiquitin-like domain-containing proteins (Ulds). As ubiquitin, eukaryotic Ubls act in conjugation pathways to modify proteins (Hochstrasser 2000; Dye and Schulman 2007). In prokarvotes, the best studied Ubls, ThiS and MoaD, display only 14 and 17 % sequence identity with ubiquitin, respectively. They are sulfur carriers involved in the biosynthesis of thiamine and molybdopterin cofactors, respectively (Marguet 2001; Rudolph et al. 2001; Wang et al. 2001). As eukaryotic Ubls, they require an activation step by an E1-like enzyme. Although this step will lead to the attachment of a sulfur atom to the C-terminus of a protein instead of binding with a protein, it resembles the ubiquitin conjugation pathways (Kerscher et al. 2006).

Archaeal genome survey has led to identify several genes encoding proteins with a  $\beta$ -grasp fold as well as E1and E2-like proteins in these organisms (Iyer et al. 2006). So, looking for the presence in archaea of an ubiquitination-like system, two ubiquitin-like small archaeal modifier proteins (SAMP1 and 2) have been recently described (Humbard et al. 2010). The authors propose that SAMP1 and 2 are conjugated to target proteins and that this process is related to the proteasome 20S from the halophilic archaeon *Haloferax volcanii*. Also, these proteins display a similar structure as Ubls as well as the di-glycine motif in the C-terminus (Jeong et al. 2011; Ranjan et al. 2011).

The ubiquitin fold has also been found in larger multidomain proteins (Hartmann-Petersen and Gordon 2004). These Ulds have been found performing several roles in eukaryotes (Kiel and Serrano 2006; Grabbe and Dikic 2009). An Uld is defined as a 45- to 80-amino acid region that resembles the folding and, occasionally, the amino acid sequence of ubiquitin. It is found in one or more repeats inside either the N-terminal or the C-terminal region of proteins, usually lacking the di-glycine motif typical of ubiquitin and Ubls (Burroughs et al. 2007a; Grabbe and Dikic 2009). As a result, they do not bind covalently with other proteins. However, they can form non-covalent interactions with proteins containing either ubiquitin-associated or ubiquitin-like binding domains (Kiel and Serrano 2006).

We have previously characterized a 124-amino acid polypeptide (P400) from the haloalkaliphilic archaeon *Natrialba magadii* (Nercessian et al. 2009). Despite not displaying significant sequence identity with any known protein, including ubiquitin, P400 shows a folding similar to that of Ubls according with in silico secondary structure prediction. Also, we obtained a 3D model of P400 similar to that of human ubiquitin 1 (pdb code: 1UBQ). These results also fit with FTIR (Fourier transform infrared spectroscopy) studies showing that the recombinant P400 is mainly built of  $\beta$ -sheets, similar to Ubls (Ordóñez et al. 2011).

Based on the recent sequenciation of the *Natrialba magadii*'s genome, this work intended to settle the gene encoding P400 and analyze its expression in cells at both different growth phases and culture conditions. Also, an exploration on its biological role was performed.

# Materials and methods

## Bioinformatic analysis

A search for the complete gene to which *p400* DNA fragment correspond was done using sequence identity searches blastP against *Natrialba magadii*'s, ATCC 43099, Shotgun complete Genome at NCBI (http://www.ncbi.nlm. nih.gov/BLAST/). Protein sequence homology searches were performed with psi-blast. Protein sequences alignment of the proteins retrieved from psi-blast were done using ClustalW (Thompson et al. 1994). Manual adjustment of the alignment was performed accordingly with psi-blast results. Secondary structure predictions were calculated with Jpred and Psipred (Cole et al. 2008; Jones 1999). Gene context analysis was done using Integrated Microbial Genomes (IMG) Data Management System (Mavromatis et al. 2009).

Biological material and growth conditions

The haloalkaliphilic archaeon, *Natrialba magadii*, ATCC 43099, was grown in culture medium as described by Tindall et al. (1984), except that casamino acids were replaced by yeast extract (5 g/l). Also *N. magadii*'s cell cultures under starvation (yeast extract was removed) or low salt (15 % w/v of NaCl) conditions were developed. *Escherichia coli* cells were grown in LB medium supplied with antibiotics when needed. Cells were grown at 37 °C under aerobic conditions and constant agitation (170 r.p.m.). Growth was measured by optical density at 600 nm (OD<sub>600</sub>).

## RNA purification

Total RNA was isolated from *N. magadii*'s cells from exponential ( $OD_{600} \sim 0.7$ ), early stationary ( $OD_{600} \sim 1.2$ ) and late stationary ( $OD_{600} \sim 2$ ) growth phases of control and treated cultures using TriZOL reagent (Invitrogen S.A.) as specified by the manufacturer. RNA was resuspended in DEPC-treated H<sub>2</sub>O and its concentration was determined using an Ultraspect 1100 spectrophotometer.

#### Northern blot analysis

RNA from different growth phases was denatured by incubation with loading buffer containing 0.2 µg/ml BrEt and 6 M glyoxal at 55 °C for 1 h. RNA samples were loaded onto a 1.5 % (w/v) agarose gel and electrophoresis was performed at 100 V for 1 h. RNA was transferred from the gel to nitrocellulose Hybord N+ membrane (Amersham S.A.) by downward capillarity action for 16 h using  $10 \times$  SSC (Ming et al. 1994). RNA was cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene S.A.) and then hybridized to gene-specific probes. Nmag 2608 probe, corresponding to the middle region of Nmag\_2608 gene, was obtained by PCR from N. magadii's genomic DNA using specific primers: F1 sense 5'-GCGGATCCA GATCTTCGTCAAA-3', R1 antisense 5'- TAAGCTTGAT CTTCGTGAAGA-3'. Also, a probe for the gene contiguous Nmag 2609 was obtained by PCR from N. magadii's genomic DNA using specific primers: F2 sense 5'-ATGT ACGTCGATCTTACCCA-3', R2 antisense 5'-TTAGT CAGCGGTCGATGCGG-3' and used to determine the possible co-expression of the gene Nmag\_2608 together with Nmag 2609 gene. 7S-specific probe for the constitutively expressed ribosomal 7S rRNA gene was obtained by PCR from N. magadii's genomic DNA using the specific primers: F3 sense 5'-AGTTGCTGATGCCGGCGTGTC-3' and R3 antisense 5'-GGTGGTCCGCTGCTCACTTC-3' borrowed from Dr. De Castro and used as loading control. All probes were radioactively labeled using Random Primers DNA Labeling System (Invitrogen S.A.) and <sup>32</sup>P-αdCTP as specified by manufacturer. For the hybridization step, membranes with cross-linked RNA samples were incubated with modified Church solution (0.5 M phosphate buffer pH 7.5, 7 % SDS and 10 mM EDTA pH 8) for 30 min at 65 °C followed by incubation with 12.5 ng of the denatured <sup>32</sup>P-labeled probe in the same solution at 65 °C for 16 h. Membranes were washed twice with 2× SSC-0.1 % SDS for 20 min followed by one wash with  $0.5 \times$  SSC-0.1 % SDS for 20 min. For detection of the hybridized RNA, membranes were exposed in an amplification film Fuji Imagining Plate Type BAS III and analyzed by the bioimaging analyser Storm 840 Amersham Biosciences. Densitometry analysis was performed using ImageQuant 5.2 software.

## Heterologous expression of Nmag\_2608 gene

The *Nmag\_2608* gene was amplified by PCR from *N. magadii*'s genomic DNA. Two versions of the gene with (F4) and without the first twenty residues of the protein N-terminal region (F4') were obtained using two sets of sense primers: F4 5'-CATATGCGACAGCTTGCCGCCC T-3' and F4' 5'-CATATGAGTCGTTCGACTCATCG CCA-3' containing restriction sites for *NdeI* enzyme

combined with the antisense primer R4 5'-TTAAG CTTGTCGTCAGCCGACTG-3' containing restriction site for *Hin*dIII enzyme. The products were digested with *Nde*I and *Hin*dIII and cloned into the expression vector pET24b(+) (Novagen), digested with the same enzymes. The resulting constructs, pET24b-*nmag2608C* and pET24b-*nmag2608T*, were transformed into *E. coli* Rosetta (DE3) competent cells, plated onto LB plates containing 50 µg/ml kanamycin and 25 µg/ml chloramphenicol, and incubated at 37 °C overnight. Positive clones were verified by colony-PCR. *E. coli* Rosetta (DE3) cells harboring each construction were grown in selective LB medium to an OD<sub>600nm</sub> of 0.5 and expression was induced by adding 0.5 mM IPTG for 3 h at 37 °C. The cell pellets and clarified medium were separated by centrifugation.

### Protein preparation

Cells were collected from different growth phase cultures of N. magadii or E. coli Rosetta strain expressing Nmag\_2608 and resuspended in buffer 100 mM Tris-HCl pH 8, 500 mM NaCl and 1 mM PMSF. The resuspended cells were sonicated and centrifuged at  $17,000 \times g$  for 30 min and the supernatant was stored as total protein extracted. Extracellular protein samples were prepared by precipitation with 50 % acetone and 1 mM PMSF of the clarified culture medium, at 4 °C for 16 h. The protein pellets were resuspended in buffer 100 mM Tris-HCl pH 8 with 500 mM NaCl or SDS loading buffer. All proteins were conserved at -20 °C. Protein concentration in the samples was determined with bicinchoninic acid using bovine serum albumin (BSA) as standard (Smith et al. 1987). Extracellular proteins from N. magadii cultures at OD<sub>600nm</sub> between 1 and 1.5 were also prepared. Cell pellets were harvested at 2-3 h intervals during the stationary phase of growth and extracellular proteins were obtained as described above.

#### SDS-PAGE and western blot assays

Total cellular and extracellular protein preparations from *N. magadii* and *E. coli* cultures expressing different versions of Nmag\_2608 protein were loaded onto 12 % polyacrylamide gels. Following electrophoresis, proteins were transferred to a nitrocellulose membrane. Immuno-detection assays were performed by incubation of membranes with anti-P400 primary polyclonal antibody (1:5,000) or anti-ubiquitin polyclonal primary antibody (1:100) (Sigma S.A.) for 16 h. The immunoreactive band was visualized after incubation with alkaline phosphatase-conjugated secondary antibody (1:10,000), using NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate disodium salt).

## Results

Identification of the gene containing P400 region: Nmag\_2608

An in silico survey of the *N. magadii*'s genome using P400 sequence as query identified the ORF Nmag\_2608 of 262 residues (blastP from NCBI). This ORF is annotated as conserved hypothetical protein without sequence similarity with any protein of known role. The protein encoded by Nmag\_2608 is 90 amino acids longer in its C-terminus than P400 and has a signal peptide in the N-terminus (SignalP; Bendtsen et al. 2004) (Fig. 1). This N-terminal region belongs to signal peptides that contain a lipobox motif L-[I/G/A]-[A/G/S]-C (Hayashi and Wu 1990) present in bacterial lipoproteins for secretion (Prosite program; Hulo et al. 2007). In Nmag\_2608, this motif contains Ser instead of Leu.

For further characterization, psi-blast sequence similarity searches using Nmag\_2608 as query against the NCBI nr database with a threshold e value of 0.05 were performed. Proteins with no assigned role from different halophilic archaea were retrieved (Fig. 2). They show good sequence identity and similarity with Nmag\_2608 (e value between e-42 and e-11 after 3 iterations). Also, all of them have a similar length to Nmag 2608, and several contain a lipoboxlike signal motif. Nmag\_2608 probably belongs to a halophilic archaeal family of secreted proteins having a putative lipoprotein signal peptide. Then, a group of proteins containing domains related to solute binding was retrieved by iteration 4 of this psi-blast search. Among them periplasmic solute-binding proteins (mainly phosphate binding proteins) with a lysR domain (pfam code PF03466); proteins with immunoglobulin fold domains (protein OM2255\_1450 glycosyl hydrolase-like protein and fibronectin type III domaincontaining proteins); and V12B01\_23145 SLBB-like proteins from Vibrio splendidus (see supplementary material).

Analysis of the secondary structure of Nmag\_2608 by prediction programs showed that, beside P400, residues in C-terminal region display a secondary structure mainly composed of  $\beta$ -sheets with an ending  $\alpha$ -helix (Fig. 2).

Fig. 2 Multiple sequence alignment of Nmag\_2608 and related halophilic proteins. Sequences were obtained by psi-blast sequence similarity search after 3 iterations (*e* value threshold 0.05) using NCBI tools (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments were done using ClustalW and manually corrected according to psi-blast results. Protein names are indicated at *left* while their lengths at *right*. The secondary structure predicted by psiPred program is indicated in the *last lane* of each alignment, where  $\beta$ -sheet is signaled by **e** and  $\alpha$ -helix by **h**. Nmag, *N. magadii*; Huta, *Halorhabdus utahensis*; Htur, *Haloterrigena turkmenica*; Hbor, *Halogeometricum borinquense*; rrnAC, *Haloarcula marismortui*; Hmuk, *Halomicrobium mukohataei*; HQ, *Haloquadratum walsbyi*; Hlac, *Halorubrum lacusprofundi*; HVO, *Haloferax volcanii*; VNG, *Halobacterium* sp.

Since similarity searches did not retrieve any protein sequence with known function (threshold below 0.005 and identity higher than 15 %), a gene context analysis of Nmag\_2608 was performed. It allows to find out functional relationships of annotated "conserved hypothetical proteins". This term refers to proteins without sequence similarity with those of known function (Mavromatis et al. 2009). Using these IMG tools, a group of nine halophilic archaeal ortholog genes to which Nmag 2608 belongs was retrieved, which showed a divergent distribution around the gene of interest. Only Hmuk\_2777 and Huta 2659 from Halomicrobium mukohataei and Halorhabdus utahensis, respectively, show a conserved gene neighborhood. Finally, Nmag\_2608 gene is found in the same chromosomal cassette with methyl-tRNA synthetase Nmag 2601, beta-lactamase Nmag 2602, pyruvate kinase Nmag\_2605, cell surface adhesin Nmag\_2606, cyclase Nmag\_2609, ATP binding protein Nmag\_2610, and several hypothetical proteins including the upstream gene Nmag 2607.

Expression of *Nmag\_2608* gene during early stationary phase of growth of *N. magadii*'s cells

The existence of *Nmag\_2608* gene encoding the P400containing protein led to corroborate its expression in *N. magadii*'s cells. Northern blot tests using P400 DNA as probe to evaluate *Nmag\_2608* expression during growth

P400 Nmag_2608	MR QL AALLL VALL VMS AGC SAFDSSPD GD R EPLS VENDEL VP GL T ED G I ND TT AF AN AH YD GL E YS E F ER T E OR V I HN ES	
P400 Nmag_2608	GD V V C T T N T T Q T V T E E A A R Y V L R V D P D S L A D P E P D A VMT R E T WR E D G D R MS I S R V T D A A G T T E F H G Q N MGM E S T S L P V S L GD V V C T T N T T Q T V T E E A A R Y V L R V D P D S L A D P E P D A VMT R E T WR E D G D R MS I S R V T D A G T T E F H G Q N MGM E S T S L P V S L	
P400 Nmag_2608	Y ELS EVEHT VS V	
P400 Nmag_2608	ELEEPDWVEEGWAELESQSADD 262	

Fig. 1 Alignment of the P400 polypeptide sequence with hypothetical protein Nmag\_2608 from *Natrialba magadii*'s genome. A blastp was performed using the amino acidic sequence of P400 against the genome of *Natrialba magadii* ATCC 43099. Nmag\_2608 correspond to the conserved hypothetical protein Nmag\_2608 sequence. *High-lighted in grey* in Nmag\_2608 sequence is a signal peptide predicted by SignalP 3.0 HMM and Prosite (signal peptide probability 1.000)

Nmag_2608		
	·····MRQLAALLLWALLVMSAGG········SAFDSSDDDDREPLSVENDELV····POLTEDGINDTT···AFANAHYDD. · EVSEFERTEQ	74
Huta_2669	MLALLWYRMSMRFLVWTLLVATAGGTTLTGSEEPETYT BAAVPTDDPADAPAQVVVP.GYVADGEIDVSWLVDAHREAL-ANQGFVWRATF	
Htur_1300	MRASPLSVIAVVALVISC.VGLPNAGENQPPDVSPDEFPNASEI	69
		77
Hbor DRAFT_3827		
rrnAC3133	NVCYSMARTMGWRLLVLIVVVTAGCGGFGF·····SDGDDPVTPADVPTSSATT·YP·····TGVGPTGVTSPS···LUGSAHGDRU·NGSGYTVTMNR····································	54
rrnAC0024	······································	85
Hmuk_2777		82
HQ,1326A	·····MSWEYFAAIAWACELAFAGY······GYVAPDDPTPTTSPAL·NS····TAISDETLADAEFP···EGFSRSEIDIATARRLSITF	72
Hmuk_0909	·····MRLGPLALVAVALL···AGOGGLVG····TSTETTYTAAVVPEPPATESTGSAIAP··BVGGARIVDAD···RLARAHRTAI·RMRBVVWQERO	81
Hbac_1301		79
	MORROFFIGYGTGYAYGSAGGLGG	
Hbac_0 102		
rrnAC0236	····MLLERRHLSLALWYLLIASAGCSGLFG·······SETGTGTSETPTSADANGPA···P·GSSG···FGAG···AIERGTFETL·SNSGFTTSLSF	77
rrnAC3368	HAGEIAVLLVLLFAGG-GAFGGAFRQTYTPVDFQSQDDRANPAYTEDA-VDARREAAEHRRAT-RRREYVLGMRL	. 75
rrnAC3416	······································	75
HQ,1800A	MARTPIIVAGVVFLMIAGC	66
rrnAC2940	······MRRGYALAVWCCLLSAGCTGLFGDN···AAARETYTDADYPTADAPTSAAQ··RA·POVYGDRYYMAT···AUGRAHVRR-SNTOYRYHHTF	84
HVO_0494	MYSNG I PYLLIAYLYLL. AGGAGG ADVADAA DADDTDTPTDGGG SADTGAS SOGD DFEL SDPERLEDAG. SFTVSY	1 75
	MITELLIVEV SLUVE CGCLGGGP	
rrnAC2054		
VNG1538H	MRRQ LAALL VYALVAYAGG	01
rrnAC2720	·····MWQVTLAVGIAUI···AGGSSFFPTSP····TADDSDDTDADVPTDSPEA·TV····AVPTSDGTVDVA···RULDRDEQAU·ASRGEHRHV··	76
2rystructure .	······································	
Nmag_2608	VINNESGDVVCTTNTTQTVTEEAARVVLRVDPDSLADPEPDAVMTRETWREDEDRMSISRVTDAAGTTEFHQNM	149
Huta_2669	NWSGQVGNVSSQ.FGT FQEITFEEGTRVVRDTNQ.DLNERGEPIYIRYSEYANGSD RUTESI RYLSGEVEVNRINVSDS	166
Htur_1300	NRTORNPSVLEDDFSHMNDTSRTLVEPDASQVLEHTTGVFSGNOSTVSNGSTEVVLSRENDWTEVRKLSPVS	141
		160
HborDRAFT_3827		150
rrnAC3133	TVRYANGSLRSHLR	163
rrnAC0024	SEF-TIGNDSSSVRREETWAVESPTRYRDEMVRMTTDSNGAVERYEQSTWADGTNWWEERDNGTVERQRGEVRFSRDKYA	164
Hmuk_2777	STSRQRANETLDTRQVVQVANESTVAVWTNERVVWDESRARVFDNYSEFQGPDDRUVEVTELDGETRQRQLGSTAPRPE	161
HQ 1326A	LRTEPV-SGVALERFRPG-AYADVQVEAGATRARFRLDVHNGYADVSQNDVYVESNVRHSBSGRNGRIAFKMTNGSVG	148
Hmuk_0909	RASPVETNESERIDTRLWVERERRYRVELSTSWRPVNTSEYTEDET.RVERDVSRAGFTYSTAPGTNV	148
	TOEDSNETTYRTVOTDAGTASVRWSDTLFGSD	147
Hbac_1301		141
Htac_0 102	A ETTHDGDTEPSPWLPSQEYESAYDLENGRQYYRQELTETEETDYSELYYADGEALYGQRIGDETQYGRQSYDRS	140
rrnAC0236	QLSTVRDGKNRSVFINR···TVAIDRESDRSLATGELVQASGDTL······ATTTYTADET··TAEERVLTRGDDRRTDYRSASPPYD·····	154
rrnAC3368	SLEWGTQIVSVDERAQGAWTATRPTP	135
rrnAC3416	WYTDSNGTVLRRSNHTRYVAYNKTTYAGQ FRQ NGTELNTFTTRIDYWTNGSIYAAQYDERVNRRSQ YKWSVRDDGPV	152
HQ 1800A	RTVTETTNVSVIQQTES TRRIDLRANQELLVATTTQQQEAQ SQ SQ - PQ SRSVQ STDQYYNNNGT - I W N QTQ SNQ SQ YA IQQQQA	152
rrnAC2940	TVMNGSGLHRRVRTRASISAGVESYTATRTISGQSVTAREIHSTWRDERAL-RRTTVNGSTSTEVVADSRGIGGPPR	160
		100
HVO_0494	SYTGYETAVTREFAADLDAERSYSR-TTSTTDGQSDAGGVEQYVADGV-TTYDVGGDDEATYTSYEGSTE	143
rrnAC2054	SYSTTEAGETATITNVYQYDLQANRSLETFTMSGPDARTDFETEYADGSAVTRYETDGEAFYQVRPQDDT	134
VNG1538H	AQSLANSTQRAETTGVVRGNAASGALFLRTQSPRGQTVAVYTDENGTAVERRAAGNRSQVRDASGRAP	135
rrnAC2720	• EQ.AGPQ.NTLD • VWIDREREIRRVRRRLGPI • • • • • • • • SNDVVLANRT • • • • • • • • • • • • • • • • • • •	136
2rystructure .	00000000000000000	
Nmag_2608		205
Huta_2669	TARLMQ RIT. APVALY LSADSVRSEP. VRIDE. ESHYRIDVRSAPD. TVNTGISNYSVTAF	
Htur_1300	······WFNESGEYYLWRGVFNNDSDLGYDFAAIDATYEREG·····VEWFQG······VPVM@YEATGVDA····LPDRWAGGENASSNFEEFSATLLL	
Hbor DRAFT_3827		210
rrnAC3133	TWRYWYHTA-ALNGRPAADYTRTVAPFHTRTRQSQQPDCTYYYIRGDRLRENTTSTAWTSRENATLVAH	210
	TWRYWYHTA-ALNGRPAADYTRTYAPFHTRTRQSQQPDBTVYYIRGDRLRENTTSTAWTSRENATLYAH	210
rrnAC3133	TWRYWYHTA·ALNGRPAA······DVTRTVAPFHTRTRQ·····SQQPDG······TVYVIRGDRLRE······NTTSTAWTSRENATLVAHD ····································	210 233
rrnAC3133 rrnAC0024 Hmuk_2777	TWRYWYHTA·ALNGRPAA······DVTRTVAPFHTRTRQ······SQQPDG······TVYVIRGDRLRE······NTTSTAWTSRENATLVAHVT    ····································	210 233 218 217
rrnAC3133 rrnAC0024 Hmuk_2777 HQ,1325A	TWRYWVHTA-ALNGRPAA    DVTRTVAPFHTRTRQ    SQQPDG    TVYVIRGDRLRE    NTTSTAWTSRENATLVAHY      YRTA    FVLNRYAVYNQSATA    VITRDG    GRVYÜIRGGGGEI    PAAERLEEFRVELUG      LDREATV    ALERFLTIDNATVS    VITRDG    GRVYÜIRGGGRTF    VTERPIRVSVSAVII      ETRFRADS    MWAVYSRILTIGEFRVQ    VTENTG    ERRIQVAITDVAV    RATOVRGYLTUG	210 233 218 217 207
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1325A Hmuk_0909	TWRYWYHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVYVIRGDRLRE  NTTSTAWTSRENATLVAHYT    YRTA  FYLNRYAVYNQSATA  VITRDG  GRYYQIRGGGGEI  PAAERLEEFRVELU    LDREATV  AIERFLTIDNATVS  VITRDG  GRYYQIRGGGREI  PAAERLEEFRVELU    ETRFRADS  AIERFLTIDNATVS  VIRDDG  QRHYELRGEQRTF  VIERDIRVSVSAVI    ETRFRADS  MWAVYSRILTIGEFRVIQ  VTENTG  ERRIVAVIDAV  RNATDVRGVITV    TARYGHQPEV  AISYLAIGSATVA  ATTVDG  QRYYQITGSADTL  PVTGEISNSVEALUA	210 233 218 218 217 207 207
rrnAC3133 rrnAC0024 Hmuk_2777 HQ 1325A Hmuk_0909 HBac_1301	TWRYWYHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVYVIRGDRLRE  NTTSTAWTSRENATLVAHYT    YRTA  FYLNRYAVYNQSATA  VITRDG  GRYYDIRGSGGEI  PAAERLEEFRYELLW    LDREATV  AI ERFLTIDNATYS  VLRDDG  GRHYDIRGSGGEI  PAAERLEEFRYELLW    ETRFRAADS  AI ERFLTIDNATYS  VLRDDG  GRHYDIRGSGGEI  PAAERLEEFRYELLW    TARYGHQPEV  AI SRYLAIGSATVA  VLRDDG  GRHYDIGSADTL  PTTSTAWTSRENATLVAHYT    ETRFRAADS  AI SRYLAIGSATVA  ATTVDG  GRYVDITGSADTL  PYTGEISNYSVEALW    TARYGHQPEV  AI SRYLAIGSATVA  ATTVDG  GRYVDITGSADTL  PYTGEISNYSVEALW    ERLQLQRPLEELDYNATE  TYTYEG  TTAYDYN TGIRD  PIRVPANNATGHYYDT	210 233 218 217 207 207 200
rrnAC3133 rrnAC0024 Hmuk_2777 HQ 1325A Hmuk_0909 Hlac_1301 Hlac_0102	TWRYWYHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVYVIRGDRLRE  NTTSTAWTSRENATLVANT    YRTA  FYLNRYAVYNQSATA  VITRDG  GRYYGIRGSGEI  PAAERLEEFRVELLUG    LDREATY  ALERFLTIDNATYS  VLRODG  GRYYGIRGSGEI  PAAERLEEFRVELLUG	210 233 218 217 207 207 200 7 218
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1325A Hmuk_0909 Hlac_1301 Hlac_0102 rrnAC0235	TWRYWVHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVVVIRGDRLRE  NTTSTAWTSRENATLVANT    YRTA  FVNRYAVYNQSATA  VITRDG  GRVVIRINGSGEI  PAAERLEEFRVELUG    LDREATV  AIERFLTIDNATVS  VLRDDG  QRYVELRGEQRTF  VTERPIRVSVSAVI    ETRFRADS  MWAVYSRILTIGEFRVIQ  VTENTG  ERRIVAITOVAV  RNATOVRGYLTQ    TARYGHQPEV  AISRYLAIGSATVA  ATTVDG  QRYVITGSADTL  PVTGEISMYSVEALW    MATOVRGYLTQ  TARYGHQPEV  AISRYLAIGSATVA  ATTVDG  QRYVITGSADTL  PVTGEISMYSVEALW    MATOVRGYLTQ  AISRYLAIGSETVE  TVTVEG  TTAVQINTGIRD  PIRVPANNATGHVVT    ADDLESAMRSEVYTG  IRVEGAGETEVEGOLUNINTYAG  TETVNG  DSVSGFEATGSNVTGFAADNAVSTNVSANGTTDTASATVHV	210 233 218 217 207 207 200
rrnAC3133 rrnAC0024 Hmuk_2777 HQ 1325A Hmuk_0909 Hlac_1301 Hlac_0102	TWRYWYHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVYVIRGDRLRE  NTTSTAWTSRENATLVANT    YRTA  FYLNRYAVYNQSATA  VITRDG  GRYYGIRGSGEI  PAAERLEEFRVELLUG    LDREATY  ALERFLTIDNATYS  VLRODG  GRYYGIRGSGEI  PAAERLEEFRVELLUG	210 233 218 217 207 207 200 7 218
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1325A Hmuk_0909 Hlac_1301 Hlac_0102 rrnAC0235	TWRYWVHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVVVIRGDRLRE  NTTSTAWTSRENATLVANT    YRTA  FVNRYAVYNQSATA  VITRDG  GRVVIRINGSGEI  PAAERLEEFRVELUG    LDREATV  AIERFLTIDNATVS  VLRDDG  QRYVELRGEQRTF  VTERPIRVSVSAVI    ETRFRADS  MWAVYSRILTIGEFRVIQ  VTENTG  ERRIVAITOVAV  RNATOVRGYLTQ    TARYGHQPEV  AISRYLAIGSATVA  ATTVDG  QRYVITGSADTL  PVTGEISMYSVEALW    MATOVRGYLTQ  TARYGHQPEV  AISRYLAIGSATVA  ATTVDG  QRYVITGSADTL  PVTGEISMYSVEALW    MATOVRGYLTQ  AISRYLAIGSATVA  ATTVDG  QRYVITGSADTL  PVTGEISMYSVEALW    MATOVRGYLTQ  AISRYLAIGSATVA  ATTVDG  QRYVITGSADTL  PVTGEISMYSVEALW    ADDLESAMRSEVYTG  INVEGGETEGES LNUWNFYSDEGEVRGEPTAKTADAFEG  DRNIPETASATVHW  GQVQ PVNESSVIDR  DRNIPETASATVHW	210 233 218 217 207 207 200 7 218
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1325A Hmuk_0909 Hiao_1301 Hiao_0102 rrnAC0235 rrnAC02358 rrnAC3416	TWRYWYHTA-ALNGRPAA  DVTRTVAPFHTRTRQ  SQQPDG  TVYVIRGDRLRE  NTTSTAWTSRENATLVANT    YRTA  FYLNRYAVYNQSATA  VITRDG  GRYYQIRGSGGI  PAAERLEEFRYELL    LDREATV  AI ERFLTIDNATVS  VITRDG  GRYYQIRGGQRTF  VTERPIRVSVSAVI    ETRFRADS  MWAVVSRILTIGEFRVIQ  VTENTG  ERRIDATV  RNATOVRGYLTW    TARYGHQPEV  AI SRYLAIGSATVA  ATTVDG  QRYVITGSADT  PVTGEISNYSVEALMA    ADDLESAMRSEYYTG  IRYEQGTAGGETEVEG  LNUNNPTSDGEGEVRGEPTAKFTADAFEG  DRNIPETYETASATVHW    GQYQ PVNESSVIDR  SLQSDINWTYAG  TETVPC  SSGSVIDRAADNAVSTNVSNGTTDASATVLW    VSGSVLDDPAR  LIEAYLATNYSVPRP  RDDRDS  CSVTLMATDPPAA  FEPADNYSVQAT    SDLSERRTIR  AVGEAVALSVADRSDSG  VVLVZ  TRFRNPDRLNTPLF  VSDPRVSVRLR	210 233 218 217 207 200 207 200 218 232 192 212
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1325A Hmuk_9999 Hiao_1301 Hiao_1301 Hiao_0102 rrnAC3358 rrnAC3358 HQ13800A	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPOTVYVIRGDRLRENTTSTAWTSRENATLVAN YRTAFYLNRYAVYNQSATAVITROGGRYV[IGGGGI]PAAERLEEFRYELU LDREATVALERFLTIDNATVSVLRDGGRYV[IGGGGT]YCRPIRVSVSAVI ETRFRAADSWWAVYSRLTIGEFRVQVTENTGGRYV[IGGARTFVTERPIRVSVSAVI TARYGHQPEVAISRYLAIGSATVAATTVDGQRYV[IGGADTLPVTGEISNYSVEAU TARYGHQPEVAISRYLAIGSATVAATTVDGQRYV[IGGADTLPVTGEISNYSVEAU TARYGHQPEVAISRYLAIGSATVAATTVDGQRYV[IGGADTLPVTGEISNYSVEAU GQVQPVNESSVIDRSLLQSGDINWTYAGTYTVETYTVEDSYSGFAADAFEGDRNIPETYETASATVHY SQVQPVNESSVIDRSLLQSLGSDINWTVAGTETVNGOSYSGFAADAFEGDRNIPETYETASATVHY SVLDPDPARLIEAYLATNYSVDRPRODRDSCSVTLMATDPPAAFEPADNYSVAAT SVLVDPPARLIEAYLANYSVDRPTYTVETRFRNPENNTSPALNATVENYSANGTTASATVHY SVLVDPPARLIERLINTIEYRRVEQQQR.	210 233 218 217 207 207 207 207 200 218 232 192 232 192 212 217
rrnAC3133 rrnAC0024 Hmuk_2777 Hq.1335A Hmuk_9999 Hiac_1301 Hiac_0 102 rrnAC3358 rrnAC3358 rrnAC3358 rrnAC3416 Hq.1300A rrnAC240	TWRYWVHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPOTVVVIRGDRLRENTTSTAWTSRENATLVAN YRTAFYLNRYAVYNQSATAVITROCGRVVIRGGQRTENTTSTAWTSRENATLVAN LDREATVAIERFLTIDNATVSVLRDCQRYVIRGGQRTEVTERPIRVSVSAVI ETRFRAADS. MWAVYSRLTIGEFRVIQVTENTCQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDCQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDCQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDCQRYVITGSADTLPVTGEISNYSVEAVI GQVQPVNESSVIDRSLLQSGDINWTYAGGTVTVECTXYDYNGIRDPIRVPANNATGHVVT SQVQPVNESSVIDRSLLQSLGSDINWTYAGTETVNCOSYSGFEATGSNVTGFAADNAVSTNVSANGTTDTASATVLX SVGVDPPARLIEAYLATNYSVDRPRODROSCSVTLMATDPPAAFEADNYSVAAT SVLDAPPARLIEAYLATNYSVDRPRODROSCSVTLMATDPPAAPPANNSVAAT SVLDAPPARLIEAYLATNYSVDRPQQRCTRFRNPDRINTPFVQNLTQTSTATVENATSTLVIC SPRAALFFEPTFNGRLIALLSATNITSIRFGOEVIKQLYGSVFIKGGSADVQNLTQTSTATVENATSLVIC SPRAALFFEPTFNGRLIALLSATNITSIRFGOEVIKQLYGSVFIKAGGSAD	210 233 218 217 207 207 207 207 218 232 192 212 212 217 235 215
rrnAC3133 rrnAC0924 Hmuk_2777 HQ.1335A Hmuk_0909 HBo_1301 HBo_1301 HBo_0102 rrnAC3256 rrnAC3358 rrnAC3346 HQ.1300A rrnAC340 HVD_0434	TWRYWVHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPOTVVVIRGDRLRENTTSTAWTSRENATLVAN YRTAFYLNRYAVYNQSATAVITROCGRVVIRGGQRTENTTSTAWTSRENATLVAN LDREATVAIERFLTIDNATVSVLRDCQRYVIRGGQRTEVTERPIRVSVSAVI ETRFRAADS. MWAVYSRLTIGEFRVIQVTENTCQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDCQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDCQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDCQRYVITGSADTLPVTGEISNYSVEAVI GQVQPVNESSVIDRSLLQSGDINWTYAGGTVTVECTXYDYNGIRDPIRVPANNATGHVVT SQVQPVNESSVIDRSLLQSLGSDINWTYAGTETVNCOSYSGFEATGSNVTGFAADNAVSTNVSANGTTDTASATVLX SVGVDPPARLIEAYLATNYSVDRPRODROSCSVTLMATDPPAAFEADNYSVAAT SVLDAPPARLIEAYLATNYSVDRPRODROSCSVTLMATDPPAAPPANNSVAAT SVLDAPPARLIEAYLATNYSVDRPQQRCTRFRNPDRINTPFVQNLTQTSTATVENATSTLVIC SPRAALFFEPTFNGRLIALLSATNITSIRFGOEVIKQLYGSVFIKGGSADVQNLTQTSTATVENATSLVIC SPRAALFFEPTFNGRLIALLSATNITSIRFGOEVIKQLYGSVFIKAGGSAD	210 233 218 217 207 207 207 207 218 232 192 212 212 217 235 215
rrnAC3133 rrnAC0024 Hmult_92777 HQ,1325A Hmult_9999 Hiao_1301 Hiao_1012 rrnAC3358 rrnAC3358 rrnAC33415 HQ,1300A rrnAC2340 HVD_0434 rrnAC2354	TWRYWVHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPOTVVVIRGDRLRENTTSTAWTSRENATLVAN YRTAFYLNRYAVYMQSATAVITROCGRVVIRGGQRTENTTSTAWTSRENATLVAN LDREATVAIERFLTIDNATVSVLRDGGRVVIRGGQRTEVTERPIRVSVSAVI ETRFRAADSMWAVYSRLTIGEFRVIQVTENTGQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDGQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDGQRYVITGSADTLPVTGEISNYSVEAVI SQQPQNESSVIDRSLLQSGDINWTVAGTVTVEGTAYQYNTGIRDPIRVPANNATGHVVT SQQVQPVNESSVIDRSLLQSGDINWTVAGTETVNGOSYSGFEATGSNVTGFAADNAVSTNVSANGTTDTASATVLQ SVGVDPDPARLIEAVLATNVSVDRPRODRDSCSVTLMATDPPAAFFAADNSVANGTTDTASATVLQ SVLDAPPARLIEAVLATNVSVDRPRODRSCSVTLMATDPPAAFFAADNSVAR SVLDPDPARLIERLINTIEYRRVEQTQRGTRFRNPRNTNTAQRSSVRAVGRUNDTASATVENTSSLSAT SPRAALFFEPTFNGRLIALLSATNITSIRFGGEVIKQLYGSVEIKGGSADGSAVATRPVDRYTNYSLSAT SPRAALFFEPTFNGRLIALLSATNITSIRFGGEVIKQLYGSVEIKGGAAAQGEPGTLEYTSFEYVVLUG SVLAAAIALSQARAYGADDDLTFAGTETVDGSVEIVGIKGGAAAAQGEPGTLEYTSFEYVVLUG	210 233 218 217 207 200 207 200 218 232 192 212 217 235 215 203
rrnAC3133 rrnAC0024 Hmuk_2777 HQ,1335A Hmuk_9999 HBc_1301 HBc_0102 rrnAC3358 rrnAC3358 rrnAC3415 HQ,1300A rrnAC3415 HQ,1300A rrnAC2340 HV0_0434 VNQ1535H	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVYVIRGDRLRENTTSTAWTSRENATLVAHT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVAIERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRILTIGEFRVIQVTENTCQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmult_92777 HQ,1325A Hmult_9999 Hiao_1301 Hiao_1012 rrnAC3358 rrnAC3358 rrnAC33415 HQ,1300A rrnAC2340 HVD_0434 rrnAC2354	TWRYWVHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPOTVVVIRGDRLRENTTSTAWTSRENATLVAN YRTAFYLNRYAVYMQSATAVITROCGRVVIRGGQRTENTTSTAWTSRENATLVAN LDREATVAIERFLTIDNATVSVLRDGGRVVIRGGQRTEVTERPIRVSVSAVI ETRFRAADSMWAVYSRLTIGEFRVIQVTENTGQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDGQRYVITGSADTLPVTGEISNYSVEAVI TARYGNQPEVAISRYLAIGSATVAATTVDGQRYVITGSADTLPVTGEISNYSVEAVI SQQPQNESSVIDRSLLQSGDINWTVAGTVTVEGTAYQYNTGIRDPIRVPANNATGHVVT SQQVQPVNESSVIDRSLLQSGDINWTVAGTETVNGOSYSGFEATGSNVTGFAADNAVSTNVSANGTTDTASATVLQ SVGVDPDPARLIEAVLATNVSVDRPRODRDSCSVTLMATDPPAAFFAADNSVANGTTDTASATVLQ SVLDAPPARLIEAVLATNVSVDRPRODRSCSVTLMATDPPAAFFAADNSVAR SVLDPDPARLIERLINTIEYRRVEQTQRGTRFRNPRNTNTAQRSSVRAVGRUNDTASATVENTSSLSAT SPRAALFFEPTFNGRLIALLSATNITSIRFGGEVIKQLYGSVEIKGGSADGSAVATRPVDRYTNYSLSAT SPRAALFFEPTFNGRLIALLSATNITSIRFGGEVIKQLYGSVEIKGGAAAQGEPGTLEYTSFEYVVLUG SVLAAAIALSQARAYGADDDLTFAGTETVDGSVEIVGIKGGAAAAQGEPGTLEYTSFEYVVLUG	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 HQ,1335A Hmuk_9999 HBc_1301 HBc_0102 rrnAC3358 rrnAC3358 rrnAC3415 HQ,1300A rrnAC3415 HQ,1300A rrnAC2340 HV0_0434 VND1538H	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVYVIRGDRLRENTTSTAWTSRENATLVAHT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVAIERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRILTIGEFRVIQVTENTCQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 Hq.1335A Hmuk_9999 Hiac_1301 Hiac_0 102 rrnAC3256 rrnAC3256 rrnAC3358 rrnAC3416 Hq.1300A rrnAC240 HV0_0434 rrnAC2054 VNG1538H rrnAC2720	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVALERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRLTIDGFRVIQVTENTGQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 HQ.1335A Hmuk_9999 Hibo_1301 Hibo_102 rrnAC3358 rrnAC3358 rrnAC3416 HQ.1800A rrnAC2340 HVD_0434 rrnAC2354 VNG1538H rrnAC2720 2rystructure .	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVALERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRLTIDGFRVIQVTENTGQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1335A Hmuk_9999 HBc_1301 HBc_0102 rrnAC3358 rrnAC3358 rrnAC3416 HQ1800A rrnAC3416 HQ1800A rrnAC2340 HV0_0434 rrnAC2954 VND1358H rrnAC2954 VND1358H rrnAC2720 2rystructure .	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVALERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRLTIDGFRVIQVTENTGQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 Hq.1326A Hmuk_9999 Hibo_1301 Hibo_102 rrnAC3368 rrnAC3368 rrnAC3368 rrnAC3368 rrnAC3368 rrnAC3368 Hq.1800A rrnAC2340 HVD_0434 rrnAC2054 VNG1538H rrnAC2720 2rystructure Nmag_2608 Huba_2669	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVALERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRLTIDGFRVIQVTENTGQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmulg2777 HQ1326A Hmulg999 Hiao_1301 Hiao_102 rrnAC3588 rrnAC3588 rrnAC3588 HQ1800A rrnAC2540 HVD_0434 rrnAC2544 VNG1538H rrnAC2720 2rystructure Nmag_2605 Htu_1300	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVALERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRLTIDGFRVIQVTENTGQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1335A Hmuk_0909 Hiao_1301 Hiao_100 rrnAC3358 rrnAC3415 HQ1500A rrnAC3415 HQ1500A rrnAC3445 HQ1500A rrnAC2540 VNG1535H rrnAC2720 2rystructure Nmag_2605 Huta_2605 Huta_2605	TWRYWYHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPDCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYNQSATAVITRDCGRYVQIRGGGGGGTPAAERLEEFRYELL LUREATVALERFLTIDNATVSVLRDDCQRYYGIRGGGGRTFVTERPIRVSVSAVI ETRRRADSMWAVYSRLTIDGFRVIQVTENTGQRYYGITGSADTLPYTGEISNYSVEAL TARYGHQPEVAISRYLAIGSATVAATTVDCQRYYGITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL CQRYQITGSADTLPYTGEISNYSVEAL 	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
rrnAC3133 rrnAC0024 Hmuk_2777 HQ1335A Hmuk_9999 Hiao_1301 Hiao_0102 rrnAC3358 rrnAC3358 rrnAC3358 rrnAC3456 HQ1800A rrnAC2400 HV0_0434 rrnAC2400 HV0_0434 rrnAC2540 VN01538H rrnAC2720 2rystructure Nmag_2605 Huta_2605 Huta_2605 Huta_2605 Huta_2605 rrnAC3133	TWRYWVHTA-ALNGRPAADVTRTVAPFHTRTRQSQQPCTVVVIRGDRLRENTTSTAWTSRENATLVANT YRTAFYLNRYAVYMQSATAVITROCGRYV[IGGGGE]PAAERLEEFRYELLC LDREATVAIERFLTIDNATVSVLRDCQRYV[IGGGC]PAAERLEEFRYELC TARYGNQPEVAISRYLTIGEFRIQVTENTCQRYV[IGGATFVTERPIRVSVSAVI TARYGNQPEVAISRYLTIGEFRIQVTENTCQRYV[IGGATLPYTGE]SNYSVEAU TARYGNQPEVAISRYLAIGSATVAATTVCQRYV[IGGATLPYTGE]SNYSVEAU TARYGNQPEVAISRYLAIGSATVAATTVCQRYV[IGGATLPYTGE]SNYSVEAU TARYGNQPEVAISRYLAIGSATVAATTVCQRYV[IGGATLPYTGE]SNYSVEAU TARYGNQPEVAISRYLAIGSATVAATTVCQRYV[IGGATLPYTGE]SNYSVEAU SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNFTSDEGEGVRGEPTAKFTADAFEGDRNIPETVETASATVLC. SOULDESAMRSEVTGSLQSLGSDINWTVAGTETVNCSVLMATDPPAA SOULDESAMRSEVYTGIRVEGTAGGETEVEGLNLWNTSDEGEGVRGEPTAKFTADAFEGOSVLMATOPPAA SOULDESAMRSEVTGSLQSLGSDINWTVAGTETVNCSVLMATDPPAA SOULDESAMRSEVTGSLQSLGSDINWTVSDRPRONSOLA SOULDESAMRSEVTGSLQSLGSDINVTSDRPSOULTGSVLMATDPPAA SOULTGAVASSLQSLGSDINVTSDRPSULVCSVLMATDPPAA SOULTGAVASSLQSLGSDINVTSDRPSULVCSVLMATDPPAA SOULTGAVASSLQSLGSDINVTSDRPSULVCSVLMATDPAA SOULTGAVASSLQSLGSDINVTSDRPSULVCSVLMATDPAA SOULTGAVASSSULVCSULVCSULVCSULASSANATRPORVTNTSSLSAT SOULTGAVASSLQSLGSDINVTSDRPSULVCSULVCSULXSANGEPGTLEVSSULVCSULXSANGEPGTLEVSSULVCSVLMATDAVSNATACSUNTAGSUVTSSRAAATRPDPSSEAAAATLHIN SOULTGAVAS	210 233 218 217 200 207 200 218 200 218 232 192 217 235 215 203 203
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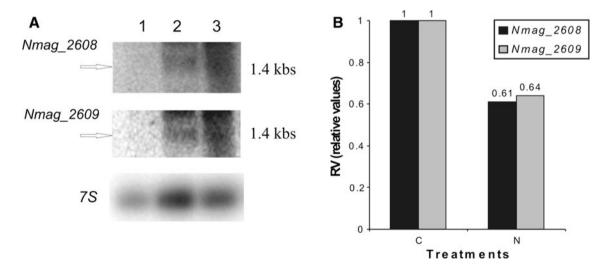
were performed. Figure 3a shows that a 1.4 kbp transcript appears at early stationary growth phase. Because this size was higher than the expected 786 bp for Nmag 2608, its genomic context was analyzed. Since it produces a 336 bp transcript in antisense mode, co-transcription of upstream Nmag\_2607 with Nmag\_2608 is unlikely. Conversely, Nmag\_2609, noted as a cyclase, is located downstream from Nmag\_2608 and transcribed in the same direction. Since Nmag\_2609 has a 666 bp mRNA, its co-transcription with *Nmag\_2608* could explain the transcript size (1.4 kbp) observed. Agreeing with the existence of a polycistronic mRNA harboring both genes, the same band was revealed with Nmag\_2609 and Nmag\_2608 probes (Fig. 3a). In other words, both genes are expressed in the same transcriptional cluster. Also, a manual search at the intergenic region between both genes for consensus archaeal promoter sequence BRE and tata box showed that these sequences are absent in the upstream region from the ATG of Nmag 2609 (data not shown).

To get information on Nmag\_2608 physiological role, northern blot tests with total RNA of cultures grown in low salt concentration (15 % NaCl) were performed. The *Nmag\_2608* transcript amount decreased 39 % in cells grown in low salt (Fig. 3b). As expected for polycistronic genes, a similar result was obtained with *Nmag\_2609* probe (Fig. 3b). Also, both *Nmag\_2608* and *2609* decreased in starved cells, even though these results were less precise (data not shown).

Localization of Nmag\_2608 protein in the extracellular protein fraction

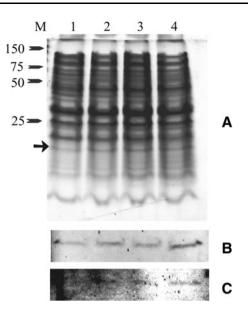
Nmag 2608 protein is predicted to contain a lipoprotein signal peptide. To corroborate its membrane localization, western blot tests of different cellular fractions of N. magadii's cultures with an antibody raised against P400 region were performed. Total cellular protein, cytosolic, and membrane fractions from N. magadii's cultures obtained at exponential, early and late stationary phase of growth did not show reactive bands (data not shown). Because northern blot analysis showed that Nmag\_2608 transcript appears only in early stationary growth phase (Fig. 3), western blot tests of extracellular protein fractions from close time intervals within this growth phase were made. As viewed in Fig. 4b a unique 17 kDa band, smaller than the expected theoretical 29 kDa mass of Nmag\_2608 complete protein, was revealed in all the samples tested. Similarly, this band was also detected by anti-ubiquitin antibody (Fig. 4c) with an intensity lower than that observed with anti-P400 antibody. Mass spectrometry analysis of the reactive band identified one peptide mass corresponding to the region between 121 and 129 of Nmag\_2608. This result confirms that the reactive secreted protein corresponds to a fragment of Nmag 2608 protein.

Since in silico analysis of Nmag\_2608 predicted that the signal for translocation of the gene product is coded by the 20 first residues of the protein, we decided to confirm this



**Fig. 3** Northern blot analysis of *Natrialba magadii*'s *Nmag\_2608* transcript. Membranes containing total mRNA from *N. magadii* were hybridized with gene-specific probes. **a** Northern blot of mRNA from different growth phases of cultures grown under normal conditions. *Lane 1* exponential, 2 early stationary, 3 late stationary phase of growth of *N. magadii* culture. *Nmag\_2608 panels: Nmag\_2609* gene internal probe labeled with <sup>32</sup>P- $\alpha$ -dCTP. *Nmag\_2609 panels: Nmag\_2609* gene probe labeled with <sup>32</sup>P- $\alpha$ -dCTP. *Arrows* indicate the detected transcript with both probes which have a relative size of

1.4 kbp. 7S panels: hybridization of the membrane with a 7S rRNA  $^{32}$ P-radioactive probe. **b** Graphical representation of relative amounts of *Nmag\_2608* and *Nmag\_2609* transcript in early stationary phase of growth for normal (*C*) and treated cultures, 15 % NaCl (*N*) of *N. magadii* obtained by densitometry analysis. The densitometry was corrected to the amounts of 7S rRNA loaded and for the treatment cultures the values were normalized to the control culture values. *Inset* indicates color reference for the *bars* in the graph. The figure is a representation of four independent experiments



**Fig. 4** Localization of Nmag\_2608 protein by western blot analysis of extracellular protein samples of *N. magadii*'s cultures. Extracellular protein preparations were obtained from clarified medium cultures from stationary growth phase grown to OD<sub>600</sub> of 1.02 (*lane 1*), 1.20 (*lane 2*), 1.27 (*lane 3*) and 1.37 (*lane 4*). Proteins were separated by SDS-PAGE (**a**) and subjected to western blot analysis against Nmag\_2608-specific anti-P400 primary antibody (**b**) or anti-ubiquitin primary antibody (**c**). The *arrow* indicates the corresponding reactive band in the gel. Molecular weight standard (*M*) positions are indicated to the *right* of the image

by heterologous expression tests. Thus, two constructs with or without the predicted N-terminus signal peptide were expressed in E. coli Rosetta cells. Western blot assays against anti-P400 were performed to intracellular and extracellular protein fractions of the IPTG-induced cultures of E. coli containing either pET24b-nmag2608C (complete) or pET24b-nmag2608T (truncated) construct. The complete version of Nmag 2608 expressing cultures displayed two bands of  $\sim$  48 and 38 kDa by SDS-PAGE in the extracellular fraction (Fig. 5a). Western blot tests showed that both bands reacted with the anti-P400 primary antibody. Also, both bands showed identity with Nmag 2608 protein sequence when analyzed by mass spectrometry (data not shown). On the other hand, the truncated version of the protein (Nmag2608Tr) expressed in E. coli was  $\sim$ 48 kDa by SDS-PAGE, which was not secreted but remained associated with the cells (Fig. 5b).

# Discussion

We previously proved that P400, a polypeptide of N. magadii, folds similar to ubiquitin family proteins (Nercessian et al. 2009; Ordóñez et al. 2011). We now characterized Nmag\_2608 protein holding P400 (Fig. 1) which belongs to a group of orthologs from different

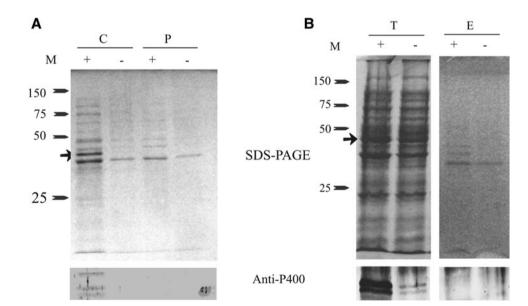


Fig. 5 Localization of complete and truncated recombinant versions of Nmag\_2608 by western blot against anti-P400. *E. coli* Rosetta cell cultures containing the construction pET24b::*Nmag2608C* or pET24b::*Nmag2608T* were incubated with (+) or without (-) 0.5 mM IPTG, and protein from cellular and extracellular fractions was prepared as described (see "Materials and methods"). **a** SDS-PAGE (12 %) and western blot with anti-P400 primary antibody assay of extracellular protein fractions from cultures expressing the

complete version Nmag2608rC (*lanes C*) and cultures containing the empty plasmid pET24b(+) (*lanes P*). **b** SDS-PAGE (12 %) and western blot with anti-P400 primary antibody assay of cellular (*lanes T*) and extracellular (*lanes E*) proteins of cultures expressing the truncated version Nmag2608rT. Molecular marker positions (*M*) are indicated in kDa at the *left* of each image. *Arrows* indicate the bands corresponding to the recombinant versions of Nmag\_2608 protein

halophilic archaeal organisms without sequence identity with any known protein. Then Nmag\_2608 and related proteins would be conserved among halophilic archaeal genomes. Bioinformatic data reveal that Nmag 2608 contains a signal peptide with the lipobox motif L-[I/G/A]-[A/ G/S]-C found in bacterial lipoproteins and archaeal proteins (Hayashi and Wu 1990; Mattar et al. 1994; Giménez et al. 2007). Then, Nmag 2608 possibly links to the cell membrane by a thioester bond of the lipobox Cys (Hayashi and Wu 1990). Scarce information is available about membrane proteins with ubiquitin fold. Downes et al. (2006) described a group of membrane-anchored proteins, membrane-anchored ubiquitin fold (MUBs) found in several eukaryotic organisms. Nevertheless, these proteins would be linked to the membrane through prenvlation of its c-terminal region, a different mechanism from the lipoproteins anchorage.

Unlike bacteria, archaea would not have a signal peptidase II to process lipoproteins signal peptides (Tokuda and Matsuyama 2004; Ng et al. 2007). However, several *Haloferax volcanii* lipoproteins have been reported to join the cell membrane through their lipobox Cys (Giménez et al. 2007). In addition, a signal peptide peptidase gene (*Nmag\_2612*) is located within the *Nmag\_2608* genomic context. The protein encoded by this gene could then take part in Nmag\_2608 processing.

It has been shown that  $\beta$ -grasp fold is stable within proteins having neither sequence nor functional similarities. This fold takes part in many biochemical tasks by association with proteins and non-protein-soluble substrates (Burroughs et al. 2007a, b). For example, NqoI class proteins, involved in polysaccharide export and predicted for secretion and anchoring into the cell membrane display a N-terminal  $\beta$ -sheet rich domain and 1–8 soluble ligand binding  $\beta$ -grasp (SLBB) domains with a  $\beta$ -grasp fold (Burroughs et al. 2007b). After 4 iterations of a psi-blast search using as query the Nmag\_2608 sequence a 326-amino acid V12B01\_23145 protein was retrieved, which holds a SLBB-like domain from residues 137-326. Despite having 13 % identity with Nmag\_2608, this domain aligns with the region holding P400 (Fig. 2 and supplementary material). Then, Nmag\_2608 resembles an SLBB domain-containing protein with ubiquitin-like structural homology. Also, a group of proteins with  $\beta$ -grasp fold, extracellular localization and possible role in proteinprotein interaction has been described in Mycobacterium species (Wang et al. 2007). Finally, like several prokaryotic and eukaryotic ubiquitin-like proteins, Nmag\_2608 displays a DGD motif in its  $\beta$ -grasp domain (Nercessian et al. 2009) also present in SLBB domain (Burroughs et al. 2007b). All together, the interaction of Nmag\_2608 with other molecules could be predicted.

Acquired bioinformatic data led to examine whether N. magadii's cells express the Nmag\_2608 gene. Although larger than expected, a 1.4 kbp transcript was detected only at early stationary phase of growth (Fig. 3). Analysis of the genomic context of Nmag 2608 suggests its co-transcription with the adjoining Nmag\_2609 gene encoding for a protein of the cyclase family. Cyclases take part in the transport and metabolism of amino acids (COG001878). distant from *Nmag\_2608* Although stop codon, Nmag\_2609 plus Nmag\_2608 can produce a transcript of  $\sim 1.4$  kbp since their sizes are 666 and 786 bp, respectively. This was corroborated by northern blot assay (Fig. 3a). Nmag\_2609 shows homology with metaldependent hydrolases involved in the tryptophan metabolism by converting N-formyl kynurenine in kynurenine and formic acid (Pabarcus and Casida 2005). This pathway is mainly responsible for the synthesis of NAD/NADP in eukaryotes (Dobrovolskya et al. 2005). Conversely, it takes part in the synthesis of siderophores and antibiotics within some bacteria (Keller et al. 2010; Kurnasov et al. 2003; Lomovskaya et al. 1998; Matthijs et al. 2004; Narui et al. 2009). Then, co-expression may suggest that Nmag\_2608 is involved in the metabolism of tryptophan. By the other way, the archaeon Methylococcus capsulatus uses the extracellular membrane-associated protein MopE for copper uptake (Helland et al. 2008). For this, a kynurenine residue formed by oxidation of tryptophan in the MopE copper-binding site is required. Then, co-expression of Nmag\_2608 with Nmag\_2609 could be necessary to change one of its three tryptophans to kynurenine for interaction with ligands of the processed gene product. This hypothesis will be considered in further research.

To get information on physiological function, Nmag\_2608 and Nmag\_2609 transcription in stressed cells was assessed. Both transcripts levels decreased nearly 37.5 % in cells grown under low salinity at stationary growth phase (Fig. 3b). These changes agree with those displayed by genes associated with the transport of metabolites across the membrane of *Halobacterium* sp. NRC-1 grown in low salinity (Coker et al. 2007). Then, cells keep proper intracellular ionic conditions by modulation of transporter genes. Agreeing with this, present data suggest that Nmag\_2608 takes part in the uptake of solutes from the environment.

To check the Nmag\_2608 localization in *N. magadii*'s cells, western blot tests using an anti-P400 antibody were performed. They revealed a reactive band in the extracellular fraction of *N. magadii* at early stationary growth phase (Fig. 4), which proves protein secretion, also confirmed by reaction of the same band with an anti-ubiquitin antibody (Fig. 4c). The size of the revealed protein result was minor than expected, suggesting a further processing besides signal peptide removal, after translocation across

the membrane. Evenmore, mass spectrometry analysis of the reactive band allowed the identification of a peptide within the P400 region of the protein. These results indicate that the ubiquitin-like domain of Nmag\_2608 is kept during processing and released to the environment.

In accordance with the above, expression of Nmag\_2608 in E. coli also produced two extracellular proteins (Fig. 5a). In this case, the 48 kDa band would correspond to the leaderless protein, while possibly, the smaller band (38 kDa) corresponds to the mature processed version of Nmag 2608. Conversely, and revealing a signal peptide need for translocation, truncated Nmag\_2608 expression produced a protein associated with cells (Fig 5b). Thus, Nmag\_2608 would be processed differently from other bacterial or archaeal lipoproteins described so far (Mattar et al. 1994; Giménez et al. 2007). On the other hand, the different sizes of the revealed protein bands compared to those expected for Nmag 2608 could be due to the an unspecific interaction of the recombinant protein in E. coli with a 20 kDa polypeptide of unknown identity. Another possibility is that this difference results from reduced migration in SDS-PAGE as reported for some halophilic proteins (Madern et al. 2000). Supporting this, the identity of these proteins as Nmag\_2608 was also confirmed by mass spectrometry.

Most haloarchaeal predicted lipoproteins are secreted by the Tat translocation system (Storf et al. 2010). Since it does not contain a Tat motif, Nmag\_2608 is most likely to be secreted by a different system (data not shown). The protein expressed in *E. coli* is also translocated outside the cell, suggesting that the same mechanism of secretion works in both bacteria and archaea. Finally, whether Nmag\_2608 interacts with solutes of the extracellular medium must be explored. Nevertheless, present results would be in good agreement with the previously described roles of many ubiquitin-like domains in protein–protein and protein–soluble ligand interactions.

To sum up, this work reports the first description of an archaeal protein containing an ubiquitin-like domain, which is processed and secreted to the extracellular medium.

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