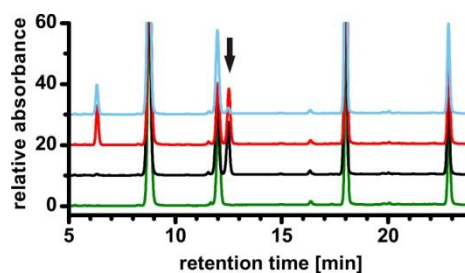


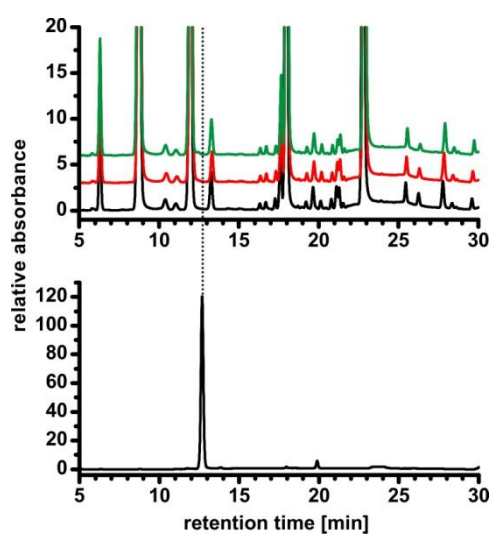
Supplementary Information

Supplementary Figure 1



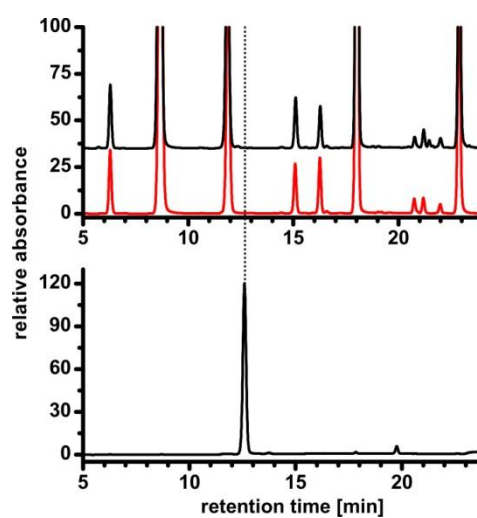
HPLC analysis of hydrolyzed T-arm substrates after methylation with *Mj*Nep1. Shown are the chromatograms for T-arm substrate without pseudouridine (green), with two pseudouridines (red) and with one pseudouridine at position 54 (blue) or 55 (black). The N1-methylpseudouridine signal is marked with an arrow.

Supplementary Figure 2.



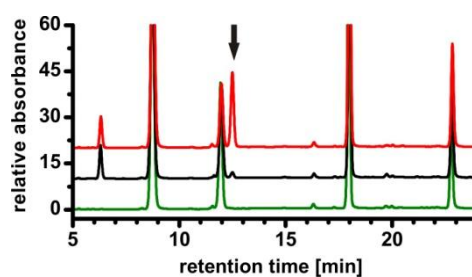
HPLC analysis of hydrolyzed tRNAs isolated from *E.coli*-BL21(DE3)-RIPL cells expressing *MjNep1* (red), *Mja_1640* (green) or no heterologous protein (black) (top) and chemically synthesized N1-methylpseudouridine (bottom) as reference.

Supplementary Figure 3



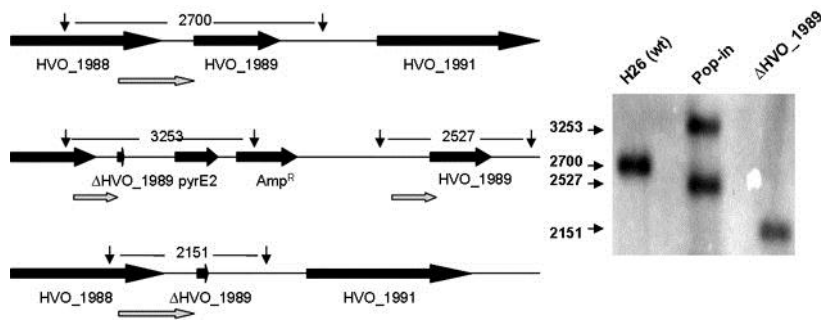
HPLC analysis of hydrolyzed tRNAs isolated from deletion mutant cells (red) and the same tRNAs after incubation with SAM and *MjNep1* (black) (top) and chemically synthesized N1-methylpseudouridine (bottom) as reference.

Supplementary Figure 4



HPLC analysis of hydrolyzed T-arm substrates after methylation with Mja_1640. Shown are the chromatograms for the T-arm substrates without pseudouridine (green), with two pseudouridines (red) and with one pseudouridine at position 55 (black). The N1-methylpseudouridine signal is marked with an arrow.

Supplementary Figure 5



Left side: Schematic overview of the genomic organization of the wild-type, the Pop-In mutant and the Pop-Out mutant of the *hvo_1989* gene. Relevant restriction sites are indicated by arrows, the probes for Southern blot analysis are indicated by open arrows and the sizes of hybridizing restriction fragments are shown.

Right side: Southern blot analysis of genomic DNA of the wild-type, the Pop-In mutant and the Pop-Out mutant.

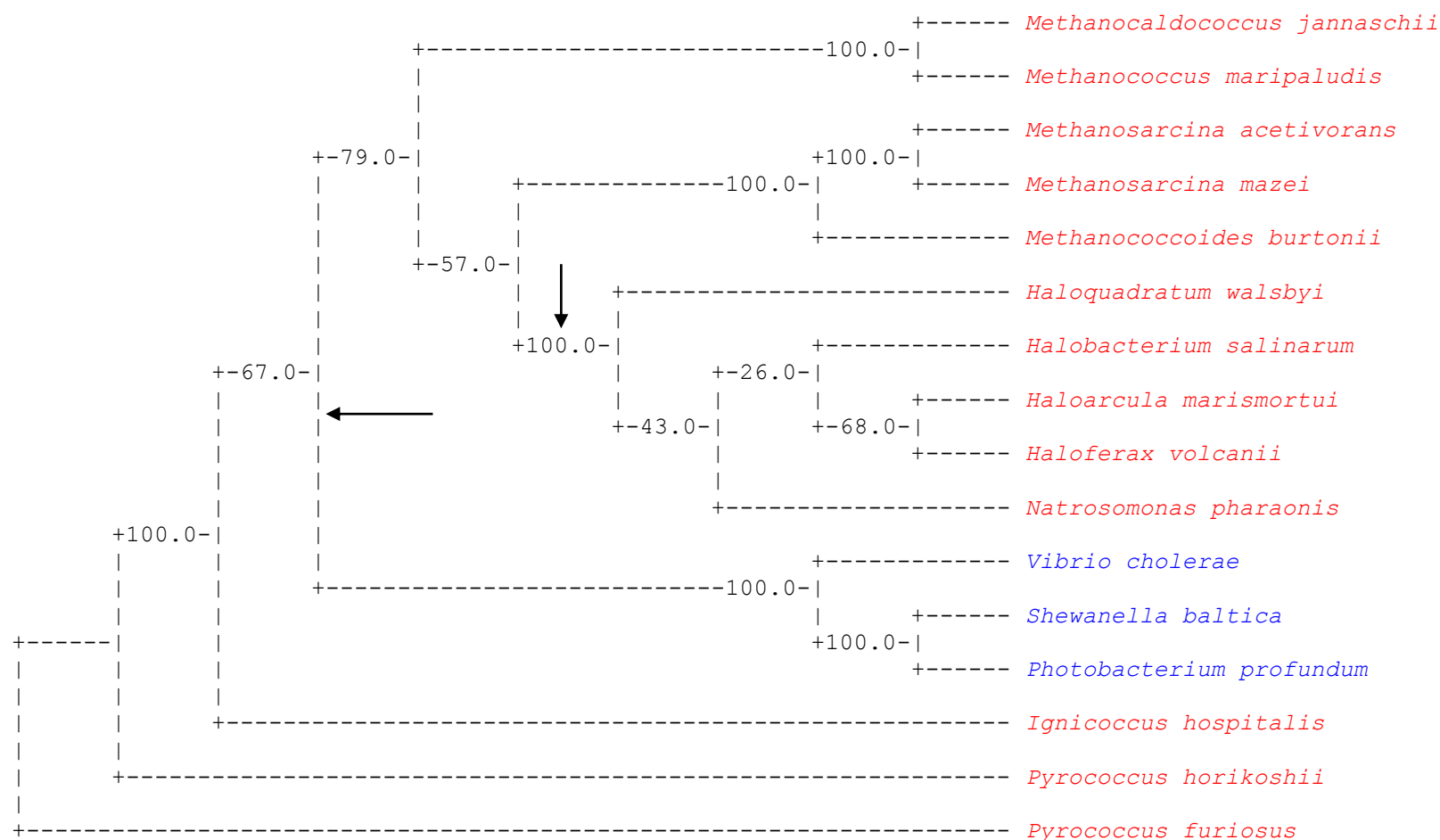
Supplementary Figure 6

Pyrococcus horikoshii	----MRVFIVKAN-EAHTDYDFSLKDLPGTSG RLD LICRALNSAFHLSHS FR KNVRVYVT	55
Pyrococcus furiosus	----MRIFIICKS-TAHTAFDYSIKDIPGTSG RLD LICRSLNAAFQLSHS FR KNVRVYTV	55
Haloferax volcanii	----MRQFIVTGHDAPTTPD-FSLDDIAGGAG RLD VLCRCVNSAFFLSHD IR EDVRVHLV	55
Haloarcula marismortui	----MRQFVIIGHDAPTTPE-FSLDDLAGAAG RLD VLCRCVTSAFFLSHA IR EDVRVHLI	55
Natronomonas pharaonis	----MRQFIVIGHDVPTDPDAVSLADLP-AG RLD VLCRCVNSAFFLSHD IR DNVRVWL	55
Haloquadratum walbyi	----MRR-----TGGFPN---LDDLAGGAG RLD ILCRCVTSALACSHG IR EDVRVHLV	46
Halobacterium salinarum	----MRQFVVLGHDAPTTPD-FPLDDLPGAAG RLD VLCRCVSAALFVSHG IR DDVEVFLV	55
Methanosarcina acetiv.	----MRDIVIIGH-KAKTSGDFSINDLPGSAG MD ILCRCVSSALFLSFG MR RDVNVHLL	55
Methanosarcina mazei	----MRDIVIIGH-KAKTSGDFSINDLPGSAG MD ILCRCVSSALFLSFG MR RDVNVHLL	55
Methanococcoides burtonii	----MRNFVIIAH-KALTGDFSINDLPGSAG MD ILCRCINSCLFLSHD IR RDVQVHLL	55
Methanocaldococcus jann.	----MREFIFKAN-KTITSSDINLKLPGSCG RLD LLCRCVSDAFFLSHD IR RDVVFYAV	55
Methanococcus maripaludis	----MKEFIIKAN-KAVTNGEINLKLPGSSG RLD LLCRCVNSAFFLSHD MR RDITIFYSV	55
Ignicoccus hospitalis	----MRTFVTRSL-TATTSPAFPKRGIASHT GRD VIARMLRAALWTERGV IR KDSSFFVT	55
Photobacterium profundum	----MRAFVLRAR--SAPTNSQLLLESVGQEA HT EILAHITLMNAIFVAQSH IR DDVIVHLV	54
Shewanella baltica	----MRAFVLRAR--SAPTDSQLFLASVGQEA HT EILAHITLMNTIFVAQSH IR NDVVVYLV	54
Vibrio cholerae	MRNTMRSFILRAR--SAPTDSQLRLDEIGGK CH EILAHCMMSLFTAQSH IR EDVVIHLV	58
	:: . : :::: :	
Pyrococcus horikoshii	LLGPPSPPKTIRFEGPKLRPKILNP DE LSIAKVIIKALNAGKN-LKGSSTKELEVLP GV	114
Pyrococcus furiosus	LYGPPDPKTIREFGAKIKPKTLNP DE VSTAKLIGKILNAGKD-IKEP-TKEREVLP GI	113
Haloferax volcanii	LGDEYT---VRFEGSEL--RLNP DE RSTAALIRKALEKREE---AIGHMPAESS P GV	106
Haloarcula marismortui	LGDEYT---VTFEGSDL--RLNP DE RSTAALIRKALEEREE---AIGHIPVETSP P GV	106
Natronomonas pharaonis	VGDEYA---IRFEGSEL--RLNP DE RSTAALVRGALAAREK---AIGSMAANPSP P GV	106
Haloquadratum walbyi	LNNQFT---VQFDGANLK--RLNP DE RSTAALIRTALENREK---AIGHMPAEST P GV	97
Halobacterium salinarum	LGDEVT---VRIDAGAVQ--YMHP DE ERNVAGLLKQALDAKTE---AIGHQEASS P GI	106
Methanosarcina acetiv.	LLGEPEPGKIIREFGLHLR--YLN DE RSSGSLIQKALQK-----TVTEKDIRST P GV	107
Methanosarcina mazei	LLGEPEPGKIIREFGLHLR--YLN DE RSSGSLIQKALLK-----NAAGQDIRST P GV	107
Methanococcoides burtonii	LLGEPEPGKIIREFDSEHVR--YLN DE RSAGSLIKKALQK-----TAGEYEVST P GV	107
Methanocaldococcus jann.	LYGQPNPPVCIKFVGSSELK--KVSP DE RNIAIFIKKALKKFEELDEEQRKDWNQST P GI	113
Methanococcus maripaludis	NYGDPNPPVTLKFVGSSELK--RVSP DE RSIALFIKKALEK-----DATELWKEST P GI	107
Ignicoccus hospitalis	LEGPPNPPLTLRFKGPELE--ADLS DE RAAVEAIRKCMLG-----ELK G CK	99
Photobacterium profundum	LESTQDFSRTISFTSSEITN-VGGF HE QALLTKIARALDLSQG---MTKEQERKVE P GI	110
Shewanella baltica	LESTHDFSRTICFDTRNICH-IGGF HE QALLTKIAKALDISRG---MTKEQTRVVDE G IT	110
Vibrio cholerae	LESTRDYSRTITVEANEISD-VGGF HE AALIALLVKALDASVG---MGKEQTRVVQ P GLT	114
	. : . : .*	*
Pyrococcus horikoshii	VSNMTFEDVIRKNIR--MNLYVLEEGG---ADIMKVKFPRNNVAFILGDHIGLSEEDLKT	169
Pyrococcus furiosus	ISRMTFEDVVRKNIK--YNLYLLEENG---VDIEKVKFPQDNVGFILGDQNGFTTEELNF	168
Haloferax volcanii	IRRMGFETTLEEAAS-DATVELHED G ---DPVVQVEPPE-NPL F VLSDDHDFTDDEAE	161

Haloarcula marismortui	LTRRGFEGTLDDVAR-RGTVVQLHED G ---DPIVGVAPPS-DPV F VLSDDHDFRDEEAAL	161
Natronomonas pharaonis	IRTQSLSGLLLESVAD-DGPLVTLHED G ---QPVVDIDPSA-EPT F VLSDDHHSFDDAETAL	161
Haloquadratum walbyi	IRRMGFAETVNRLKT-ETTPQLHET G ---TPIVDIDPPD-DPL F VLSDDHSLTNDIEIQ	152
Halobacterium salinarum	VSKRGFEAVLDVAVS---GPVVALHED G ---DALAAVDPLA-DPV F VLSDDHRDFTAAEASL	159
Methanosarcina acetiv.	VRNGDLNTLLASFE--GRTLFLYLRED G ---EDIRGLDREIRDPV F ILGDHMGVTEEEEEKQ	162
Methanosarcina mazei	TRTGDLNLSLLASFE--GRTLLYLRED G ---KDFREIAREIQDPV F ILGDHTGVTEEEEEKQ	162
Methanococcoides burtonii	IRSGNLGTLTLLNEFKDAGRRLIYLHED G ---EDIRELSDLTNNAV F ILGDHMGVTEEEEQQL	164
Methanocaldococcus jann.	VRRLGFRNLVLEKLEEGKNIYYLHM G ---EDVENVDIE--NPV F IIGDHIGIGEEEDERF	168
Methanococcus maripaludis	SSKREFRDIILEKKNEGKRIFYLHLN G KPLEDFEFKDD--DFL F VLDGHIGIGDEDEEF	165
Ignicoccus hospitalis	RTKESFEEVLKSLG--MKVYVLS E D G ---EDVVSFEFPK-SAA F LLG-----SQHDLEL	147
Photobacterium profundum	VRTVSFEKLVQELAE-DYQLFMMDDK G ---TNIREQEFEG-NPC F LLTDHIPMPKKSFN	165
Shewanella baltica	VSTISFEKLVQDLAV-DYQLFMMDDK G ---TSIREQEFVG-NPC F LLTDHIPMPKKSFN	165
Vibrio cholerae	VRTISFEALLGELAE-HHSLYMMDDK G ---DSIRDIKIGP-NPC F ILTDHIPMPKKSNGS	169
	: : : * . . *: :	
Pyrococcus horikoshii	LEGIAK-KISIG P KAYLTSHVISYVNIHL D RIGVP-----	203
Pyrococcus furiosus	LEGLVP-KITIG P KPYLTSHVIAFVNIYL D RIGIP-----	202
Haloferax volcanii	LAAAADERVRL G PEILHADHSITVAHNYL D TAGYSRY-----	198
Haloarcula marismortui	LADRADERVSL G PKALHADHSITVAHNYL D TAGFERY-----	198
Natronomonas pharaonis	LAAHADRRVSL G PEPLHADHAITVAHNYL D TDGYNRY-----	198
Haloquadratum walbyi	LGDIAEHQIRL G PMILHADHAITVAHNYL D TNGYNQY-----	189
Halobacterium salinarum	LADASDHRVRV G PTVLHADHAITVAHNHL D TAGFDAY-----	196
Methanosarcina acetiv.	LLEAGAKIISV G PISLHSNHCITLLHNE L DRAEAERGEIPGGEKLAGE	211
Methanosarcina mazei	LLEAGAQVISV G PISLHSNHCITLIHNE L DRAEAERGEIPGGNISRPED	211
Methanococcoides burtonii	IKEHEAKTISL G PIPLHADHCIIILNNE I DRNLSGKSQ-----	202
Methanocaldococcus jann.	LDEIKAKRISL S PLELHANHCITIIHNVL D KKRICEI-----	205
Methanococcus maripaludis	LEEIGAEEKISL S PLELHADHCIIILVHNIL D RLK-----	198
Ignicoccus hospitalis	PPWLEAERVSL G PKSYLASHCIYYLHYVL D LKLDPSRRPGS-----	189
Photobacterium profundum	LKRLGATKISL G PKMLFASQCVVLIHNE L DINM-----	198
Shewanella baltica	LKRLGAQKISL G PKMLFASQCVVLIHNE L DINQ-----	198
Vibrio cholerae	MKRLGVEKISL G PKMLFASQCVTLIHNE I DHQEAGW-----	205
	: :.* : : : : :*	

Multiple sequence alignment of 13 proteins from euryarchaea and 3 proteins from gamma proteobacteria bacteria. Two amino acids in the active side that are different in euryarchaea and gamma proteobacteria are shown in red. Universally conserved amino acids are shown in blue.

Supplementary Figure 7



Consensus tree of methylases of 13 euryarchaea (red) and 3 bacteria (blue) generated with ProtDist, Fitch, and CONSENSUS of the PHYLIP 3.67 program package. 100 bootstrap replications were performed and the bootstrap values are indicated at the nodes. Notably, the tree is very similar to the 16 S rRNA tree and haloarchaea as well as euryarchaea form monophyletic groups (arrows).

Supplementary Table 1

sequence	theoretical mass [Da]	observed mass [Da]	mass difference between fragments [Da]
5'-GG	709.1	709.2	
5'-GGG	1054.2	1054.2	345.0
5'-GGGG	1399.2	1399.1	344.9
5'-GGGGG	1744.3	1744.2	345.1
5'-GGGGGΨ	2050.3	2050.1	305.9
5'-GGGGGΨ-mΨ	2370.3	2370.1	320.0
5'-GGGGGΨ-mΨ-C	2675.4	2675.1	305.0
5'-GGGGGΨ-mΨ-CA	3004.4	3004.0	328.9
5'-GGGGGΨ-mΨ-CAA	3333.5	3333.1	329.1
5'-GGGGGΨΨ	2356.3	2356.1	306.0
5'-GGGGGΨΨC	2661.3	2661.1	305.0
5'-GGGGGΨΨCA	2990.4	2990.1	329.0
5'-GGGGGΨΨCAA	3319.4	3319.0	328.9
CUC-3'	855.2	855.2	
CCUC-3'	1160.2	1160.2	305.0
CCCUC-3'	1465.2	1465.2	305.0
UCCCUC-3'	1771.3	1771.2	306.0
AUCCCUC-3'	2100.3	2100.1	328.9
AAUCCCUC-3'	2429.4	2429.1	329.0
AAAUCCCUC-3'	2758.4	2758.1	329.0
CAAAUCCCUC-3'	3063.5	3063.1	305.0
mΨ-CAAAUCCCUC-3'	3383.5	3383.0	319.9
Ψ-CAAAUCCCUC-3'	3369.5	3369.0	305.9

Masses of the T-arm analog fragments determined by MALDI mass spectrometry after methylation with *Mj*Nep1 and partial acidic hydrolysis. Given are theoretical and observed mass of the methylated and unmethylated fragments and the observed mass differences between two adjacent fragments (theoretical values are G 345.0 Da, U and Ψ 306.0 Da, C 305 Da, A 329.1 Da, mΨ 320 Da). Fragments of modified and unmodified RNA are observable due to incomplete methylation. The T-arm analog containing two pseudouridines was used.