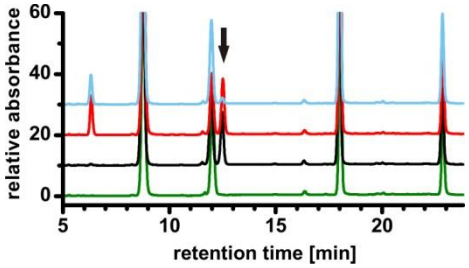
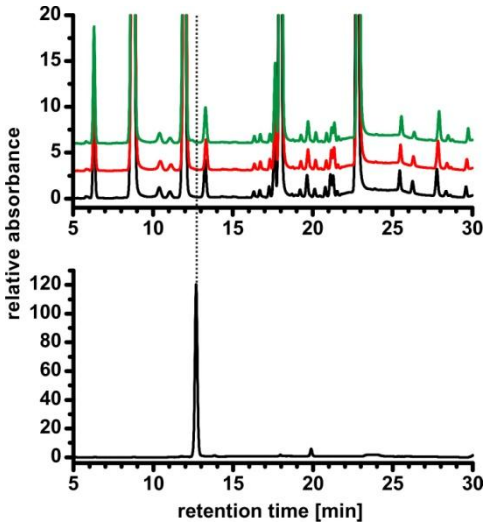
Supplementary Information

Supplementary Figure 1



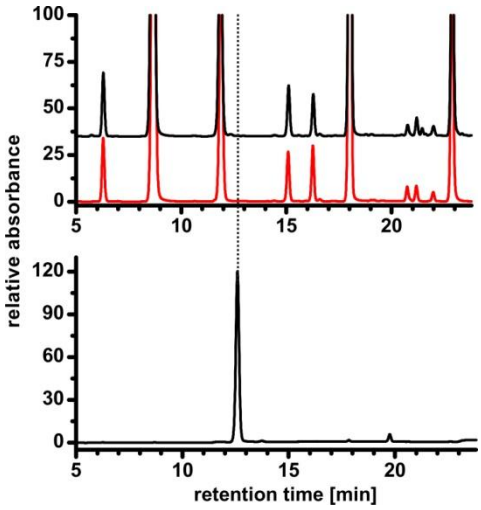
HPLC analysis of hydrolyzed T-arm substrates after methylation with *Mj*Nep1. Shown are the chromatograms for T-arm substrate without pseudouridine (green), with two pseudouridines (red) and with one pseudouridine at position 54 (blue) or 55 (black). The N1-methylpseudouridine signal is marked with an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

Supplementary Figure 2.



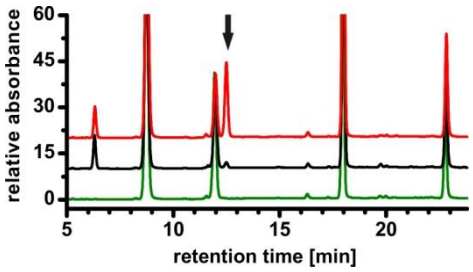
HPLC analysis of hydrolyzed tRNAs isolated from *E.coli*-BL21(DE3)-RIPL cells expressing *Mj*Nep1 (red), Mja_1640 (green) or no heterologous protein (black) (top) and chemically synthesized N1-methylpseudouridine (bottom) as reference.

Supplementary Figure 3



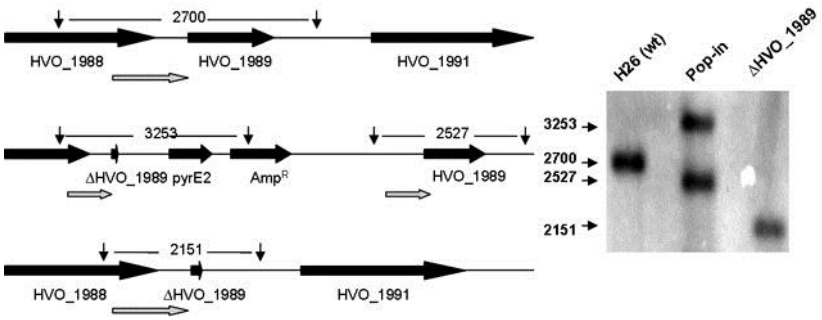
HPLC analysis of hydrolyzed tRNAs isolated from deletion mutant cells (red) and the same tRNAs after incubation with SAM and *Mj*Nep1 (black) (top) and chemically synthesized N1-methylpseudouridine (bottom) as reference.

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.

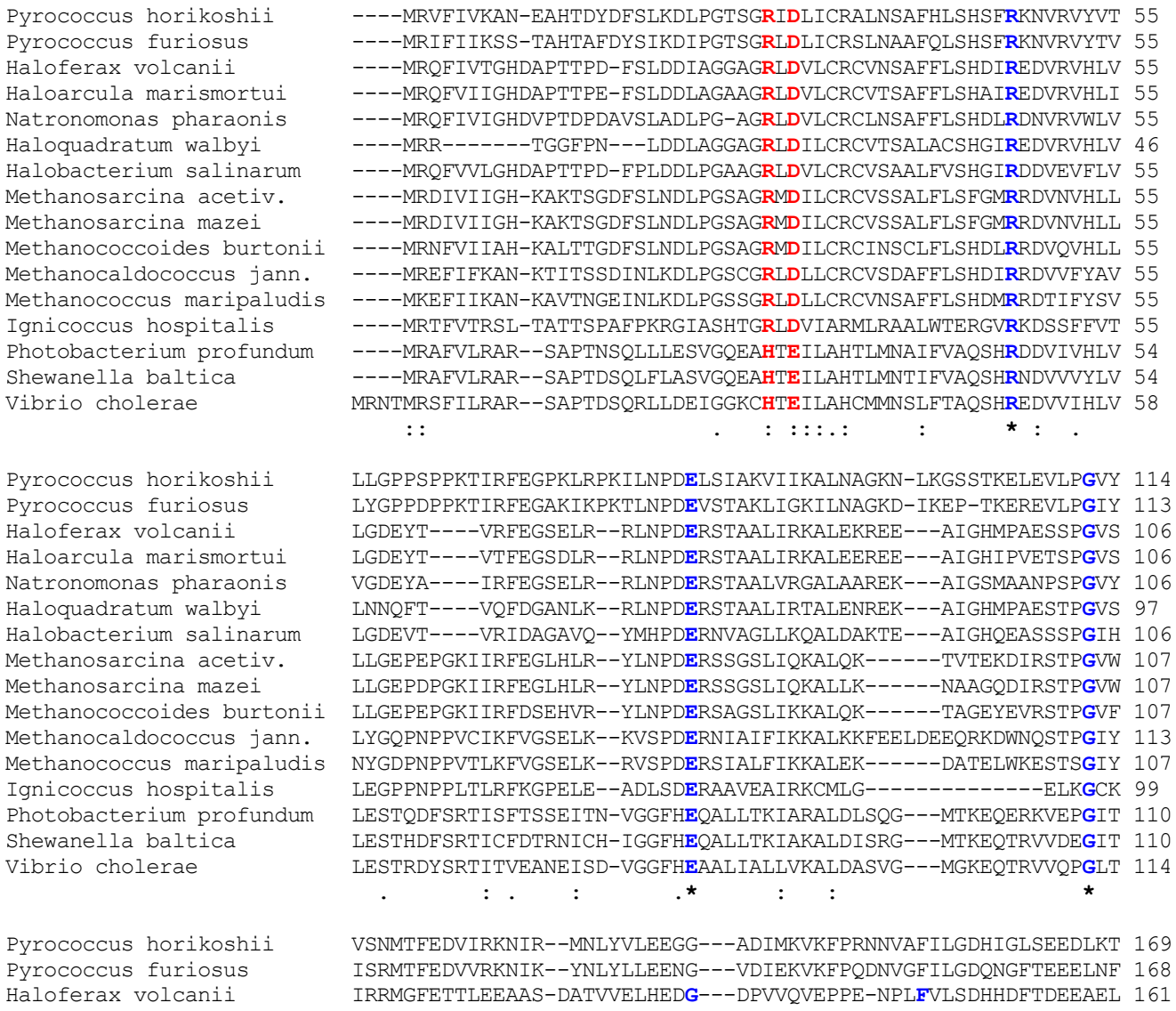
Supplementary Figure 5



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



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Haloarcula marismortui     LTRRGFEGTLDDVAR-RGTVVQLHEDG---DPIVGVAPPS-DPVFVLSDHHDFRDEEAAL 161
Natronomonas pharaonis     IRTQSLSGLLESVAD-DGPLVTLHEDG---QPVVDIDPSA-EPTFVLSDHHSFDDAETAL 161
Haloquadratum walbyi       IRRMGFAETVNRLKT-ETTPIQLHETG---TPIVDIDPPD-DPLFVLSDHHSLTNDEIEQ 152
Halobacterium salinarum    VSKRGFEAVLDAVS---GPVVALHEDG---DALAAVDPLA-DPVFVLSDHRDFTAAEASL 159
Methanosarcina acetiv.     VRNGDLNTLLASFE--GRTLFYLREDG---EDIRGLDREIRDPVFILGDHMGVTEEEEKQ 162
Methanosarcina mazei       TRTGDLNSLLASFE--GRTLLYLREDG---KDFREIAREIQDPVFILGDHTGVTEEEEKQ 162
Methanococcoides burtonii  IRSGNLGTLLNEFKDAGRRLIYLHEDG---EDIRELSDLTNNAVFILGDHMGVTEEEEQL 164
Methanocaldococcus jann.   VRRLGFRNLVLEKLEEGKNIYYLHMNG---EDVENVDIE--NPVFIIGDHIGIGEEDERF 168
Methanococcus maripaludis  SSKREFRDIILEKKNEGKRIFYLHLNGKPLEDFEFKDDE--DFLFVLGDHIGIGDEDEEF 165
Ignicoccus hospitalis      RTKESFEEVLKSLG---MKVYVLSEDG---EDVWSFEFPK-SAAFLLG-----SQHDLEL 147
Photobacterium profundum   VRTVSFEKLVQELAE-DYQLFMMDKKG---TNIREQEFEG-NPCFLLTDHIPMPKKSFNS 165
Shewanella baltica         VSTISFEKLVQDLAV-DYQLFMMDKKG---TSIREQEFVG-NPCFLLTDHIPMPKKSFNT 165
Vibrio cholerae            VRTISFEALLGELAE-HHSLYMMDKKG---DSIRDIKIGP-NPCFILTDHIPMPKKSGNS 169
                                :   :           :   *     .       .  *::         .

Pyrococcus horikoshii      LEGIAK-KISIGPKAYLTSHVISYVNIHLDRIGVP-------------- 203
Pyrococcus furiosus        LEGLVP-KITIGPKPYLTSHVIAFVNIYLDRIGIP-------------- 202
Haloferax volcanii         LAAAADERVRLGPEILHADHSITVAHNYLDTAGYSRY------------ 198
Haloarcula marismortui     LADRADERVSLGPKALHADHSITVAHNYLDTAGFERY------------ 198
Natronomonas pharaonis     LAAHADRRVSLGPEPLHADHAITVAHNYLDTDGYNRY------------ 198
Haloquadratum walbyi       LGDIAEHQIRLGPMILHADHAITIAHNYLDTNGYNQY------------ 189
Halobacterium salinarum    LADASDHRVRVGPTVLHADHAITVAHNHLDTAGFDAY------------ 196
Methanosarcina acetiv.     LLEAGAKIISVGPISLHSNHCITLLHNELDRAEAERGEIPGGEKLRAGE 211
Methanosarcina mazei       LLEAGAQVISVGPISLHSNHCITLIHNELDRAEAERGEIPGGNISRPED 211
Methanococcoides burtonii  IKEHEAKTISLGPIPLHADHCIILINNEIDRNLSGKSQ----------- 202
Methanocaldococcus jann.   LDEIKAKRISLSPLELHANHCITIIHNVLDKKRICEI------------ 205
Methanococcus maripaludis  LEEIGAEKISLSPLELHADHCIILVHNILDRLK---------------- 198
Ignicoccus hospitalis      PPWLEAERVSLGPKSYLASHCIYYLHYVLDLKLDPSPRRPGS------- 189
Photobacterium profundum   LKRLGATKISLGPKMLFASQCVVLIHNELDINM---------------- 198
Shewanella baltica         LKRLGAQKISLGPKMLFASQCVVLIHNELDINQ---------------- 198
Vibrio cholerae            MKRLGVEKISLGPKMLFASQCVTLIHNEIDHQEAGW------------- 205
                                  : :.*    :.: :   :  :*
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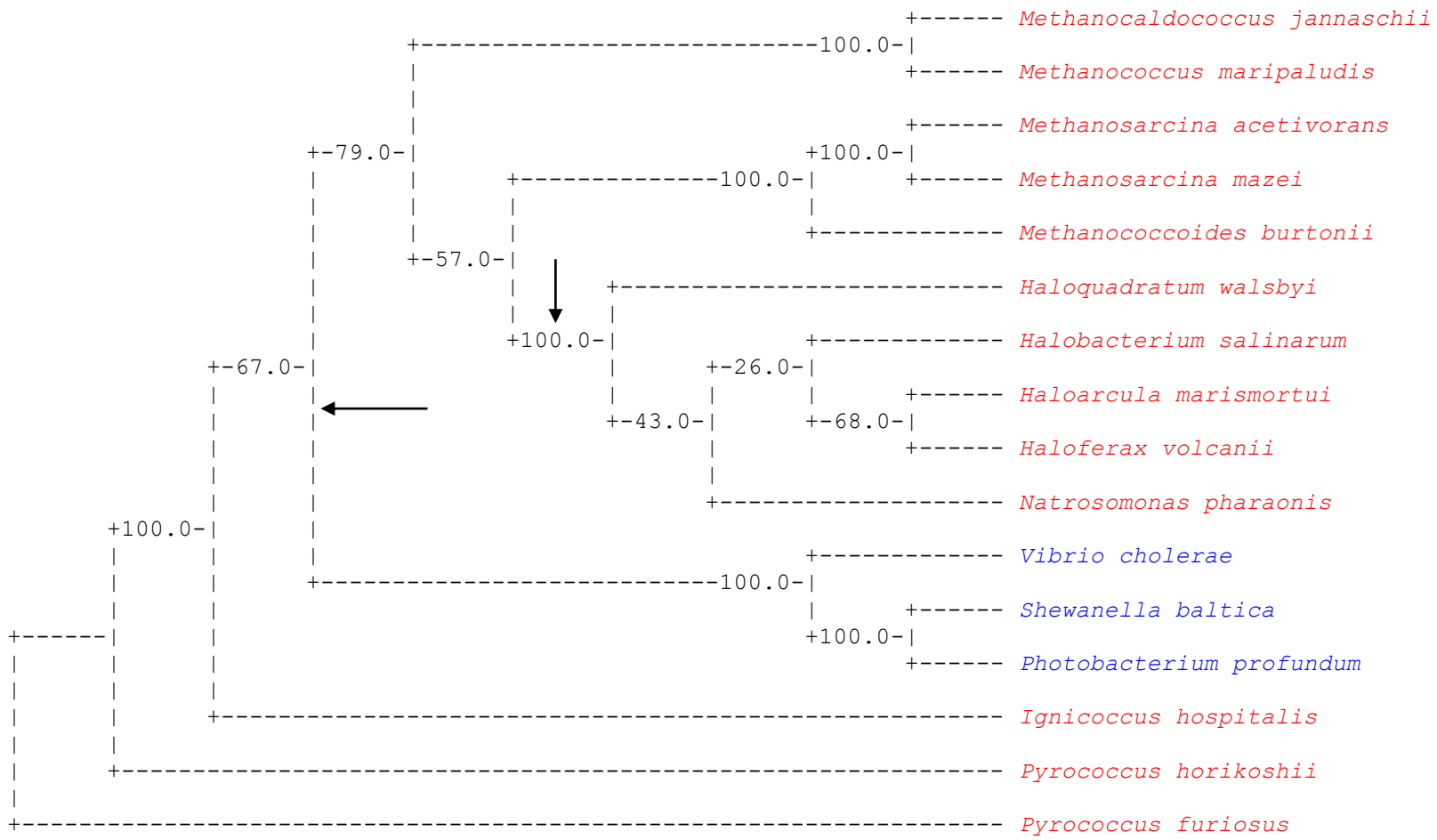
Multiple sequence alignment of 13 proteins from euryarchaea and 3 proteins from gamma proteobacteria bacteria. Two amino acids in the active side that are different in euryarchaea and gamma proteobacteria are shown in red. Universally conserved amino acids are shown in blue.

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 fragmants [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.