Supplementary Data

The flavoprotein Mcap0476 (RlmFO) catalyzes m^5U1939 modification in *Mycoplasma capricolum* 23S rRNA

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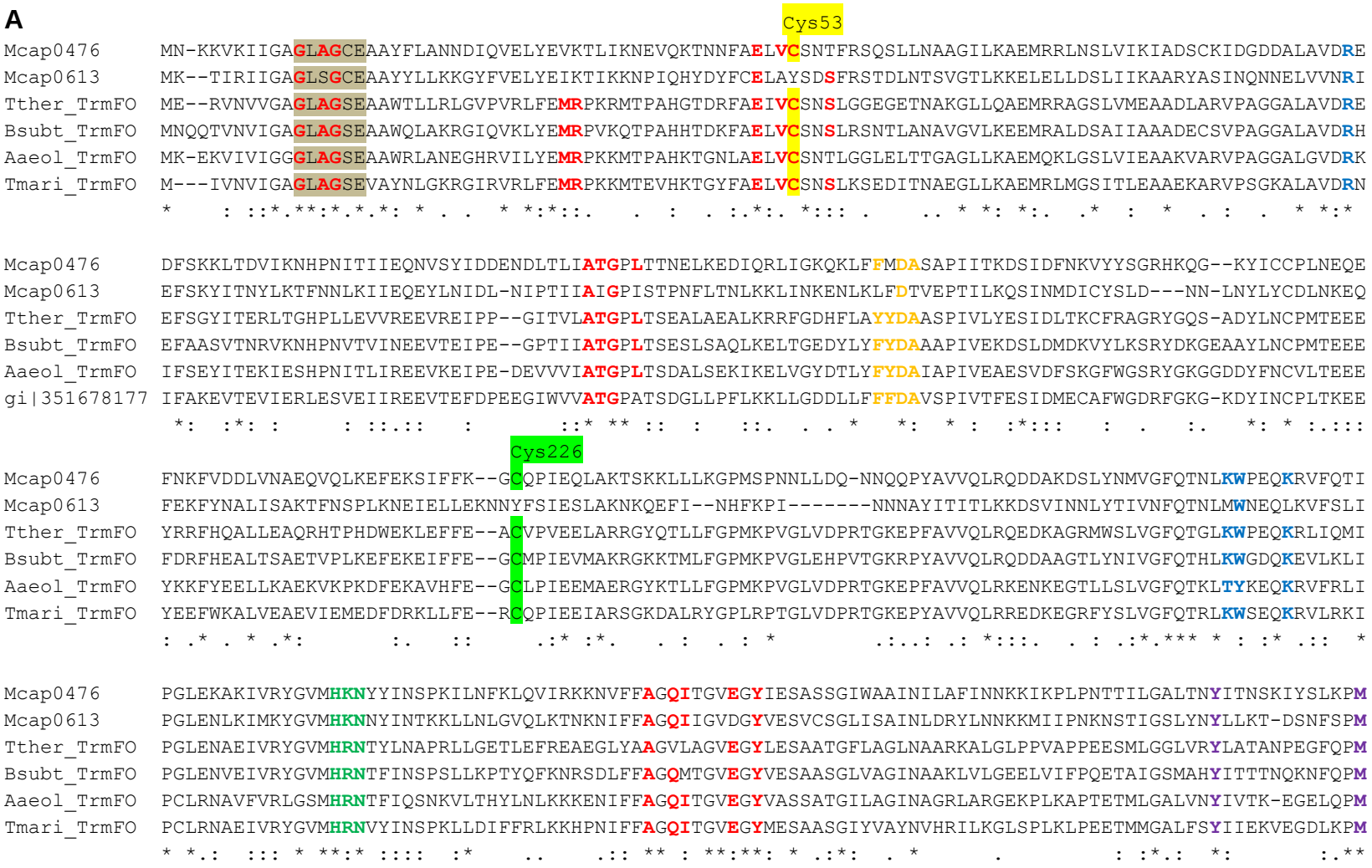
References

A


Cys53
Cys226
Mcap0476
Mcap0613
Tther_TrmFO
Bsubt_TrmFO
Aaeol_TrmFO
Tmari_TrmFO
gi|351678177

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Mcap0476      KCNLGILEQENKY-QSNDKFYS--YNNSKNSLENYIEQLNKI----LRTNI
Mcap0613      RINWALVDMIGGF-ELSDNSKKIYSKRAIKLIKQYLKKI----------NT
Tther_TrmFO   YANWGLVPPVEGRMGKKE-KRQAMYRRGLEAFSAWLSGLNPPLPRPEAALV
Bsubt_TrmFO   NANFGLLKELPVKIKNKKERNEQYANRAIETIQTIS----------KTI
Aaeol_TrmFO   NPVFGLLPPLEKKVRDKKKRKELMAKRALETMEKWIKENNL-VPEGLVKVC
Tmari_TrmFO   YANFGLLPPLKVRVKDKFEKRKKLAERAIETMKKFLEEN----------PW
                  .::         . :  .   ... : :.
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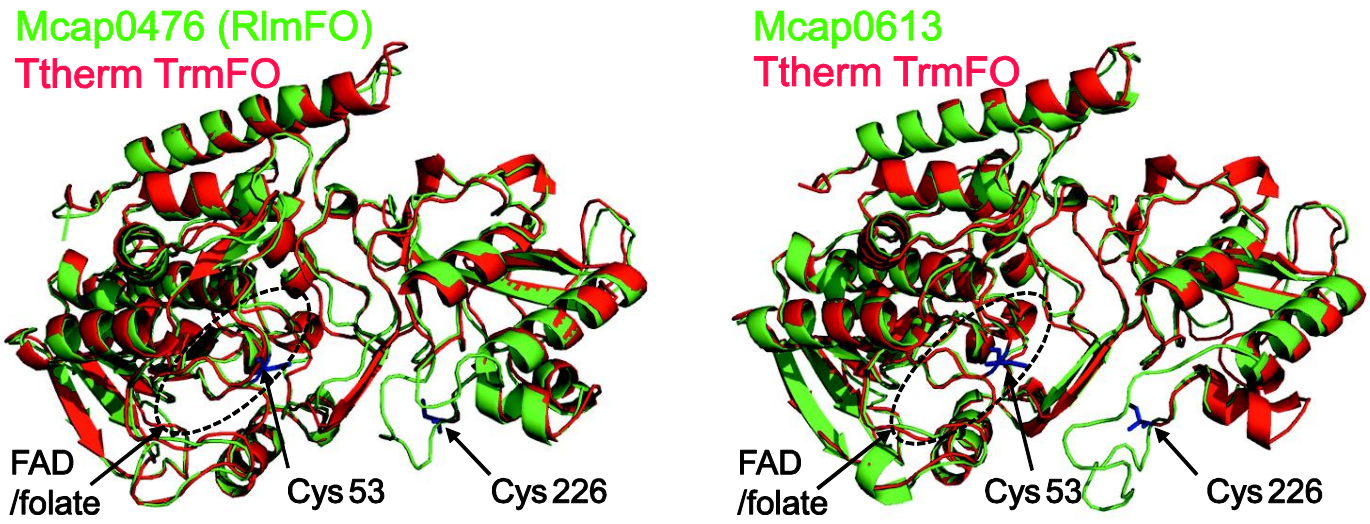
B

	Mcap0476	Mcap0613	Ttherm	Bsub	Aaeol	Tmari
Mcap0476		39.0	38.4	46.5	43.4	44.0
Mcap0613	59.4		28.4	37.7	29.4	32.7
Ttherm	60.0	50.4		50.9	52.7	51.3
Bsub	67.3	56.7	69.0		52.9	49.3
Aaeol	64.2	51.7	69.2	67.6		54.8
Tmari	63.9	54.9	67.3	68.6	72.3	

C

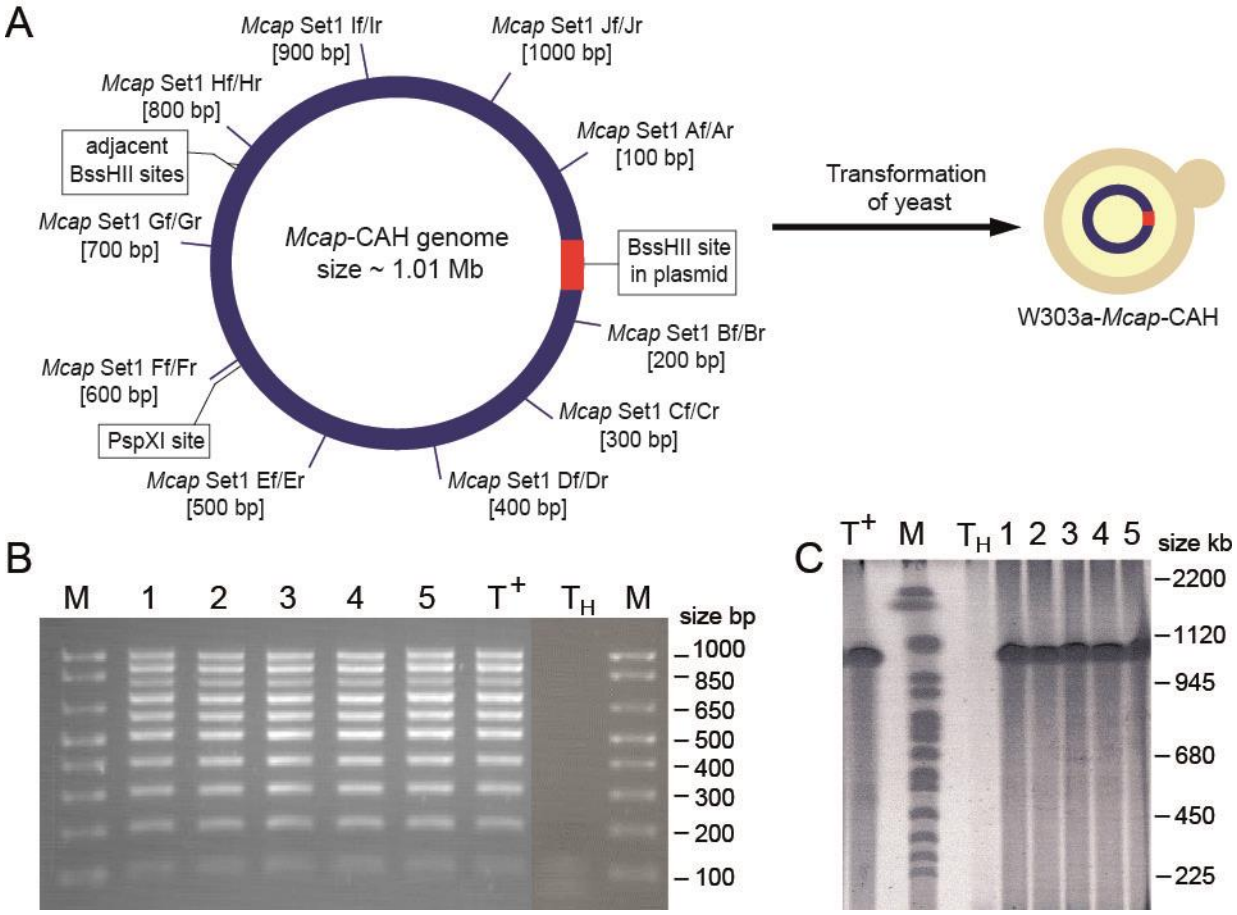


Supplementary Figure 1. Comparison of TrmFO-like proteins.

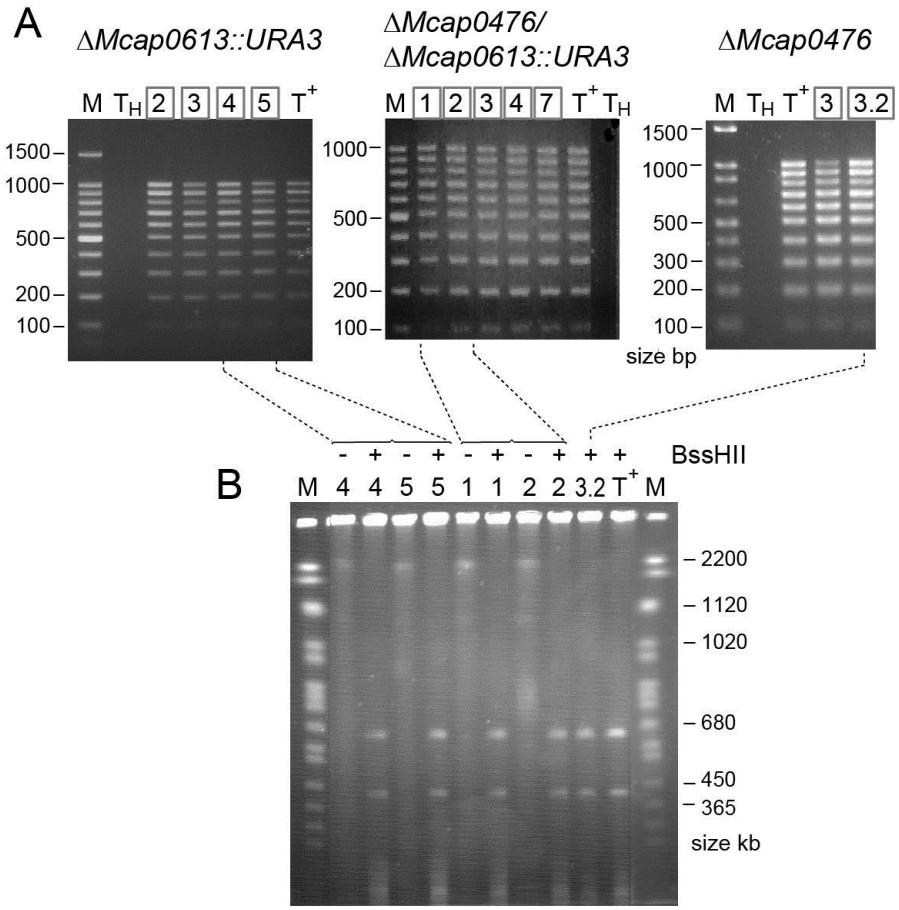
(**A**) Multiple TrmFO sequence alignment generated using ClustalW. TrmFO sequences from *T. thermophilus* (Tther_TrmFO), *B. subtilis* (Bsubt_TrmFO; the numbering system for this protein is used here), *Aquifex aeolicus* (Aaeol_TrmFO) and *Thermotoga maritima* (Tmari_TrmFO) are aligned against Mcap0476 and Mcap0613 proteins. The highlighted residues are thought to be involved in the binding of FAD (red), folate (green), GSH (orange) (1,2), tRNA (blue), and the formation of the domain interface (purple). The signature motif for the GSH reductase family (brown highlight) is shown at the N-terminus. Identical residues (*), conserved substitutions (:), and semi-conserved substitutions (.) are indicated under the sequences. Cys53 (yellow highlight) and Cys226 (green highlight) have been reported to play a major catalytic role in *B. subtilis* TrmFO (2).

(**B**) Identity and similarity between TrmFO-like sequences are indicated respectively as percentages above and below the diagonal, and were calculated from pairwise alignments using the EMBOSS Needle program. This program is available on the EBI web server: http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=emboss_needle&context=protein and uses the Needleman-Wunsch alignment algorithm. Parameters: Matrix, EBLOSUM62; Gap open, 10.0; Gap extend, 0.5; End Gap Penalty, false; End Gap Open Penalty, 10.0; End Gap Extension Penalty, 0.5. Abbreviations as in the sequence alignment.

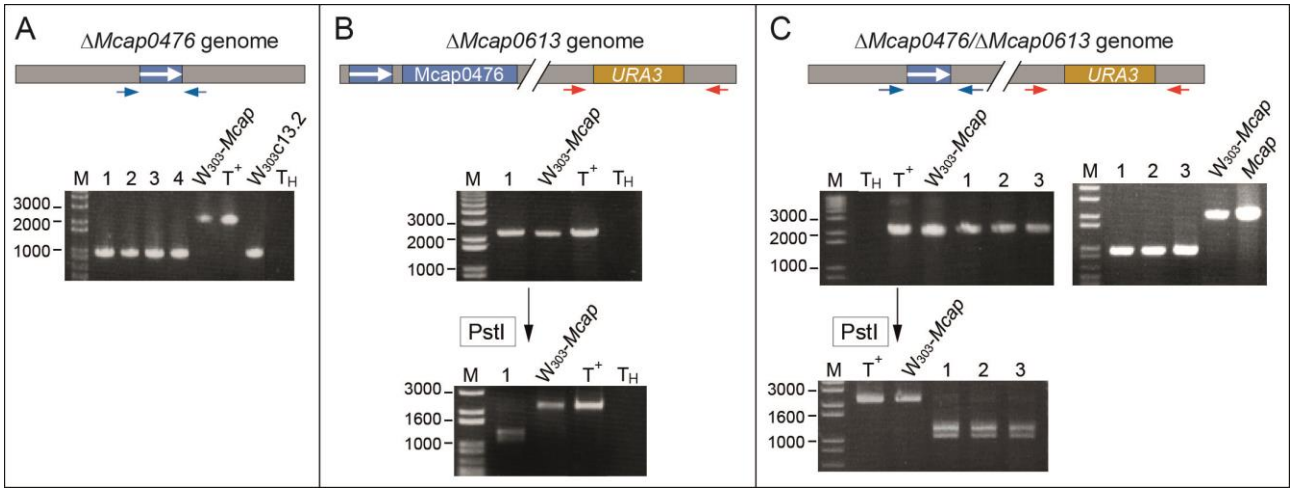
(**C**) Structures for the Mcap0476 (RlmFO) and Mcap0613 proteins were calculated using I-TASSER (3). The closest published match to these models is the crystal structure (PDB 3G5R) of Tther TrmFO (1). The I-TASSER RlmFO and Mcap0613 models (green) are shown superimposed onto Tther TrmFO (red), and can be seen to have essentially the same fold. Both *Mcap* proteins lack an AdoMet binding site (4), but possess structures resembling the N^5, N^{10}-methylenetetrahydrofolate (folate) and FAD binding regions seen in Tther TrmFO (dashed ellipse). Modelling FAD into the structures using I-TASSER gave a good fit in the TrmFO, RlmFO and Mcap0613 proteins. Folate could be easily docked into the TrmFO structure, although docking of folate into the corresponding regions of the RlmFO and Mcap0613 proteins would require some structural rearrangement. The estimated accuracy of the model for RlmFO: confidence (C) score 1.32, template modelling (TM) score 0.90 ± 0.06. For Mcap0613: C-score 1.50; TM-score 0.92±0.06. An alternative modelling approach using Phyre2 (5) gave essentially the same folds for the RlmFO and Mcap0613 proteins (not shown). The catalytically important residues Cys53 and Cys226 (*B. subtilis* TrmFO numbering) are present in both Tther TrmFO and RlmFO, but absent in Mcap0613. These cysteines are 20 Å apart, and it has been suggested for TrmFO that dimerization occurs upon tRNA binding bringing Cys53 and Cys226 of the two monomer units into close proximity (2).



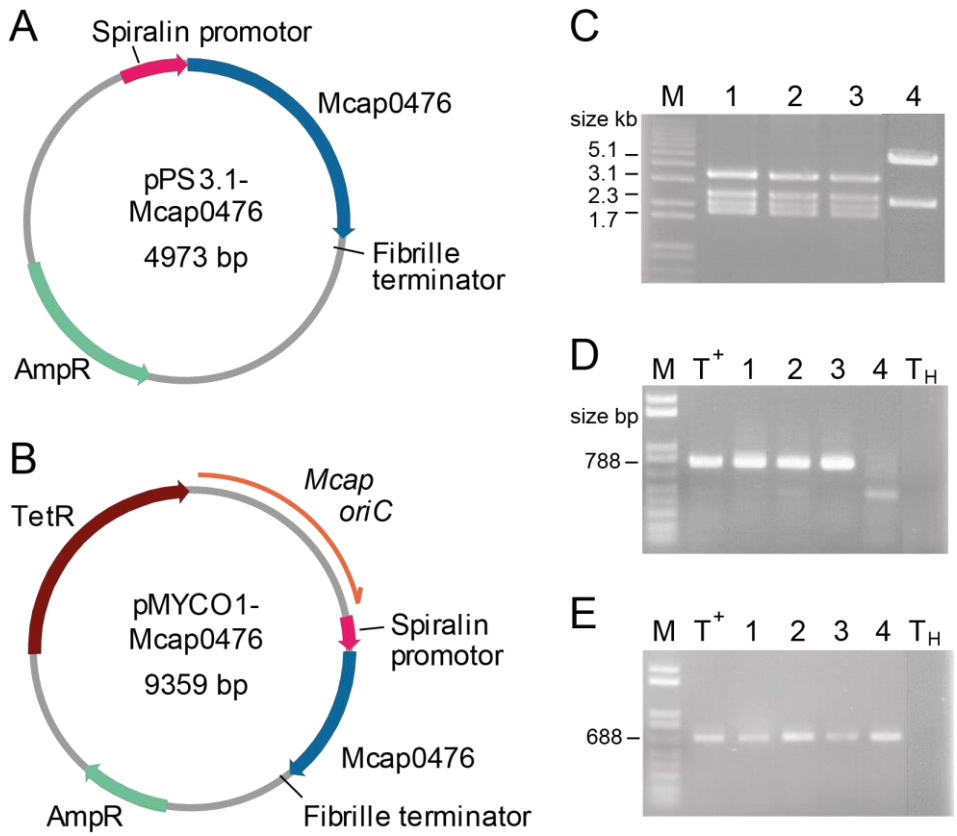
Supplementary Figure 2. Cloning of the *Mcap*-CAH genome in yeast. (**A**) *Mcap*-CAH was obtained by recombination of the plasmid pSD4-puro-CAH-Δ*Mcap0050* (red rectangle) into the *Mcap*-wt chromosome providing the elements for selection in yeast and mycoplasma (6). The map of the *Mcap*-CAH genome indicates the relative positions of pSD4-puro-CAH-Δ*Mcap0050*, PCR priming sites and BssHII and PspXI restriction sites. The engineered genome was isolated in an intact state from *Mcap*-CAH cells and transferred into yeast spheroplasts to form the yeast clone W303a-Mcap-CAH, where the *Mcap*-CAH genome was maintained as a centromeric plasmid. (**B**) *Mcap* genome structures were assessed by multiplex PCR and (**C**) pulsed-field gel electrophoresis (PFGE). The annealing sites for the set of ten pairs of PCR primers (Supplementary Table 1) are spaced at intervals of about 100 kb around the chromosome. The sizes of the PCR products from each adjacent primer pair differ in increments of 100 bp, such that the intact *Mcap* genome produces a ladder of ten bands. The multiplex PCR pattern is illustrated here with five yeast clones. Yeast clones were analyzed further by PFGE and produced bands matching the expected size of the PspX1-linearized *Mcap*-CAH genome. Clone 2 of W303a-*Mcap*-CAH was used in subsequent steps. Abbreviations: M, DNA size ladders of 100-1,000 bp (Invitrogen) in panel B, or 225–2,200 kb *S. cerevisiae* chromosomal DNA ladder (Bio-Rad) in panel C; T^+, DNA template from *Mcap*-wt cells; T_H, negative control without DNA.



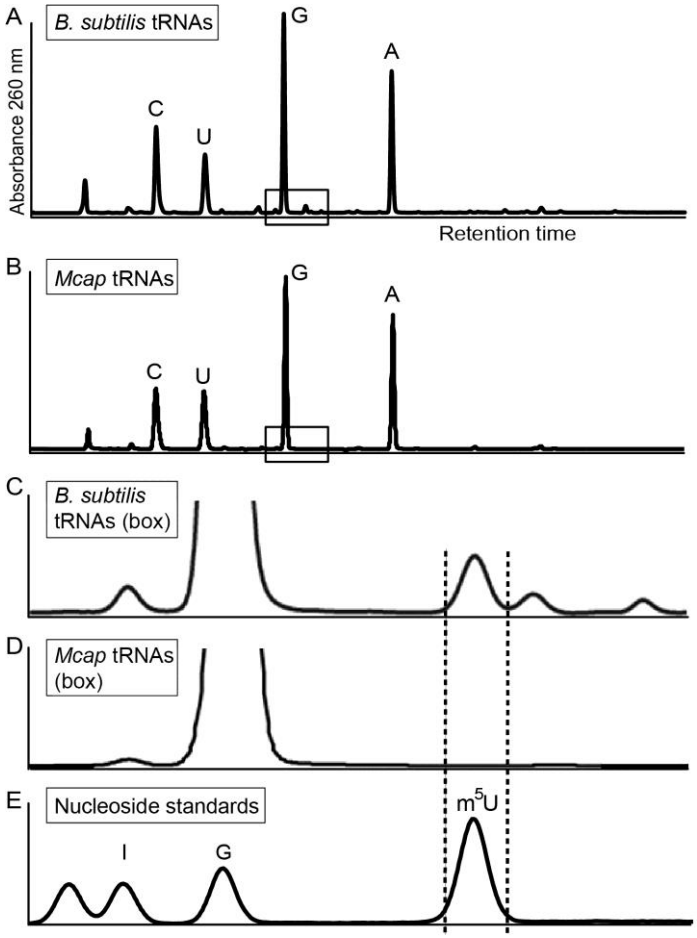
Supplementary Figure 3. Multiplex PCR and PFGE analyses of *Mcap* genomes in yeast. (**A**) Multiplex PCR analyses of yeast clones harboring *Mcap*-CAH genomes with single and double knockouts (Figure 2). The band patterns from these candidates are consistent with correctly structured genomes, and W303-Δ*Mcap0476* (clone 3.2), W303-Δ*Mcap0613*::URA3 (clones 4 and 5) and W303-Δ*Mcap0476*/Δ*Mcap0613*::URA3 (clones 1 and 2) were taken for PFGE analysis (dashed lines). (**B**) PFGE served as one of the final checks of *Mcap*-CAH genome structure and size. After removal of yeast genomic DNA, agarose plugs were either mocked digested (-) or digested with BssHII (+) and separated by PFGE. The *Mcap*-CAH genome contains three BssHII sites (Supplementary Figure 2), two of which are too close together to be distinguished by PFGE; digestion of the selected clones with BssHII gave rise to the expected bands of 389 kb and 621 kb. Bands in the mock digestion are the linear form of the *Mcap*-CAH genome caused by mechanical breaks, and a ~2.2 Mb band from residual yeast DNA. Abbreviations as in Supplementary Figure 2.



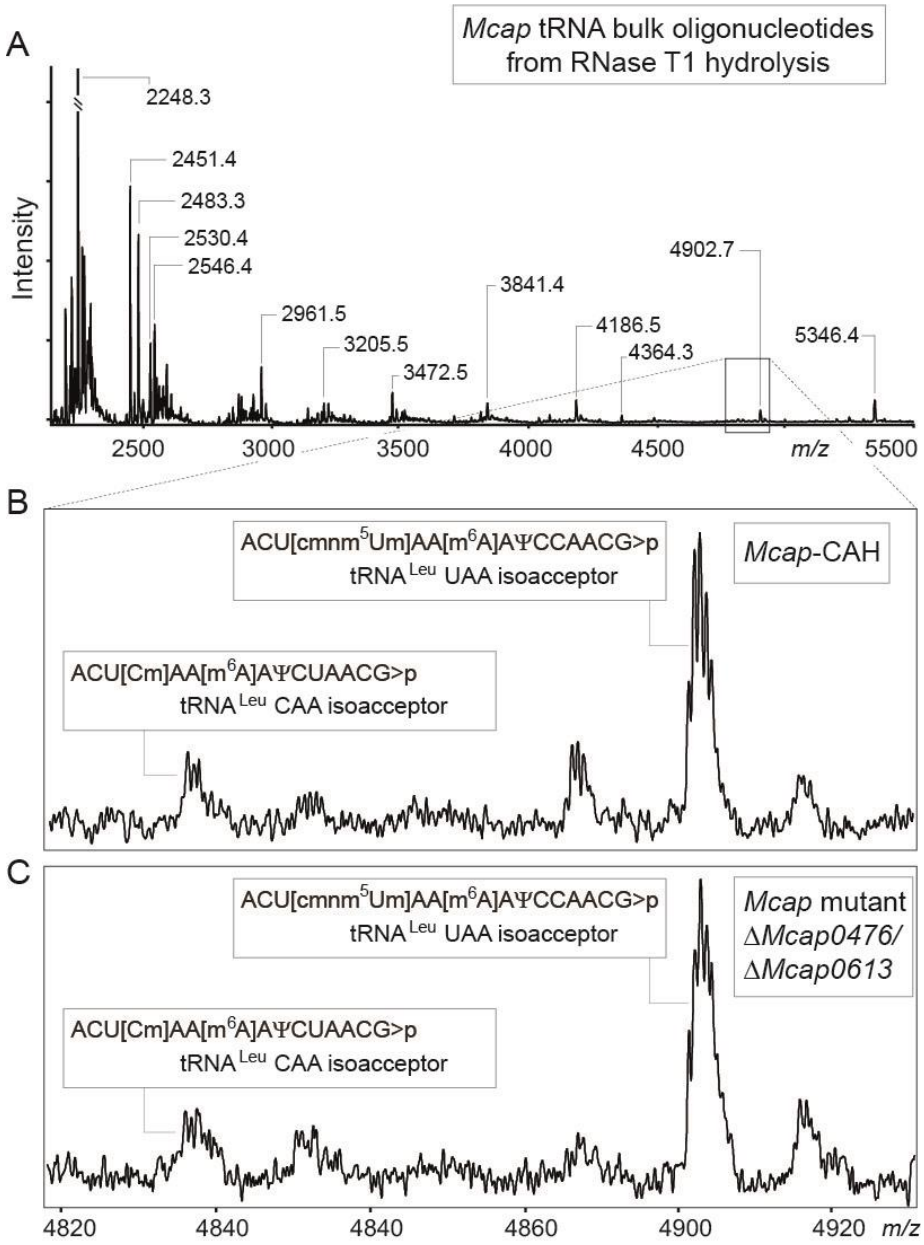
Supplementary Figure 4. Analyses of *Mcap*-CAH genomes after transplantation back into *Mcap* cells. Intact *Mcap*-CAH mutant genomes were isolated from the yeast recombinants W303-Δ*Mcap0476* clone 3.2, W303-Δ*Mcap0613::URA3* clone 4 and W303-Δ*Mcap0476*-Δ*Mcap0613*::URA3 clone 2. The genomic DNA was transplanted back into *Mcap*-wt recipient cells as described above. Deletion of Mcap0476 (Figure 2A) and/or the replacement of Mcap0613 by the URA3 cassette (Figure 2B) was then verified by PCR as shown here for the single knock-out mutants (**A**) *McapΔ0476* and (**B**) *Mcap-Δ0613* and (**C**) the double mutant *McapΔ0476/ΔMcap0613*. Deletion of Mcap0476 was tested for in panels A and C using the PCR primer pair TrmFO15a and TrmFO16 (Supplementary Table 1) positioned on each side of Mcap0476 (blue arrows). PCR products of 2 kb indicated that Mcap0476 was intact in W303-*Mcap-CAH*, whereas the 1 kb band from clones 1, 2, 3 and 4 showed that Mcap0476 had been seamlessly deleted. Replacement of Mcap0613 by *URA3* in panels B and C was tested with the PCR primer pair TrmFO25b and TrmFO26 (red arrows). Both the *URA3* and Mcap0613 genes produce fragments of 2.3 kb, but can be differentiated by PstI cleavage of *URA3* into bands of 1.0 and 1.3 kb. These mutants were further analyzed by multiplex PCR and PFGE (not shown). Abbreviations; W303-*Mcap*, DNA purified from W303 yeast strain containing a *Mcap*-CAH genome; W303-cl3.2, DNA purified from yeast strain W303-Δ*Mcap0476* (clone 3.2); other abbreviations as in Supplementary Figure 2.



Supplementary Figure 5. Complementation of the *ΔMcap0476* mutant by expression of Mcap0476 from a plasmid. (**A**) Cloning of the Mcap0476 gene into plasmids pPS3.1 and (**B**) pMYCO1 is described in Materials and Methods. Δ*Mcap0476* cells were transformed with the pMYCO1-Mcap0476 plasmid to express the wild-type Mcap0476 enzyme in this knock-out strain. (**C**) After transformation, plasmids were isolated from tetracycline resistant colonies and their structures were tested by PstI restriction analysis. Positive clones produce digestion products of 3.1, 2.3, 2 and 1.7 kb (lanes 1, 2, 3) whereas the empty pMYCO1 plasmid produces two bands at 5.1 and 2.3 kb (lane 4). (**D**) The clones were analyzed by RT-PCR (Materials and Methods) to screen for Mcap0476 expression. Clones 1, 2 and 3 expressed Mcap0476 mRNA giving rise to a cDNA product 788 bp. (**E**) All the four clones produced the Mcap0613-specific cDNA of 688 bp, showing that Mcap0613 mRNA was still been transcribed. Clone 3 was selected for further analysis by MS. AmpR, ampicillin resistance gene; TetR, tetracycline resistant gene; *oriC*, origin of replication; other abbreviations as in Supplementary Figure 2.

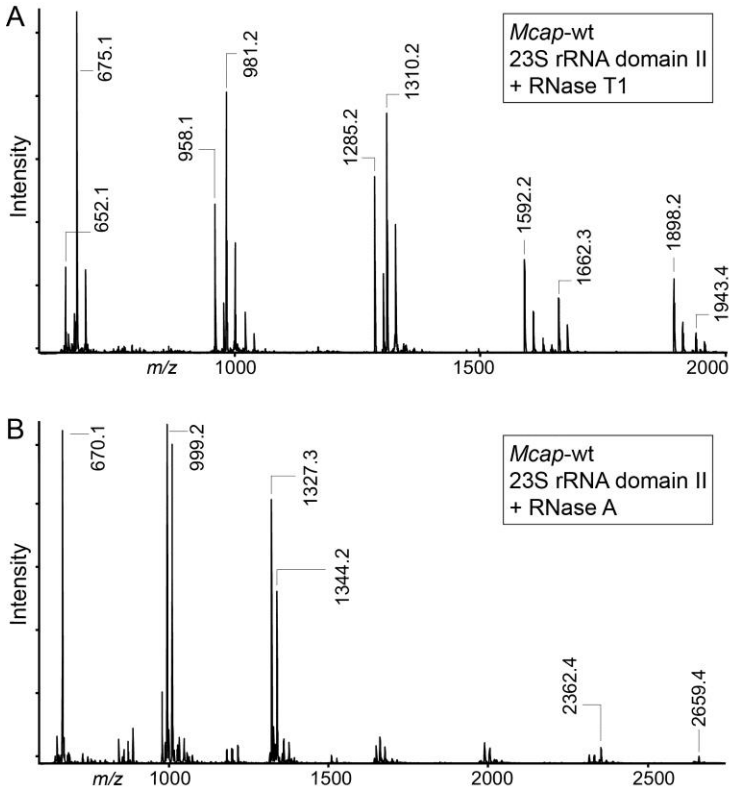


Supplementary Figure 6. HPLC profiles of tRNA nucleosides from bulk *Mcap* tRNA. (**A**) The digestion products of total tRNAs from the wild-type *B. subtilis* strain 168 (7) showing the positions of the unmodified nucleosides. (**B**) HPLC analysis of nucleosides derived from *Mcap*-CAH tRNAs. (**C**) Enlargement of the boxed region containing the *B. subtilis* m^5U modification. (**D**) Enlargements of the same region for the *Mcap* tRNA nucleosides showing absence of m^5U consistent with earlier reports for this species (8,9). (**E**) Nucleoside standardization mixture; the fractions corresponding to the retention times for inosine (I), guanosine and m^5U are indicated.



Supplementary Figure 7. (**A**) MALDI-MS analyses of bulk *Mcap* tRNAs after RNase T1 digestion. Oligonucleotides in the range *m/z* 2300 to 5500 are shown, corresponding to heptamers and larger. The cmnm^{5}U34 modification in the tRNA^{Leu} UAA isoacceptor migrates at *m/z* 4902.7 in a 15-nucleotide fragment ACU[cmnm^{5}Um]AA[m^{6}A]AΨCCAACGp (boxed). This fully modified fragment is clearly evident in (**B**) the wild-type spectrum and also in (**C**) the spectrum from *ΔMcap0476/ΔMcap0613*, as well as from the individual *ΔMcap0476* and *ΔMcap0613* knock-out strains (not shown). The corresponding fragment from the tRNA^{Leu} CAA isoacceptor anticodon at *m/z* 4833.7 lacks this modification, as do all tRNAs with C34 (10). The UCU[cmnm^{5}Um]CA[m^{6}A]AACCGp (*m/z* 3933.5) fragment from tRNA^{Trp} contains cmnm^{5}U34 in

the wild-type and all the knock-out strains (not shown). All *m/z* values given are for monoisotopic, singly-protonated oligonucleotides with a 2´-3´-cyclic phosphate (11). Most, but not all, of the large RNase T1 fragments could be assigned to predicted *Mcap* tRNA fragment masses including modifications (12). However, some peaks could not be assigned and a few expected peaks were missing indicating that the published list of modifications for *Mcap* tRNAs (12,13) is not fully comprehensive.



Supplementary Figure 8. MALDI-MS analyses of *Mcap* 23S rRNAs domain II showing the lack of modification at nucleotide U747. (**A**) Spectrum after RNase T1 digestion. Nucleotides U747 and G748 are in the trimer UUG>p at *m/z* 958, and both are unmodified. The *m/z* 958 fragment also contains U746, and the presence of a Ψ746 synthase ortholog in *Mcap* (Figure 3) indicates that this uridine is probably isomerized. Nucleotide G745 is in the fragment UAUUCG>p at *m/z* 1898 and is unmodified. (**B**) Spectrum after RNase A digestion where unmodified G748 is in the fragment GAAAAGACp at *m/z* 2659.

Supplementary Table 1. Additional PCR primer pairs used in this study

Primers for multiplex PCR of *Mcap* genome			
Primer name	**Sequence (5´→ 3´)**	**Location in *Mcap* genome**	**Size product (bp)**
Mcap Af	ATGAACCTAAACGATATTTTAAAAGAACTC	1	100
Mcap Ar	TATTAATTGTTTTAATATAGTCGTTATACACAG	100	
Mcap Bf	CTAACAGATCCAGCACCAGCTAC	100 001	200
Mcap Br	TCTTGATATTTTTGCAAAGTAATTTGTACTT	100 200	
Mcap Cf	AATTATTGGAATTCAACCAGTAAGTGATG	200 021	300
Mcap Cr	AATTTAGTTGTATTTAAATTAACTCCTTCTTT	200 320	
Mcap Df	TTTGATTGTTTCAAATTAACTTTTTAATTGCC	300 011	400
Mcap Dr	GAAATTGGAAAAATTTCTAAAACAATTTCTG	300 410	
Mcap Ef	CGGCTTGAAAGGCTATTAATGCATCAAC	399 931	510
Mcap Er	TTGCTGTAGTTACTCATAATCAAAATGTTG	400 440	
Mcap Ff	AAGAAGTATAACTCACATACATAGGCG	500 101	600
Mcap Fr	ACATATTTAACATTAATTGTAAATTTTGATTATCTTC	500 700	
Mcap Gf	TATTGGTAGATAAGCATGATCAGTACC	600 061	700
Mcap Gr	AATTAGGAAGAGAACCTAATGCTGAAG	600 760	
Mcap Hf	AGCTCCAGAAATTACTACAGTTGTTAAG	703 451	800
Mcap Hr	TCCATATTATATTATTGCTCCGCAAATAGC	704 250	
Mcap If	CTAAATCTAAAATTCCACCCTCACTAATAG	800 061	900
Mcap Ir	TGCAAGAACTGTTAAAGGTCCAAGAAAAAC	800 960	
Mcap Jf	TTAAAGGATTATTTGTTGCTGGTGATGTTA	900 201	1000
Mcap Jr	AAATCAACCTAATAATAAGGCAATTGATTCATAG	901 200	
PCR primers for investigating *Mcap* genes			
TrmFO15a	GAAGTTGGTGGTTTAACTCCAAG	5´-Mcap0476	1 kb, 2 kb or
TrmFO16	CTGAAATTACTCAAGCTTCACC	3´-Mcap0476	3.3 kb
TrmFO25b	GAGTTTGTCAAATTCCATTATCTG	5´-Mcap0613	2.3 kb
TrmFO26	CCACTTGCATTGCCATCATTG	3´-Mcap0613	

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