**Supplemental Material**

**Table S1.** Strains and plasmids used in this study.

| Strain or plasmid | Phenotype, genotype and/or description[a] | Source or Reference |
|---|---|---|
| **Strains:** | | |
| ***E. coli*** | | |
| DH5α | *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | Life Technologies |
| GM2163 | *dam-13::*Tn *9 dcm-6 hsdR2 leuB6 his-4 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 rpsL136 tonA31 tsx-78 supE44* McrA$^-$ McrB$^-$ | New England Biolabs |
| ***H. volcanii*** | | |
| H26 | DS70 *pyrE2* | (1) |
| HM1052 | H26 *hvo_0558* (*ΔubaA*) | This study |
| HM1053 | H26 *hvo_1864*[b] *(ΔmoaE*) | This study |
| HM1041 | H26 *hvo_2619* (*Δsamp1*) | This study |
| HM1042 | H26 *hvo_0202* (*Δsamp2*) | This study |
| HM1055 | H26 *hvo_2177* [c] (*Δhvo_2177* encoding Ubl β-grasp protein with a diglycine motif) | This study |
| HM1067 | HM1042 *hvo_2619* (*Δsamp1 Δsamp2*) | This study |
| HM1088 | H26 *hvo_0559* (*ΔubaB*) | This study |
| HM1096 | HM1067 *hvo_2177* (*Δsamp1 Δsamp2 Δhvo_2177*) | This study |
| **Plasmids:** | | |
| pJAM202c | Ap$^r$; Nv$^r$; *H. volcanii-E.coli* shuttle plasmid vector | (2) |
| pTA131 | Ap$^r$; pBluescript II carries P$_{fdx}$-*pyrE2* with MCS | (1) |
| pJAM809 | Ap$^r$; Nv$^r$; pJAM202 carries P2*rrnA-hvo1862-StrepII* (KpnI site upstream of StrepII coding sequence) | (3) |
| pJAM816 | Ap$^r$ Nv$^r$; pJAM809 carries *psmB-StrepII* | (3) |
| pJAM947 | Ap$^r$; Nv$^r$; pJAM202c carries P2$_{rrn}$-*Flag-hvo_2619* (Flag-SAMP1)[d] | (4) |
| pJAM949 | Ap$^r$; Nv$^r$; pJAM202c carries P2$_{rrn}$-*Flag-hvo_0202* (Flag-SAMP2) | (4) |
| pJAM957 | Ap$^r$; Nv$^r$; pJAM202c carries P2$_{rrn}$-*hvo_0558-StrepII* (UbaA-StrepII) | This study |
| pJAM977 | Ap$^r$; Nv$^r$; pJAM202c carries P2$_{rrn}$-*Flag-hvo_2177* (Flag-HVO_2177) | This study |
| pJAM993 | Ap$^r$; Nv$^r$; pJAM202c carries P2$_{rrn}$-*Flag-hvo_2619 hvo_0558* (Flag-SAMP1, UbaA-StrepII) | This study |
| pJAM994 | Ap$^r$; Nv$^r$; pJAM202c carries P2$_{rrn}$-*Flag-hvo_2619 hvo_0558* (Flag-SAMP1, UbaA-StrepII C188S) | This study |

| | | |
|---|---|---|
| pJAM995 | $Ap^r$; $Nv^r$; pJAM202c carries $P2_{rrn}$-*Flag-hvo_0202 hvo_0558* (Flag-SAMP2, UbaA-StrepII) | This study |
| pJAM996 | $Ap^r$; $Nv^r$; pJAM202c carries $P2_{rrn}$-*Flag-hvo_0202 hvo_0558* (Flag-SAMP2, UbaA-StrepII C188S) | This study |
| pJAM1116 | $Ap^r$; $Nv^r$; pJAM202c carries $P2_{rrn}$-*hvo_0558-StrepII C188S* (UbaA-StrepII C188S) | This study |
| pJAM1119 | $Ap^r$; $Nv^r$; pJAM202c carries $P2_{rrn}$-*hvo_1864 -StrepII* (MoaE-StrepII) | This study |
| pJAM959 | $Ap^r$; pTA131-based pre-knockout plasmid for *hvo_0558* (UbaA)[e] | This study |
| pJAM960 | $Ap^r$; pTA131-based knockout plasmid for *hvo_0558* (UbaA)[f] | This study |
| pJAM1108 | $Ap^r$; pTA131-based pre-knockout plasmid for *hvo_2619* (SAMP1) | This study |
| pJAM1115 | $Ap^r$; pTA131-based knockout plasmid for *hvo_2619* (SAMP1) | This study |
| pJAM1107 | $Ap^r$; pTA131-based pre-knockout plasmid for *hvo_0202* (SAMP2) | This study |
| pJAM1109 | $Ap^r$; pTA131-based knockout plasmid for *hvo_0202* (SAMP2) | This study |
| pJAM1112 | $Ap^r$; pTA131-based pre-knockout plasmid for *hvo_2177** (β-grasp protein) | This study |
| pJAM1117 | $Ap^r$; pTA131-based knockout plasmid for *hvo_2177** (β-grasp protein) | This study |
| pJAM1113 | $Ap^r$; pTA131-based pre-knockout plasmid for *hvo_1864* (MoaE) | This study |
| pJAM1114 | $Ap^r$; pTA131-based knockout plasmid for *hvo_1864* (MoaE) | This study |
| pJAM1120 | $Ap^r$; pTA131-based pre-knockout plasmid for *hvo_0559* (RHD) | This study |
| pJAM1127 | $Ap^r$; pTA131-based knockout plasmid for *hvo_0559* (RHD) | This study |

[a]Abbreviations: $Ap^r$, ampicillin resistance; $Nv^r$, novobiocin resistance; MCS, multiple cloning site; RHD, rhodanese domain protein.
[b]HVO_1864, MoaE includes N-terminal MoaE and C-terminal MobB domains.
[c]HVO_2177, defined in this study as a Ubl β-grasp protein with C-terminal diglycine motif and N-terminal methionine analogous to residue number 22 of the current genome annotation (5).
[d]Flag-, N-terminal Flag-tag fusion; -StrepII, C-terminal StrepII fusion.
[e]Pre-knockout plasmids were generated in two steps: (i) the target gene with approximately 500 bp of DNA flanking the 5'- and 3'-end of the gene was amplified from genomic DNA by PCR and (ii) PCR products were cloned into plasmid vector pTA131 using restriction enzymes BamHI and HindIII and T4 DNA ligase.
[f]Knockout plasmids were generated by inverse PCR using the preknockout plasmid as a template and primers up and downstream of the target gene (Inverse FW and RV, see Table S2). The resulting PCR product was self-ligated to generate the knockout plasmid which carried the flanking DNA with a markerless in-frame deletion of the target gene in pTA131. Knockout plasmids were transformed into parent H26 (*ΔpyrE2*) or an H26-derived mutant by selection for growth on uracil (pop-in), and deletion strains were enriched from these integrants by selection for resistance to 5-fluoroorotic acid (5-FOA) (pop-out).

**Table S2.** Primers used in this study.

| Primer Pair[a] | Primer Sequence (5'-3')[b] | PCR Product/Description | Source or Reference |
|---|---|---|---|
| SAMP1-530 HindIII FW<br>SAMP1-530 BamHI RV | 5'-CTAAGCTTGGCACCGACACCGACGCG-3'<br>5'-TCAGGATCCACCGAGGACACGATGCCGATTC-3' | genomic region including *samp1* and 0.5 kb flanking 5' and 3' of this gene; generated using *H. volcanii* DS70 genomic DNA as template; includes BamHI and HindIII sites for cloning into pTA131 to generate pJAM1108 | This study |
| SAMP1-Inverse FW<br>SAMP1-Inverse RV | 5'-CGCGCGGTCGTCCCCCCG-3'<br>5'-ACTCCCGTCTCGTCGCCCGGC-3' | *samp1*-knockout plasmid pJAM1115 generated by inverse PCR using pJAM1108 as template | This study |
| SAMP1-Confirm FW<br>SAMP1- Confirm RV | 5'- CGGCACCGTCGCGGTCGCGATTCG-3'<br>5'- CGGCGTCCAGACCTACGACGGGCT-3' | used to confirm *Δsamp1* mutants by PCR; primers anneal both 5' and 3' of *samp1* by 0.7 kb | This study |
| SAMP1-530 HindIII FW<br>SAMP1-Inverse RV | 5'-CTAAGCTTGGCACCGACACCGACGCG-3'<br>5'-ACTCCCGTCTCGTCGCCCGGC-3' | 0.5-kb probe generated by PCR using pJAM1115 as template; probe used to confirm *Δsamp1* mutants by Southern blot | This study |
| HVO_2619 KpnI up (SAMP1-Internal FW)<br>HVO_2619 BlpI down (SAMP1-Internal RV) | 5'-AAGGTACCGAGTGGAAGCTGTTCGCCGACCTCG-3'<br>5'-TTAATGCTCAGCCTAGCCGCCGCTGACCGG-3' | 0.25-kb fragment carrying *samp1* coding region generated; used to screen for Δ*samp1* mutants | (4) |
| SAMP2-520 BamHI FW<br>SAMP2-520 HindIII RV | 5'-TCAGGATCCAGAACTGCTCCATCGTCCGG-3'<br>5'-CGAAGCTTGGTCTCGGTGTGCCATGG-3' | genomic region including *samp2* and 0.5 kb flanking 5' and 3' of this gene; generated using *H. volcanii* DS70 genomic DNA as template; includes BamHI and HindIII sites for cloning into pTA131 to generate pJAM1107 | This study |
| SAMP2-Inverse FW<br>SAMP2-Inverse RV | 5'-TAGTCGGCGGTATGGAAGACAC-3'<br>5'-CATGGTCGCTCGTGGGTC-3' | *samp2*-knockout plasmid pJAM1109 generated by inverse PCR using pJAM1107 as template | This study |
| SAMP2-Confirm FW<br>SAMP2-Confirm RV | 5'- GTAGACCAGCGCGTCGAGGCCGTC-3'<br>5'- GCCTGCTGGACGACCTGCACGTCG-3' | used to confirm Δ*samp2* mutant by PCR; primers anneal both 5' and 3' of *samp2* by 0.7 kb | This study |
| SAMP2-Inverse FW<br>SAMP2-520 HindIII RV | 5'-TAGTCGGCGGTATGGAAGACAC-3'<br>5'-CGAAGCTTGGTCTCGGTGTGCCATGG-3' | 0.5-kb probe generated by PCR using pJAM1109 as template; probe used to confirm Δ*samp2* mutants by Southern blot | This study |

| | | | |
|---|---|---|---|
| HVO_0202 KpnI up (SAMP2-Internal FW)<br>HVO_0202 BlpI down (SAMP2-Internal RV) | 5′-CGTTGGTACCATGAACGTGACCGTCGAGG-3′<br>5′-TTAATGCTCAGCTACCCGCCTTTGATGAGG-3′ | 0.2-kb fragment carrying *samp2* coding region generated; used to screen for Δ*samp2* mutants | (4) |
| HVO_2177-520 HindIII FW<br>HVO_2177-520 BamHI RV | 5′-CGAAGCTTGACCATCATCACATCTGACACACGG-3′<br>5′-TAGGATCCACGAGATACACCGCCGCG-3′ | genomic region including *hvo_2177* and 0.5 kb flanking both 5′ and 3′ of this gene generated using *H. volcanii* DS70 genomic DNA as template; includes BamHI and HindIII sites for cloning into pTA131 to generate pJAM1112 | This study |
| HVO_2177-Inverse FW<br>HVO_2177-Inverse RV | 5′-GCG**g**CGACAGCGGGCCTTC-3′<br>5′-GCCACGTCGCCGTTATTCGGGACAGTAATTCAAA-3′ | *hvo_2177* -knockout plasmid pJAM1117 generated by inverse PCR using pJAM1112 as template | This study |
| HVO_2177-Confirm FW<br>HVO_2177-Confirm RV | 5'- GGTTCGGTCACGCGCTTCTCTCCG-3'<br>5'- GGATTGCCGGCTTTTTCCCTCCCG-3' | used to confirm *Δhvo_2177* mutants by PCR; primers anneal both 5′ and 3′ of *hvo_2177* by 0.7 kb | This study |
| HVO_2177 KpnI up (HVO_2177-Internal FW)<br>HVO_2177 BlpI down (HVO_2177-Internal RV) | 5′-AAGGTACCAAA**a**G**a**CTCCG**t**GTCCTCGCCGCGAC-3′<br>5′-TTAATGCTCAGCATCAGCCCCCCGCGACC-3′ | 0.34-kb fragment carrying *hvo_2177* coding region generated; used to screen for Δ*hvo_2177* mutants | (4) |
| HVO_2177 alt start KpnI up<br>HVO_2177 BlpI down | 5′-GGGGTACCATGGAGCTCGAATTACGCTTCTTCGC-3′<br>5′-TTAATGCTCAGCATCAGCCCCCCGCGACC-3′ | 0.28-kb fragment carrying *hvo_2177* coding region generated using *H. volcanii* genomic DNA as template; KpnI and BlpI sites included for cloning into pJAM939 to generate pJAM977 | This study |
| HVO_2177-Inverse FW<br>HVO_2177-520 BamHI RV | 5′-GCG**g**CGACAGCGGGCCTTC-3′<br>5′-TAGGATCCACGAGATACACCGCCGCG-3′ | 0.5-kb probe generated by PCR using pJAM1117 as template; probe used to confirm Δ*hvo_2177** mutants by Southern blot | This study |
| HVO_0558 FW<br>HVO_0558 RV | 5′-ATGACGCTCTCACTCGACGCCAC-3′<br>5′-CCTGCCGCTGGAGGTTGCTC-3′ | used to detect *ubaA*-specific transcript by qPCR and RT-qPCR | This study |
| Hvo_0558 BamHI FW<br>Hvo_0558 HindIII RV | 5′-TTATGGATCCCAGAAGTGACTCAGAACGGCGACG-3′<br>5′-CTAAGCTTACGTGGTTCAGGACGGGTGCGGTG-3′ | genomic region including *ubaA* (HVO_0558) and 0.5 kb flanking 5′ and 3′ of this gene generated using *H. volcanii* DS70 genomic DNA as template; includes BamHI and HindIII sites for cloning into pTA131 to generate pJAM959 | This study |

| | | | |
|---|---|---|---|
| HVO_0558-Inverse FW<br>HVO_0558-Inverse RV | 5’-GGAGAGCGCGTCTCGGC-3’<br>5’-ATCCCGAGGTTGGCGTCG-3’ | *ubaA*-knockout plasmid pJAM960 generated by inverse PCR using pJAM959 as template | This study |
| HVO_0558-Confirm FW<br>HVO_0558-Confirm RV | 5'-GCGGTGTGGATATACCGCGAC-3'<br>5'-TGGAAGCTGCGATTGAGGAGC-3' | used to confirm Δ*ubaA* mutants by PCR; primers anneal both 5’ and 3’ of *ubaA* by 0.7 kb | This study |
| HVO_0558-BamHI FW<br>HVO_0558 -Inverse RV | 5’-TTATGGATCCCAGAAGTGACTCAGAACGGCGACG-3’<br>5’-ATCCCGAGGTTGGCGTCG-3’ | 0.5-kb probe generated by PCR using pJAM960 as template; probe used to confirm Δ*ubaA* mutants by Southern blot | This study |
| HVO_0558 NdeI FW<br>(HVO_0558-Internal FW)<br>HVO_0558 KpnI Strep RV<br>(HVO_0558-Internal RV) | 5’-TTCCTTACATATGACGCTCTCACTCGACGCCACCC-3’<br>5’-CCGGTACCGTCGAGGCTGATTGCGCAG-3’ | 0.8-kb DNA fragment carrying *ubaA* coding region generated using *H. volcanii* genomic DNA as template; NdeI and KpnI sites included for cloning into pJAM809 to generate pJAM957 for synthesis of UbaA-StrepII in *H. volcanii*; also used to screen for Δ*ubaA* mutants | This study |
| Hvo_0558 C188S FW<br>Hvo_0558 C188S RV | 5’-GTCCCCGACAGCGCGACGACCGGC-3’<br>5’-GCCGGTCGTCGCGCTGTCGGGGAC-3’ | Used for site-directed mutagenesis with pJAM957 as template to generate plasmid pJAM1116 encoding UbaA-StrepII C188S | This study |
| HVO_1864-500 HindIII FW<br>HVO_1864-500 BamHI RV | 5’-TTAAGCTTGAGCACGCTTCCGCCGATG-3’<br>5’-TCGGATCCCACTTCTCGATGGACAGGTC-3’ | genomic region including *hvo_1864* and 0.5 kb flanking 5’ and 3’ of this gene generated using *H. volcanii* DS70 genomic DNA as template; includes BamHI and HindIII sites for cloning into pTA131 to generate pJAM1113 | This study |
| HVO_1864- Inverse FW<br>HVO_1864-Inverse RV | 5’-TGAGACGGCGCGGATAACTC-3’<br>5’-GAGGACGTGCATACCCGAAG-3’ | *hvo_1864*-knockout plasmid pJAM1114 generated by inverse PCR using pJAM1113 as template | This study |
| HVO_1864-Confirm FW<br>HVO_1864-Confirm RV | 5'- CGCCGCGATGAGCAGGCG-3'; 5'-<br>AGTCGCGTCTCGGTTCGGTTTCCG-3' | used to confirm Δ*hvo_1864* mutants by PCR; primers anneal both 5’ and 3’ of *hvo_1864* by 0.7 kb | This study |
| HVO_1864-500 HindIII FW<br>HVO_1864-Inverse RV | 5’-TTAAGCTTGAGCACGCTTCCGCCGATG-3’<br>5’-GAGGACGTGCATACCCGAAG-3’ | 0.5-kb probe generated by PCR using pJAM1114 as template; probe used to confirm Δ*hvo_1864* mutants by Southern blot | This study |

| | | | |
|---|---|---|---|
| HVO_1864 NdeI FW<br>(HVO_1864 Internal FW)<br>HVO_1864 KpnI Strep RV<br>(HVO_1864 Internal RV) | 5’-TTCCTTACATATGCACGTCCTCGGAATCGTCGGC-3’<br>5’-CCGGTACCGCGGTCGTGGACCCAGAAC**tc**-3’ | 0.8 kb DNA fragment carrying MoaE (HVO_1864) coding region generated using *H. volcanii* DS70 genomic DNA as template; includes NdeI and KpnI sites for cloning into pJAM816 to generate pJAM1119 encoding MoaE-StrepII; also used to screen for Δ*hvo_1864* mutants | This study |
| HVO_0559 BamHI FW<br>HVO_0559 HindIII RV | 5’-TTGGATCCACGTCCGAACCCGCG-3’<br>5’-CTAAGCTTCCGCGGTCTCACCAACGC-3’ | genomic region including *hvo_0559* and 0.5 kb flanking 5’ and 3’ of this gene generated using *H. volcanii* DS70 genomic DNA as template; includes BamHI and HindIII sites for cloning into pTA131 to generate pJAM1120 | This study |
| HVO_0559 Inverse FW<br>HVO_0559 Inverse RV | 5’-GTCACTTCTGCGGACGCTTTTCGACACC-3’<br>5’-ACCCCACGTGAACGGGCGGA-3’ | *hvo_0559*-knockout plasmid pJAM1127 generated by inverse PCR using pJAM1120 as template | This study |
| HVO_0559 Confirm FW<br>HVO_0559 Confirm RV | 5’-CCGACCGTGCCGGGGAGGACG-3’<br>5’-AAGGCCGGCCGCCGCACGAAG-3’ | used to confirm Δ*hvo_0559* mutants by PCR; primers anneal both 5’ and 3’ of *hvo_0559* by 0.7 kb | This study |
| HVO_0559 BamHI FW<br>HVO_0559-Inverse RV | 5’-TTGGATCCACGTCCGAACCCGCG-3’<br>5’-ACCCCACGTGAACGGGCGGA-3’ | 0.5-kb probe generated by PCR using pJAM1127 as template; probe used to confirm Δ*hvo_0559* mutants by Southern blot | This study |
| qRT*dmsA* FW<br>qRT*dmsA* RV | 5'-CAAGGCTGGGGAAGCGACT-3'<br>5'-CGCTCGTGTACTTGCTCGTGTCGAC-3' | used to detect *dmsA*-specific transcript by RT-PCR | This study |
| tRNA-Lys-UUU probe | 5’-CGGGCTGGGAGGGACTTGAACCCCC-3’ | used as probe for detection of *H. volcanii* $tRNA^{Lys}_{UUU}$ | This study |

[a]SAMP1, HVO_2619; SAMP2, HVO_0202; UbaA, HVO_0558.

[b]Restriction site mutations are underlined; silent mutations that enabled primer optimization are in lowercase and bold.

**Table S3.** Frequency of target gene knockout in *H. volcanii* strains.

| Strain | Genotype | Knockout Frequency |
|---|---|---|
| HM1041 | H26[a] *hvo_2619* | 35.7% (10/28) |
| HM1042 | H26 *hvo_0202* | 2.8% (1/36) |
| HM1052 | H26 *hvo_0558* | 9.4% (12/127) |
| HM1053 | H26 *hvo_1864* | 14.8% (4/27) |
| HM1055 | H26 *hvo_2177* | 66.7% (8/12) |
| HM1067 | HM1042 *hvo_2619* | 23.1% (3/13) |
| HM1088 | H26 *hvo_0559* | 23.1% (3/13) |
| HM1096 | HM1067 *hvo_2177* | 23.1% (3/13) |

[a]Parent strain H26 and its derivatives HM1042 and HM1067 were used as hosts for homologous recombination and deletion of target gene as indicated.

**Supplemental Figure Legends**

**Figure S1.** Multiple amino acid sequence alignment of *H. volcanii* UbaA (HVO_0558) with representative members of the E1/MoeB/ThiF superfamily. Members of the superfamily selected for alignment included: (i) yeast Uba4p required for Ahp1p conjugation and the 2-thiolation of 5-methoxycarbonylmethyl-2-thiouridine ($mcm^5s^2U$) in tRNAs (6), (ii) *Thermus thermophilus* TtuC essential for the synthesis of thiamine, MoCo and the 2-thioribothymidine ($s^2T$) of tRNAs (7), (iii) *Escherichia coli* ThiF of thiamine biosynthesis (8) and (iv) *E. coli* MoeB and human MOCS3 required for MoCo biosynthesis (9). The conserved glycine rich motif related to the NTPase P-loop is indicated. Conserved active site residues required for hydrolysis of ATP and adenylation of the C-terminal carboxyl group of β-grasp proteins are indicated by •. Conserved cysteine residues required for: i) formation of E1-Ub thioester intermediates in protein conjugation and formation of a ThiF-ThiS acyldisulfide in thiamine biosynthesis are indicated by ♦ and ii) coordination of $Zn^{2+}$ for structural integrity are indicated by ▲. Identical and similar amino acid residues are highlighted in black and grey, respectively. Abbreviations: Hvo, *H. volcanii*; Sc, *Saccharomyces cerevisiae;* Ec, *Escherichia coli*; Hs, *Homo sapiens*; Tt, *Thermus thermophilus.* GI numbers for bacterial and eukaryal proteins in this alignment include: 226713013 (Sc_Uba4p), 22001810 (Hs_MOCS3), 215840725 (Tt_TtuC), 89110046 (Ec_ThiF) and 16128794 (Ec_MoeB) where N-terminal domains are indicated by N.

**Figure S2.** *ubaB* encodes a rhodanese domain (RHD) protein and is divergently transcribed from *ubaA*. A. Multiple amino acid sequence alignment of UbaB (HVO_0559) with select RHD proteins. *H. volcanii* is predicted to encode at least 6 RHD-proteins in addition to UbaB (*i.e.*, HVO_1947, HVO_2772, HVO_0024, HVO_0025, HVO_1483 and HVO_1365). Conserved active

site cysteines required for sulfurtransferase activity are highlighted in red and indicated by ♦. Identical and similar amino acid residues are highlighted in black and grey, and C- and N-terminal domains are indicated by C and N, respectively. GI numbers for bacterial and eukaryal RHD proteins in this alignment include: 226713013 (ScUba4p), 22001810 (HsMOCS3), 85675438 (EcSseA) and 62288133 (EcGlpE). B. Schematic representation of the *ubaA* and *ubaB* gene organization in *H. volcanii* and select haloarchaea. Linkage of *ubaA* and *ubaB* in genomic neighborhoods is conserved in *H. volcanii* (HVO), *Halogeometricum borinquense* (Hbor) and *Halorubrum lacusprofundi* (Hlac).

**Figure S3.** Dendrograms of the C-terminal MoaE (A) and N-terminal MobB (B) domains of HVO_1864 with representative proteins. Gene symbols and/or locus tags are indicated. Protein domains were aligned with Clustal W, and Mega 4.0 was used to generate the dendrograms by neighbor-joining and p-distance methods with 1000 bootstrap replicates and pairwise deletion. MobB-MoaE fusion proteins are indicated by •. *H. volcanii* MoaE (HVO_1864) is indicated by **. Abbreviations: B, Bacteria; E, Eukarya; EA, Euryarchaeota; CA, Crenarchaeota; KA, Korarchaeota.

**Figure S4.** Southern blots confirm markerless deletion of target genes in the mutant strains: A, HM1041 (H26 *samp1*) and HM1052 (H26 *ubaA*); B, HM1042 (H26 *samp2*) and HM1053 (H26 *moaE*); C, HM1055 (H26 *hvo_2177*); and D, HM1088 (H26 *ubaB*). 2'-Deoxyuridine-5'-triphosphate coupled by an 11-atom spacer to digoxigenin (DIG-11-dUTP) was used to label the dsDNA probes used for Southern blot as previously described (10). Genomic DNA from parent and mutant strains was cleaved with restriction enzymes as indicated. Molecular masses (kb) of

DIG-labeled DNA standards and bands hybridizing to the DIG-labeled probes are indicated on left and right, respectively.

**Figure S5.** PCR confirmation of markerless deletion of target genes in the mutant strains: A, HM1041 (H26 *samp1*); B, HM1042 (H26 *samp2*); C, HM1055 (H26 *hvo_2177*); D, HM1053 (H26 *moaE*); E, HM1052 (H26 *ubaA*); F, HM1067 (HM1042 *samp1* or H26 *samp2 samp1*); G, HM1096 (HM1067 *hvo_2177* or H26 *samp2 samp1 hvo_2177*); and H, HM1088 (H26 *ubaB*). PCR included reactions using 'internal' primers specific for the coding region of the target gene (left) and 'confirm' primers annealing outside of the genomic region cloned into the plasmids used for homologous recombination and gene knockout (right). PCR product specificity for markerless deletion was confirmed by DNA sequencing. Details on PCR primer sequences used in this analysis are presented in Suppl. Table S2.

PCRs with 'confirm' and 'internal' primer pairs were consistent with in-frame knockout of each target gene with one exception. While 'confirm' PCR (Suppl. Fig. 5E, right) and Southern blot (Suppl. Fig. S4-E) were consistent with knockout of the *ubaA* gene, internal regions of *ubaA* could be PCR-amplified from HM1052 with the primer pairs: (i) HVO_0558 FW and HVO_0558 RV and (ii) HVO_0558 internal FW and HVO_0558 internal RV (see Suppl. Table S2 and Fig. S5-E). However, the PCR products (specific to *ubaA* based on size and DNA sequence) were reproducibly generated at lower levels from the genomic DNA of HM1052 compared to parent H26. To further investigate this finding, H26 was retargeted for *ubaA* knockout, and 11 independently isolated *ubaA* mutant strains were identified by 'confirm' PCR. Like HM1052, all of these *ubaA* mutant strains were positive for internal regions of *ubaA* based on the detection of 'internal' PCR end-products (albeit at low levels). To further address this finding, RT-qPCR

was performed using primers specific for *ubaA* and *ribL* with total RNA isolated from H26 and HM1052 grown aerobically to log-phase in GMM-alanine medium at 42°C. While *ribL*-specific transcripts were at comparable levels in both strains, *ubaA*-specific transcripts were only detected for parent H26. Thus, HM1052 (H26 *ubaA*) was used for this study based on the deletion of *ubaA* from its genomic context in this strain and undetectable levels of *ubaA*-specific transcript generated by this strain.

For RT-qPCR, total RNA (0.1 µg) was used as template to generate cDNA with the iScript cDNA synthesis kit (Bio-Rad). This cDNA served as the template for PCR with iQ SYBR Green Supermix (BioRad) and primer pairs specific for the coding region of *ubaA* and *ribL* (see Table S2). *H. volcanii* 'wild-type' DS70 genomic DNA (0.016, 0.08, 0.4, 2.0 and 10 ng), isolated as previously described (10), served as a standard. Negative controls without the cDNA synthesis step were included to confirm RNA samples were free of genomic DNA contamination. PCR (50 µl) was subjected to 40 amplification cycles: denaturation at 95°C for 30 sec, annealing for 1 min (at 70.5 and 55°C for *ubaA* and *ribL*, respectively) and elongation at 72°C (for 21 and 17 sec for *ubaA* and *ribL*, respectively) using an iCycler (BioRad). All assays were performed in biological triplicate with the means ± standard deviations (SD) calculated.

**Figure S6.** Cell lysate separated by SDS-PAGE and stained with Coomassie blue reveals equal amounts of protein loading for the α-Flag IB presented in Fig. 2. *H. volcanii* strains (indicated above and presented in Fig. 2) were grown aerobically to stationary phase in N-limiting medium at 42°C. Protein loading was determined by $OD_{600}$ of cell culture (0.065 units per lane). Cell lysate was separated by SDS-PAGE separation for immunoblot and total protein stain with Coomassie blue. Strains and/or plasmids in Fig. 6A to D indicated above each lane correspond

to those presented in Fig. 2A to D, respectively. Migration of protein molecular mass standards (kDa) indicated on left. See Materials and Methods for details.

**Figure S7.** Site-directed variant UbaA C188S and UbaA proteins are synthesized at comparable levels in *H. volcanii* parent H26 (wt) and *ubaA* mutant strain HM1052 (*ΔubaA*). UbaA and UbaA C188S proteins were synthesized with C-terminal StrepII tags from cells grown aerobically to stationary phase in GMM-Ala (200 rpm, 42°C). Cell lysate was separated by 12% SDS-PAGE and analyzed by α-StrepII immunoblot as described in Materials and Methods. Appropriate strains indicated above each lane. Migration of protein molecular mass standards (kDa) indicated on right.

**Figure S8.** Mutant strains of this study were not highly impaired in either growth rate or cell yield under standard aerobic conditions (ATCC 974 complex medium at 42°C, 200 rpm). *H. volcanii* strains (indicated on right) were grown three times to log phase in 2-ml medium in 13 × 100 mm tubes and used as an inoculum (to an $OD_{600}$ of 0.01-0.02) for final analysis of growth rate and cell yield in 20-ml medium in 250-ml baffled Erlenmeyer flasks. Growth was monitored over time by an increase in $OD_{600}$.

**Figure S9.** UbaB is not required for anaerobic growth with DMSO as the terminal electron acceptor. *H. volcanii* strains (indicated on right) were grown anaerobically on rich medium (YPC) supplemented with glucose and DMSO (at 42°C) as described in Materials and Methods. Growth was monitored over time by an increase in $OD_{600}$.

**Figure S10.** *dmsA*-specific transcript, encoding the catalytic subunit of DMSO reductase, is present in parent as well as *ubaA* and *samp1* mutant strains. Strains (indicated on top) were

grown to log-phase in rich medium (YPC) under aerobic conditions (200 rpm), supplemented with glucose and DMSO and incubated overnight at 42°C as described in Materials and Methods. RT-PCR analysis was similar to RT-qPCR described above (Suppl. Fig. S5) with the following exception: PCRs were with Phusion DNA polymerase, unlabeled deoxyribonucleotide triphosphates (dNTPs) and primer pairs specific for the coding region of *dmsA* (see Table S2 for primers). PCR was subjected to 40 amplification cycles: denaturation at 95°C for 30 sec, annealing for 1 min (at 63.2°C) and elongation at 72°C (14 sec) using an iCycler (BioRad). Products were separated by 2% (w/v) agarose gel electrophoresis in TAE buffer and stained with ethidium bromide. Negative controls without the cDNA synthesis step were included to confirm RNA samples were free of genomic DNA contamination.

**Figure S11.** SAMP2-conjugate levels are increased in rich medium during growth on DMSO compared to growth in the presence of oxygen. 'Wild type' cells expressing either Flag-SAMP1 or Flag-SAMP2 (H26-pJAM947 and H26-pJAM949) were grown at 42°C to stationary phase in rich medium (YPC) supplemented with 2% (w/v) glucose in the presence of either oxygen (200 rpm) or 100 mM DMSO as indicated. Cells were harvested and analyzed by α-Flag IB (A) with equivalent protein loading confirmed by staining parallel gels for total protein with Coomassie Blue (B) as described in Materials and Methods. Migration of protein molecular mass standards (kDa) indicated on left. Protein loading was determined by $OD_{600}$ of cell culture (0.065 units per lane). Based on Coomassie Blue staining, the total protein loaded per lane was comparable; however, significant differences were observed in the banding pattern of proteins that correlated with the type of terminal electron acceptor available.

**Figure S12.** UbaA and SAMP2 are required for wild type growth at high temperature (50°C). *H. volcanii* strains (indicated on right) were grown thrice in ATCC 974 medium to log-phase (2-ml in 13 x 100 mm tubes at 42°C, 200 rpm). Cells were either: (A) diluted to 0.1 $OD_{600}$, plated on ATCC 974 medium in serial dilutions (as indicated above) and incubated at 42°C or 50°C (as indicated below), or (B) inoculated into ATCC 974 medium (20 ml in 250-ml baffled Erlenmeyer flasks) to a final $OD_{600}$ of 0.01 to 0.02 for analysis of growth rate and cell yield at 50˚C (200 rpm). Similar analysis was performed in liquid culture at 42˚C with no detectable differences in growth rate or cell yield between strains (see Suppl. Fig. S8).

## References for Supplemental Data


1. Allers T, Ngo HP, Mevarech M, Lloyd RG (2004) Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. *Appl Environ Microbiol* 70: 943-953.

2. Zhou G, Kowalczyk D, Humbard MA, Rohatgi S, Maupin-Furlow JA (2008) Proteasomal components required for cell growth and stress responses in the haloarchaeon *Haloferax volcanii*. *J Bacteriol* 190: 8096-8105.

3. Humbard MA, Zhou G, Maupin-Furlow JA (2009) The N-terminal penultimate residue of 20S proteasome a1 influences its $N^a$-acetylation and protein levels as well as growth rate and stress responses of *Haloferax volcanii*. *J Bacteriol* 191: 3794-3803.

4. Humbard MA, Miranda HV, Lim JM, Krause DJ, Pritz JR, Zhou G, Chen S, Wells L, Maupin-Furlow JA (2010) Ubiquitin-like small archaeal modifier proteins (SAMPs) in *Haloferax volcanii*. *Nature* 463: 54-60.

5. Hartman AL, Norais C, Badger JH, Delmas S, Haldenby S, Madupu R, Robinson J, Khouri H, Ren Q, Lowe TM *et al.* (2010) The complete genome sequence of *Haloferax volcanii* DS2, a model archaeon. *PLoS One* 5: e9605.

6. Pedrioli PG, Leidel S, Hofmann K (2008) Urm1 at the crossroad of modifications. 'Protein Modifications: Beyond the Usual Suspects' Review Series. *EMBO Rep* 9: 1196-1202.

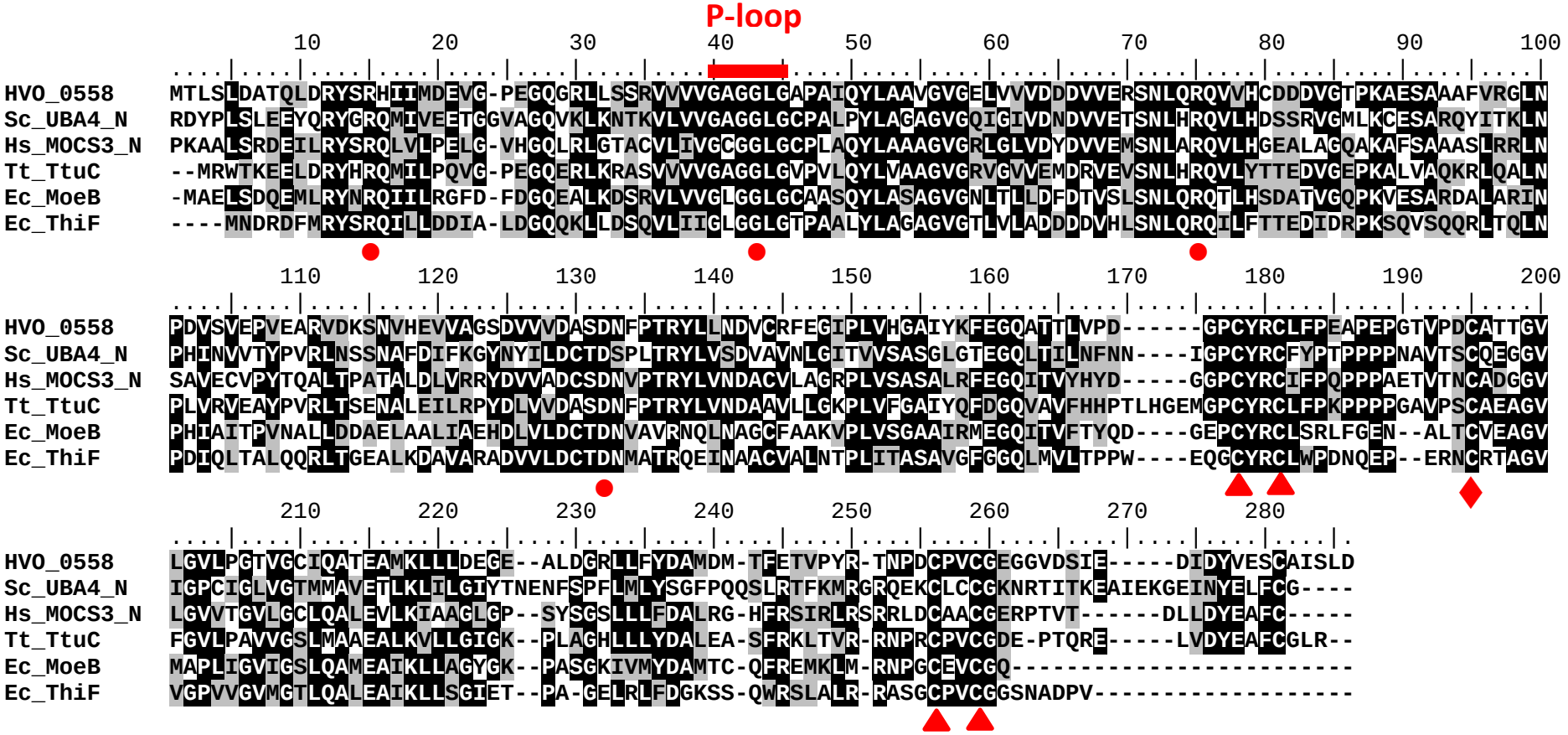
7. Shigi N, Sakaguchi Y, Asai S, Suzuki T, Watanabe K (2008) Common thiolation mechanism in the biosynthesis of tRNA thiouridine and sulphur-containing cofactors. *EMBO J* 27: 3267-3278.

8. Jurgenson CT, Begley TP, Ealick SE (2009) The structural and biochemical foundations of thiamin biosynthesis. *Annu Rev Biochem* 78: 569-603.

9. Schwarz G, Mendel RR, Ribbe MW (2009) Molybdenum cofactors, enzymes and pathways. *Nature* 460: 839-847.

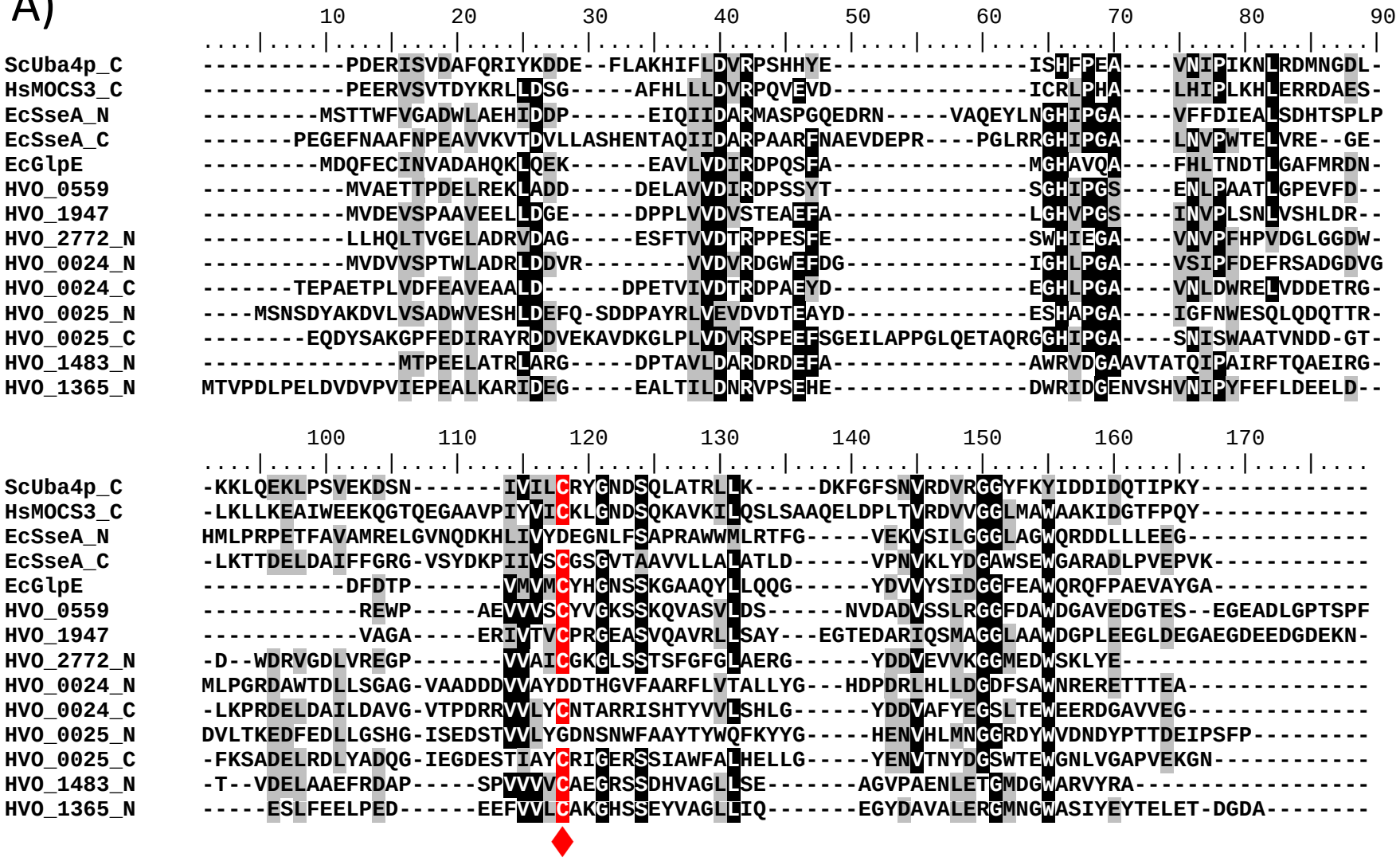
10. Rawls KS, Yacovone SK, Maupin-Furlow JA (2010) GlpR represses fructose and glucose metabolic enzymes at the level of transcription in the haloarchaeon *Haloferax volcanii*. *J Bacteriol* 192: 6251-6260.

Suppl. Figure S1.



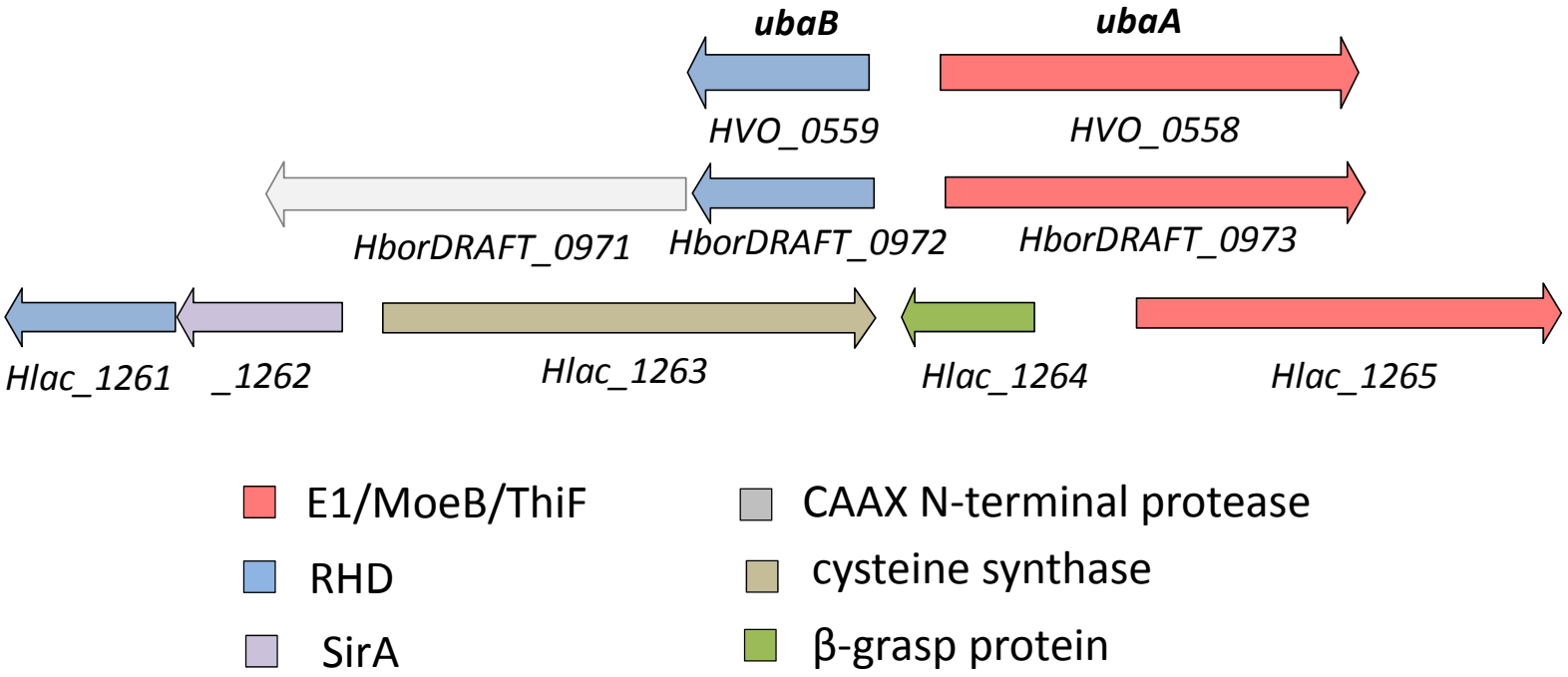
Suppl. Figure S2.

A)



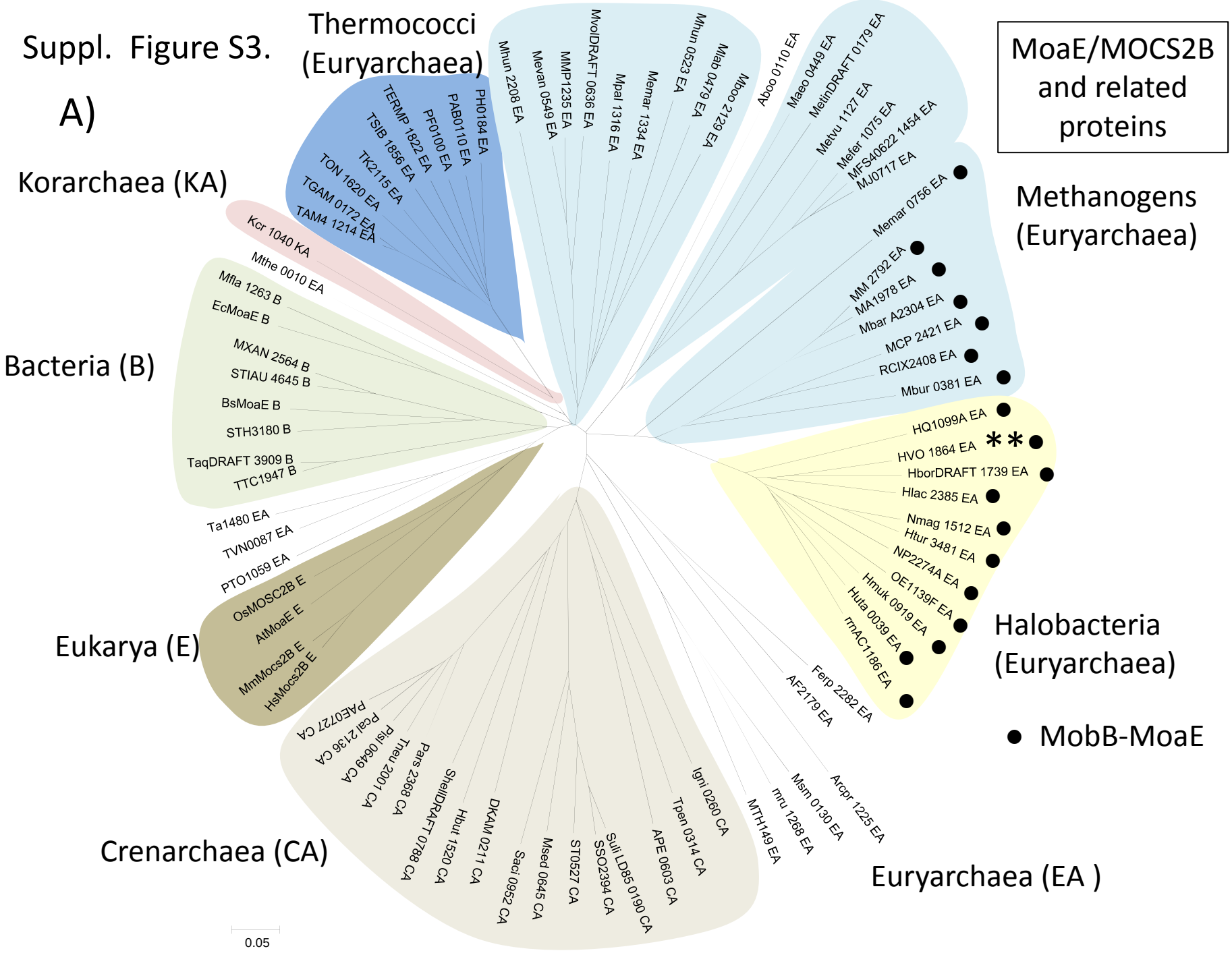
Suppl. Figure S2.

B)



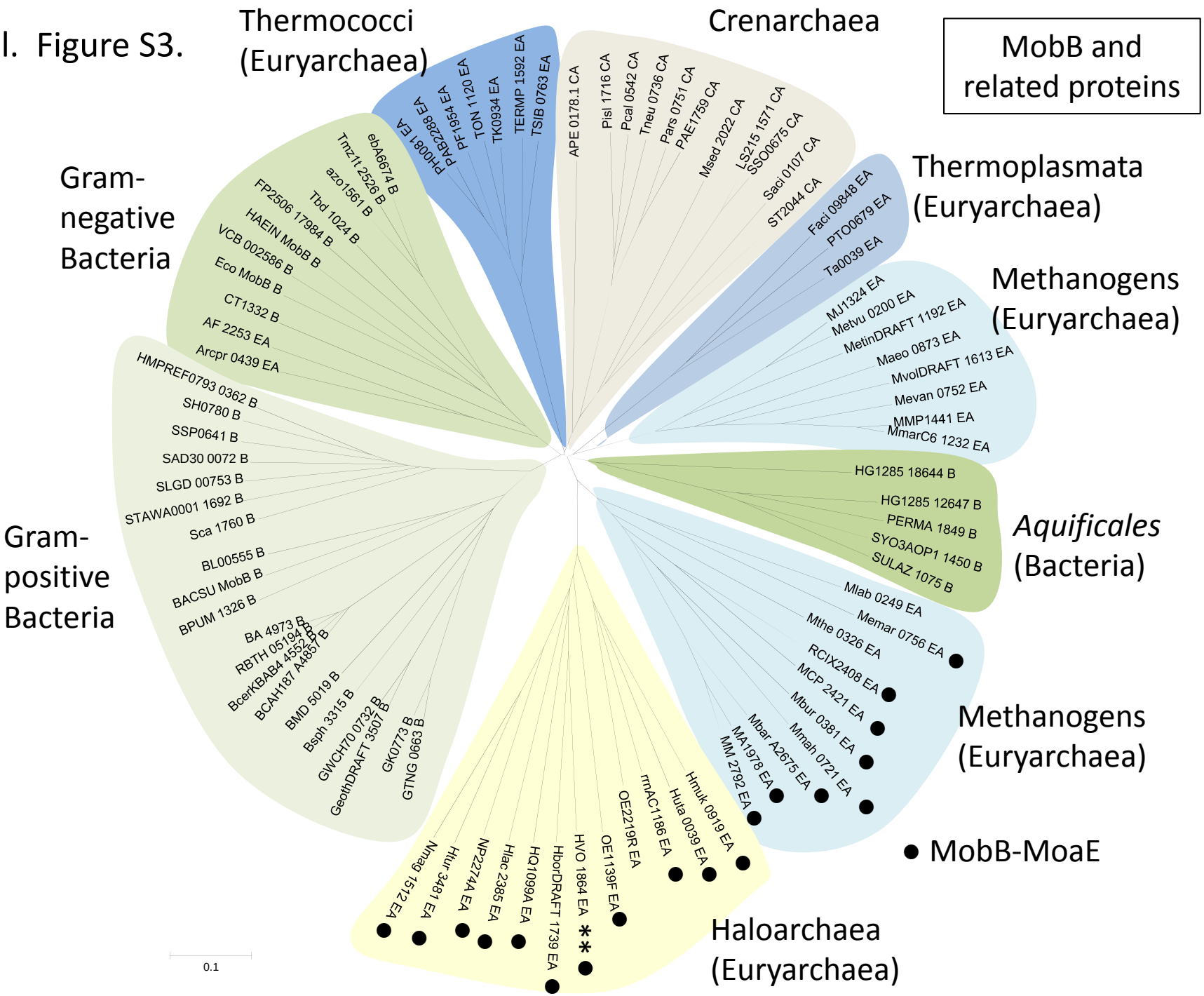
Suppl. Figure S3.

A)

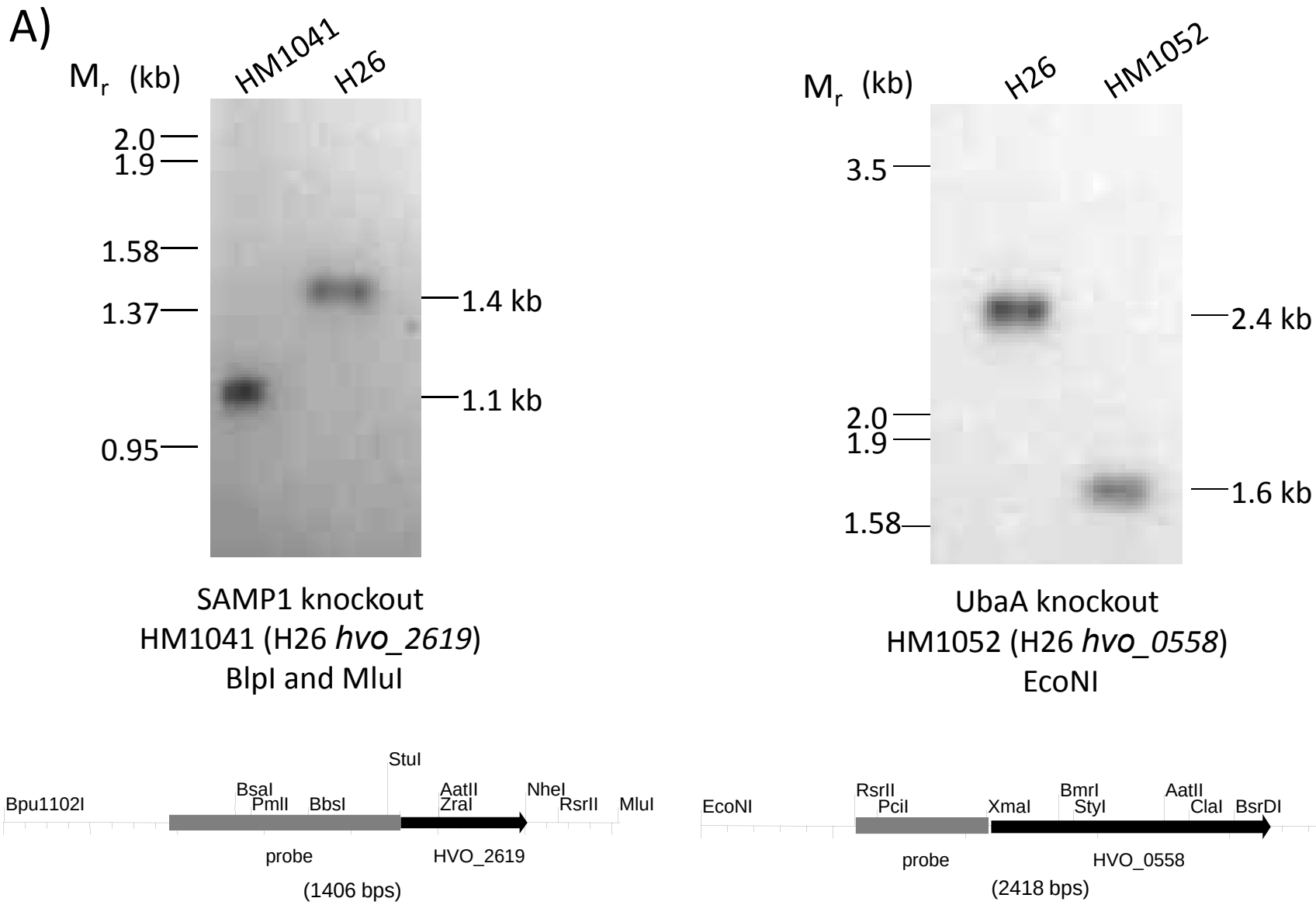


Suppl. Figure S3.

B)

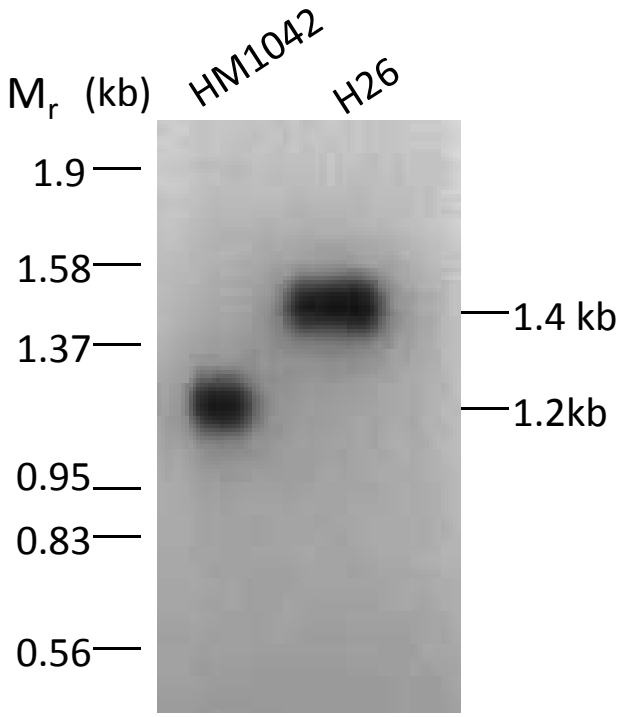


Suppl. Figure S4.

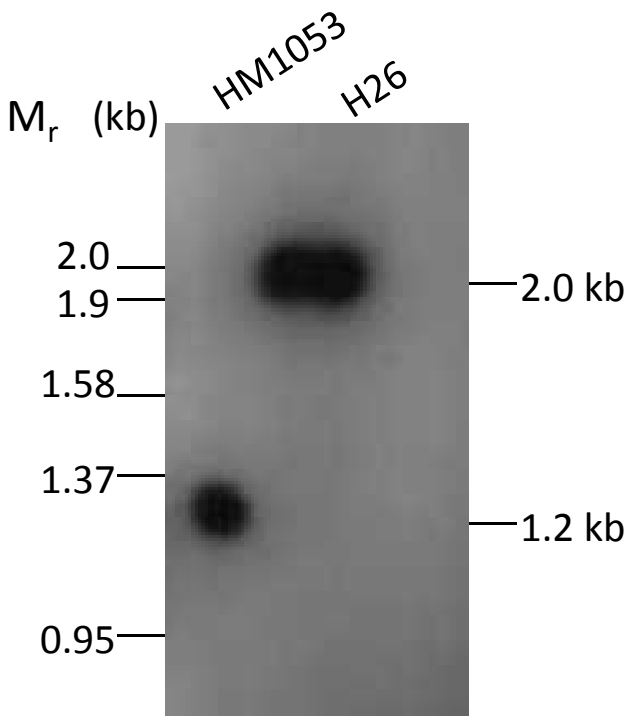


Suppl. Figure S4.

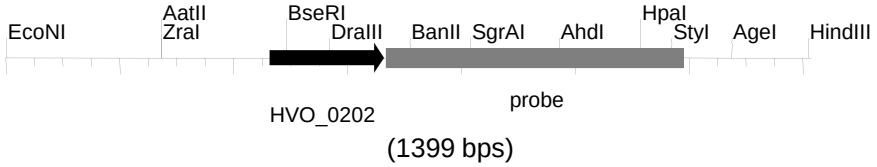
B)



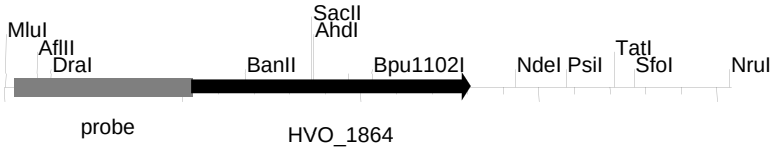

SAMP2 knockout
HM1042 (H26 *hvo_0202*)
EcoNI and HindIII




MoaE knockout
HM1053 (H26 *hvo_1864*)
NruI and MluI



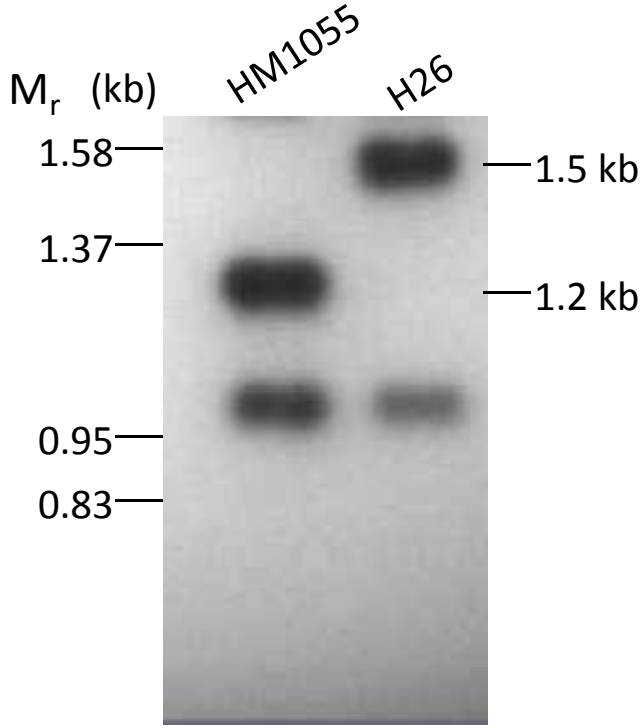

(1399 bps)



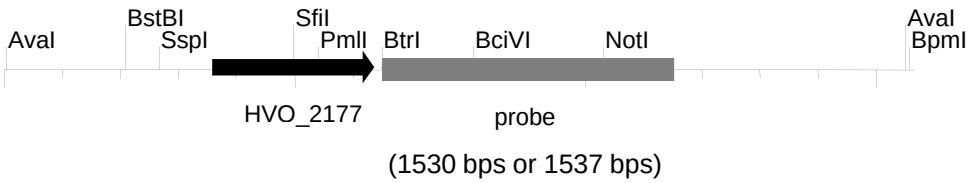

(2032 bps)

Suppl. Figure S4.

C)



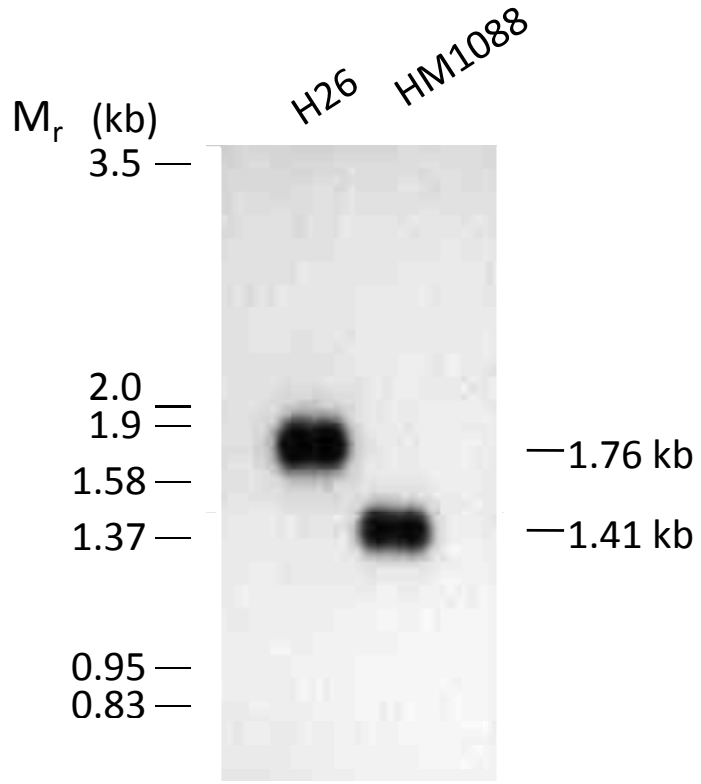

Ubl β-grasp knockout
HM1055 (H26 *hvo_2177*)
BpmI and AvaI



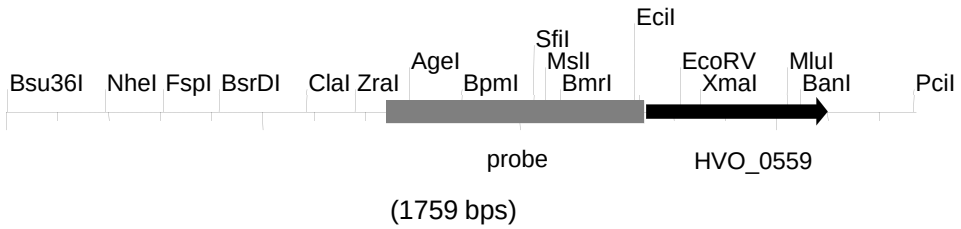

(1530 bps or 1537 bps)

Suppl. Figure S4.

D)

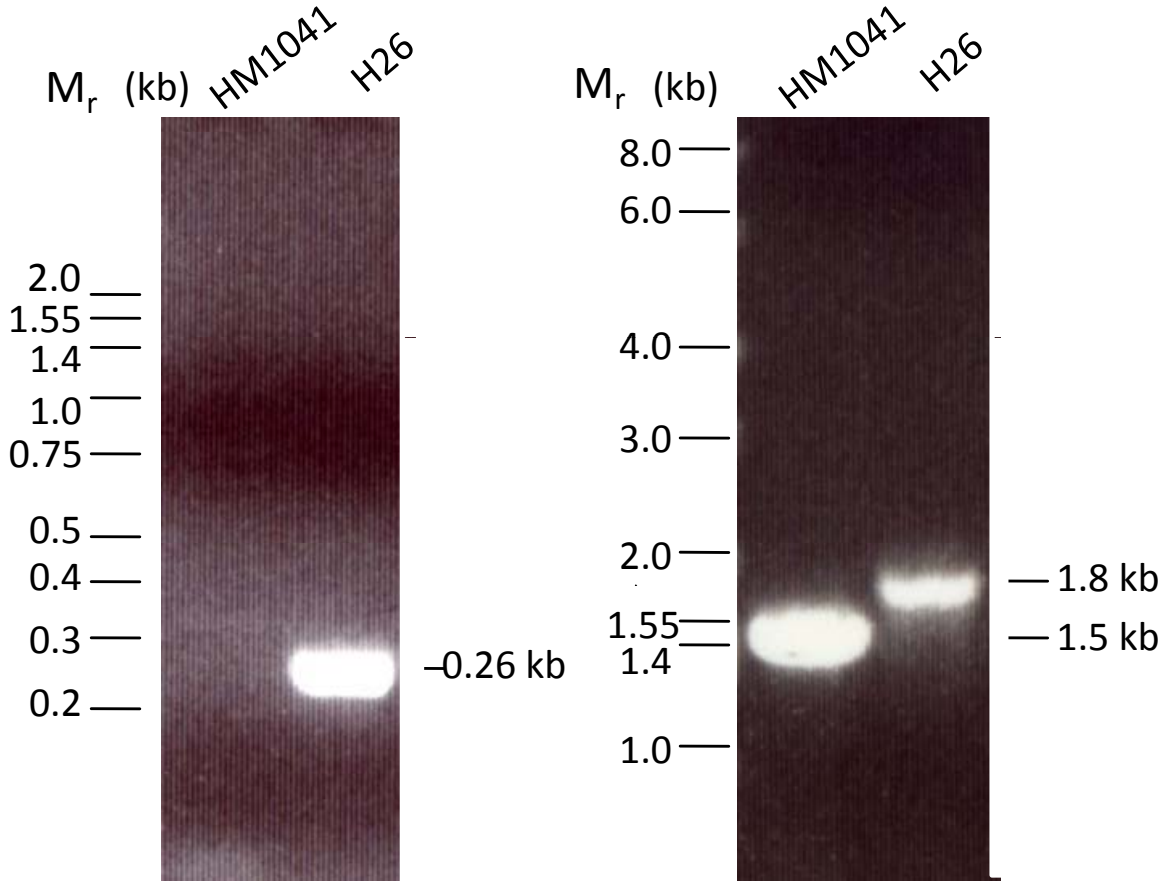



UbaB knockout
HM1088 (H26 *hvo_0559*)
Bsu361 and PciI



Suppl. Figure S5.

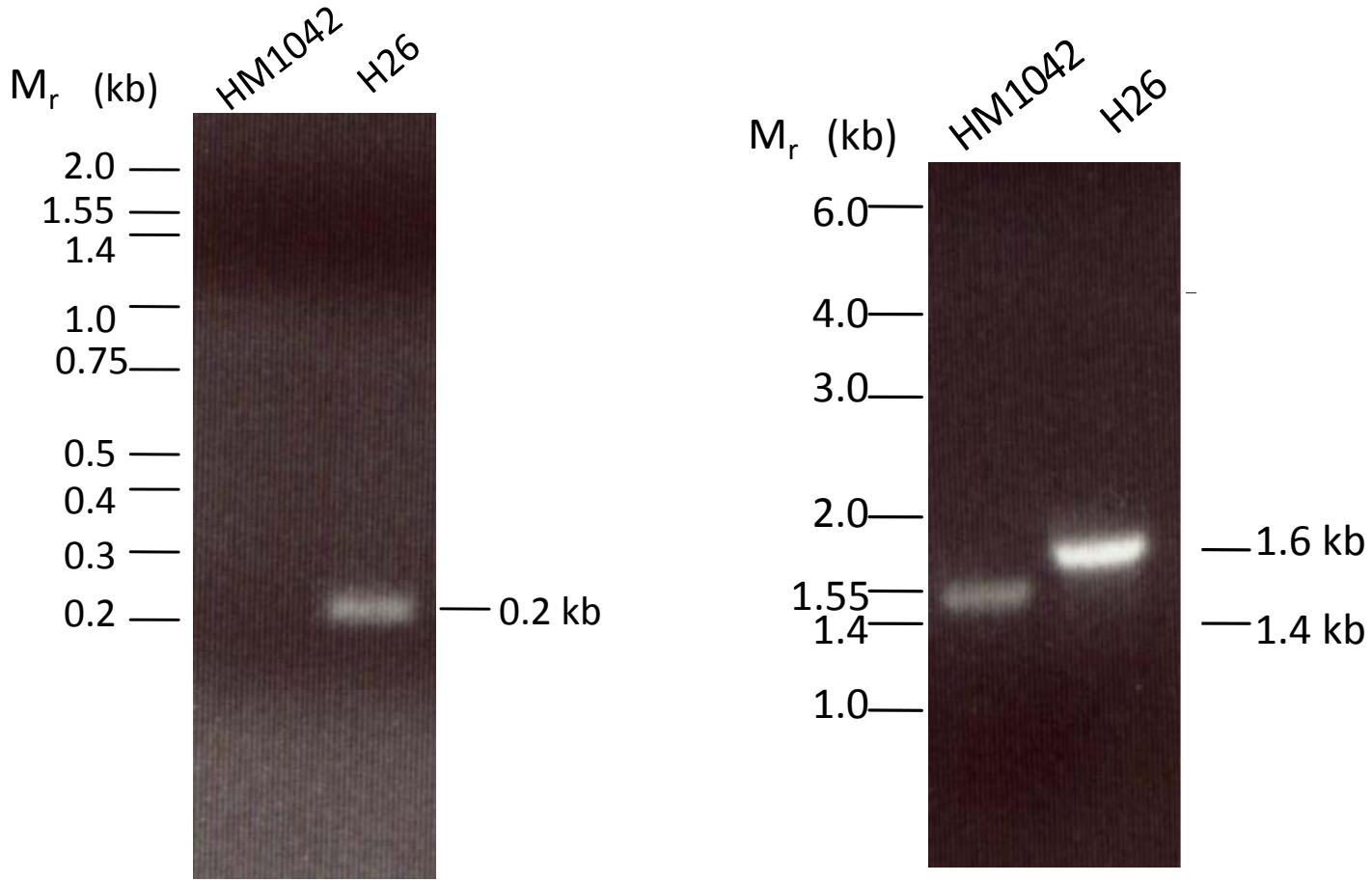
A)




SAMP1 knockout
HM1041 (H26 *hvo_2619*)

Suppl. Figure S5.

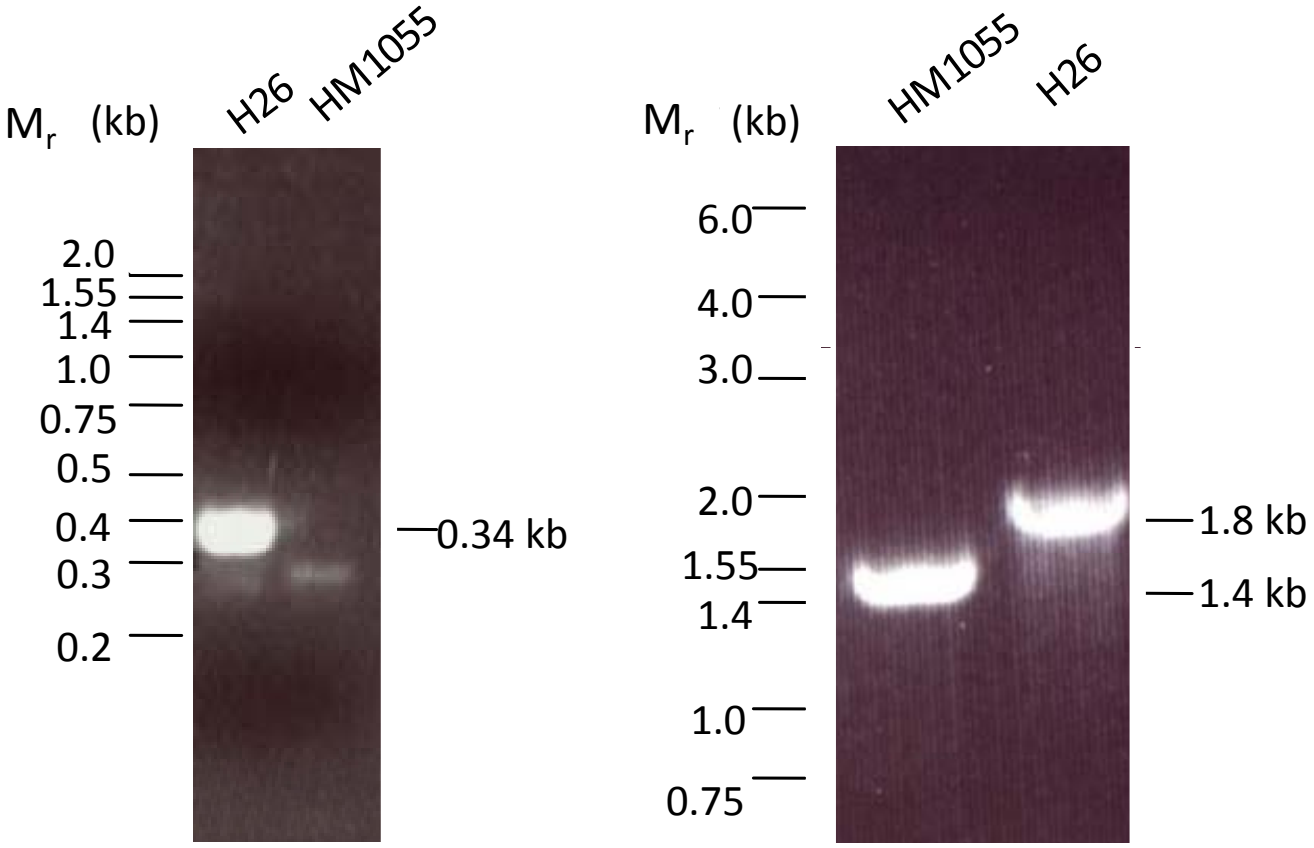
B)




SAMP2 knockout
HM1042 (H26 *hvo_0202*)

Suppl. Figure S5.

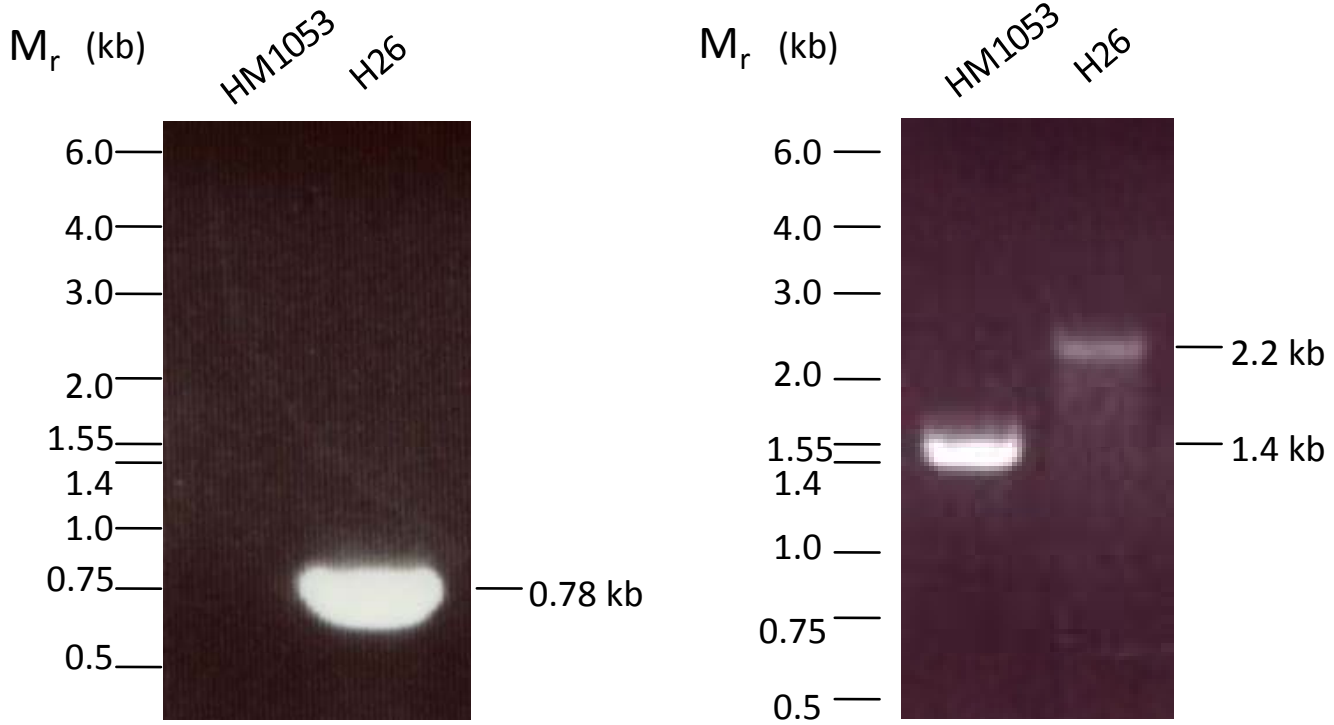
C)




Ubl β-grasp HVO_2177 knockout
HM1055 (H26 *hvo_2177*)

Suppl. Figure S5.

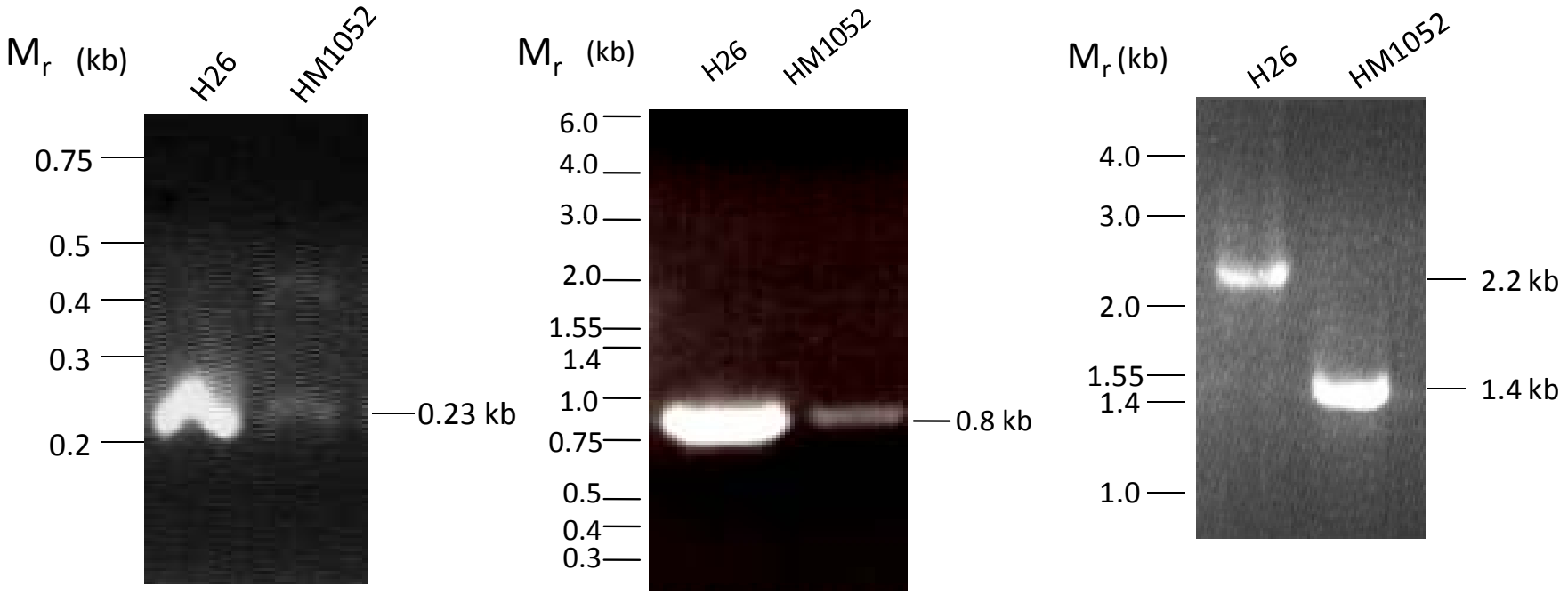
D)




MoaE knockout
HM1053 (H26 *hvo_1864*)

Suppl. Figure S5.

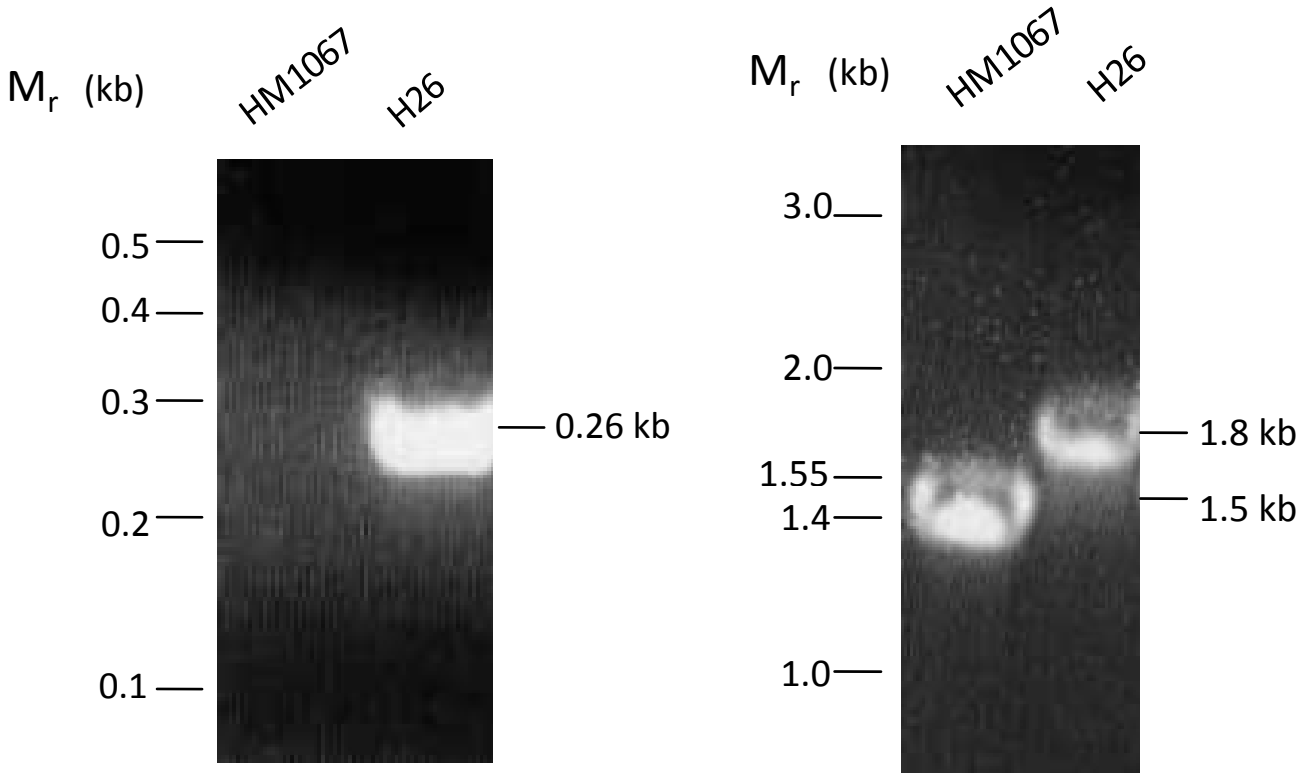
E)




UbaA knockout
HM1052 (H26 *hvo_0558*)

Suppl. Figure S5.

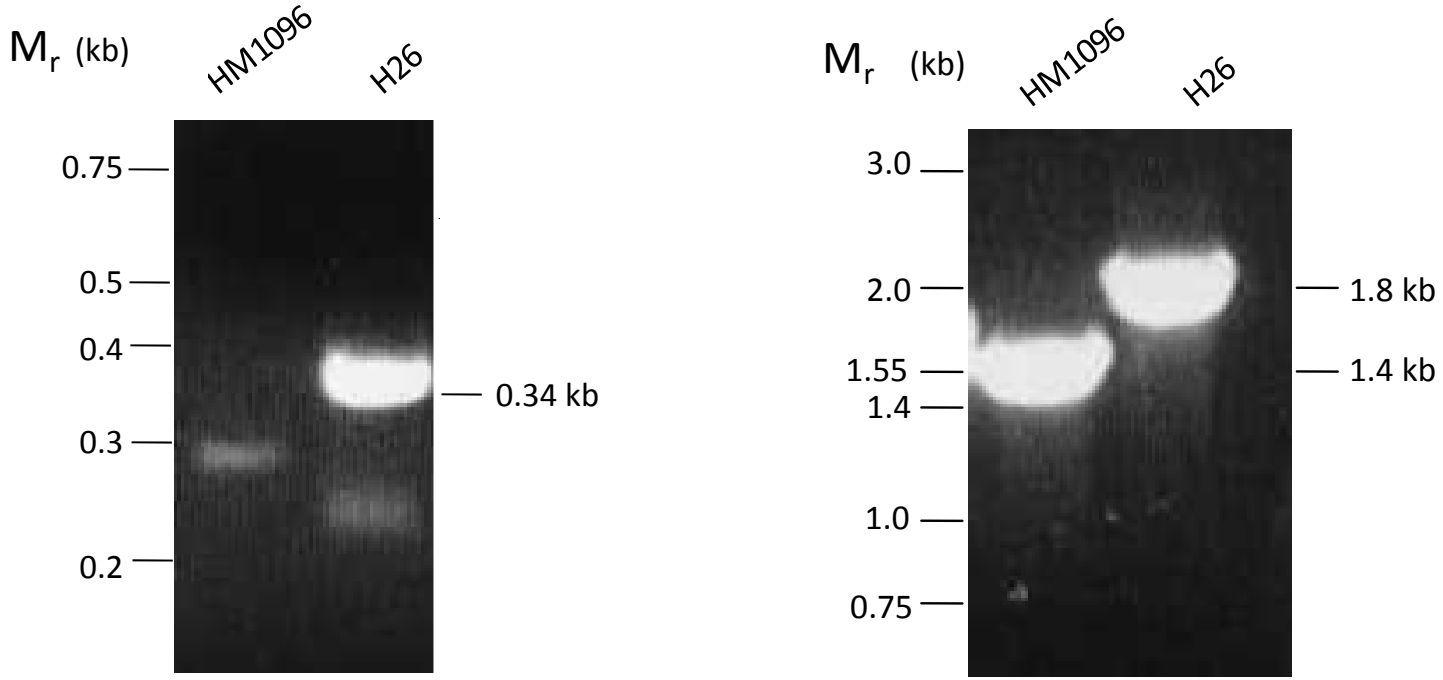
F)




SAMP1 and SAMP2 double knockout
HM1067 (HM1042 *hvo_2619*)

Suppl. Figure S5.

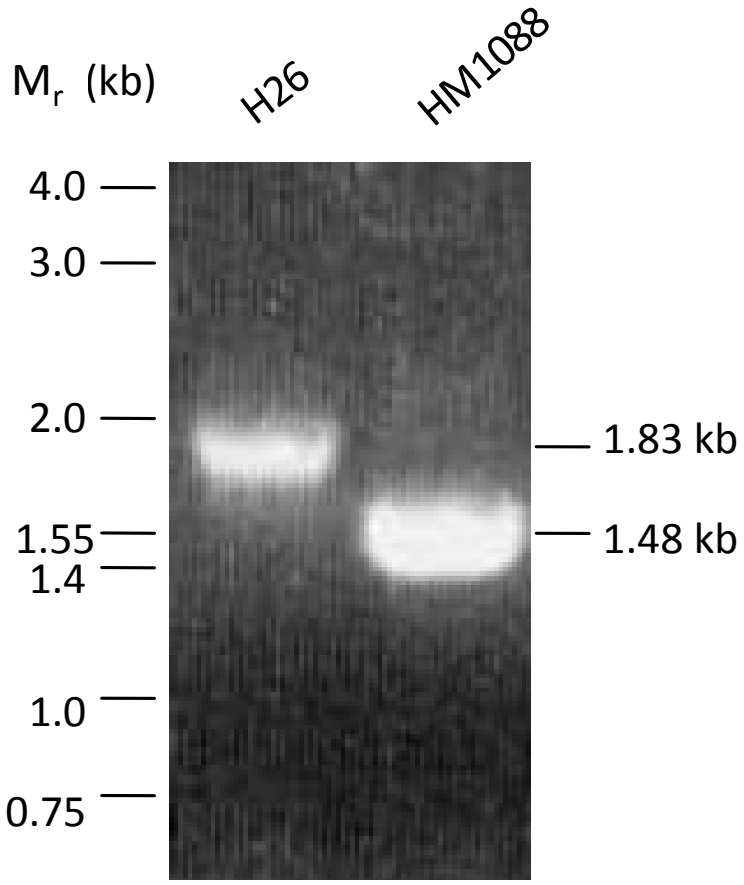
G)




SAMP1, SAMP2 and Ubl β-grasp HVO_2177 triple knockout
HM1096 (HM1067 *hvo_2177*)

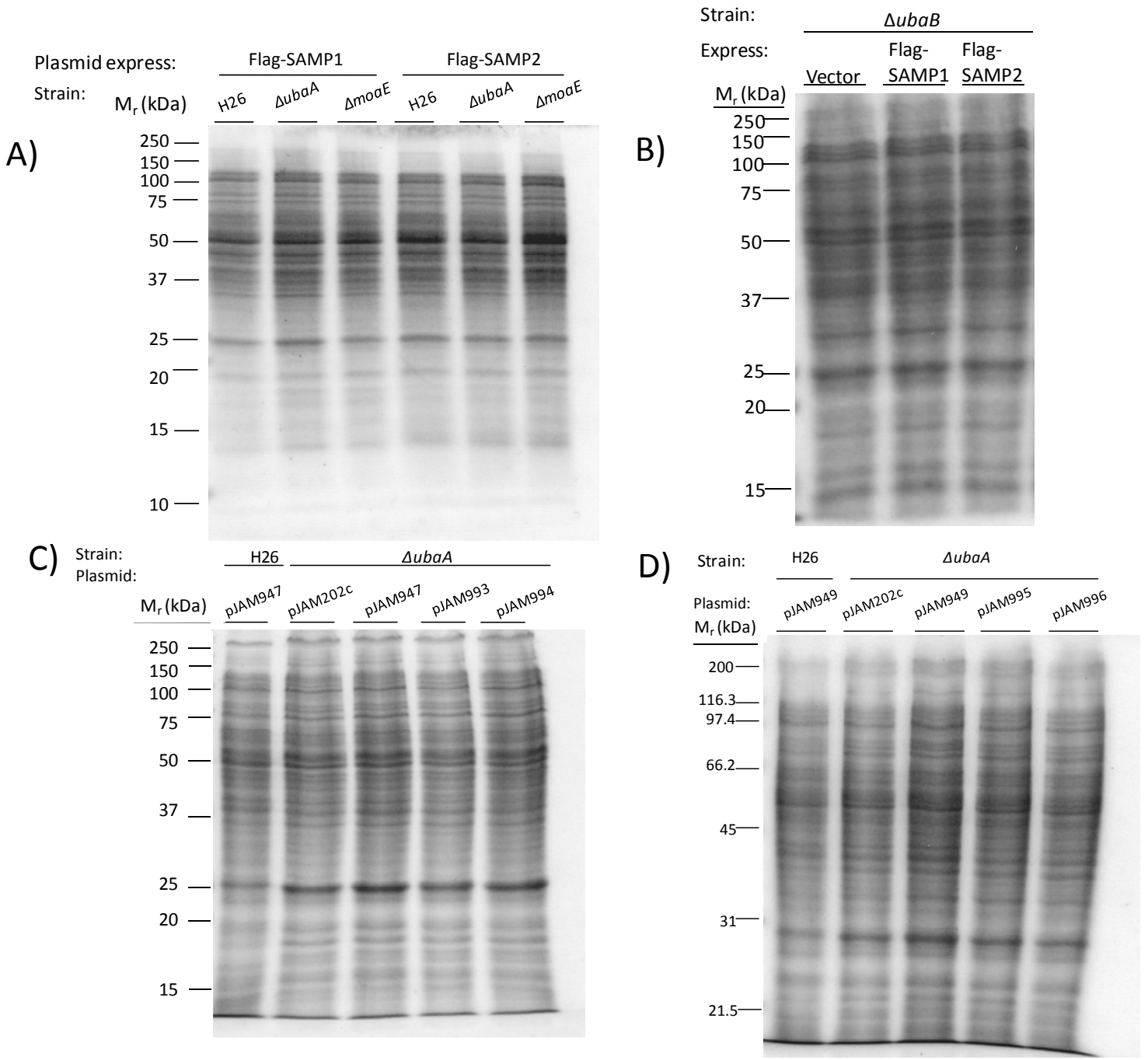
Suppl. Figure S5.

H)



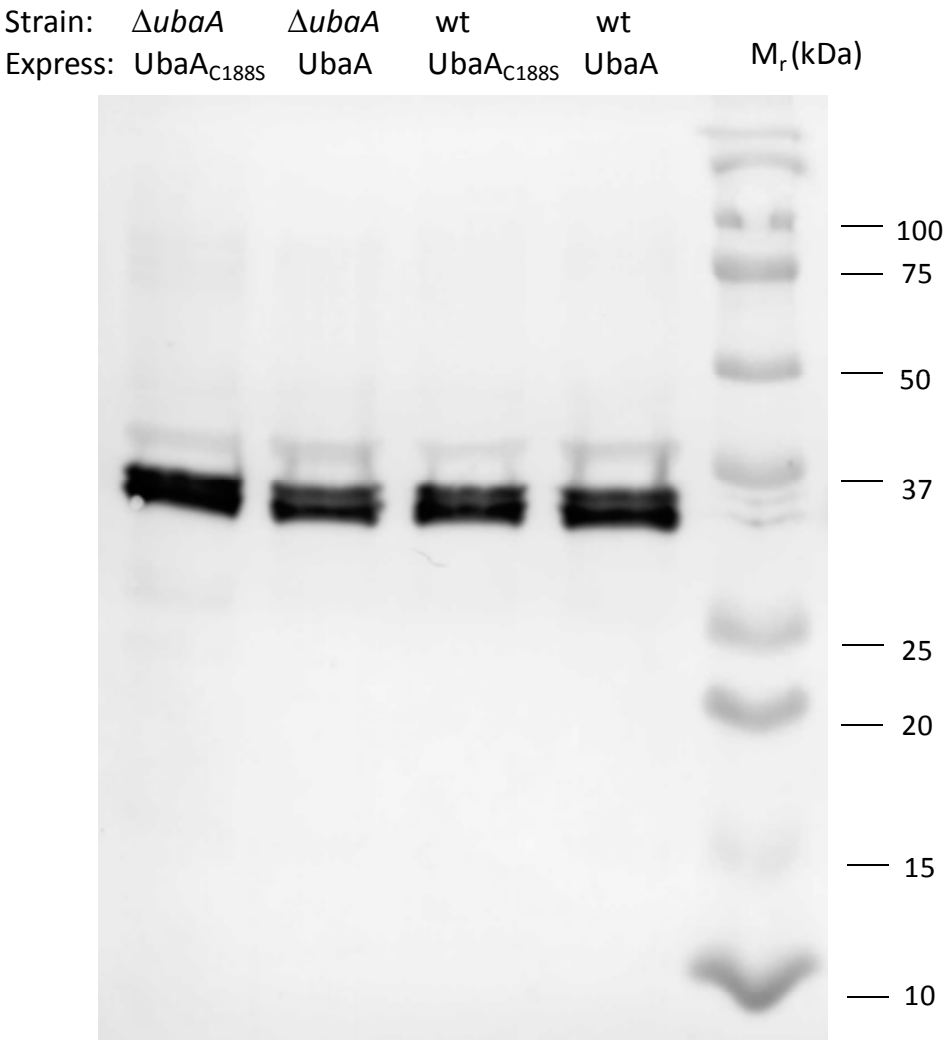

UbaB knockout
HM1088 (H26 *hvo_0559*)

Suppl.
Figure S6.



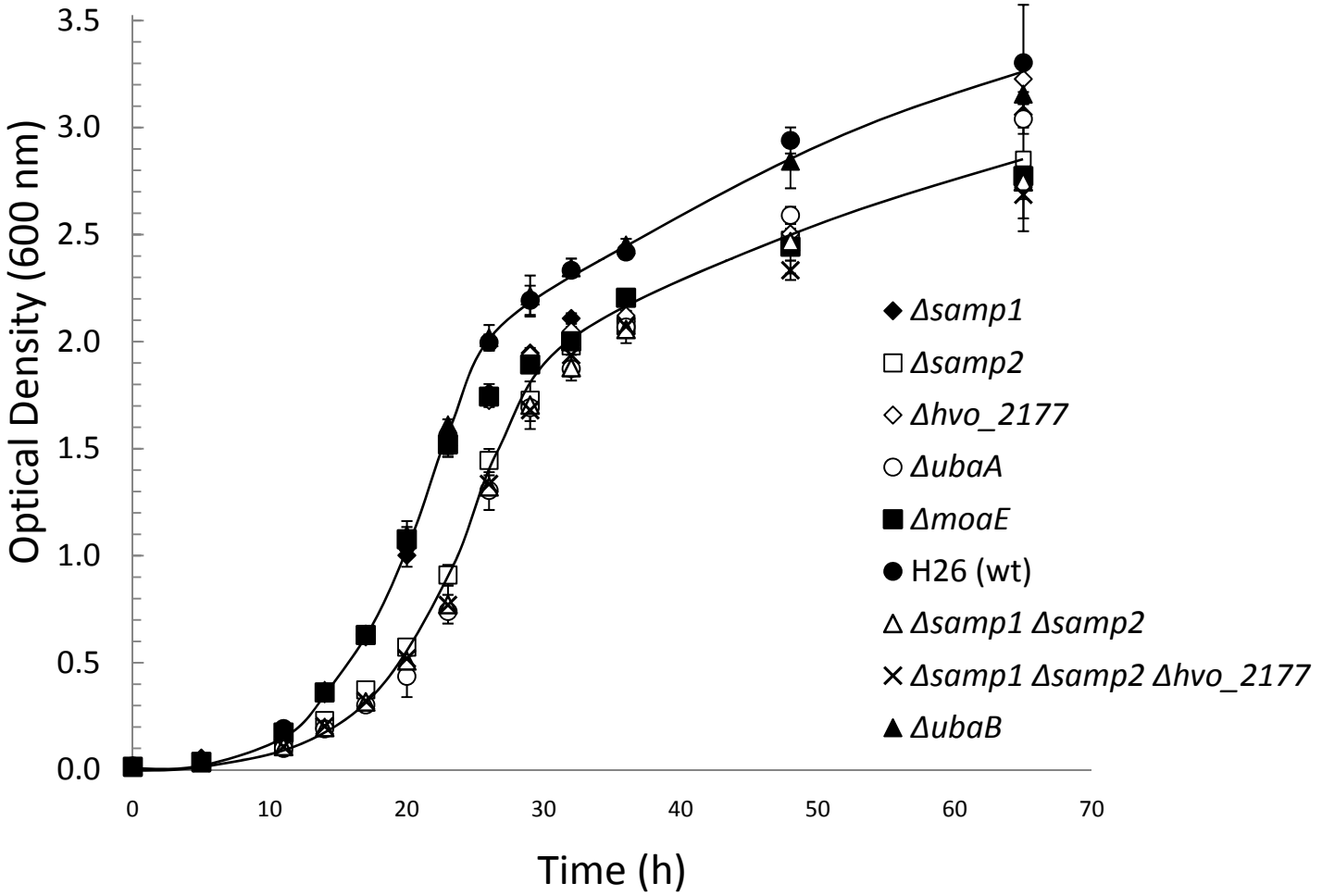

Coomassie Blue

Suppl. Figure S7.

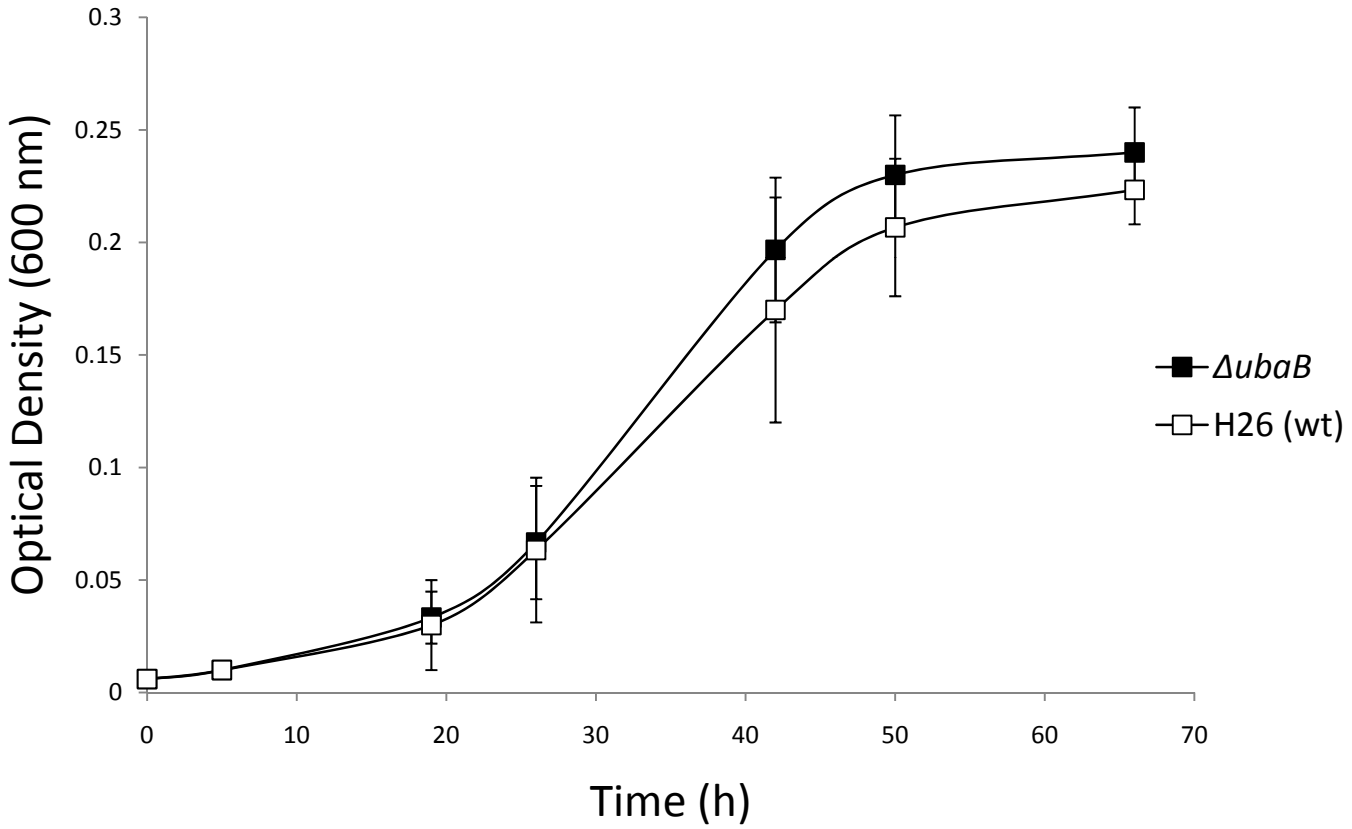



α-StrepII immunoblot

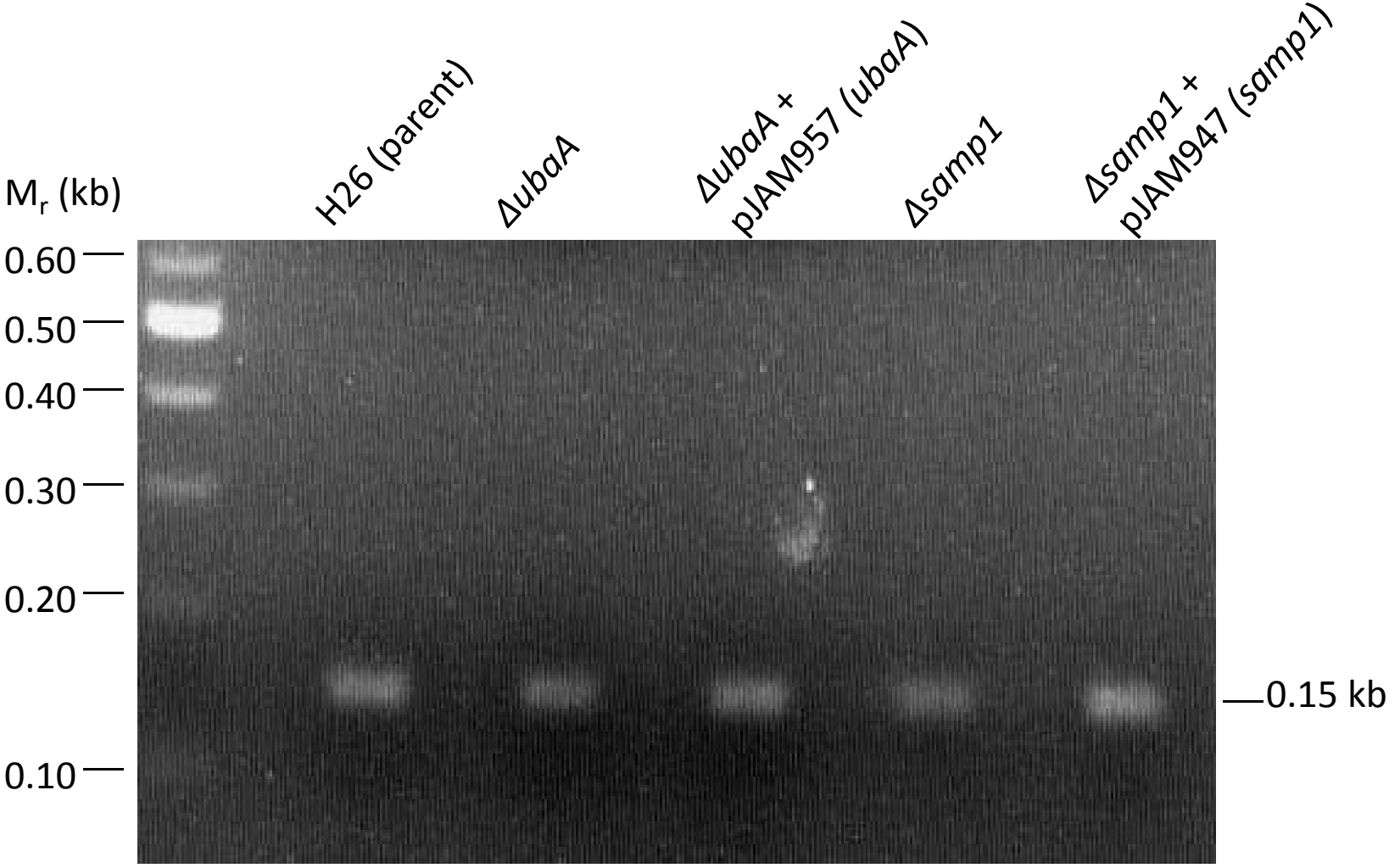
Suppl. Figure S8.



Suppl. Figure S9.

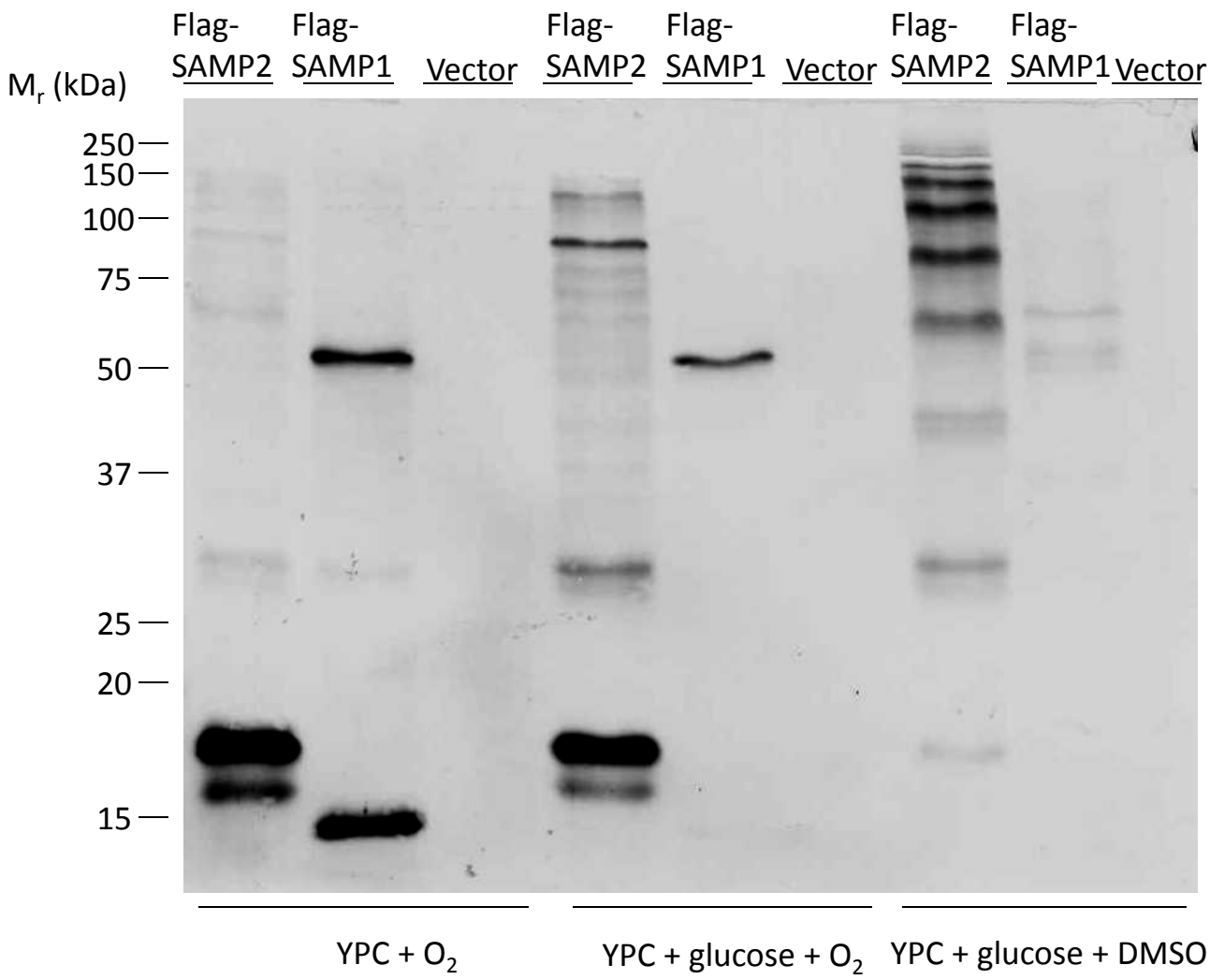


Suppl. Figure S10.



Suppl. Figure S11.

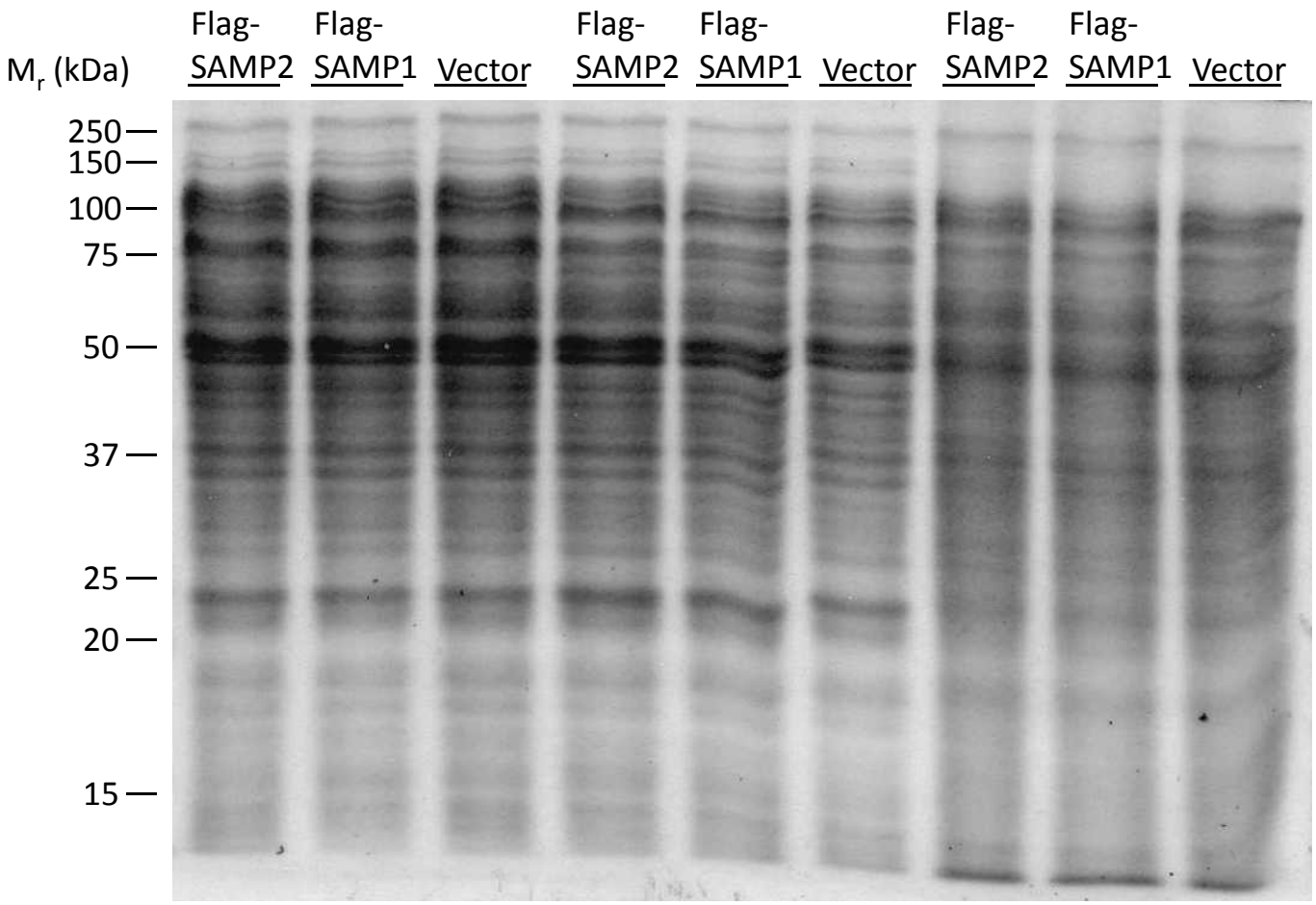
A)




α-Flag immunoblot

Suppl. Figure S11.

B)




Coomassie Blue

Suppl. Fig. S12.