

Figure S1. Proposed central metabolic pathways in *T. maritima* depicting ATP formation and hydrogen formation. **GK-** Glucose kinase, **PGI-** Phosphoglucose isomerase, **PFK-** Phosphofructokinase, **FBA-** Fructose-1,6-bisphosphate aldolase, **TIM-** Triose-phosphate isomerase, **G6PDH-** Glucose-6-phosphate dehydrogenase, **ilvD-** Phosphogluconate dehydratase, **gnd-** 6-phosphogluconate dehydrogenase, **KDG-** 2-keto-3-deoxygluconate, **KDPG-** 2-Keto-3- deoxy-6-phosphogluconate, **GAP-** Glycerldehyde -3-phosphate, **1,3 BPG-** 1,3- bisphosphoglycerate, **GAPDH-**Glyceraldehyde-3phosphate dehydrogenase, **H₂ase-** Hydrogenase, **Ldh-** lactate dehydrogenase, **PFOR-** Pyruvate Ferredoxin oxidoreductase, **Pta-**Phosphate acetyltransferase, **AckA-** Acetate kinase.

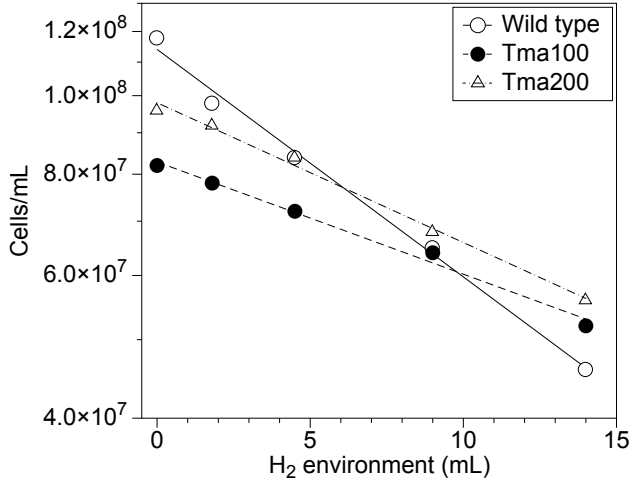


Figure S2. Hydrogen resistance in various strains of *T. maritima*. Tubes inoculated with 4×10^6 cell of wild type, Tma100 and Tma200 were injected with varying amounts of H₂ (mL) in the headspace without any positive pressure. Tubes were incubated at 80°C for 24h.

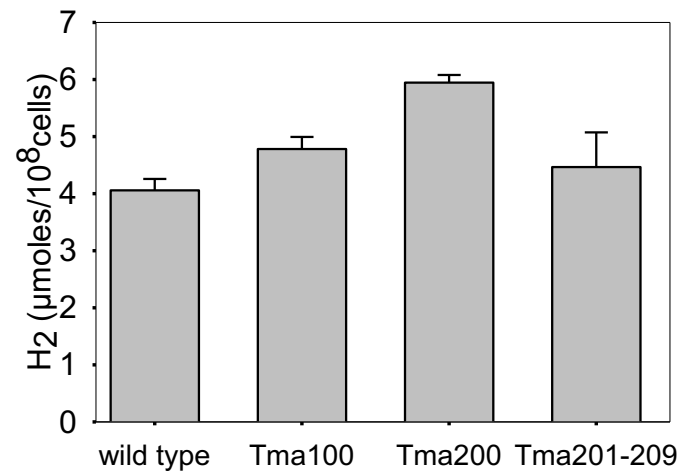


Figure S3. Comparison of H₂ production by Tma100 and Tma200 in batch cultures. Hydrogen was normalized to 10⁸ cell/mL for all three strains. The error bars represent the standard deviations from two biological replicates.

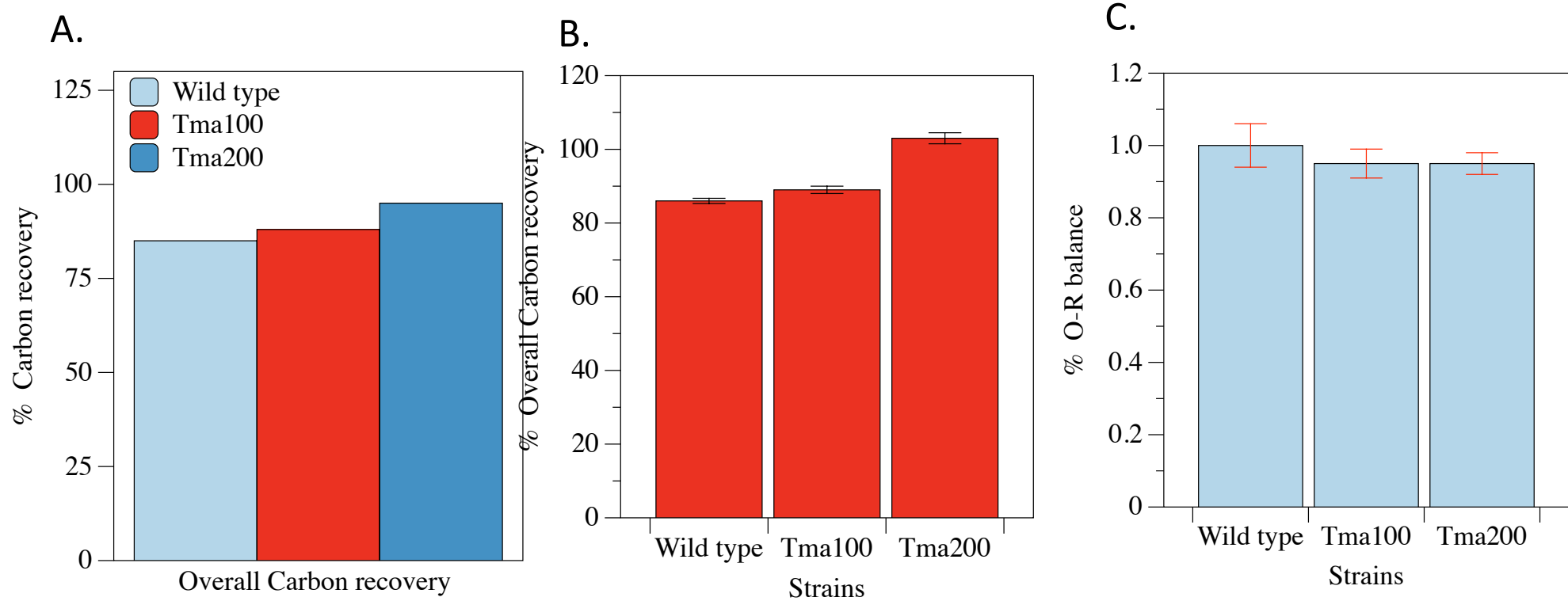


Figure S4. Carbon recovery. Carbon recovery in 30th hour of fermentation (panel A). The overall carbon recovery calculated as the ratio of maltose consumed to products (acetate, lactate, biomass and CO₂) formed (panel B). Oxidation reduction (O-R) balance (panel C)

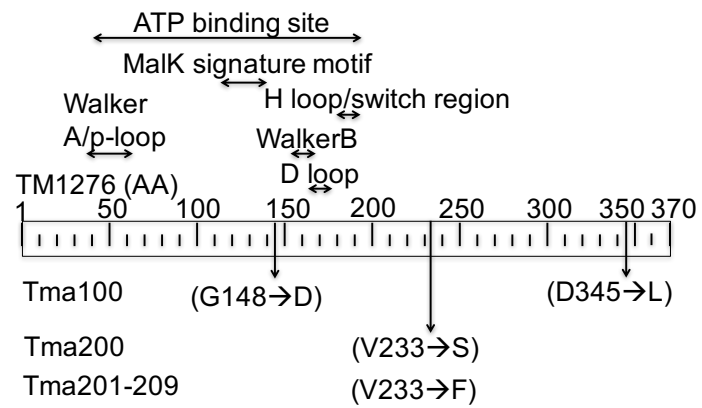


Figure S5. Domain structure and mutations of *malK3*. The diagram shows the features and predicted domains of *malK3*.

Substitution mutations are indicated by vertical arrows for respective strains along with their corresponding coordinates.

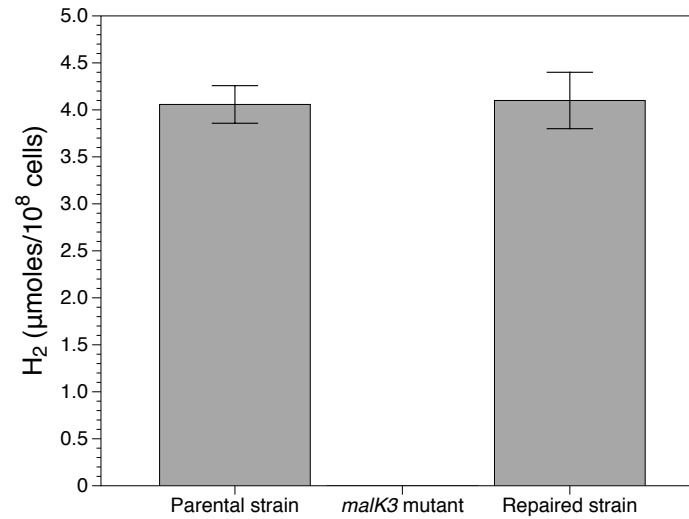


Figure S6. Hydrogen production from a fully functional *malk3* strain and a strain with a complete loss of function mutation in the *malk3* gene. All strains were grown in defined medium and incubated at 80°C for 24h and H₂ analysis was performed by GC. The error bars represent the standard deviations from two biological replicates.

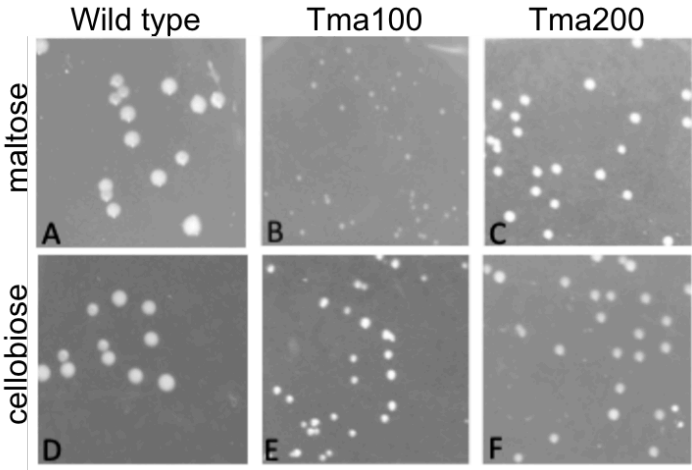


Figure S7. Colony size variation in *T. maritima* strains. Strains were grown on complex medium plates supplemented with 0.1% maltose (panel A, B and C) or 0.1% cellobiose (panel D, E and F), respectively.

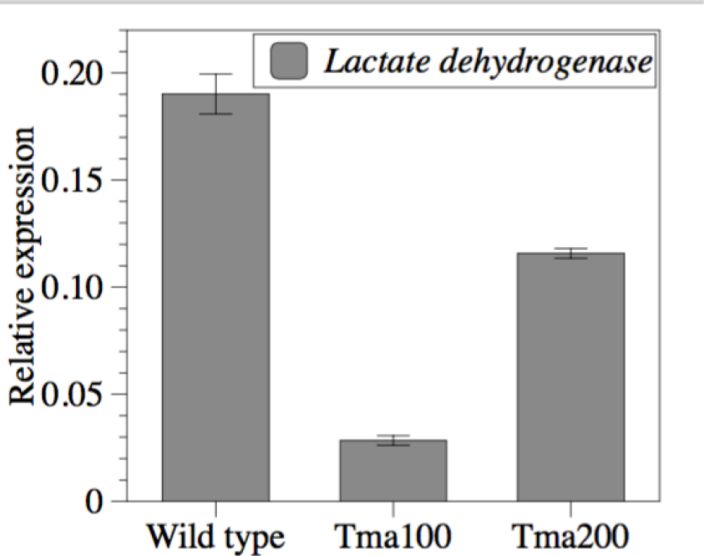


Figure S8. Relative transcript abundance. Lactate dehydrogenase (*ldh*) gene expression in various strains of *T. maritima*.

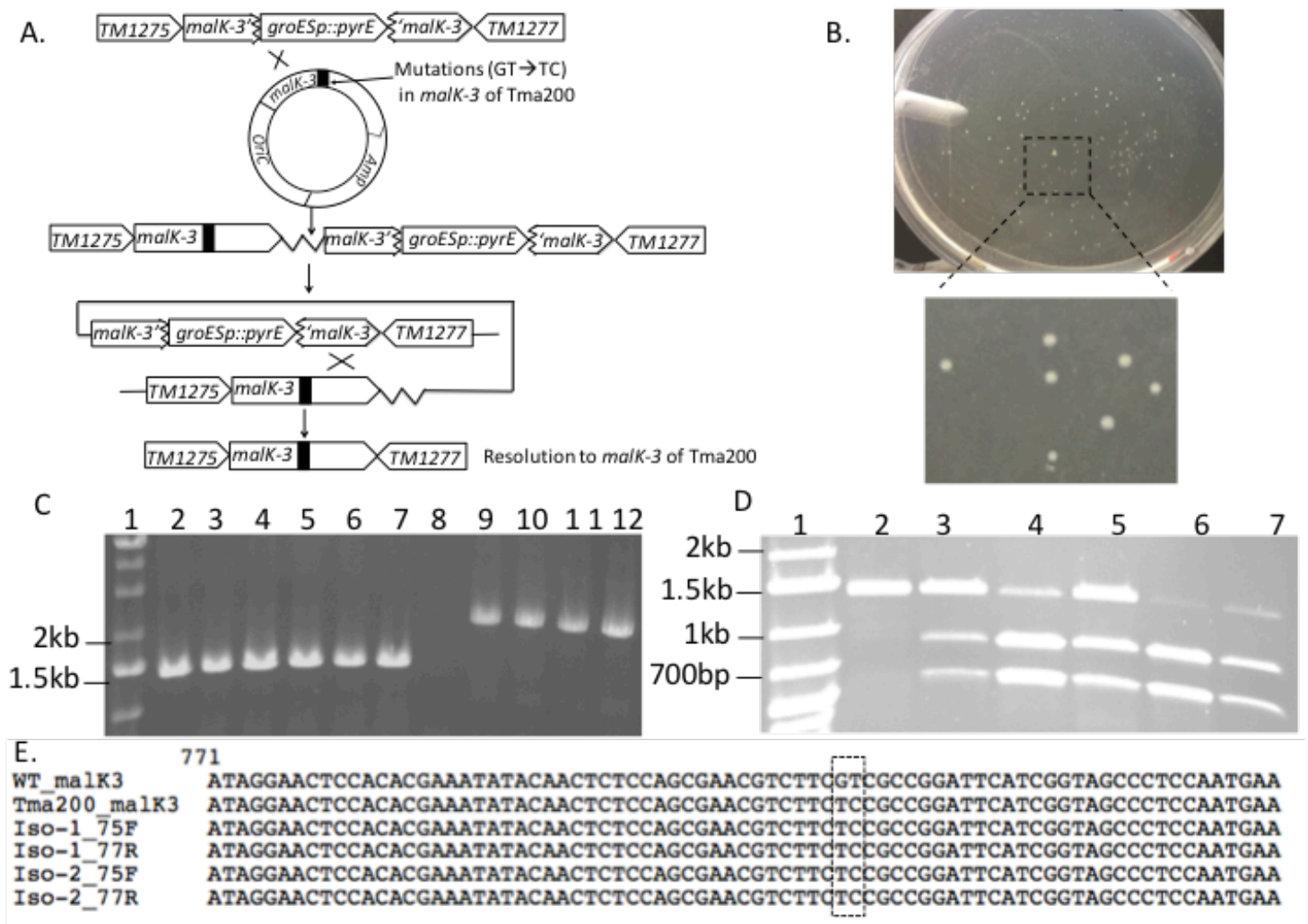


Figure S9. Reconstruction of an excess H₂ trait by replacing the disrupted *malK3* allele of the *malK3* mutant with the *malK3* of Tma200. **9A.** A schematic of crossover at the disrupted *malK3* allele of the *malK3* mutant and resolution into a strain possessing *malK3* allele of Tma200. **9B.** Colony phenotype of the strain repaired with *malK3* of Tma200. **9C.** PCR amplification of the *malK3* locus of the bigger colonies (lanes 2-7) and small colonies (lanes 8-12). **9D.** Restriction digestion of the PCR amplicon of bigger colonies with *Acil* (lane 3-7) and wild type (lane-2). **9E.** Sequencing of the *malK3* locus of the two isolates representing mutations in the *malK3* identical to the *malK3* of Tma200 strain.

1 **Table S1. Growth, cell yield and metabolites during exponential growth in**
2 **bioreactors^a.**

	Wild type	Tma100	Tma200
Generation time (minutes)	65	100	86
Biomass (mg cdw/L)	86.96 ± 0.45	47.55 ± 0.10	71.17 ± 0.01
H ₂ (mM)	10.07 ± 0.01	6.48 ± 0.03	12.15 ± 0.13
H ₂ (mM)/g cdw	115.80 ± 0.23	135.64 ± 0.49	170.71 ± 2.60

3 ^aParameters were obtained from studies using 3 L fermenters and values were
4 determined during the initial growth phase (0-5 h).

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Table S2. Lactate excretion.

Strain	Lactate (5h) (mM)	Lactate (30 h) (mM)
Wild type	0.17±0.05	12.32±0.05
Tma100	<0.1mM	<0.1mM
Tma200	<0.1mM	2.92± 0.15

9 ^aLactate was estimated from studies using 3 L fermenters after
10 the times indicated.
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Table S3. Summary of confirmed mutations.

Mutant Strain	Substitution/ Deletion	Location of mutation/total length (AA)	ORF (numbering according to (1))	Gene Annotation
Tma100	G → A (Gly → Glu)	148/369	TM1276	Maltose/maltodextrin transport ATP-binding protein (MalK) of an ABC transporter
Tma100	G → A (Glu → Lys)	345/369	TM1276	Maltose/maltodextrin transport ATP-binding protein (MalK) of an ABC transporter
Tma100 and Tma200	G → A (Trp → Stop)	229/614	TM0460	Peptide ABC transporter substrate binding protein
Tma200	GT → TC (Val → Ser)	233/369	TM1276	Maltose/maltodextrin transport ATP-binding protein (MalK) of an ABC transporter
Tma200	G → A (Ala → Phe)	1045/1690	TM0459	RNA polymerase, beta subunit
Tma200	~10kb deletion	Deletion from 5' of TM1322-3' of TM1332	TM1322 – TM1331	Six hypothetical proteins, two astB/chuR-related protein and two lacI family transcriptional regulator

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Table S4. Comparative analysis of inherited mutations.

Gene name/gene locus tag	Mutations in NC_000853.1, NC_023151.1 and NC_021214.1	Mutations in laboratory wild type (<i>T. maritima</i>), Tma100 and Tma200
Transcriptional regulator, LacI family ^a (TM0299)	NM	Arg→His (Corresponds to coordinate-317059 of NC_000853.1)
Molecular chaperone ^a (groEL) ^a (TM0506)	NM	Glu→Lys (Corresponds to coordinate-533154 of NC_000853.1)
(Sugar kinase, pfkB family) ^a (TM0828)	NM	Ala→Thr (Corresponds to coordinate -854666 of NC_000853.1)
Oxaloacetate decarboxylase, beta subunit ^a (TM0880)	NM	Lys→Phe (Corresponds to coordinate-903233 of NC_000853.1)
Intergenic region Between ^a (TM1145) and ^a (TM1146)	NM	Intergenic region-no protein (Corresponds to coordinate-1158469 of NC_000853.1)
Intergenic of malE ^a (TM1839) and alpha amylase ^a (TM1840)	NM	Intergenic region- no protein (corresponds to 1815578 of NC_000853.1)

17 NM-no mutation, ^a Gene locus tag as per NC_000853.1.

18 References

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