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Identification of the ATPase Subunit of the Primary Maltose Transporter in the Hyperthermophilic Anaerobe *Thermotoga maritima*

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ABSTRACT *Thermotoga maritima* is a hyperthermophilic anaerobic bacterium that produces molecular hydrogen (H_2) by fermentation. It catabolizes a broad range of carbohydrates through the action of diverse ABC transporters. However, in *T. maritima* and related species, highly similar genes with ambiguous annotation obscure a precise understanding of genome function. In *T. maritima*, three putative *malK* genes, all annotated as ATPase subunits, exhibited high identity to each other. To distinguish between these genes, *malK* disruption mutants were constructed by gene replacement, and the resulting mutant cell lines were characterized. Only a disruption of *malK3* produced a defect in maltose catabolism. To verify that the mutant phenotype arose specifically from *malK3* inactivation, the *malK3* mutation was repaired by recombination, and maltose catabolism was restored. This study demonstrates the importance of a maltose ABC-type transporter and its relationship to sugar metabolism in *T. maritima*.

IMPORTANCE The application and further development of a genetic system was used here to investigate gene paralogs in the hyperthermophile *Thermotoga maritima*. The occurrence of three ABC transporter ATPase subunits all annotated as *malK* was evaluated using a combination of genetic and bioinformatic approaches. The results clarify the role of only one *malK* gene in maltose catabolism in a nonmodel organism noted for fermentative hydrogen production.

KEYWORDS genetic systems, ABC transporter, maltose, hyperthermophile, homologous recombination, hydrogen

The ATP-binding cassette (ABC) transporter gene family forms a large group. These transporters support the efficient transport of a wide variety of substrates in an ATP-dependent manner. Maltose transporters are intensively studied and usually belong to the ABC transporter family. They are composed of a substrate binding protein (encoded by *malE*), transmembrane proteins (encoded by *malF* and *malG*), and an ATP-hydrolyzing enzyme (encoded by *malK*) present as a homodimer (1, 2). Subunits of ABC transporters are frequently identified because they are contiguous with related subunits encoded within operons, and because of their pattern of regulated gene expression responding to substrate availability. However, in the case of *malK*, the ATPase subunit is not typically contiguous with its other subunits (*malE*, *malF*, and *malG*) (3–5). In addition, its transcriptional expression is not responsive to maltose availability (3, 6). This makes the identification of *malK* dependent on other approaches. The *malK* gene has been studied in model organisms, including *Escherichia coli* and *Salmonella enterica*, where only one copy is present (5–7). However, in hyperthermophilic bacteria and archaea, *malK* occurs in multiple copies (8–10).

T. maritima is a well-studied hyperthermophilic bacterium that belongs to the order

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Thermotogales. It uses simple and complex carbohydrate substrates for growth without catabolite repression (11–14). Maltose is a preferred carbon source for *T. maritima* (15), and its transport is mediated by the maltose ABC transporter rather than the phosphoenolpyruvate phosphotransferase system (12, 16). *T. maritima* has three *mal* operons (*mal1*, *mal2*, and *mal3*) (12, 17). The maltose-inducible nature and protein abundance of the subunits of the *mal2* operon (*malE2*, *malF2*, and *malG2*) support their role as maltose transporters (9, 18). *T. maritima* also has three annotated *malK* sequences, including *malK1* (*THMA_0427*), *malK2* (*THMA_1258*), and *malK3* (*THMA_1301*) (12, 19). None of the annotated *malK* genes were upregulated when maltose was employed as an carbon-inducing source (9, 20).

The historic lack of a *T. maritima* genetic system has limited the understanding of maltose transport. In this study, a reverse genetics approach guided by bioinformatic analysis was used to investigate the relative importance of putative *malK* genes. A similar approach may be used to study the metabolic function of other genes with identical annotations.

RESULTS

Occurrence and comparisons of MalK-like proteins from *T. maritima*. Some bacteria have evolved a single copy of *malK* (21, 22). However, prior studies (9, 19) and four individual wild-type genome sequences of *T. maritima* (accession numbers AE000512, CP007013.1, CP004077, and CP011107.1) indicated the presence of three *malK*-like genes in this organism. In contrast to *T. maritima*, other thermophilic and hyperthermophilic organisms had various numbers of *malK* genes, as shown in Table S1 in the supplemental material. While the majority of the hyperthermophilic members of the class *Thermotogae* have a total of three *malK*-like genes, one or two *malK*-like genes are present in mesophilic members of the *Thermotogales* (Table S1).

To clarify the significance of the *T. maritima malK* genes, genetic experimental strategies were pursued. To simplify their designation, they were renamed here *malK1* (*THMA_0427*), *malK2* (*THMA_1258*), and *malK3* (*THMA_1301*). Since the N-terminal region of MalK interacts with transmembrane ABC protein subunits and plays an important role in ATP hydrolysis, this region is highly conserved (23). Previously characterized MalK proteins from *Thermococcus litoralis* (24) and *Thermus thermophilus* (10) were used for comparison to the putative MalK of *T. maritima* and the nonfunctional $MalK_2$ of *T. thermophilus*. While the N termini of all these MalK proteins exhibited several common motifs, including Walker A and B sequences, a "signature" motif, and Q and H loops (25), the intervening region between Walker A and Walker B was divergent for MalK1, MalK2, and $MalK_2$ of *T. thermophilus* (Fig. 1). In addition, MalK3 of *T. maritima* possessed several conserved residues that were common in the active MalK of *T. litoralis* and *T. thermophilus*. Although the sequence divergence in MalK1 and MalK2 might make them unlikely to interact with MalF and MalG, the presence of conserved motifs would allow them to act as ATPase subunits for other ABC-type transporters (26–28).

Disruption of *malK* by homologous recombination. Analysis of the *T. maritima* transcriptome indicated that *malK3* expression was not responsive to the addition of maltose (20). Moreover, studies employing 14 monosaccharides and polysaccharides (arabinose, glucose, mannose, rhamnose, ribose, xylose, galactomannan, glucomannan, β-1,3–β-1,4-glucan, laminarin, pustulan, starch, β-xylose, and chitin) did not lead to upregulation of either of the *malK* genes of *T. maritima* (9).

In addition, *malK3* was not contiguous with other ABC transporter subunits, including *malE*, *malF*, and *malG*. To test which, if any, of the *malK* genes mediated maltose transport, targeted gene disruptions were constructed using the P_{groES}::$pyrE_{TAF}$-selectable marker and a uracil auxotroph (PBL3004) deleted for *pyrE*, as described previously (29). To avoid counterselection for maltose catabolism, consequently, cells were cultivated in complex medium (CM) using cellobiose. This was followed by subculturing cells in defined medium (DM) containing cellobiose without uracil to enable enrichment of Pyr^+ recombinants. The clonal isolates were recovered using solid DM containing cellobiose. Five isolates were genotyped from each transformation

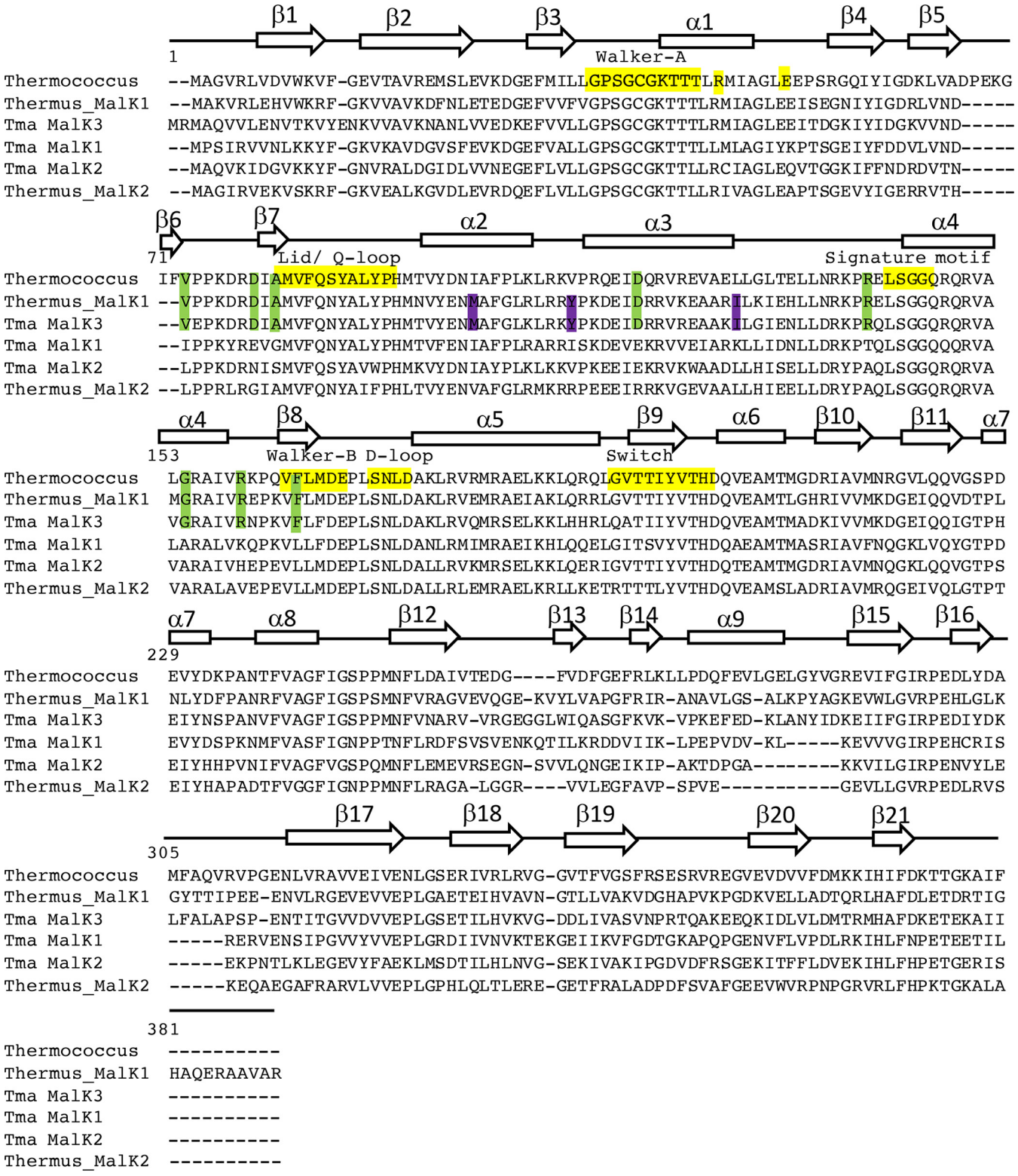

FIG 1 Structural alignment of MalK from *Thermococcus litoralis* (24) and *Thermus thermophilus* (10). Secondary structure elements of *T. litoralis* MalK are indicated and numbered. Yellow-shaded sequences represent conserved motifs, and green-shaded areas show conserved residues in MalK3 and MalK of *T. litoralis* and *T. thermophilus*. The purple shading indicates conserved residues common between MalK3 and MalK of *T. thermophilus*. Residues of MalK1 and MalK2 of *T. maritima* and MalK2 of *T. thermophilus* that were common to the MalK of *T. litoralis* and *T. thermophilus* and MalK3 of *T. maritima* are not shaded.

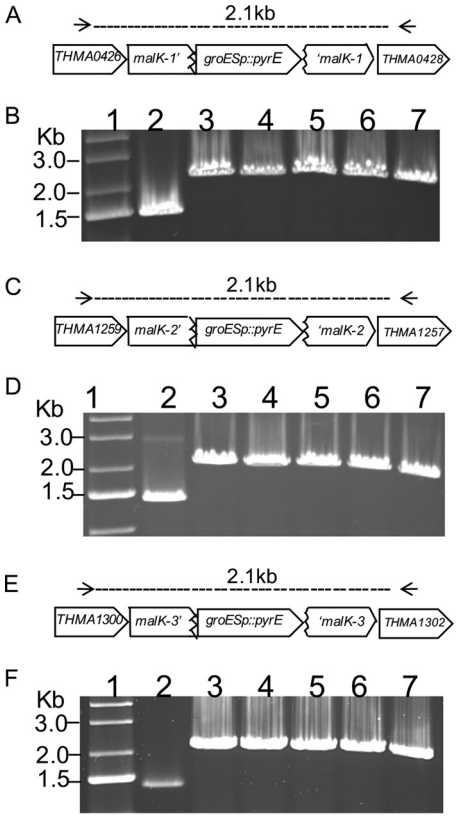

FIG 2 Disruption of the *malK* loci. (A, C, and E) Schematic representation showing a disrupted copy of *malK1* (A), *malK2* (C), and *malK3* (E). Primers outside each *malK* locus are indicated by horizontal arrows. (B, D, and F) Agarose gels depicting PCR amplicons of the *malK1* (B), *malK2* (D), and *malK3* (F) genomic regions for five isolates (lanes 3 to 7) compared to the wild-type strain (lane 2). Lane 1 shows a DNA molecular marker.

using PCR and DNA sequencing (Fig. 2). In all cases, a larger PCR product of the target *malK* gene was identified resulting from insertion of the selectable marker that increased the amplicon size by 763 bp relative to the size of the undisrupted allele. The possibility of homologous recombination at the P_{groES} locus due to homology encoded within the selectable marker was excluded by checking the size of the P_{groES} locus; all isolates retained an intact P_{groES} sequence (Fig. S1D). In addition, the *pyrE* deletion allele was retained in all recombinants, as indicated by PCR and DNA sequencing of this region (Fig. S1B).

Identification of the primary maltose transporter ATPase subunit. If any of the *malK* genes were required for maltose transport, mutant derivatives would be unable to grow using maltose as the sole source of carbon and energy. Therefore, growth of all three mutants was examined in DM with the addition of either maltose or cellobiose. Only the *malK3* mutant failed to grow using maltose. In contrast, all mutants grew normally using cellobiose (Fig. 3A). Colony formation of the *malK3* mutant was then examined relative to the parental wild type using CM or DM and either maltose or cellobiose. After 3 days of incubation at 80°C, the mutant failed to form colonies on maltose plates using either DM, while colonies were apparent in both media containing cellobiose (Fig. 3B). While these results indicated that *malK3* was required for maltose catabolism, they also suggested that *malK1* and *malK2* were not and were therefore incorrectly annotated.

To better understand the possible functions of *malK1* and *malK2*, additional tests were conducted. The *malK1* gene is located within an inositol operon and upstream of a xylose isomerase gene, so the *malK1* mutant was tested for phenotypes related to inositol and xylose catabolism. No growth defect was observed on xylose (Fig. S2) and,

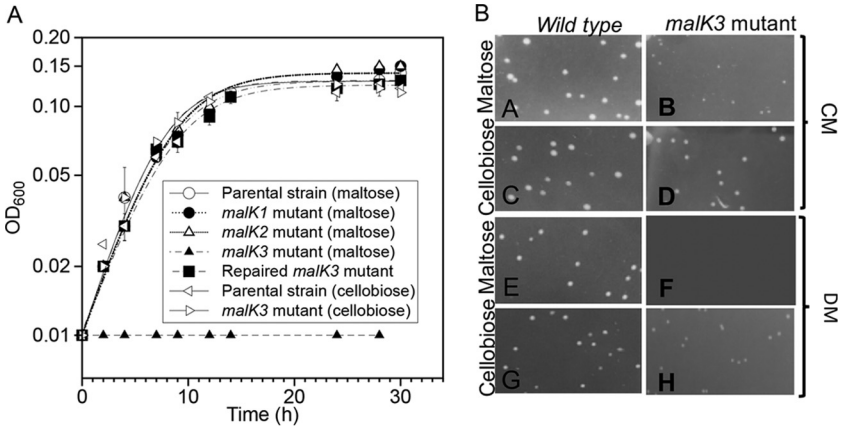

FIG 3 Growth phenotypes. Growth in tubes of the wild type, *malK1*, *malK2*, and *malK3* mutants, and the repaired *malK3* mutant using DM (A), or on plates containing CM and DM (B) with added maltose or cellobiose, as indicated. Error bars represent results from two biological replicates.

in our hands, none of the strains, including the parental strain, exhibited growth using inositol as reported previously (30). The presence of an α-mannosidase located in the 5′ direction of *malK2* suggested that this *malK* could instead be involved in mannose transport. However, no growth defect was apparent using this sugar. To test whether MalK1 or MalK2 might be involved in transporting maltose oligosaccharides, no growth defect was observed when the *malK1* and *malK2* mutants were grown on these oligosaccharides. To further test if MalK1 and MalK2 can transport α-linked glucose polysaccharide (starch) or β-linked glucose polysaccharide (carboxymethylcellulose [CMC]), the *malK1* and *malK2* mutants were tested on these polysaccharides, but no growth defect was observed (data not shown). These results suggest that the *malK1* and *malK2* genes are not required for maltose transport or the other tested sugars and that *malK3* is specific for maltose catabolism (Fig. 4). This is consistent with transcriptomics studies where none of the *malK*-like genes showed upregulation in the presence of maltose (20).

Homologous repair of *malK3*. To confirm that the maltose defect in the *malK3* mutant was associated with *malK3* disruption, the strain was complemented with the wild-type *malK3* allele. Prior to repair, the mutant was evaluated on maltose CM plates and formed microcolonies relative to the parental wild-type strain, presumably due to the presence of trace amounts of other carbon sources. The mutant small colony size was then used as a screen for repair of the *malK3* disruption mutation. To accomplish this, natural transformation was conducted as described previously (29) using a wild-type allele of *malK3* and enrichment in DM supplemented with maltose and uracil. The enrichment samples were then plated on CM-maltose plates and resulted in the formation of two colony sizes (Fig. 5A). With additional incubation, the larger colonies continued to increase in size, but the small colonies did not change size. PCR and DNA sequencing demonstrated that large colonies encoded the wild-type allele, while small colonies retained the still-disrupted *malK3* allele (Fig. 5B). One of the three isolates (PBL3027) encoding a wild-type allele was examined in DM containing maltose and grew normally relative to the wild-type strain (Fig. 3A). This result confirmed that the isolate had regained the capacity to catabolize maltose as a result of the repair of the *malK3* disruption mutation.

DISCUSSION

The identification of genes involved in carbon catabolism is important for efforts to develop *T. maritima* as a host for synthesis of improved biohydrogen. The binding protein-dependent ATP-binding cassette (ABC) transporters are the major route for the assimilation of carbon and energy sources in other hyperthermophilic microbes, including the archaea *Sulfolobus solfataricus* (31) and *Thermococcus litoralis* (32) and the

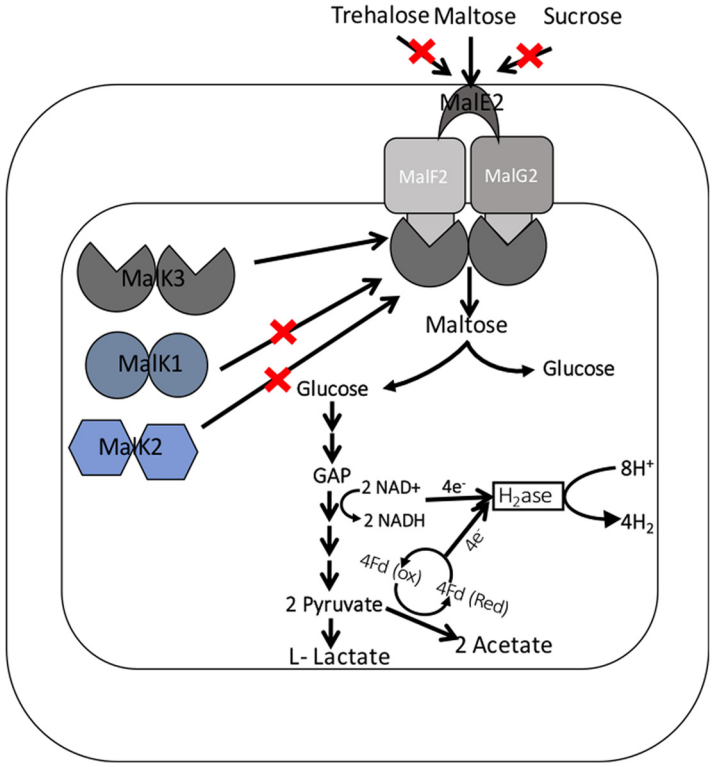

FIG 4 A proposed schematic of maltose transport and its relationship to metabolism in *T. maritima*. Maltose is transported via the maltose ABC transporter, and no other similar sugars are transported through the maltose transporter. GAP, glyceraldehyde-3-phosphate; H_2ase, hydrogenase; Fd, ferredoxin.

bacteria *Thermoanaerobacter thermosulfurigenes* (33), *Thermoanaerobacter ethanolicus* (34, 35), *Alicyclobacillus acidocaldarius* (36), and *Thermus thermophilus* (10). Interestingly, among these organisms, *T. maritima* possesses the second highest number of transporter genes that belong to ABC transporters (12, 37, 38). Although various transcriptomics studies have been done in *T. maritima* to clarify the identity of carbon transporters (9, 13), the *malK* gene of the maltose transporter was not clear in part due to the lack of a genetic system to identify the required genes.

T. maritima produces 4 mol H_2 from per mol glucose through an electron-confurcating hydrogenase (39) and utilizes maltose efficiently (15). However, the identity of the ATPase subunit required for maltose transport was complicated by the presence of three *malK*-like genes (9, 19) and four wild-type genome sequences (accession numbers AE000512, CP007013.1, CP004077, and CP011107.1). In *E. coli*, maltose transport involves a complex signal transduction cascade triggered by maltose association with MalE that then binds MalF/MalG, followed by ATP hydrolysis catalyzed by MalK (40, 41). Therefore, a functional MalK must have highly conserved residues to achieve interactions with MalF and MalG (7, 42, 43). In this regard, the Q loop of MalK that remains in close proximity to MalF and MalG (44, 45) and the sequence between the Q loop and the signature motif help distinguish MalK from its homologs (2, 7, 42). A protein sequence alignment of *malK3* with the experimentally validated MalK protein sequence of a thermophilic bacterium, *Thermus thermophilus* (32), and a hyperthermophilic archaeon, *Thermococcus litoralis* (24), suggests that positions V73, D79, A81, D114, R135, and G148 are conserved and were evident in *malK3* of *T. maritima*. Furthermore, *malK2* of *Thermus thermophilus* that was not involved in maltose transport (32) and both *malK1* and *malK2* of *T. maritima* lack these conserved residues. A mutation of L86F in the *malK* gene of *Salmonella enterica* serovar Typhimurium that maps inside the Q loop has been shown to exert maltose-defective growth (7), and this conserved residue is variant in *malK2* in both *T. maritima* (L86V) and *T. thermophilus* (L86I), suggesting that these MalK-like proteins might not interact with MalF and MalG.

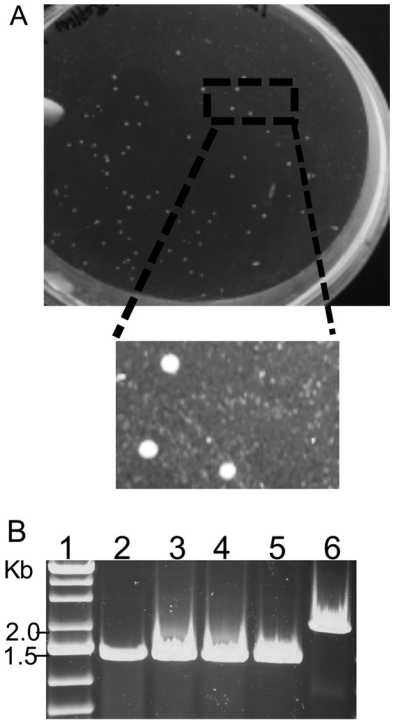

FIG 5 Repair of the *malK3* mutation. (A) A CM maltose petri plate showing colonies of the *malK3* mutant repaired with the wild-type *malK3* allele. (B) An agarose gel depicting PCR amplification of the *malK3* locus from three large colonies (lanes 3 to 5), one small colony (lane 6), and the wild type (lane 2). Lane 1 shows a DNA molecular marker.

A genetic approach to clarify the functional *malK* from *malK1* and *malK2* in *Thermus thermophilus* HB27 (10) provided insight. The availability of a homologous recombination method for *T. maritima* (29) allowed for the use of a similar approach. *T. maritima* possesses three *mal* operons (*mal1*, *mal2*, and *mal3*) (12, 17), but only the *mal2* operon is induced at the transcriptional level by the addition of maltose (9). The *mal2* operon is also missing a *malK* subunit. Perhaps, the noncontiguous MalK3 interacts with the ABC transporter subunits of the *mal2* operon. However, MalK1 and MalK2 cannot interact with the maltose ABC transporter subunit, because the *malK3* mutant remained unable to catabolize maltose despite the presence of wild-type MalK1 or MalK2. While the *mal1* and *mal3* operons might use either MalK1 or MalK2 to transport a different sugar, no growth defect was observed when these *malK* mutants were cultivated using various sugars. Predicting the function of a transporter based on the affinity of the substrate binding subunit to various substrates may be deceptive. However, gene knockout studies can clarify the actual substrate that can be transported by the transporter through the manifestation of a defective phenotype, such as a loss of sugar catabolism. Although MalE2 of *T. maritima* was shown to bind maltose and trehalose (8), the gene disruption data presented here do not support the uptake of trehalose by the maltose transporter of *T. maritima*. Instead, these findings are consistent with *Alicyclobacillus acidocaldarius*, where MalE (SBP) bound trehalose, sucrose, lactose, and glucose efficiently, but the complete transporter was found to transport primarily maltose (36).

In *T. maritima*, H_2 synthesis is a function of maltose transported by the maltose transporter (15). Manipulating the amount of transported maltose through the maltose transporter employing genetic engineering is likely to change the maltose metabolism and the amount of formed H_2. The continued use of genetics to investigate gene function in *T. maritima* will promote the development of this organism as a model hyperthermophile, and specifically for the production of biohydrogen with this species.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
PBL3004	*T. maritima pyrE*-129	29
PBL3023	PBL3004 *malK1′*::P_{groES}::$pyrE_{TAF}$::*′malK1*	Current study
PBL3024	PBL3004 *malK2′*::P_{groES}::$pyrE_{TAF}$::*′malK2*	Current study
PBL3025	PBL3004 *malK3′*::P_{groES}::$pyrE_{TAF}$::*′malK3*	Current study
PBL3027	PBL3025, wild-type *malK3*	Current study
Plasmids		
pBN1312	pUC19 P_{groES}::$pyrE_{TAF}$	29
pBN1324	pUC19 *malK1′*::P_{groES}::$pyrE_{TAF}$::*′malK1*	Current study
pBN1325	pUC19 *malK2′*::P_{groES}::$pyrE_{TAF}$::*′malK2*	Current study
pBN1326	pUC19 *malK3′*::P_{groES}::$pyrE_{TAF}$::*′malK3*	Current study
pBN1327	pUC19 wild-type *malK3*	Current study

MATERIALS AND METHODS

Strains and cultivation. The strains, plasmids, and primers used in this study are shown in Tables 1 and 2. *Thermotoga maritima* MSB8 (GenBank accession number CP011107.1) and its derivatives were cultured under anaerobic conditions at 80°C in either complex medium (CM) or defined medium (DM), as described previously (29). CM was prepared as described previously (46). DM also was prepared as described previously (47) but lacked yeast extract and tryptone. *T. maritima* and its mutants were cultivated in Hungate tubes or serum bottles supplemented with 0.5% (15 mM) maltose or cellobiose, unless otherwise indicated. The uracil auxotroph (PB3004) was grown in DM containing 5 μg/ml uracil. Tubes were sealed with butyl rubber stoppers (Bellco Biotechnology) and crimped with metal collars, and the headspace was exchanged with N_2. Growth was monitored spectrophotometrically at a wavelength of 600 nm. Sterile 1-ml syringes attached to 20.5-G needles were used for inoculation at an initial cell density of OD_{600} of 0.02, unless otherwise indicated. All tubes were incubated anaerobically at 80°C overnight, unless otherwise specified.

Bioinformatic analysis of *T. maritima* MalK. The deduced protein sequence of *malK3* of *T. maritima* was used to retrieve paralogs from *T. maritima* using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. More than 50% identity was used as a criterion to predict reliable MalK-like proteins. Additionally, these sequences were used as queries to retrieve orthologs from the genomes of archaea and other bacteria. The *malK* sequences annotated as spermidine/putrescine transporter subunits were

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)	Restriction site[a]
P1 (P_{groES} F)	CAGAAAGAGGGCGCTTCAA	BamHI
P2 (pyrE R)	TTATTTTTTAATGAATCTACTTCCT	BamHI
P3 (THMA0427, malK1′)	ATGCCCAGTATCAGGGTTGTG	EcoRI
P4 (THMA0427, malK1′)	CTGAAGGTGTTTTATTTCCGCC	SacI
P5 (THMA0427, ′malK1)	CAGGAACTTGGAATCACCTCTGT	SalI
P6 (THMA0427, ′malK1)	TTACAGAATCGTTTCCTCCGTTT	SphI
P7 (THMA1258, malK2′)	TTGGCGCAGGTGAAAATAGA	EcoRI
P8 (THMA1258, malK2′)	CTTTTTCAGCTCGCTTCTCATC	SacI
P9 (THMA1258, ′malK2)	CTTCAGGAAAGAATTGGAGTCACC	SalI
P10 (THMA1258, ′malK2)	TCATGAGATTCTCTCTCCCGTCT	SphI
P11 (malK3′ F)	GTGAGAATGGCTCAGGTTGTCC	SacI
P12 (malK3′ R)	GCTTCTCATCTGCACTCTGAGTTT	KpnI
P13 (′mal3 F)	GAGCTCAAGAAGCTCCACCA	PstI
P14 (′mal3 R)	TCAGATAATAGCCTTCTCCGTTTCT	SphI
P15 (wt malK3 F)	GTGAGAATGGCTCAGGTTGTCC	EcoRI
P16 (wt malK3 R)	TCAGATAATAGCCTTCTCCGTTTCT	KpnI
P17 (THMA426F)	GACTCTTTCCAAGTACGTGAAAGG	NA
P18 (THMA428 R)	TGCCCCGTGCTATCACA	NA
P19 (THMA1257)	TTCAACCGCAGCTGTTTGG	NA
P20 (THMA1259)	TTCCAGCACACATTCTGAACAC	NA
P21 (THMA1300 F)	TGGTCGTGACAAAGAACGGT	NA
P22 (THMA1302 F)	GGACGGACTCAAGGACTACG	NA
P23	CTGTGGGAGAGGACACCCT	NA
P24	AGAACGATTCCTCCCTCTGTC	NA
P25 (pyrE F)	GTGATAAAGGAAATCCTCGAGAAAA	NA
P26 (pyrE R)	TCATTTCAATCCCCTGCTTCCCGGT	NA

[a]NA, not applicable.

not considered in this study to ensure functional clarity. The region surrounded by Walker A and Walker B of the MalK protein has been reported to be important for transmembrane subunit interactions and ATP binding (23). Therefore, analysis of this region was used to characterize all three MalK proteins of *T. maritima*.

Construction and repair of *malK* mutations. Mutant construction employed the *pyrE*-selectable marker that was derived from *Thermosipho africanus* and fused to P_{groESL} (P_{groES}::$pyrE_{TAF}$), as described previously (29). This cassette was cloned at the BamHI site of the pUC19 (using P1 and P2 primers) and was named pBN1312. A *malK1* disruption cassette (pBN1324) was constructed by amplifying the N-terminal region of the coding region of *malK1* (540 bp) (using P3/P4) and the C-terminal region (540 bp) (using P5/P6) and cloning these fragments at the EcoRI/SacI and SalI/SphI sites of pBN1312, respectively. Similarly, the *malK2* (pBN1325) and *malK3* (pBN1326) disruption cassettes were constructed by amplifying the N-terminal regions of either *malK2* (534 bp) (using P7/P8) or *malK3* (531 bp) (using P11/P12) and cloning them at the EcoRI/SacI and SacI/KpnI sites of pBN1312, respectively, followed by insertion of the amplified C-terminal regions of *malK2* (534 bp) (using P9/P10) and *malK3* (579 bp) (using P13/P14), respectively, at the SalI/SphI and SalI/SphI sites of pBN1312 (Table 1). The *malK3* repair construct (pBN1327) resulted from cloning the open reading frame of *malK3* of the wild-type strain (using P15 and P16 primers) at the EcoRI/KpnI site of pUC19. Ligation, transformation, and selection of *E. coli* DH5α transformants were performed as described previously (48).

The *T. maritima* uracil auxotroph (PBL3004) (29) and the *malK3* mutant (PBL3025) were used as recipients for natural transformation, as described previously (29). Disruption constructs for *malK1*, *malK2*, and *malK3* were used to transform PBL3004. Transformed cells of *T. maritima* were cultivated initially at 80°C for 18 to 24 h under nonselective conditions by inoculating cells into tubes containing 10 ml of CM supplemented with 0.5% cellobiose. Genetic selection was performed subsequently in DM containing 0.5% cellobiose lacking uracil. Liquid enrichments were used to isolate clonal populations by plating on DM plates supplemented with cellobiose. Five colonies from each successful transformation carried out with *malK1*, *malK2*, and *malK3* disruption constructs were pursued for genotypic analysis. Selected colonies were grown in tubes containing CM, and genomic DNA was prepared as described previously (49, 50). Putative disruption mutations located at the *malK1*, *malK2*, and *malK3* loci were screened by PCR using outside primers P17/P18, P19/P20, and P21/P22 to detect the P_{groES}::$pyrE_{TAF}$-disrupted alleles of *malK1*, *malK2*, and *malK3*. Primers outside the *malK1*, *malK2*, and *malK3* loci were used to exclude the possibility of single-crossover intermediates at the *malK* loci. To rule out the occurrence of recombination at the native P_{groES} locus, the P_{groES} locus in all disrupted mutants was amplified by PCR using primers flanking P_{groES} (P23/P24). The *malK3* disruption mutation was repaired using a two-step enrichment process. Transformed cells were propagated in CM supplemented with cellobiose and then in DM supplemented with maltose. Selection for maltose utilization was then performed using CM supplemented with uracil and maltose to enrich for cells containing the repaired *malK3* allele. Since the *malK3* mutant formed microcolonies only on CM plates with added maltose, and the repair of the *malK3* mutation was anticipated to result in larger colonies, the population was examined for variations in colony size. A mixture of colony sizes was apparent, and three large colonies and one small colony were pursued for genotypic analysis using PCR. Using primers complementary to the flanking genes, the repaired wild-type and parental disrupted alleles were distinguished. Small colonies encoded the disrupted "large" *malK3* allele, while large colonies encoded the small undisrupted *malK*3 allele. The presence or absence of a maltose-specific growth phenotype in the *malK1* (PBL3023), *malK2* (PBL3024), and *malK3* (PBL3025) mutants was determined by cultivating these mutants in DM supplemented with maltose. Growth was measured periodically by measuring the optical density at 600 nm (OD_{600}) of each tube. To observe the maltose-specific colony phenotype, these cells were also plated on CM and DM plates containing maltose. To test if the phenotype of the PBL3025 was solely dependent on maltose, cellobiose was used as an alternative carbon source in the tubes; the parental strain (PBL3004) was used as a control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00930-17.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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Table S1. MalK orthologs[a].

Organism name	MalK3	MalK2[b]	MalK1[b]
Thermotoga maritima	*THMA1301*	*THMA1258* (53%)	*THMA0427* (50%)
Thermotoga naphthophila	*Tnap_1518*	*Tnap_1560* (53%)	*Tnap_212* (50%)
Thermotoga petrophila	*Tpet_1495*	*Tpet_1537* (53%)	*Tpet_500* (50%)
Thermotoga sp RQ2	*TRQ2_1543*	*TRQ2_1586* (53%)	*TRQ2_1453* (42%)
Thermotoga neapolitana	*CTN_1296*	*CTN_1340* (50%)	*CTN_249* (50%)
Thermotoga sp RQ7	*TRQ7_07955*	*TRQ7_0817*(50%)	*TRQ7_02745*(50%)
Pseudothermotoga thermarum	*Theth_0983*	*Theth_1625* (51%)	*Theth_1475* (48%)
Pseudothermotoga lettingae	*Tlet_1280*	*Tlet_0086* (47%)	
Fervidobacterium nodosum	*Fnod_0163*	*Fnod_1592* (44%)	
Fervidobacterium pennivorans	*Ferpe_0412*	*Ferpe_0169* (41%)	
Marinitoga piezophila	*Marpi_0817*	*Marpi_2099* (48%)	
Thermosipho melanesiensis	*Tmel_1161*	*Tmel_1702* (49%	
Kosmotoga olearia	*Kole_0287*	*Kole_0983* (49%)	
Mesotoga prima	*Theba_0827*		
Spirochaeta thermophila DSM 6578	1228 (37%)	0139 (39%)	2286 (37%)

Thermoanaerobacterium thermosaccharolyticum DSM 571	*Tthe_0613*	<40%	
Thermoanaerobacter mathranii	*Tmath_1760*	*Tmath_0271*(51%)	
Thermococcus sp. ES1	*TES1_1663*	*TES1_0529* (71%)	*TES1_1822* (56%) and *TES1_1184* (50%)
Marinithermus hydrothermalis	*Marky_1332*	*Marky_0939* (49%)	
Thermus thermophilus HB27	*TTC_0211*	*TTC_0611* (50%) ($MalK_2$)	
Thermus thermophilus SG0.5JP17-16	*Ththe16_0581*	*Ththe16_0993* (51%)	*Ththe16_2043* (51%)
Clostridium saccharolyticum WM1	*Closa_0793*	*Closa_0101* (48%)	*Closa_2083* (41%)
Ruminiclostridium thermocellum ATCC 27405	*Cthe_1862*	*Cthe_1587* (41%)	
Geobacillus thermodenitrificans	*GTNG_0543*	*GTNG_3226* (40%)	
Geobacillus	*GTCCBUS3UF*	*GTCCBUS3UF5_*	

thermoleovorans	*5_7000*	*22910* (47%)	
Halothermothrix orenii	*Hore_00450*	*Hore_03330* (45%)	*Hore_03320* (43%)

[a]MalK3 of *T. maritima* was used to find orthologous MalK3-like protein sequences in hyperthermophilic and non thermophilic microbes. [b] % identity to MalK3.

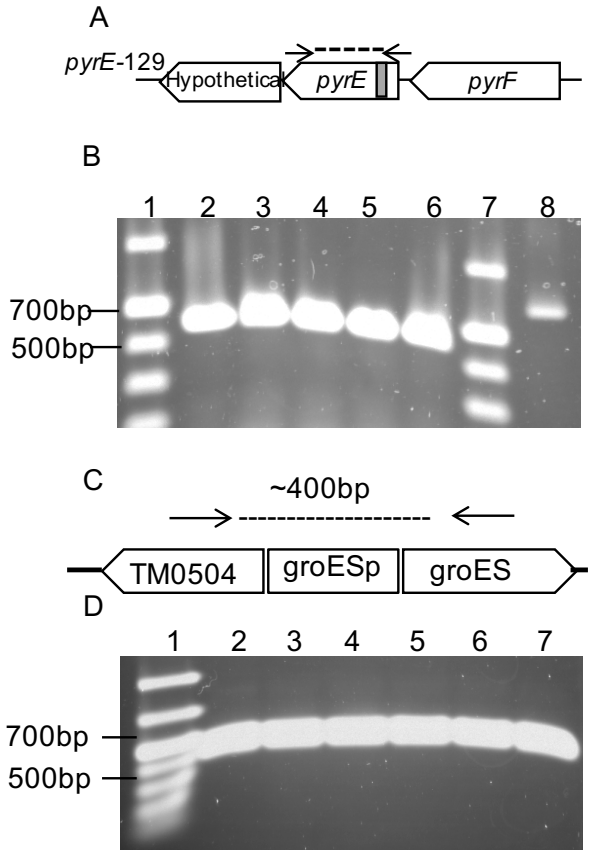

Figure S1. Lack of recombination at the *pyrE* and *groESp* loci. (A) The *pyrE* ORF in wild type. (B) An agarose gel depicting PCR amplicon of the *pyrE* genomic region in 5 isolates (Lanes 2-6) compared to the *wild type* (Lane 8), amplified by the indicated primers as horizontal arrows. (C) The genomic environment around *groESp* in the wild type and uracil auxotroph strains respectively. (D) An agarose gel showing PCR amplicons of the *groESp* genomic region in 5 isolates (Lanes, 3-7) compared to the wild type (Lane, 2), amplified by primers flanking *groESp*.

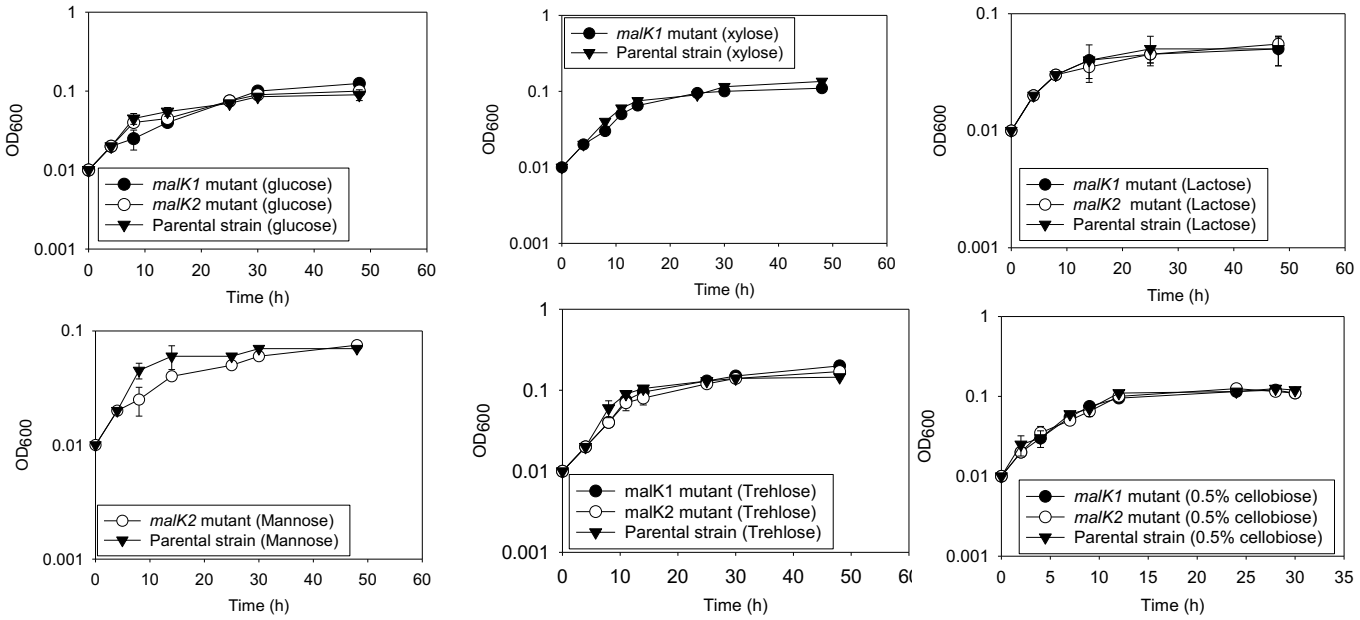

Figure S2. Growth physiology of *malK* mutants in DM supplemented with sugars. All tubes were incubated at 80°C and cell densities were determined at the times indicated.