

Ligands of Thermophilic ABC Transporters Encoded in a Newly Sequenced Genomic Region of *Thermotoga maritima* MSB8 Screened by Differential Scanning Fluorimetry^{∇†}

Nathalie Boucher and Kenneth M. Noll*

Department of Molecular and Cell Biology, University of Connecticut Storrs, Connecticut 06269-3125

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The chromosome of *Thermotoga maritima* strain MSB8 was found to have an 8,870-bp region that is not present in its published sequence. The isolate that was sequenced by The Institute for Genomic Research (TIGR) in 1999 is apparently a laboratory variant of the isolate deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 3109) in 1986. This newly sequenced region from the DSMZ culture was located between TM1848 (*cbp*, cellobiose phosphorylase) and TM1847 (the 3' end of a truncated ROK regulator). The new region contained seven genes: a beta glucosidase gene (*bglA*), three trehalose ABC transporter genes (*treEFG*), three xylose ABC transporter genes (*xylE2F2K2*), and the 5' end of a gene encoding the ROK regulator TM1847. We present a new differential scanning fluorimetry method using a low pH that was necessary to screen potential ligands of these exceptionally thermostable periplasmic substrate-binding proteins. This method showed that trehalose, sucrose, and glucose stabilized TreE, and their binding was confirmed by measuring changes in intrinsic fluorescence upon ligand binding. Binding constants of 0.024 μ M, 0.300 μ M, and 56.78 μ M at 60°C, respectively, were measured. XylE2 ligands were similarly determined and xylose, glucose, and fucose bound with K_d (dissociation constant) values of 0.042 μ M, 0.059 μ M, and 1.436 μ M, respectively. Since there is no discernible phenotypic difference between the TIGR isolate and the DSMZ isolate despite the variance in their genomes, we propose that they be called genomovars: *T. maritima* MSB8 genomovar TIGR and *T. maritima* MSB8 genomovar DSM 3109, respectively.

In the course of examining the evolution of maltose ABC transporters among members of the order *Thermotogales*, we noticed that many of the species for which genome sequences were determined have three members of this family (16). The genome of the first sequenced species, *Thermotoga maritima* strain MSB8, was exceptional in having only two *mal* operons, *mal1* and *mal2*. The arrangement of genes adjacent to the *mal3* orthologs in several *Thermotoga* species is similar to that near TM1848 in the *T. maritima* genome, but there is no Mal3 transporter in that region of the sequenced *T. maritima* genome. In 1994, Liebl et al. (8) published the sequence of a portion of a *malE* gene that they found adjacent to a beta glucosidase gene (*bglA*) that they cloned and characterized in the same study. When we searched GenBank for homologs of the MalE3 proteins of the *Thermotoga* species, we found that Liebl et al.'s MalE was a strong match, more so than the *T. maritima* MalE1 and MalE2. A *bglA* gene is upstream of the *malE3* in all the other *Thermotoga* species, so we speculated that the region containing the *T. maritima* *mal3* ortholog had been deleted during cultivation of that strain (16). This report confirms that hypothesis and demonstrates that the isolate that was sequenced was a laboratory variant of *Thermotoga maritima* MSB8 and should be designated as genomovar TIGR.

The original isolate deposited at the DSMZ culture collection, genomovar DSM 3109, contains the complete third *mal* transporter operon. Another ABC transporter operon was also found, as was Liebl et al.'s *bglA* gene, which was also missing from the *T. maritima* MSB8 genomovar TIGR genome sequence published in 1999 (14).

To examine the functions of these two new transporters, we sought a method to screen the binding properties of their periplasmic substrate-binding proteins. Differential scanning fluorimetry (DSF) can provide a rapid screening assay, but these highly thermostable proteins do not denature at temperatures achievable by commonly used instruments. We present here a modification of typical DSF methods that can be applied to binding studies of extremely thermostable proteins. This assay allowed us to more easily screen many sugars and to narrow the number of ligands needed to measure ligand-binding affinities using intrinsic fluorescence. These results expand our knowledge of the physiological capabilities of this organism and also provide a cautionary tale for genome-sequencing projects in that cultivation can create genome variants that may confuse investigations of metabolic function.

MATERIALS AND METHODS

Strains. *Thermotoga maritima* MSB8 genomovar DSM 3109, *Thermotoga petrophila* RKU-1 (DSM 13995), and *Thermotoga naphthophila* RKU-10 (DSM 13996) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Thermotoga maritima* MSB8 genomovar TIGR and *Thermotoga* sp. strain RQ2 were kindly provided by Karl O. Stetter. *Thermotoga neapolitana* NS-E was provided by the late Holger W. Jannasch.

PCR amplification and DNA sequencing. The region between TM1848 and TM1847 was amplified by PCR using the IDUUltra *Taq* DNA polymerase enzyme

* Corresponding author. Mailing address: University of Connecticut, Department of Molecular and Cell Biology, Unit 3125, 91 N. Eagleville Rd., Storrs, CT 06269-3125. Phone: (860) 486-4688. Fax: (860) 486-4331. E-mail: kenneth.noll@uconn.edu.

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kit (ID Labs Biotechnology, Inc.) using the primers listed in Table S1 in the supplemental material, and the products were amplified using an MJ Mini thermocycler.

The 10-kb PCR product from *T. maritima* MSB8 genomovar DSM 3109 was sequenced using a primer-walking method. After amplification as described above, the purified PCR product was sequenced using the primers listed in Table S2 in the supplemental material. To increase the reliability of the sequences, at least two identical sequences were obtained and sequencing was performed on both DNA strands. All sequencing was done by capillary electrophoresis on a 3130xl genetic analyzer (Applied Biosystems).

Fragments (1,478 bp) of the 16S rRNA genes from both *T. maritima* MSB8 genomovars were sequenced using two sets of primers (see Table S1 in the supplemental material) and the same PCR conditions used above but with IDproof *Taq* DNA polymerase (ID Labs Biotechnology, Inc.). The purified PCR products (QiaQuick PCR purification kit; Qiagen) were used as templates in sequencing reactions containing a single primer (see Table S1) and BigDye Terminator v3.1 (Applied Biosystems).

Prediction software. Open reading frames (ORFs) were predicted using GeneMark (10) and ORF Finder. The operons were predicted by FGENSEB. The transcription and translation terminators were predicted by FindTerm and RibEX (1). Protein transmembrane helices were predicted by the TMHMM Server v2.0 (7).

Cloning, expression, and purification of TreE and XylE2. The putative substrate-binding protein-encoding genes *treE* and *xylE2* from *T. maritima* MSB8 genomovar DSM 3109 were amplified using the IDProof *Taq* DNA polymerase enzyme kit (ID Labs Biotechnology, Inc.) with the primers listed in Table S1 in the supplemental material. The genes were cloned into the pET15b vector and the pET15b-*treE* and pET15b-*xylE2* plasmids were transformed in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL competent cells (Stratagene) for expression. All clones were validated by sequencing. The periplasmic substrate-binding protein genes *malE1* and *malE2* were cloned as previously described (13).

The recombinant proteins were extracted and purified according to the following protocol for the ligand screening. Cells were grown in an LB medium containing ampicillin (50 µg/ml) for 4 to 5 h and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 16 h at 24°C. In a typical experiment, cell pellets of 0.1 g to 0.5 g were washed with 5 ml of phosphate buffer and lysed using the B-PER bacterial protein extraction kit (Pierce), and the overexpressed His-tagged proteins were purified with a nickel-Sepharose high-performance column (GE) according to the manufacturer's protocol. The recombinant TreE tended to aggregate, especially at 4°C. This phenomenon was also observed for the related trehalose/maltose binding protein (TMBP) from *Thermococcus litoralis* (5). To prevent this, the cell lysate containing TreE was treated with RNase A (Qiagen) and DNase I (Pierce) (5). The proteins were dialyzed against 2 liters of buffer (20 mM sodium phosphate, pH 7.4) at room temperature.

Differential scanning fluorimetry screening of ligand binding. The samples were mixed to a final volume of 20 µl, according to the following preparation unless otherwise indicated: 2.5 µg protein, 4 µl 100 mM citric acid buffer (pH 3.50 to 3.75), 0.032 µl 5,000× Sypro orange (Invitrogen), 1.2 µl 2.5 M NaCl, and 2 µl ligand, typically with a final concentration of 10 mM ligand. The final concentrations of cellobiose, mannan, pullulan, and sorbitol were 25 µM, 0.225% (wt/vol), 0.5% (wt/vol), and 0.1% (wt/vol), respectively. The fluorescence intensities were measured using an iCycler IQ real-time detection system with excitation at 490 nm and emission at 520 nm. The samples were heated from 25 to 94°C with a heating rate of 0.5°C per min. All the assays were done in triplicate.

The midpoint temperature of the unfolding transition (T_m) was obtained with the program GnuPlot from curve fitting to a Boltzmann equation (15) as follows:

$$y = L + \frac{(U - L)}{1 + \exp\left(\frac{T_m - x}{a}\right)}$$

where L , U , and a are the minimum and maximum fluorescence intensities and the slope of the curve, respectively. The ΔT_m of the protein for a specific ligand was calculated as the difference between the T_m values of the ligand-bound and ligand-free proteins.

Sugar purity. The purity of the sugars was at least 98% unless noted otherwise. Arabinose, melibiose, raffinose, cellobiose, sorbitol, tagatose, lactose, ribose, trehalose, maltose, L-fucose, xylose, fructose, galactose, L-rhamnose, myo-inositol, glucose, sucrose, mannan, mannose, α-1,4-maltotetraose (95%), and pullulan were obtained from Sigma-Aldrich. The xyloglucan oligosaccharide (95%), β-1,4-mannotriose (95%), and β-1,4-mannotetraose (95%) were supplied from Megazyme, and the α-1,4-maltotriose (97%) was from ICN.

Intrinsic fluorescence spectroscopy. All fluorescence measurements were performed using an SLM Aminco-Bowman 2 spectrofluorimeter. The protein sam-

ples were incubated at 60°C and the temperature of the cuvette with the sample was equilibrated at 60°C for 5 min. Fluorescence emission spectra were measured at an excitation wavelength of 280 nm for XylE2 and 295 nm for TreE, and the emission intensities were measured over the wavelength range of 300 to 400 nm. The dissociation constants were measured by adding increasing amounts of selected carbohydrates into a stirred cuvette at 60°C. In the experiments, 3-µl portions of different stock solutions of the carbohydrate were added to 1.5 ml of 0.45 µM or 0.08 µM protein solution (20 mM sodium phosphate, pH 7.4). After the addition of the sugar, the sample was stirred for 2 min to reach equilibrium and then the fluorescence intensity at the predetermined emission wavelength was recorded for 45 s.

The K_d values of TreE for trehalose and XylE2 for glucose and xylose were obtained from curve fitting using KaleidaGraph to the equation accounting for ligand depletion as follows: $F = F_0 + \Delta F/2P_t [(K_d + P_t + L_t) - (K_d + P_t + L_t)^2 - 4P_t L_t]^{1/2}$, where F is the measured fluorescence at ligand concentration L_t and P_t is the total protein concentration (3, 19). This formula was used since the protein concentration was greater than the measured K_d . Other K_d values were obtained from curve fitting using GraphPad Prism to the following equation: $F = (F_{\max} \cdot L)/(K_d + L)$, where F is the measured fluorescence at ligand concentration L .

Nucleotide sequence accession number. The genomic sequences between TM1848 and TM1847 of genomovar DSM 3109 were deposited in GenBank as JF907620.

RESULTS AND DISCUSSION

Identification of the two genomovars of *Thermotoga maritima* MSB8. PCR amplifications with oligonucleotides that hybridized to the loci TM1848 (*cbp*) and TM1847 (ROK) were performed using DNA isolated from the *T. maritima* strain used to prepare DNA for the TIGR sequencing project (14) and from the strain obtained from the DMSZ collection (DSM 3109). The amplification from the inoculum used for the TIGR sequencing project gave a fragment of 1,216 bp, as predicted from the genome sequence, while the amplification from the DMSZ strain gave a fragment of approximately 10 kb (see Fig. S1 in the supplemental material). Other *Thermotogales* species gave fragments of sizes predicted by their reference genome sequences (see Fig. S1).

To ensure that both cultures were genuinely *T. maritima* MSB8, the 16S rRNA gene of each culture was sequenced. Both sequences were identical and matched with 100% identity to the sequence of the 16S rRNA gene deposited in 1987 as *Thermotoga maritima* MSB8 DSM 3109 (M21774.1). This demonstrated that these two cultures represent genomic variants or genomovars of *T. maritima* MSB8. We propose to designate the strain deposited at the DSMZ collection as *Thermotoga maritima* MSB8 genomovar DSM 3109 while the variant used by TIGR for its genome-sequencing project will be referred as *Thermotoga maritima* MSB8 genomovar TIGR.

Seven ORFs in the region between TM1848 and TM1847 deleted from genomovar TIGR. The sequence of the deleted region was completed by primer walking using the 10-kb PCR product from *T. maritima* MSB8 genomovar DSM 3109 as the template. This genomovar was found to have an additional 8,870 bp of genomic DNA between the loci TM1848 (*cbp*) and TM1847 (ROK). This region is missing from the genome of the genomovar TIGR between the positions 1,827,804 and 1,827,805. The new sequence has been deposited in GenBank as JF907620. This additional genomic DNA contains seven ORFs as predicted by GeneMark.hmm 2.4. A comparison of this region with the syntenic regions of other *Thermotoga* species shows that they all have similar genes at this location (Fig. 1), suggesting that genomovar DSM 3109 lost 8,870 bp of

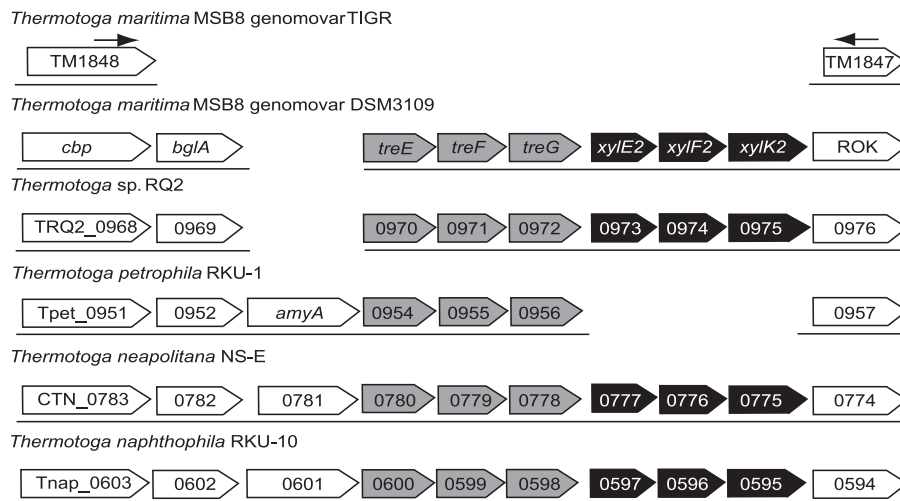


FIG. 1. Gene organization in syntenic regions of *Thermotoga* species in the vicinity of the TM1848 and TM1847 orthologs. The arrows represent the primer annealing sites used for PCR amplification of the 10-kb region. The *tre* operon is represented by gray boxes, while the *xyl2* operon is represented by black boxes.

genomic DNA, giving rise to genomovar TIGR during laboratory cultivation.

The first ORF encodes a beta glucosidase (BglA) that was first characterized in 1994, before publication of the genome sequence (8). The sequence matches with 100% identity to the sequence in GenBank deposited by W. Liebl (gi395290). The region downstream of *bglA* contains a palindromic sequence that suggests the presence of a rho-independent transcription terminator.

The next three ORFs encode a putative member of the maltose ABC transporter family and will be designated as a trehalose ABC transporter system (Tre). *T. maritima* has two known maltose operons, *mal1* and *mal2*, that were previously characterized (13). As we demonstrate below, the newly discovered *mal3* operon encodes a transporter with a binding preference for trehalose, so we propose that this be called the *tre* operon. It encodes a periplasmic substrate-binding protein (TreE) and two transmembrane proteins (TreF and TreG) (Fig. 1). Like the *mal1* and *mal2* operons and several other ABC transporter operons in *T. maritima* and other organisms, such as the *E. coli* *malEFG* operon, the *tre* operon contains no gene encoding an ATP binding protein. The newly discovered *tre* operon is only distantly related to the two *mal* *T. maritima* operons and is more closely related to the *mal3* operons previously found in most other *Thermotogales* species, which are also related to the archaeal *Thermococcus litoralis* and *Pyrococcus furiosus* trehalose/maltose transporters (16) (data not shown). Unlike those archaeal trehalose/maltose binding proteins, TreE does not contain a lipid binding site at its N terminus that anchors it to the membrane (5). Both TreF and TreG proteins have six putative transmembrane helices.

The fourth, fifth, and sixth ORFs encode a second putative ABC transporter system containing a periplasmic substrate-binding protein, a transmembrane protein with eight putative transmembrane helices, and an ATP-binding protein with two nucleotide-binding domains (Fig. 1). The substrate-binding protein of this ABC transporter system is homologous to TM0114 (XylE1), a substrate-binding protein that binds both

glucose and xylose (12, 20). This suggests that this ABC transporter operon should be named *xyl2*.

TM1847 encodes a putative transcription factor that belongs to the ROK (Repressor, ORF, Kinase) family. However, the 5' portion of the gene is deleted in genomovar TIGR. As in other *Thermotoga* species, genomovar DSM 3109 contains a full ORF likely to encode a functional protein (Fig. 1) that could possibly regulate the Xyl2 transporter.

Binding properties of TreE and XylE2. (i) Thermal stabilities. To identify the ligands that interact with the substrate-binding proteins encoded in this region, we used differential scanning fluorimetry (DSF). DSF analysis of ligand binding is based on the concept that proteins are stabilized by their ligands, and the difference between the denaturation temperatures of the ligand-bound and ligand-free forms, their ΔT_m , can be measured (9, 11, 15, 17). A ΔT_m close to zero indicates no thermal stability change in the presence of a ligand, while a positive value points to an interaction between the protein and the ligand. DSF can be performed in a real-time PCR thermocycler that records the increase of fluorescence intensity of the dye Sypro orange as the dye binds to hydrophobic residues that are revealed as the protein unfolds (15).

The substrate-binding proteins from *T. maritima* are extremely thermostable, and their unfolding temperatures (T_m) are typically above 94°C, the temperature limit for the PCR thermocycler (2, 20, 23). The T_m can be lowered using mildly denaturing conditions. We determined that an acidic buffer was the best denaturant for these thermophilic substrate-binding proteins. Using a citric acid buffer (pH 3.50 to 3.75), the T_m of MalE1 was linearly correlated with the pH, with an R^2 of 0.99 (see Fig. S2 in the supplemental material).

Prior to using this technique with the newly discovered substrate-binding proteins, we tested the ligand-induced stability of recombinant MalE1 and MalE2 from *T. maritima*. These proteins were previously characterized and their apparent dissociation constants (K_d) were determined using fluorescence spectroscopy (13, 23). MalE1 binds maltotriose, maltose, and mannotetraose with respective K_d values of 0.008 μ M, 24 μ M,

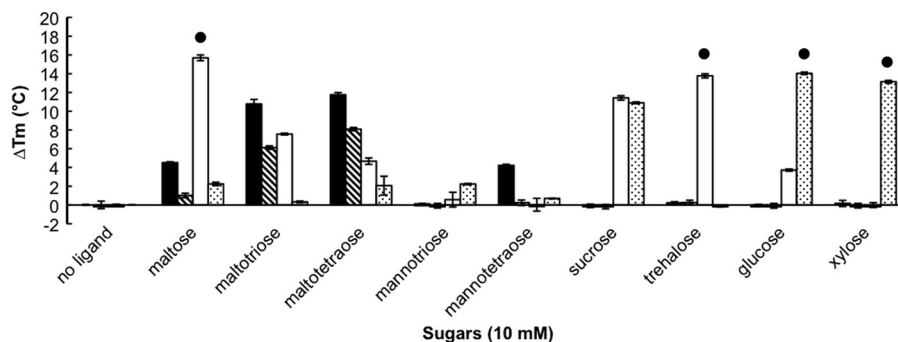


FIG. 2. Thermal stability of the following recombinant proteins of *T. maritima* determined by differential scanning fluorimetry: MalE1 (black), MalE2 (crosshatched), TreE (white), and XylE2 (stippled). ●, actual ΔT_m is greater than or equal to the value indicated.

and 38 μM (13). At 10 mM sugar, we observed that maltotriose, maltose, and mannottetraose increased the thermal stability of MalE1 with ΔT_m values of 10.77°C, 4.52°C, and 4.22°C, respectively (Fig. 2; also see Table S3 in the supplemental material). MalE2 binds maltose, maltotriose, and trehalose with respective K_d values of 8.4 μM , 11 μM , and 9.5 μM , but mannottetraose was not a ligand (13). At 10 mM sugar, we observed that maltose and maltotriose increased the thermal stability of MalE2 with ΔT_m values of 1.02°C and 6.85°C, respectively, while the ΔT_m value in the presence of 10 mM mannottetraose was 0.24°C (Fig. 2; also see Table S3). Although trehalose binds to MalE2 (13), it did not increase the thermal stability of MalE2 ($\Delta T_m = -0.05^\circ\text{C}$) (Fig. 2; also see Table S3). Trehalose is stable at high temperatures and low pHs (4), and so hydrolysis does not account for our observation. Instead, it is likely that the tertiary structure of MalE2 did not offer sufficient contacts between the sugar and the binding pocket to increase its stability in the presence of trehalose. Trehalose could have remained bound to the unfolded MalE2, leading to a lower ΔT_m value (9). Interestingly, maltotetraose, a sugar that was not previously tested, stabilized both MalE1 and MalE2 with ΔT_m values of 11.75°C and 8.67°C, respectively (Fig. 2; also see Table S3). No change in the thermal stability of MalE1 and MalE2 was recorded in the presence of other sugars (see Table S3). Thus, our ligand-screening assay gave no false-positive results and one false-negative result and revealed one potentially new ligand for these two previously studied proteins.

To determine the protein-ligand interactions of the newly identified substrate-binding proteins, the genes encoding TreE and XylE2 were expressed in *E. coli*. We screened 26 sugars with each protein by using DSF (see Table S3 in the supplemental material). We observed that the thermal stability of TreE was increased the most in the presence of 10 mM trehalose and maltose (Fig. 2; also see Tables S3 and S4). The upper values of the unfolding transition curves for both proteins were above 94°C, so their exact ΔT_m values could not be determined. TreE was also stabilized with 10 mM sucrose, maltotriose, maltotetraose, and glucose with ΔT_m values of 11.41°C, 7.57°C, 4.67°C, and 3.72°C, respectively (see Table S3). XylE2 was best stabilized by 10 mM glucose and xylose (Fig. 2; also see Tables S3 and S5) and was also stabilized with 10 mM sucrose, 10 mM L-fucose, 2.5 mM cellobiose, and 10

mM *myo*-inositol with ΔT_m values of 10.89°C, 8.82°C, 6.03°C, and 5.41°C, respectively (see Table S3).

(ii) Intrinsic fluorescence spectroscopy. Fluorescence spectroscopy was used to verify that the substrate-binding proteins bind the sugars identified with DSF and to measure their binding affinities. The K_d values were determined at 60°C, the highest temperature that the instrument could reach. TreE binds trehalose, sucrose, and glucose with K_d values of 0.024 μM , 0.300 μM , and 56.78 μM , respectively (Table 1). Fluorescence emission spectra were measured at an excitation wavelength of 280 nm and 295 nm to monitor aromatic residues and tryptophan, respectively. Despite this, no fluorescence changes were observed with maltose and maltotriose (data not shown). TreE's unfolding temperature (ΔT_m) was dependent on maltose concentration (see Table S4 in the supplemental material), and phylogenetic analyses show that its closest relatives transport maltose (data not shown). Taken together, these results suggest that TreE binds maltose. Changes in intrinsic fluorescence cannot always be observed during binding as found with glucose binding by *T. maritima* XylE1 (12, 20).

XylE2 binds glucose, xylose, and L-fucose with K_d values of 0.059 μM , 0.042 μM , and 1.436 μM , respectively (Table 1). Although sucrose, *myo*-inositol, and cellobiose stabilized XylE2 to heat denaturation, the K_d values for these ligands were relatively high at 116.4 μM , 56.47 μM , and 3.955 μM , respectively. It is likely these K_d values resulted from binding by the high-affinity ligand glucose contained as minor impurities in the sucrose, *myo*-inositol, and cellobiose rather than binding by those compounds.

TABLE 1. Apparent K_d values at 60°C of TreE and XylE2 for different sugars as measured by changes in intrinsic fluorescence upon ligand binding

Protein	Sugar	K_d value at 60°C (μM)
TreE	Trehalose	0.024
	Sucrose	0.300
	Glucose	56.78
XylE2	Glucose	0.059
	Xylose	0.042
	L-Fucose	1.436

***T. maritima* strain MSB8 genomovars.** The term genomovar was introduced by R. Rosselló in 1991 to describe different variants, or genomic groups, of *Pseudomonas stutzeri* (18). The term genomovar can be used for those strains exhibiting genomic variations yet with no detectable phenotypic differences that would allow them to be classified as separate species (21, 22). We demonstrated that the *T. maritima* isolate used for the genome-sequencing project published in 1999 (14) is a variant that lost 8,870 bp of genomic DNA between the loci TM1848 and TM1847 when compared to the isolate deposited in the DSMZ strain collection in 1986 (6). Both genomovars of *T. maritima* MSB8 have the same 16S rRNA gene sequence and no discernible phenotypic differences. We propose to designate the type strain (DSM 3109) as *T. maritima* MSB8 genomovar DSM 3109 and the sequenced variant as *T. maritima* MSB8 genomovar TIGR. The 8,870-bp region is largely syntenic with regions from other *Thermotoga* species that also have a *bglA* gene and a putative *tre* operon, suggesting that *T. maritima* MSB8 genomovar TIGR lost these genes.

A sequencing project for *T. maritima* genomovar DSM 3109 is in progress at the Joint Genome Institute (JGI). Upon its completion, the genome of the original isolate *T. maritima* MSB8 genomovar DSM 3109, deposited at the DSMZ culture collection, will be available in GenBank. The sequence of the genomovar DSM 3109 will likely provide new insights into the genetics, physiology, and metabolism of *Thermotoga maritima* MSB8.

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