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Whole-Genome Expression Profiling of *Thermotoga maritima* in Response to Growth on Sugars in a Chemostat

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To provide data necessary to study catabolite-linked transcriptional networks in *Thermotoga maritima*, we used full-genome DNA microarray analysis of global transcriptional responses to growth on glucose, lactose, and maltose in a chemostat. A much larger number of genes changed expression in cells grown on lactose than on maltose, each relative to genes expressed in cells grown on glucose. Genes encoding putative oligopeptide transporters were often coregulated with adjacent glycosidase-encoding genes. Genes encoding enzymes catalyzing NADH oxidation were up-regulated on both lactose and maltose. Genes involved in iron and sulfur metabolism were differentially expressed in response to lactose. These data help define the sets of coregulated genes and suggest possible functions for their encoded products.

Thermotoga maritima belongs to a lineage of hyperthermophilic heterotrophs that, as indicated by many measures, diverged from the common ancestor of bacteria before other heterotrophic bacteria (1, 2). *T. maritima* grows on a variety of sugars transported by ATP-binding protein cassette (ABC) transporters but not by phosphoenolpyruvate phosphotransferase systems (10, 17). In many organisms catabolite transport is linked to transcriptional control of gene expression, but little is known about such regulation in *T. maritima* or other members of the *Thermotogales*. A close relative, *Thermotoga neapolitana*, prioritizes its carbon sources in a cyclic AMP (cAMP)-independent manner and regulates expression of some of its carbohydrases in response to its carbon source (16, 18, 30). The genome sequence of *T. maritima* shows no evidence of cAMP-dependent regulatory systems, suggesting that this mechanism is absent from this lineage. These facts show that *T. maritima* may provide a unique example of how mechanisms that regulate the transcription of genes in response to carbon source arose in organisms with relatively simple catabolite transporters. Consequently, information gleaned from examinations of regulatory phenomena in *T. maritima* may shed light on how complex catabolite-responsive regulatory networks arose in other bacterial lineages. The study described here helps lay the groundwork for such examinations by observing the global transcriptional response to three sugar growth substrates under highly controlled growth conditions.

Seven percent of the *T. maritima* open reading frames (ORFs) encode proteins involved in the metabolism of sugars, more than twice the fraction found in other bacteria and archaea (17). Thus the transcription of a significant fraction of its genes can be expected to respond to carbon source availability. The transcriptional responses of a selected set of *T. maritima* genes have been examined with DNA microarrays containing a subset of genes (3). Those analyses targeted many of the genes that comprise that 7% of the genome. One of our goals was to identify transcriptional responses of genes that are not obvious components of sugar catabolism networks, including genes encoding putative transcriptional regulatory proteins and a set of transporter genes with closest sequence homologies to archaeal genes. These transporters were annotated as oligopeptide ABC transporters (17), but evidence gathered for some archaeal homologs suggests that they may actually transport sugars (8, 11). To observe the patterns of transcriptional responses of genes predicted to respond to catabolites as well as those of unknown function requires the global view of expression afforded by full-genome DNA microarrays. The analyses presented here are the first reported examination of global gene expression in *T. maritima* using a genome DNA microarray representing 1,865 ORFs (99% of the ORFs identified in its genome sequence).

Continuous culture provides cells of a uniform physiological state for global transcription analyses. For this investigation we chose to grow cells in continuous culture to provide cells grown at the same specific growth rate regardless of the supplied growth substrate. Cells grown under controlled, reproducible conditions are especially important for studies of whole-genome expression since growth rate can play a major role in transcriptional control systems (19, 20). Our continuous-culture apparatus was constructed from common laboratory components. Details of its construction and operation can be obtained online at http://www.tigr.org/microarray/Thermotoga.

T. maritima cells were grown in a medium slightly modified from that used for *T. neapolitana* (6). Cells were grown at experimentally determined limiting ammonium chloride (0.15 g/liter) and excess sugar (5 g/liter) concentrations. We used nitrogen rather than carbon limitation since limitation for a particular sugar can lead to expression of genes necessary for growth on other substrates and so confound interpretation of gene expression patterns (7, 12, 21).

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The continuous-growth apparatus consisted of a 250-ml growth chamber with no gas headspace; the culture was mixed with a Teflon-coated stir bar. Anaerobic medium kept under a blanket of nitrogen was fed into the reactor, where it was maintained at 77°C. The medium pH was maintained by 100 mM HEPES at pH 7, as determined by monitoring the effluent. Cells were grown at a dilution rate of 0.1 h^{-1}. When the culture reached steady-state growth, cells were collected from the reactor effluent in 12-ml aliquots in sterile tubes immersed in a dry ice-ethanol bath to immediately freeze the cells (<1 min). Total RNA was extracted from six separate cultures (two for each carbon source) with Trizol reagent (Invitrogen, Carlsbad, Calif.), treated with RQ1 DNase (Promega, Madison, Wis.), and purified with a QIAGEN RNeasy minikit.

Microarray data show that growth on lactose elicits a more diverse transcriptional response than growth on maltose. The entire microarray procedure is described in detail at http://www.tigr.org/microarray/Thermotoga. Genomic DNA from the *T. maritima* strain MSB8 sequenced at The Institute for Genomic Research (TIGR) was used as a template for amplification of unique internal segments of 1,865 ORFs (representing 99% of the total 1,877 identified *T. maritima* ORFs), which were printed onto UltraGAPS microarray slides (Corning Life Sciences, Acton, Mass.) with a Molecular Dynamics Generation III array spotter (Amersham Biosciences, Piscataway, N.J.). Hybridization probes were prepared by indirect labeling of total RNA with either Cy3 or Cy5 dyes, leading to production of 3 μg of cDNA with 150 to 170 pmol of dye nucleotide incorporated per microgram of cDNA produced. All hybridizations were done as previously described (14) in pairs of flip-dye experiments with 13 total hybridizations for the comparison of maltose versus glucose and 16 for the comparison of lactose versus glucose. TIFF images of the hybridized arrays were analyzed with TIGR-Spotfinder, version 2.0.2, software (www.tigr.org/software), and the data sets were normalized by applying the LOWESS algorithm (block mode; smooth parameter: 0.33) with TIGR-MIDAS, version 2.2 (www.tigr.org/software), software. Flip-dye replicate spots that had Cy3-to-Cy5 ratios beyond 2.0 standard deviations from the average ratio were discarded, and statistical analysis was performed on $\log_2$-transformed signal ratios of the remaining replicates by using the Significance Analysis of Microarrays (version 1.21; EXCEL add-in version) procedure developed by Tusher et al. (28) and available at www-stat.stanford.edu/~tibs/SAM/index.html. The false-discovery rate was 1.5%, and only genes exhibiting at least a twofold change in expression are reported.

Dramatically different patterns of gene expression were observed in cells grown on lactose and maltose in comparison with cells grown on glucose, demonstrating that *T. maritima* responds very differently to these two disaccharides. Detailed microarray results are available at http://www.tigr.org/microarray/Thermotoga. In maltose-grown cells, the expression of only 24 genes changed, while 106 genes were differentially regulated after cells switched to growth on lactose. This shows that, at the transcriptional level, maltose catabolism in *T. maritima* is not very different from glucose catabolism, while more adjustments are required when lactose is the carbon source. We can say with confidence that the observed changes in gene expression were in response to the carbon source provided and not to a more general catabolite starvation response because cell growth was limited by nitrogen in all cases and not by the supplied sugar. We cannot, however, conclude that the responses were for lactose and maltose specifically, but may be responses to those general classes of sugars (1,4-β-galactosides and 1,4-α-glucosides, respectively).

Genes one might predict to respond to the supplied sugar did not always do so. First, although *galK* (galactose kinase, TM1190) and *galT* (uridylyltransferase, TM1191) were up-regulated in lactose-grown cells compared with glucose-grown cells, the epimerase gene, *galE* (TM0509), was not. This may reflect the need for GalK and GalT for galactose catabolism, while GalE may play an additional, constitutive role in lipopolysaccharide or exopolysaccharide synthesis. Second, as described below, the expression of several genes encoding sugar hydrolases unrelated to lactose changed in response to growth on lactose, a phenomenon similarly observed in *T. maritima* cells grown on cellulose (3). Finally, genes one might expect to be up-regulated by maltose growth, encoding enzymes involved in the conversion and storage of maltose to malto-oligosaccharides and/or maltodextrin and the conversion of glucose-1-phosphate to glucose-6-phosphate (*mgtA* [TM0364] and *mmtA* [TM0767, TM1168, and TM0184]) were not noticeably induced by maltose. This perhaps reflects a constitutive role for them in storage polysaccharide synthesis. Surprisingly, the expression of genes encoding α-glucosidases was also not affected by growth on maltose. Apparently genes specific for maltose catabolism are not subject to catabolite repression by glucose as are some genes necessary for lactose catabolism.

Validation of microarray expression data by complementary methods. We used real-time quantitative PCR to provide an independent assessment of gene expression for selected genes. Real-time PCR was performed with QuantiTect SYBR Green PCR reagents (QIAGEN) and a Bio-Rad iCycler iQ thermal cycler. Selected genes found to be differentially expressed by the microarrays were examined along with six control genes with microarray ratios around 1 (i.e., unchanged) (*lysC1*, TM0547; *gap*, TM0688; *rpsB*, TM0762; *eno*, TM0877; *deoD2*, TM1737; and *recA*, TM1859). The correlation coefficients of the standard curve were typically at least 0.995. The starting quantities of transcript determined by the iCycler software were normalized with the geNorm applet for MS Excel (29). The mean expression ratios of the differentially expressed genes obtained by real-time PCR are listed in Table 1. Real-time PCR analyses showed that the relative expression patterns of all the examined genes agreed with those measured with DNA microarrays. Real-time PCR showed that *pdhD* (TM0381) and *nox* (TM0379) are up-regulated in both lactose and maltose at low levels, in qualitative agreement with microarray analyses. Microarrays showed that the increase in expression of *pdhD* in maltose-grown cells was only 1.61-fold (data not shown), while the increases of its expression in lactose and that of *nox* in both lactose and maltose were slightly greater than 2-fold.

We used Northern blot hybridizations to further confirm the microarray data and gain information about operon structures of selected genes. Total RNA used in Northern hybridizations and dot blotting was extracted as previously reported (18). Digoxigenin-labeled DNA hybridization probes were visualized with DIG Chem-Link reagent (Roche) on X-ray film. Five blots were sequentially hybridized to the specific probes for

TABLE 1. Summary of changes in expression of genes examined by DNA microarray, real-time PCR, and Northern and dot blot hybridizations

ORF, product	Change in expression[a] for growth on:							
	Lactose vs glucose				Maltose vs glucose			
	Microarray[b]	Real-time PCR[c]	Northern blot	Dot blot	Microarray[b]	Real-time PCR[c]	Northern blot	Dot blot
TM0031	0.30	0.60 ± 0.10	ND	ND	0.24	0.05 ± 0.02	ND	ND
TM0115	7.10	ND	ND	+	No change	ND	ND	∅
TM0310	4.05	18.91 ± 2.42	ND	+	No change	0.87 ± 0.49	ND	∅
TM0379, Nox	2.79	1.24 ± 0.06	ND	ND	2.49	1.7 ± 0.62	ND	ND
TM0381, PdhD	2.22	1.79 ± 0.83	ND	ND	No change	2.92 ± 1.79	ND	ND
TM1190, GalK	6.41	19.58 ± 11.13	No change	+	No change	0.56 ± 0.18	No change	∅
TM1191, GalT	6.65	ND	∅	ND	No change	ND	∅	ND
TM1192, GalA	6.23	ND	∅	ND	No change	ND	∅	ND
TM1193, LacZ1	5.45	11.13 ± 2.20	∅	+	No change	1.11 ± 0.64	∅	∅
TM1194	5.46	ND	∅	ND	No change	ND	∅	ND
TM1195, LacZ2	7.95	25.01 ± 4.97	++	+	No change	0.91 ± 0.23	∅	∅
TM1196	6.30	ND	+	ND	No change	ND	∅	ND
TM1197	7.16	ND	+	+	No change	ND	∅	∅
TM1198	6.79	ND	∅	ND	No change	ND	∅	ND
TM1199	8.26	ND	++	ND	No change	ND	+	ND
TM1200	2.83	ND	+	ND	No change	ND	No change	ND
TM1201	5.52	ND	+	ND	No change	ND	∅	ND
TM1202, MalG1	5.45	ND	+	ND	No change	ND	∅	ND
TM1203, MalF1	7.28	ND	+	ND	No change	ND	No change	ND
TM1204, MalE1	5.82	26.54 ± 6.48	+++	+++	No change	1.16 ± 0.46	No change	+
TM1216	3.55	3.36 ± 1.36	ND	ND	3.53	4.13 ± 2.20	ND	ND
TM1837, MalF2	<2	ND	ND	∅	No change	ND	ND	∅
TM1839, MalE2	2.40	41.57 ± 9.41	ND	++	3.28	26.12 ± 9.43	ND	++
TM1840, AmyA	<2	ND	ND	∅	No change	ND	ND	∅

[a] Symbols and abbreviations used in the table: +, up-regulation; ∅, signal below detection; ND, not determined (gene expression was not examined using that method). The degree of change was assessed arbitrarily; in the following definitions, x is the determined ratio for the respective method. No change, $2 > x > 0.5$ (microarray) or $1 > x > 0.5$ (real-time PCR); +, $4 > x \geq 2$ (microarray), $5 > x > 1$ (real-time PCR), or above background and stronger than the signal in glucose or no signal in glucose (dot blot and Northern blot); ++, $6 > x \geq 4$ (microarray), $10 > x > 5$ (real-time PCR), or good signal intensity (dot blot and Northern blot); +++, $x \geq 6$ (microarray), $x > 10$ (real-time PCR), or signal intensity is oversaturated (dot blot and Northern blot).

[b] The false-discovery rate was 1.5%.

[c] The expression ratios (± standard deviations) were calculated as means of the starting quantities of transcripts obtained with cells grown on either lactose or maltose divided by the means of those obtained from glucose-grown cells (from three assays).

three genes, and all blots were probed with the *gap* gene (TM0688) control in the last hybridization to ensure the presence of equivalent amounts of total RNA in all lanes. The expression of the genes in the cluster TM1190 to TM1204 was of particular interest because of their apparent operon structure yet varied encoded functions. Qualitative assessment of signal intensities indicated higher levels of expression in lactose of *malEFG1* (TM1204 to TM1202), *lacZ2* (TM1195), a *lacI* homolog (TM1200), the genes encoding the arabinogalactan endo-1,4-β-galactosidase (TM1201), and three of the five subunits of an oligopeptide ABC transporter (TM1199, TM1197, and TM1196) (Table 1). Except for the transcript of *galK* (TM1190), which showed very weak signal intensities, transcripts of the genes located at the 3′ end of this 15-gene cluster (*lacZ1*, *galA*, and *galT*; TM1193 to TM1191, respectively), could not be detected. The transcripts of TM1198 and TM1194, encoding the membrane-spanning and the ATP-binding subunits of the oligopeptide ABC transporter, also were not detected.

A third independent measure of gene expression in response to growth substrate was provided by dot blot analyses of 11 genes. The selection of the genes for dot blot hybridization was based on their annotated functions, rather than on the expression patterns detected by microarray hybridization to avoid choosing a data set biased by previous results. Serial dilutions of PCR products of the *gap* gene served as standards for normalization and quantification. PCR products of the 11 selected genes were spotted in six replicates and hybridized with total RNA labeled with digoxigenin (DIG Chem-Link; Roche). Chemiluminescence signal intensities were quantified with Molecular Analyst software (Bio-Rad), and intensity values were normalized by linear regression of the *gap* standard curve. Significant differential expression was detected with only two genes: those encoding the putative maltose binding proteins. A large increase in expression level of *malE1* (TM1204) was observed in cells grown on lactose, while *malE2* (TM1839) expression increased in both lactose- and maltose-grown cells, but to a lesser extent than expression of *malE1* (Table 1). Although expression levels of the remaining nine genes were below the range of the standard curve, qualitative assessments of their expression indicated up-regulation in lactose of *galK*, *lacZ1*, and *lacZ2* as well as genes encoding a sugar ABC transporter ATP-binding protein subunit (TM0115), an oligopeptide ABC transporter membrane-spanning subunit (TM1197), and a β-galactosidase (TM0310). The expression of *malG2* (TM1837) and *amyA* (TM1840) in all three conditions was below the detection limit. Expression of *bglA*, encoding a β-glucosidase, was also examined. This gene was found in *T. maritima* by Liebl et al. (13) (accession number X74163) but was not reported later in the annotated full-genome sequence

(17). A *bglA* transcript was detected, and its expression in response to all three carbon sources was at the same low level.

In summary, the relative levels of expression of genes in lactose- and maltose-grown cells versus levels in glucose-grown cells examined by all four methods showed good agreement (Table 1). Overall the patterns of increased or decreased expression levels were consistent across methods although for a few genes the change of gene expression seemed to be at the borderline of detection by real-time PCR. The differences in the degrees of expression between the methods are to be expected due to the differences in their sensitivities (15).

Coregulation of putative oligopeptide transporter genes with adjacent glycosidase genes. Genes encoding xylanase, xylosidase, β-galactosidases, α-glucosidase, α-galactosidase, β-glucosidase (*bglB*), and laminarinase (*lamA*) were differentially expressed in coordination with adjacent genes encoding putative oligopeptide ABC transporters. ORFs in three of these apparent operons (TM0071 to TM0075, TM1194, TM1196 to TM1199, and TM0027 to TM0031) were reported to be most closely related to those encoding archaeal transporters (17). Their coregulation with the above glycosidases suggests that in *T. maritima* the encoded transporter proteins may function as sugar transporters (in addition to or instead of oligopeptides). *Sulfolobus solfataricus* maltose and cellobiose binding proteins were shown to be homologous to oligopeptide binding proteins (8), as were a cellobiose binding protein of *Pyrococcus furiosus* (11) and an α-galactoside binding protein of *Rhizobium meliloti* (9). A number of other genes encoding putative oligopeptide transporters in *T. maritima* have previously been observed to be up-regulated in cells grown on mannose and cellulose (3). That study also showed that *lamA* and *bglB* were highly expressed in cells grown on laminarin and barley glucan while very low expression was seen in cells grown on glucose, starch, xylose, and β-xylan.

The region from *malE1* to *galK* (TM1204 to TM1190) contains one "archaeal" oligopeptide ABC transporter operon as well as genes encoding a bacterial maltose ABC transporter, a regulatory protein (LacI homolog), and several enzymes involved in galactoside catabolism. All 15 putative genes are transcribed in one direction (*malE1* to *galK*) with little intergenic space (some even overlap). We found that all these genes are up-regulated in lactose-grown cells, raising the possibility they are a single operon. Our Northern blots, however, detected numerous transcripts resulting from this region (not shown). The LacI homolog TM1200 could be an important transcriptional control element for this collection of genes including genes TM1194 and TM1196 to TM1199, derived from archaea. The archaeal transporter genes have clearly come under the control of bacterial promoters and regulatory elements, and the process of regulatory amelioration of horizontally transferred genes is an interesting subject for future investigations.

Possible regulatory links between sugar availability and intracellular redox processes. The expression of several genes with no obvious functional connection to catabolite availability was coordinately regulated. For example, in lactose-grown cells *pdhD* (TM0381), apparently encoding a dihydrolipoamide dehydrogenase (DHLD), was up-regulated. This gene was also previously shown to be differentially expressed in *T. maritima* cells grown on barley glucan, starch, cellulose, and xylose (3). In aerobes and facultative anaerobes this enzyme is a component of pyruvate and α-ketoglutarate dehydrogenases and is also involved in the biosynthesis of glycine and serine. *T. maritima* lacks both of these dehydrogenases (17, 25) and DHLD activity in glycine and serine biosynthesis does not explain the increase in expression of *pdhD* in lactose-grown cells. Connections between lipoamide dehydrogenase and galactose or maltose transport have been observed in *Escherichia coli* (24) and *Streptococcus pneumoniae* (26). *T. maritima* may also utilize this protein in a similar unknown process involving sugar metabolism and/or transport.

It is noteworthy that many of the genes whose expression was increased in both lactose- and maltose-grown cells encode enzymes involved in NADH oxidation, including six NADH dehydrogenase subunit-encoding genes and a *nox* gene encoding an NADH oxidase. In *T. neapolitana*, this NADH oxidase was shown to catalyze the reduction of polysulfide with electrons from NADH (4, 5). NAD-dependent redox enzymes may be differentially expressed as part of a mechanism controlling the activities of sugar hydrolases in response to changes in anabolic reduction charge. NAD^+ was shown to be an allosteric activator of the activities of an α-glucosidase (TM1834) and an α-glucuronidase (TM0752) (22, 23, 27) in *T. maritima*. Although TM1834 was not differentially expressed in the cells we examined, TM0752 was up-regulated in lactose-grown cells. Other sugar hydrolases may be similarly activated by NAD^+ so that anabolic reduction charge could play a role in controlling the catabolism of sugars.

Several genes encoding proteins involved in iron and sulfur metabolism were also differentially expressed in response to lactose. Down-regulated were genes in two apparent operons encoding proteins involved in the mobilization of sulfur atoms from cysteine to iron-sulfur clusters (Suf, TM1368 to TM1371), thiamine (ThiH), biotin (biotin synthetase, TM1269), and methionine (cystathionine gamma-synthase, TM1270). Also down-regulated were genes encoding an apparent ferrous ion transporter (FeoA and FeoB). However, several genes encoding proteins containing iron-sulfur centers were up-regulated in response to lactose, including two ferredoxins (TM1289 and TM1815), an unidentified iron-sulfur cluster binding protein (TM1291 and TM1292), and an NADH dehydrogenase (TM1211 to TM1216). These findings point to a complex regulatory interaction between carbon source availability and secondary metabolite (iron and sulfur) transformations.

Summary. Using four methods, including the first full-genome DNA microarrays, we examined the expression of 1,865 ORFs identified in the annotated genome sequence of *T. maritima* in cells grown on glucose, lactose, and maltose. This study provides a framework toward understanding its underlying mechanisms of regulating gene expression in response to growth substrate. Since cells were grown in nitrogen-limited continuous cultures, the observed patterns of gene expression were in response to the provided sugars. A much larger number of genes changed expression in cells grown on lactose than on maltose, each relative to genes expressed in cells grown on glucose. Among the few genes that were up-regulated on both disaccharides, those encoding enzymes carrying out NADH-requiring reactions were notable. Genes involved in iron and sulfur metabolism were differentially expressed in response to lactose. Further investigations are needed to determine

whether DHLD activity is related to sugar transport. This examination of the expression of virtually all the identified ORFs comprising the genome of *T. maritima* in response to growth substrate represents an important step toward learning how expression of its genes is coordinately regulated. This study provides testable hypotheses of possible functions for the products of many *T. maritima* genes and provides information necessary for future investigations into the evolution of mechanisms of catabolite-linked transcriptional controls in this hyperthermophile.

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