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ORIGINAL PAPER

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# Cloning and transcriptional analysis of the *Thermoanaerobacter ethanolicus* strain 39E maltose ABC transport system

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**Abstract** *Thermoanaerobacter ethanolicus* strain 39E is a Gram-positive thermophile that converts sugars resulting from plant carbohydrate polymer degradation into ethanol. A putative maltose ATP-binding cassette (ABC) transport operon was isolated with genes encoding for the integral membrane components (*malF* and *malG*); the ATP-binding protein (*malK*); and a partial gene for the maltose-binding protein (*malE*). This operon is unlike most other maltose transport operons, which do not contain a contiguous *malK* gene. Sequence analysis showed that the individual genes in the putative operon possessed a considerable range of similarities to their respective homologs in other eubacteria and archaea. MalK had 52% amino-acid identity and over 70% similarity with its homolog from the archaeon *Thermococcus litoralis*, while the membrane components and binding protein exhibited much less similarity with a range of other thermophilic eubacteria. Transcript was not detected in maltose-, glucose-, or xylose-grown cells using Northern blotting, but RT-PCR showed that *malFGK* were expressed in cells grown on maltose or xylose. Based on these results, the strain 39E maltose operon may be subject to glucose catabolite repression.

**Key words** ABC transport · Bioconversion · Maltose · *Thermoanaerobacter ethanolicus* · Transport · Transcript analysis

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## Introduction

*Thermoanaerobacter ethanolicus* produces considerable amounts of ethanol from a wide range of polymeric and soluble carbohydrates (Lynd 1989; Wiegel et al. 1983; Wiegel and Ljungdahl 1981). The type strain JW200 (Wiegel et al. 1979) and strain 39E (Zeikus et al. 1980) (formerly *Clostridium thermohydrosulfuricum*) were both first isolated from Yellowstone Park. Strain 39E was reassigned as *T. ethanolicus* (Lee et al. 1993), although both strains are very closely related to *Thermoanaerobacter brockii* (Breves et al. 1997). Genomic sequencing is probably required to further the phylogeny of the genus *Thermoanaerobacter* (J. Wiegel, personal communication).

Both strains, JW200 and 39E, have been studied in some detail and their high specific rates of ethanol production make the organism an attractive candidate for use in bioconversion processes (Hyun et al. 1985; Lacis and Lawford 1992). Previous physiological work in our laboratory (Jones et al. 2000) showed that strain 39E possessed a high-affinity binding protein and transport system specific for maltose and maltotriose, the major degradation products arising from starch hydrolysis. Maltose transporters have been characterized to varying degrees in other thermophiles (Liebl et al. 1997; Sahm et al. 1996; Xavier et al. 1996). All were binding-protein-dependent systems, a subfamily within the ATP-binding cassette (ABC) transporter superfamily (Higgins 1992). These systems are composed of a periplasmic or membrane-associated binding protein that transfers the substrate to integral membrane components which mediate ATP-dependent uptake, with substrate affinity and specificity mediated in large measure by the binding protein (Boos and Shuman 1998). The goals of the present experiments were to clone, sequence, and characterize the transcription of the genes responsible for maltose uptake in *T. ethanolicus* strain 39E.

## Materials and methods

### Primer design and DNA amplification parameters

Since the *T. ethanolicus* maltose transport system was previously biochemically defined as capable of transporting multiple sugars (Jones et al. 2000), primers were designed using a Megalign (DNAStar, Madison, WI, USA) alignment of the following proteins: *Escherichia coli* MalK [gi7428823], *Bacillus subtilis* YurJ [gi2635752], *Rhodobacter sphaeroides* SmoK [gi2338762], and *Mycobacterium tuberculosis* SugA [gi7442501]. Primers were designed from residues 37–44 and 229–238 of the alignment (ATPrelU and ATPrelD, respectively). *Bam*HI and *Xba*I restriction sites (underlined) were engineered into the 5′-ends of ATPrelU GTGGATCCNWSNGGNTGYGGNAA RWS and ATPrelD TTTTCTAGANWSDATR AAANC CNGCNACRAA, respectively. The predicted size of amplification product from *T. ethanolicus* genomic DNA was 603 bp.

Amplification reactions contained 200 µM dNTPs, 1.5 mM $MgCl_2$, 50 pmol ATPrelU and ATPrelD, 1.5 U *Taq* DNA polymerase, and 50 ng *T. ethanolicus* strain 39E (ATCC 33223) genomic DNA in reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.3)]. Samples were cycled for 1 cycle of 95°C for 5 min, 44°C for 1 min, 72°C for 1 min; 28 cycles of 95°C for 1 min, 44°C for 1 min, 72°C for 1 min; and 1 cycle of 95°C for 1 min, 44°C for 1 min, 72°C for 5 min. Negative control reactions contained all components except DNA. There were never any products from these control reactions.

### PCR product cloning and sequencing

The single DNA band (approximately 630 bp long) resulting from the PCR was isolated and purified using SeaPlaque GTG low-melting agarose (FMC, Rockland, ME, USA) and the Geneclean II kit (Bio101, La Jolla, CA, USA), then double digested with *Bam*HI and *Xba*I, and ligated to similarly-digested pUC18 to create pATP. The nucleotide sequence of pATP, purified using a Midi-Prep kit (Qiagen, Chatsworth, CA, USA), was determined to verify that it encoded a portion of an ATP-binding protein. DNA sequencing was performed using standard protocols (Ausubel et al. 1996). Computational analysis of DNA and protein sequences were performed with Lasergene, BLAST (Altschul et al. 1990), and ClustalW (Thompson et al. 1994).

### Isolation of putative transport maltose operon

Preliminary sequence analysis showed that the ATPrel PCR product most likely encoded a portion of the *T. ethanolicus malK* gene. A *T. ethanolicus* strain 39E genomic DNA library was screened as previously described to isolate the remainder of *malK* and any contiguous genes (Erbeznik et al. 1998). The ATPrel PCR product was used as the template in random primed labeling reactions (Life Technologies, Gaithersburg, MD, USA) and served as the probe for use in library screens as well as in Northern blotting.

### RNA extraction

Cells were grown to mid-logarithmic phase and harvested in RNase-free centrifuge tubes. Cell pellets were resuspended in RNAlater storage solution (Ambion, Austin, TX, USA) to an optical density (600 nm) of 50 and stored at –80°C. Total RNA was extracted using the RNAqueous kit (Ambion) following the manufacturer's protocol for Gram-negative bacteria. RNA was quantitated by spectrophotometry and samples were routinely electrophoresed to assess the quality of individual preparations.

### Northern analyses

A 1 µg aliquot of each RNA sample was electrophoresed under denaturing conditions and then transferred to a nylon membrane (Sambrook et al. 1989). The blots were prehybridized at 42°C for 1 h in NorthernMax solution (Ambion), hybridized overnight in the same solution at 42°C with probes adjusted to $1 \times 10^6$ counts per minute (CPM)/ml, washed twice for 15 min at 42°C in 2× SSC (300 mM NaCl, 30 mM sodium citrate), 0.1% SDS and exposed to film (Biomax MS; Eastman Kodak, Rochester, NY, USA) for 2 h.

### Reverse transcriptase-PCR (RT-PCR)

RT-PCR was used to analyze the transcription of the putative maltose operon. RNA was first treated with DNaseI to remove any residual DNA and then reverse transcribed into cDNA using the ThermoScript system (Life Technologies). A 2 µl aliquot of the cDNA synthesis reaction served as the template for amplification reactions that contained: 200 µM dNTPs, 1.5 mM $MgCl_2$ 25 pmol each primer, and 1 U *Taq* DNA polymerase. Reactions were amplified as follows: an initial denaturation at 95°C for 5 min; 23 cycles of 95°C for 15 s, 50°C for 15 s, 72°C for 30 s; and a terminal extension at 72°C for 2 min. Negative control reactions consisted of cDNA synthesis reactions that lacked reverse transcriptase. There were never any products visualized from control amplification reactions.

## Results

### PCR amplification with ATPrel primers and product sequencing

*T. ethanolicus* 39E genomic DNA was used as the template in an amplification reaction containing primers specific for

a portion of the putative *malK* gene. The amplification reaction produced a single DNA band of approximately 630 bp, which was close to the size predicted (603 bp) by the alignment used to design the primers (data not shown). The PCR product was sequenced and a database search revealed that there was a $1 \times 10^{-63}$ smallest sum probability of the PCR product encoding a portion of a sugar ATP-binding protein (data not shown). Therefore, this PCR product (ATPrel) was used as a probe to screen a *T. ethanolicus* genomic library in an attempt to clone, sequence, and characterize the whole gene and the genes that are immediately adjacent.

## Library screening and identification of additional maltose genes

The genomic library screen using ATPrel as a probe yielded two independent positive clones from 20,000 recombinant colonies. Each clone contained an identical 3.1 kb DNA insert, and Southern hybridization and PCR confirmed that this fragment (named pREL19) contained DNA homologous to the ATPrel PCR product (data not shown). Sequence analysis revealed that pREL19 contained DNA-encoding putative permease and ATP-binding protein genes from an ABC transport system (Fig. 1). In order to isolate the remainder of the operon, a 500 bp probe complementary to the 5′-end of pREL19 was amplified using PCR. A 1.4 kb clone (named pMALG), overlapping pREL19, was isolated from the *T. ethanolicus* genomic DNA library using this probe. pMALG contained DNA encoding the C-terminal 70 amino acid residues of a gene encoding a putative substrate-binding protein, the entire coding region of *malF*, and a portion of *malG* that overlapped with the pREL19 clone (Fig. 1). All sequences have been deposited in GenBank (accession number 12061563).

Since pMALG did not contain the 5′-end of the putative operon (namely, the 5′-end of the *malE* gene), another probe (MALEF), which straddled the region between the *malE* and *malF* genes, was designed from the 5′-end of pMALG. Unfortunately, multiple library screening resulted in the reisolation of the pMALG clone. It appeared that the established *T. ethanolicus* genomic DNA library did not contain a clone with the 5′-end of the putative maltose operon. Therefore, inverse PCR was used in an attempt to isolate the remaining portion directly from 39E genomic DNA. However, the PCR product obtained did not encode the remainder of *malE* (data not shown). A genome-walking protocol using PCR (GW-PCR) technique (Morris et al. 1995) also failed to amplify the remainder of *malE* (data not shown).

## Sequence analysis of the maltose operon

The sequence of pREL19 and the overlapping pMALG clone revealed four complete and two partial open reading frames (ORFs) with different polarities (Fig. 1) and organized in three transcriptional units. One partial and three full ORFs (*malE*, *malF*, *malG* and *malK*, respectively) are oriented in the same direction without any obvious transcriptional initiation signals or transcriptional terminators separating them. Thus, it is likely that they constitute an operon. These genes appear to encode the archetypal ABC sugar transport system, namely, a substrate-binding protein, two integral membrane proteins, and an ATP-binding protein. Downstream from the last gene of this putative operon, there is a palindromic sequence corresponding to an mRNA hairpin loop followed by a series of T nucleotides typical of a rho-independent terminator. One full and one partial ORF lie downstream of *malK*. These genes did not show any significant similarities to any other genes in Genbank. However, hydropathy plots of both ORFs indicated that they most likely encoded cytoplasmic proteins (data not shown).

## Amino acid sequence comparison

The deduced amino acid sequence of the individual genes in the putative *malEFGK* operon of *T. ethanolicus* revealed a considerable range of similarities to homologs in other eubacteria and archaea (Figs. 2 and 3). The putative *T. ethanolicus* MalK has over 70% similarity with its closest homolog, while the membrane components

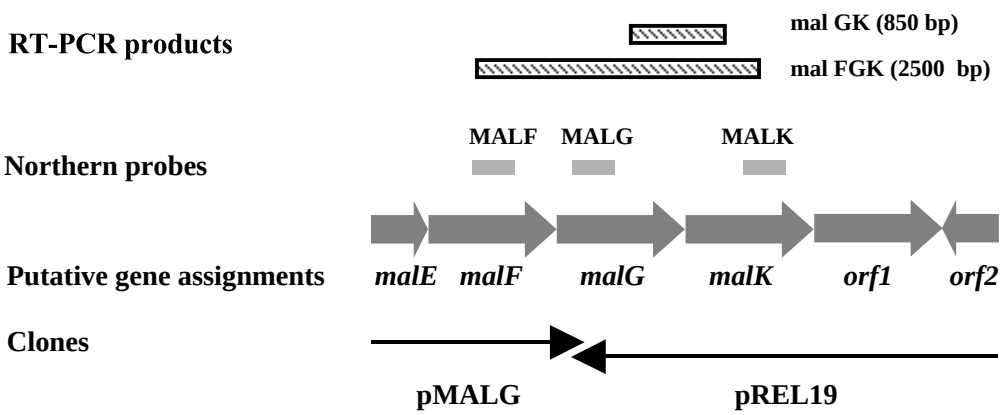


**Fig. 1.** Analysis of the pMALG and pREL19 clones. *Arrows* donate the direction of open reading frames (*ORF*). Putative gene assignments and probes used for Northern analysis (*solid boxes*) are also shown. PCR products spanning *malG* and *malK*, and *malF* and *malK* were used in RT-PCR analysis (*stippled boxes*)

**Fig. 2.** **A** Alignment of *T. ethanolicus* MalF with *Synechocytis* sp. (*Syne*) ORF [gi7442700]; *Thermotoga maritima* (*Tmar*) UgpF [gi7442716] and ORF [gi7442721]; *Bacillus subtilis* (*Bsut*) ORF [gi7442699]; *Klebsiella oxytoca* (*Koxy*) CymB [gi2126120]. **B** Alignment of *T. ethanolicus* MalG with *Thermotoga maritima* (*Tmar*) UgpG [gi7442820]; *Pyrococcus horikoshii* (*Phor*) ORF [gi7442817]; *Synechocytis* sp. (*Syne*) LacG [gi7442789]; *Bacillus subtilis* (*Bsut*) YvdI [gi7442787] and AmyC [gi7442796]. Conserved residues are indicated by an *asterisk*. Functionally conserved residues are represented by a *colon* or a *period* to indicate probable and possible conservation, respectively. Regions in *bold* type denote conserved hydrophilic segments common to sugar transport membrane proteins

**A**

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Teth MalF   TFYLPAVASGVTLSLVWFIMYDPTPDGLLNKFLSLFGFPN-INWLGRMDTALFSLMLMTY 172
Syne orf    AYYTPVITSIVVAGIAWKALYASN--GILNQILALLGFSDGIPWLTSPNLALWSVMVVTV 170
Tmar UgpF   LIFTPYAISPAIAGVLWSFLLNPVVGHVNYILSKLF--GLQVEWLTTKPYALIAVIIATV 173
Tmar orf    FFYWPYMMPAVAGTTMWKWLLSYDIGLLNHILRTLG--LPPVPWLIKPFPALFSIALLRT 91
Bsut orf    ILILPWAVPGFVTILIFAGLFNDSFGAMNHDILAFFG-IDPLPWMTDANWSRLALILMQG 284
Koxy CymB   VFILPYAIPAFVTLLIFRLLLNG-IGPVN-STLNSWG-IDSIGFLSDPLIAKMTVIAVSV 290
                *   .      :  :      :             : ::    :   :

Teth MalF   IGGHGSGIILYLASLGGIPKTIYEAAEIDAAGTWSKFRNITWPLLKPITLYMLITGIIG- 231
Syne orf    WKGLGYYMVIYLAGLQAIPQELYEAGAIDGADGWRQHWDITIPLMRPYCFLVGVLSSIS- 229
Tmar UgpF   WKTLPFDIIFYLAGLQDIPQELIEASLVEGANSWARTWKIVFPLLSPITFYLVIMNLVSF 233
Tmar orf    WGMTGLLMVMFITGLQNVPQDYIEAAMIDGANRWQRFWYVVFPAMS-NTNLLVLMTAIAH 150
Bsut orf    WLGFPYIFLVSTGVLQSIPDDLYEAATIDGASVFSKLRYITLPMVFIAMAPIIITQFTF- 343
Koxy CymB   WVGAPYFMLLITGAMTNIPRDLYEASEVDGASKFQQFREITLPMVLHQVAPSLVMTFAH- 349
                ::.    :  :*    **. ::.*  : :   :. * :        :

Teth MalF   SSQAFMTAYLMTSGGP--------NHATTTIALIWQDAFEYFDFGVAAAESFVLALI 280
Syne orf    ALKVFEEVYIMTQGGPL-------NASKTVVYYVYERAFQDLEMNYASAIGLVLFLV 279
Tmar UgpF   MFSSFAIIDVTTKGGPG-------NYTTTLIYRLYLDAFAFQKIGPAAAQSVILFLI 283
Tmar orf    TLRSFAGVYVLTTGGPG-------YATTIIPLYIYQTAFTQFRIGYSYAASVIYFLI 200
Bsut orf    NFNNFNIIYLFNGGGPAVTGS-TAGGTDILVSWIYKLTMQSSQYSLAAALTILLSVF 399
Koxy CymB   NFNNFGAIYLLTEGGPINPEYRFAGHTDILITWIYKLTLDFQQYQIASVISIIIFLF 406
                *      . ***          :      ::  ::       : .   :  :.
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**B**

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Teth MalG     ATVITLSNVLFASMAGYPFAKLKFPGGNTIFWILISTIMIPGQ-VTLIPL 146
Tmar UgpG     ASLITLGKLTTGALAGFAFSHFNFKSKKIMFATLFATLFLPAETVMILPL 126
Phor orf      AGITVTGNIIFSSMAGYAFARLKFPGRDVIFSSLLSLLMIPMF-VTLVPN 129
Syne LacG     SSITVALNLFFCALAAYPLARLNFVGRDMTLAAIVATLMIPFQ-LIMIPL 121
Bsut YvdI     ALSVMVLQVTIVTLAGYSYSRYRFAGRKKSLIFFLIIQMVPTM-AALTAF 126
Bsut AmyC     TGFSALLLIIFGSLAAYPLARRETKLNKAVFALLISIMIIPPL-TSMVPL 124
                    :   :: .:  :       .  :  ..   ::*     : .

Teth MalG     YVLVINVFNLADTYFAIILPQLVS--VYNIFLMKQYMTS-IPSTLIDAAR 193
Tmar UgpG     FLIMKTFG-WVNTYWALTIPFMAS--ATNTFLMRQHFLT-IPRELQDAAL 172
Phor orf      YIIIYKLG-LVDNIFGLSLLSLVS--VSSIFLMRQYFTS-LPNEIFEAAR 175
Syne LacG     YILAVNLG-LRNSYVGIIFPSLAS--AFGIFLLRQAFQG-VPKELEEAGR 167
Bsut YvdI     YVLAMLIG-ALDQYWFLTAIYIGGGIPMNTWLMKGYFDT-VPREIDEAAR 174
Bsut AmyC     YRMVVDAG-MVNTHAIAIFINTAAYMPLTVFLYSGFIRSTIPKELVEAAR 173
              :          :           .      ::        :*  : ::.

Teth MalG     IDACSEFGIFWKVIFPLAKPGIAVMAIFTFVSQWNDFFWPFLVTKTSAMR 243
Tmar UgpG     IDGASYMQFFWKVLIPLSKHMLAGASIINFVYAWNMYLWPLIVSMEDKMK 222
Phor orf      LDGCGPIKSFFYIALPLAKPALGAVAVYQFLGAWNAFIGPLIFLRSPENF 225
Syne LacG     IDGCRELGIWWHIMIPAIRPALVTLAIFVFIGAWSDFLWPLIVLDQPEYY 217
Bsut YvdI     IDGAGHLRIFASIVLPLVKPMLAVQALWAFMAPFGDYLLTKFLLRSPERL 224
Bsut AmyC     IDGAGMLKIFFTIVFPLLKPITATICIISCVFIWNDYQFAIFFLQDQKVQ 223
              :*..        :  *  :       :   :  :. :    :.

Teth MalG     TIQVGLAS-FKFADSTLYGPMMAGAVIASIPMFILFFSLQKYFLQGITIGAIKG 296
Tmar UgpG     TVQVGVKMLMQAESANNWGVIMAGTVVALAPTVVMFLALQNLFVKSLVRSGMKG 276
Phor orf      TLPVGLSFAFQRSMWTEYTPIIAGSIVAAAPTILLFVALNKYLIRGIVVTGGKG 279
Syne LacG     TLPLGVAQLS-SALDFDWRLIAAASVIAIAPIIVLFLFVQRYIIPSEASSGVKG 270
Bsut YvdI     TIAVGLQSFISNPQQQKVALFAAGAILAALPICVLFFFLQKNFVSGLTAGGTKG 278
Bsut AmyC     TLTVAMAG-FFGENANNLHLVAAAALMAMLPMVVLFLALQKYFIAGLSSGAVKG 276
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exhibited only 25%–30% similarity with a range of other thermophilic eubacteria.

The putative MalF and MalG permease proteins had predicted molecular weights of 34.4 and 34.2 kDa, respectively. They were composed mainly of hydrophobic and polar amino acids (over 70% of the residues in each protein). These proteins showed similarity to several integral cytoplasmic membrane proteins involved in sugar transport (Fig. 2) and contain a conserved hydrophilic segment with the consensus sequence EAA—G———I-LP (Mourez et al. 1997). There is no particular enrichment of identity apart from the EAA motif, which is typical of integral membrane proteins from binding-protein-dependent transport systems. The lack of overall sequence identity for the permease proteins has been noted by others (Horlacher et al. 1998). On the basis of the EAA signature, MalF and MalG can be assigned to the disaccharide subcluster of bacterial binding-protein-dependent permeases (Saurin and Dassa

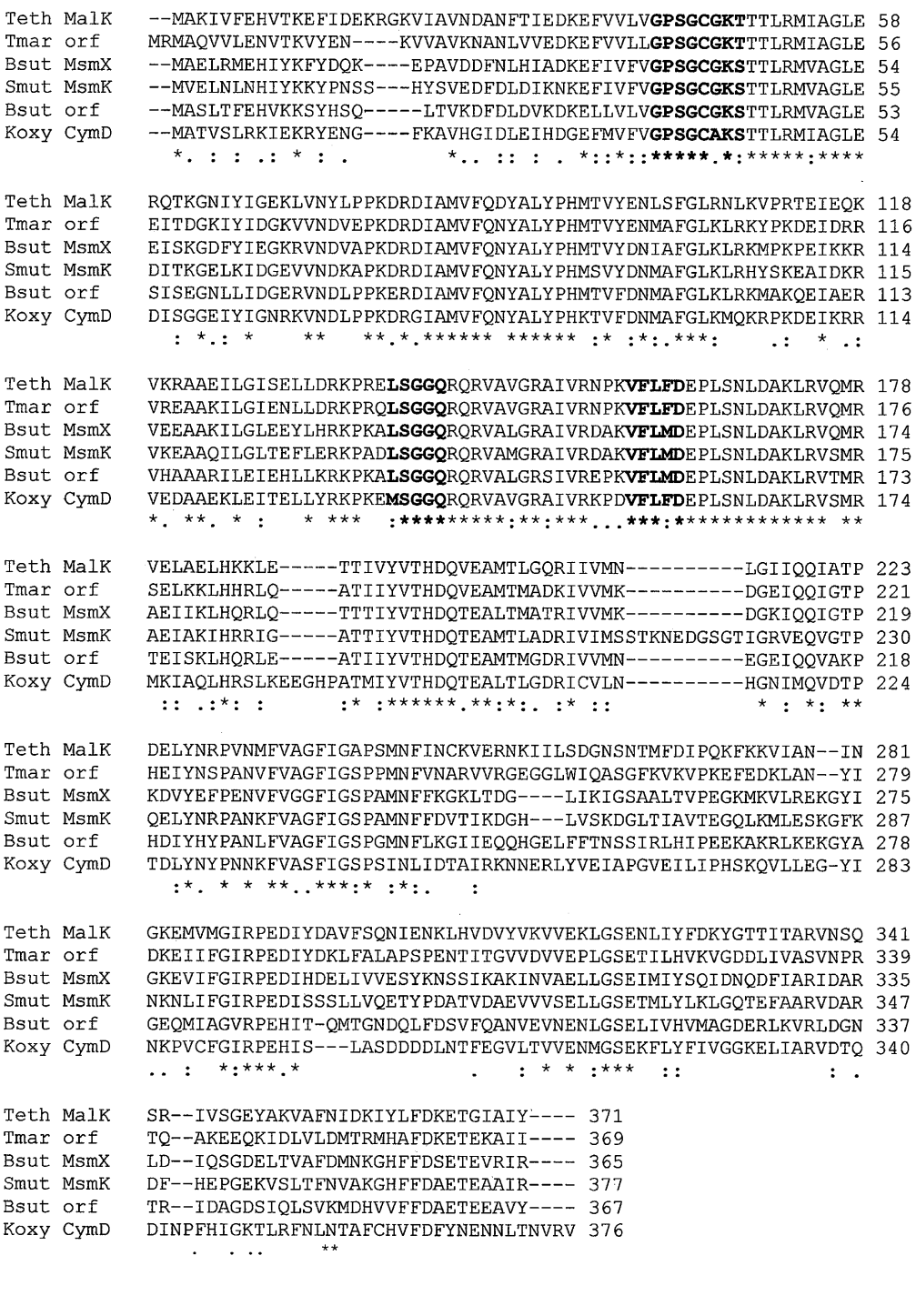


**Fig. 3.** Alignment of *T. ethanolicus* MalK with *Thermotoga maritima* (*Tmar*) ORF [gi4981834]; *Bacillus subtilis* (*Bsut*) MsmX [gi7442505] and ORF [gi2635752]; *Streptococcus mutans* (*Smut*) MsmK [gi282320]; *Klebsiella oxytoca* (*Koxy*) CymD [gi854321]. Conserved residues are indicated by an *asterisk*. Functionally conserved residues are represented by a *colon* or a *period* to indicate probable and possible conservation, respectively. Regions in *bold* type denote recognized functional domains

1994); this subcluster contains proteins involved in the transport of short (di- and tri-) oligosaccharides, including malto-oligosaccharides, raffinose-melibiose, and lactose.

The strain 39E MalK protein has a predicted molecular weight of 42.2 kDa and nearly 100 of its 371 residues are charged, a proportion similar to its closest homolog from *Thermococcus litoralis*, with which it shares 52% amino-acid identity (Horlacher et al. 1998). A multiple sequence alignment of MalKs from different bacterial and archaeal species revealed that conserved regions were found throughout the entire sequence, although conservation was somewhat less prominent in the carboxy-terminal region (Fig. 3). The highly conserved regions include the recognized functional domains of MalK, i.e., Walker sites A (GPSSGCGKT) and B (VFLMD), and the ABC motif LSGGQ (linker peptide) (Schneider and Hunke 1998).

The C-terminal 60 amino acid of the putative MalE protein, located directly upstream from MalF, showed no similarity to maltose-binding proteins from a range of other thermophiles (data not shown). This was not surprising, since this portion of MalE proteins is not at all conserved. However, this C-terminal segment aligned

well with Block BL01037, the bacterial extracellular solute-binding proteins family 1 signature sequence (Henikoff et al. 1999).

## Northern blot analysis

In an effort to determine whether the putative maltose genes were transcribed as a single operon, RNA prepared from maltose-, glucose-, and xylose-grown cells was hybridized (in individual reactions) with DNA probes that were complementary to *malF*, *malG*, or *malK*. Gene expression was not detected in RNA samples when they were hybridized with any of the maltose-specific probes (Fig. 4). However, this did not seem to be an analysis problem, since the same RNA samples hybridized with a xylose isomerase probe (Fig. 4, lanes 4–8) in a manner previously described (Erbeznik et al. 1998). Therefore, the lack of hybridization with the maltose-specific probes was probably not due to overall degradation of the mRNA.

One possible explanation of these results was that the putative maltose operon actually encoded proteins responsible for the transport of a different carbohydrate. One of the other consistently high similarities to the putative maltose operon in strain 39E was the *cym* locus from *Klebsiella oxytoca*, which encodes a binding-protein-dependent transport system specific for cyclomaltodextrins (Fiedler et al. 1996). Since previous work has shown that *T. ethanolicus* strain 39E possesses a cell-bound cyclomaltodextrinase (Saha and Zeikus 1990), it was logical to hypothesize that the bacterium might have a transport system for uptake of this class of cyclic carbohydrates. Results showed that strain 39E actually grew more quickly on α- or β-cyclomaltodextrin than on maltose (maximal growth rates of 0.22, 0.17, and 0.13, respectively). However, Northern analysis once again failed to show expression in samples from cyclomaltodextrin-grown cells using any of the probes tested (data not shown).

To rationally determine which other substrates to test as possible inducers of the cloned operon, the similarities of the strain 39E-deduced amino acid sequences with transporters other than maltose were reexamined. When this was done, proteins functioning as ABC transporters for ribose, galactose, mannitol, mannose, and lactose proteins were all found to align with varying degrees of similarity with the putative MalF, MalG, and MalK proteins (lower similarities than the proteins shown in Figs. 2 and 3). When RNA was isolated from *T. ethanolicus* cultures growing on these sugars, as well as on starch or α-trehalose, none of the maltose-specific probes hybridized to these RNAs in Northern hybridizations (data not shown).

## RT-PCR analysis

Since Northern analyses failed to show expression when using a variety of possible inducing substrates and DNA probes, RT-PCR was attempted. This technique has the advantage of being far more sensitive than Northern blotting. cDNA was prepared from maltose-, glucose-, and

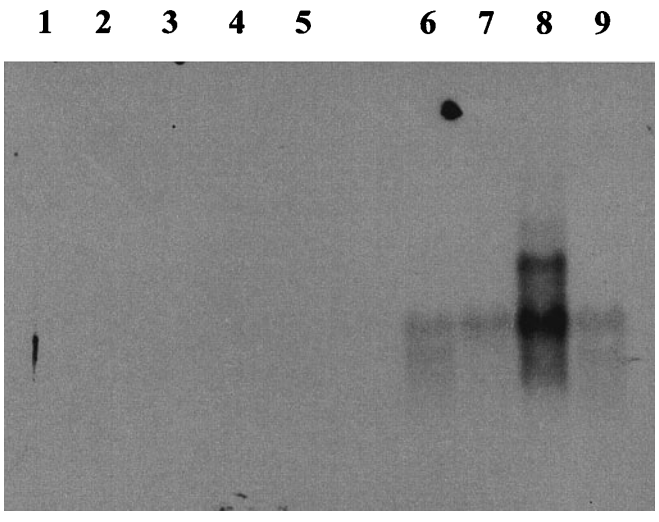


**Fig. 4.** Northern blot of *T. ethanolicus* total RNA (1 μg in each lane) isolated from cells grown on different carbohydrates and hybridized with radiolabeled maltose operon (MalF) or xylose isomerase (XI300) PCR products (lanes 2–5 and 6–9, respectively). *Lane 1* molecular size markers; *2,6* RNA isolated from cells grown on glucose; *3,7* maltose; *4,8* xylose; *5,9* mannitol. Probes that were complementary to *T. ethanolicus malG* and *malK* genes also failed to hybridize to RNA from glucose-, maltose-, or xylose-grown cells (see text)

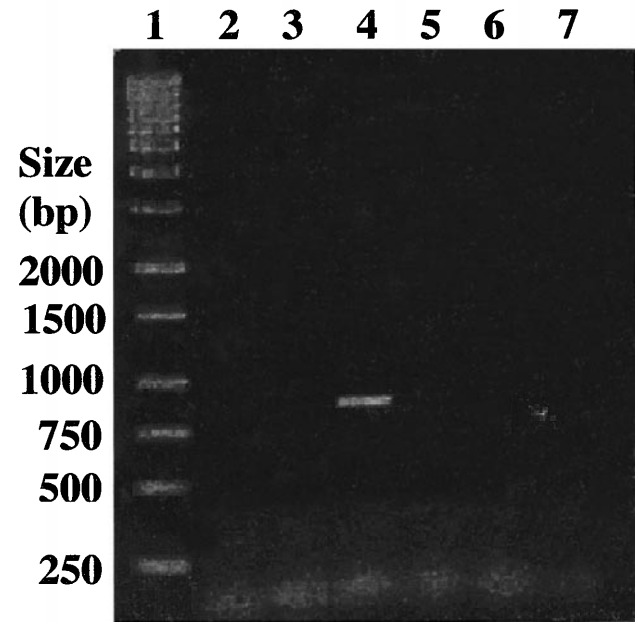


**Fig. 5.** RT-PCR of *T. ethanolicus* RNA isolated from glucose-, maltose-, or xylose-grown cultures amplified with PCR primers specific for *malG* and *malK*. *Lane 1* standards; *lanes 2 and 3* RNA from glucose cultures amplified with and without reverse transcriptase (RT), respectively; *lanes 4 and 5* maltose RNA with and without RT; *lanes 6 and 7* xylose RNA with and without RT

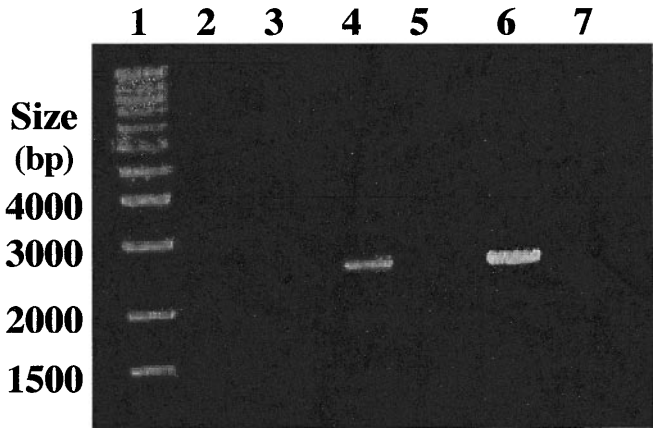


**Fig. 6.** RT-PCR of *T. ethanolicus* RNA isolated from glucose-, maltose-, or xylose-grown cultures amplified with PCR primers specific for *malF*, *malG* and *malK*. *Lane 1* standards; *lanes 2 and 3* RNA from glucose cultures amplified with and without reverse transcriptase (RT), respectively; *lanes 4 and 5* maltose RNA with and without RT; *lanes 6 and 7* xylose RNA with and without RT

xylose-grown cells, and amplified with primers specific for a PCR product that spanned *malG* and *malK*. Using these primers, a PCR product of the predicted size (850 bp) was detected only in maltose-grown cells (Fig. 5; lane 4).

Another set of primers was used to amplify cDNA derived from a *malFGK* transcript. However, no PCR product was initially detected (predicted size 2.5 kb). The cDNA synthesis parameters were then altered by reducing the reaction temperature from 50° to 42°C; this change generally results in fuller length cDNA fragments produced from RNA (Life Technologies). Using these new parameters, a PCR product that spanned the *malF* through *malK* genes was detected in cells grown on maltose or xylose (Fig. 6; lanes 4 and 6). In contrast, product was not detected in amplifications using cDNA derived from cells grown on glucose (Fig. 6; lane 2) or cyclomaltodextrins (data not shown). Based on these results, it appears that these genes are transcribed as an operon in the absence of glucose.

## Discussion

Previous work suggested that although *T. ethanolicus* strain 39E (formerly *Clostridium thermohydrosulfuricum*) grew on maltose, it did not have a discrete maltose transport system (Hyun and Zeikus 1985; Hyun et al. 1985). However, more recent experiments have shown that strain 39E does indeed transport the disaccharide, that this activity is specific to maltose-grown cells, and that transport most probably occurs via an ABC type binding protein-dependent mechanism (Jones et al. 2000), similar to the systems in the thermophiles *Thermococcus litoralis* (Xavier et al. 1996) and *Thermoanaerobacterium thermosulfurigenes* (Sahm et al. 1996).

Protein sequence analysis showed that the putative operon cloned from strain 39E contained regions of conservation with ABC transport systems specific for maltose and other disaccharides. The *T. ethanolicus* genes showed the greatest similarity to maltose transport genes from the archaeon, *Thermococcus litoralis*, even though the substrate ranges and affinities of the two systems are different (Horlacher et al. 1998; Jones et al. 2000). In addition, the maltose transport operons in *Thermococcus litoralis* and other thermophiles, *Thermoanaerobacterium thermosulfurigenes* and *Alicyclobacillus acidocaldarius*, do not include *malK* (Horlacher et al. 1998; Sahm et al. 1996; Hulsmann et al. 2000). In fact, *malK* does not form an operon with *malEFG* in any other maltose ABC transport system, except in several species of *Mycobacterium* (Borich et al. 2000). Thus, the genetic organization of the *T. ethanolicus* maltose transporter is different from those of its closest homologs.

Several nonmaltose transport systems exhibit sequence similarity to the putative 39E maltose transporter. The *msm* operon from *Streptococcus mutans* (McLaughlin and Ferretti 1996) encodes an ABC transport system that is specific for the uptake of oligomeric starch degradation products such as raffinose (Tao et al. 1993). The strain 39E genes also showed similarity to the *Klebsiella oxytoca cym* locus, which encodes a cyclomaltodextrin ABC transport system (Fiedler et al. 1996). *T. ethanolicus* had previously been shown to have a cell-associated cyclomaltodextrinase (Saha and Zeikus 1990); we determined for the first time that the organism actually grows on α- and β-cyclomaltodextrins. Since no transcripts were detected using maltose gene probes in cells grown on these substrates, it is probable that *T. ethanolicus* has a discrete system for cyclomaltodextrin uptake that is separate from the maltose transport system. Overall, based on this sequence analysis and similarities to other systems, it was reasonable to conclude that the genes isolated from *T. ethanolicus* did comprise most of a maltose transport operon.

Earlier experiments showed that glucose-grown cells do not transport maltose (Jones et al. 2000), and so it was predicted that *mal* genes would not be detected in cells grown on the monosaccharide. Although Northern analysis failed to detect expression of any RNA species, *malGK* and *malFGK* transcripts were detected in maltose- and xylose-grown cells using RT-PCR. In contrast, neither transcript was detected by RT-PCR in cells grown on glucose. Therefore, *mal* gene expression may be subject to glucose catabolite repression. In *Thermococcus litoralis*, mRNA for all transport components was detected in the presence of maltose and α-trehalose (Xavier et al. 1996; Greller et al. 1999), but there was no comparison with cells grown on other carbohydrates. Similar to *T. ethanolicus*, maltose transport gene expression was not detected in *Thermoanaerobacterium thermosulfurigenes* in the presence of glucose (Sahm et al. 1996). Overall, it seems that maltose transport systems in thermophiles are subject to repression by glucose.

In the Gram-positive organisms thus far studied, glucose repression is controlled by a *trans*-acting regulator

protein, MalR. In *Streptococcus pneumoniae*, *mal* gene transcription is repressed in the presence of glucose (Puyet et al. 1993), and MalR also differentially regulates the *malXCD* and *malMP* operons according to physiological conditions (Nieto et al. 1997). A maltose repressor gene was located directly downstream of *malG* in *Alicyclobacillus acidocaldarius*, apparently in the same operon (Herrman et al. 1996; Hulsmann et al. 2000). In this organism, maltose transport was detected when cells were grown on maltose plus glucose, but not on glucose alone. Although additional work is needed; given the overall similarity of these maltose transport genes (Hulsmann et al. 2000; Puyet et al. 1993), it seems likely that *T. ethanolicus* may regulate its maltose operon using a similar repressor protein.

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