Sugar metabolism and regulation in the hyperthermophilic archaeon

*Thermococcus litoralis*

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Chapter 1

General Introduction

The kingdom of archaea

Earth was produced by supernova explosion in solar system 4.5 billion years ago. Life on earth dated from about 3.8 billion years ago. At early time, the earth would have suffered frequent massive meteorite impacts some of which sufficiently large to heat the oceans to > 110°C (Nisbet and Sleep 2001). The early atmosphere contained abundant CO₂ and sulfur gasses, but low oxygen (Kasting 1993). The standard view clearly implies (but does not prove) that the early microbial community is chemotrophic and hot (Woese 1987). Only hyperthermophilic organisms, which live optimally at 80–100°C in water, could have evolved.

The biological evidence, based on 16S rRNA sequence analysis which rooted the universal phylogenetic tree, suggested that hyperthermophiles could have been the common ancestor (Nisbet and Sleep 2001). The hyperthermophilic genera represent the shortest lineages, and are close to the root of the phylogenetic tree (Stetter 1996). This tree divides the living world into three domains: bacteria, archaea, and eukaryote. Except for the organisms belonging to two bacterial genera, Thermotoga and Aquifex, all the other hyperthermophiles isolated up to date belong to the domain Archaea (Woese et al. 1987, 1990). Phylogenetically, archaea fall into three distinct kingdoms: Crenarchaeota, Euryarchaeota and Korarchaeota. The kingdom Crenarchaeota consists of hyperthermophiles or thermoacidophiles. Some example genera are Sulfolobus, Desulfurococcus, Pyrodictium, Thermoproteus and Thermofilum. The kingdom Euryarchaeota includes hyperthermophiles (e.g. genera Pyrococcus and Thermococcus), methanogens, halophiles and thermophilic methanogens (Woese 1987). The kingdom Korarchaeota are those uncultured microbes based on 16S rRNA sequences from terrestrial hot springs.

Hyperthermophiles grow in so-called extreme conditions that can occur in a great variety of geothermal heated environments, such as terrestrial solfataric field, hot springs, shallow and deep submarine hydrothermal vents, and oil wells (Stetter 1996, 1999). The thermophilic environments are found as a result of volcanic activity or movements of earth’s crust at
tectonically active site. Organisms growing at 60–80°C are called moderately thermophilic, and those growing above 80°C are called hyperthermophiles. These organisms are so well adapted to the thermophilic environments that some can not grow below 80°C, and most have a minimum growth temperature 60°C (Stetter 1982; Huber et al. 1989). The highest growth temperature of an unnamed archaeon (coccoid) could reach 121 °C (Kashefi and Lovley 2003).

The phylogenetic tree of three domains of life (modified from Morell (1997)).

**Adaptation of microorganisms to extreme conditions**

Some archaea grow under high temperature, low pH, or high salt concentration. In our view, these harsh environments create a series of challenges to the structure and composition of microbial membranes, proteins and DNA (Brown and Doolittle 1997).

The membranes of archaea differ in composition and structure from those of eukaryotes or bacteria in four important ways. First, the non-polar chains are joined to a glycerol backbone by ether rather than ester linkage. The ether linkage is more resistant to hydrolysis. Second, the alkyl chains are highly methyl-branched isoprenyl chains rather than linear fatty acyl chains. They are built up from repeats of a fully saturated five-carbon fragment. The branched, saturated hydrocarbons are more resistant to oxidation. Third, archaea glycerol
ethers contain 2,3-sn-glycerol, which differs from the 1,2-sn-glycerols found in the two other domains. Fourth, some lipids in archaea are tetraethers for which ester have no comparable structures (Gambacorta et al. 1993; Koga et al. 1993). The ability of archaeal lipids to resist hydrolysis and oxidation may help these organisms to withstand the extreme conditions, such as high temperature, low pH and salt concentration.

Proteins normally denature when temperature approach 100°C. Archaeal proteins have developed several ways to cope with high temperatures, e.g., increasing ion-pair contents, forming higher-order oligomers, decreasing the length of surface loops and flexibility at room temperature, optimizing electrostatic and hydrophobic interactions, and changing amino acids to increase internal hydrophobicity and helix property of residues in α-helix (Danson and Hough 1998; Brown and Doolittle 1997). Purified enzymes from hyperthermophiles usually show extraordinary heat stability in vitro, for example, an amylase from Pyrococcus woesei is still active at 120°C (Greybowska et al. 2004). At the upper temperature border of growth of hyperthermophiles, the function of heat-shock proteins appears to become essential. At 108°C, about 80% of soluble protein of a crude extract of Pyrodictium occultum consisted of a heat inducible molecular chaperone designated thermosome (Minuth et al. 1998). Extreme environmental conditions require optimized interactions within the protein, at the protein-solvent boundary, or with the influence of extrinsic factors such as compatible solutes. These compounds were found to accumulate intracellular in some hypethermophiles in response to temperature and salt stress. Some of theses compatible solutes may have a role in the protection of cell components against thermal denaturation, such as di-myo-inositol-1,1'-phosphate, glycine betaine, mannosylglycerate or trehalose (Lamosa et al. 1998). These compounds are potential agents for biotechnological application in stabilizing mesophilic proteins.

DNA is especially vulnerable to high temperature, radiation, oxidative damage and desiccation. DNA is subject to denaturation and chemical modification at temperature of >70°C. However, DNA of hyperthermophiles such as Pyrococcus furiosus is known to be more stable in vivo than that of mesophile E.coli (Diruggiero et al. 1997). Monovalent and divalent salts are recognized to enhance the stability of nucleic acids because theses salts screen the negative charges of the phosphate groups. KCl and MgCl₂ protect the DNA from depurination and hydrolysis (Daniel and Cowan 2000). Thermal resistance of DNA double helix appears to be improved in hyperthermophiles by reverse gyrase, a unique type I DNA topoisomerase that causes positive superwist for stabilization (Forterre et al 1996). Archaeal hyperthermophiles possess histones phylogenetically related to the eukaryotic core histones.

The metabolic patterns of archaea

The majority of hyperthermophiles are strictly anaerobes, and most of them depend on the reduction of sulfur compounds for optimal growth (Schönheit and Schäfer 1995). Many isolates have been obtained in anoxic and sulfur-rich environments. However, the metabolism of hyperthermophiles is diverse and includes obligate or facultative autotrophs and heterotrophs.

The hypethermophiles with autotrophic metabolism are chemolithoautotrophs and obtain energy from the oxidation of H2 (or some case S0) coupled to the reduction of S0, SO4^{2-}, CO2, and NO3^−, but rarely O2. CO2 fixation proceeds via the reductive citric acid cycle or via the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway (Schönheit and Schäfer 1995).

Peptides, amino acids and carbohydrates serve as carbon and energy source of the hyperthermophiles with heterotrophic metabolism. These substrates are completely oxidized to CO2 by external electron acceptors (S0, SO4^{2-}, S2O3^{2-}, NO3^− or O2) in the respiratory microorganisms or converted to acetate and several organic acids in the fermentative microorganisms (Stetter 1996). Several archaea have been reported to perform respiration either aerobically with oxygen as a terminal electron acceptor (e.g. *Sulfolobus*, *Pyrobaculum*, thermoplasma and halophilic archaea) or anaerobically with alternative terminal electron acceptors, such as nitrate (e.g. *Pyrobaculum*), sulphur (e.g. *Thermoproteus*), sulphate (e.g. *Archaeoglobus*) or CO2 (e.g. *Methanogens*) (Schönheit and Schäfer 1995).

Several archaea are able to grow on saccharides (e.g. starch, glycogen, pullulan, maltodextrin, maltose, cellulose) as carbon sources and only a few hyperthermophic archaea e.g. *sulfolobus*, can grow on monosaccharide (e.g. glucose, arabinose) (Table 1). Both fermentation and respiratory on carbohydrates by archaeal species have been reported (Schönheit and Schäfer 1995). The anaerobic archaeal genera *Pyrococcus*, *Thermococcus*, *Desulfovirgococcus* and aerobic genus *Sulfolobeus* and *Thermoproteus* have been investigated on their sugar metabolism pathways and possible metabolic regulations (Verhees et al. 2003; de Vos et al. 1998).
<table>
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<th>Organisms</th>
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<th>Carbohydrate substrates</th>
<th>Metabolism types and pathways</th>
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<tr>
<td><strong>Sulfolobales</strong></td>
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<tr>
<td><em>Solfolobus solfataricus</em></td>
<td>80</td>
<td>Starch, dextrin, xyloglucan, maltose, sucrose, lactose, glucose, xylose</td>
<td>Aerobic, Modified ED</td>
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<tr>
<td><em>Pyroccus furiosus</em></td>
<td>100</td>
<td>Starch, pullulanan, glycogen, maltose, cellobiose, lactose, melibiose</td>
<td>Anaerobic, EM</td>
<td>Kengen et al. (1996); Driskill et al. (1999)</td>
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<tr>
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<td>Starch, maltose, cellobiose, glucose</td>
<td>Anaerobic</td>
<td>Barbier et al. (1999)</td>
</tr>
<tr>
<td><em>Pyroccus woesei</em></td>
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<td>EM</td>
<td>Kengen et al. (1996)</td>
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<tr>
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<td>Sucrose</td>
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<td><em>Thermococcus litoralis</em></td>
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<tr>
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<td><em>Thermococcus fumicolans</em></td>
<td>85</td>
<td>Maltose</td>
<td>Anaerobic</td>
<td>Godfroy et al. (1996)</td>
</tr>
</tbody>
</table>

ED: Entner-Doudoroff pathway. EM: Embden-Meyerhof pathway
Sugar transport and metabolism pathways in archaea

Sugar transporters

General speaking, polysaccharides are first hydrolyzed into monomers or oligomers extracellularly, then are transported into the cell and degraded into glucose. Three main classes of transporters are found in mesophilic bacteria for sugar uptakes (Dills et al. 1980): 1) secondary transporter, in which the sugar is transported across the membrane in the combination of protons or sodium ions (Jung 2001). 2) phosphoenol/pyruvate (PEP)-dependent phosphotransferase systems (PTS), in which sugar transport and sugar phosphorylation occur simultaneously with the consumption of PEP (Erni 2001). 3) ATP binding cassette (ABC) transporter, which are composed of a cytoplasmic membrane binding protein for sugar binding, two membrane transport domains, and an ATPase subunit. The ATP is consumed while sugar transport takes place (Boos and Eppler 2001).

So far only ABC-type transporters were found for sugar uptake in hyperthermophilic archaea. Analysis of genome sequence and biochemical studies indicated that archaea are devoid of PTS system, and as well in the genome of two thermophilic bacteria, T. maritima and A. aeolicus (Koning et al. 2001). Secondary transporter gene are abundant in archaeal genome, however, none of those transport systems have been identified for sugar uptakes. They seem to involve in uptake of inorganic substrates (Koning et al. 2002).

The ABC transporters belong to two main families, the sugar transporters (CUT-family) and the di/oligopeptide transporter (Opp-family) (Schneider et al. 2001). Both families exist in archaea. The two families differ in substrate specificity and in architecture of the transport complex. The first family, like the maltose/trehalose transporter of T. litoralis, maltodextrin transporter of P. furiosus, arabinose and glucose transporters of S. solfataricus, exhibits typical domain composition of CUT1 (CUT subfamily) (Schneider 2001). These transporter systems are composed of a MalE, MalF, MalG and Malk encoding a binding protein, two integral membrane proteins and an ATP-binding subunit.

The second family of archaeal ABC transporter for carbohydrate uptake is homologues to the di/oligopeptide transporter family of mesophilic bacteria, e.g. cellobiose/β-glucan-oligomer transporter of P. furiosus (Koning et al. 2001), the maltose/maltodextrin and cellobiose/cello-oligomer transporters of S. solfataricus (Elferink et al. 2001). This transporter family is particularly abundant in the genomes of hyperthermophilic organisms, and the genes of enzymes involving in sugar metabolism often cluster together. In hyperthermophilic bacteria T. maritima these transporters have been implicated in peptide transport rather than sugar
transport which was suggested that sugar and peptide metabolism are coordinately regulated (Nelson et al. 1999).

Studies in intact cells have demonstrated that hyperthermophiles mediated sugar uptake has very high affinity. The \( \text{Km} \) values have been reported about 20 nM for the TMBP of trehalose/maltose transporter in \( T. \) \( \text{litoralis} \) (Xavier et al. 1996) and 175 nM for CbtA when transport \( \beta \)-gluco-oligomers (e.g. cellobiose) in \( P. \) \( \text{furiosus} \) (Koning et al. 2001). The high affinity of the binding proteins to their substrates could reflect that the organic substrate concentrations are usually low in the very hostile environments. In mesophilic bacteria, only some substrates which appear very low concentrations in nature environment are absorbed in nanomolar levels, e.g. vitamin and iron (Koster et al. 2001).

**Embden–Meyerhof (EM) pathway in archaea**

Transported oligosaccharides are hydrolyzed further to hexose (e.g. glucose, galactose, mannose, and fructose) and pentose (e.g. xylose and arabinose). Subsequently, these monosaccharides are being oxidized via a well-conserved set of central metabolism pathway. A series of reactions are involved in the degradation of glucose to pyruvate by the Embden-Meyerhof (EM) pathway, which is also called glycolysis. It is the general route for glucose degradation in all domains of life. Some microorganisms use an alternative pathway of glucose degradation, for instance, the Entner-Doudoroff (ED) pathway (Romano and Conway 1996). In addition, some organisms are capable of alternative route and bypass in sugar degradation, including the oxidative pentose phosphate pathway (Sprenger et al. 1995). In archaea, glucose is metabolized to pyruvate via variants of two main sugar catabolic routes: the modified EM pathway or ED pathway (Verhees et al. 2003). Subsequently, pyruvate is converted into acetyl-CoA and \( \text{CO}_2 \) by ferredoxin oxido-reductase. \( P. \) \( \text{furiosus} \) and other obligatory anaerobic fermenting archaea mainly produce acetate and \( \text{CO}_2 \) as end products via an archaea-specific acetyl-CoA synthase (She et al. 2001; Musfeldt et al. 1999). \( S. \) \( \text{solfataricus} \) and other respiring archaea completely oxidize acetyl-CoA to \( \text{CO}_2 \) via citric acid cycle, coupled with respiratory oxidation of NAD(P)H and ferredoxin. (Romano and Conway, 1996). The net reaction of the classical EM pathway is:

\[
\text{Glucose} + 2\text{ADP} + 2\text{P}_i + 2\text{NAD}^+ \rightarrow \text{2 pyruvate} + 2\text{ATP} + 2\text{NADH} + 2\text{H}^+ 
\]

The modified–EM pathway in the members of the Thermococcales contains the ADP-dependent hexokinase and phosphofructokinase instead of ATP-type kinase (Kengen et al. 1994; Tuininga et al. 1999). In the step of glyceraldehyde-3-phosphate (GAP) oxidation, a
distinct enzyme catalyzes GAP into 3-phosphoglycerate by a single-step (Mukund and Adams 1993), whereas in bacteria and eukaryotes, the GAP is converted into 3-phosphoglycerate by a coupled NAD⁺-dependent GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and the ATP-generating phosphoglycerate kinase (PGK). The general view of the net reaction in *P. furiosus* (Verhees et al. 2003) could be described as:

\[
\text{Glucose} + 4 \text{fd}^{\text{ox}} \longrightarrow 2 \text{pyruvate} + 4 \text{fd}^{\text{red}} + 4 H^+
\]

(fd is the single-electron carrier ferredoxin, which could be either oxidized (fd^{ox}) or reduced (fd^{red})).

The classical ED pathway of bacteria (and a few eukaryal micro-organisms) consists of nine enzymes, and starts with the phosphorylation of either glucose or its oxidized derivative gluconate. The generated 2-keto-3-deoxy-6-phosphogluconate (KDPG) is converted by an aldol cleavage resulting in the formation of GAP and pyruvate. The net reaction of the bacterial ED pathway:

\[
\text{Glucose} + \text{ADP} + \text{Pi} + \text{NADP}^+ \longrightarrow 2 \text{pyruvate} + \text{ATP} + \text{NADH} + \text{NADPH} + 2H^+
\]

In halophilic archaea, glucose is oxidized to gluconate and then converted to 2-keto-3-deoxygluconate (KDG) by glucose dehydrogenase and dehydratase. Only KDG is phosphorylated into 2-keto-3-deoxy-6-phosphogluconate (KDPG) by KDG kinase (De Rosa et al. 1984; Wood 1987). Such a pathway (via KDPG) results in a similar net reaction with classical ED pathway in bacteria. On the other hand, thermoacidophilic archaea *Sulfolobus*, *Thermoplasma* and *Halobacterium* apply a modified-ED pathway. In this case, KDG undergoes aldol cleavage without phosphorylation to produce glyceraldehyde and pyruvate. Glyceraldehyde is converted to glycerate and 2-phosphoglycerate which is further degraded to pyruvate by enolase and pyruvate kinase. This modified pathway does not result in net generation of ATP (Budgen and Danson 1986; Danson 1989). The net reaction is:

\[
\text{Glucose} + 2 \text{NAD(P)}^+ \longrightarrow 2 \text{pyruvate} + 2 \text{NAD(P)}H + 2H^+
\]

**Sugar kinases involved in archaeal glycolysis**

Sugar kinases play important roles for the carbohydrate metabolism. Archaeal sugar kinases reported so far include glucokinase (Koga et al. 2000), fructokinases (Rangaswamy and Altekar 1994; Qu et al. 2004), phosphofructokinase (Ronimus and Morgan 2001) and galactokinase (Verhees et al. 2002). The glucose kinases and phosphofructokinase of *Pyrococcus* and *Thermococcus* species are ADP-dependent. The ADP-dependent glucose
kinase (ADP-GLKs) is not related to the classical ATP-dependent enzyme of bacteria (GLK) or eukaryotes (hexokinase) (Koga et al. 2000). The ATP-dependent phosphofructokinase (ADP-PFKs) is paralogues of the ADP-GLKs, sharing the same Cluster of Orthologous Groups (COG 4809) (Verhees et al. 2003). A bifunctional enzyme (MJ 1604) from hyperthermophilic archaeon *Methanococcus jannaschii*, is ADP-GLK/PFK (Sakuraba et al. 2002). The gene encoding the ADP-GLK/PFK is adjacently oriented with a predicted glucose isomerase (MJ 1605) as well as a glycogen synthase (MJ 1606), which suggests that ADP-GLK/PFK play roles in glycol synthesis.

Two ATP-dependent fructokinases have been characterized in the halophilic archaeon *Halobacterium vallismortis* (Rangaswamy and Altekar 1994) and the hyperthermophilic archaeon *T. litoralis* (Qu et al. 2004). The enzymatic reactions performed by the two enzymes are completely different. The fructokinase in *H. vallismortis* forms fructose-1-P, and the latter forms fructose-6-P. The fructokinase of *T. litoralis* belongs to the ribokinase family which is a large family of prokaryotic and eukaryotic carbohydrate kinases including fructokinase, ribokinase, 1-phosphofructokinase, archaeal 6-phosphofructokinase and adenosine kinase. Since the amino acid sequence of fructokinase from *H. vallismortis* is unavailable, the comparison of the amino acid sequence similarity between these two fructokinases can not be performed.

**Regulation of sugar transport and metabolism in archaea**

**Regulation of transporter system**

Regulation happens at transcription, translation or protein levels. In the transcription level, most appreciated in prokaryotes, transcription of genes is regulated by regulator binding or releasing which are caused by the presence of inducer. When transcription is constitutive, translation of mRNA can be regulated by mRNA stability. The protein activity is regulated by specific degradation or phosphorylation or other modifications.

Expression of ABC transporters are strongly regulated in most organisms. Maltose/trehalose transporter in *T. litoralis* (Horbacher et al. 1998) and cellobiose transporter in *P. furiosus* (Koning et al. 2001) are tightly regulated with the presence of sugars in the medium. On the contrary, in *S. solfataricus*, the binding proteins for glucose and trehalose seem to be expressed constitutively, and binding proteins for cellobiose and maltose are only slightly up regulated when cells are grown in the presence of such sugars (Elferink et al. 2001).
Regulation of the glycolysis pathway

Regulation of the glycolysis pathway is a very complex process. Modulation of the glycolytic flux relies on the coordinated triggering of multiple events, both at enzyme level (regulation of enzymatic activity by allosteric effectors or by covalent modification) and at DNA level (modulation of gene expression).

Classical control sites of glycolysis pathway are the unidirectional conversions, which are catalyzed by phosphofructokinase (PFK) and pyruvate kinase (PYK). Usually, PFK is allosterically controlled by fructose-6-P and fructose-2,6-bisphosphate. However the PFK in *P. furiosus* (Tuininga et al. 1999) and pyrophosphate-dependent PFK in *Thermoprotens tenax* (Siebers et al. 1998) are not controlled allosterically. Similarly, the pyruvate kinase of hyperthermophilic archaea *Archaeoglobus fulgidus*, *Aeropyrum penix* and *Pyrobaculum aerophilum* seems to be non-allosteric (Johnsen et al. 2003).

In *P. furiosus*, expression of fructose-1,6-bisphosphate aldolase is much higher in maltose-grown cells than pyruvate-grown cells (Siebers et al. 2001). Compared to peptone-grown cells, glucose-6-P isomerase (PGI), ADP-phosphofructokinase (PFK) and glyceraldehydes-3-phosphate ferredoxin oxidoreductase (GAPOR) are up-regulated in maltose-grown cells (Schut et al. 2003). These results indicated that the regulation of the glycolytic flux in *P. furiosus* appears in enzyme expression rather than allosteric regulation of enzyme activity.

Catabolite repression in archaea

Catabolite repression includes transient repression by glucose, a carbon source hierarchy and a global mode of regulation (Magasanick and Neidhardt 1987; Saier 1996). All of these have been detected only in *S. solfataricus*, which is the best studied halothermophiles. This is an aerobic or facultative organism that grows chemoheterotrophically on reduced carbon compounds (including glucose) and peptides by aerobic respiration at an optimal temperature of 80°C (De Rosa et al. 1975; Grogan et al. 1989; Schönheit and Schäfer 1995). Glucose oxidation is via ED pathway (De Rose et al. 1984; Selig et al. 1997). *S. solfataricus* is selected as a model organism because it is aerobic and it is readily cultivated in liquid and solid defined medium. The isolation of different phenotype mutants is also available. *S. solfataricus* harbors a catabolite repression-like system analogous to that observed in the members of bacterial and eukaryotic domains (Bini and Blum 2001). This system controls expression of three genes for carbohydrate utilization. They are α-glucosidase or maltase (MalA) for degradation of α-1,4-linked polysaccharides, the α-amylase (AmyA) for
hydrolyzing starch, dextrin and ω-cyclodextrin, and the β-glycosidase (LacS) for cleaving β-linked oligosaccharides, e.g. cellobiose or lactose (Haseltine et al. 1996; Rolfsmeier et al. 1998; Haseltine et al. 1999). Transcription levels of three genes varied in response to growth on different carbon sources (Rolfsmeier et al. 1998) and coordinately regulated. Maximum gene expression occurred during growth on sucrose as the sole carbon source, and the minimum expression existed during growth on sucrose plus yeast extract which contains asparagine and aspirate. Since the three target glycosyl hydrolase genes are separated by a minimum of 25 Mb in the genome of S. solfataricus, they are physically unlinked. This supports the hypothesis that coordinated gene expression relies on a global gene regulatory system (Bini and Blum 2001). The isolated mutants who are suffered to catabolite repression alter the expression of three glycosyl hydrolases to the same extent. It suggests that a transacting factor regulatory factor is necessary for the expression of lacS and other catabolite repression-regulated genes.

cAMP is a mediator in signal transduction pathway of catabolite repression in bacteria and eukaryotes. In gram-negative bacteria, cAMP binds to CRP protein and forms a CRP-cAMP complex to control transcription of specific promoter involving in sugar transport and metabolism. cAMP has been found in some archaea species, e.g. Methanobacterium thermoautotrophicum, Halobacterium volcanii and Sulfolobus solfataricus (Leichtling et al. 1986). Interestingly, adenylate cyclase, the enzyme responsible for the cAMP formation, was not found in archaeal genome for its homologous protein. It is unclear which enzyme is responsible for cAMP synthesis.

**Maltose/trehalose and maltodextrin transporter system in T. litoralis**

*T. litoralis* is a hyperthermophilic marine archaeon, which was originally isolated from deep submarine hydrothermal vents (Neuner et al. 1990). *T. litoralis* belongs to the order Thermococcales, which also includes the Pyroccous genus.

*T. litoralis* grows optimally at 85 °C under anaerobic conditions. In many aspects of growth and metabolism, *T. litoralis* resembles *P. furiosus*, who is the best-characterized hyperthermophilic archaeon. The *T. litoralis* was firstly reported as a heterotrophic organism only grown on peptone and pyruvate, but not on sugars (Neuner et al. 1990). Later, starch, maltose, cellobiose or sucrose were found to be utilized as carbon sources by *T. litoralis* (Andreotti et al. 1994; Kletzin et al. 1995; Ma et al. 1994; Oshima et al. 1994; Rinker et al. 1996). *T. litoralis* cells produce extracellular amylolytic enzymes having both α-1,4 and α-
1,6 hydrolytic activities (Brown and Kelly 1993), and an intracellular α-glycosidase (Kelly and Adams 1994). The polysaccharide utilization in *T. litoralis* suggests that degradation is initiated with the hydrolytic activity of extracellular enzymes to produce a series of oligosaccharides. These oligosaccharides are further transported into the cell, hydrolyzed into glucose and catabolized by a modified Embden-Meyerhof pathway (Brown et al. 1993; Kelly et al. 1994).

**Maltose/trehalose transporter and maltose metabolism**

The maltose and trehalose transporter in *T. litoralis* is the first report of sugar ABC transporter in archaea (Xavier et al. 1996). The *malE* encoding the TMBP (*T. litoralis* maltose/trehalose binding protein), the *malF* and *malG* encoding two integral membrane proteins, the *malK* encoding the ATP-subunit of *T. litoralis* transport system had been cloned and sequenced (Horlacher et al. 1998). The genes of *T. litoralis* transport system display similar properties with the counterpart of some bacterial ABC transport system in their amino acid sequence and organization. The further genetic studies revealed that the maltose/trehalose ABC transporter oriented at the 5’-end of a 16 kb DNA fragment and together with a putative iron/thiamin transporter at the 3’-end. This bacterial-like composite transposon is almost identical in *P. furiosus* and *T. litoralis* with only 153 nucleotide differences by a possible event of lateral gene transfer (DiRuggiero et al. 2000).

**MalE (TMBP):** MalE (TMBP) is a binding protein in trehalose/maltose ABC transporter, which binds substrate from environment and delivers to the membrane domain of the transporter. TMBP exhibits about 40% homology in amino acid sequences with the maltose/maltodextrin binding protein in *E. Coli*. It contains a lipid anchor connected to the membrane in its amino-terminus, which is similar to that in gram-positive bacteria (Tam and Saier 1993). The protein shows high affinity to both trehalose and maltose (17 and 22 nM, respectively), and a Vmax of 3–7 nmol min⁻¹ mg⁻¹ protein at 85°C (Xavier et al. 1996). The three dimensional structure of TMBP in *T. litoralis* is very similar to that in *E. coli* (Diez et al. 2001). The substrate binding pocket is formed by two lobes that connect via a flexible hinge. Each lob binds to one of the two membrane domains (Boos and Shuman 1998). The catalytic site of TMBP can only accommodate maltose and trehalose, but not maltotriose. TMBP expression is observed when cells are grown on maltose, trehalose and yeast extract (which contains trehalose) (Horlacher et al. 1998). Expression is also observed upon growth on the substrates containing α-glucosides such as maltotriose and starch, even though these
substrates are not recognized by TMBP. These substrates are most likely hydrolyzed by extracellular \( \alpha \)-amylase and amylopullulanases that release glucose, short maltodextrin and inducer maltose (Koning et al. 2002).

**MalF and MalG:** The genes of \( \textit{malG} \) and \( \textit{malF} \) encode two internal membrane proteins. The deduced amino acid sequences of the inner membrane proteins are also homologous to the other MalF and MalG protein sequences of \( G^+ \) and \( G^- \) bacteria. Both proteins have a sequence identity around 30% with the correspondent protein from \( E. \textit{coli} \).

The conserved EAA loop usually found in membrane components of ABC transporter is presented in thermococcal MalF and MalG. Moreover, MalG of \( T. \textit{litoralis} \) shows entire sequence homology to its counterpart of \( E. \textit{coli} \) including six trans-membrane segments.

**MalK:** The ATP-hydrolyzing subunit of the ABC trehalose/maltose transporter is named as MalK. The domains involving in the hydrolysis of ATP and the interaction with the membrane domains, and a carboxyl-terminal domain have been characterized in \( T. \textit{litoralis} \) (Greller et al. 1999, Diederichs et al., 2000). The structure of MalK shows a dimer formation. The carboxy-terminal domain of MalK predominately composes of \( \beta \)-sheets and partly shows structural homology with the oligonucleotide/oligosaccharide binding fold. This domain is similar with MalK of \( E. \textit{coli} \) which participates in the binding with MalT, a central activator for stimulating transcription of all \( \textit{mal} \) genes (Boos and Shuman 1998). The MalT–like protein has not been found in archaeal genomic sequence.

**Maltose metabolism**

Metabolic strategy of maltose in \( T. \textit{litoralis} \) shows similar way to that in \( E. \textit{coli} \). Two enzymes, 4-\( \alpha \)-glucanotransferase and maltodextrin phosphorylase involve in maltose degradation in \( T. \textit{litoralis} \) (Xavier et al. 1999). These two enzymes have been purified, cloned and sequenced (Jeon et al. 1997) which appear same properties on substrate specificity and catalytic property with those of \( E. \textit{coli} \). The first enzyme produces glucose and a series of maltodextrin from maltose by the transferring reaction. The second enzyme then degrades maltodextrin longer than three glucose residues into glucose-1-P (Xavier et al. 1999). Consequently, the glucose and glucose-1-P are converted into glucose-6-P by a hexokinase and a phosphoglycomutase.
Maltodextrin transporter system and maltodextrin metabolism

A gene cluster containing maltodextrin ABC transporter in *T. litoralis* had been reported (Imamura et al. 2004). It contains a typical ABC transporter including maltodextrin binding protein (MdxE), two transmembrane permases MdxF and MdxG, an ATPase subunit MdxK, an extracellular amylopullulanase, and a putative phosphor-sugar mutase. Only maltodextrins longer than maltotriose are considered to be transported by this maltodextrin transporter, even though maltose also binds to MdxE with low affinity. The purified recombinant amylopullulanase prefers to hydrolyze pullulan containing α-1,6 linkage than starch or maltodextrins containing α-1,4 linkage. Maltotriose is yielded from pullulan hydrolysis, whereas glucose and maltodextrins (mainly from maltotriose to maltoheptose) are produced from starch hydrolysis by amylopullulanase.

Aims and outline of this research

A trehalose/maltose transporter system, which transports maltose and trehalose into the cell, orients on a 16 kb bacterial-like transporson in *Thermococcus litoralis* that appears in nearly identical sequence in *Pyrococcus furiosus* as the result of a lateral gene transfer. Unlike the typical ABC transporter system, the genes encoding the enzymes for maltose utilization are neither located in this gene cluster encoding this maltose/trehalose transporter nor under the control of maltose being transported. The trehalose-utilization enzymes could not be detected in the cell extract of *T. litoralis*. The binding protein (TMBP) is induced by the presence of maltose and trehalose in the medium, which implies that a transcriptional regulator could be in the transporter system. Meanwhile three genes in this gene cluster encoding the transporter system are still not identified. The aims of this research were to explore the functions of these three unidentified genes in this gene cluster, and to investigate the interaction of sugar transport, metabolism and regulation in this trehalose/maltose transport system.

In chapter 2, a novel trehalose glycosyltransferring snythase was identified and characterized. This enzyme is involved in trehalose metabolism and it is regulated by TrmB. This is the first report of such kind of enzyme.

In Chapter 3, the first ATP-dependent fructose-6-P forming fructokinase (FrK) in archaea was identified and characterized. This single gene (*frk*) is oriented divergently to the gene cluster encoding the trehalose/maltose ABC transporter.
The expression of TMBP (binding protein) of the trehalose/maltose ABC transporter is induced in maltose and trehalose grown-cells. It could be a result of a transcriptional regulator encoding in this transporter system. In order to search transcriptional regulator, all genes in this gene cluster have been investigated. A sugar-specific transcriptional repressor of the trehalose/maltose ABC transporter (TrmB) has been identified. The properties of TrmB were described in Chapter 4.

By present research, all genes located in the trehalose/maltose transporter gene cluster of *T. litoralis* have been identified and characterized. The sugar metabolism and regulation in this transport system will be discussed in Chapter 5.

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Chapter 2

TreT, a novel trehalose glycosyltransferring synthase of the hyperthermophilic archaeon *Thermococcus litoralis*

Qiuhao Qu, Sung-Jae Lee, and Winfried Boos

**ABSTRACT**

The gene cluster in *Thermococcus litoralis* encoding a multicomponent and binding protein-dependent ABC transporter for trehalose and maltose contains an open reading frame of unknown function. We cloned this gene (now called *treT*), expressed it in *E. coli*, purified the encoded protein and identified it as an enzyme forming trehalose and ADP from ADP-glucose and glucose. The enzyme can also use UDP- and GDP-glucose but with less efficiency. The reaction is reversible and ADP-glucose plus glucose can also be formed from trehalose and ADP. The rate of reaction and the equilibrium are favoring the formation of trehalose. At 90°C, the optimal temperature for the enzymatic reaction, the half-maximal concentration of ADP-glucose at saturating glucose concentrations is 1.14 mM and the Vmax is 160 units/mg protein. In the reverse reaction, the half-maximal concentration of trehalose at saturating ADP concentrations is 11.5 mM and the Vmax was estimated to be 17 units/mg protein. Under non-denaturating in vitro conditions the enzyme behaves as a dimer of identical subunits of 48 kDa. As the transporter encoded in the same gene cluster, TreT is induced by trehalose and maltose in the growth medium.

**ABBREVIATIONS:** EIIBC enzyme II BC; PTS, phosphotransferase system.
INTRODUCTION

Trehalose synthesis in response to osmotic stress is observed in many organisms. For instance, in *Escherichia coli* trehalose is formed by the gene products of *otsA* and *otsB* catalyzing the transfer of glucose from UDP-glucose onto glucose-6-P (trehalose-6-P synthase) followed by the formation of trehalose (trehalose-6-P phosphatase) (Strøm and Kaasen 1993). This is the usual pathway for trehalose synthesis in most organisms. Another enzymatic reaction, catalyzed by the *treS* gene product, transforms maltose into trehalose in an equilibrium reaction (Tsusaki et al. 1997). A third possibility is realized in some hyperthermophilic organisms. Here, the terminal α(1-4)-linked unit of a linear maltodextrin is converted into an α,α(1-1) linkage by maltooligosyltrehalose synthase (encoded by *treY*). The terminal trehalose is then released by an additional enzyme, maltooligosyltrehalose trehalohydrolase (encoded by *treZ*) (Maruta et al. 1996). Formally, trehalose phosphorylase (Eis and Nidetzky 1999) forming glucose and glucose-1-P from trehalose may also be regarded as a trehalose-synthesizing enzyme since the reaction is reversible at least *in vitro*. Yet, there is little doubt that the function of trehalose phosphorylase *in vivo* is in trehalose degradation rather than synthesis.

Trehalose metabolism, aside from the function of trehalose phosphorylase, is usually achieved by trehalase, an enzyme hydrolyzing trehalose to glucose (Boos et al. 1987; Hirimburegama et al. 1992; Horlacher et al. 1996). In Gram-negative enteric bacteria such as *E. coli*, degradation of trehalose is initiated by its uptake via an EIIBC of the phosphotransferase system under simultaneous phosphorylation to trehalose-6-P, followed by cytoplasmic hydrolysis of the latter to glucose and glucose-6-P mediated by trehalose-6-P hydrolase (Rimmmele and Boos 1994; Klein et al. 1995).

The hyperthermophilic archaeon *Thermococcus litoralis* accumulates trehalose in response to high osmolarity when grown in the presence of yeast extract (Lamosa et al. 1998) that contains trehalose. Indeed, trehalose induces a high affinity and binding protein-dependent ABC transporter for trehalose and maltose that is most likely responsible for the accumulation of trehalose. This operon is also induced under conditions of elevated temperature (Shockley et al. 2003). Thus, it is understood that the accumulation of trehalose occurs in response to osmotic and, possibly, heat stress. However, trehalose must also be metabolized in *T. litoralis* albeit slowly since trehalose is used up in the stationary phase of growth after its accumulation. The enzymes for trehalose synthesis and degradation have not been identified in *T. litoralis*. Dialyzed
cellular extracts obtained from cells grown in the presence of trehalose do not hydrolyze or otherwise modify trehalose. In the past we have been studying the function of proteins encoded by a gene cluster in *Thermococcus litoralis* that appears in nearly identical sequence in *Pyrococcus furiosus* as the result of a lateral gene transfer (DiRuggiero et al. 2000). This cluster contains genes for a binding protein-dependent ABC transporter for trehalose and maltose (Xavier et al. 1996; Horlacher et al. 1998; Diederichs et al. 2000; Diez et al. 2001; Greller et al. 2001) as well as a gene encoding the maltose-inducible repressor for the operon (Lee et al. 2003). The last unidentified gene in this cluster, now called *treT* (appearing in identical sequence in *P. furiosus*, PF 1742), shows homology to trehalose phosphorylases from fungi. Here we report that the enzyme exhibits a novel activity to reversibly transfer the glucose moiety of ADP-glucose onto glucose to form trehalose.

**MATERIALS AND METHODS**

**Materials**

$^{14}$C-trehalose (600-720 µCi/µmol) and purified *E.coli* trehalase were obtained from Trenzyme GmbH, Germany (www.trenzyme.com). All other commercial chemicals and enzymes used in this study were purchased from Sigma.

**Cloning of treT, overexpression and purification of the recombinant protein**

Two primers were designed based on the annotated sequence of the putative trehalose synthase (protein ID: AAG45375, PF1742). The forward primer was 5’-CGGGATCCATGTATGAGGTAACGAAGTTGGAGTTTTGGA-3’ and the reverse primer was 5’-GCGTCGACAAAAAGAATTTTAGTAATCAAGTACCTCTCAAG-3’ with the BamHI and Sall restriction site in bold, respectively. The chromosomal DNA was used as a template for PCR prepared as previously described (Lee et al. 2003). The PCR product was cloned into plasmid pET24a(+) (Novagen, Inc.) encoding a C-terminal His-tag. The resulting plasmid was transformed into *E. coli* strain BL21. The transformed strain was grown at 28°C in 4 L NZA medium (10 g NZ-amine A (Sheffield product Inc.), 5 g yeast extract, and 7.5 g NaCl in 1 L distilled water) and was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside overnight after the OD$_{578}$ of the culture had reached 1.0.
The cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.5 containing 5 mM MgCl₂ and 500 mM NaCl. The suspension was extracted by passing it three times through a French pressure at 16,000 p.s.i and 4°C. The cell debris was removed by centrifugation at 19,000 x g for 10 min. The supernatant was heated to 80°C for 10 min and centrifuged at 19,000 x g for 20 min. The supernatant was loaded onto a Ni-NTA affinity column equilibrated with 50 mM Tris-HCl, pH 7.5 as the first step of purification. The column was washed twice with the same Tris-HCl buffer containing 20 mM and 50 mM imidazole, respectively. Bound protein was subsequently eluted with 200 mM imidazole and passed through a desalting column (Econo-Pac 10 DG, BioRad laboratories) to remove the imidazole. The preparation was purified further by molecular sieve chromatography on a Superdex 200 column (Amersham Biosciences) at 4°C equilibrated with PBS buffer containing 1 mM β-mercaptoethanol and 5 mM MgCl₂ to remove contaminants (as the second step of purification). Purified proteins were analyzed in 12% SDS-polyacrylamide gels and Western blots using anti-His-tag antibodies from mouse (Qiagen).

**Molecular mass determination**

Gel filtration chromatography was performed on a Superdex 200 column (Amersham Biosciences) at 4°C equilibrated with PBS buffer containing 1 mM β-mercaptoethanol and 5 mM MgCl₂. The low molecular mass calibration kit (Amersham Biosciences) was used consisting of bovine pancreas chymotrypsinogen A (25 kDa), hen egg albumin (43 kDa), bovine pancreas serum albumin (67 kDa), and rabbit muscle aldolase (158 kDa).

**Thin layer chromatography (TLC)**

Enzyme reaction mixtures were prepared in a total volume of 100 µl at 80°C. In the trehalose synthesis (forward) reaction, 10 mM glucose, 10 mM ADP-glucose, 20 mM MgCl₂ and 0.5 µg enzyme were incubated in 50 mM Tris-HCl, pH 6.5. Samples were removed at different time intervals and 6 µl were spotted onto silica gel plates (type 60, Merck) which were then developed with butanol:ethanol:water (5:3:2) as solvent. Sugar-containing compounds were visualized by dipping the dried plate into methanol containing 5% H₂SO₄ followed by charring at 120°C for 5 min.
Enzymatic trehalose degradation (reverse reaction) was done in 100 µl containing either 10 mM unlabeled trehalose or 47 µM 14C-trehalose and 10 mM ADP in the presence of 20 mM MgCl₂ at 80°C. Samples were removed at different time intervals and separated by TLC. The products formed were analyzed by charring or by autoradiography. The control experiments were performed in the trehalose synthesis as well as the degradation reaction with either denatured enzyme or no enzyme in the mixtures described above. The enzyme was denatured by autoclaving at 130°C for 30 min.

HPLC
Trehalose formation was also determined using a high pH-anion exchange chromatography (Dionex Corp., Mississauga, Ontario) with pulsed amperometric detection. 100 µl reaction mixture containing 8 mM ADP-glucose, 10 mM MgCl₂ and varying glucose concentrations in 50 mM Tris-HCl, pH 6.5 were incubated at 90°C. The reactions were started with the enzyme and stopped when still linear by adding cold buffer to a volume of 1 ml. The mixture was then kept on ice for 20 min. A Dionex carboPac MA-1 analytical column (4 × 250) equilibrated with 100 mM sodium acetate and 500 mM NaOH for the separation of monosaccharides and disaccharides was injected with 20 µl reaction mixture. The quantities of glucose and trehalose were calculated by comparison with sugar standards (1 mM).

Kinetic constants
Trehalose synthase activity (initial rates) was measured in both directions with discontinuous coupled enzyme assays. For trehalose synthesis, the half-maximal substrate concentration and Vmax for ADP-glucose was determined following ADP formation (method 1). 100 µl reaction mixture containing 20 mM glucose, 10 mM MgCl₂ and varying ADP-glucose concentrations in 50 mM Tris-HCl, pH 6.5 were incubated for 7 min at 90°C. The reaction was started by adding enzyme and stopped by adding cold buffer to a final volume of 980 µl. After cooling on ice for 20 min, the ADP produced in the reaction mixture was determined spectrophotometrically by adding 0.8 units pyruvate kinase and 1.35 units lactate dehydrogenase in a mixture, 0.3 mM NADH and 2 mM phosphoenolpyruvate at 25°C. The decrease in the absorption at 340 nm was followed in a total volume of 1 ml.
The half-maximal glucose concentration for ADP-glucose-dependent trehalose formation was determined using HPLC (see above) to follow the initial rate of trehalose formation. The reverse reaction (trehalose degradation) was measured by following the initial rate of glucose formation (method 2). 100 µl reaction mixture contained 5 mM ADP, 20 mM MgCl₂ and varying trehalose concentrations in 50 mM Tris-HCl, pH 6.5 at 90°C. The reaction was started by adding 0.5 µg pure enzyme and stopped by adding 380 µl cold buffer. Then the following coupled enzyme assay was performed at 25°C: 0.5 units of hexokinase and glucose-6-P dehydrogenase, a final concentration of 0.2 mM ATP, 1 mM MgCl₂ and 0.4 mM NADP⁺ were added to a final volume of 500 µl. The NADPH produced was measured at 340 nm.

For determining substrate specificity instead of glucose potential sugar acceptors were used in a concentration of 10 mM in the forward reaction. In the reverse reaction trehalose was replaced by potential disaccharide substrates at 10 mM.

All tests were done in duplicate and all auxiliary enzymes were not rate-limiting. One unit of enzyme activity is defined as 1 µmol substrate formed per min at 90°C. Specific enzyme activity is referred to as units per mg protein.

**Induction by different sugars**

*T. litoralis* was cultivated in Bacto Marine Broth medium (Lamosa et al. 1998) supplemented only with peptone (5 g/l) as a control or with peptone plus maltose (3 g/l), trehalose (3 g/l) or sucrose (3 g/l) for TreT induction. Cells were grown overnight at 80°C, harvested by centrifugation (8,000 rpm, 4°C) and extracted by sonication in an anaerobic chamber under a N₂:H₂ (95:5, v:v) atmosphere in 50 mM Tris-HCl, pH 7.5 containing 5 mM MgCl₂ and 150 mM NaCl. Thin layer chromatography to measure trehalose formation was performed as described above. For Western Blotting, the uninduced and induced cell extracts were loaded onto a 12% SDS-PAGE gel, transferred and blotted with a Fluorotrans transfer membrane (Pall Europe Limited, England) and incubated with anti-TreT antibodies raised in rabbit.
RESULTS

treT encodes an enzyme involved in trehalose metabolism as indicated by sequence similarity

Fig. 1 shows the operon encoding the ABC transporter for trehalose and maltose (Horlacher et al. 1998) and the gene for an ATP-dependent fructokinase (frk) in T. litoralis (Qu et al. 2004). This gene cluster is contained in a 16 kb fragment of T. litoralis that appears to be the result of a horizontal gene transfer (of a composite transposon) between T. litoralis and P. furiosus (DiRuggiero et al. 2000). The operon encoding the ABC transporter is induced by maltose and trehalose with TrmB acting as a transcriptional repressor (Lee et al. 2003). From its encoded transport proteins and profile of induction by maltose and trehalose, one would assume that the role of the gene cluster is in sugar metabolism. In this respect it was of interest what treT, the only remaining gene of unidentified function, would encode. NCBI BLAST results revealed a conserved domain found in glycosyl transferases near the C-terminus of TreT. The five best hits concern hypothetical proteins from other hyperthermophiles, for instance, PH1035 of Pyrococcus horikoshii (77% identity) or TM 0392 of Thermotoga maritima (53% identity). Also, there is high sequence similarity with two enzymes with trehalose phosphorylase activity from fungi, Pleurotus sajor-caju (31% identity) and Grifola frondosa (30% identity). Interestingly, TreT of T. litoralis also has low sequence similarity to a sucrose synthase from potato (EC 2.4.1.13; 25% identity). This enzyme performs the reversible transfer of glucose via UDP-glucose onto fructose to form sucrose.

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**Fig. 1.** The T. litoralis ABC transporter gene cluster and the products of its genes. Arrows indicate the direction of transcription of the individual genes.
Fig. 2. Sequence comparison. Multiple alignment of the amino acid sequence of TreT with those of a hypothetical protein from *P. horikoshii* (PH1035), trehalose phosphorylase from *P. sajor-caju* (EC 2.4.1.64), trehalose synthase from *G. frondosa* (EC 2.4.1.64), and sucrose synthase from potato. Asterisks indicate identical amino acids; colons, high amino acid conservation; dots, low conservation in all five proteins as given by Clustal W.

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<th>Protein</th>
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<td>TreT_ <em>T. litoralis</em></td>
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Fig. 2 shows a comparison of the deduced amino acid sequence of TreT with four other protein sequences including a hypothetical protein from *P. horikoshii*, trehalose phosphorylase from fungi and sucrose synthase from potato, as given by Clustal W multiple sequence alignment.

Cloning and heterologous expression of *treT* and purification of the product. Amplified chromosomal *treT* DNA from *T. litoralis* was digested with two restriction endonucleases and cloned under IPTG control into expression vector pET 24a(+) as a C-terminal his-tag version. Fig. 3A shows an SDS-PAGE analysis of total cellular proteins with and without induction by IPTG. Cellular extracts of IPTG-induced cells were heated to 80°C for 10 min prior to purification of the clarified extracts by Ni-NTA affinity chromatography. TreT is a protein of 413 amino acids with a calculated molecular mass of 48,146 Da. After the first purification step, two bands of approx. 48 and 37 kDa were seen in 12% SDS polyacrylamide gels. Molecular sieve chromatography under non-
terminal his-tag version. Fig. 3A shows an SDS-PAGE analysis of total cellular proteins with and without induction by IPTG. Cellular extracts of IPTG-induced cells were heated to 80°C for 10 min prior to purification of the clarified extracts by Ni-NTA affinity chromatography. TreT is a protein of 413 amino acids with a calculated molecular mass of 48,146 Da. After the first purification step, two bands of approx. 48 and 37 kDa were seen in 12% SDS polyacrylamide gels. Molecular sieve chromatography under non-denaturing conditions led to the purification of the larger protein exhibiting a molecular mass of 104,000 Da (Fig. 3C) indicating that TreT is a dimer of two identical polypeptides of 48 kDa.

Fig. 3. Purification and molecular mass determination of TreT. A. 12% SDS-PAGE of TreT. Lane 1, protein standards; lane 2, uninduced cell extract; lane 3, induced cell extract; lane 4, TreT after Ni-NTA affinity chromatography; lane 5, TreT further purified by molecular sieve chromatography on Superdex 200. B. Western blot analysis of TreT purification using anti-His-tag antibodies. Lane 1, protein standards; lane 2, TreT after Ni-NTA affinity chromatography; lane 3, purified single band of TreT after molecular sieve chromatography; lane 4, uninduced cell extract; lane 5, induced cell extract. C. Gel filtration chromatography of TreT on Superdex 200. Protein standards used: 1, rabbit muscle aldolase (158 kDa), 2, bovine pancreas serum albumin (67 kDa), 3, hen egg albumin (43 kDa), 4, bovine pancreas chymotrypsinogen A (25 kDa).
**TreT is an ADP-glucose-dependent trehalose glycosyltransferring synthase.** We performed several tests with purified TreT to elucidate its enzymatic activity. We tested several disaccharides (maltose, sucrose, trehalose, lactose) and polysaccharides (linear maltodextrins, pullulan) and combinations of polysaccharides with disaccharides for their ability to act as substrates for a putative hydrolytic, phosphorolytic or transfer activity of the enzyme. We also used glucose-6-P as acceptor for a possible glucosyltransfer from UDP-glucose or glucose-1-P to form trehalose-6-P or trehalose. None of these tests gave a positive result. However, when incubating ADP-glucose alone with the enzyme at 85°C a product was slowly formed that could be identified by thin layer chromatography (TLC) as trehalose. ADP-glucose in the absence of the enzyme was then found to be unstable at this temperature and the buffer conditions used to slowly form free glucose. We then realized that the slow formation of trehalose was due to the transfer of glucose from ADP-glucose onto the glucose that had been liberated from ADP-glucose. Indeed, when glucose was included in the reaction mixture from the beginning on, fast and time-dependent formation of trehalose could be observed (Fig. 4A). The identity of the formed product with trehalose was established by the action of *E. coli* periplasmic trehalase (Boos et al. 1987). This enzyme is specific for α,α-(1-1) glucosyl glucoside (trehalose) and does not hydrolyze α− or β-paranitrophenyl glucosides nor maltose, sucrose, lactose or cellobiose (Fig. 4A).

The trehalose synthesizing reaction never went to completion indicating that it is reversible. At high ADP-glucose concentration (10 mM) and trace amounts of ^14^C-glucose the reaction went to near completion. This demonstrates its reversible nature. When starting the reaction with trehalose and ADP (i.e., the reverse reaction) we observed the formation of ADP-glucose and glucose (Figs. 4C and D). Using ^14^C-trehalose the newly formed radioactive spot (aside from glucose) migrated identically
Fig. 4. Identification of enzymatic products in the trehalose synthesis and degradation reactions by TLC. Samples of the incubation mixture were taken after different time intervals in the presence of enzyme.

**A. Forward reaction** in the presence of 10 mM glucose and 10 mM ADP-glucose. Lanes 1-6, 0, 5, 10, 15, 20 and 30 min incubation; lane 7, sample from lane 6 treated with *E. coli* trehalase for 1 h at RT; lanes 8-12, sugar standards: glucose, trehalose, ADP-glucose, glucose-1-P and glucose-6-P; lane 13, sugar standards, from top to bottom: glucose, trehalose and ADP-glucose; lanes 14-23, cellobiose, maltose, sucrose, lactose or trehalose incubated with *E. coli* trehalase, samples taken immediately after the addition of enzyme and after 1 h at RT each. The arrow indicated the trehalose hydrolysis reaction with TreA.

**B.** Forward reaction in the presence of $^{14}$C-glucose and 10 mM ADP-glucose. Lanes 1-8, reaction mixture incubated for 0, 3, 5, 10, 15, 20, 25 and 30 min; lane 9, $^{14}$C-glucose; lane 10, $^{14}$C-trehalose.

**C.** Reverse reaction using 10 mM trehalose and 10 mM ADP (lanes 1-5) or 50 mM phosphate (lanes 6-10) after incubation for 0, 5, 10, 20 and 40 min, respectively. Lanes 11-13, sugar standards: lane 11, glucose, trehalose, ADP-glucose (from top to bottom); lane 12, glucose-1-P; lane 13: glucose-6-P.

**D.** Reverse reaction using $^{14}$C-trehalose and 10 mM ADP. Lane 1, $^{14}$C-glucose; lane 2, $^{14}$C-trehalose; lanes 3-10, reaction mixture incubated for 0, 1, 2, 5, 10, 20, 40 and 60 min.
and can be superimposed with chemically stained ADP-glucose (charring is not shown). No enzymatic reaction can be detected when phosphate is used instead of ADP (Fig 4C). Using autoclaved TreT as a control in both the trehalose synthesis and degradation reactions, no product formation can be detected (not shown). In the ADP-glucose forming reaction the amount of enzyme needed to obtain the same rate of reaction is ten times higher than for trehalose formation. Under these conditions, the reaction starts fast towards ADP-glucose but the equilibrium is shifted strongly in the direction of trehalose formation. This can also be seen when the reaction is started with trace amounts of radiolabeled trehalose and 10 mM ADP (Fig. 4D).

**Fig. 5. Identification of enzymatic products of the forward reaction by HPLC.**

The retention time of standard trehalose and glucose were 16.35 min and 22.00 min, respectively. Three enzyme mixtures were prepared with increased glucose concentration at 3.5 mM, 5.0 mM and 7.5 mM but constant ADP-glucose (8 mM) at 90°C for 7 min. Three peaks appeared at different retention times: the injection peak, trehalose (16.35 min) and glucose (22.00 min).

The formation of free glucose in this reaction is not caused by unspecific degradation of trehalose at the assay temperature since trehalose is stable under the conditions used (not shown). The stability of trehalose at high temperature had been demonstrated previously (Higashiyama 2002). Trehalose synthesis was also followed using HPLC. At varying
initial glucose concentrations (3.5, 5, and 7.5 mM) but identical TreT concentrations, a new peak emerged which corresponded to standard trehalose (16.35 min retention time). The trehalose concentration obtained in this way after 7 min increased in response to the initial glucose concentration (Fig. 5).

![Graph A]

**Fig. 6. Kinetic analysis of the forward and the reverse reaction.** The initial rate of trehalose formation was used to determine the concentration dependence of ADP-glucose (*A*) and glucose (*B*) in the forward reaction. In the reverse reaction, the formation of ADP was followed to determine the concentration dependence of trehalose (*C*). Values for Km and Vmax were extrapolated from the figures shown.
Kinetic constants, temperature dependence and substrate specificity of TreT. The kinetics of trehalose glycosyltransferring synthase activity were measured in discontinuous two-step coupled assays. The half-maximal concentration of ADP-glucose at saturating glucose concentrations was 1.14 mM (Fig. 6A) and that for glucose at saturating ADP-glucose concentrations was 6.2 mM (Fig. 6B) as determined by HPLC. The Vmax for the purified enzyme was 160 units per mg protein for ADP-glucose at saturating glucose concentration and 220 units per mg protein for glucose at saturating ADP-glucose concentration. In the reverse reaction, the half-maximal concentration of trehalose was 11.5 mM and the Vmax was estimated to be 17 units per mg protein (Fig. 6C). Thus, under saturating substrate concentrations the enzymatic reaction is strongly favored towards the formation of trehalose.

The effect of temperature (from 30-100°C) on the enzymatic activity was measured with method 1 at pH 6.5. The optimum for the trehalose forming reaction was 90°C (Fig. 7) with 65% enzymatic activity remaining at 100°C. TreT showed a narrow pH optimum range around pH 6.5. At pH 7.5, only 13% of the activity at pH 6.5 remained.

Sugar specificity was tested in the directions of disaccharide synthesis as well as degradation. Fructose, mannose, galactose, xylose, 2-deoxy-glucose, glucosamine, α-methylglucoside, sorbitol, G-1-P, G-6-P, F-1-P, and F-6-P were unable to replace glucose as an acceptor for the glucosyl transfer from ADP-glucose when tested with method 1. Also, maltose, lactose and sucrose were unable to be cleaved in the presence of ADP when tested with HPLC. Phosphate and PEP could not substitute for ADP in trehalose degradation as tested by using $^{14}$C trehalose as substrate followed by TLC and
autoradiography. Thus, TreT appears to be specific for the synthesis and the degradation of trehalose.

With UDP-glucose or GDP-glucose replacing ADP-glucose, only about 6% and 5% of TreT activity remained while TDP-glucose was inactive. Method 1 was used to test the influence of cations (at 20 mM concentration). The enzyme needs Mg\(^{2+}\) for its activity (set as 100%) which can be replaced to some extent by CaCl\(_2\) (91%), MnCl\(_2\) (78%), CoCl\(_2\) (71.6%) and NiCl\(_2\) (57%). No enzymatic activity could be seen with ZnCl\(_2\).

![TLC analysis of TreT induction by different sugars.](image)

**Fig. 8. TLC analysis of TreT induction by different sugars.** *A.* Trehalose formation by extracts of *T. litoralis* grown on peptone (lanes 2-6) and peptone plus sucrose (lanes 7-11) after 0, 5, 10, 20 and 40 min incubation, respectively. *B.* Trehalose formation by by extracts of trehalose-induced (lanes 2-6) or maltose-induced cells (lanes 7-11) after 0, 3, 5, 7 and 10 min incubation. Lane 1 in *A* and *B*, sugar standards: glucose, trehalose, ADP-glucose.

**TreT is induced by maltose and trehalose in the growth medium.** We obtained cellular extracts from *T. litoralis* grown in peptone alone (as a control) and in peptone
plus maltose, trehalose or sucrose. The dialyzed extracts at identical protein concentrations were tested for their ability to form trehalose from ADP-glucose and glucose. Trehalose formation was monitored by TLC and is shown in Fig. 8. When grown only on peptone, trehalose formation was hardly detectable (Fig. 8A). Induction was highest with maltose in the growth medium (Fig. 8B), less efficient with trehalose and even less with sucrose although with the latter it was clearly higher than in the extract of uninduced cells (grown on peptone alone). The induction of TreT by maltose and trehalose in the growth medium could also be detected in Western blots with anti-TreT antibodies from rabbit (not shown). The induction pattern of \textit{treT} was identical to that of \textit{malE}, one of the genes encoding the ABC transporter for trehalose and maltose, consistent with the notion that these genes form an operon together with \textit{treT} (Selig et al. 1997; Greller et al. 1999).

**DISCUSSION**

We describe an enzyme, TreT, from the hyperthermophilic archaeon \textit{T. litoralis} that produces trehalose by the transfer of glucose from ADP-glucose (or, less effectively, UDP-glucose and GDP-glucose) onto the 1-position of glucose to form trehalose and ADP. The trehalose produced is identical with \(\alpha,\alpha\)-trehalose since \textit{E. coli} trehalase was able to hydrolyze it to glucose. Such an enzymatic activity for the synthesis of trehalose has not yet been described for any organism. Instead, when activated glucose in the form of UDP-glucose is being used in the synthesis of trehalose the transfer of the glucosyl moiety always occurs onto glucose-6-P to form trehalose-6-P followed by enzymatic hydrolysis of the latter to trehalose (for instance, by the enzymes encoded by the \textit{E. coli otsA otsB} genes). In this way, unidirectional formation of trehalose is assured. The trehalose synthesizing reaction catalyzed by TreT is reversible. Thus, ADP-glucose and glucose can be formed from ADP and trehalose.

What is the physiological role of TreT? From the position of \textit{treT} within a gene cluster encoding an ABC transporter for trehalose (and maltose) one would be inclined to interprete the function of TreT as a trehalose-degrading enzyme rather than a trehalose-synthesizing enzyme. This appears also sensible in light of the rather high half-maximal concentration for glucose of 6.2 mM. It would be unlikely that growing \textit{T. litoralis} contains such high internal glucose concentrations. With a Km of 0.7 mM for glucose, the ADP-dependent hexokinase (Selig et al. 1997) would effectively reduce the
concentration of free internal glucose and prevent any noticeable trehalose formation by TreT. One could argue that the metabolism of maltose which also induces TreT synthesis would provide ample amounts of free internal glucose (Xavier et al. 1999) needed for trehalose formation by TreT. However, trehalose formation was not observed when *T. litoralis* grew on maltose (Lamosa et al. 1998). The accumulation of trehalose present in the medium via the high affinity and binding protein-dependent ABC transporter will result in high internal trehalose concentrations (Lamosa et al. 1998) which could subsequently be used for glucose formation by TreT, followed by hexokinase-dependent phosphorylation and glycolysis (Selig et al. 1997). The fact that the formation of ADP-glucose is a necessary by-product of this pathway opens the possibility that it may be used for glucose polymer formation in the form of glycogen. Indeed, the major pathway of cellulose biosynthesis in plants and cyanobacteria follows this strategy (Porchia et al. 1999). There, sucrose is used by an enzyme that forms UDP-glucose from UDP and sucrose. UDP-glucose is then used as a donor for cellulose biosynthesis (Sebkova et al. 1995). There is only residual sequence similarity of these types of enzymes with TreT described here. In contrast, significant sequence identity is seen with enzymes that are often described as putative trehalose synthases and a few are characterized as authentic trehalose phosphorylases. These enzymes reversibly cleave trehalose by phosphorolysis to form glucose-β-1-P and glucose (Eis et al. 2001). They usually function as trehalose-degrading enzymes rather than trehalose-synthesizing enzymes. The high similarity of these enzymes to TreT is intriguing. One is inclined to describe the ADP-glucose formation by TreT also as a phosphorolysis in which not free Pi but the β-phosphoryl group of ADP is the active agent hydrolyzing trehalose.

**ACKNOWLEDGEMENTS**

We acknowledge the contribution of Peter Kroth who made us aware of the similarity of the TreT function to the plant enzymes forming UDP-glucose from sucrose in cellulose biosynthesis. We thank Reinhold Horlacher for providing 14C-trehalose and purified trehalase, Xiangzhen Li for preparing *T. litoralis* cell extract, Christoph Mayer for measuring sugars with HPLC, Irena Hendekovic for kinetic analyses and E. Oberer-Bley for critically reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.
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Chapter 3

Molecular and biochemical characterization of a fructose-6-phosphate-forming and ATP-dependent fructokinase of the hyperthermophilic archaeon Thermococcus litoralis

Qiuhao Qu, Sung-Jae Lee, and Winfried Boos

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ABSTRACT

Close to an operon encoding an ABC transporter for maltose and trehalose, Thermococcus litoralis contains a gene whose encoded sequence showed similarity to sugar kinases. We cloned this gene, now called frk, and expressed it as a C-terminal His-tag version in E. coli. We purified the recombinant protein, identified it as an ATP-dependent and fructose-6-phosphate-forming fructokinase (Frk) and determined its biochemical properties. At its optimal temperature of 80°C the apparent $K_m$ and $V_{max}$ values of Frk were 2.3 mM and 730 U/mg protein for fructose at saturating ATP concentration, and 0.81 mM and 920 U/mg protein for ATP at saturating fructose concentration. The enzyme did not lose activity at 80°C for 4 hours. Under denaturating conditions in SDS-PAGE it exhibited a molecular mass of 35 kDa. Gel filtration chromatography revealed a molecular mass of 58 kDa indicating a dimer under non-denaturating in vitro conditions.

ABBREVIATIONS: Frk, fructokinase; P, phosphate; PBS, phosphate buffered saline; PTS, phosphotransferase system.
INTRODUCTION

*Thermococcus litoralis* is a hyperthermophilic marine archaeon which grows optimally at 85°C on peptone under anaerobic conditions (Neuner et al. 1990). The organism can utilize carbohydrates such as maltose, trehalose, and sucrose as well as starch but no monosaccharides except mannose (Rinker and Kelly 1996; Xavier et al. 1996). A binding protein-dependent maltose/trehalose ABC transporter in *T. litoralis* has been described (Xavier et al. 1996; Horlacher et al. 1998; Diederichs et al. 2000; Diez et al. 2001; Greller et al. 2001) and the enzymology for maltose metabolism has been studied (Xavier et al. 1999). Next to the operon harboring the ABC transporter genes a divergently oriented single gene encodes a putative sugar kinase. This gene as well as the operon are contained in a 16 kb DNA fragment that shows the typical features of a composite transposon. This whole cluster of genes was found in nearly identical sequence in *Pyrococcus furiosus*, and the functional form indicated a recent lateral gene transfer between the two organisms (DiRuggiero et al. 2000).

The deduced amino acid sequence of the putative sugar kinase, now called *frk*, revealed similarity to ATP-fructokinases that are part of the ribokinase family of sugar kinases (Bork et al. 1993) such as the *E. coli* ScrK protein (Schmid et al. 1988). The latter is involved in the metabolism of fructose which is internally released from sucrose phosphate taken up by a specific PTS. Sucrose has been reported to be also taken up by *T. litoralis* (Rinker and Kelly 1996). In archaea, specifically in the hyperthermophilic *Pyrococcus* species that are related to *T. litoralis*, no PTS transporter has been recognized so far, indicating that sucrose transport in *T. litoralis* would be performed by an active transport system. *frk* and the trehalose/maltose transport genes (*malE* operon) share a common divergent promoter but no obvious promoter elements can be recognized in front of *frk*. A transcriptional regulator (TrmB) for the *malE* operon has been identified (Lee et al. 2003). Footprint analysis revealed that TrmB binds to two sites in this promoter region, binding to one of them results in transcriptional repression of the *malE* operon. The other site consists of an inverted repeat sequence positioned at 73 to 40 bp in front of *frk* and has not been analyzed so far. Binding of TrmB to these two sites is prevented by maltose which supposedly acts as inducer, qualifying TrmB as a transcriptional repressor for the *malE* operon. However, TrmB also binds sucrose competitively with maltose (S.-J. Lee, unpublished results), indicating the involvement of TrmB in the regulation of sucrose (and therefore fructose) metabolism. Thus, it
became relevant to determine the enzymatic activity of the \textit{frk} gene product and its possible regulation by TrmB.

In this paper we report that the gene product of \textit{frk} in \textit{T. litoralis} is a fructokinase (Frk) forming fructose-6-P in an ATP-dependent manner. We purified the enzyme and determined its kinetic parameters. We found that the enzyme exhibited a high specific activity only for fructose.

**MATERIALS AND METHODS**

**Cloning, overexpression, purification and molecular mass determination.** Chromosomal DNA of \textit{T. litoralis} was prepared as previously described (Lee et al. 2003). PCR was performed with chromosomal DNA as template and primers encompassing \textit{frk}. Based on the annotated gene sequence (sugar kinase, AF307053), the two primers were 5'- CGGAATTCCATGCTATTTATTTACTTTAA-3' (forward primer with \textit{EcoR} I restriction site in bold) and 5'- CGGGATCCCGGAAGAAAGCTGAATTTCCT-3', (reverse primer with \textit{BamHI} recognition site in bold). The PCR product was digested with \textit{EcoRI} and \textit{BamHI} and cloned into plasmid pCS19 (Spiess et al. 1999) encoding a C-terminal 6xHis tag. The resulting plasmid was named pSL149 and transformed into \textit{E. coli} strain DHB4 (Boyd et al. 1987). The transformant was cultivated at 30°C in 2 L NZA medium (10 g NZ-amine A (Sheffield product Inc), 5 g yeast extract, and 7.5 g NaCl in 1 L distilled water) containing 200 µg/ml ampicillin. When the OD\textsubscript{600} reached 0.5 to 0.7, the cells were induced with 0.1 mM isopropyl-\textbeta-D-thiogalactopyranoside, followed by growth for 5 more hours.

Cells were harvested by centrifugation and resuspended in 20 ml 50 mM Tris-HCl, pH 7.5 containing 5 mM MgCl\textsubscript{2} and 500 mM NaCl. The suspension was passed three times through a French pressure cell at 16,000 p.s.i. and centrifuged at 19,000 x g for 10 min. The supernatant was heated at 80°C for 10 min and the precipitant removed by centrifugation (19,000 x g, 20 min).

The supernatant was loaded onto a Ni-NTA affinity column equilibrated with 50 mM Tris-HCl, pH 7.5 and washed with the same buffer containing 20 mM imidazole. Bound protein was eluted with 200 mM imidazole in this buffer. For final purification, samples of 250 µl were applied to a Superdex 200 HR 10/30 column (24 ml bed volume,
Amersham Biosciences) equilibrated with PBS, pH 7.4, and 5 mM MgCl₂. The protein was eluted with the same buffer. This preparation showed a single band on 12% SDS-PAGE and was stored at -80°C in PBS without loss of activity.

Molecular mass determination of Frk was done by gel filtration chromatography at 4°C on a Superdex 200 column (Amersham Biosciences) equilibrated with PBS containing 1 mM β-mercaptoethanol and 5 mM MgCl₂. The column was calibrated with the low molecular mass calibration kit (Amersham Biosciences) containing bovine pancreas chymotrypsinogen A (25 kDa), hen egg albumin (43 kDa), bovine pancreas serum albumin (67 kDa), and rabbit muscle aldolase (158 kDa). The dimeric nature of Frk was corroborated by chemical crosslinking: 2.4 µg purified Frk was incubated with 10 mM dimethyl suberimidate for 1 hour in 50 mM HEPES buffer (pH 7.5) in a total volume of 18 µl. Two microliters of 1 M Tris-HCl buffer, pH 8.0 was added to stop the reaction. The preparation was analyzed by SDS-PAGE for dimer formation.

**HPLC analysis.**
Sugars, sugar phosphates and sugar bisphosphates were analyzed by high pH-anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD). The HPAEC/PAD was performed with a Dionex BioLC-system (Dionex Corp., Mississauga, Ontario) equipped with a quaternary gradient pump (GS50), a pulsed electrochemical detector (PED) with gold electrode (ED-50), and an autosampler (AS50). Samples were separated on a Dionex CarboPac PA-1 analytical column (2 x 250 mm) equipped with an amino trap precolumn previously equilibrated in 100 mM NaOH. The response factors of the PAD for the sugars and sugar phosphates were determined by repeated injections (20 µl) of standard mixtures (10-200 nmol). The enzyme reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM fructose, 5 mM ATP, and 20 mM MgCl₂ were incubated for 2-60 min at 60°C. The reaction was stopped by cooling to 4°C and removing the enzyme by ultrafiltration through microcon YM-10 (Millipore, Inc.).

**Thin layer chromatography (TLC).**
10 µl samples from the reaction mixture (30 µg fructokinase, 20 mM fructose, 25 mM ATP, 100 mM MgCl₂ in 50 mM Tris-HCl, pH 7.5 in a total volume of 200 µl) were applied after different time intervals onto silica gel plates (type 60, Merck) and developped with butanol:ethanol:water (5:3:2) as solvent. To visualize the spots, the
plate was dipped into methanol containing 5% H₂SO₄. The carbohydrate spots were visible after drying and charring at 120°C for 5 min.

**Enzymatic assays.**

ATP-fructokinase activity was determined by measuring the formation of fructose-6-P in a two step assay (assay 1). We used 100 µl samples of 50 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 12.5 mM MgCl₂ and varying fructose concentrations. After preincubation at 80°C or at the different temperatures for 5 minutes, the reaction was started by adding purified enzyme and stopped with 50 mM (final concentration) cold EDTA. Fructose-6-P was then quantified at 25°C by adding 0.4 mM NADP⁺, 0.5 U glucose-6-P dehydrogenase (EC 1.1.1.49) and 0.5 U glucose-6-P isomerase (EC 5.3.1.9) to a final volume of 1 ml. The NADPH produced was determined at 340 nm. Using this method, the Kₘ and Vₘₐₓ values at 80°C for the formation of fructose-6-P and the temperature-dependence of the enzymatic activity were determined. All assays were performed in duplicate.

The Kₘ and Vₘₐₓ values for ATP hydrolysis were determined by measuring ADP formation. In a two step assay (assay 2) the reformation of ATP from ADP by pyruvate kinase was followed spectrophotometrically via the oxidation of NADH by lactate dehydrogenase. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM fructose and varying amounts of ATP. The assay was started by the addition of fructokinase and stopped by chilling. Then, 5 mM phosphoenolpyruvate, 0.15 mM NADH, 5.0 U lactate dehydrogenase and 3.5 U pyruvate kinase were added and the reaction was allowed to proceed at 25°C. The decrease in the absorption at 340 nm was measured. It was ensured that all auxiliary enzymes were not rate-limiting. One unit of enzyme activity is defined as 1 µmol of fructose-6-P formed per minute. Specific activity is referred to units per mg protein. Protein determination was done with the BCA (Bicinchoninic Acid) protein assay kit from Sigma.

**Temperature dependence and thermal stability.**

The temperature dependence of fructokinase activity was measured from 30°C to 100°C in the standard reaction system (assay 1). To test thermostability, the enzyme was incubated in sealed vials at 80°C for 10, 20, 30, 40, 50, 60, 120, 180, and 240 min. The vials were then cooled on ice for 1 hour. The remaining enzyme activity was tested at 80°C. Untreated enzyme was used as control.
**pH dependence and specificity of substrate, cation and phosphoryl group donor.** Possible substrates were tested by measuring ADP formation as described above (assay 2). The following sugars were used: D-fructose, D-glucose, D-glucosamine, D-mannose, D-galactose, 2-deoxy-D-glucose, D-fructose-6-P, D-fructose-1-P, and D-glucose-6-P. To test divalent cation specificities, CoCl₂, ZnCl₂, MnCl₂, NiCl₂, SrCl₂, and CaCl₂ were used instead of MgCl₂ at equal concentration at 80°C (12.5 mM, assay 1). To analyze the specificity of phosphoryl group donors, GDP, CTP, ADP, GTP, pyrophosphate, and polyphosphate were used instead of ATP at equal concentration at 80°C (2.5 mM, assay 1). For pH dependence, the enzyme was assayed in two buffer systems. From pH 4-8, 100 mM Tris base was adjusted to the desired pH with phosphoric acid. For the pH range of 9-10 a 1:1 mixture of 50 mM Tris base and 50 mM CAPS buffer (cyclohexylamino-1-propane-sulfonic acid) was used and adjusted with NaOH to the desired pH. Both buffers contained 0.1 mM CaCl₂. The pH of these buffers was measured at room temperature and at 80°C; the difference was at most 0.5 pH units.

**Fructose binding.** Purified enzyme (10 µM in 100 µl) was mixed with 0.14 µM ¹⁴C-fructose and unlabeled fructose (the final concentration ranging from 10 µM to 1 mM) in Tris-phosphate buffer, pH 7.5. Reaction mixtures were incubated at 80°C for 10 min, stopped by the addition of 2 ml ice-cold saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5, and kept on ice for 10 min (Richarme and Kepes 1983). They were then filtered through cellulose nitrate membrane filters (Schleicher and Schüll, pore size 0.45 µm) and washed with 2 ml 95% saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5. Bound radioactivity was measured in a scintillation counter. The Kᵰ value was defined as the fructose concentration at half-maximal binding of fructokinase.

**RESULTS**

**Purification of fructokinase.** A 936 bp DNA fragment containing frk was amplified by PCR and cloned into plasmid pCS19, yielding pSL149. The plasmid-encoded protein was expressed in E. coli DHB4 and purified from cellular extracts by heating to 80°C followed by Ni-NTA affinity chromatography and gel filtration. About 5 mg of the
purified enzyme was obtained from 1 liter bacterial culture. It was stored in PBS at –80°C and did not lose its activity for 3 months. The purified protein showed a single band on SDS-PAGE with an apparent molecular weight of 35,000, very close to the calculated molecular mass of 36,221 (His-tag version; see below, Fig. 1B).

**Fig. 1. Molecular mass determination of Frk.** A. Molecular sieve chromatography on Superdex 200 at 4°C. The apparent molecular mass of Frk is 58 kDa. Arrows indicate molecular mass standards. B. SDS-PAGE of purified Frk crosslinked with suberimidate. Crosslinked Frk has a molecular mass of about 70 kDa. Lane M, molecular mass standards; lane 1, purified His-tag Frk without crosslinking; lane 2, crosslinked protein without additions; lane 3, Frk incubated with ATP / Mg²⁺ prior to crosslinking; lane 4, Frk incubated with ATP / Mg²⁺ and fructose (5 mM) prior to crosslinking.

**Molecular mass.** When gel filtration chromatography was used to estimate the molecular mass, the protein appeared as a symmetrical peak calibrated at about 58 kDa irrespective of the initial concentration (ranging from 0.05 to 1.0 mg/ml) and regardless of the presence of ATP (2.5 mM) and Mg²⁺ (10 mM) in the running buffer or of heating the protein prior to gel filtration (Fig. 1A). Crosslinking with suberimidate followed by SDS-PAGE gave small amounts of a product exhibiting 70 kDa. Again, the presence of ATP or Mg²⁺ or different heating protocols prior to the crosslinking had no effect on its result (Fig. 1B). This indicated that the enzyme is a dimer of two identical subunits.

**Sequence similarity.** The deduced amino acid sequence of *frk* showed similarity to other fructokinases of the ribokinase family of archaea and bacteria. High similarity was noticed to the putative ATP-fructokinases of *Pyrococcus abyssi* (PAB0482, 58% identity) and *Pyrococcus furiosus* (PF1458, 54% identity). The latter is a second and putative fructokinase in addition to the one encoded in the 16 kb transposon insertion (Diruggiero et al. 2000) described in this publication. Low similarity was observed to
fructokinases from the Gram-negative bacterium Rhizobium leguminosarum biovar trifolii (34% identity) (Fennington and Hughes 1996), the ATP-dependent phosphofructokinase of the archean Aeropyrum pernix (26% identity) (Hansen and Schönhheit 2001) and ribokinase of E. coli (26% identity). There was no significant similarity to the ATP-dependent fructokinases of Lactococcus lactis (Thompson et al. 1991) and Zymomonas mobilis (Zembrzuski et al. 1992) which belong to the ROK (Repressor, ORF, Kinase) family. Fig. 2 shows the alignment of some of these kinases that belong to the ribokinase family including their conserved regions that are connected to the ATP-dependent kinase activity.

Identification of enzymatic products. To identify the product of fructose phosphorylation, samples of the incubation mixture containing purified enzyme, fructose, Mg\(^{2+}\) and ATP were analyzed by thin layer chromatography (TLC) (Fig. 3A). While fructose was used up, a compound was formed with similar migration properties on TLC as fructose-6-P, but different from fructose-1-P and fructose-1,6-phosphate.

Fig. 2. Sequence alignment. Multiple alignment of the amino acid sequences of fructokinases from T. litoralis, P. abyssi (putative, PAB0482), P. furiosus (putative, PF1458, the "second" fructokinase) and R. leguminosarum, phosphofructokinase from A. pernix and ribokinase from E. coli. The asterisks indicate identical amino acids in thermococcal Frk and any of the other sugar kinases. The proposed sequence motifs involved in ATP binding and substrate recognition are shaded and bold/italics, respectively. The three XXX indicate the conserved sequence AGD at positions 253, 254 and 255 in the crystal structure of E. coli ribokinase (Sigrell et al. 1998). Frk, fructokinase; FrkII, fructokinase II; PFK, phosphofructokinase; RK, ribokinase.

| Frk-T. litoralis | -----MIYAIHELIDFIKAKE-----GLKLDVFREFEKEHGPPFANVLLGRLAGKSA |
| Frk-P. abyssi | -MYLMSCIGELLVDVIAETE-----GLKLDVFREFEKEHGPPFANVLLGRLAGFDC |
| FrkII-P. furiosus | ----MIAFGEVLIIDFAAETE-----GLKLDVFREFEKEHGPPFANVLLGRLAGFDC |
| Frk-Rhizobium | -----MILCCGALIDMLPRDT-----TLGEEKGAPYAIANTAIALGRLGPITA |
| PFK-A. pernix | --MSEKILVHLILDVLGRHVRKRFIPGGELDDNEIIRLTAYTAAAGMSVLAKLDGDY |
| RK-E. coli | MQNAGSLVVLGSLNDHPLNS---FTTPGEVTVGHQVADFEGKGANQAVAAGRSANIA |

| Frk-T. litoralis | LISKVGDPPFGEFLIEELKKERVKTEKYYIKDNTKHTG1FVQLIGAKFKPEFLIDGAVYFN |
| Frk-P. abyssi | LVSVGDPFFFFGFLVESLRENKVTG1KDEEKHTG1FVQLTGPSFPLYDGGAYFN |
| FrkII-P. furiosus | LSVKGDPPFGEFLIRKLEEGVNTGVLIDNEKHTG1FVQLTGPSFPLYDGGAYFN |
| Frk-Rhizobium | FFTG1ADMMGELI1LETILKSNFDVSICAFTFRPST---FAVVLNQATAYFCDEGAGR |
| PFK-A. pernix | AMGADVGLDESFNFI1TTLRYGNLTDHVFVRKEVQTSCLFLTRNFPGFRVHLGILGAT |
| RK-E. coli | FIACGIDS1GEGSRQQLATDNI1TPSVV12GESTGVALIFVNGEGENVGI1HAGANAA |
Using HPLC under alkaline conditions, three compounds could be identified as the products of phosphorylation. All increased with time (Fig. 3B) and initial fructose concentration (Fig. 3C). The main peak, (product 1, about 85% of the total phosphorylation products) was eluting at 42.6 min close to the retention time of standard fructose-6-P (42.7 min, Fig. 3C). The two additional peaks (products 2 and 3, about 12% and 3%, resp.) were eluted at 38.7 and 45.8 min. Treatment of the product mixture by alkaline phosphatase yielded only free fructose but no other sugar (Fig. 3D). Thus, the additional phosphorylated products 2 and 3 could only be fructose phosphates. Conceivably, product 3 could be fructose-1,6-diphosphate (retention time 46.9 min) being formed by the phosphorylation of fructose-6-P. Yet, this is excluded as well since fructose-6-P was not a substrate for the enzyme and did not form the slower migrating compound. Taken together, this led us to conclude that products 2 and 3 are fructose-2-P and fructose-3-P.
Fig. 3. Analysis of the products of fructose phosphorylation. A. Thin-layer chromatographic analysis of enzymatic fructose phosphorylation. The reaction was performed at 60°C with 30 µg fructokinase in 200 µl 50 mM Tris-HCl, pH 7.5, 20 mM fructose, 25 mM ATP, 100 mM MgCl₂. 6 µl aliquots were applied onto TLC plates at different time intervals. Standards: lane 1, fructose; lane 2, fructose-6-P; lane 3, fructose-1-P; lane 4, ATP; lane 5, ADP; lanes 6-13, incubation mixture at 0, 10, 20, 30, 40, 60, 120 min and after 24 h. B. HPLC analysis of the phosphorylation reaction mixture after different time intervals. Samples were taken at 0, 5, 30 and 60 min after starting the reaction. 1, 2, and 3 indicate fructose-6-P and fructose-2- or -3-P. C. HPLC analysis of product formation at different initial fructose concentrations. The reaction was performed for 30 min at 60°C at 2, 4, 6, 8 and 10 mM fructose. The reaction mixture contained 10 µg purified enzyme in 200 µl 50 mM Tris-HCl, pH 7.5, 10 mM ATP and 50 mM MgCl₂. 1, 2, and 3
indicate fructose-6-P and fructose-2- or -3-P. D. HPLC analysis of the unknown fructose phosphates after treatment with alkaline phosphatase. The reaction mixture described in Fig. 3C (10 mM fructose, 30 min incubation time) was treated with alkaline phosphatase and analyzed by HPLC. 1, reaction mixture after treatment with alkaline phosphatase; 2, standard fructose; 3, reaction mixture without alkaline phosphatase treatment. Note: sugar phosphates are off scale in this analysis.

**Enzyme characteristics.** The rate of the enzymatic reaction was determined by measuring the formation of fructose-6-P via the formation of NADPH (isomerization to glucose-6-P and its oxidation by NADP⁺ and glucose-6-P dehydrogenase; assay 1). Using this two step assay, typical Michaelis-Menten kinetics were observed. At 80°C, the apparent \( K_m \) and \( V_{max} \) values for fructose at saturating ATP concentrations were 2.3 mM and 730 U/mg protein. The apparent \( K_m \) and \( V_{max} \) values for ATP at saturating fructose concentrations (assay 2) were 0.81 mM and 920 U/mg protein. The difference in the \( V_{max} \) values must be due to the formation of fructose-2- and -3-P. Only the formation of fructose-6-P contributes to the determination of the kinetic constants for fructose in the above assay.

Frk showed highest activity with ATP. Other nucleoside triphosphates can substitute for ATP: ITP (73%), GTP (62%), UTP (16%), and CTP (16%) can act as phosphoryl donors. ADP, GDP, UDP, PEP, polyphosphate, and pyrophosphate were inactive.

The enzyme required divalent cations for its activity. MgCl₂ was most effective, but could be partially substituted by Mn²⁺ (65.2%) and Co²⁺ (43.7%). Very low enzyme activity was observed with Ni²⁺ (8.7%) and Sr²⁺ (1.0%), and no activity with Ca²⁺ and Zn²⁺.

**Fig. 4.** Effect of temperature on the specific activity of purified Frk. The assay was performed under the conditions of \( V_{max} \) for fructose and ATP.
Frk specifically catalyzed the phosphorylation of D-fructose to fructose-6-P. Other monosaccharides, such as mannose, glucose, D-fructose-6-P, glucosamine, 2-deoxy-D-glucose, D-galactose, D-fructose-1-P, and D-glucose-6-P did not serve as substrates. At 30°C the activity of the enzyme was very low (less than 1% of its maximal activity at 80°C). It increased rapidly above 60°C, exhibited an optimum at 80°C and 11.7% of that value at 100°C (Fig. 4). The thermostability was determined by pre-incubation at 80°C from 10 min to 4 hours. The enzyme did not lose any activity for 4 hours at 80°C. It was active at pH values from 6.0 to 9.0 and had optimal activity between pH 7.5 and 8.

**Fig. 5. Fructose-binding assay.** A. 0.14 µM ¹⁴C-fructose in a test volume of 100 µl was incubated with increasing amounts of fructokinase at 80°C for 10 min. B. 10 µM purified fructokinase was incubated with 0.14 µM ¹⁴C-fructose and increasing amounts of unlabeled fructose. Half-maximal binding occurred at 0.25 mM fructose and defined the Kₐ.

**Fructose binding.** To determine the affinity of Frk for fructose in the absence of ATP hydrolysis we measured binding of ¹⁴C-fructose in a test used for periplasmic binding proteins (Richarme and Kepes 1983). In order to form an enzyme-substrate complex, the enzyme was heated up with ¹⁴C-fructose at 80°C for 10 min prior to precipitation with ice-cold saturated ammonium sulfate. Fructose-binding was linearly dependent on enzyme concentration (Fig. 5A) and showed Michaelis-Menten kinetics (Fig. 5B). The Kₐ for fructose was 0.25 mM, about 10 times lower than the Kₘ for fructose phosphorylation. Maximal binding showed a stoichiometry of 0.8 per monomeric protein.
DISCUSSION

In this publication, we report the characterization of Frk, an ATP-dependent fructokinase, from *T. litoralis* as well as *P. furiosus* frk, the gene encoding this enzyme, is positioned on a 16 kb transposon-like DNA fragment that occurs with identical sequence in both organisms (DiRuggiero et al. 2000). Comparison of the amino acid sequence of Frk showed similarity to those kinases that belong to the ribokinase type (Bork et al. 1993), a large family of prokaryotic and eukaryotic carbohydrate kinases including fructokinase, ribokinase, 1-phosphofructokinase, archaeal 6-phosphofructokinases and adenosine kinase. In contrast, the *T. litoralis* fructokinase did not exhibit significant sequence similarity to the ADP-dependent glucokinase from the same organism (Ito et al. 2001) even though the crystal structures of the latter enzyme as well as of that of ribokinase from *E. coli* (Sigrell et al. 1998) reveal a fold similar to the one in the protein of the ATP-dependent ribokinase family to which the fructokinase of *T. litoralis* belongs.

Conserved domains in Frk were recognized in the C-terminal section and are indicated in Fig. 2. The shaded sequence has been associated with ATP-binding by others (Aulkemeyer et al. 1991; Fennington and Hughes 1996). Based on the crystal structure of *E. coli* ribokinase (Sigrell et al. 1998), Asp 255 is conserved in a number of kinases as the catalytic residue, Ala253 and Gly254 (indicated by XXX in Fig. 2) are conserved to form the anion pocket. The other two motifs, shown in bold/italics in Fig. 2, are conserved in four fructokinases, but not in ribokinase and phosphofructokinase. These two motifs were suggested to represent the fructose recognition site (Fennington and Hughes 1996).

The enzyme showed substrate specificity only for fructose and was inactive towards a range of other sugars tested. Some fructokinases are known to have limited substrate specificity towards mannose, for example, the fructokinases from *L. lactis* (Thompson et al. 1991), *Z. mobilis* (trace activity on mannose) (Scopes et al. 1985; Zembrzuski et al. 1992), and *E. coli* (Sebastian and Asensio 1972) (Table 1). The *K*ₘ of the *T. litoralis* enzyme for ATP at saturating fructose concentrations was 0.81 mM. This is in keeping with other ATP-dependent sugar kinases, for instance, a phosphofructokinase from *Aeropyrum pernix* which has a *K*ₘ of 0.68 mM (Hansen and Schönheit 2001). Other sugar kinases of hyperthermophilic archaea are ADP-dependent (Ronimus and Morgan 2003) which has been explained by the instability of ATP at high temperatures. Thus, the
high affinity of galactokinase of P. furiosus for ATP (K_m 8 µM, V_max 41.9 units/mg protein) was interpreted by the low availability of ATP (Verhees et al. 2002). The apparent discrepancy to the low affinity of T. litoralis fructokinase and other ATP-dependent kinases from hyperthermophiles which also have a low affinity for ATP may possibly be explained by the rather high V_max of these enzymes, thus ensuring efficient phosphorylation even at low ATP concentrations.

**Table 1.** Comparison of the biochemical properties of fructokinases from different organisms.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Thermococcus litoralis</th>
<th>Lactococcus lactis</th>
<th>Zymomonas mobilis</th>
<th>Rhizobium leguminosarum</th>
<th>Streptococcus mutans</th>
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<td>-</td>
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<tr>
<td>mannose</td>
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<td>(Zembrzuski et al. 1992)</td>
<td>(Fennington and Hughes 1993)</td>
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</table>

*ROK, Repressor, ORF, Kinase; data from ExPASy, Enzyme nomenclature database, http://us.expasy.org/enzyme/

** Trace activity
The $K_m$ for fructose phosphorylation (2.3 mM) was about 10 times higher than the $K_d$ (0.25 mM) for fructose binding in the absence of ATP. The reason for this discrepancy is unclear at present. If one does not evoke conformational changes upon binding ATP it may simply be due to the high turnover of the enzyme which limits the access of substrate under phosphorylation conditions.

Since *T. litoralis* appears to be unable to grow on fructose the role of the cytoplasmic fructokinase must be connected to the metabolism of sucrose. The latter has been reported to be a carbon source for *T. litoralis* (Rinker and Kelly 1996). Since phosphotransferase-type transporters are absent in archaea this implies that sucrose is taken up by the cell without phosphorylation and cleaved internally to free fructose. Sucrose is no substrate for the maltose/trehalose transporter even though TrmB, the negative regulator of the corresponding operon, does recognize this sugar. This implies that it is the putative operon encoding sucrose degradation that is controlled by TrmB. In contrast, *frk* located adjacent to the gene cluster encoding the maltose/trehalose ABC transporter appears not to be regulated by TrmB as judged by Western blotting of the enzyme or Northern blotting of the cognate RNA after growth in the presence of different possible inducers.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the help of Christoph Mayer in analysing sugars by HPLC. We thank E. Oberer-Bley for her help with the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

**REFERENCES**


Chapter 4

TrmB, a sugar-specific transcriptional regulator of the trehalose/maltose ABC transporter from the hyperthermophilic archaeon *Thermococcus litoralis*

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**ABSTRACT**

We report the characterization of TrmB, a protein of 38.800 apparent molecular weight, that is involved in the maltose-specific regulation of a gene cluster in *Thermococcus litoralis*, malE malF malG orf trmB malK, encoding a binding protein-dependent ABC transporter for trehalose and maltose. TrmB binds maltose and trehalose half-maximally at 20 µM and 0.5 mM sugar concentration, respectively. Binding of maltose but not of trehalose showed indications of sigmoidality and quenched the intrinsic tryptophan fluorescence by 15% indicating a conformational change upon maltose binding. TrmB causes a shift in electrophoretic mobility of DNA fragments harboring the promoter and upstream regulatory motif identified by footprinting. Band shifting by TrmB can be prevented by maltose. In vitro transcription assays with purified components from *Pyrococcus furiosus* have been established to show pmalE promoter-dependent transcription at 80 °C. TrmB specifically inhibits transcription and this inhibition is counteracted by maltose and trehalose. These data characterize TrmB as a maltose-specific repressor for the trehalose/maltose transport operon of *Thermococcus litoralis*.

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INTRODUCTION

The hyperthermophilic archaeon *Thermococcus litoralis* belongs to the order *Thermococcales* of euryarchaeota. It grows optimally at 85 °C under anaerobic conditions and was originally isolated from deep submarine hydrothermal vents (Neuner et al. 1990). This organism can use starch, maltose, cellobiose or sucrose as carbon sources (Rinker and Kelly 1996). The enzymology of maltose utilization is known (Xavier et al. 1999). Trehalose and maltose are transported by a high affinity and binding protein-dependent ABC transporter with a Km of approximately 20 nM (Xavier et al. 1996; Horlacher et al. 1998). As a result of lateral gene transfer, the same system with nearly identical sequence is present in *Pyrococcus furiosus* (DiRuggiero et al. 2000). The latter organism has a second, very similar maltodextrin-specific transporter that is absent in *T. litoralis*. This ABC transporter does not recognize trehalose (DiRuggiero et al. 2000; Evdokimov et al. 2000; Diez et al. 2001; Koning et al. 2002). The genes encoding the trehalose/maltose transporter in *T. litoralis* are clustered, most likely forming an operon, and are positioned divergently to another gene which shows homology to genes encoding sugar kinases (Fig. 1). The trehalose/maltose transport genes are induced by the presence of maltose or trehalose in the growth medium (Xavier et al. 1996; Horlacher et al. 1998) as are the identical genes in *P. furiosus* (DiRuggiero et al. 2000). The components of the transporter have been purified (Horlacher et al. 1998; Greller et al. 1999; Greller et al. 2001) and the cognate binding protein as well as the ABC subunit have been crystallized and their structures solved (Diederichs et al. 2000; Diez et al. 2001). So far, nothing was known about the mechanism of induction. In contrast, the regulation of the homologous system in *E. coli*, one of the most thoroughly studied binding protein-dependent ABC transporters, is well understood (Boos and Shuman 1998). The central feature is the function of MalT, a transcriptional activator that needs the cAMP/CAP complex, the inducer maltotriose and ATP for the specific activation of σ70-programmed RNA polymerase (Raibaud and Richet 1987; Raibaud et al. 1989; Richet et al. 1991). In addition, MalK, the ATP-hydrolyzing subunit of the ABC transporter, is closely connected to the control of MalT activity (Boos and Böhm 2000; Böhm et al. 2002).

The basic transcriptional machinery in archaea appears to be more similar to eukaryotes than to prokaryotes (Thomm 1996; Bell and Jackson 1998). Thus, transcription initiation requires three promoter elements, the TATA box centered at –26 / -27 from
the start point of transcription, a transcription factor B recognition element (BRE) comprising two adenines upstream of the TATA box, and an initiation element (INR) around the transcription initiation site. Correspondingly, the minimal factors needed to facilitate binding of the eukaryotic polymerase II-like archaeal RNA polymerase (RNAP) to the promoter are TATA box binding protein (TBP) and transcription factor binding protein (TFB) (Hausner et al. 1991; Soppa 1999; Hausner and Thomm 2001). One would therefore expect that regulation of transcription in archaea would follow eukaryotic rather than prokaryotic schemes (Struhl 1999). Yet, the few examples of archaeal transcriptional regulation point to a bacterial-like regulation. MDR1 (metal-dependent repressor) was found to inhibit RNAP function in Archaeoglobus fulgidus (Bell et al. 1999) and Lrs14 regulates its own expression by preventing the binding of TBP at the corresponding BRE-TATA box in Sulfolobus sp. (Bell and Jackson 2000). Homologues to the bacterial Lrp/AsnC family of transcriptional regulators were found in Methanococcus jannaschii and Pyrococcus furiosus (Napoli et al. 1999; Brinkman et al. 2000; Enoru-Eta et al. 2000; Dahlke and Thomm 2002). In contrast to the growing understanding of the basic machinery of transcription initiation, the understanding of the specific regulation of this process, especially for genes involved in sugar uptake and metabolism, is not well understood (Voorhorst et al. 1999).

Here we report the characterization of TrmB (transcriptional regulator of mal operon) as a maltose/trehalose-specific gene regulator (repressor) for a cluster of genes encoding a high affinity ABC transporter as well as TrmB itself.

MATERIALS AND METHOD

Purification of the transcriptional regulator TrmB

Chromosomal DNA from T. litoralis was prepared as previously described (Ausubel et al. 1987). PCR was performed using chromosomal DNA as template and primers encompassing trmB. 5'-ends carried NcoI and BamHI restriction sites for convenient cloning. After digestion with the corresponding restriction enzyme, the fragment was ligated into plasmid pCS19 (Spiess et al. 1999) containing a C-terminal 6 x His tag. The resulting plasmid was named pSL152. It is ampicillin resistant, contained the lacIq gene and expressed trmB under IPTG-inducible promoter control. E. coli strain SF120 (Baneyx and Georgiou 1991) was transformed with pSL152 selecting for ampicillin resistance. For TrmB expression, cells were grown in NZA medium (10 g NZ-amine A,
Sheffield Product Inc), 5 g yeast extract, 7.5 g NaCl per liter] at 30 °C. 0.2 mM IPTG was added at OD 0.5-0.7, cells were grown for 5 more hours at 37 °C and harvested by centrifugation. The pellet was resuspended in 20 ml Ni-NTA affinity solution (buffer I; 50 mM sodium phosphate, 300 mM NaCl, pH 7.5), ruptured in a French pressure cell at 16,000 p.s.i., and centrifuged for 30 min at 17,300 X g. The supernatant was heated to 80 °C for 10 min. After centrifugation of the precipitated proteins, the supernatant was loaded onto a Ni-NTA superflow column from Qiagen equilibrated with buffer I. The column was washed with 50 mM imidazole and eluted with 250 mM imidazole, both in the same buffer. TrmB-containing fractions were pooled and dialyzed against 50 mM Tris, 200 mM sodium chloride, pH 7.5, and subsequently against 200 mM sodium phosphate buffer, pH 7.5. The final concentration of purified TrmB was about 1.0 mg/ml. It was kept frozen at –70 °C.

**EMSA (electrophoretic mobility shift assay)**

Labeled DNA was obtained from appropriate PCR products and end labeled with T4 polynucleotide kinase (PNK, MBI Fermentas) according to the instructions of the manufacturer. The labeled DNA was purified using mini Quick Spin Columns (Roche). The binding buffer was 25 mM HEPES, 150 mM potassium glutamate, 10% glycerin, 1 mM DTT, pH 7.5. All samples contained about 40 nCi labeled DNA (0.5-1 µM) and 1 µg poly(dI•dC)•poly(dI•dC) competitor DNA (Roche, Germany) per 10 µl. TrmB and sugar were added in the concentrations indicated in the legends to the figures. The reaction samples were incubated at 70 °C for 10 min, mixed with 3 µl loading buffer (25 mM HEPES, 150 mM potassium-glutamate, pH 7.5 containing 50% glycerol and 0.05% bromophenol blue), loaded directly onto 8% native PAGE gels and ran at room temperature under a constant voltage of 200 V.

**Footprint and primer extension assays**

For footprinting, the DNA fragment containing the target promoter region was produced using two primers in the PCR. The first, starting in the putative sugar kinase, is 5'CCCAAGCCTTCTCAGACCAACTACA (coding strand); the second, starting in malE, is 5'TATGTCGTGGCCACCAATG (non-coding strand). The primers were 5'-labeled with 32P γ-dATP and PNK. The labeled PCR product (approx. 20,000 cpm) was incubated with TrmB at 70 °C for 10 min in binding buffer, and digested with 0.005
units DNaseI for 5 min at 37 °C. The reactions were stopped with Stop buffer containing 20 mM EDTA, 0.6 M sodium acetate, pH 5.2, and put on liquid nitrogen immediately. The reaction samples were extracted with phenol / chloroform and chloroform / isoamylalcohol, precipitated with 0.1 vol. 3 M sodium acetate and 2.5 vol. 96% ethanol, and centrifuged. The pellet was washed once with 70% ethanol. It was dried and resuspended in loading solution (0.3% bromophenol blue and Xylene cyanol FF, 10 mM EDTA, pH 7.5, 97.5% deionized formamide) and applied directly onto a 6% polyacrylamide sequencing gel containing 7 M urea.

For primer extension analysis total RNA was isolated (DiRuggiero and Robb 1995) from T. litoralis cells at different growth phases (6 and 12 h), with and without maltose induction (0.2%). Growth was in modified marine culture medium (DiRuggiero et al. 2000). The start point for the 5’-end labeled primer was 90 nucleotides downstream of the malE translation start site. The sequence ladder was prepared from sequencing reactions with the same labeled primer. 50 µg RNA together with the labeled primer (150,000 cpm) was precipitated with 0.1 vol. 3 M sodium acetate and 3 vol. 96% ethanol. The precipitate was centrifuged at 14,000 g for 30 min in an Eppendorf centrifuge and washed with 70% ethanol. After resuspension in 10 µl annealing buffer (50 mM Tris HCl, pH 7.5, 200 mM KCl, 2.5 mM EDTA) the sample was denatured at 85 °C for 3 min and hybridized for 10 min at 55 °C. The primer extension reaction was started by adding prewarmed AMV reverse transcriptase mixture (Promega; 1 unit). After 30 min. at 37 °C, the sample was precipitated with sodium acetate and ethanol as before and resuspended in 10 µl loading solution containing formamide.

**Sugar binding assay**

100 µl 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 µM or 5 µM TrmB were incubated with 0.1 µCi 14C-maltose (0.65 µM) or 14C-trehalose (1.1 µM). Prior to the addition, the sugars had been mixed with unlabeled maltose or trehalose to reach a final chemical concentration between 2 and 100 µM for maltose and 2 µM and 10 mM for trehalose. Incubation was for 5 min at 80 °C. The assay was stopped with 2 ml ice-cold saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5, and kept on ice for 10 min. The suspension was then filtered through cellulose nitrate membrane filters (Schleicher und Schüll; pore size 0.45 µm) and washed with 2 ml 95% saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5. Bound radioactivity was determined in a scintillation counter.
in vitro transcription assay

DNA was obtained from pMLP. This plasmid is derived from pUC19 and carries an EcoRI-BamHI fragment starting in the putative sugar kinase gene and ending in malE (Fig.1A). The experimental conditions for the in vitro transcription assay (Hethke et al. 1996) are as follows. 300 ng linearized pMLP DNA harboring the intergenic region (digested with EcoRI) in 40 mM HEPES buffer, pH 7.3, 250 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.44 mM of each ATP, GTP, CTP, 0.002 mM UTP and 2 µCi [γ-32P] UTP were mixed with transcriptional components from Pyrococcus furiosus, i.e., 25 ng TFB (recombinant), 200 ng TBP (recombinant) and 110 ng RNAP. Maltose and trehalose were added to a final concentration of 25 µM to 100 mM, each. TrmB was added to final concentrations as indicated in the legend to figure 8.

Fluorescence-based analysis of conformational change

We used 25 mM Hepes, 300 mM potassium glutamate, pH 8.0, as buffer and 20 µg TrmB in a 1 ml sample at 50 °C. Measurements were done with a Perkin Elmer Model 650-40 instrument at 284 nm excitation and 336 nm emission (40). Sugars were added in 10 µl portions to a final concentration of 0.2 mM maltose and 2 mM trehalose, respectively.

RESULT

Purification of the transcriptional regulator TrmB

The search for the specific gene regulator of the trehalose/maltose transport gene cluster was initiated by the observation that a lateral gene transfer between T. litoralis and P. furiosus had taken place: a 16 kb DNA fragment harboring this gene cluster had been transferred, probably in recent times (DiRuggiero et al. 2000). Since the trehalose-specific induction of the transporter had been maintained after the transfer, it seemed likely that the elements of induction were also present on the 16 kb DNA fragment. Therefore, we cloned all the genes contained on it as his-tag variants, which, as judged from their sequence, might be candidates to encode transcriptional regulators. We expressed these genes in E. coli, purified the corresponding proteins, and tested them in a gel shift assay using a DNA fragment spanning the putative regulatory elements of
the operon (see below). We obtained a positive signal with the protein encoded by the gene directly proximal to malK located within the cluster of transport genes (Fig. 1). We named the gene trmB (transcriptional regulator of the mal operon).

**Fig. 1. Genetic scheme of the trehalose/maltose ABC transport operon of T. litoralis.**

**A.** Gene cluster encoding the binding protein-dependent ABC transporter for trehalose/maltose and the divergent promoter region between malE and the putative sugar kinase. **B.** Sequence of the intergenic region. Bold letters indicate binding sites for the transcriptional regulator TrmB as seen by footprinting (see Fig. 4); BRE, transcription factor B recognition element; RBS, ribosome binding site; +1, transcription start site of malE; ‘cat’ and ‘atg’ indicate the methionine start codon for the putative sugar kinase and maltose binding protein, MalE, respectively.

Sequence homology searches revealed that TrmB (338 amino acids and 38.838 calculated molecular weight) belongs to the DUF118 gene family of Pfam (Sanger institute, UK). Weak homology (30% identity over 159 consecutive amino acids) could be recognized with CymJ of Klebsiella oxytoca, YrhO of Bacillus subtilis (26% identity over 165 consecutive amino acids) and AF1232 from Archaeoglobus fulgidus (27% identity over 194 consecutive amino acids). High homology was recognized with PH1034 of Pyrococcus horikoshii (71% identity over the full size protein) as shown in...
These proteins exhibit sequence similarity mostly at the N-terminus and contain a distinct helix-turn-helix motif. CymJ of *K. oxytoca* is also positioned within an operon encoding a binding protein-dependent ABC transporter specific for cyclodextrins (DiRuggiero et al. 2000). The function of CymJ has not been elucidated except that it is dispensable for transport.

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**Fig. 2.** Multiple protein sequence alignment of *T. litoralis* TrmB.

*TrmB* is shown together with its related homologs: *Pyrococcus horikoshii* PH1034, *Archaeoglobus fulgidus* AF1232, *Klebsiella oxytoca* CymJ, and *Bacillus subtilis* YrhO. An asterix indicates amino acid identity; colon, high conservation; dots, low conservation. The putative helix-turn-helix motif was predicted by the PHD program.

TrmB, as a C-terminal His-tag version, was overexpressed (from plasmid pSL152) in *E. coli* SF120 that is characterized by the lack of several proteases. Growth was in rich medium (NZA) at 30 °C and using 0.2 mM IPTG as inducer. Prior to Ni-NTA affinity chromatography, the cell-free extract was heated to 80 °C for 10 min to remove most of the *E. coli* proteins. After Ni-NTA chromatography the protein exhibited one major band in SDS-PAGE with an apparent molecular weight of 38.800. The protein easily
precipitated after dialysis overnight in Tris buffer, pH 7.6 containing 200 mM NaCl. The precipitate could be solubilized in 200 mM sodium phosphate, pH 7.5, even without the addition of NaCl, and revealed a pure protein preparation. From 1 liter culture 4 mg pure protein was routinely obtained.

**EMSA (electrophoretic mobility shift assay) of TrmB**

The intergenic region, upstream of the trehalose/ maltose transport operon, contained an inverted repeat (TTTACTTTAXGXA), BRE (transcription factor B recognition element), a potential TATA box, and a putative RBS (ribosome binding site) (Fig. 1B). To determine the DNA elements that can be recognized by TrmB, we used electrophoretic mobility shift assays (EMSA). Fig. 3B shows EMSA with different concentrations of TrmB and DNA encompassing the entire intergenic region (region 1). 1.5 µM TrmB was sufficient to completely shift the DNA. We noticed that after binding TrmB a minor portion of the bound DNA never entered the gel, possibly indicating the formation of higher oligomeric forms of TrmB. We prepared three different size fragments of the promoter region. The region 1 fragment contained the entire intergenic region, region 2 the BRE-TATA box but not the inverted repeat sequence, and region 3 only the inverted repeat sequence as well as the proximal region leading into the gene encoding the putative sugar kinase (Fig.3A). When these three different labeled DNAs were used as EMSA probes, all were retarded by TrmB binding, the least effective one being region 3 (Fig. 3C). Thus, the intergenic region must contain at least two binding sites for TrmB.

**Detection of TrmB binding sites by footprinting**

To define the correct binding sites for TrmB, we used protection from DNaseI digestion in the presence of TrmB (Fig. 4). We used two samples labeled at one end: the coding and non-coding sequence (left and right in Fig. 4, respectively). We could observe two binding sites for TrmB. One is the promoter-proximal part of the inverted repeat sequence, 5′CTTTAGGGATGTTTGTACTTAAAGTAAATAAATA, and the other is the promoter itself, the BRE-TATA box, 5′CAAAATATATATACCTTTTAGTATATACA (Fig. 1B). Therefore, TrmB binding to the promoter region will inhibit the binding of transcriptional components TBP and TFB and will thus prevent recruitment of RNA polymerase, essentially by repressing
the initiation of transcription of the *malE* operon. When 100 µM maltose was added, TrmB no longer protected the DNA from DNaseI digestion (Fig 4). The addition of 2.5 mM trehalose gave the same effect (data not shown). Thus, TrmB binding to the promoter elements is prevented by these sugars acting as inducers.

![Fig. 3. Electrophoretic mobility shift assay (EMSA) with TrmB.](image)

**A** DNA fragments used in this study. The open box indicates the inverted repeat sequence, the filled box the BRE-TATA box of *malE*. **B**, EMSA with labeled DNA consisting of the entire intergenic region (Reg1); the final concentrations of TrmB were 0, 0.5, 1.25, 1.5, 2.0, and 2.5 µM. **C**, Comparison of EMSA with fragments of different sizes of the intergenic region (Reg1, Reg2, Reg3). Purified TrmB was added to each fragment at 0, 1.25, and 2.5 µM final concentration, respectively. 1 µg poly(dI•dC)•poly(dI•dC) was present in these reactions as competitor DNA.

**TrmB binds maltose and trehalose with different affinities**

Sugar binding assays were done by ammonium sulfate precipitation in the presence of ¹⁴C-labeled sugar (Berrier et al. 2000). Maltose-binding was linearly dependent on TrmB concentration (Fig. 5A), and TrmB was half-maximally saturated at 20 µM maltose (Fig. 5B). The binding of maltose showed indications of sigmoidal behavior at two concentrations of TrmB (2.5 and 5 µM; only the data for 5 µM are shown in Fig. 5B and C). This can hardly be recognized in the Michaelis-Menten plot shown in Fig.
5B. However, it can easily be seen when only the amount of TrmB-bound radioactivity (but not the chemical amount of bound maltose) is plotted against the total maltose concentration (labeled plus unlabeled) as seen in Fig. 5C. Since the amount of radioactive maltose in all assays is the same (61,000 cpm) the expected behavior for a non-cooperative (Michaelis-Menten type) maltose-binding would be such that the number of TrmB-bound radioactivity would only decline when increasing amounts of unlabeled maltose are present in the assay. However, as seen in Fig. 5C, the amount of labeled maltose bound to TrmB increased with increasing total maltose concentration before declining, suggesting positive cooperative binding. To better visualize this cooperativity in the calculated binding data of Fig. 5B and to show the difference to a purely non-cooperative, Michaelis-Menten type binding behavior, we added a theoretical Michaelis-Menten type binding curve using the concentration of half-maximal binding (20 µM) as the "Kd" and 1.8 µM as maximally TrmB-bound maltose. Now the difference of maltose binding to TrmB and a purely Michaelis–Menten type binding is obvious.

Fig. 4. **Footprint analysis of the intergenic promoter region (encompassing Reg1).**

The DNaseI protection assay was done on the coding strand (left) and non-coding strand (right). TrmB protected the BRE-TATA box (a, d) and part of the inverted repeat sequence (b, c) (compare with Fig. 1B). Final concentrations of added TrmB were 0, 1.25, 2.5, and 5 µM. C, G, and A indicate the sequence ladder. M, 100 µM maltose was added to the assay containing 5 µM TrmB.
When radioactive trehalose was used in the binding assay, half-maximal binding was only observed at concentrations higher than 0.5 mM. But in contrast to maltose as substrate, the TrmB-bound radioactivity only declined with the addition of increasing amounts of unlabeled trehalose giving no indication of cooperative binding for trehalose (not shown). Binding of labeled trehalose was abolished in the presence of unlabeled maltose (data not shown). Also, binding of maltose (at 0.65 µM concentration) could be chased by increasing amounts of trehalose (Fig. 5C), showing initially (at lower trehalose concentration) the same phenomenon of cooperative binding of maltose with increasing amounts of unlabeled trehalose. Using the intrinsic tryptophan fluorescence of TrmB (284 nm excitation and 336 nm emission) we observed 15% reduction in fluorescence at 80°C upon addition of 200 µM maltose but no effect with trehalose (data not shown).

Fig. 5. Sugar binding by TrmB.

**A**, 1.6 µM labeled sugars (maltose and trehalose) were incubated at 80 °C with increasing amounts of TrmB as indicated. The protein was precipitated with ammonium sulfate in the cold, filtered and counted. **B**, Maltose-binding. 20 µg purified TrmB (5 µM) was incubated at 80 °C with 0.65 µM radioactive maltose and increasing amounts of unlabeled maltose. The concentration of TrmB-bound maltose was calculated and is shown as filled circles. Maximally, 1.8 µM maltose was bound to TrmB. Half-maximal binding occurs at 20 µM maltose. For comparison, a theoretical Michaelis-Menten type binding curve with 20 µM as Kd and 1.8 µM maximally bound TrmB is added. **C**, The cpm's of maltose bound to TrmB derived from the experiment shown in Fig. 5B are plotted against the total maltose concentration (filled squares). In contrast to Michaelis-Menten type binding behavior, the bound cpm's increase with unlabeled maltose concentration. The same experiment with 0.65 µM radioactive maltose was done with increasing concentrations of unlabeled trehalose (open squares).
Maltose and trehalose affect TrmB-mediated EMSA differently

When maltose was included in the TrmB-mediated EMSA it prevented the retardation of labeled region 1 DNA. The concentration of maltose which elicited the half-maximal effect was comparable to that which is needed to half-maximally saturate TrmB in the *in vitro* binding assay (20-50 µM). Surprisingly, trehalose showed the opposite effect. At concentrations comparable to half-maximal saturation of binding to TrmB (0.5 mM) there was no discernable effect in the EMSA. However, at 5-10 mM trehalose, the stability of the TrmB-DNA complex was clearly increased and EMSA of region 1 became more effective (Fig. 6).

**Fig. 6.** Trehalose and maltose affect TrmB-mediated EMSA with labeled region 1. 1.25 µM purified TrmB was used in this experiment. Trehalose and maltose were added at concentrations of 1, 2.5, 5, 10 mM, and 5, 10, 20, 50 µM, respectively. 0, no sugar added.

Transcription start site by primer extension

To determine the transcriptional start point in the direction of *malE*, we prepared total RNA of cells harvested at early (6 h) and mid-log (12 h) phase grown in peptone (uninduced conditions) and peptone plus maltose (induced conditions). As primer we used DNA starting 90 base pairs downstream of the translational start site of *malE*. Also, we used a primer starting within *gdh* (encoding glutamate dehydrogenase) as control for a constitutively expressed gene. Fig. 7 shows the two transcription start sites in the *malE* direction. The major start site is 26 nucleotides downstream of the BRE-TATA box (AAATATA). The transcript is stronger when RNA was isolated from early log cultures than from mid-log cultures (Fig. 7). The transcript is not detectable when cells were grown under non-inducing conditions (data not shown).
Fig. 7. Determination of the transcription start site of malE.

Primer extension analysis was done with 50 µg T. litoralis total RNA. Lanes 1 and 2 show the minor (a) and major (b) transcription start site of malE; lanes 3-6 show the transcription start site of the gene for glutamate dehydrogenase as positive control (c). Total RNAs were prepared from different carbon sources, i.e., peptone (lanes 3, 4) and peptone plus maltose (lane 1, 2, 5, 6), and growth phases, i.e., early log phase (lanes 1, 3, 5) and mid-log phase (lanes 2, 4, 6). G, A, T and C contain dideoxy sequencing reactions primed with labeled malE primer. The sequences of the two transcriptional start sites, a and b, are indicated.

In vitro transcription assay of malE

To determine the function of TrmB in the transcription of the malE promoter, we used the established in vitro transcription assay with purified components (RNA polymerase, TBP and TFB) from P. furiosus. As a control, we also used a constitutively transcribed gene encoding glutamate dehydrogenase of P. furiosus. The templates used produced a transcript of 110 bases for the malE operon and of 89 bases for the gene encoding glutamate dehydrogenase (Fig. 8). When TrmB was present in increasing concentrations, the transcript of malE was reduced concomitantly. The presence of maltose prevented this inhibition at a concentration of 100 µM (Fig. 8A). Surprisingly, trehalose had the same effect as maltose albeit at 25-fold higher concentration (above 2.5 mM; Fig. 8B). At this and even higher concentrations, trehalose had not prevented the retardation of region 1 DNA during EMSA.
Fig. 8. *in vitro* transcription assay of *malE*.

*malE* transcription was performed at 80 °C using basic transcriptional components of *P. furiosus*. As template, linearized pMLP DNA containing the *T. litoralis malE* promoter was used. Lane 1, control assay with plasmid containing the gene for glutamate dehydrogenase (*gdh*) of *P. furiosus*; lanes 2 and 3, *malE* transcription in the presence of maltose (A) or trehalose (B) at 5 mM concentration each, but in the absence of TrmB; lanes 4-6, *malE* transcription in the presence of 0.8-3.2 µg TrmB; lanes 7-14, *malE* transcription in the presence of 3.2 µg TrmB and 25, 100, 250 µM, 1, 2.5, 10, 25, and 100 mM maltose (A) or trehalose (B). Inhibition of transcription by TrmB is overcome with 100 mM maltose (A, lane 8) and 2.5 mM trehalose (B, lane 11), respectively. The *malE* (110 b) and *gdh* (89 b) transcripts are indicated by arrows.

**Discussion**

In this publication we report the characterization of TrmB, a novel archaeal substrate-specific transcriptional regulator controlling the maltose/trehalose-specific induction of the binding protein-dependent ABC transporter in *Thermococcus litoralis*. TrmB binds maltose and trehalose with 20 µM and >500 µM half-saturating concentration, respectively, and is able to interfere with *in vitro* transcription of the *malE* promoter. In line with the binding properties of TrmB, maltose (100 µM) and trehalose (2.5 mM) were shown to prevent TrmB-mediated transcription inhibition. Thus, TrmB could be
characterized as a transcriptional repressor. Like other archaeal repressors, TrmB protected the TATA box as well as the adjacent BRE site in DNaseI protection assays. Thus, substrate-specific gene regulation appears to be based on the prevention of TBP binding and subsequent polymerase recruitment. The role of the inducer is to prevent TrmB from binding to the TATA box. In this function, maltose is much more effective (50-100 µM) than trehalose (2.5 mM). This appears physiologically reasonable since maltose is metabolized rather efficiently by \textit{T. litoralis} (Xavier et al. 1999) and will never reach significant concentrations \textit{in vivo}, whereas trehalose is accumulated, preferentially at high osmolarity, to high internal concentrations (Lamosa et al. 1998).

In \textit{E. coli}, MalT, the central gene activator of the maltose system, interacts with the C-terminal extension of MalK, the ATP hydrolizing subunit of the ABC transporter, whose expression is controlled by MalT (Boos and Böhm 2000; Böhm et al. 2002). \textit{T. litoralis} MalK exhibits the same C-terminal regulatory extension as \textit{E. coli} MalK. However, our attempts to demonstrate an interaction between isolated MalK of \textit{T. litoralis} and TrmB were unsuccessful. Possibly, an interaction between these two proteins, if it occurs at all, is restricted to MalK when complexed with the membrane components of the transporter and occurs only in the process of actively transporting substrate.

There is something curious about the effect of trehalose on TrmB and its interaction with the intergenic DNA region. Aside from the observation that binding of TrmB to trehalose is much weaker than to maltose, trehalose binding, in contrast to maltose binding, does not show signs of cooperativity and does not induce a conformational change that is detectable by fluorescence measurements. TrmB-mediated EMSA of the entire intergenic region (region 1 in Fig. 1) is prevented by maltose at concentrations that reflect its binding characteristics to TrmB but not by trehalose, even at concentrations exceeding the saturation of trehalose binding by TrmB; it even makes TrmB/DNA complex formation seemingly stronger even though trehalose binding to TrmB prevents footprinting and interferes with TrmB-mediated malE transcription. Presently, we have no sensible explanation for this curious effect of trehalose binding. TrmB elicits incomplete band shift of the BRE-TATA box alone. The same is seen with the inverted repeat motif. Thus, binding of TrmB to region 1 containing both the BRE-TATA box as well as the inverted repeat must be enhanced by cooperativity between the two sites.
TrmB, when complexed with maltose, cannot recognize a potential binding site in the entire fragment of region 1. Since DNaseI protection assays with TrmB showed not only protection of the TATA box but also of the upstream inverted repeat, we conclude that binding of TrmB to the inverted repeat and BRE-TATA box is affected by maltose and trehalose. It remains to be seen whether binding of TrmB to both elements is involved in the regulation of *malE* transcription. Alternatively, TrmB binding to the inverted repeat could be involved in the transcriptional regulation of the divergently oriented gene encoding the putative sugar kinase.

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Chapter 5

General Discussion

The trehalose/maltose transporter in *Thermococcus litoralis* together with a gene cluster encoding a putative iron/thiamin transporter is located in a 16 kb bacteria-like transposon. This entire cluster of genes was found in nearly identical sequence in *Pyrococcus furiosus*, and the functional form indicated a recent lateral gene transfer between the two organisms (DiRuggiero et al. 2000). By the end of this research, all 7 genes distributed in the trehalose/maltose gene cluster in *T. litoralis* have been identified and characterized (Horlacher et al. 1998; Xavier et al. 1996; Grelle et al. 1999; Lee et al. 2003; Qu et al. 2004a, b). The system includes an ABC transporter for maltose and trehalose uptake, two enzymes for sugar metabolism and one transcriptional regulator. The trehalose/maltose ABC transporter resembles bacterial ABC transporters in the organization and amino acid similarity for individual components. Four genes encode the binding protein (TMBP, MalE), two membrane integral components (MalF and MalG) and an ATPase subunit (MalK) in this gene cluster, respectively.

![Diagram](image)

Fig 1. The gene cluster of the trehalose/maltose ABC transporter system is located on a 16kb bacteria-like transposon.

**Maltose and trehalose metabolism in *T. litoralis***

In *T. litoralis*, the maltose utilization is performed by two key enzymes, maltodextrin phosphorylase and 4-α-glucanotransferase. Surprisingly, the two genes are not located in this trehalose/maltose transporter gene cluster and their expression is not induced by the presence of their substrates maltose or trehalose, although the phosphorylase expression was two-fold induced by maltose or maltodextrins (Xavier et al. 1999).
Maltodextrin ranging from maltotriose to maltoheptose is transported by another maltodextrin transporter (Imamura et al. 2004). The constitutive expression of maltose degrading enzymes seems to be necessary also for utilization of maltodextrins which are transported by the maltodextrin transporter in _T. litoralis_.

Trehalose acts as a stress protectant and a sole carbon source in _E. coli_ by development of two entirely different systems coordinately (Strom and Kaasen 1993). In _E. coli_, endogenous trehalose synthesis is performed by two genes (_otsA_ and _otsB_) encoding two enzymes, trehalose-6-P synthase and trehalose-6-P phosphatase. _otsA_ and _otsB_ constitute an operon which is induced by osmotic stress and dependent on the sigma factor RpoS. Trehalose metabolism under osmotic stress is regulated by a same osmotic inducible periplasmic trehalase TreA (Boos et al. 1987). On the other hand, generally, trehalose induces TreB (PTS-dependent enzymeII^Tre_ for its uptake as trehalose-6-P (Klein et al. 1995) followed by internal hydrolysis with trehalose-6-P hydrolase (TreC) to glucose and glucose-6-P. TreB and TreC form an operon which is controlled by adjacent repressor, TreR.

Trehalose/maltose ABC transporter is induced by trehalose and maltose. Trehalose is accumulated in cells during growth in the medium with yeast extract containing trehalose. After its accumulation, trehalose was metabolized slowly in the stationary phase of growth (Lamosa et al. 1998). In our research, a novel trehalose glycosyl transferring synthase (TreT) was identified involving in trehalose metabolism in _T. litoralis_, and in _P. furiosus_ as well. This enzyme produces trehalose by the transfer of glucose from ADP-glucose (or, less effectively, UDP-glucose and GDP-glucose) onto the 1-position of glucose to form trehalose and ADP. The reaction is reversible, however the rate of synthesis is ca. 10-fold higher than that of degradation (Qu et al. 2004b). The *in vivo* function of TreT whatever for synthesis or degradation is still unclear. From the position of _treT_ within a gene cluster encoding an ABC transporter for trehalose (and maltose) one would be inclined to interpret the function of TreT as a trehalose degrading enzyme rather than a trehalose-synthesizing enzyme. This appears also sensible in light of the rather high half-maximal concentration for glucose of 6.2 mM. It would be unlikely that growing _T. litoralis_ contains such high internal glucose concentrations. With a Km of 0.7 mM for glucose, the ADP-dependent hexokinase (Selig et al. 1997) would effectively reduce the concentration of free internal glucose and prevent any noticeable trehalose formation by TreT. One could argue that the metabolism of maltose which also induces TreT synthesis would provide ample
amounts of free internal glucose needed for trehalose formation by TreT. In fact, a microarray analysis indicated that *P. furiosus* cells can elevate expression level of *malE* (6.5-fold) and *treT* (2.4-fold) under heat shock stress (increasing 5°C, 30 minutes) (Shockley et al. 2003). These might imply that one endogenous trehalose metabolism system is used to response to high osmolarity and intracellular trehalose utilization in *T. litoralis*. However, trehalose formation was not observed when *T. litoralis* grew on maltose (Lamosa et al. 1998).

It is speculated that the accumulation of trehalose present in the medium via the high affinity and binding protein-dependent ABC transporter would result in high internal trehalose concentrations which could subsequently be used for glucose formation by TreT, followed by hexokinase-dependent phosphorylation and glycolysis. Another product of trehalose degradation by TreT, ADP-glucose, could be incorporated into the pathway of oligosaccharides synthesis when some maltose remains in the cell simultaneously. For example in *E. Coli*, glucose from ADP-glucose is transferred to maltose and to higher oligosaccharides of maltodextrin series (Fox et al. 1976). Maltodextrin could be utilized as carbon source by *T. litoralis* cells, and then free ADP produced by this pathway could be used for next cycle of trehalose degradation.

**Fructose metabolism in T. litoralis**

The *frk* gene is oriented divergently to the operon encoding an ABC transporter for maltose and trehalose with similarity in amino acids to those proteins of the ribokinase family. This gene was cloned, expressed and identified as an ATP-dependent fructose-6-phosphate-forming fructokinase (Frk), in solution it becomes a dimer. At its optimal temperature of 80°C the major product is fructose-6-P (about 85%) and combined with two minor products either to be fructose-2-P or fructose-3-P (about 15%, totally). Fructose catabolism pathway was only proposed in a halophilic archaeon, *Halococcus saccharolyticus*. This pathway involves a fructokinase which phosphorylates fructose to fructose-1-P, which is further converted into fructose-1,6-biphosphate by a fructose-1-P kinase, finally entering a EM-type of glycolytic pathway (Johnsen et al. 2001)

**Regulation of trehalose/maltose in the transport system**

The gene *trmB* is located in the trehalose/maltose transport gene cluster in between *treT* and *malK*. Its gene product (TrmB) exhibits low homology with CymJ of *K.*
*oxytoca* which acts as a potential regulator within an operon encoding a binding protein dependent ABC transporter for cyclodextrin (Pajatsch et al. 1998a; Pajatsch et al. 1998b). Footprint analysis revealed that TrmB binds two sites in the promoter region of *malE*. One of them is in the BRE-TATA box of promoter region of *malE*, and another is in an inverted repeat upstream of *malE* TATA box region and close to *frk* encoding an ATP-fructokinase. TrmB binds to those two regions which can be prevented by maltose (100 µm) and trehalose (2.5 mM). Sugar binding assay revealed that maltose binds to TrmB much more effective than trehalose with the half-saturating concentration of 20 µm and > 500 µm, respectively. The binding of TrmB to those two sites causes the transcriptional repression in *malE* direction, which was proved by *in vitro* transcriptional assay. However transcription of *frk* is not affected by TrmB detected with Western blot and Northern blot (Qu et al. 2004a), although TrmB binds to the second inverted repeat in front of *frk*.

Microarray analysis of heat shock effect on gene expression of *P. furiosus* showed that the *malE* and *treT* gene expression were up-regulated during 1-h heat shock stress (Shockley et al. 2003). The phenomenon implies that TrmB is dissociated from promoter region of *malE* and led to an increased expression under heat shock stress. Interestingly, sucrose and maltotriose also bind to TrmB and the binding of sucrose is inhibited by maltose. Gel-shift assay revealed that TrmB binds to promoter regions of some genes including the putative regulator or enzymes. TrmB seems to be a potential global regulator for sugar metabolism in *T. litoralis* (S. J. Lee, unpublished data).

*P. furiosus* is the best-studied archaeon in metabolism and transcriptional regulation. A complete-genome DNA (2065 open reading frames) microarray of *P. furiosus* has been used to study the gene regulation under different carbon sources (maltose and peptone). This is very useful in understanding gene regulation in *T. litoralis*, whose genome sequence is absent. Microarray analysis showed that the genes encoding maltose/trehalose ABC transporter (PF 1740-PF1742) (>10-fold) and the maltodextrin transporter (PF1938-PF1933) (>5-fold) are up-regulated in maltose-grown cells. This phenomenon suggests that their gene products presumably work together to metabolize maltose as well as other maltodextrins derived from starch, even though the binding proteins of maltose/trehalose and maltodextrin transporters show different specificities. The gene expression of enzymes involving in glycolytic pathway in *P. furiosus* is also regulated in maltose grown cells. Glucose-6-P isomerase (PGI) (>4-fold), ADP phosphofructokinase (PFK) (>5-fold), and glyceraldehyde-3-P Fd oxidoreductase
(GAPOR) (>6-fold) are strongly up-regulated. ADP-glucokinase, the key enzyme for the first step of glucose utilization is slightly up-regulated in maltose grown cell (> 2-fold). It is most likely that maltose uptake and intracellular degradation elevates the glucose content in vivo, and therefore may increase the gene transcription for glucose utilization. Other genes, which are also up-regulated in maltose-grown cells, include those responsible for biosynthesis of 12 amino acids, ornithine, and citric acid cycle intermediates including those enzymes involving in the production of acyl and aryl acids and 2-ketoacids, and three genes encoding the enzymes for gluconeogenesis. Microarray data revealed that P. furiosus can readily adapt to changes in carbon sources. The information from P. furiosus provides important references for studying the regulation of gene expression under different carbon sources in T. litoralis.

E. coli MalK is regulated by MalT, the central activator of such maltose/maltodextrin system. MalK-MalT interaction represses the transporter system when its substrate is not being transported. Transport of any maltodextrin by maltose/maltodextrin ABC transporter will free MalT from its inactive state. The E. coli MalK exhibits an N-terminal domain for ATP hydrolysis and MalFG binding and a C-terminal regulatory domain for sending a signal to MalT by a protein-protein interaction. The crystal structure analysis revealed that thermococcal MalK also contains a C-terminal regulatory domain which is similar to that in E. coli. However, the search for a MalT-like protein in archaeal genome was unsuccessful. Our experimental evidence indicated that TrmB does not bind MalK (Lee et al. 2003). Expression of malT itself is also controlled by the presence of glucose via cAMP-CRP complex and by the global regulator MIC (Boos and Böhm 2000). Catabolite repression system is well-studied in bacteria and Eukaryotes, which includes transient repression, permanent repression and inducer exclusion. The archaeal catabolite repression-like system was only reported in S. solfataricus when cell grew on different carbon sources. Further research is necessary on revealing whether a carbon source hierarchy or a global regulator in sugar metabolism exist in T. litoralis.
References


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Summary

A novel trehalose glycosyl transferring synthase (TreT) from the hyperthermophilic archaeon *T. litoralis* was identified. This enzyme produces trehalose by the transfer of glucose from ADP-glucose (or, less effectively, UDP-glucose and GDP-glucose) onto the 1-position of glucose to form trehalose and ADP. Such an enzymatic activity in the synthesis of trehalose has not yet been described for any other organisms. It catalyzes a reversible reaction for trehalose synthesis and degradation by converting glucose and ADP-glucose to trehalose and ADP. The enzyme properties were characterized biochemically. The physiological function of TreT *in vivo* is still not completely understood.

An ATP-dependent and fructose-6-phosphate-forming fructokinase (Frk) was also identified and characterized in *T. litoralis*, which gene locates divergently to the trehalose/maltose transporter. The biochemical properties of this enzyme were characterized in this study.

TrmB is a novel archaeal sugar-specific transcriptional repressor regulating the expression of trehalose/maltose ABC transporter dependent on the presence of maltose or trehalose in medium. TrmB binds maltose much stronger than trehalose. TrmB binds to the DNA fragments of the TATA box promoter region and upstream motif of *malEFG*-operon. TrmB does not bind to MalK.

Two enzymes (TreT and FrK) have been identified in this research work and their expressional regulation by TrmB also has been investigated. The further interesting work can focus on the sucrose or trehalose metabolism pathway and the net work of sugar metabolism regulation in *T. litoralis*. 
Zusammenfassung


Das erste Protein (TreT) ist eine neuartige Glucosyl-transferierende Trehalosesynthase, die den Glucosylrest von ADP-Glucose auf freie Glucose überträgt und dabei Trehalose synthetisiert. Diese Enzymaktivität wurde bisher noch in keinem Organismus beschrieben. Die Reaktion ist reversibel; das Enzym katalysiert auch die Spaltung von Trehalose mit Hilfe von ADP und unter Bildung von Glucose und ADP-Glucose. Die physiologische Rolle des Enzyms ist noch unklar, aber mehrere Gründe sprechen dafür, dass TreT am Abbau von Trehalose beteiligt ist.

Das zweite Enzym (Frk) ist eine Fructokinase, die Fructose unter ATP-Spaltung zu Fructose-6-Phosphat phosphoryliert. Es ist das erste Enzym dieser Art, das in Archaea beschrieben wurde.

Das dritte Protein ist TrmB. Es hat keine enzymatische Funktion, ist aber an der Regulation der Genexpression beteiligt: es reguliert sowohl die Expression des *malEFG*-Operons wie auch seines eigenen Gens. Es wurden Bindungsstudien von TrmB an die Promoterregion des Operons durchgeführt, ebenso Bindungsstudien von Trehalose und Maltose an TrmB. Beide Zucker wirken *in vivo* als Induktoren des Systems, was TrmB als einen transkriptionellen Repressor des Systems charakterisiert.
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