Biochemical evidence for the presence of two α-glucoside ABC-transport systems in the hyperthermophilic archaeon \textit{Pyrococcus furiosus}

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Summary

The hyperthermophilic Archaeon \textit{Pyrococcus furiosus} can utilize different carbohydrates, such as starch, maltose and trehalose. Uptake of α-glucosides is mediated by two different binding protein-dependent ABC-type transport systems. The maltose transporter also transports trehalose, while the maltodextrin transport system mediates the uptake of maltotriose and higher malto-oligosaccharides but not maltose. Both transport systems are induced during growth on their respective substrates.

Introduction

The hyperthermophilic anaerobic Archaeon \textit{Pyrococcus furiosus} can grow heterotrophically on some sugars, such as cellobiose, maltose and starch (Fiala and Stetter, 1986; Kengen \textit{et al.}, 1993). Maltose and starch metabolism has been studied in some detail (de Vos \textit{et al.}, 1998) but virtually nothing is known about uptake of these carbohydrates into the cell. \textit{P. furiosus} produces a number of starch-hydrolyzing enzymes, such as an extracellular amylopullulanase (Dong \textit{et al.}, 1997) and α-amylase (Jorgensen \textit{et al.}, 1997), and an intracellular α-amylase (Laderman \textit{et al.}, 1993), and α-glucosidase (Costantino \textit{et al.}, 1990). The extracellular enzymes hydrolyze starch into smaller oligosaccharides, which are then transported into the cell via so far not known mechanisms, and intracellularly hydrolyzed to glucose. Glucose is further metabolized by the modified Embden-Meyerhof pathway (Schäfer \textit{et al.}, 1994) that involves an ADP-dependent glucokinase and phosphofructokinase (Kengen \textit{et al.}, 1994).
In bacteria, maltose and maltodextrins are usually transported via ATP-binding cassette (ABC) transporters (Ehrmann et al., 1998). In archaea and thermophilic bacteria, ABC-transporters seem to play a dominant role in sugar transport. The trehalose/maltose transporter of *Thermococcus litoralis* has been biochemically characterized and the trehalose/maltose binding protein (TMBP) and ATP binding subunit MalK have been functionally expressed in *E. coli* (Greller et al., 1999; Horlacher et al., 1998). This system mediates the uptake of trehalose and maltose but not of maltodextrins. In contrast, a single maltose, trehalose and maltodextrin transporter has been described for the thermophilic bacterium *Thermoanaerobacter ethanolicus* (Jones et al., 2000). The genome of *P. furiosus* contains a gene cluster that encodes an ABC-type transporter that is nearly identical to the trehalose/maltose transporter of *T. litoralis* (DiRuggiero et al., 1999; Horlacher et al., 1998). This system mediates the uptake of trehalose and maltose but not of maltodextrins.

### Table 1. Homology between *P. furiosus* and *E. coli* maltose/maltodextrin transporter clusters

|                | *Escherichia coli* |
|----------------|--------------------|
|                | MalE  | MalF  | MalG  | MalK  |
| *Pyrococcus furiosus* |       |       |       |       |
| PF1938         | 28a (46)b | 35 (56) |
| PF1937         |       | 33 (55) |
| PF1936         |       | 47 (63) |
| PF1933         |       | 28 (44) |
| PF1739         |       | 36 (54) |
| PF1740         |       | 41 (63) |
| PF1741         |       | 46 (64) |
| PF1744         |       | 46 (64) |

*a*Identity; *b*Similarity

*Figure 1.* Genetic organisation of the neighbourhood of the genes encoding the maltodextrin (A) (indicated in grey) and trehalose/maltose (B) (indicated in black) transporters.
In addition, it contains an operon that encodes a homolog of the maltose/maltodextrin transporter of *E. coli*. Since the second transporter was found to be induced upon growth of *P. furiosus* on maltose, it was assumed that *P. furiosus* contains two maltose transport systems, one specific for maltose and trehalose and another one specific for maltose and maltodextrins (DiRuggiero et al., 2000). Here, we show that *P. furiosus* contains a trehalose/maltose and a maltodextrin transport system. The latter system is specific for maltodextrins only and is not involved in maltose uptake.

**Results**

**Identification and heterologous expression of two ORFs involved in sugar binding**

The complete genome sequence of *P. furiosus* shows two gene clusters that encode binding protein-dependent ABC transporters that are homologous to the maltose/maltodextrin transport operon of *E. coli* (Table 1, Fig. 1). One of the clusters (PF1739-PF1741, PF1744) is identical (99-100% amino acid sequence identity) to the trehalose/maltose transport operon found in the related archaeon *Thermococcus litoralis* (data not shown). The other cluster PF1933, PF1936-PF1938 shows a high degree of homology to the *E. coli* maltose transport operon (Table 1). As observed for the possible trehalose/maltose transporter cluster, the gene encoding the ATPase subunit of the second transporter is not located in the operon that contains the genes encoding the binding protein and two permeases. Instead, two ORFs separate the second permease and the ATPase subunit, one of which encoded an amylolpullulanase (Fig. 1). To determine if the two gene clusters specify maltose transporters, the respective binding proteins, PF1938 and PF1739 were cloned into an *E. coli* expression vector behind the *trc*-promoter.

![Figure 2. Functional expression of PF1938 and PF1739 in E. coli BL21/RIL. \(^{14}C\)-Maltose (A), \(^{14}C\)-maltotriose (B) and \(^{14}C\)-trehalose (C) binding activities were measured before (-) and after (+) induction of the promotor.](image-url)
and transformed to *E. coli* strain BL21 (DE3)/RIL. Although this *E. coli* strain contains an endogenous periplasmic maltose/maltodextrin binding protein, upon expression of the respective binding proteins a significantly elevated level of $[^{14}C]$-maltose, $[^{14}C]$-trehalose or $[^{14}C]$-maltotriose binding at 60 °C was found (Fig. 2). At this temperature, the endogenous *E. coli* proteins precipitate leading to low background binding levels.

The expressed binding protein encoded by PF1739 binds $[^{14}C]$-maltose and $[^{14}C]$-trehalose, whereas PF1938 only binds $[^{14}C]$-maltotriose. Therefore, it seems that PF1739 encodes a trehalose/maltose binding protein (TMBP), while PF1938 encodes a maltodextrin binding protein (MDBP).

### Induction of binding proteins

To study the functional expression of the maltose and maltotriose binding proteins in *P. furiosus*, binding studies using $[^{14}C]$-maltose (Fig. 3B), $[^{14}C]$-maltotriose (Figure 3C) and $[^{14}C]$-trehalose (Fig. 3D) were performed using membranes isolated from *P. furiosus* cells grown on different substrates. The binding studies were performed in triplicate.
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grown on trehalose, maltose, maltotriose and starch (Fig. 3A). The mRNA levels and substrate binding activities reproducibly did not match completely. Taken together, the results suggest that both TMBP and MDBP are functionally expressed when cells are grown on maltose and maltotriose, whereas in starch and trehalose-grown cells, TMBP is most prominently present.

Maltose uptake

*P. furiosus* cells grown on maltose readily accumulate $[^{14}C]$-maltose under anaerobic conditions at 80 °C (Fig. 4). The transient uptake levels indicate rapid metabolism of the labelled substrate in the cell. Maltose uptake shows a steep temperature dependence, and uptake is hardly detectable at temperatures below 40 °C (results not shown). Initial rates of maltose uptake were used to determine the affinity of the transport system. The $K_m$ for maltose uptake is 30-40 nM at 80 °C. This
transporter is therefore a high affinity transport system.

The effect of a 100-fold excess non-labeled maltotriose and trehalose on maltose uptake was studied (Fig. 4). \[^{14}\text{C}\text{-maltose}\] Maltose uptake was not inhibited by the non-labelled maltotriose, but was effectively reduced by a 100-fold excess trehalose. This observation indicates that maltose uptake is mediated by a system that also accepts trehalose as a substrate.

**Purification of the binding proteins**

A high level of maltose binding was observed in membranes derived from maltose-grown cells. These membranes were solubilized with Triton X-100, and the binding protein was purified to homogeneity by concanavalin A (ConA) affinity chromatography and subsequent MonoQ anion exchange chromatography using the \[^{14}\text{C}\text{-maltose}\] binding at 60 °C to monitor the purification (Fig. 5). The maltose binding activity corresponded with a 45-kDa protein. To characterize the substrate specificity of this protein, an inhibition assay was used where addition of excess of non-labeled substrate leads to a decrease in binding of \[^{14}\text{C}\text{-maltose}\] substrate, while addition of a non-labeled non-substrate does not lead to a decrease of \[^{14}\text{C}\text{-maltose}\] substrate binding. Addition of a 10-fold excess of non-labelled trehalose or maltose completely abolished the \[^{14}\text{C}\text{-maltose}\] binding, while an excess glucose, maltotriose or maltotetraose was without effect (Fig. 6A). The 45-kDa binding protein was therefore identified as TMBP, which only has maltose and trehalose as substrates.

The maltodextrin binding protein was partially purified from Triton X-100 solubilized membranes derived from cells grown on maltose using ConA chromatography. The binding of \[^{14}\text{C}\text{-maltotriose}\] was used to monitor the purification. The substrate specificity of MDBP was studied using the inhibition assay as explained above. The \[^{14}\text{C}\text{-maltotriose}\] binding activity of the active fraction was effectively inhibited by maltotriose and maltotetraose, but not by glucose or maltose (Fig. 6B). Therefore,
this binding protein is a maltodextrin binding protein, MDBP.

Glycosylation of the binding proteins

Both TMBP and MBP appear as glycosylated proteins as they bind to the ConA affinity column that specifically binds terminally mannosylated and glucosylated glycoproteins. The purified TMBP could be stained with the glycoprotein specific PAS-stain. To identify the carbohydrate moieties, purified TMBP was hydrolyzed and the released sugars were labelled with the fluorophore AMAC. After polyacrylamide gel electrophoresis only glucose molecules could be observed, suggesting that TMBP is glycosylated with glucose moieties (Fig. 7).

Discussion

Pyrococcus furiosus contains two operons involved in \(\alpha\)-glucoside transport, a trehalose/maltose and a maltodextrin transport system. Both systems are members of the ATP-binding cassette family of transporters. The maltodextrin transport operon shows homology to the maltose/maltodextrin transporter of \textit{E. coli}. Surprisingly, however, this system is not involved in the uptake of maltose but binds higher malto-oligosaccharides only. The binding protein was heterologously expressed in \textit{E. coli} to confirm the substrate binding specificity. Recently, the crystallization of the \textit{P. furiosus} maltodextrin binding protein has been reported in the presence of maltose (Evdokimov et al., 2001). Surprisingly, maltotriose was found in the binding pocket. This result is now confirmed by our biochemical data. Apparently, the maltodextrin binding protein exhibits such an extremely high binding affinity for maltotriose that it binds the minute amount of contaminants present in the maltose solution. This is consistent with the hypothesis, that hyperthermophilic organisms utilize highly efficient ABC-transporters to survive conditions where the substrate concentrations are very low (Elferink et al., 2001).

The trehalose/maltose transporter is identical to the system described in the related archaeon \textit{T. litoralis} (DiRuggiero et al., 2000). Also, the binding protein of this transporter has been crystallized and its structure has been solved (Diez et al., 2001). The trehalose/maltose transport operon in both organisms is flanked by inverted repeats. In \textit{T. litoralis} a hypothetical transposon is located upstream...
of this fragment. It was hypothesized that this fragment was acquired recently by one of the organisms by lateral gene transfer (DiRuggiero et al., 2000). Because the maltotriose transporter does not transport maltose, it could very well be that *T. litoralis* acquired the trehalose/maltose transporter from *P. furiosus* as maltose and trehalose are the only two sugars known so far to be used for growth by *T. litoralis* (Neuner et al., 1990). On the other hand, genes encoding the trehalose/maltose transporter are present neither in *P. horikoshii* nor *P. abyssi*. Although the maltodextrin transporter is present in *P. abyssi*, this organism is unable to grow on starch or other sugars (Erauso et al., 1993). It is not known if *P. glycovorans* (Barbier et al., 1999), which can grow on sugars, contains the trehalose/maltose transporter. It could therefore very well be that both *P. furiosus* and *T. litoralis* acquired the genes encoding the trehalose/maltose transporter from a third organism.

Both MDBP and TMBP are induced by growth on α-glucosides. Surprisingly, MDBP was found to be induced also after growth on maltose. This induction could be caused by the presence of small amounts of maltotriose in the maltose solution. Maltotriose induces not only MDBP but also TMBP. This most likely relates to extracellular α-glucosidases that cleave the maltotriose into glucose and maltose. The latter induces the trehalose/maltose transport system. When *P. furiosus* is grown on starch, the substrate is first extracellularly cleaved by an amylolpullulanase, which cleaves starch into maltodextrins (Dong et al., 1997) (See Fig. 8). The maltodextrins are subsequently hydrolysed to smaller subunits (G2-G7) by an extracellular α-amylase (Jorgensen et al., 1997). Therefore, when utilizing starch as carbon source, both maltose and small malto-oligosaccharides are formed. Once transported inside, the malto-oligosaccharides are most likely further hydrolysed to maltose by the intracellular α-amylase. In this respect, maltose metabolism in *P. furiosus* and *T. litoralis* differ. In the latter organism, maltose is hydrolysed by MalP and 4-α-glucanotransferase, similar to the system found in *E. coli* (Xavier et al., 1999). In *P. furiosus*, however, maltose is hydrolysed...
by α-glucosidase to glucose, which then enters a modified Embden-Meyerhof pathway. The concerted action of the two transport systems for α-glucosides permits <i>P. furiosus</i> to efficiently utilize these components.

**Experimental procedures**

**Organisms and growth conditions**

<i>P. furiosus</i> Vc1 (DSM 3638) was grown routinely at 80 °C in modified <i>Methanococcus</i> medium (Kengen et al., 1993) under anaerobic conditions in the presence of 5 mM of the indicated carbohydrate or with 0.2% (w/v) pyruvate. For growth on peptone, the medium was supplemented with 1% (w/v) elemental sulphur. <i>Escherichia coli</i> DH5α (Hanahan, 1983) and BL21/RIL (Stratagene, La Jolla, USA) were grown in LB supplemented with the appropriate antibiotics at 37 °C.

**Chemicals**

Sugars were from Merck (Darmstadt, Germany). [14C]-Maltose (516 mCi/mmol) was purchased from Amersham-Radiochemicals (Little Chalfont, Buckinghamshire, United Kingdom), [14C]-maltotriose (850 mCi/mmol) and [14C]-trehalose (850 mCi/mmol) were kind gifts from Prof. Winfried Boos, Konstanz, Germany.

**Transport and binding studies**

Cells grown overnight in 50 ml medium were harvested under anaerobic conditions, washed once in growth medium without carbon-source, resuspended, and stored at room temperature until use. Transport assays were performed anaerobically at 80 °C using a continuous flow of N2-gas. Cells were used at 10 µg protein/ml, and transport was initiated by the addition of the radiolabelled substrate to a final concentration of 10 µM. Samples were taken at different time points, filtered over BA85 nitrocellulose filters (Protran; Schleicher & Schuell, Dassel, Germany), and washed twice with growth medium without carbon source. The radioactivity retained on the filters was determined by liquid scintillation counting. Kinetic constants were estimated from triplicate measurements of the initial uptake rate determined after 10 s.

Binding studies were performed using the method described by Richarme and Kepes (Richarme and Kepes, 1983). Basically, 1 µM radiolabelled substrate was added to isolated <i>P. furiosus</i> membranes or to purified binding protein (10 µg protein/ml). Binding assays were performed in 100 µl volume at 60 °C. After 3 min, the binding reactions were terminated by the addition of 2 ml ice-cold 0.1 M LiCl, filtered over BA85 nitrocellulose filters and washed once with 2 ml 0.1 M LiCl. The radioactivity retained on the filters was determined as described above.

**Purification of binding proteins**

Cells were harvested and resuspended in 50 mM Tris-HCl pH 7.5, and broken by a single passage through a French Pressure cell at 600 lb/in^2. Membranes were collected by centrifugation for 45 min at 100,000 x g at 4 °C. The pellet was resuspended in 50 mM Tris-HCl pH 7.5, washed once and solubilized in 0.5% (v/v) Triton X-100 for 30 min at 37 °C. Non-solubilized material was removed by centrifugation (350,000 x g, 15 min, 4 °C), and the supernatant was collected and applied onto a concanavalin A (ConA)-Sepharose (Pharmacia, Roosendaal, The Netherlands) column equilibrated with buffer A (25 mM Tris-HCl pH 7.4, 500 mM NaCl, and 0.05% [v/v] Triton X-
10). The column was washed thoroughly with buffer A, and bound glycoproteins were eluted using buffer A supplemented with 250 mM α-methyl-mannopyranoside. Fractions were dialyzed overnight against buffer B (25 mM Tris pH 6.8, and 0.05% [v/v] Triton X-100), measured for substrate binding activity as described above and fractions containing maltose or maltotriose binding activity were pooled, and applied to a HR5/5 MonoQ column (Pharmacia, Uppsala, Sweden), pre-equilibrated with buffer B. Proteins were eluted with a linear gradient of 0 to 500 mM NaCl in buffer B. Fractions were analysed by SDS-PAGE and assayed for binding activity, pooled and stored at –80 °C.

Cloning and expression of binding proteins

Oligonucleotide primers were designed based on the nucleotide sequence of the complete PF1938 and PF1739 genes as found in the P. furiosus database (http://www.genome.utah.edu). PF1938 and PF1739 were amplified by PCR (forward 5’-cccccgatatcatgaggagagcaacatacgcc-3’, reverse 5’-cccccggatccttatccttgcatgttgtta-3’; forward 5’-cccccgatatcatgaatgtcaagaaggtactgc-3’, reverse 5’-ccccctctagattagctgtattgtttaac-3’, respectively), and the resulting 1.35 kb fragments were ligated in pBSKS (Stratagene, La Jolla, California, USA) to yield pSMK10 and pSMK11. The inserts were transferred to the expression vector pET302 (van der Does et al., 1998) to yield pSMK14 and pSMK16, containing PF1938 and PF1739, respectively, with an amino-terminal terminal hexa-histidine tag.

For expression, E. coli BL21 (DE3)/RIL was used that expresses plasmid encoded tRNAs for the amino acids leucine, isoleucine and arginine with rare codons. Cells transformed with pSMK14 or pSMK16 were grown to an OD at 660 nm of 0.8, and induced for 2 h with 0.5 µM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested and broken by French Press treatment at 800 lb/in². The membrane and soluble fraction were collected by centrifugation (350,000 x g, 20 min, 4 °C), and analysed by SDS-PAGE and Western Blotting using His-tag antibodies (Dianova GmbH, Hamburg, Germany), and by [14C]-maltose or [14C]-maltotriose binding assays at 37 and 60 °C.

Total RNA isolation and Northern analysis

Total RNA was isolated from exponentially growing P. furiosus cells using the TRIZOL Reagent (Gibco BRL Life Technologies, Breda, The Netherlands). For Northern blot analysis, 10 µg total RNA was separated on formaldehyde-agarose (1%) gels, and transferred to a Zeta-probe membrane (Biorad, Veenendaal, The Netherlands) by capillary blotting. Primers were designed according to the gene sequences present in the P. furiosus (http://www.genome.utah.edu/) database. Probes for PF1938 (forward: 5’-cccccgatatcatgaggagagcaacatacgcc-3’, reverse: 5’-tgccatgtattcttccgc-3’), PF1739 (forward: 5’-cccccgatatcatgaatgtcaagaaggtactgc-3’, reverse: 5’-cccccctctagattagctgtattgtttaac-3’, respectively), and the resulting 1.35 kb fragments were ligated in pBSKS (Stratagene, La Jolla, California, USA) to yield pSMK10 and pSMK11. The inserts were transferred to the expression vector pET302 (van der Does et al., 1998) to yield pSMK14 and pSMK16, containing PF1938 and PF1739, respectively, with an amino-terminal terminal hexa-histidine tag.

Sugar analysis

For the identification of the sugar molecules present on the glycosylated binding proteins, 20 µg protein was dialyzed overnight against demineralized water. Sugar moieties were hydrolyzed from the protein by incubation
for 5 h in 2 N trifluoroacetic acid (TFA) or 3 h in 4 N HCl at 100 °C. Released saccharides were labelled with 2-aminoacridone (AMAC) and analyzed by gel electrophoresis (Jackson, 1994).

Other techniques

N-terminal amino acid sequence analysis was performed with the purified protein blotted on PVDF membrane by NAPS (Nucleic Acid/Protein Service Unit, Vancouver, Canada). DNA sequencing was performed by BioMedisch Technologisch Centrum (BMTC, University of Groningen, The Netherlands). Glycoproteins in SDS-PAGE were stained using Periodic Acid-Schiff (PAS) (Sigma) as described (McGuckin and McKenzie, 1958). Protein concentrations were determined using the DC Biorad Kit (BIORAD, Veenendaal, The Netherlands).

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