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Chapter 2

# Cellobiose uptake in the hyperthermophilic Archaeon *Pyrococcus furiosus* is mediated by an inducible, high-affinity ABC-transporter

Sonja M. Koning, Marieke G. L. Elferink, Wil N. Konings and Arnold J. M. Driessen

## Summary

**The hyperthermophilic archaeon *Pyrococcus furiosus* can utilize different β-glucosides, like cellobiose and laminarin. Cellobiose uptake occurs with high affinity ($K_m$ = 175 nM) and involves an inducible binding protein-dependent transport system. The cellobiose binding protein (CbtA) was purified from *P. furiosus* membranes to homogeneity as a 70-kDa glycoprotein. CbtA not only binds cellobiose but also cellotriose, cellotetraose, cellopentaose, laminaribiose, laminaritriose and sophorose. The *cbtA* gene was cloned and functionally expressed in *E. coli*. *CbtA* belongs to a gene cluster that encodes a transporter that belongs to the Opp-family of ABC-transporters.**

## Introduction

*Pyrococcus* species are anaerobic hyperthermophilic Archaea that are able to grow heterotrophically on a range of substrates. *P. furiosus* (Fiala and Stetter, 1986) and *P. glycovorans* (Barbier *et al.*, 1999) have been reported to grow on various sugars including the β-glucoside cellobiose (Kengen *et al.*, 1993). On the other hand, *P. abyssi* ST549 is unable to grow on cellobiose (Godfroy *et al.*, 2000), despite the presence of a β-glucosidase (Ladrat *et al.*, 1997). *P. furiosus* also utilizes the β-glucoside polymer laminarin (Gueguen *et al.*, 1997), and metabolism of β-glucosides has been studied in some detail. Cellobiose is intracellularly hydrolysed to two glucose molecules by the β-glucosidase CelB (Kengen *et al.*, 1993), while laminarin is first cleaved by the extracellular β-glucosidase, LamA, to

yield smaller oligo-glucosides. These are subsequently transported into the cell via an unknown mechanism, and further hydrolysed by CelB to glucose. The combined activity of CelB and LamA results in the complete hydrolysis of laminarin to glucose (Gueguen *et al.*, 1997). Glucose is further metabolised by the modified Embden-Meyerhof pathway (Schäfer *et al.*, 1994), which involves a glucokinase and phosphofructokinase which are both ADP-dependent (Kengen *et al.*, 1994).

To understand the energy yields during growth on β-glucosides, the mechanism of sugar uptake needs to be elucidated. In bacteria, cellobiose enters the cell either via a phospho*enol*pyruvate-dependent phospho-transferase system (Helaszek and White, 1991; Kajikawa and Masaki, 1999) or via a binding protein-dependent ATP-binding cassette (ABC)-transporter (Schlosser *et al.*, 1999). Analysis of the completed genome sequences of a variety of Archaea demonstrates that phospho*enol*pyruvate-dependent phospho-transferase systems are absent in these organisms. In the thermoacidophile *Sulfolobus solfataricus*, sugars appear to be transported into the cell mainly via binding protein-dependent ABC-transporter systems (Albers *et al.*, 1999a; Elferink *et al.*, 2001). In the hyperthermophile *Thermococcus litoralis* a trehalose/maltose ABC-transport system has been described biochemically (Xavier *et al.*, 1996). The trehalose/maltose binding protein, TMBP, and the ATPase subunit, MalK, have been functionally expressed in *E. coli* (Greller *et al.*, 1999; Horlacher *et al.*, 1998). These binding protein-dependent transport systems exhibit an unusually high affinity for the sugar, with a $K_m$ in the submicromolar range. In the hyperthermophilic bacterium *Thermotoga maritima,* a high-affinity binding protein-dependent ABC-transport system has been

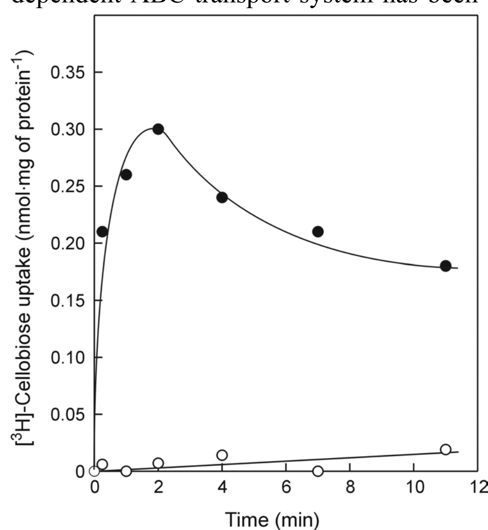


**Figure 1.** Cellobiose transport in *P. furiosus* cells. Cells were grown on cellobiose (●) or maltose (○) as carbon- and energy source, and accumulation of 10 μM [$^3$H]-cellobiose was assayed under anaerobic conditions at 80°C.

described for maltose, trehalose and maltotriose (Wassenberg *et al.*, 2000). The abundance of such high-affinity transport systems in thermophilic organisms (both bacteria and archaea) suggests that they play a major role in sugar utilization in the nutrient-poor extreme environments in which these organisms thrive. In an effort to understand the metabolism of cellobiose

in *P. furiosus*, we now report on a binding protein-dependent ABC-transport system for oligo β-glucosides.

## Results

### Cellobiose uptake by *Pyrococcus furiosus*

*P. furiosus* cells grown on cellobiose readily accumulate [$^3$H]-cellobiose when incubated at 80 ºC under anaerobic conditions (Fig. 1). Uptake was completely abolished by aerobic conditions (results not shown). Cellobiose was strongly temperature-dependent. Below 40 ºC, hardly any cellobiose was transported into the cell, while above 90 ºC, rapid metabolism resulted in a rapid decrease of the accumulated radioactivity. At 80 ºC, uptake of cellobiose occurred with a $K_m$ of 175 nM indicating the presence of a high-affinity transport system. Cellobiose transport activity was found only in cells grown on cellobiose and was not observed in maltose-grown cells (Fig. 1). Uptake of [$^3$H]-cellobiose was completely inhibited by a 10-fold excess of non-labelled cellobiose and cellotriose, but not by glucose, maltose or lactose. These data suggest that *P. furiosus* contains a high-affinity transport-system for cellobiose and cellotriose.

### Isolation and characterization of a cellobiose binding protein

Membranes were isolated from cellobiose- and maltose-grown *P. furiosus* cells, and incubated with [$^3$H]-cellobiose. A high level of [$^3$H]-cellobiose binding was observed with membranes derived from cellobiose-grown cells, while membranes of maltose-grown cells showed only background binding (Fig. 2A). In contrast

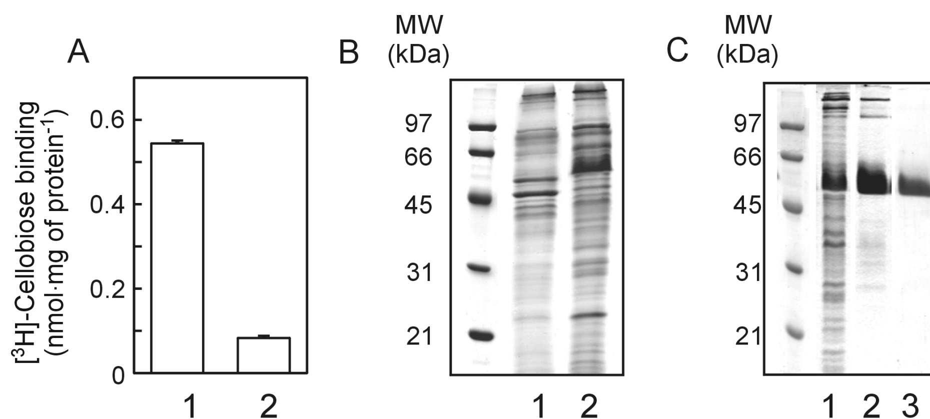


**Figure 2.** *P. furiosus* contains a cellobiose binding protein. (A) [$^3$H]-cellobiose binding to membranes derived from cells grown on cellobiose (lane 1) or maltose (lane 2). Binding studies were performed as described. (B) Coomassie-stained SDS-PAGE gel. Comparison between membranes derived from maltose-grown cells (lane 1) and membranes derived from cellobiose-grown cells (lane 2). (C) Coomassie-stained gel. Purification of Cbp. Lane 1, membranes; 2, ConA fraction; 3, purified Cbp.

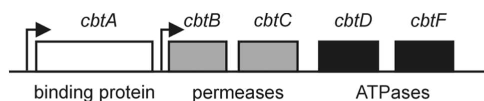


**Figure 3.** Genetic organization of the genes encoding the cellobiose transport system. The proteins encode the genes are: *cbtA*, the extracellular binding protein; *cbtB* and *cbtC*, permease domains; and *cbtD* and *cbtF*, the cytosolic ATP binding domains. The arrows indicate putative promotor regions. Boxes in the same colour indicate homologous functions.

to cellobiose uptake, binding was not oxygen-sensitive. Membranes derived from maltose- or cellobiose-grown cells were analysed by SDS-PAGE and coomassie brilliant blue staining. Comparison revealed the presence of a unique and abundant 55-kDa protein in the membranes of cellobiose-grown cells (Fig. 2B). The cellobiose binding protein was purified from Triton X-100 solubilized membranes using [$^3$H]-cellobiose binding at 60 ºC to monitor the purification. The protein could be purified to homogeneity by concanavalin A affinity chromatography followed by MonoQ anion exchange chromatography. The binding activity corresponded to the 55-kDa protein which is now termed CbtA (Fig. 2C). Since CbtA binds to concanavalin A, while it is eluted with α-methyl-mannopyranoside, it appears to be glycosylated. The glycosylation was verified with a Periodic Acid-Schiff (PAS) staining of the protein after SDS-PAGE (not shown).

Purified CbtA binds cellobiose with a $K_d$ of 45 nM and a $B_{max}$ of 0.7 nmol.mg

**Table 1.** Substrate specificity of the cellobiose binding protein CbtA and growth of *P. furiosus* on various sugars

| **Competing substrate** | **Residual [$^3$H]-cellobiose binding[a] % of control** | **Growth[b]** |
|---|---|---|
| Cellobiose | 11 ± 1 | + |
| Cellotriose | 12 ± 2 | + |
| Cellotetraose | 9 ± 1 | + |
| Cellopentaose | 10 ± 0. 7 | + |
| Laminaribiose | 17 ± 3 | + |
| Laminaritriose | 12 ± 1 | n. d. |
| Sophorose | 33 ± 15 | + |
| Gentiobiose | 109 ± 26 | - |
| β,β-Trehalose | 89 ± 16 | - |
| Maltose | 100 ± 17 | + |
| Glucose | 85 ± 8 | - |

[a]Binding of 200 nM [$^3$H]-cellobiose to the purified CbtA was measured in the absence and presence of 2 μM of the indicated non-labelled sugar substrates. Experiments were performed in triplicate with indicated standard error of the mean

[b]Growth in the presence of 0. 1 % (w/v) of the indicated sugar as sole carbon and energy source. +, growth; -, no growth; n. d., not determined. Growth experiments were performed in duplicate.

protein$^{-1}$ at 60 ºC. The substrate specificity of CbtA was determined by means of competition for [$^3$H]-cellobiose binding. In 10-fold excess, non-labelled cellobiose, cellotriose, cellotetraose and cellopentaose appeared to be effective competitors for [$^3$H]-cellobiose binding to CbtA (Table 1). Competition was also observed with laminaribiose and laminaritriose, both building blocks of the polymer laminarin. The disaccharide sophorose was less effective as inhibitor, while the disaccharides β,β-trehalose and gentiobiose were ineffective. All of the sugars that effectively competed with [$^3$H]-cellobiose binding to CbtA also supported growth of *P. furiosus* (Table 1). These data suggest that CbtA is a broad-specificity β-glucoside binding protein.

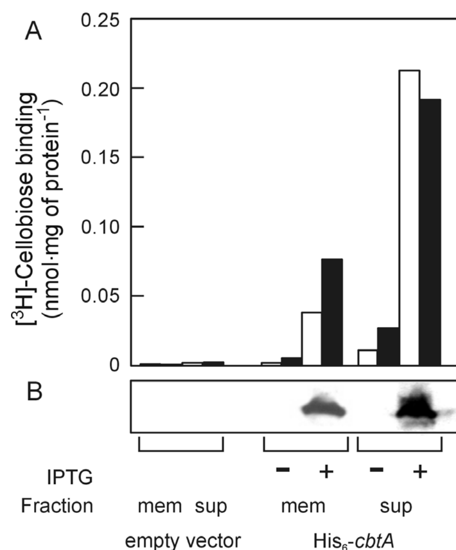


**Figure 4.** Expression of *P. furiosus* CbtA in *E. coli* SF120/1244. (A) Cellobiose binding activity at 37ºC (white bars) and 60ºC (black bars) using 500 nM [$^3$H]-cellobiose. (B) Western blot detection of $His_6$-CbtA using His-antibodies in the membrane (mem) and supernatant (sup) of cell lysates after (+) and before (-) induction. *E. coli* SF120/1244 cells were used that were co-transformed with p1244, harbouring the rare tRNA genes, and pET302 (empty vector) or pSMK4 containing the *cbtA* gene with an N-terminal hexa-histidine tag.

**Cloning and heterologous expression of CbtA**

N-terminal amino acid sequence analysis by Edmann degradation of the purified CbtA yielded the amino acid sequence QEQELPR. Database searches of the *P. furiosus* genome (http://wit.mcs.anl.gov/) identified an ORF (RPF00252) with an exact match. This ORF contained an additional 20 amino acids at the N-terminus, predicted to form a typical signal sequence. The calculated molecular mass of the mature protein is 70-kDa, which is substantially larger than the 55-kDa estimated for the purified CbtA by SDS-PAGE. This discrepancy is due to an incomplete denaturation of CbtA in SDS. After boiling for 30 minutes in 2 % SDS, CbtA migrated as a 70-kDa protein on SDS-PAGE. Hydropathy analysis of CbtA indicates the presence of a hydrophobic domain at the carboxyl-terminus that possibly functions as a membrane anchor. Strikingly, the hydrophobic domain is preceded by a serine/threonine-rich region that may function as a flexible linker to connect the catalytic domain to the membrane-anchoring region. Homology searches revealed that the protein belongs to the OppA-family of binding proteins,

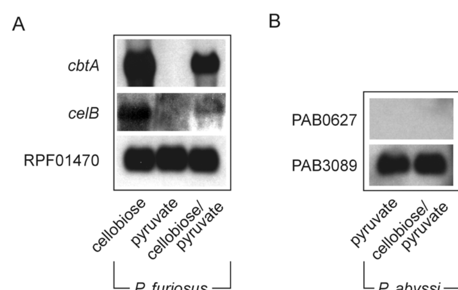


**Figure 5.** Northern blot analysis of total RNA extracted from *P. furiosus* (A) or *P. abyssi* (B) cells grown on cellobiose, pyruvate or pyruvate/cellobiose. Histones (RPF01470, PAB3089) were used as internal controls for the total amount of RNA.

with the highest homology to putative binding protein of various thermophilic Archaea and Bacteria. The *cbtA* gene is part of a gene cluster that includes four other genes (Fig. 3). The products of two of these ORFs, i.e., *cbtB* (RPF00251) and *cbtC* (RPF00250), are homologous to OppB and OppC, respectively. These proteins constitute the permease domain of the Opp system. The other two ORFs, *cbtD* (RPF00249) and *cbtF* (RPF00248), encode gene products that are homologous to OppD and OppF, the ATP-hydrolysing subunits of the transport system. Upstream of *cbtA* and *cbtB* a putative TATA-box is observed.

The *cbtA* gene was cloned with an N-terminal hexa-histidine-tag into an *E. coli* expression vector behind the *trc*-promoter and transformed to *E. coli* strain SF120 together with p1244 (13). The latter plasmid bears tRNAs for the amino acids leucine, isoleucine and arginine, with codons that are rarely used by *E. coli*. Since *E. coli* uses a phospho*enol*pyruvate transferase system for cellobiose uptake (Fox and Wilson, 1968), functional expression of CbtA could conveniently be determined by [$^3$H]-cellobiose binding studies (Fig. 4A). While binding of cellobiose was absent in the soluble and membrane fraction of the lysed parental strain, significant binding levels were observed in the cells upon the induction of CbtA expression. The binding activity correlates with the presence of the protein in the various fractions as evidenced by immunoblotting using a hexa-histidine-tag antibody (Fig. 4B). These results demonstrate that the *P. furiosus cbtA* gene encodes a cellobiose binding protein, and that this protein can be functionally expressed in *E. coli*.

**Cellobiose uptake by *Pyrococcus abyssi***

*P. abyssi* GE5 is unable to grow on cellobiose (Erauso *et al.*, 1993), but does contain a gene cluster that is highly homologous to the *cbt*-cluster of *P. furiosus*. ORFs PAB0627, PAB0628, PAB2363, PAB0630 and PAB0631 of the *P. abyssi* GE5 chromosome resemble *cbtA*, *cbtB*, *cbtC*, *cbtD* and *cbtF*, respectively. The gene products are more than 79 % identical. To determine if this putative cellobiose transport system is expressed in *P. abyssi,* cells were grown on pyruvate in the presence of cellobiose. Northern blotting shows that under these growth conditions, both *cbtA* and the structural gene of the β-glucosidase, *celB*, are

expressed in *P. furiosus*, while no expression is seen when cells are grown on pyruvate only (Fig. 5A). Under similar growth conditions, it was not possible to detect the expression of the *P. abyssi* homolog of the *P. fusiosus* CbtA, PAB0627 (Fig. 5B). Membranes derived from *P. abyssi* cells grown on pyruvate, and a combination of pyruvate and cellobiose were inactive for [$^3$H]-cellobiose binding, while a high binding activity could be observed in membranes derived from *P. furiosus* cells grown on cellobiose and the combination of cellobiose and pyruvate. In cells grown solely on pyruvate only minor binding activity could be detected (Fig. 6). These data suggest that *P. abyssi* is defective in the expression of the cellobiose transport system.

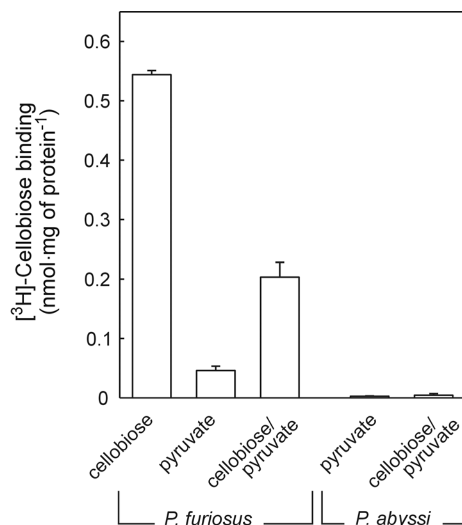


**Figure 6.** [$^3$H]-cellobiose binding to *P. furiosus* and *P. abyssi* membranes derived from cells grown on cellobiose, pyruvate or pyruvate/cellobiose.

## Discussion

Here we show that *P. furiosus* contains a high-affinity binding-protein-dependent ABC-transport system for the uptake of cellobiose and most other β-glucosides. The cellobiose binding protein, CbtA, is a 70-kDa glycosylated protein. Strikingly, it is homologous to the di- and tri-peptide binding proteins of the OppA-family. So far, only the α-galactoside binding protein AgpA of *Rhizobium meliloti* was known to be a sugar-binding member of this family (Gage and Long, 1998), that also includes nickel, opine, heme and substituted sugar transporters (Tam and Saier, 1993). The gene cluster encoding the cellobiose transporter includes genes that encode two distinct ATPases and two membrane domains. This architecture corresponds to what is generally observed for members of the Opp family of oligopeptide ABC transporters. ABC-transporters for sugars usually contain only a single ATPase subunit that is thought to function as a homo-dimer.

Databank searches revealed the presence of many putative binding proteins of other thermophilic Archaea and bacteria that are homologous to the cellobiose ABC transport system of *P. furiosus*. Nine out of eleven operons encoding ABC transporters present in the genome of the hyperthermophilic bacterium *Thermotoga maritima* encode members of the OppA-family. It has been suggested that these

transport systems are oligopeptide transporters (Nelson *et al.*, 1999), but based on our current finding and location of these operons in the vicinity of genes that are involved in sugar metabolism, it is more likely that some of these transporters are sugar transporters. The cellobiose transport system of the thermoacidophilic archaeon *S. solfataricus* also belongs to the Opp transporter family (Elferink *et al.*, 2001). Again, genes encoding sugar metabolising enzymes are located in the vicinity of the transport operon. In this respect, the gene upstream of the *cbtA* gene in *P. furiosus* encodes a β-mannosidase. Its specific physiological role is unclear (Bauer *et al.*, 1996).

Like oligopeptide binding proteins, CbtA binds a broad range of polymeric substrates. In contrast, sugar-binding proteins usually exhibit a narrow substrate specificity that is often limited to monosaccharides. Therefore, it may well be that the substrate binding pocket of CbtA more-or-less resembles that of the OppA-family of binding proteins that can accommodate a range of short and long oligopeptides (Detmers *et al.*, 2000; Lanfermeijer *et al.*, 2000).

The *cbt* gene cluster contains two putative TATA-boxes, i. e., one upstream of *cbtA* and one upstream of the *cbtB* gene. The latter promoter most likely controls expression of the *cbtBCDF* genes. Northern analysis revealed larger amounts of *cbtA* transcript as compared to the *cbtBCDF* transcripts. The presence of two promoter regions presumably relates to the need for binding protein in excess to the transporter domains to allow efficient scavenging of the substrate at the external surface of the membrane.

*P. abyssi* GE5 harbours a gene cluster that shares a very high degree of homology with the *cbtABCDF* genes of *P. furiosus*. However, *P. abyssi* GE5 does not grow on cellobiose (Godfroy *et al.*, 2000), which has been attributed to the lack of a gene encoding a β-glucosidase, CelB, needed to hydrolyse cellobiose to glucose (http://www.genoscope.cns.fr/Pab/).
Another *P. abyssi* strain, i. e., ST549 does exhibit β-glucosidase activity (Ladrat *et al.*, 1997) but is also unable to grow on cellobiose. Our data with *P. abyssi* GE5 indicates that the putative cellobiose transporter is not expressed when cells are grown on pyruvate in the presence of cellobiose. These conditions do, however, result in expression of the *ctbABCDF* genes of *P. furiosus*. It seems likely that *P. abyssi* GE5 is defective in a response regulator that triggers induction of the genes involved in cellobiose metabolism, including the transport system.

*P. furiosus* has not been reported to grow on cellulose, although the endoglucanase, EglA, exhibits hydrolytic activity against carboxymethyl cellulose (Bauer *et al.*, 1999). The organism, however, does grow on different cello-oligomers. EglA is an extracellular protein that exhibits a greater affinity for cellopentaose and cellohexaose as compared to the shorter cello-oligomers (Bauer *et al.*, 1999). The long cello-

oligomers will most likely first be hydrolysed extracellularly to yield cellobiose, cellotriose or cellotetraose. These compounds are then transported into the cell, and hydrolysed to glucose by CelB (Kengen *et al.*, 1993) and possibly other proteins. *P. furiosus* can also grow on the β-1,3-glucose polymer laminarin. This possibly involves the hydrolysis of laminarin into smaller laminari-oligomers by the extracellular enzyme LamA. The laminari-oligomers enter the cell via the cellobiose transport system, and are than hydrolysed to glucose by the intracellular β-glucosidase CelB (Gueguen *et al.*, 1997). Our studies show that *P. furiosus* is able to grow on the β-glucoside sophorose. This compound also inhibits cellobiose binding to CbtA, and therefore it is likely that sophorose enters the cells via the cellobiose transporter. Sophorose metabolism requires an intracellular β-glucanase, but such an enzyme has not yet been reported for *P. furiosus*. The β-glucosides gentiobiose and β,β-trehalose are not recognized by CbtA, nor is *P. furiosus* able to grow on these substrates. CelB was shown to hydrolyse β-1,6-glycosidic bonds (T. Kaper, personal communication). Therefore, the inability of *P. furiosus* to grow on gentiobiose might be due to a lack of transport activity.

Summarizing, *P. furiosus* contains a binding-protein-dependent ABC transport system for the uptake of cellobiose, and a range of β-glucosides. This system is homologous to the OppA-family of ABC transporters that mainly used for oligopeptide transport. These transporters share the property that they are involved in the transport of oligomeric compounds such as oligosaccharides and oligopeptides.

## Experimental procedures

### *Organisms and growth conditions*

*Pyrococcus furiosus* Vc1 (DSM 3638) and *Pyrococcus abyssi* GE5 (CNCM I-1302) were grown routinely at 80 ºC in modified *Methanococcus* medium (Kengen *et al.*, 1993) under anaerobic conditions in the presence of 5 mM carbohydrate or 0.2 % (w/v) pyruvate. For *P. abyssi*, the medium was supplemented with 1 % (w/v) elemental sulphur. Continuous monitoring of growth of *P. furiosus* on different sugars was performed in the Cary 100 spectrophotometer (Varian, Mulgrave, Victoria, Australia) in micro-cuvets under a $N_2/CO_2$ atmosphere at 90 ºC. Cells were grown in 750 µl medium supplemented with 0. 1 % (w/v) sugar as indicated, and growth was monitored at 660 nm for 15 hours. Cells grown on laminarin were followed for 48 hours. *Escherichia coli* DH5α (Hanahan, 1983) and SF120 (Baneyx and Georgiou, 1991) were grown in LB supplemented with the appropriate antibiotics at 37 or 25 ºC, respectively.

### *Chemicals*

Laminaribiose and laminaritriose were purchased from Dextra Laboratories (Reading, United Kingdom), sophorose was obtained from Sigma (Steinheim, Germany), and all other sugars were from Merck (Darmstadt, Germany). [$^3$H]-Cellobiose was purchased from Amersham-Radiochemicals (Little Chalfont, Buckinghamshire, United Kingdom).

***Transport and binding studies***

Cells grown overnight in 50 ml medium were harvested under anaerobic conditions, washed once in growth medium without carbon-source, and after resuspention stored at room temperature until use. Transport assays were performed anaerobically at 80 ºC under continuous flow of $N_2$-gas using 10 µg of cell protein/ml. [$^3$H]-Cellobiose was added to a final concentration of 10 µM and at different time points samples were taken and washed twice with medium without carbon source using BA85 nitrocellulose filters (Protran; Schleicher & Schuell, Dassel, Germany). The radioactivity retained on the filters was determined with FilterCount (Packard Bioscience B.V., Groningen, The Netherlands). The kinetic parameters of transport were estimated from triplicate measurements of the uptake for 10 seconds. For binding studies, 1 µM [$^3$H]-cellobiose was added to *P. furiosus* membranes or the purified protein (10 µg of protein per ml). Binding studies were performed aerobically at 60 ºC. After 3 minutes incubation, reactions were terminated by the addition of 2 ml ice-cold 0. 1 M LiCl, samples were filtered and washed once with 2 ml 0. 1 M LiCl. The radioactivity retained by 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 pass through a French Pressure cell at 600 lb/in$^2$. Membranes were collected by ultracentrifugation for 45 minutes at 100,000 x g at 4 ºC. The pellet was resuspended in 50 mM Tris-HCl pH 7.5, and washed once. Membranes were solubilized using 0.5 % (v/v) Triton X-100 for 30 minutes at 37 ºC. Non-solubilized material was removed by centrifugation (350,000 x g, 15 minutes, 4 ºC), and the supernatant was applied to 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-100). The column was washed thoroughly with buffer A, and bound glycoproteins were eluted using buffer A supplemented with 250 mM α-methyl-mannopyranoside. Fractions with cellobiose binding activity were pooled, dialysed overnight against buffer B (25 mM Tris pH 6. 8, and 0.05 % (v/v) Triton X-100), and applied on 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. The cellobiose binding protein (CbtA) eluted at 120 mM NaCl. Active fractions were analysed by SDS-PAGE, pooled and stored at –80 ºC.

***Cloning and expression of CbtA***

Oligonucleotide primers were designed based on the nucleotide sequence of the complete *cbtA* gene present in the *P. furiosus* database (http://wit.mcs.anl.gov/). The gene was amplified by PCR (forward 5'-ccccgatatcatgaagagactcgttggtgtac-3', reverse 5'-cccccggatccttaagatcttctcctcctt-3'), and the resulting 1.8 kb fragment was ligated in pBSKS (Stratagene, La Jolla, California) to yield pSMK3 which was transformed to DH5α (Hanahan, 1983). pSMK3 was digested with *Bsp*HI and *Bam*HI, and the insert was ligated into the expression vector pET302 (van der Does *et al.*, 1998) to yield pSMK4 containing the *cbtA* gene with an N-terminal hexa-histidine tag. These expression plasmids were co-transformed with p1244 (Kim *et al.*, 1998) into *E. coli* SF120 (Baneyx and Georgiou, 1991). The plasmid p1244 harbours tRNAs for the amino acids leucine, isoleucine and arginine with rare codons. Cells were grown to an OD at

660 nm of 0.8, and subsequently induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours. Cells were harvested by centrifugation, and broken by French Press treatment at 800 lb/in$^2$. Lysed cells were separated in a membrane and soluble fraction by ultra-centrifugation (350,000 x g, 20 min, 4 ºC). The different fractions were analysed by SDS-PAGE and Western Blotting using His-tag antibodies (Dianova GmbH, Hamburg, Germany), and assayed for [$^3$H]-cellobiose binding at 37 and 60 ºC.

***Total RNA isolation and Northern analysis***

Total RNA was isolated from exponentially growing *P. furiosus* and *P. abyssi* cells using the TRIZOL Reagent (Gibco BRL Life Technologies, Breda, The Netherlands). *P. abyssi* RNA was treated with DNase I to remove co-isolated DNA. For Northern blot analysis, 10 µg of total RNA was separated on formaldehyde-1.1% agarose 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://wit.mcs.anl.gov/) and *P. abyssi* (http://www.genoscope.cns.fr/Pab/) databases. Probes for *cbtA* (forward: 5'-cgccctcatgaagagactcgttggtgt-3'; reverse: 5'-aaccttaacctcttggagcc-3'), *celB* (forward: 5'-ctggtttccagtttgagatggg-3'; reverse: 5'-tggctttggaaaaattcttgccc-3'), RPF01470 (forward: 5'-atgggagaattgccaattgc-3'; reverse: 5'-tcagctcttaattgcgagc-3'), PAB0627 (forward 5'-atggaaaaactagtggcagccatagttg-3'; reverse 5'-tgagaccctctttgagaaccaccc-3') and PAB3089 (forward 5'-atgggagagttgccaattgc-3'; reverse 5'-tcagctcttaatagccaac-3') were DIG-labelled using PCR on genomic DNA. Detection was done with DIG-AP antibodies (Boehringer Mannheim, Germany) and CDP-Star (Tropix Inc., Bedford, USA).

***Other techniques***

For the determination of the N-terminal sequence of Cbp, the purified protein was electro-eluted from SDS-PAGE and freeze-dried. Protein sequencing was performed 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 $D_C$ Biorad Kit (BIORAD, Veenendaal, The Netherlands).

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