Identification of a system required for the functional surface localization of sugar binding proteins with class III signal peptides in *Sulfolobus solfataricus*

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Summary

The hyperthermophilic archaeon *Sulfolobus solfataricus* contains an unusual large number of sugar binding proteins that are synthesized as precursors with a class III signal peptide. Such signal peptides are commonly used to direct archaeal flagellin subunits or bacterial (pseudo)pilins into extracellular macromolecular surface appendages. Likewise, *S. solfataricus* binding proteins have been suggested to assemble in higher ordered surface structures as well, tentatively termed the bindosome. Here we show that *S. solfataricus* contains a specific system that is needed for the functional surface localization of sugar binding proteins. This system, encoded by the *bas* (**b**indosome **a**ssembly **s**ystem) operon, is composed of five proteins: *basABC*, three homologues of so-called bacterial (pseudo)pilins; BasE, a cytoplasmic ATPase; and BasF, an integral membrane protein. Deletion of either the three (pseudo)pilin genes or the *basEF* genes resulted in a severe defect of the cells to grow on substrates which are transported by sugar binding proteins containing class III signal peptides, while growth on glucose and maltose was restored when the corresponding genes were reintroduced in these cells. Concomitantly, Δ*basABC* and Δ*basEF* cells were severely impaired in glucose uptake even though the sugar binding proteins were normally secreted across the cytoplasmic membrane. These data underline the hypothesis that the *bas* operon is involved in the functional localization of sugar binding proteins at the cell surface of *S. solfataricus*. In contrast to surface structure assembly systems of Gram-negative bacteria, the *bas* operon seems to resemble an ancestral simplified form of these machineries.

Introduction

Sulfolobus solfataricus is a thermophilic archaeon which grows optimally at 80°C and a pH value of 3–4. It was originally isolated from a hot and sulphur-rich water pool in a solfataric field nearby Naples, Italy (Zillig *et al.*, 1980). The natural environment of *S. solfataricus* is relatively poor in carbohydrate substrates and therefore *Sulfolobus* needs optimized machineries for the uptake of these resources. Studies on sugar uptake showed that *S. solfataricus* is equipped with a range of binding protein-dependent ATP binding cassette (ABC) transporters (Elferink *et al.*, 2001). The glucose and arabinose binding protein, GlcS and AraS respectively, exhibit a very high affinity in the nanomolar range to their substrates (Albers *et al.*, 1999; Elferink *et al.*, 2001) allowing cells to efficiently scavenge these substrates from the environment.

N-terminal amino acid sequencing of isolated sugar binding proteins of *S. solfataricus* demonstrated that these proteins are equipped with class III signal peptides (Elferink *et al.*, 2001; Albers and Driessen, 2002). Such signal peptides are commonly used for the assembly of subunits of type IV pili, which are involved in surface attachment, DNA transfer and twitching motility, and the archaeal flagellum. A critical step in the assembly process of these proteins is the cleavage of the very short positively charged N-terminus by a specialized membrane bound signal peptidase at the cytosolic face of the membrane (Strom and Lory, 1992; Bardy and Jarrell, 2002). The remaining hydrophobic domain of the signal peptide is subsequently used as a scaffold for the assembly of the maturated protein into a macromolecular surface structure (Cohen-Krausz and Trachtenberg, 2002; Craig *et al.*, 2003; 2006). *S. solfataricus* expresses a type IV pilin signal peptidase, PibD, which processes both FlaB, the subunit of the flagellum, and the sugar binding proteins which contain a class III secretory signal peptide (Albers *et al.*, 2003). Analysis of the cleavage specificity of PibD showed that it is able to process all predicted substrate proteins present in the *S. solfataricus* genome (Albers

et al., 2003). Processing of the signal peptides of the substrate binding proteins by PibD further implies that the binding proteins might be assembled into a macromolecular structure in analogy with type IV pili or flagellin subunits. This structure has tentatively been termed the bindosome.

In Gram-negative bacteria specialized systems are responsible for the translocation of proteins or DNA molecules across the outer membrane. This general secretion pathway (GSP) comprises different secretion systems that have been classified into five groups (Pugsley, 1993). One of these groups includes a complex machinery of up to 30 subunits which are necessary for the correct assembly of type IV pili (Bally *et al.*, 1992; Hobbs and Mattick, 1993). Type II secretion systems contain so-called pseudopili that have been proposed to push substrate proteins across the outer membrane (Py *et al.*, 2001). Interestingly, type II secretion systems share homologous subunits with type IV pili assembly systems (Wolfgang *et al.*, 2000). One of the core components of these systems is a cytoplasmic ATPase that provides the energy for pilus formation. PilP and PilT from *Pseudomonas aeruginosa* drive pilus assembly and retraction respectively, and enhance the surface attachment and twitching motility of the cells (Whitchurch *et al.*, 1991). Typical examples of ATPases from type II secretion systems are PulE of *Klebsiella oxytoca* and EpsE of *Vibrio cholerae* which energize pullulanase and cholera toxin secretion respectively (Overbye *et al.*, 1993; Possot and Pugsley, 1994; Sandkvist *et al.*, 1995). All these secretion ATPases are cytoplasmic proteins but they appear membrane associated through the attachment to specific membrane proteins of the particular secretion complex. The structure of the N-terminal domain of EspE in association with the cytoplasmic domain of the membrane integral protein EspL has been elucidated and key amino acids needed for complex formation have been identified (Sandkvist *et al.*, 1995; Abendroth *et al.*, 2005). Strikingly, these ATPase assembles into a hexameric ring, an essential feature of their function (Robien *et al.*, 2003).
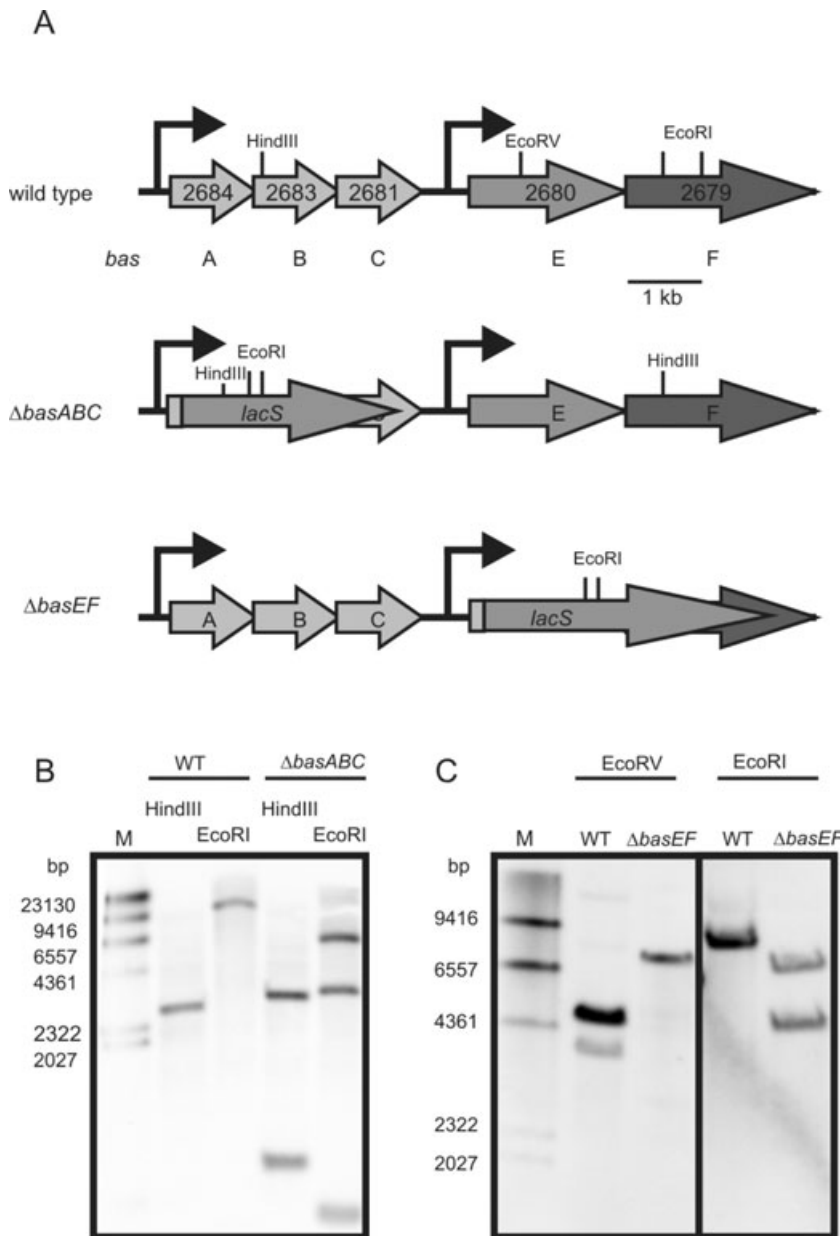
In contrast to bacteria, archaea generally, do not contain an outer membrane, but instead are surrounded by a proteinaceous layer, the S-layer. However, archaea do contain homologues of bacterial secretion ATPases but for many of these systems the function is unknown. In *Halobacterium* and *Methanococcus* it has been demonstrated that FlaI, an ATPase of the flagellum operon, is essential for flagella formation (Patenge *et al.*, 2001; Thomas and Jarrell, 2001; Thomas *et al.*, 2002). The *S. solfataricus* P2 genome contains five secretion ATPases (Albers and Driessen, 2005). One of the genes, *flaI*, is located in the flagella operon and is most likely involved in flagella assembly. Two other operons containing the ATPases SSO0120 and SSO2680 are expressed under all tested growth conditions in which the sugar binding proteins with class III signal peptides are expressed. These two operons also encode a gene for an integral membrane protein that is homologous to GspF, the inner membrane protein of type II secretion systems, and in addition, these operons contain genes encoding for small pilin-like proteins (Albers and Driessen, 2005). Comparative genomics with the genomes of two other *Sulfolobus* species, *S. acidocaldarius* and *S. tokodaii* (Kawarabayasi *et al.*, 2001; Chen *et al.*, 2005), indicates that in these species, the secretion ATPase operon which includes SSO2680 is absent. Strikingly, these species also lack most of the sugar binding proteins equipped with a class III signal peptide (Szabó *et al.*, 2007a). Therefore, the SSO2680 containing operon may be a likely candidate for the surface localization of the sugar binding proteins in *S. solfataricus*. Here we have studied the effect of the deletion of the genes encoding the (pseudo-)pilin proteins, the structural ATPase and the membrane protein of the SSO2680 operon on the growth of *S. solfataricus* on different sugars. In addition, we have analysed the uptake of glucose in the deletion strains. Our data suggest that the SSO2680 operon is required for the functional assembly of sugar binding proteins at the cell surface.

Results

Targeted disruption of the structural genes of the SSO2680 operon

The SSO2680 operon of *S. solfataricus* consists of five genes (Fig. 1A): the *basA* (SSO2684), *basB* (SSO2683) and *basC* (SSO2681) that encode small pilin-like proteins with a class III signal peptide and a processing site that matches the specificity of PibD, the type IV signal peptidase of *S. solfataricus* (Albers *et al.*, 2003); *basE* (SSO2680) that encodes a VirB11 homologue with the typical Walker A/B sites and GspE motif common to all secretion ATPases that function in type II secretion and type IV pilin assembly systems. BasE is a thermostable Mn^{2+} dependent ATPase (Albers *et al.*, 2006a); and *basF* (SSO2679) that encodes a membrane protein with 10 predicted transmembrane domains. Together with BasE, BasF probably constitutes the core of an assembly system in the cytoplasmic membrane of *S. solfataricus*. BasF contains the conserved GspF cytoplasmic loops that likely serve as docking sites for BasE. The Bas operon is the only Gsp-like operon that lack homologues in the genomes of *S. acidocaldarius* and *S. tokodaii*, two *Sulfolobus* species that also lack most class III signal peptide containing sugar binding proteins (Albers *et al.*, 2006b; Szabó *et al.*, 2007a). Therefore, the Bas operon seems the most likely candidate for the assembly of sugar binding proteins at the cell surface of *S. solfataricus*. To

A

determine its role in this assembly process, two knockout strains were constructed: a triple knockout deleting the three putative pilin genes, Δ*basABC*, and a double knock-out strain of the core components of the system, the Δ*basEF*.

LacS, a β-galactosidase, is essential for growth of *S. solfataricus* on lactose as sole carbon source. *lacS* can be used for the targeted disruption of genes in *S. solfataricus* PBL2025 strain (Schelert *et al.*, 2004) by homologous recombination. For this purpose, the flanking regions of *basABC* and *basEF* were cloned and fused to the 5′ and 3′ ends of a *lacS* cassette containing the gene with its own promoter and terminator region. Subse-

quently, *S. solfataricus* cells were transformed with the corresponding plasmids, selected for growth on lactose medium, plated and single colonies were analysed. The successful deletion of the target genes in the positive clones was confirmed by PCR (Fig. 2) and Southern blot analysis (Fig. 1B and C). To exclude the possibility that the deletion of the genes had any polar effects on the expression of downstream genes, RT-PCR was used to verify the expression of the various *bas* genes in the deletion strains (Fig. 2A and B). *basE* and *basF* were expressed in the Δ*basABC* strain, but the levels seem to be slightly decreased when compared with wild-type levels (Fig. 2A). Furthermore, RT-PCR confirmed the lack

A

B
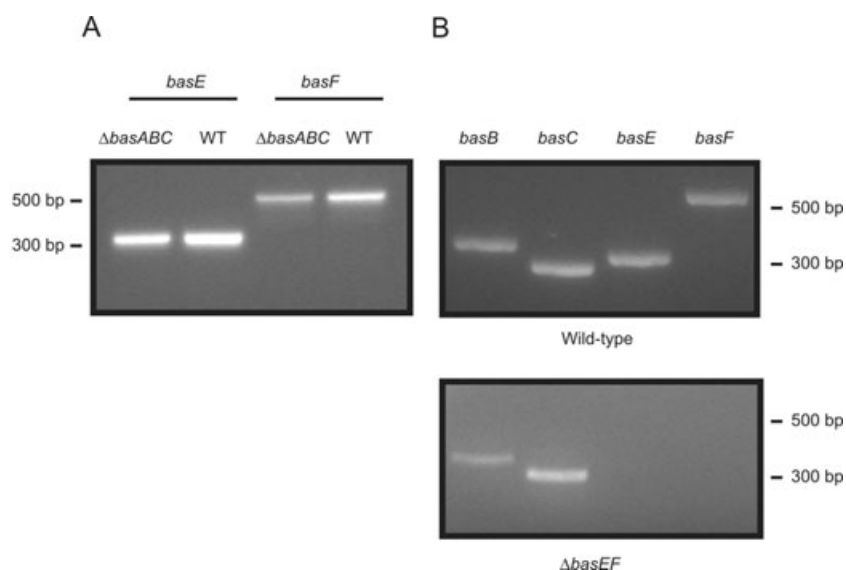
Wild-type

ΔbasEF

Fig. 2. Expression of the *bas* operon genes in the deletion strains.
A. Expression levels of *basE* and *basF* in the wild-type and Δ*basABC* strain.
B. Expression levels of *basB*, *C*, *E* and *F* in the wild-type and Δ*basEF* strain. Shown are the results of gene-specific PCRs on cDNA isolated from the strains indicated grown on tryptone media. PCRs on isolated total RNAs yielded no products with the same set of primers. Sizes of DNA marker are indicated.

of *basE* and *basF* expression in the Δ*basEF* strain, whereas normal expression levels of *basB* and *basC* were observed (Fig. 2B). These data confirm the deletion of the *basABC* and *basEF* genes in the respective strains and are consistent with the notion that these gene clusters are controlled by separate promoters (Albers and Driessen, 2005).

Characterization of ΔbasABC *and* ΔbasEF *strains*

Growth of the Δ*basABC* and Δ*basEF* strains on tryptone, glucose, arabinose and maltose supplemented Brock medium was compared with that of the wild type (Fig. 3). Both the Δ*basABC* and Δ*basEF* strains were still able to grow in tryptone medium. While the growth of the Δ*basABC* strain was only slightly influenced, Δ*basEF* cells showed a longer lag phase. However, both strains reached nearly the same final optical density (OD) levels as the wild type (Fig. 3A). To determine if the growth of the deletion strains is affected on sugars that are substrates of binding proteins synthesized with a type class III signal peptide, growth on glucose (dependent on GlcS, glucose binding protein) and arabinose (dependent on AraS, arabinose binding protein) was monitored. The Δ*basEF* strain hardly grew on glucose while no growth could be observed on arabinose (Fig. 3B and C). Also, the Δ*basABC* strain showed a severe growth defect on glucose and arabinose although the effect was less pronounced than with the Δ*basEF* cells.

Sulfolobus solfataricus exhibits at least two ABC-type uptake systems for maltose or maltose-oligomers. One system (SSO3053–3059) is involved in uptake of preferably maltose-oligomers and its binding protein, SSO3053, contains a typical class I type signal peptide (Elferink *et al.*, 2001). The second ABC transporter (SSO1168–

1171) is located upstream of the α-amylase gene and its binding protein is induced upon growth on maltose or starch (Rolfsmeier *et al.*, 1998). The binding protein, SSO1171, contains a class III signal peptide. Both deletion strains were found to grow on maltose but as compared with the wild-type strain, a significantly longer lag phase was observed when cells were transferred from a tryptone- to a maltose-containing medium (Fig. 3D). Once adjusted to maltose-containing medium, cells from the deletion strain transferred into fresh maltose medium showed a growth rate equal to that of the wild type (Fig. 3E). This suggests that at the first transfer, growth on maltose depends on SSO1171, the binding protein that contains the class III signal peptide consistent with earlier expression studies (Rolfsmeier *et al.*, 1998). However, upon prolonged exposure to maltose, expression of the alternative maltose transport system and the accompanying binding protein, SSO3053 with a class I signal peptide is induced. Taken together, these data indicate that the *bas* operon is essential for growth of *S. solfataricus* on sugars that are transported into the cell via binding proteins synthesized with a class III signal peptide.

Glucose uptake by the ΔbasABC *and* ΔbasEF *deletion strains*

To determine if the failure or reduction of growth of the Δ*basEF* and Δ*basABC* strains on arabinose and glucose is due to a reduced substrate transport activity, D-[^{14}C]-glucose uptake experiments were performed at pH 3 and 60°C using a filter-based assay (Albers *et al.*, 1999). Glucose uptake by Δ*basEF* and Δ*basABC* cells was significantly decreased compared with the wild type strain (Fig. 4). As with the growth of these cells on glucose, the effect was most pronounced for the Δ*basEF* cells.
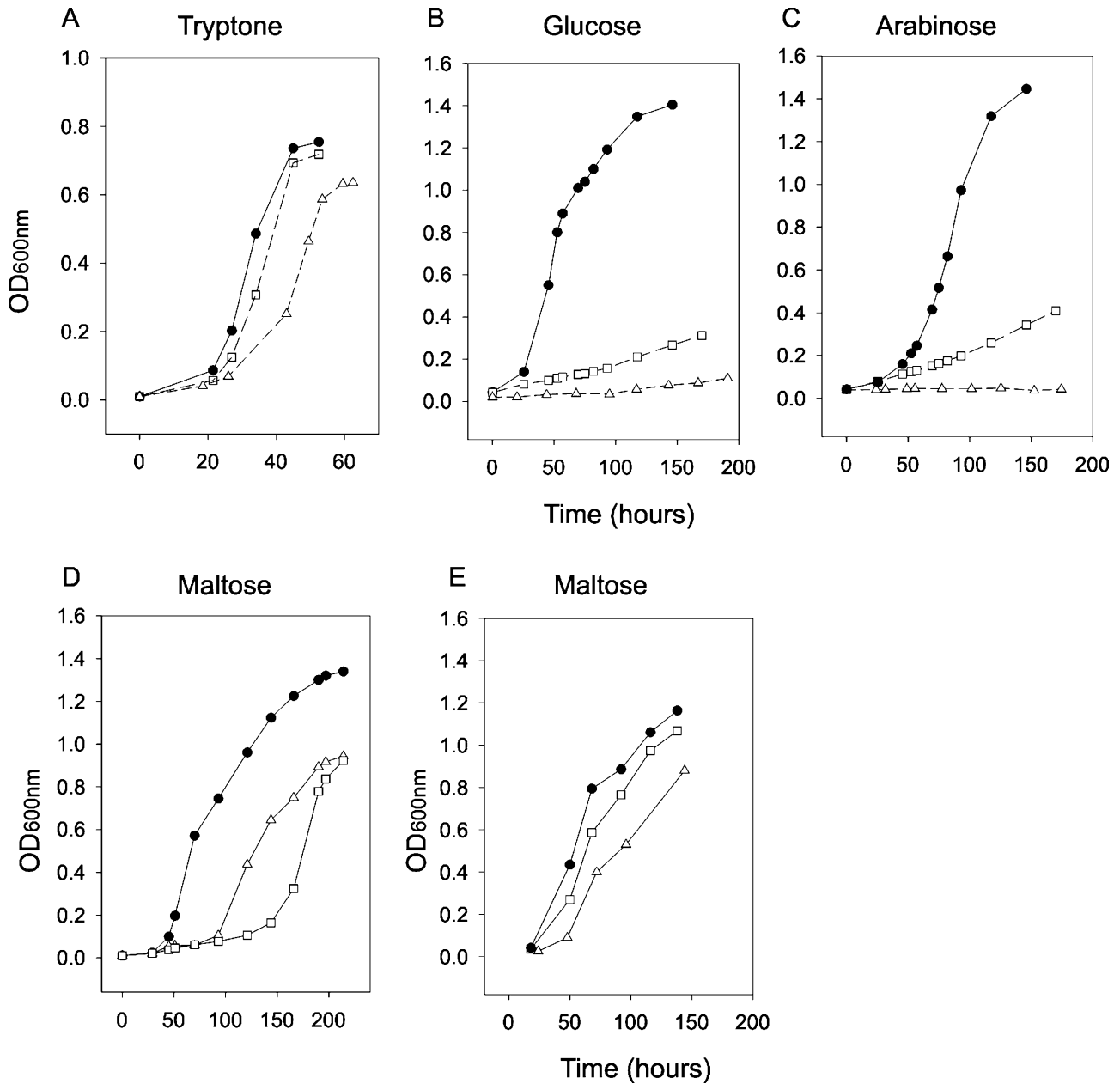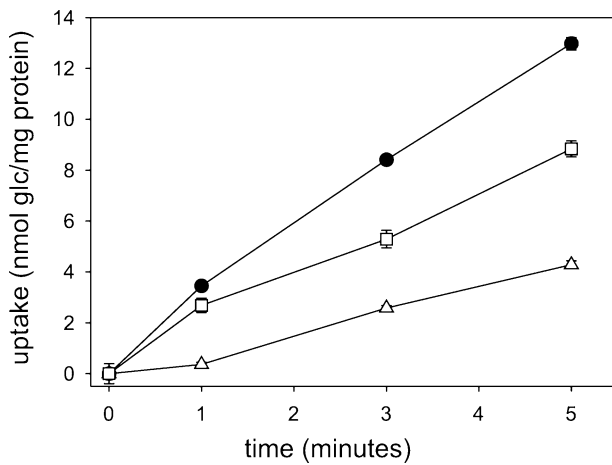
Fig. 3. Growth of the *bas* operon deletion strains on different substrates. Growth of the wild-type (filled circles), Δ*basABC* (open rectangles) and Δ*basEF* strain (open triangles) were monitored on tryptone (A), glucose (B), arabinose (C), maltose (D). Growth curves of cells pre-grown on maltose and which were inoculated freshly into maltose medium are shown in E.

To show that the reduced levels of glucose transport were not a result of decreased levels of glucose transporter components, membranes were isolated form the deletion and wild type strains grown on tryptone medium. Glucose binding experiments with isolated membranes showed the same GlcS D-[^{14}C]-glucose binding activity with all three strains (Fig. 5B). Binding proteins were isolated from detergent-solubilized membranes by lectin affinity chromatography. The amount of GlcS protein in the deletion strains was similar to that of the wild type (Fig. 5C). To detect AraS,

cells were grown in the presence of arabinose. As shown in Fig. 5C, the level of AraS in the Δ*basEF* strain was similar as observed in the wild type. However, a pronounced increase of the AraS levels was observed in the Δ*basABC* cells, suggesting that the limited growth of this deletion strain on arabinose is related to increased levels of arabinose transporter. Finally, Western blot analysis demonstrated that GlcV, the ATPase of the ABC glucose transporter, is normally expressed in Δ*basEF* cells, while slightly higher expression levels are observed in the

Fig. 4. Glucose uptake in the *bas* operon deletion strains. [^{14}C]-Glucose uptake was measured in wild-type (filled circles), Δ*basABC* (open rectangles) and Δ*basEF* (open triangles) cells.

Δ*basABC* strain (Fig. 5A). Overall, these results demonstrate that the reduced glucose uptake activity and the growth defect of the knockout strains on glucose and arabinose are not caused by change of expression levels of the different transporters.

Growth complementation of the Δbas*ABC and* Δbas*EF strains*

For complementation of the deletion strains, the various *bas* genes were cloned into a virus-based vector (Jonuscheit *et al.*, 2003; Albers *et al.*, 2006a). *basE* (pSVA15), *basF* (pSVA77), both genes (pSVA75), or *basABC* (pSW104) were cloned into pMJ05 (Table 1). Next, Δ*basEF* cells grown on tryptone medium were electroporated with the corresponding plasmids, and the expression of the plasmid encoded genes was induced by the addition of arabinose. Growth was continued on tryptone medium in the presence of a low concentration of arabinose. Expression of *basEF*, but not of *basE* or *basF* separately, resulted in faster growth of the deletion strain on 0.1% tryptone (data not shown). To determine if growth of the Δ*basEF* cells on glucose could be restored, cells were grown on glucose minimal medium in presence of 0.06% arabinose. Δ*basEF* transformed with pSVA75 (*basEF*) exhibited growth on glucose after a lag phase of 60 h (Fig. 7A), whereas no growth was observed with cells transformed with pSVA15 (*basE*) or pSVA77 (*basF*), although the proteins were expressed (data not shown). Also, the growth of the Δ*basABC* strain on glucose was restored by pSW104 which supports the expression of
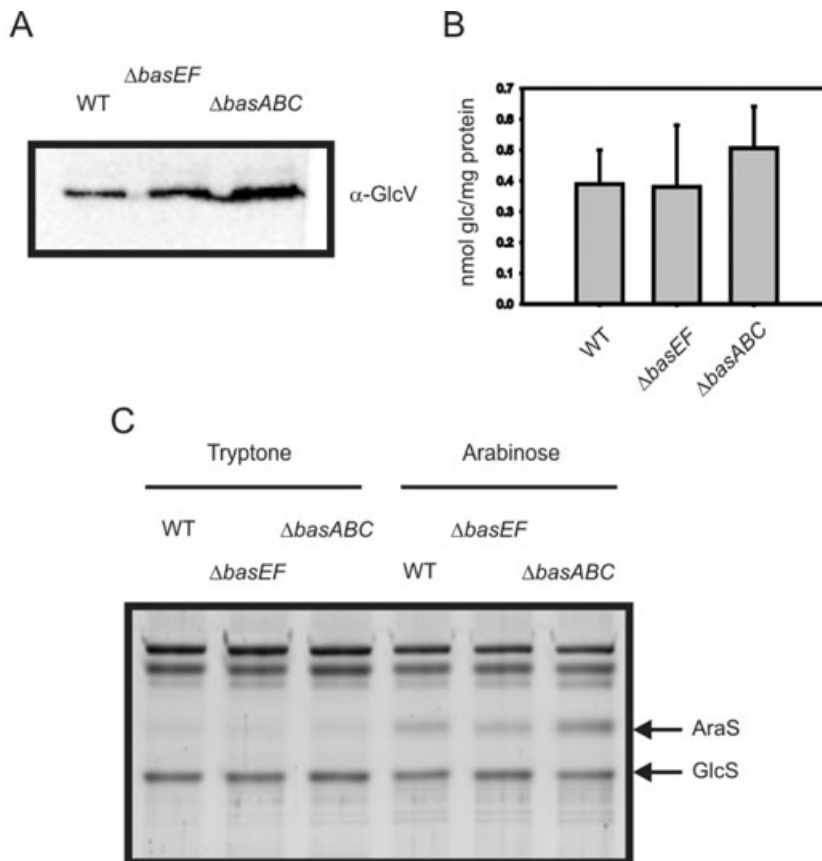
Fig. 5. Expression levels of glucose and arabinose ABC transporter subunits.
A. Membranes isolated from the wild-type and both knockout strains were separated on SDS-PAGE and the presence of GlcV, the ATPase of the glucose transporter, was detected by Western blot analysis.
B. D-[^{14}C]-glucose binding assay in membranes isolated from the wild-type and both deletion strains.
C. Protein profiles of ConA fractions isolated from Triton X-100 solubilized membrane fractions of the wild-type strain and the deletion strains grown on tryptone or tryptone/arabinose and analysed by SDS-PAGE.

Table 1. Microbial strains and plasmids.

Strain or plasmid	Genotype or sequence	Source or derivation
Strains		
PBL2025	Δ(SSO3004–3050)	PBL2000 (Worthington *et al.*, 2003)
Δ*basABC*	*basEF::lacS*	PBL2025 (Schelert *et al.*, 2004)
Δ*basEF*	*basABC::lacS*	PBL2025 (Schelert *et al.*, 2004)
Plasmids		
pET2268	*lacS*	pET401 (K. van Wely, unpublished)
pSVA26	*basEF::lacS*	pET2268 (Szabó *et al.*, 2007b)
pSVA78	*basABC::lacS*	pET2268 (Szabó *et al.*, 2007b)
pMZ1	C-terminal strep-10x histag	pSVA5 (Albers *et al.*, 2006a)
pSVA15	*basE*-10x histag	pMJ05 (Albers *et al.*, 2006a)
pSVA69	*basEF*	pMZ1 (this study)
pSVA73	*basF*	pMZ1 (this study)
pSVA75	*basEF*	pMJ05 (Albers *et al.*, 2006a)
pSVA77	*basF*	pMJ05 (Albers *et al.*, 2006a)
pSW004	*basABC*	pMZ1 (this study)
pSW104	*basABC*	pMJ05 (Albers *et al.*, 2006a)

basABC (Fig. 7B). Interestingly, complementation of growth was not dependent on the presence of the inducer arabinose (Fig. 7A), consistent with studies on the *araS* promoter that indicated that even in the absence of arabinose, low levels of expression are observed (M. Jonuscheit, pers. comm.). Apparently, these low levels of expression suffice to restore the growth of Δ*basEF* cells. Growth of the Δ*basABC* and Δ*basEF* cells on 0.4% arabinose minimal medium could partially be restored by the respective genes, but not as efficient as for growth on glucose (data not shown). This is most likely due to the massive induction of the production of recombinant proteins due to the high arabinose concentration, which negatively affects growth. For overexpression of proteins in *S. solfataricus* normally 0.4% of arabinose is used (Albers *et al.*, 2006a), but in case of the expression of *basEF* a concentration higher than 0.06% already slowed down growth even of wild type cells (data not shown). Also growth on maltose could be restored. Expression of *basABC* and *basEF* in the Δ*basABC* and Δ*basEF* cells resulted in a pronounced reduction of the lag phase while similar OD levels were reached in the stationary phase of growth as compared with the wild type (Fig. 7C).

The Δ*basEF* strain showed a reduced rate of glucose uptake (Fig. 4). The reintroduction of the *basEF* genes (Fig. 6), but not of *basE* or *basF* alone (data not shown), into these cells resulted in a restoration of the glucose transport activity (Fig. 6). The level of glucose uptake in the Δ*basEF* strain containing pSVA75 increased with the amount of arabinose present in the medium used for induction. Arabinose at 0.02% and 0.04% restored the glucose uptake activity to 25% and 60% of wild-type levels respectively. Taken together these data demonstrate that the *bas* operon is required for the functional assembly of sugar binding proteins at the cell surface of *S. solfataricus*.

Discussion

A recent *in silico* based method used to predict pilin-like proteins in archaeal genomes has led to the identification of a great number of possible subunits of cell surface appendages of unknown structure and organization (Szabó *et al.*, 2007a). This group of proteins also includes the flagellins, the subunits of the archaeal flagellum. Flagella are not assembled by a system that is homologous to the type III systems used in bacteria, but by systems that are more similar to bacterial type IV pilin assembly systems (Ng *et al.*, 2006). *S. solfataricus* contains a high number of substrate binding proteins with class III signal peptides. Purified GlcS, AraS and TreS from *S. solfataricus* membranes have been shown by
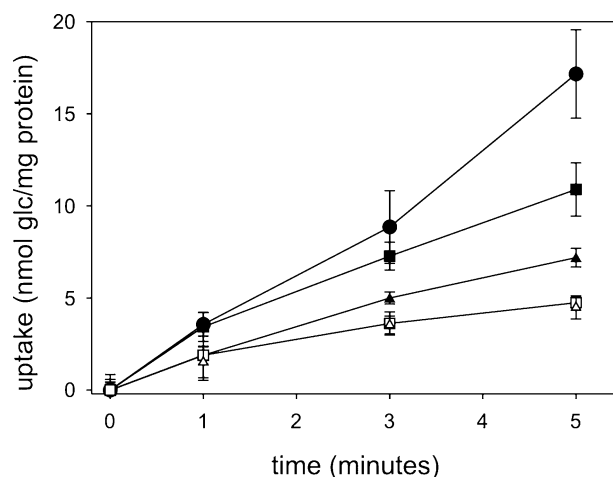
Fig. 6. Glucose uptake in Δ*basEF* strain after complementation with plasmid pSVA75. Glucose uptake of Δ*basEF* cells (open rectangles) were compared with Δ*basEF* cells transformed with the plasmid without the addition of arabinose (open triangles), with 0.02% arabinose (filled triangles), 0.04% of arabinose (filled rectangles) and wild-type strain cells (filled circles).
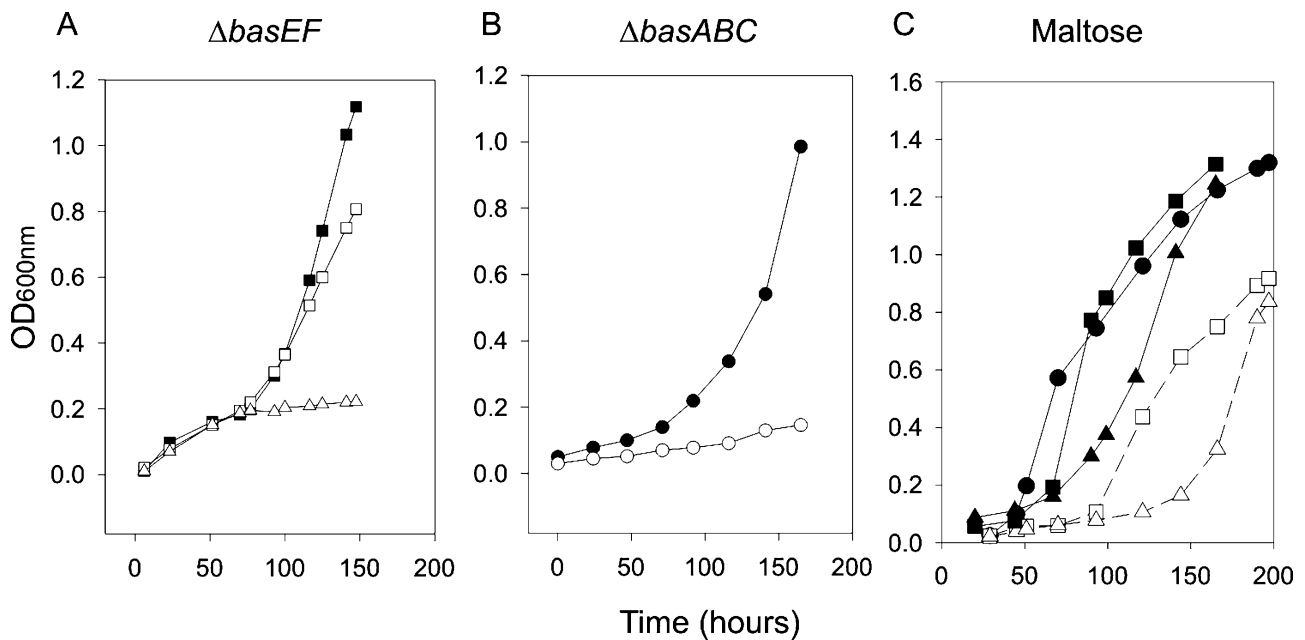
Fig. 7. Growth complementation of the *bas* operon deletion strains on sugar minimal media.
A. Growth of Δ*basEF* cells (open triangles), Δ*basEF* cells complemented with pSVA75 without the addition of arabinose (open rectangles) and Δ*basEF* cells complemented with pSVA75 with the addition of arabinose (filled rectangles) on glucose minimal medium.
B. Growth of Δ*basABC* cells on glucose either transformed (filled circles) or not transformed (open circles) with plasmid pSW104.
C. Growth of wild type (open circles), Δ*basABC* (open triangles) and Δ*basEF* (open rectangles) cells on maltose. Δ*basABC* (filled triangles) cells and Δ*basEF* (filled rectangles) cells were transformed with pSW104 or pSVA75 respectively.

N-terminal sequencing to exhibit class II signal peptides (Albers *et al.*, 1999; Elferink *et al.*, 2001). The precursors of these sugar binding proteins and the flagellin of *S. solfataricus* are substrates for PibD, the membrane-bound type IV signal peptidase (Albers *et al.*, 2003). Moreover, mutational analysis of the signal peptide of GlcS demonstrated that all predicted class III signal peptides containing proteins in *S. solfataricus* are substrates for PibD (Albers *et al.*, 2003). Because of the analogy with other class III signal peptide containing protein substrates, we have previously suggested that the sugar binding proteins are assembled in a macromolecular cell surface-associated structure, which was termed the bindosome. Although attempts were underway to identify the nature of the bindosome by electron microscopy or blue native PAGE of isolated binding proteins from the membrane of *S. solfataricus* the exact structure of the bindosome is still unknown. It may resemble a pilus-like structure or represent some other degree of organization possibly associated with the S-layer (Fig. 8). Here we have now shown that the *bas* operon is involved and essential for the correct localization of the binding proteins to the cell surface of *S. solfataricus*.

The *bas* operon of *S. solfataricus* is composed of five genes that are homologues to proteins of bacterial type II secretion and type IV pilin assembly systems. Deletion of the core of this system, *basEF*, that encode an
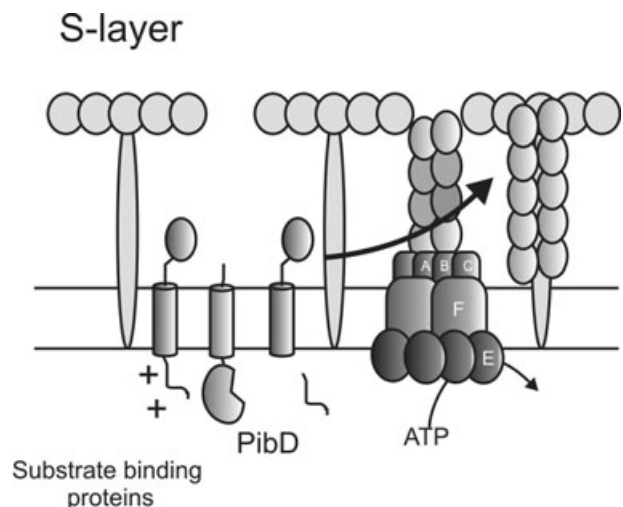
Fig. 8. Model of bindosome assembly. The binding protein precursors are after translocation and insertion into the membrane, processed by PibD and then assembled into the bindosome by the Bas system. At the expense of ATP by BasE, binding proteins are assembled at the BasF platform and integrated into the bindosome with the help of the pseudopili genes. The bindosome either forms a pseudopilus-like structure or binding proteins are associated with the S-layer in a specific manner. The exact structure of these complexes is unknown.

ATPase and an integral membrane protein respectively, severely interferes with growth of *S. solfataricus* on sugars such as glucose that are transported into the cell via binding protein-dependent ABC transporters (Fig. 3). Although in the Δ*basEF* strain, the glucose ABC transporter is normally expressed and the glucose binding protein, GlcS, is present at the membrane and active in glucose binding (Fig. 5), uptake of glucose was dramatically impaired (Fig. 4). Both the growth defect and the transport deficiency could be complemented by re-introduction of both *basE* and *basF* into the Δ*basEF* cells. No complementation was observed when the cells were transformed with only the single genes. These data strongly indicate that the Bas system is involved in the functional assembly of the bindosome. BasE is an ATPase (Albers and Driessen, 2005) and ATP is likely needed to provide the energy for the assembly of the binding proteins. With type II secretion and type IV pili systems, inactivation of the cytoplasmic ATPase(s) generally results in an abolishment of the secretion of substrate proteins or the assembly of the pilus (Wu *et al.*, 1997; Anantha *et al.*, 1998; Wolfgang *et al.*, 1998). In the *V. cholerae* toxin secretion system, the ATPase EpsE forms a complex with the membrane protein EpsL (Abendroth *et al.*, 2005). In a similar manner, BasE may interact with the membrane protein BasF. Indeed, BasE seems to strongly interact with BasF as it can be co-purified with BasE from detergent-solubilized *S. solfataricus* membranes (B. Zolghadr, unpubl. data).

Compared with the Δ*basEF* deletion strain, the deletion of the *basABC* genes led to an intermediate, although pronounced phenotype showing growth inhibition on sugar minimal media (Fig. 3) and a reduction of glucose uptake (Fig. 4). *basABC* are small proteins that are synthesized as precursors with a predicted class III signal peptide. They appear to resemble pilus subunits, and possibly form a (pseudo) pilus. Because of the intermediate phenotype, they appear not essential for bindosome assembly but rather fulfil an accessory role. The pseudopilus of type II secretion systems is thought to form a piston which pushes substrate proteins from the periplasm through an outer membrane pore, the secretin. Therefore, the deletion of the major pseudopilins results in a loss of secretion whereas removal of the minor pseudopilins yields severely reduced secretion (Possot *et al.*, 2000; Durand *et al.*, 2005). In the Δ*basABC* strain, the effect seems to be much less severe than observed for bacterial type II secretion systems. This may point at a difference in function: in the bacterial type II secretion systems the pseudopilus is necessary to eject the substrate through the outer membrane, whereas in *S. solfataricus* we envision the pseudopili to be involved in the formation or positioning of the bindosome structure. Possibly, the binding proteins have a high tendency for self-assembly once the N-terminal positive charges have been removed by PibD. Pseudopili may only be needed to guide and regulate this process. Interesting, Δ*basABC* cells exhibit a low level of growth on arabinose, and these cells show an elevated level of AraS (Fig. 5C). Moreover, membranes of these cells showed an increased level of glucose binding activity and of GlcV protein, the nucleotide binding protein of the glucose ABC transporter (Fig. 5A and B). Possibly, this compensates for the reduced incorporation of binding proteins into the bindosome. This is, however, not observed for GlcS. Unlike AraS, the expression of GlcS is not regulated (Elferink *et al.*, 2001; Lubelska *et al.*, 2006). We also noted some smaller growth defect of the Δ*basABC* cells on tryptone medium. It should, however, be emphasized that in *S. solfataricus* several other binding proteins, with unknown function, are equipped with a class III signal peptide (Szabó *et al.*, 2007a).

In contrast to *S. tokodaii* and *S. acidocaldarius* which only possess a few of the binding proteins with a predicted class III signal peptide, *S. solfataricus* can grow on a wide variety of sugars. What could be the advantage of a bindosome structure? The bindosome may allow a more efficient retrieval of sugars from the environment and enrich sugars from the medium between the S-layer and the cytoplasmic membrane. The habitat of *Sulfolobus* species is relatively poor in carbon sources and therefore it is crucial for these organisms to capture substrates as efficient as possible from the surrounding medium. The periplasm of Gram-negative bacteria is mostly gel-like with slow diffusion of binding proteins (Brass *et al.*, 1986), although this view has recently been challenged in a study in which the diffusion of an exogenous reporter protein GFP was reported (Mullineaux *et al.*, 2006). Binding proteins are highly abundant in the periplasm and it was suggested that the more rapid diffusion of substrates than predicted for a gel-like environment can occur because of a network of binding proteins. Little is known about the composition and organization of the archaeal 'periplasm', but one may envisage a more ordered structure of binding proteins in the bindosome will provide a kinetic advantage for sugar acquisition and subsequent channelling to the ABC transporters. This putative archaeal surface structure assembly system contains much fewer components than the bacterial counterparts which contain additional components for outer membrane translocation or other membrane subunits. Therefore, future biochemical studies on the Bas system will likely provide novel insights in the mechanisms of assembly by this group of conserved prokaryotic systems.

Experimental procedures

Materials

D-Arabinose was purchased from Sigma (St Louis, USA).

Strains

Sulfolobus solfataricus PBL2025 (Schelert *et al.*, 2004) was grown aerobically at 80°C in the medium described by Brock (Brock *et al.*, 1972), adjusted to pH 3 with sulphuric acid and supplemented with 0.1(w/v)% of tryptone and/or different sugars at 0.4% (w/v) as sole carbon and energy source. For growth on minimal medium, *Sulfolobus* cells were first grown in medium containing 0.1% tryptone and 0.4% of the sugar of interest, and subsequently transferred to minimal medium supplemented with sugar of interests as sole carbon and energy source. Growth of cells was monitored by measuring the OD at 600 nm. For the propagation of plasmids *E. coli* strain DH5α was used. For the virus containing plasmids ElectroMAX™ *E. coli* Stbl4™ cells (Invitrogen, Germany) were used.

Cloning and expression

Plasmid pET2268, which contains the *lacS* cassette for selection, was used as base for the deletion mutant constructs (Szabó *et al.*, 2007b). The 1006 bp upstream region of *basE* was amplified using primers (5′-CCCGGTACCCCATATATCTAGGTCTATTATTAGC-3′ and 5′-CCCCCATGGTAATAAGAGGGCTTGCCGAAAAGGGAG-3′) containing a KpnI and NcoI restriction site respectively. The 1192 bp downstream region of *basF* was amplified using primers (5′-CCCGGATCCCTAGAAATAAGAAATCGTATGAGATTATAC-3′ and 5′-CCCCCCCCCGCGGCCGCCAGCTACACCACTACAAGATTTC-3′) containing a BamHI and NotI restriction site respectively. After digestion of the PCR products and pET2268 with the mentioned restriction enzymes the upstream and downstream flanking regions were ligated into pET2268 yielding the *basEF* knockout plasmid pSVA26. The same procedure was used for the construction of the *basABC* deletion plasmid pSVA78. The 733 bp upstream region was amplified using primers (5′-GGGGGGTACCAGATAGGCTATCTACTTCG-3′ and 5′-GGGGGCCATGGCCTCAGAAATACCTCTC-3′) containing a KpnI and NcoI restriction site respectively. The 756 bp downstream region of *basC* was amplified using primers (5′-CCCCGGATCCGTTATCGACCAATAAG-3′ and 5′-CCCCCCCCGCGGCCGCGTTCCCCTACTTATTTC-3′) containing a BamHI and NotI restriction site respectively.

Plasmid pMZ1 is a variant of the pSVA5 virus expression entry vector, with an excisable C-terminal tandem Strep-II/10xHIS epitope tag and additional restriction sites for flexible cloning. It was constructed by inserting an artificial fragment into the NcoI and ApaI sites of pSVA5. An artificial DNA fragment was generated by PCR with overlapping oligonucleotides OZ104 (5′-GGGGGGGAATTCCCATGGTACCTGCAGGATCCGGATGGAGTCATCCACAATTTG-3′) and OZ105 (5′-GGGGGGGAATTCGGGCCCTCAATGATGGTGATGATGGTGATGATGGTGATGCTTCTCAAATTGTGGATGACTCCATCCG-3′). The PCR reaction was performed without additional template. The product was digested with NcoI and ApaI and ligated into pSVA5 cut with the same enzymes. Successful insertion of the fragment was confirmed by DNA sequencing.

For the construction of expression plasmids, the *basF* gene was amplified from *S. solfataricus* P2 genomic DNA with primers (5′-CCCCCTCATGATAAGAGTGACTGAGAG-3′ and 5′-CCCCCGGATCCTATCGAGAAGAATTTCAAGATTATCG

G-3′) containing a BspHI and a BamHI restriction site respectively. Primers used for the amplification of the *basEF* product were 5′-CCCCCTCATGATACTTATTGGTAAAAAGAAATCACAC-3′ (BspHI) and 5′-CCCCCGGATCCTATCGAGAAGAATTTCAAGATTATCGG-3′ (BamHI). The PCR products were digested with the respective restriction enzymes, and ligated into pMZ1 prepared with the same enzymes resulting in plasmids pSVA73 and pSVA69 respectively. Both constructs were then cut with BlnI and EagI, and subsequently ligated into pMJ05 treated with the same enzymes yielding the virus vector based expression plasmids pSVA77 and pSVA75 respectively.

Gene inactivation procedure

For gene inactivation, *S. solfataricus* strain PBL2025 (Schelert *et al.*, 2004) was used. Essentially, cells were electroporated with either pSVA26 or pSVA78 and inoculated in lactose minimal medium for selection. Growth occurred after 12–14 days and cells were then plated on lactose plates. After 7 days of growth cells were sprayed with Xgal and blue colonies were inoculated into tryptone medium. Genomic DNA was isolated from these cells and tested for successful deletion of the target genes as described (Schelert *et al.*, 2004).

cDNA synthesis

Total RNA was extracted from 50 ml of the *S. solfataricus* culture grown on 0.1% tryptone (w/v) and harvested at early growth phase (OD$_{600}$ 0.2–0.3). The RNA isolated was followed as previously described (Brinkman *et al.*, 2002). The cDNA was prepared as followed: 5 μg of Random hexamers (Qiagen) was added to 20 μg of RNA and the mixture was incubated for 10 min at 72°C and cooled down on ice rapidly. To the mixture was 5 μM of dNTP mixture (dATP, dGTP, dCTP and dUTP from sigma), 10 mM DTT, 5× RT buffer and 400 U superscript II reverse transcriptase from Invitrogen added and total reaction volume was adjusted to 20 μl with RNase-free water. The cDNA synthesis was carried out at 42°C for 2 h.

Southern blotting

Genomic DNA (8 μg) was digested with the appropriate enzymes and separated on 0.8% agarose gel. The gel was equilibrated in 20× SSC and the DNA was transferred overnight to a positively charged nylon membrane (BIO-RAD, the Netherlands). DNA hybridization was performed in standard hybridization buffer. PCR products of both *lacS* and *basE* for the *basEF* deletion mutant or *lacS* and *basC* for the *basABC* deletion mutant were digoxigenin-labelled with the HighPrime Kit (Roche, the Netherlands). Detection was performed as recommended by the manufacturer using a LumiImager (Roche, the Netherlands).

Sulfolobus solfataricus *membrane isolation*

Cells were harvested at OD$_{600}$ ~1 and resuspended in 20 mM MES buffer pH 6.5, containing 100 mM NaCl, 1 mM PMSF and a small amount of DNase I. Cells were broken by French pressure treatment at 25 kPsi (Constant Cell Disruption System, Daventry, UK). Unbroken cells were removed by

centrifugation at 10 000 r.p.m. and 4°C for 15 min, and cyto-plasmic membranes were recovered from the supernatant by ultracentrifugation at 100 000 *g* and 4°C for 1.5 h. Membrane pellets were resuspended in 20 mM MES buffer pH 6.5, and 100 mM NaCl, frozen in liquid nitrogen and stored at −80°C.

Purification of binding proteins by ConA chromatography

Isolated membranes from *S. solfataricus* PBL2025, Δ*basABC* and Δ*basEF* cells were incubated in 10 ml ConA buffer (20 mM MES, pH 7.5, 100 mM NaCl and 1% Triton X-100) for 1 h at room temperature. Solubilized membrane proteins were passed onto a 1.5 ml ConA sepharose column equilibrated with 10 ml ConA buffer. The flow through was reloaded two times and stored on ice for further analysis by 10% SDS-PAGE gel. The column was washed with 30 ml ConA buffer, and bound membrane proteins were eluted with ConA buffer supplemented with 0.05% Triton X-100 and 150 mM methyl-α-D-mannopyranoside. Eluted fractions were stored at 4°C and analysed by 10% SDS-PAGE gel.

Glucose binding and uptake assay

The binding of D-[^{14}C]-glucose (250 Ci mmol^{-1}) to the con-cavaline A enriched sugar binding protein fraction of *S. solfataricus* was measured by the addition of 10 μl of the fraction to 90 μl Brock medium with 3.4 μM D-[^{14}C]-glucose. The mixture was incubated at 60°C for 2 min and the reaction was stopped by addition of 2 ml of ice-cold 0.1 mM ammo-nium sulphate and filtration through a 0.45 μM pore size nitrocellulose filter. The filter was dissolved in scintillation fluid and radioactivity was determined with scintillation counter (FLO-scint A, Packard Instruments, Groningen, the Netherlands). For the glucose uptake assay, 10 μl of the cell suspension was added to 90 μl of Brock medium containing 34 μM D-[^{14}C]-glucose. The mixture was incubated at 60°C, and glucose uptake was stopped at regular time intervals by the addition of 2 ml of ice-cold 0.1 mM LiCl solution. The material was filtered on 0.45 μM pore size nitrocellulose filters. Filters were dissolved with scintillation fluid and the radioactivity was measured by scintillation counting (FLO-scint A, Packard Instruments, Groningen, the Netherlands).

Acknowledgements

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