Analysis of ATPases of putative secretion operons in the thermoacidophilic archaeon *Sulfolobus solfataricus*

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INTRODUCTION

Gram-negative bacteria use a wide variety of complex mechanisms to secrete proteins across their membranes or to assemble secreted proteins into surface structures. As most archaea only possess a cytoplasmic membrane surrounded by a membrane-anchored S-layer, the organization of such complexes might be significantly different from that in Gram-negative bacteria. Five proteins of *Sulfolobus solfataricus*, SSO0120, SSO0572, SSO2316, SSO2387 and SSO2680, which are homologous to secretion ATPases of bacterial type II, type IV secretion systems and the type IV pilis assembly machinery, were identified. The operon structures of these putative secretion systems encoding gene clusters and the expression patterns of the ATPases under different growth conditions were determined, and it was established that all five putative ATPases do show a divalent cation-dependent ATPase activity at high temperature. These results show that the archaeal secretion systems are related to the bacterial secretion systems and might be powered in a similar way.

Around 4.5% of the genome of the thermoacidophilic archaeon *S. solfataricus* encodes proteins predicted to be secreted to the external medium (Albers & Driessen, 2002). These proteins contain putative secretory signal peptides, double arginine-containing signal peptides that bear the signature for the twin arginine export pathway, lipoprotein signal peptides, and type IV pilin-like signals. This latter group is exceptionally large in *S. solfataricus* and consists of 16 proteins (Albers et al., 2003). In addition to the flagellin subunit, this group includes precursors of both characterized and predicted substrate-binding proteins that function as extracellular components of ABC transporters (Albers et al., 2003; Albers & Driessen, 2002). GlcS, the glucose-binding protein, AraS, the arabinose-binding protein, and TreS, the trehalose-binding protein, have been isolated from *S. solfataricus* membranes and N-terminal sequenced. This has confirmed that they are processed in the same manner as the flagellin (Albers et al., 1999; Elferink et al., 2001). Moreover, by means of an *in vitro* assay it has been shown that PilB, the type IV pilin peptidase of *S. solfataricus*, processes both the flagellin and the glucose-binding protein precursors (Albers et al., 2003).

Sequences of primers used to identify specific transcripts are listed in Supplementary Table S1 with the online version of this paper at http://mic.sgmjournals.org.
Proteins containing type IV pilin signal peptides can be assembled into multimeric structures, such as the archaeal flagellum or type IV pili. In both cases, the positive short N-terminal part of the signal peptide is cleaved from the precursor, and this is believed to prevent transmembrane translocation of the protein before processing. The mature proteins are subsequently assembled by dedicated secretion systems into the flagellum or pilus structure (Bardy et al., 2003; Mattick, 2002). The structures of both multimeric complexes have been solved (Cohen-Krausz & Trachtenberg, 2002; Craig et al., 2003; Samatey et al., 2001). This shows that the N-terminal hydrophobic domain of both proteins is used as a scaffold for the assembly of the subunits. Considering the role of the signal peptide in achieving the oligomeric state of these proteins, it is an intriguing question why a certain group of substrate-binding proteins, which are part of an ABC transporter, are equipped with a type-IV-pilin signal peptide. We hypothesize that these proteins assemble into a multimeric complex using the hydrophobic domain at the N-terminus as a scaffold, so that the sugar-binding domain is facing outwards. We tentatively call such a complex the 'bindosome'. Preliminary data indeed suggest that the binding proteins are contained in a large complex, but little is known about its exact size and structure (S. V. Albers, unpublished results). By analogy with the bacterial systems, we expect that the subunits of such a putative bindosome are assembled by a dedicated secretion system with homology to the bacterial type II, type IV, or type IV pilin secretion systems. One common component of the Gram-negative bacterial systems is a cytoplasmic ATPase. The latter strain carries the pACYC-RIL plasmid, which encodes additional tRNAs for rare codons (Stratagene).

To identify the secretion system(s) involved in the assembly of type IV pilin-like proteins in *S. solfataricus*, we screened the genome and identified five putative candidate genes encoding secretion ATPases. We determined which genes are co-transcribed with the secretion ATPases to form a functional secretion system. The putative secretion ATPases were expressed in *Escherichia coli*, purified and shown to exhibit ATPase activity. The possible function of these systems is discussed.

**METHODS**

**Strains and growth conditions.** *Sulfolobus solfataricus* P2 (DSM 1617, obtained from DSMZ, was grown aerobically at 80 °C in the medium described by Brock et al. (1972) supplemented with 0.4% tryptone, 0.4% glucose, 0.4% arabinose or 0.4% maltose. Rich medium contained 0.2% tryptone, 0.1% yeast extract and 0.2% sucrose. The medium was adjusted to pH 3 with sulfuric acid. *E. coli* strain DH5α was used for all cloning steps. *E. coli* strain C43 (DE3) (Miroux & Walker, 1996) was used for the overproduction of protein. The latter strain carries the pACYC-RIL plasmid, which encodes additional tRNAs for rare codons (Stratagene).

**RNA isolation and analysis.** Total RNA was isolated from *S. solfataricus* cells by using TRIZOL reagent (Invitrogen). Cell pellets were resuspended in TEN buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). Lysis was achieved by incubation for 5 min at room temperature in equal amounts of TEN buffer supplemented with 1-6% sodium N-lauroylsarcosine and 0-12% Triton X-100 (v/v). Subsequent steps were performed according to the protocol of the manufacturer. Isolated total RNA was treated for 1 h with RNase-free DNase at 37 °C. Isolated total RNA (1 μg) was added to one RT-PCR Ready-to-Go bead (Amersham). Sequences of primers used to identify specific transcripts are listed in Supplementary Table S1. The genes were amplified with primers containing the appropriate restriction sites to ligate the PCR products into the expression vector pBADHisA (Stratagene).

**Cloning and plasmid construction.** Chromosomal DNA was isolated as described previously (Albers et al., 1999). Vectors and plasmids used in this study are summarized in Table 1. Oligonucleotide primers for the genes *sso0120, sso0572, sso2316* (flat), *sso2387* and *sso2680* were designed based on the genome sequence of *S. solfataricus* P2 (http://www-archbac.u-psud.fr/projects/sulfolobus/) and listed in Supplementary Table S1. The genes were amplified with primers containing the appropriate restriction sites to ligate the PCR products into the expression vector pBADHisA (Stratagene). The C-terminal 6 × His epitope tags of the reverse primers were introduced in-frame to the gene products. All gene products were sequenced.

**Expression of recombinant proteins in *E. coli***. *E. coli* C43 (DE3) cells containing the RIL plasmid (Stratagene) and pET2270, pET2271, pET2272, pET2273 or pET2274 were grown in dYT medium (per litre: 16 g tryptone, 10 g yeast extract and 5 g NaCl), supplemented with ampicillin (50 μg ml⁻¹) and chloramphenicol (30 μg ml⁻¹), at 37 °C for 3 h and then shifted to 25 °C to decrease inclusion body formation. At OD₆₀₀~0-8, protein expression was induced by the addition of 0.01% arabinose. After 6 h incubation, the cells were collected, resuspended in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, and frozen in liquid nitrogen.
**Table 1.** Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>pBADhisA</td>
<td>Arabinos inducible expression vector</td>
<td>Invitrogen</td>
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<tr>
<td>pET 2270</td>
<td>pBADAHis carrying SSO2680his as NcoI–XhoI fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pET 2271</td>
<td>pBADAHis carrying SSO0572his as NcoI–KpnI fragment</td>
<td>This study</td>
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<tr>
<td>pET 2272</td>
<td>pBADAHis carrying SSO1200his as XhoI–EcoRI fragment</td>
<td>This study</td>
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<tr>
<td>pET 2273</td>
<td>pBADAHis carrying SSO2387his as NcoI–EcoRI fragment</td>
<td>This study</td>
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<tr>
<td>pET 2274</td>
<td>pBADAHis carrying SSO2316his (FlaIhis) as NcoI–XhoI fragment</td>
<td>This study</td>
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**Purification of recombinant proteins.** Cells were rapidly thawed and 1 mM PMSF and DNase were added. Subsequently cells were broken in a cell disruptor at 30000 p.s.i. (207000 kPa). After a low spin for 15 min at 8000 g to remove unbroken cell material and inclusion bodies, a high spin for 1 h at 100000 g was performed to pellet the membranes. In the case of FlaI, the supernatant was adjusted to 100 mM NaCl and then subjected to heat incubation for 20 min at 70°C. The sample was cooled on ice and precipitated proteins were spun down. The supernatant was applied to a nickel-loaded chelating column (Pharmacia). An imidazole gradient from 5 to 250 mM was applied, and the protein was found to be eluted at around 230 mM imidazole. Fractions containing FlaI were pooled and dialysed overnight at 4°C against 20 mM Tris/HCl, pH 7.5, 100 mM NaCl and 10% (v/v) glycerol. The crude membranes containing the other four recombinant proteins were subjected to a sucrose gradient to isolate inner-membrane vesicles. The inner-membrane vesicles were solubilized at a protein concentration of 5–50 mg ml⁻¹ using 1% (w/v) N-dodecyl-β-maltosides (DDM), for 30 min at room temperature. After spinning down the aggregates and non-solubilized proteins, the supernatant was applied to a nickel-loaded chelating column. After extensive washing, the proteins were eluted with an imidazole gradient from 5 to 250 mM imidazole. Fractions containing the recombinant proteins were pooled and dialysed overnight at 4°C against 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10% (v/v) glycerol, 0–5% (w/v) DDM. SSO0572 was dialysed against the same buffer containing 20 mM Tris/HCl, pH 9, to prevent precipitation.

**Fractionation of Sulfolobus cells.** *S. solfataricus* cells grown on 400 ml minimal medium containing only the desired carbon source were collected by centrifugation, and resuspended in 3–5 ml of 10 mM Tris/HCl, pH 7.5, 1 mM EDTA. PMSF (1 mM) was added and cells were lysed by sonication (8 times, 15 s on at 80% amplitude, 45 s off) using a Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments) equipped with a microprobe. The samples were centrifuged for 30 min at 100000 g at 4°C, and the supernatant and pellet were taken to be the cytoplasmic and membrane fractions, respectively. Membranes were resuspended in 300 μl of 10 mM Tris/HCl, pH 7.5.

**ATPase activity measurements.** Purified proteins (3 μg) were pre-heated at 60°C for 2 min in 100 μl assay buffer (25 mM MES, pH 6.5, 150 mM NaCl and 1 mM MgCl₂). Reactions were initiated by adding 1 mM ATP, GTP, CTP or UTP and stopped after 15 min by freezing in liquid nitrogen. For measurements in the presence of other divalent cations, MgCl₂ was replaced by either MnCl₂ or CaCl₂. The amount of released inorganic phosphate was determined using a colorimetric method (Lanzetta et al., 1979). The data were corrected for non-enzymic ATP hydrolysis. For the autophosphorylation assay, the proteins were incubated in assay buffer containing Mn²⁺ with 50 μM ATP [300 mCi (4-81 GBq) of [γ-32P]ATP ml⁻¹] for 45 min at 50°C. The proteins were run on a 12% SDS-PAGE and analysed by phosphor imaging.

**Other methods and materials.** DDM was from Anatrace. All other chemicals were purchased from Sigma. Polyclonal antisera were raised in chickens against purified secretion ATPases by Agrisera. Protein concentration determinations were carried out using the DC Kit (Bio-Rad).

**RESULTS**

**Identification, alignments and description of proteins**

A BLAST search (Altschul et al., 1990) of the *Sulfolobus* genome sequence was performed with bacterial representatives of type II (PulE, GspE), type IV (VirB11, HP0525) and type IV pilin (PiIT) secretion ATPases. This search identified five genes, sso0120, sso0572, sso2316, sso2387 and sso2680, encoding ORFs that may be involved in the secretion of protein substrates in *S. solfataricus*. All proteins contain part of the GspE family signature, but they fall into two distinct groups: the first group contains SSO0572; in the other group, all proteins are homologues of the type IV secretion ATPases, such as VirB11 and the Flp-pilus-assembling ATPase CpaF. The latter are members of COG0630 (Tatusov et al., 2000), which contains ATPases predicted to be involved in secretion processes. SSO0572 is different, and belongs to the COG1855 family, which has been defined as containing PiIT family-like ATPases. This division has also been found by Figurski and coworkers (Planet et al., 2001), who performed a phylogenetic analysis of secretion NTPases. These authors propose that secretion NTPases can be divided into the ‘Type II’ and the ‘Type IV’ family. The ‘Type II’ family also contains members that are involved in the assembly of type IV pilin components and differ from type II secretion systems, although their ATPases exhibit 30–50% homology to members of the GspE subfamily (Robien et al., 2003). Since the *S. solfataricus* genomic sequence was only partially available at the time of that study, the analysis by Figurski only includes SSO0572 and SSO0120. SSO0572 was included in the ‘Type II’ family of secretion NTPases and assigned as an uncharacterized archaeal protein distinct from bacterial PiIT, PiIB or GspE homologues. SSO0120 clustered in the ‘Type IV’ family in the subfamily of TadA homologues. TadA proteins have been shown to be essential for fibril formation and non-specific tight adherence to surfaces in *Actinobacillus actinomycetemcomitans* (Kachlany et al.,
TadA is often found in conjunction with the integral membrane proteins TadB and TadC. One group of archaeal homologues of TadA are the FlaI proteins, the ATPases of the flagellin operon, which are always accompanied by FlaJ, an integral membrane protein homologous to TadB/TadC. Therefore, not only SSO0120, but also SSO2316 (FlaI), SSO2387 and SSO2680 belong to the 'Type IV' family of NTPases. All five proteins share the Walker A and B motifs and two conserved regions, termed Asp and His boxes (Fig. 1). The greatest diversity is found in the N-termini, which are abundant in negatively charged amino acids. The N-termini are believed to be needed for the interaction of the ATPases with integral membrane components of the secretion systems (Yeo et al., 2000). The members of the GspE and PilB family show a conserved tetracysteine motif (Possot & Pugsley, 1997), which is involved in metal binding (Robien et al., 2003). However, none of the putative secretion ATPases contains such a motif. The predicted protein size varies between 55 kDa and 66 kDa, and they are relatively hydrophilic, so that they are predicted to be cytoplasmic proteins. In an earlier edition of the S. solfataricus genome, SSO2387 had a length of 583 amino acids, but the current sequence shows a frame shift at base pair position 271. This leads to a downstream stop codon, thereby deleting more than half of the C-terminal part of the protein. The 583 amino acid SSO2387 was described by Lower & Kennelly (2003) as a protein-serine kinase. Indeed, we cloned SSO2387 and also did not find the frame shift indicated in the S. solfataricus database. The protein contains an active site that shows resemblance to eukaryotic serine/threonine kinases, and an autophosphorylation activity was demonstrated for a recombinant SSO2387 (see also below). However, the homology to secretion ATPases was not discussed, although this protein shows all the typical motifs that characterize this family. None of the other four secretion ATPases.
ATPases possesses residues that would assign them as kinases.

Operon composition

To identify the genes that are co-transcribed with the five putative secretion ATPases and to define the members of the operons, primers were designed that matched the 3' and 5' sequence of neighbouring genes. RT-PCR was performed on RNA isolated from cells grown on rich medium. The results are shown in Fig. 2. In all cases, several genes were found to be co-transcribed. The smallest operon contained only three genes, including the ATPase gene sso0572. The other two genes are predicted to encode for a glutamine amidotransferase (sso0571) and a protein involved in pyrodoxine biosynthesis (sso0570). On the basis of the identity of the co-transcribed genes, it is unclear if the proteins of this operon are involved in secretion of proteins. However, the ATPase is mainly membrane bound in *Sulfolobus* (see below) and might therefore be part of a protein complex encoded elsewhere on the genome. The composition of the flagellin operon is very similar to that described for other archaea and comprises a minimal set of proteins, such as FlaB, the structural component of the flagellum, FlaH and FlaI, putative ATPases, and the integral membrane protein FlaJ. Interestingly, all archaea exhibit several copies of FlaB genes, whereas *S. solfataricus* and *Sulfolobus tokodaii* only contain a single copy. Although a transposon is present between SSO2322 and SSO2319, thereby destroying SSO2321, the downstream genes SSO2319...

**Fig. 2.** Composition of the putative secretion operons. RT-PCR analysis of the co-expressed genes. Total RNA (1 µg) derived from tryptone-/yeast-/sucrose-grown cells was used as a template. The primers were designed to match the 3' and 5' sequence of neighbouring genes. The bars under the genes indicate the product that should be amplified if the two neighbouring genes are co-transcribed. The number under the bar indicates the corresponding lane on the agarose gel. White arrows represent ORFs containing type IV pilin-like signal peptides; black arrows represent secretion ATPases; light-grey arrows represent predicted integral membrane proteins; dark-grey arrows indicate putative ORFs. M, marker lane; C, control for contaminating DNA: the sample was treated in the same way as the RT-PCRs, except that the reverse transcriptase step was omitted.
to FlaJ are still transcribed. SSO2386, the only integral membrane protein of the SSO2387 operon, is homologous to FlaJ, the membrane protein of the flagellin operon. The ATPase SSO2387 and SSO2386 might form the minimal necessary core of a secretion machinery. The putative function of the other two genes is unknown. The operons containing SSO0120 and SSO2680 are the most promising candidates for secretion systems involved in putative bindosome assembly. In both cases, the ATPase gene is followed by integral membrane proteins, SSO0119 and SSO2679, which are homologous to TadB/TadC, membrane components of the bacterial tight adherence system involving the production of fibrils (Kachlany et al., 2000).

**Expression and localization in S. solfataricus**

To determine the conditions under which the various secretion systems are expressed, *S. solfataricus* cultures were grown on different defined media containing glucose, arabinose, maltose or tryptone as sole carbon source. Samples were taken in the mid-exponential and stationary growth phases. RNA was isolated and analysed by RT-PCR using primers directed against the five different secretion ATPases (Fig. 3). SSO0572 and SSO0120 were expressed in all media tested, although the expression levels of SSO0120 varied depending on the growth phase. SSO2680 showed a similar behaviour, but was not detected in exponentially growing cells grown on arabinose. SSO2387 mRNA could not be detected under all tested growth conditions, while FlaI mRNAs could be found only in the stationary growth phase of arabinose- and maltose-grown cells. We noted, however, that FlaB, the structural protein of the flagellum, is expressed only under stress conditions such as carbon-source limitation or pH change (Z. Szabó, unpublished results). FlaB can be detected very easily under these conditions, but it is still difficult to demonstrate expression of the accessory flagellum genes, presumably because of low mRNA levels. Summarizing, the expression of four of the secretion ATPases could be demonstrated under the chosen growth conditions. SSO2387 was expressed when the cells were grown on rich medium containing tryptone, sucrose and yeast extract (see Fig. 2). Interestingly, in two of the operons examined, other small proteins, SSO2681, SSO0117 and SSO0118, with type IV pilin-like signal peptides are present (Fig. 3). These might either function as pseudopilins comparable to bacterial type II secretion systems (Pugsley, 1993b) or represent a structural subunit of a pilus-like structure. As shown in Fig. 4, the pattern of expression of the putative structural gene SSO2681 was comparable to that of the ATPase SSO2680 (Fig. 3) under the conditions tested, but, based on PCR, product yield appeared to be higher than that of the ATPase. Expression was especially

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**Fig. 3.** Expression of secretion ATPases in *S. solfataricus*. RT-PCR using primers directed against the indicated secretion ATPase. Each reaction contained 1 μg total RNA of cells grown under the indicated conditions. As a control for loading, total RNAs were run on a TBE agarose gel and stained with ethidium bromide to visualize the 16S and 23S rRNAs. H, high OD600 (~2-5); L, low OD600 (~0-5).

**Fig. 4.** Expression of type IV pilin signal peptide containing proteins from secretion ATPase operons. (a) Expression and (b) N-terminal sequences of SSO2680, SSO0118 and SSO0117. Expression studies were conducted as described in the legend to Fig. 3. The putative type IV prepilin peptidase cleavage site is indicated by an arrow. The signal sequences correspond to the consensus sequence, which was previously determined for the type IV prepilin signal peptidase, PibD, of *S. solfataricus* (Albers et al., 2003). The hydrophobic part of the signal peptide is underlined.
high in the stationary growth phase when the cells were grown on sugars, which suggests that this operon might be involved in the assembly of the bindosome. SSO0118 was prominently expressed only in the stationary phase of arabinose- and maltose-grown cells and exponentially growing tryptone cells, in which SSO0120 was also more highly expressed. SSO0117 could only be detected in the stationary growth phase of maltose-grown cells. This could mean either that SSO0118 is a major component of a pseudopilin-like structure and SSO0117 only a minor subunit or that SSO0118 is the preferred substrate, in contrast to SSO0117 at the tested conditions.

To study the ATPase protein levels and their localization in *S. solfataricus*, membranes and cytoplasm were isolated from cells grown under the same conditions as the RT-PCR experiments. Antibodies were raised against the purified ATPases (see below) and were used to detect expression. The antibodies against SSO0120, SSO0572, Flal and SSO2387 gave satisfactory results in Western blotting. As with the RT-PCR experiments, SSO2387 could not be detected. Also, Flal could not be detected. As the mRNA levels of Flal were low, protein expression may have been below the detection level of Western blotting. However, homologously expressed 6× His-tagged Flal in *Sulfolobus* localizes solely to membranes (S. V. Albers, unpublished results). Consistent with the RT-PCR measurements, SSO0572 was expressed under all tested conditions and appeared mainly membrane bound, with some protein found in the cytoplasmic fraction of stationary-phase cells (Fig. 5). SSO0120 gave weak signals in the membrane fractions. At least for SSO0572, SSO0120 and Flal, the hydrophilic ATPases co-localized with *Sulfolobus* membranes.

**Heterologous expression and activity of secretion ATPases**

The five putative secretion ATPases were cloned and expressed heterologously in *E. coli*. Except for Flal, all proteins were expressed mainly in inclusion bodies. Flal could be purified from the cytoplasm of *E. coli* using a heat step and nickel affinity chromatography (see Fig. 6a). Several attempts were made to purify the other proteins from inclusion bodies, but no activity could be detected after refolding procedures. Adjustment of growth conditions, such as temperature, time of induction of expression and concentration of arabinose, also did not result in more soluble cytoplasmic protein. In the case of EpsE, deletion of the N-terminus resulted in the expression of soluble protein (Robien et al., 2003). Therefore, N-terminal deletions of the *Sulfolobus* proteins were constructed, but were either not expressed or were still in inclusion bodies. Next to the protein in inclusion bodies, a small part of the protein was found in membrane fractions. Finally, the proteins were isolated from *E. coli* inner-membrane vesicles after solubilization of the membranes and affinity chromatography (see Fig. 6a).

We measured the ATP hydrolysing activities of the purified proteins and found that all five proteins are highly active with Mn$^{2+}$ as divalent cation (Fig. 6b), while in most cases Ca$^{2+}$ also supported activity. Strikingly, except for SSO0572, Mg$^{2+}$ supported only poorly the ATPase activity of these proteins. The ATPase activity of SSO2387 is stimulated by both Mn$^{2+}$ and Ca$^{2+}$. SSO2387 has been described to be only marginally active with Ca$^{2+}$ in the autophosphorylation and phosphorylation of exogenous proteins (Lower & Kennelly, 2003). However, these authors used 5 M urea with non-refolded protein,

Fig. 5. Localization of secretion ATPases in *S. solfataricus*. Cytoplasm and membranes from cells grown on the different substrates were separated on SDS-PAGE, immunblotted and analysed with the indicated antibodies. H, high OD$_{600}$ (~2-5); L, low OD$_{600}$ (~0.5); C, antibody control on the appropriate purified protein.
and this might explain the difference in their observations. In this study, the proteins showed the highest activity at 60 °C (data not shown), but higher temperatures could not be tested due to the presence of 0.05% DDM, which has a cloud point at 60 °C. In the presence of Mn$^{2+}$, all proteins showed the highest activity with ATP, while UTP, GTP and CTP were only poor substrates (Fig. 6c). The proteins were also tested for autophosphorylation. For that purpose, they were incubated with $[^{32}P]$ATP for 45 min at 55 °C and separated on SDS-PAGE. Only SSO2387 showed an autophosphorylation activity (Fig. 6d). These data demonstrate that all five secretion ATPases are highly active with Mn$^{2+}$–ATP.

**DISCUSSION**

Here we present a first investigation of putative archaeal secretion and assembly systems similar to bacterial type II, IV secretion systems and type IV pili assembly systems. As most archaea only possess a cytoplasmic membrane surrounded by a membrane-anchored S-layer, the organization of such complexes might be significantly different from that in Gram-negative bacteria. We identified five proteins of *S. solfataricus* that are homologous to secretion ATPases of bacterial type II, type IV secretion systems and the type IV pili assembly machinery. We determined the operon structures of these putative secretion system-encoding gene clusters and the expression patterns of the ATPases under different growth conditions, and we established that all five ATPases are equipped with a divalent cation-dependent ATPase activity.

The bacterial type II and IV secretion systems usually contain between 12 and 15 subunits (Cascales & Christie, 2003; Christie, 2001; Sandkvist, 2001). The putative systems identified in *S. solfataricus* seem to contain a much lower

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**Fig. 6.** Purification and ATPase activity of the secretion ATPases. (a) Coomassie blue-stained SDS-PAGE of purified secretion ATPases. The sizes of the protein marker are indicated in kDa. (b) The purified secretion ATPases were tested for ATP hydrolysis in the presence of Mg$^{2+}$, Mn$^{2+}$ or Ca$^{2+}$. (c) Hydrolysis of different nucleotides in the presence of Mn$^{2+}$ and (d) autophosphorylation activity with $[^{32}P]$ATP. Results shown in (b) and (c) are the mean of three experiments.
number of components, although we do not know whether or not other genes outside of the operons contribute to the secretion systems as well. In all operons, the secretion ATPases were accompanied by integral membrane proteins with homology to TadB, one of the membrane proteins of the tight adherence system (Kachlany et al., 2000). Moreover, three operons contain proteins with a type IV pilin signal peptide. FlaB, the subunit of the archaeal flagellum, is likely to be the substrate of the FlaHJJ system. SSO2681 and SSO0117/0118 might be substrates for their cognate secretion systems or might have a similar function as so-called pseudopilins. The latter are thought to form a translocation piston to push proteins across protein pores in the outer membrane of Gram-negative bacteria (Mattick et al., 1996; Sandkvist, 2001; Shevchik et al., 1997). In the archaeal system, such a piston needs to bridge the distance between the cytoplasmic membrane and the S-layer. It has been described that the S-layer of Sulfolobus species contains pores of 3–5 nm (Koenig, 1988; Prüschenk et al., 1987). These could be the exit points for secreted proteins, thereby eliminating the need for specific outer-membrane pore proteins, which in Gram-negative bacteria are formed by the secretin family of proteins (Thanassi, 2002).

The ATPase activities measured for the five recombinant secretion ATPases are comparable to the ones characterized in bacteria. *Aquificus aeolicus* PilT, plasmid R388 TrwD, Actinobacillus actinomycetemcomitans TadA and Legionella pneumophila DotB display a similar low activity of 1–15 nmol mg⁻¹ min⁻¹ (Bhattacharjee et al., 2001; Herdendorf et al., 2002; Rivas et al., 1997; Sexton et al., 2004). The low activity may be due to the lack of an interaction with other components of the secretion systems, possibly the integral membrane components and/or the substrate proteins. The addition of phospholipids has been shown to increase the activity of HP0252, TadA or RP4 TrbB two- to tenfold (Bhattacharjee et al., 2001; Krause et al., 2000). The ATPase activity is dependent on the presence of divalent cations, with an apparent preference for Mn²⁺. SSO0572 was slightly more active with Ca²⁺ than Mn²⁺. A preference for Mn²⁺ has also been demonstrated for the autophosphorylation or protein phosphorylation activity of SSO2387 by Lower & Kennelly (2003). However, in contrast to this initial report, we observe that Ca²⁺ is almost as stimulatory as Mn²⁺. The current assay is not performed in 5 M urea which might explain the low activity with Ca²⁺ observed by Lower & Kennelly. SSO2387 has been proposed to function as a protein kinase, but its homology to secretion ATPase was not previously noted, despite the fact that it contains all conserved motifs. Nevertheless, SSO2387 was the only ATPase that exhibited an autophosphorylation activity when incubated with ATP. VirB11 was shown to have ATPase and autophosphorylation activity (Christie et al., 1989), and with EpsE (Sandkvist et al., 1995) autophosphorylation was also demonstrated. The function of the autophosphorylation activity during protein secretion, however, remains obscure. We tried to assess the oligomeric behaviour of these ATPases, since in bacteria they form a hexameric complex that interacts with the membrane components of the secretion systems (Herendorn et al., 2002; Krause et al., 2000; Savvides et al., 2003). However, the proteins precipitated upon concentration, which precluded further oligomerization experiments.

For many of the described systems of the main terminal branch in Gram-negative bacteria, the ATPases play an essential role in the biological function, that is, protein secretion, pilus assembly or pilus retraction. For the archaeal flagella secretion system, FlaI was shown to be essential for the assembly of the flagellar structure in *Halobacterium salinarum* and *M. voltae* (Patenge et al., 2001; Thomas et al., 2002). The production of FlaB, the structural subunit of the flagellum, was not affected and the protein was normally processed by the peptidase in the FlaI knockout strain (Thomas et al., 2002). The same was noted in *M. voltae* for the FlaJ knockout strain (Thomas et al., 2002). FlaJ is a membrane-spanning protein, which might form the minimal secretion pore with FlaI. Apart from SSO572, all secretion ATPases are either preceded or followed by a protein that shows some similarity to FlaJ. Because of this operon organization, we speculate that the ATPase and the membrane protein constitute the minimal cores of the secretion systems.

Except for the FlaHJJ system, the substrates of the other systems in *S. solfataricus* are unknown. Since the operons containing SSO0120 and SSO2680 are expressed under all conditions under which the sugar-binding proteins are expressed, it appears that these two are the best candidates to form an assembly machinery of the ‘bindosome’. When we searched the genome of *S. tokodaiii*, a close relative of *S. solfataricus*, for the presence of ABC transporters, we found a variety of homologues of the systems which contain substrate-binding proteins belonging to the di/oligopeptide cluster in *S. solfataricus*. We could not identify any homologues to the transport systems of *S. solfataricus* from which the substrate-binding proteins are synthesized as precursors containing a type IV pilin-like signal peptide. Strikingly, a comparison between the two species of the presence of the various secretion operons showed that *S. tokodaiii* contains all the operons except the SSO2680 operon. Therefore, we consider this as the most likely candidate for the assembly of the bindosome. This operon also contains a putative substrate gene, SSO2681, that is expressed under all growth conditions. This protein is synthesized as a precursor with a type IV pilin-like signal sequence, but has no further homology to any known proteins.

Further functional elucidation will depend on gene deletions in *S. solfataricus* to identify the substrates and subunits of the different secretion systems. In this respect, a method for gene inactivation in *S. solfataricus* has very recently been reported (Worthington et al., 2003). This may now enable a more direct test of the involvement of SSO2680 and the other ATPases in the secretion of the binding proteins.
ACKNOWLEDGEMENTS

We thank Z. Szabó for critical reading of the manuscript. S. V. A. was supported by a VENI grant from the Dutch Organization for Scientific Research (NWO).

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