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In vitro signal sequence cleavage assay for type IV pilin proteins of the archaeon S. solfataricus

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Summary

Archaeal flagellins exhibit type IV pilin like signal peptides. In the thermoacidophilic archaeon S. solfataricus not only the flagellin, but also eight substrate-binding proteins, contain type IV pilin signal peptides. In the vicinity of the flagellin gene, genes are located that encode for homologues of a bacterial type II or IV secretion system. However, a prepilin peptidase could not be identified, and therefore an in vitro cleavage assay for the flagellin FlaB and the glucose-binding protein GlcS of S. solfataricus was established. This system consists of E. coli membranes containing expressed preFlaB or preGlcS mixed with S. solfataricus membranes. After detergent solubilization and incubation at 55°C, specific cleavage of both proteins was observed. This system can be used for the purification and identification of the type IV pilin signal peptidase of S. solfataricus.

Introduction

Many Gram-negative bacteria express type IV pili at their cell surface. These filamentous structures play an important role in cell-cell contacts, antigeneticy and motility (Wall and Kaiser, 1999). Prepilin are processed at an unusual site at their N-termini leaving the hydrophobic domain of the signal peptide attached to the mature protein. This domain is involved in the assembly of the pilus, in particular the subunit-subunit interaction (Forest et al., 1999; Parge et al., 1995). More than 12 genes are necessary for the proper secretion and assembly of the pilus (Nunn, 1999). Among these gene products there are polytopic cytoplasmic membrane proteins, a cytosolic ATPase that is thought to drive the secretion process, outer membrane proteins that form an oligomeric ring-like structure to extrude the pilus from the cell surface, and a specific prepilin peptidase.

Flagellins from methanogens have been shown to contain type IV signal peptides (Bayley et al., 1998; Kalmokoff and Jarrell, 1991), and in analogy, homologous flagellin genes are present in most archaean genomes. Flagellins show a very high sequence identity at their mature N-termini, in particular a conserved hydrophobic stretch of amino acids. In bacterial flagella, regions of high conservation are observed at both the N- and C-termini that are important for export and polymerization of the flagellum (Jones and Aizawa, 1991). However, bacterial flagellins are not synthesized with typical signal peptides and are secreted via a type III secretion machinery (Macnab, 1999). Archaeal flagellins on the other hand contain an unusual signal peptide that is usually very short (4-18 amino acids) and rich in positively charges amino acids (See Fig. 5). These signal
peptides show the same features as Gram-negative type IV pilin signal peptides (Faguy et al., 1994). In Pseudomonas aeruginosa prepilins are processed by a cytoplasmic leader peptidase, PilD (Strom, Nunn, and Lory, 1994). This enzyme has been characterized in some detail. PilD is bifunctional: it does not only remove the signal peptide, but, at the same time, N-methylates the N-terminus of the pilin (Strom, Nunn, and Lory, 1993). The similarity of archaeal flagellin signal peptides with type IV pilin signal peptides suggests that a PilD homologue must be present in archaea. However, so far such an homologue could not be identified by extensive database searches.

Recently, a series of sugar-binding proteins from the thermoacidophilic archaeon S. solfataricus have been purified and characterized (Chapter 2) (Elferink et al., 2001). N-terminal amino acid sequence analysis of some of the purified binding proteins suggested an unusual processing event in which a short stretch of amino acids with a net positive charge is removed leaving the protein anchored to the membrane by means of a N-terminal transmembrane segment. The N-terminus does not correspond to a typical signal peptide but rather is homologous to the type IV pilin class of signal peptides (Chapter 5). This suggests that S. solfataricus contain a PilD-like processing peptidase that could be involved in both the processing of flagellins and a subset of solute-binding proteins. In analogy to the Methanococcus voltae flagellin (Bayley and Jarrell, 1999), an in vitro cleavage assay was developed that allows the monitoring of the processing of the S. solfataricus flagellin and the glucose-binding protein.

**Results**

**Expression of preFlaB and preGlcS.**

The flagellin of S. solfataricus was identified in the corresponding database using the N-terminus of the mature S. shibatae flagellin (Faguy et al., 1996) (Fig. 2). Interestingly, S. solfataricus seems to contain only one flagellin gene, whereas the S. shibatae flagellum is composed of two subunits as commonly observed in archaea (Thomas et al., 2001). Downstream of the S. solfataricus flaB, the integration of a transposon has occurred that seems to disrupt an ORF that may encode for the second flagellin (Fig. 1). It appeared that the integration of the transposon deleted the original promotor and N-terminus of the second flagellin gene, flaA. Further downstream of the transposase, 4 ORFs are located, FlaGHJ, which are most likely involved in the biogenesis of the flagellum. FlaHJ represent the minimum core of flagella associated proteins (Thomas et al., 2001). Except for Aeropyrum pernix and Archaeoglobus fulgidus, other archaea contain the additional proteins FlaCDEFG. The function of FlaG is unknown. FlaI is homologous to PilT of type IV pilus systems and proteins involved bacterial type II secretion. FlaI contains a Walker A motif consistent with a role in ATP hydrolysis in the secretion process. FlaJ is a polytopic membrane protein with 7 putative transmembrane segments that could form the translocation pore. For FlaH, no homologous bacterial proteins could be identified, but the deletion of the flaH gene of M. voltae results in cells that are unable of producing flagella (Thomas et al., 2001).

The genes encoding the precursor forms of FlaB and GlcS (Chapter 2), the glucose binding protein, with the type IV pilin signal peptides (Fig. 1A) were cloned by PCR and expressed in E. coli with a C-terminal 6xhistidine-tag. FlaB was expressed as a 34-kDa band, which corresponds to the molecular mass on the precursor form. GlcS was also expressed but appeared unstable, as it was...
readily degraded. Removal of an internal NcoI insert of 821 bp of the glcS gene yielded a truncated form of preGlcS, preGlcS(Δ821) with a molecular mass of 36-kDa (Fig. 2B). Both proteins co-fractionate with the membranes.

**In vitro cleavage of preFlaB and preGlcS**

Solubilized *E. coli* membranes containing either preFlaB or preGlcS(Δ821) were mixed with solubilized *S. solfataricus* membranes and incubated at 55°C. Processing at the N-terminus of preFlaB and preGlcS(Δ821) should result in the removal of 18 and 12 amino acid residues, respectively (Fig. 2A), and leave a product that can be detected with an antibody directed against the C-terminal his-tag. A typical result of a cleavage assay resulting in processing of both proteins is depicted in Fig. 2B. Processing was observed only when the *E. coli* membranes were mixed with the *S. solfataricus* membranes and incubated at a high temperature. At 37°C, only residual cleavage was observed (Fig. 3, 4). The processing of preFlaB yielded two products, tentatively assigned as FlaB1 and FlaB2 (Fig 3, 4). The upper band, FlaB1, most likely resembles mature FlaB. The identity of the second protein band, FlaB2, is unclear. It occurred simultaneously with FlaB1 when the membranes were incubated at 55°C. The protease inhibitors EDTA or PMSF had no effect on the processing of preFlaB (Fig. 3, 4). With preGlcS(Δ821), processing resulted in a protein product with a slightly higher mobility on SDS-PAGE (Fig. 2B). Processing was slightly increased by EDTA. The presence of Triton X-100 was essential for the processing activity (Fig. 4), but it determined the temperature limit of the assay. At 55 and 60 °C cleavage occurred, but at 65°C the activity was completely lost. The cloud point of Triton X-100 is around 64°C, and above this temperature, the membranes were precipitated. This most likely also explains the observed loss of processing activity of the processing of the *Methanococcus jannaschii* flagellins at 80°C as compared to 60°C (Correia and Jarrell, 2000). Triton X-100 could not be exchanged by the detergent dodecylmaltoside (Fig. 4A). When the

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**Fig. 2.** Cleavage assay with preGlcS(Δ821) and preFlaB. A: N-terminal amino acid sequences of preGlcS and preFlaB. The cleavage site is indicated by an arrow. B: In vitro processing of preGlcS(Δ821) and preFlaB. The position of the precursors and cleavage products are indicated. In both cases *E.coli* membranes only show one band upon detection and *Sulfolobus* membranes did not contribute to the signal.

**Fig. 3.** In vitro processing of preFlaB. A: Processing was determined in 25 mM MES, pH 6.5, 0.25 % Triton X-100, 150 mM KCl and as indicated: KCl was exchanged for NaCl, Triton X-100 was exchanged for dodecylmaltoside (DDM), or 1 mM MgCl2 was added. Similar results were obtained with CaCl2 and MnCl2. B: Time course of processing. EDTA and PMSF were both added at 1 mM final concentration. S, mixed membranes incubated on ice for 30 min.
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S. solfataricus membranes were substituted for Pyrococcus furiosus membranes no processing was detected. These data indicate S. solfataricus membranes contain a processing peptidase for the precursors of flagellin and the glucose binding protein.

**Type IV pilin signal peptides in S. solfataricus**

The database of S. solfataricus P2 was scanned for the presence of proteins with a type IV pilin signal peptide. This screen identified the three sugar-binding proteins, including GlcS, that previously by N-terminal amino acid sequencing had been shown to possess a type IV pilin signal peptide (Elferink et al., 2001; Albers et al., 1999). The screen also identified FlaB, and five additional proteins. These proteins exhibit a high similarity to solute-binding proteins, and each is organized in an operon that in that in addition contains genes encoding an ABC-type of transporter (Elferink et al., 2001).

Alignment of all 9 proteins of S. solfataricus containing a type IV pilin signal peptide shows the conservation of the N-termini of these proteins (Fig. 5). At −1 position of the cleavage site only glycine and alanine residues are found. A positive charged residue always precedes this position. This can either be an arginine or lysine. The +1 position is in most cases a leucine or isoleucine residue or exceptionally a phenylalanine. In binding proteins +2 position mostly contains a hydroxylated amino acid, such as serine and threonine, followed by a very hydrophobic stretch of residues. The latter likely anchors the binding proteins to the membrane, while in flagellins it is involved in the assembly of the flagellum (Jones and Aizawa, 1991). Based on these aligned sequences, we propose that the consensus sequence for the processing site of the S. solfataricus type IV pilin proteins corresponds to [KR]2 [GA]1 [IL]1 [STA]2.

**Discussion**

Here we describe an assay for the in vitro processing of two different S. solfataricus proteins, FlaB and GlcS, which both contain a type IV pilin signal peptide. Processing could be detected after mixing of E. coli membranes containing either the expressed preFlaB or a C-terminal truncate of preGlcS with S. solfataricus membranes. Processing was strictly dependent on the presence of the S. solfataricus membranes suggesting that they contain the putative processing enzyme. The assay conditions were optimized with respect to detergent concentration and incubation temperature. The preFlaB is cleaved into two products. The largest product, FlaB1 likely corresponds to the mature FlaB, while the identity of the smaller product, FlaB2 is unclear. N-terminal sequencing should reveal the exact identity of the products. Preliminary results of the purification of the processing peptidase indicate, however, that the cleavage activity resulting in the FlaB2 and FlaB1 products are separable. The latter activity coincides with the processing of preGlcS (data not shown). These data indicate that another peptidase of S.
In vitro cleavage assay

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sofarticus, which is different from the type IV pilin signal peptidase, is responsible for the processing of preFlaB yielding FlaB2. In addition to GlcS, the arabinose and trehalose-binding protein of S. sofarticus are equipped with a type IV pilin signal peptide (Elferink et al., 2001). Database analysis revealed six additional binding proteins. The type IV pilin signal peptide has been reported in archaea only for flagellins (Bayley and Jarrell, 1998). Therefore, Jarrell and coworkers, (1999) proposed that the signal leader peptidase will accept only a limited number of substrates, specifically only flagellins. Based on the consensus-processing site derived from the alignment of the protein sequences, there is no immediate need to assume that flagellins and binding proteins are processed by different enzymes.

Archaeal flagellins are most likely are secreted by a system similar to bacterial type II systems, to which type IV pilin secretion is very homologues. In Gram-negative bacteria, these systems are involved in secretion of folded toxins and hydroxylases to the external medium (Pugsley, 1993). The substrates are first translocated in a Sec-dependent manner across the cytoplasmic membrane and upon folding and assembly, they are secreted across the outer membrane via a specific pore complex (Nouwen et al., 2000). Archaea, however, lack an outer membrane and instead are surrounded by a proteineous S-layer. The base of the archaeal flagellum has been proposed to be anchored to the cytoplasmic membrane via a polar cap (Thomas et al., 2001), but it is not known how it crosses the S-layer. In bacteria, the crystalline array of the S-layer is distorted by the flagellum (Sleytr and Beveridge, 1999). The question arises why sugar-binding proteins are equipped with type IV pilin signal peptides? The binding proteins require detergent extraction of S. sofarticus membranes suggesting that they are anchored to the membrane. The hydrophobic domain at the N-terminus could function as such anchor but it is unclear why the excess

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**Fig. 5.** Type IV pilin signal peptides of S. sofarticus. A: Sequences of different archaeal flagellins. B: Proteins of S. sofarticus containing type IV pilin signal peptides. Hydrophobic domains are underlined and the cleavage site is indicated by −1/+1. Positive charged amino acids in the signal peptide are shown in white. N-termini of proteins with bold number have been determined. Hypo, hypothetical proteins; PBP, phosphate-binding protein.
positive charge at the N-terminus is removed by the processing event. The loss of these residues will weaken the strength of membrane anchoring (Andersson and von Heijne, 1994), and this phenomenon is used during the insertion process of pilins into the pilus. In analogy to the assembly of the flagellum (Parge et al., 1995) one may speculate that sugar-binding proteins integrate into a pseudo-flagellum like structure that extends from the cytoplasmic membrane and possibly crosses the S-layer to effectively scavenge sugars from the external medium.

Summarizing, we have developed an assay that can be used to monitor the purification of the membrane-bound type IV pilin signal peptidase of *S. solfataricus*. This assay can be used as a tool to identify the protein and characterize its substrate specificity. Future studies should as to whether the same enzyme processes the flagellins and binding proteins, and if binding proteins are assembled into a higher order structure.

**Materials and Methods**

**Materials**

Anti-histag antibody was obtained from Dianova (Hamburg, Germany). *E. coli* BL21-CodonPlus-RIL strain containing a plasmid that expresses tRNA genes for argU, ileY, leuW was from Stratagene (Amsterdam, The Netherlands), and PVDF membranes were from Biorad (Hercules, U.S.A.).

**Cloning and Plasmid construction**

Chromosomal DNA from *S. solfataricus* was prepared by CsCl-buoyant density centrifugation (Schleper et al., 1995). Oligonucleotide primers for the amplification of flaB and glcS were designed on basis of the genome sequence of *S. solfataricus* (http://niji.imb.nrc.ca/sulfolobus/). The forward primer for flaB (924) (5'- CCCCCGAATTCATGAAGAATACCCTATA AAGATG) and reverse primer (5'- CCCCCCGATCCCTTTCA AGAGATGATTAAGTGC) contained a BspHI and BamHI endonuclease restriction site, respectively. The digested PCR product was ligated into the expression vector pET324chis yielding pET2150. Using this vector a C-terminal 6x histag is added to the expressed protein. pET324chis was constructed by the ligation of an BamHI/ HindIII fragment from pAMP42 (provided by Antonia Picon) containing a C-terminal 6x histidine tag into the expression vector pET324 (Van der Does et al., 1996). The expression of pET2150 yielded a protein that was unstable and readily degraded. Therefore an internal Ncol insert (position 702-1523) was cut out and the vector was religated yielding pET2152 (Fig. 1). The product was designated GlcS(Δ821).

**Expression**

pET2152 and pET2162 were transformed in *E. coli* BL21-CodonPlus-RIL. Transformants were inoculated in 5 ml LB+ medium (LB medium and 6 g/l of yeast extract) supplemented with ampicillin and chloramphenicol (80 µg/l each) and incubated at 37°C overnight. 2 ml of the overnight cultures were diluted into 1 l of LB + medium and grown until an OD 600 of 0.8. Expression was induced by adding 0.1 mM isopropyl b-D-thiogalactopyranoside (IPTG) and growth was continued for 2 hours. Cells were harvested by centrifugation and stored at –80°C.

**Preparation of membranes**

*E. coli* cells were suspended in 20 mM MES, pH 6.5, containing a small amount of DNase I and RNase, and subsequently passed through a French pressure cell at 800 psi. Unbroken cells were removed by a low spin at 3.000 x g for 20 min at 4°C, and membranes were collected from the supernatant by centrifugation (100,000 x g for 45 min at 4°C). Outer membranes were removed by sucrose gradient centrifugation. The cytoplasmic membranes were washed twice with 20 mM MES, pH 6.5, and stored in liquid nitrogen. *S. solfataricus* culture conditions and preparation of the membranes are described elsewhere (Albers et al., 1999).

**Cleavage assay**

*E. coli* membranes (5 µg of protein) either containing pFlaB or pGlcS(Δ821) and *S. solfataricus* membranes (10 µg of protein) were preincubated each 10 min in assay buffer (25 mM MES, pH 6.5, 0.25 % Triton X-100, 150 mM KCl) at room temperature for solubilization. Both samples were mixed and incubated for 30 min at 55°C, unless indicated otherwise. Aliquots were dissolved in SDS-sample buffer and analyzed by 17.5 % SDS-PAGE and western blotting on PVDF membranes, and
subsequent detection with anti-his antibodies observed cleavage.

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