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Research paper

N-Glycosylation of the archaellum filament is not important for archaella assembly and motility, although *N*-Glycosylation is essential for motility in *Sulfolobus acidocaldarius*

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ABSTRACT

N-Glycosylation is one of the predominant posttranslational modifications, which is found in all three domains of life. *N*-Glycosylation has been shown to influence many biological aspects of proteins, like protein folding, stability or activity. In this study we demonstrate that the archaellum filament subunit FlaB of *Sulfolobus acidocaldarius* is N-glycosylated. Each of the six predicted *N*-Glycosylation sites within FlaB are modified with the attachment of an N-glycan. Although, it has been previously shown that *N*-Glycosylation is essential for motility in *S. acidocaldarius*, as defects in the *N*-Glycosylation process resulted in none or reduced motile cells, strains lacking one to all six *N*-Glycosylation sites within FlaB still remained motile. Deletion of the first five *N*-Glycosylation sites in FlaB did not significantly affect the motility, whereas removal of all six *N*-Glycosylation sites reduced motility by about 40%. Transmission electron microscopy analyses of non glycosylated and glycosylated archaellum filament revealed no structural change in length. Therefore *N*-Glycosylation does not appear to be important for the stability and assembly of the archaellum filament itself, but plays a role in other parts of the archaellum assembly.

1. Introduction

Microorganisms form a variety of cellular appendages, such as flagella/archaella or type IV pili. These structures allow organisms to interact with their surrounding environment either by tethering to surfaces, or by active cell movement such as twitching, gliding and swimming [24]. The ability to move actively is beneficial for cell survival, as it allows the change from an unfavorable environment towards more favorable conditions. The most common motility structures are the filamentous surface appendages termed flagella in Bacteria and archaella in Archaea. Although both motility structures share the same function, the archaeal and bacterial motility structures are structurally unrelated [22].

The bacterial flagellum motor consists of two parts: a non-rotational membrane bound part (stator) and a rotor to which the hook and the filament are linked. The rotary motor is powered either by the proton- or the sodium-motive force [31]. More than 50 proteins are known to be involved in the biosynthesis and assembly of the flagellum [10]. In contrast to the flagellum, the archaellum structurally resembles bacterial type IV pili, which are comprised of only around 15 proteins. The archaellum assembly apparatus is composed of FlaJ, a membrane protein homologous to the bacterial type IV pilus protein PilC. Moreover, the archaellum contains the ATPase FlaI, which is necessary for assembly and rotation of the filament [38] (Fig. 1A) and is homologous to PilB in bacterial type IV pili. The filament forming subunit, the archaellin FlaB, is initially produced as precursor protein, which includes a type-IV pilin-like signal sequence [16,42]. These pre-archaellins are processed prior to assembly into the archaellum by the action of a specific signal peptidase PibD/FlaK [3,9]. In contrast to flagella, in which the filament subunits are assembled on the distal tip after passing through an internal channel, the archaellins assemble at the base of the archaellum filament similar to the assembly of type-IV pili.

In Archaea and Bacteria the individual subunits of the motility filament are posttranslationally modified by glycosylation. Glycosylation of flagellins has been demonstrated for a number of Gram-negative bacteria, including *Pseudomonas aeruginosa* [4,39], *Helicobacter pylori* [25], *Aeromonas* spp. [37], *Caulobacter crescentus*

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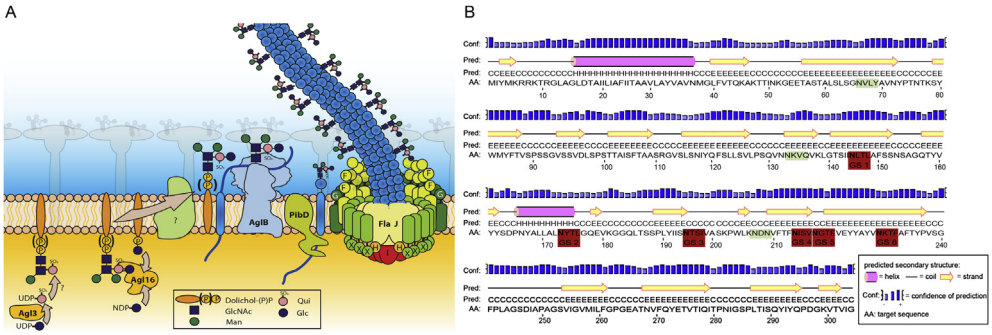

Fig. 1. Biosynthesis pathway and predicted secondary structure of the archaellin FlaB. A) The N-linked tribranched hexasaccharide of the archaellin, is synthesized on the lipid carrier dolichol-(pyro?) phosphate. Agl3 and Agl16 are essential for the full N-glycan biosynthesis, creating UDP-sulfoquinovose (Qui) or transferring the terminal glucose onto the premature lipid linked oligosaccharide, respectively. The lipid linked oligosaccharide is translocated across the membrane by an unknown flippase and the glycan is transferred onto the secreted target protein (FlaB) by the oligosaccharyltransferase AglB. The pre-archaellin FlaB is processed by the peptidase PibD and assembled into the archaella filament. B) Secondary structure prediction of FlaB using PSIPRED [13]. Primary sequence of FlaB from *S. acidocaldarius* with the six predicted *N*-Glycosylation sequons (marked red), including the targeted Asn residues (bold), three nonconserved sites (marked in green), and the predicted secondary structure: α-helices (pink, H), β-strands (yellow, E) and coiled (black line, C) are displayed. The archaeal class III signal peptide cleavage site, between G_{11}/L_{12}, has been predicted using FLAFIND [42].

[29], *Shewanella oneidensis* [12], *Campylobacter* species [15], and *Clostridium botulinum* [45]. Glycosylation of the flagellar filament in Gram-positive bacteria has only been described in *Clostridium* [45] and *Listeria* [40]. All these flagellins are O-glycosylated, in which glycans are sequentially added to the hydroxyl oxygen group of serine and threonine. In addition to O-linked glycosylation, members of the ε-Proteobacteria, like *Campylobacter* and *Helicobacter* species, as well as a subset of Δ-Proteobacteria, including *Desulfovibrio or Wollinella* species, are also able to modify their proteins via N-linked glycosylation [36]. During *N*-Glycosylation the N-linked oligosaccharide is first assembled on an isoprene-based lipid carrier from which the oligosaccharide is transferred *en bloc* onto an asparagine residue found within a conserved Asn-x-Ser/Thr motif (where x is any amino acid except proline) of a target protein. In contrast to Bacteria, the archaellins or archaeal pilins have been shown to be modified with N-linked glycans, rather than with O-glycans [23] [26,33–35,44,47].

Both N- and O-glycosylation have been shown to be crucial for the function of flagella and archaella. In Bacteria, defects in glycosylation process are known to compromise the function of flagella, either due to problems with their assembly and motility [6,17,21] as well as reduced pathogenicity for a number of bacterial pathogens [30], e.g. in *Campylobacter jejuni* [21] and *P. aeruginosa* [5]. In Archaea, defects in *N*-Glycosylation influence the function of the archaellum. In *Haloferax volcanii, Methanococcus voltae, Methanococcus maripaludis,* for example, deletion of *aglB,* whose encoded product the oligosaccharyltransferase is the central enzyme of the *N*-Glycosylation pathway, resulted in non-motile cells, lacking any archaella filament [1,14,44,46]. In *Sulfolobus acidocaldarius*, *N*-Glycosylation mutants, which exhibited a reduced *N*-Glycans size, lacking one or three terminal sugar residues, were severely effected in motility, as the cells showed no or extremely low motility [33,34]. While these previous studies showed that the function of archaella is dependent on *N*-Glycosylation, the question arose whether the observed loss of motility is caused directly by the loss of *N*-Glycans on the archaellins or indirectly by glycosylation defects of other parts of the archaellum. In this study we elucidated the effect of the loss of *N*-Glycosylation sites within the archaellin FlaB, and its effect on motility.

2. Materials and methods

2.1. Strains and growth conditions

All strains of *Sulfolobus acidocaldarius* including MW001 (Δ*pyr*E) [48], MW156 (Δ*aapF*) [20], MW451 (Δ*aapF*, *flaB*:taa) [28], MW451 complementation strains carrying plasmids with the different *flaB* mutations, MW039 (Δ*agl3*) [34], and MW043 (Δ*agl16*) [33] were grown at 75 °C in Brock medium with pH 3 [11]. The medium was supplemented with 0.1% (w/v) NZ amine and 0.1% (w/v) dextrin as carbon and energy source. Selection gelrite (0.6%) plates were supplemented with the same nutrients (as above), with the addition of 10 mM $MgCl_2$ and 3 mM $CaCl_2$. For growth of uracil auxotrophic mutants (MW001, MW156, MW451, MW039, and MW043) 10 μg ml^{-1} uracil was added to the medium. Cell growth was monitored by measuring the optical density at 600 nm.

2.2. Construction of *flaB* complementation plasmids

For the construction of the complementation plasmids the nucleotide sequence of *flaB* was synthesized containing two non-codon modifying nucleotide exchanges, which led to the loss of the intrinsic *Nco*I and *Bam*HI restriction sites. The synthetic *flaB* was cloned into pSAV1481, an improved high copy vector based on pMZ1 (Zolghadr et al., 2007), using the restriction enzymes *Nco*I and *Eag*I, cutting at the 5′and 3′ of the synthesized *flaB,* respectively. The resulting plasmid pSVA1280 was used as a template for oligonucleotide directed mutagenesis of *flaB*.

In general, a PCR reaction with a specific forward and reverse primers (Table S1), carrying a nucleotide exchange within the *flaB* sequence, were used to amplify the full template plasmid. The resulting PCR amplified plasmids were digested with *Dpn*I to remove the methylated template plasmid pSVA1280. After purification, each construct was transformed into *E. coli* DH5α, and plated on selective LB-plates containing 50 μg ml^{-1} ampicillin. Mutagenesis was confirmed by sequencing of the entire *flaB* gene. To avoid restriction in *S. acidocaldarius* each plasmid was methylated by transformation in *E. coli* ER1821 cells containing pM.EsaBC4I (available from NEB), which expresses a methylase. Plasmids pSVA1281-1286, each carrying a single alanine substitution, and the plasmids pSAV1298–pSVA1302, carrying two to a total of six asparagine to alanine substitutions in the predicted *N*-Glycosylation sites, were generated (Table S2).

For expression in *S. acidocaldarius* the different *flaB* versions (unmodified, single alanine/glutamine substitution, or multiple alanine substitutions) were cloned into the expression vector pSVA1450. For this each *flaB* version was PCR amplified with the specially designed primers, which incorporated at the 5′ ends of the forward primer (4171) and the reverse primer (4172) the restriction site *Nco*I and *Eag*I, respectively (Table 1). PCR fragments derived from the different *flaB* derivates were cloned into the expression vector