AglB, catalyzing the oligosaccharyl transferase step of the archaeal \(N\)-glycosylation process, is essential in the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*

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**Abstract**

*Sulfolobus acidocaldarius*, a thermo-acidophilic crenarchaeon which grows opti-
mally at 76°C and pH 3, exhibits an astonishing high number of \(N\)-glycans linked to the surface (S-) layer proteins. The S-layer proteins as well as other surface-exposed proteins are modified via \(N\)-glycosylation, in which the oligosaccharyl transferase AglB catalyzes the final step of the transfer of the glycan tree to the nascent protein. In this study, we demonstrated that AglB is essential for the viability of *S. acidocaldarius*. Different deletion approaches, that is, markerless in-frame deletion as well as a marker insertion were unsuccessful to create an *aglB* deletion mutant. Only the integration of a second *aglB* gene copy allowed the successful deletion of the original *aglB*.

**Introduction**

The asparagine (\(N\))-linked protein glycosylation is one of the most predominant co- and posttranslational protein modifications, which is found in all three domains of life (Larkin and Imperiali 2011). In Eukarya \(N\)-glycosylation is an essential process, which is evolutionarily highly conserved from yeast to human (Lehle et al. 2006). It is estimated that more than half of all eukaryotic proteins are glycoproteins (Apweiler et al. 1999; Zielinska et al. 2010). The biological functions of protein glycosylation span a broad spectrum, for example, as it is influencing protein folding and stability, intra and extracellular recognition, or enzyme activity, which can be crucial for the development and survival of an organism (Varki 1993; Helenius and Aebi 2004; Caramelo and Parodi 2007).

The key enzyme of the \(N\)-glycosylation process is the oligosaccharyl transferase (OTase). The OTase is catalyzing the last step of this process by transferring the fully assembled \(N\)-glycan en bloc from a lipid pyrophosphate donor onto a selected asparagine within a specific \(N\)-glycosylation recognition site Asn-x-Thr/Ser of a nascent protein, where x can be any residue except proline (Gavel and Vonheijne 1990).

In eukaryotes, this step is catalyzed in the endoplasmic reticulum (ER) lumen by the multimeric OTase complex. The OTase complex from *Saccharomyces cerevisiae* is composed of 8 nonidentical membrane subunits: Wbp1, Swp1, Stt3, Ost1, Ost2, Ost3 or Ost6, Ost4, and Ost5 (Kelleher and Gilmore 2006). The Stt3p subunit contains the catalytic site, however, Stt3p alone is not sufficient for the OTase process (Yan and Lennarz 2002; Nilsson et al. 2002).
The detailed function of the other subunits of the OTase complex is not fully understood, but they are thought to regulate and influence the modification of the N-glycosylation sites (Lennarz 2007). For Ost3p and Ost6p, which are homologous, interchangeable subunits, it has been shown that the presence of either one of the interchangeable subunits affects glycosylation occupancy of a subset of N-glycosylation sites in yeast, and they specify the interaction with different translocation complexes (Schwarz et al. 2005; Schulz and Aebi 2009; Schulz et al. 2009). In contrast to the huge eukaryal heteromeric OTase complex, only the ortholog of Stt3p is needed for the OTase reaction in lower Eukarya, for example, Leishmania major, or in the prokaryotic system (Glover et al. 2005a; Igura et al. 2008; Hese et al. 2009).

The bacterial N-glycosylation process is not common, and orthologs of OTase key enzymes have been found only in a few species of delta- and epsilonproteobacteria (Nothaft and Szymanski 2010, 2013). So far Campylobacter jejuni is the only bacterium for which the bacterial N-glycosylation pathway has been studied in detail (Szymanski and Wren 2005), besides studies of N-glycosylation pathway in Helicobacter pullorum (Jervis et al. 2010) and an atypical protein N-glycosylation process in Haemophilus influenza, lacking an OTase (Gross et al. 2008).

The bacterial OTase PglB is a membrane protein, composed of an N-terminal segment with 9–13 predicted transmembrane domains (TD) and a soluble C-terminal periplasmic domain which comprises a highly conserved site (WWDYG) important for the activity. This topology is similar to the experimentally defined topology of the eukaryal Stt3p (Kim et al. 2005). However, only the single PglB enzyme is necessary to fulfill the N-glycan transfer, in contrast to the eukaryal multimeric OTase complex (Glover et al. 2005b). The periplasmic domain possesses a mixture of α/β folds, which has been observed in the crystal structures of the soluble domain of C. jejuni PglB (Maita et al. 2010). However, the isolated periplasmic domain of PglB was insufficient to catalyze the OTase reaction on its own, implying that the transmembrane segments are needed for the catalytic action. Indeed the isolation and the crystal structure of the full OTase from Campylobacter lari together with its bound acceptor peptide, revealed new insights into the molecular basis of the N-linked glycosylation mechanism (Lizak et al. 2011).

In contrast to Bacteria, the archaeal OTase AglB is found in almost all sequenced Archaea (Magidovich and Eichler 2009; Maita et al. 2010; Kaminski et al. 2013), which underlines the broad distribution of the N-glycosylation process and the importance of this protein modification within the Archaea. Although AglB proteins exhibit a low overall sequence homology to the Stt3 orthologs, all AglBPs possess the highly conserved WWDYG motif (Magidovich and Eichler 2009; Maita et al. 2010) (Fig. 1). Like the bacterial PglB and in contrast to the eukaryal Stt3p, AglB is the only enzyme needed for the OTase reaction (Igura et al. 2008). The crystal structure of the C-terminal soluble domain of AglB from Pyrococcus furiosus was the first structure of an OTase (Igura et al. 2007). However, like the soluble part of the bacterial PglB, the soluble part of AglB was also impaired in its function. Analyses of the crystal structure and the alignment of AglB were used to identify a second conserved motif, the DxxK, where x can be any residue (Maita et al. 2010). The crystal structure revealed that this motif lies in structural proximity to the WWDYG motif and is thought to interact with the WWDYG and coordinate the target peptide (Igura et al. 2008; Lizak et al. 2011). The importance of this motif was shown by in vivo mutational studies of the Asp and Lys residue, which showed that this motif was catalytically important in yeast and in L. major (Igura et al. 2008; Hese et al. 2009). The bacterial PglB is missing this DxxK motif; however, a different MxxI motif acts as the counterpart of the DxxK sequence (Maita et al. 2010; Matsumoto et al. 2012).

So far it has been demonstrated in three archaeal species that N-glycosylation is not essential for cell viability as AglB was successfully deleted in Haloferax volcanii, Methanococcus maripaludis, and Methanococcus voltae (Chaban et al. 2006; Abu-Qarn et al. 2007; Vandyke et al. 2009). Although the deletion of aglB resulted in nonmotile cells, AglB was not essential for cell growth in these euryarchaeas. In this study, we have tested whether aglB can be deleted from the genome of the crenarchaeon S. acidocaldarius and demonstrated that it is essential for viability in this organism.

Materials and Methods

Strains and growth conditions

The strain Sulfolobus acidocaldarius MW001 (ΔpyrE) (Wagner et al. 2012), S. solfataricus P2 (Zillig et al. 1980) and S. islandicus E233S1 (ΔpyrE, Δhus) (Deng et al. 2009) and the derived mutant strains (Table 1) were grown in Brock medium at 75°C, pH 3 adjusted using sulfuric acid. The medium was supplemented with 0.1% w/v NZ-amine and 0.1% w/v dextrin as carbon and energy source (Brock et al. 1972). First and second selection gelrite (0.6%) plates were supplemented with the same nutrients (as shown above), with the addition of 10 mmol/L MgCl₂ and 3 mmol/L CaCl₂. In addition, 10 µg mL⁻¹ uracil and 100 µg mL⁻¹ 5-fluoroorotic acid (5-FOA) were added for second selection plates. For the growth of the uracil auxotrophic mutants, 10 µg mL⁻¹ 5-FOA were added.
Figure 1. Topology model of AglB from Sulfolobus acidocaldarius. (A) The model is based on the TMHMM prediction server 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Transmembrane segments are indicated in yellow boxes. Asparagine residues within a predicted glycosylation site are indicated by red circle, the N-glycan attachment sites in S. acidocaldarius are not confirmed. External loops are shown in bold numbers. The conserved WWDYG and DxxK motif within the external loops 7 are circled in red. (B–D) Structural alignment of full AglB from S. acidocaldarius with the crystal structure of the soluble domain of AglB from Pyrococcus furiosus (pdb: 2zagD). (B) Alignment was performed by the SWISS-MODEL program (http://swissmodel.expasy.org); Modeled are the residues 473–749 of the template (pdb: 2zagD) (Igura et al. 2007). Estimated per-residues inaccuracy is visualized using a color gradient from blue (more reliable regions) to red (potentially regions), indicating the low sequence identity of 17.67%. (C) Modeled structure of AglB from S. acidocaldarius compared with (D) the crystal structure of P. furiosus (AA 473–749); the conserved WWDYG and DxxK motifs are shown in blue and yellow, respectively. (E) Alignment of the WWDYG and DxxK/MxxI motifs and their flanking regions of selected OTase from the three domains of life. The amino acid sequences of the STT3, PglB, and AglB proteins were retrieved from the InterPro database, and aligned with the ClustalW program. In 2014, over 1434 sequences are grouped to the family IPR003674, including 844 Stt3, 225 PglB, and 358 AglB; in 2012, 827 sequences were grouped to this family; (530 Stt3, 96 PglB, and 201 AglB). Representative sequences from eukaryal Stt3 proteins and the bacterial PglB from Campylobacter jejuni were selected. The AglB sequences from the crenarchaeota S. acidocaldarius DSM639 and S. solfataricus P2; the euryarchaeota Methanococcus voltae strain A3, M. maripaludis strain A2 and P. furiosus DSM 3638 Hfx. volcanii DS2, Archaeoglobus fulgidus DSM4304; from the nanaarchaeota N. equitans strain Kin4-M; from the korarchaeota K. cryptofilum strain OPF8; and from the thaumarchaeota Nitrosopumilus maritimus SCM1 were selected for the alignment. Highlighted amino acids belong to the WWDYG or DxxK/MxxI motif.
uracil was added to the medium. Cell growth was monitored by measuring the optical density at 600 nm.

Construction of deletion plasmids

To verify the predicted function of aglB participation in the last step of the N-glycosylation pathway, attempts to isolate a markerless deletion mutant of aglB in S. acidocaldarius MW001 and S. islandicus E233S1 were made using the methods previously described (Deng et al. 2009, Wagner et al. 2012). Briefly the strains MW001 and E233S1, in which the genes for the uracil biosynthesis were disrupted, were transformed with the plasmid pSVA1203 or pSVA1204, respectively (Table 1). For constructing the plasmid pSVA1203, 800–1000 bp of the up- and downstream regions of S. acidocaldarius aglB (saci1274) were PCR amplified. At the 5’ end of the upstream forward primer (1725) and of the downstream reverse primer (1726) the restriction site ApaI and BamHI were introduced, respectively (Table 2). The upstream reverse primer (1712) and the downstream forward primer (1713) were designed to incorporate each 15 bp of the reverse complement strand of the other primer, resulting in a 30 bp overlapping stretch. For constructing the plasmid pSVA1204, 800–1000 bp of the up- and downstream fragments of S. islandicus aglB were PCR amplified. The upstream forward primer (1727) included the restriction site for PstI and the reverse primer (1718) contained at its 5’end 15 bp of the reverse complement strand of the downstream forward primer. The downstream forward primer (1719) incorporated as well 15 bp of the reverse complement strand of the upstream reverse primer at its 5’end, leading to a 30 bp overlapping stretch of both internal primers. The upstream reverse primer (1728) was designed to incorporate a BamHI restriction site at 5’end. All up- and downstream fragments were fused by an overlapping PCR, using the 3’ ends of the up- and downstream fragments as reverse primers.

For constructing the plasmid pSVA1241, used for the deletion of saci1162, encoding an α amylase, by the integration of aglB (saci1274), the upstream region of saci1162, the full-length aglB, and downstream region of saci1162 were amplified with the following primer 1891 + 1892, 1893 + 1894, and 1895 + 1896, respectively. At the 5’ end of the upstream forward primer (1891) and of the downstream reverse primer (1896), the restriction site ApaI and BamHI were introduced, respectively. The upstream reverse primer (1892) and the aglB forward primer (1893) were designed to incorporate each 15 bp of the reverse complement strand of the other primer, resulting in a 30 bp overlapping stretch. The aglB reverse primer (1894) and the downstream forward primer (1895) were designed to incorporate each 21 bp of the reverse complement strand of the other primer, resulting in a 42 bp overlapping stretch. The upstream, aglB, and downstream fragments were fused by an overlapping PCR, using the 3’ ends of each fragments as primers.

The overlap PCR fragments were purified and digested with ApaI and BamHI (Δsaci1274 and ( saci1162:: aglbB)), or PstI and BamHI (Δsire1141) and ligated in the predigested plasmid pSVA407, containing pyrEF (Wagner et al. 2012). The obtained deletion plasmids pSVA1203 (Δsaci1274), pSVA1204 (Δsire1141), and pSVA1241 (saciI162::aglB) were transformed into E. coli DH5α and selected on LB-plates containing 50 μg ml⁻¹ ampicillin. The accuracy of the plasmids was ascertained by sequencing. In order to avoid restriction in S. acidocaldarius and S. islandicus the plasmids were methylated by transformation in E. coli ER1821 cells containing pM.EsaBC4I (available from NEB), which expresses a methylase.

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**Table 1.** Strains and plasmids used in this study.

| Strains                  | Genotype                          | Reference                  |
|--------------------------|-----------------------------------|----------------------------|
| Sulfolobus acidocaldarius|                                   |                            |
| MW001 S. acidocaldarius DSM 639, ΔpyrE | Wagner et al. (2012)          |
| MW098 MW001 with saci1162::aglB | This study                      |
| MW099 MW001 with aglB, saci1162::aglB | This study                      |
| S. islandicus E233S1    | S. islandicus Wild type           | Deng et al. (2009)         |
| Rey15A S. islandicus    |                                  | Contursi et al. (2006)     |
| S. solfataricus P2      | S. solfataricus Wild type         | Zillig et al. (1980)       |
| pSVA407 Gm L3244        | Gene targeting plasmid, pGEM-T Easy backbone, pyrEF cassette of S. solfataricus | Wagner et al. (2012)       |
| pSVA1203 In-frame deletion of aglB (saci1274) cloned into pSVA407 with NotI, BamHI | This study          |
| pSVA1204 In-frame deletion of aglB (sire1141) cloned into pSVA407 with PstI, BamHI | This study          |
| pSVA1241 Integration plasmid, saci1162::aglB cloned into pSVA407 with ApaI, BamHI | This study          |
| pSVA1244 aglB::pyrEF-aglB-down, cloned into pUC19 with EcoRI, KpnI | This study          |
| pSVA1266 Expression plasmid of agl3 cloned into pSVA1450 with Ncol, EagI | Meyer et al. (2011)       |
| pSVA1274 Expression plasmid of agl16 cloned into pSVA1450 with Ncol, EagI | Meyer et al. (2013)       |

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Constructing the plasmid for the linear aglB<sub>up</sub>-pyrEF-aglB<sub>down</sub> fragment integration

In order to further underline the essential property of aglB in *S. acidocaldarius*, a disruption of the aglB gene by the direct homologous integration of the pyrEF cassette was performed. For this approach, 1200 bp of the aglB upstream region, the full 1525 bp of the pyrEF cassette, and 1061 bp of the aglB downstream region were PCR amplified. For the amplification of the aglB upstream region the upstream forward primer (1802) incorporated an EcoRI restriction site, whereas the upstream reverse primer (1803) incorporated 15 bp of the reverse complementary strand of the pyrEF forward primer. The pyrEF forward primer (1804) incorporates 15 bp of the reverse complementary strand of the upstream reverse primer, leading to a 30 bp overlapping stretch. The pyrEF reverse primer (1783) was designed to incorporate 15 bp of the reverse complementary strand of the downstream forward primer. The aglB downstream forward primer (1785) incorporated 15 bp of the reverse complementary strand of the pyrEF reverse primer at its 5'end. The aglB downstream reverse primer (1785) possesses a KpnI restriction site at the 5'end. The three PCR fragments were fused via an overlap PCR using the 3' overlapping ends of each PCR fragment. The amplified 3765 bp overlap PCR fragment was further amplified using the outer primers (1802 and 1785), digested with EcoRI and KpnI and ligated into the pUC19 vector, predigested with the same restriction enzymes. The obtained plasmid pSVA1244 (aglB<sub>up</sub>-pyrEF-aglB<sub>down</sub>) was transformed into *E. coli* DH5α and selected on LB-plates containing 50 µg mL<sup>-1</sup> ampicillin. The accuracy of the plasmid was verified by sequencing. Before transformation in *S. acidocaldarius* the plasmid was digested with KpnI and EcoRI, to create the linear aglB<sub>up</sub>-pyrEF-aglB<sub>down</sub> fragment.

Transformation and selection of the deletion mutant in *S. acidocaldarius*

Generation of competent cells was performed based on the protocol of Kurosawa and Grogan (Kurosawa and Grogan 2005). Briefly *S. acidocaldarius* strain MW001 was grown until an OD<sub>600</sub> between 0.1 and 0.3 in Brock medium supplemented with 0.1% w/v NZ-amine and 0.1% dextrin. Cooled cells were harvested by centrifugation

### Table 2. Primers used in this study.

| Primer | Sequence (5'-3') | Restriction site |
|--------|------------------|-----------------|
| sac1162::aglB | CTCACTGGGCCGGACCAAGAGCGACAAAGAG | ApaI |
| 1891 | ATT TCAAGATGCTAAACGGATAGGTTCTCTG | |
| 1892 | CTTACAGGTACTTAAAGCGATAGGTTCTCTG | |
| 1893 | CTTAATCATTTTATTTATGCAAAATGATTACTAGGATATAGGTTCTCTG | |
| 1894 | TATCACTGTTTCATAATAAATAATGATATTTACAGCTACCGGAGGAAAGAG | |
| 1895 | CGCCGGAGGACTCAGTAAACACGCGATAGGTTCTCTG | |
| 1896 | TTGCAGACAAGGCTTATATCC | |
| 1897 | CGCCGAGGATCCGCTACAGGAAATGCTTCTGACAG | |
| 1706 | CCCCGTCGACTACGGAAATGCTTCTGACAG | |

In-frame deletion of aglB (saci1274)

| Primer | Sequence (5'-3') | Restriction site |
|--------|------------------|-----------------|
| 1725 | ATAAAGAATGGCCGAGCTATTAGAATAGTACACCACCTAAATACT | NotI |
| 1712 | CTAATGCGAGCCGAGTTTAAGGAGGTTCCTG | |
| 1713 | ATGCAAAGTCTTAAAGTAAACACGCGATAGGTTCTCTG | |
| 1726 | CCCCGCCGGATCTTACGGAATGCTTCTGACAG | |

In-frame deletion of aglB (sIRE1141)

| Primer | Sequence (5'-3') | Restriction site |
|--------|------------------|-----------------|
| 1727 | CCCACTCTGACGTCATTGGAGGTAAAGAG | PstI |
| 1718 | AACCAGGGCTGTGTTACTAAACTGACGGTCTCTTAGAGGTAAAGAG | |
| 1719 | ATGCAACTGATTAAAGTAAACACGCGATAGGTTCTCTG | |
| 1728 | CCCCGGAGGATCTTACGGAATGCTTCTGACAG | |

aglB-pyrEF

| Primer | Sequence (5'-3') | Restriction site |
|--------|------------------|-----------------|
| 1802 | CCCACTGACTACCCGAGGTAAGAGATACAC | EcoRI |
| 1803 | CTCAAACTTTAAGGACCGAGTAAATGCTATG | |
| 1804 | TTCCTATAGGTTTAGGAGGTACACTTG | |
| 1783 | GTTGTTACGGAGAGGAGTCCCGGCGGCTTACCCAC | |
| 1784 | CGTCCGATCTCCCTCGTAAGACTCAGCGATAGGTTCTCTG | |
| 1785 | CCCACTGAGACTACGTTTCTACAGGAAAGAG | KpnI |

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(2000g at 4°C for 20 min). The cell pellet was washed three times in 50 mL, 10 mL, and 1 mL of ice cold 20 mmol/L sucrose (dissolved in demineralized water) after mild centrifugation steps (2000g at 4°C for 20 min). The final cell pellet was resuspended in 20 mmol/L sucrose to a final OD600 of 10 and stored in 50 μL aliquots at −80°C. 400–600 ng of methylated pSVA1203, pSVA1204, pSVA1241 plasmids or the linearized ablB sucrose to a final OD600 of 10 and stored in 50 μL aliquots at 75°C. Before plating, the sample and incubated at 75°C for 30 min under mild shaking conditions (150 r.p.m.). When placed, the sample was mixed with 100 μL of heated 2x concentrated recovery solution and two aliquots of 100 μL were spread onto two different gelrite plates containing Brock medium supplemented with 0.1% NZ-amine and 0.1% dextrin. After incubation for 5–7 days at 75°C large brownish colonies were used to inoculate 50 mL of Brock medium containing 0.1% NZ-amine and 0.1% dextrin, each culture was incubated for 3 days at 78°C. Each culture, which has been confirmed by PCR to contain the genomically integrated plasmid, was grown in Brock medium supplemented with 0.1% NZ-amine and 0.1% dextrin until an OD of 0.4. Aliquots of 40 μL were spread on second selection plates, supplemented with 0.1% NZ-ame and 0.1% dextrin and 10 μg mL−1 uracil, were incubated for 5–7 days at 78°C. Newly formed colonies were streaked out on new second selection plates to ensure that they were formed from single colonies, before each colony was screened for the genomic absence, presence or modification of the aglB gene by PCR.

Results

S. acidocaldarius aglB is localized next to genes encoding the translation machinery and separated from genes coding for GTases

The OTase AglB in S. acidocaldarius, encoded by saci1274, was first identified by bioinformatic methods, studying the distribution of the OTase among Archaea (Magidovich and Eichler 2009). AglB is a conserved membrane protein with 22% sequence identity to the eukaryal Stt3p from S. cerevisiae, 20% sequence identity to the bacterial PglB from C. jejuni, and 22% sequence identity to the archaeal AglB from P. furiosus. Apart from this low sequence homology, the protein possesses a similar topology with other identified OTases. Saci1274 possesses 15 predicted TD and a soluble C-terminal domain, which is enclosed between the TD 14 and 15. The eukaryal Stt3p contains 11 predicted TD and a soluble C-terminal domain (Kim et al. 2005), whereas the bacterial PglB possesses 13 N-terminal TD and C-terminal periplasmic domain (Lizak et al. 2011). Furthermore, the highly conserved WDGY motif and the DxxK motif were found within the protein sequence of Saci1274 (Fig. 1), which strengthens the proposed function of Saci1274 as an OTase (Magidovich and Eichler 2009; Kaminski et al. 2013).

In contrast to the gene organization of C. jejuni and Hfx. volcanii, for which the gene coding for the OTase is found directly located next to genes coding for GTases participating in the N-glycosylation process, no genes coding for a GTase were located directly near to aglB (saci1274) (Fig. 2), as previously reported (Magidovich and Eichler 2009; Kaminski et al. 2013). Only a newly identified GTase (Saci1262) is found with distance of a few genes to aglB (Fig. 2), which shows homologies to MurG, an N-acetylglucosamine transferase.

Deletion of aglB by markerless in-frame deletion was unsuccessful, supporting an essential role of AglB in S. acidocaldarius and S. islandicus

Due to the low sequence similarity to the known OTase, we wanted to demonstrate the predicted function of AglB in vivo with a markerless deletion mutant of aglB in the genome of S. acidocaldarius. The aglB deletion mutant should be impaired in the final step of glycosylation process, resulting in the nonglycosylation of known glycoproteins, as it was shown for Hfx. volcanii, M. voltae, and M. maripaludis (Chaban et al. 2006; Abu-Qarn et al. 2007; Vandyke et al. 2009). The genomic integration by homologous recombination of the plasmid pSVA1203 via either the up- or downstream region of the aglB and the selection pyrEF genes was confirmed by PCR using isolated genomic DNA of strains grown on the first selection plates and the external primers of the up- and downstream region (Fig. 3A). The two amplified fragments correspond to the full-length aglB (3500 bp) and the ∆aglB (1280 bp) PCR products (Fig. 3A). The DNA from the background strain MW001 and the plasmid pSVA1203 were used as a PCR control, showing either the full-length aglB or the ∆aglB PCR fragment. A second recombination step, resulting in the loop out of the integrated plasmid, would produce colonies containing either the wild type genome sequence or the deleted version (Wagner et al. 2009, 2012). Screening of more than 200 second selection colonies by PCR did not reveal any...


\[ \Delta aglB \] strain (Fig. 3A). Furthermore, the modification of the second selection condition; for example, a higher pH of 4 or a reduced incubation temperature of 60°C, did not lead to the preferred deletion strain. This result indicated that \( aglB \) might be essential in \( S. acidocaldarius \).

To test whether we were not able to obtain any \( aglB \) mutant in \( S. acidocaldarius \) due to a problem caused by alterations in the genetic neighborhood, we wanted to create a markerless \( aglB \) deletion mutant in the \( S. islandicus \) strain E233S1 (Deng et al. 2009). \( S. islandicus \) shows a different \( aglB \) upstream gene organization, lacking the gene coding for the tRNA-Ala (Fig. 2). The genomic integration of the plasmid pSVA1204, incorporating only the up- and downstream regions of \( aglB \) and the selection genes \( pyrEF \), was confirmed by PCR (Fig. 3B). The DNA from the background strain \( S. islandicus \) E233S1 and the plasmid pSVA1204 were used as a PCR control. A PCR performed with genomic DNA from the strain, with plasmid pSVA1204 incorporated into the genome, generated two PCR fragments corresponding to the up- and downstream region containing either the full-length \( aglB \) or \( \Delta aglB \). Screening of 100 sec selection colonies for the presence of the \( \Delta aglB \) version, revealed only the presence of strains possessing the full-length \( aglB \) wild type version (Fig. 3B), as it was shown for \( S. acidocaldarius \) (Fig. 3A). This result further strengthens the idea that the oligosaccharyltransferase AglB is essential for the viability and survival of \( Sulfolobus \) species.

**Disruption of \( aglB \) by the insertion of the \( pyrEF \) selection marker resulted in a lethal phenotype**

As the procedure of markerless in-frame deletion resulted in any \( aglB \) deletion mutants, we wanted to enforce the deletion of \( aglB \) by a single homologous recombination step disrupting the \( aglB \) gene by the insertion of the \( pyrEF \) selection cassette. Furthermore, the PCR fragment of the upstream region of \( aglB \) was changed leaving the first 163 bp on the \( aglB \) untouched, thereby avoiding the interference with the transcription start of the tRNA-Ala, found directly upstream of \( aglB \). The linearized \( aglB_{up-pyrEF-aglB_{down}} \) fragment was transformed into competent MW001 cells, cells were subsequently streaked on a first selection plate. After 9 days of incubation only tiny colonies were detected on the first selection plate. However,
none of these colonies were able to grow in liquid first selection medium or on a second first selection plate. This result showed that the direct disruption of \textit{aglB} leads to a lethal phenotype, which furthermore strengthens the idea that \textit{aglB} is essential for the viability of \textit{S. acidocaldarius}.

**Successful deletion of \textit{aglB} in a \textit{S. acidocaldarius saci1162::aglB} background strain**

So far every attempt to create a deletion mutant of \textit{aglB} failed. These attempts included the markerless in-frame deletion procedure at low temperature (60°C) as well as a direct homologous recombination with \textit{pyrEF} disrupting the \textit{aglB} gene. All these attempts hinted at an essential role of AglB in \textit{S. acidocaldarius}. To confirm the essential properties of AglB, a second copy of \textit{aglB} was integrated in exchange for \textit{saci1162}, encoding an \textit{\alpha}-amyrase, which has been shown to be not essential in \textit{S. acidocaldarius} (Worthington et al. 2003; Gristwood et al. 2012). The integration of the plasmid pSVA1241, used for the homologous recombination and integration of the second \textit{aglB} gene copy, can occur in different ways (i) via the upstream \textit{aglB} region (Fig. 4A), (ii) the downstream \textit{aglB} region or (iii) directly by the \textit{aglB} region (Fig. 4B). To confirm upstream integration of the plasmid pSVA1241, a PCR was performed using a forward primer against the upstream region of the \textit{saci1162} (Primer 991) and a reverse primer against the internal region of \textit{aglB} (Primer 1713) (Fig. 4A). Furthermore, the homologous recombination of the plasmid via the \textit{aglB} gene was tested by a PCR using a forward primer against \textit{pyrEF} cassette (Primer 1896) and a reverse primer against the downstream region of the original \textit{aglB} (Primer 1706) (Fig. 4B). The PCR result showed that the colonies 139 and 167 have integrated the plasmid pSVA1241 via a homologous recombination of the upstream region of the \textit{\alpha}-amyrase (\textit{saci1162::pSVA1241}), as it is shown in Figure 4A. For other tested colonies, the PCR using a forward primer binding to the internal site of the plasmid and a reverse primer binding to the downstream region of \textit{aglB} showed that integration of the plasmid occurred via the \textit{aglB} region, within the colonies 135–138, 165, 166, and 168 (Fig. 4B). The fact that most of the selected strains...
showed integration directly via the $aglB$ site, is most likely due to the larger size of $aglB$ compared to the $\alpha$-amylase upstream region. However, none of the selected colonies showed an integration of the downstream region. The colony 167, in which the plasmid integrated via the $\alpha$-amylase upstream region ($saci1162::pSVA1241$), was used for second selection. Colony PCR, using forward and reverse primer outside of the $\alpha$-amylase ($saci1162$) region with genomic DNA from obtained second selection colonies, showed that the colonies 002, 005-009, 012, and 014 successfully integrated the second copy of $aglB$ by replacing the $saci1162$ gene (Fig. 4C). These colonies showed the calculated PCR fragment size of 3977 bp corresponding to the $saci1162$::$aglB$ mutant region, as it is shown in the plasmid control (Fig. 4C). The colony 002, in which the $\alpha$-amylase was substituted by $aglB$ ($saci1162::aglB$), was termed strain MW098 and used as a background strain to create an $aglB$ knockout of the original gene site. First selection colonies of MW001 and MW098 ($saci1162::aglB$) transformed with the $aglB$ deletion plasmid pSVA1203 were confirmed by PCR (data not shown), and used for second selection procedure. Selected colonies were screened for the presence of an $aglB$ deletion mutant. PCR revealed two colonies (colony 14 and 16) originating from the MW098 ($saci1162::aglB$) background strain which showed the calculated PCR fragment size of 1280 bp corresponding to the $\Delta aglB$ region (Fig. 5B). The colony 16 was selected as the new strain MW099 ($\Delta aglB$, $saci1162::aglB$). However, none of the 40 newly selected colonies in the MW001 background showed any fragment corresponding to the $\Delta aglB$ region by PCR amplification, which showed only the PCR product corresponding to the full $aglB$ (3500 bp) (Fig. 5A), as it was shown before (Fig. 3A). The results highlight that the difficulty generating a $\Delta aglB$ mutant in a MW001 background does not originate from the genetic approach, in which the recombinant plasmid can only be used in a $saci1162::aglB$ background to create the $\Delta aglB$ mutant, clearly demonstrates that $aglB$ is essential for the survival of $S. acidocaldarius$.

**Deletion of $aglB$ in the $saci1162::aglB$ background strain was not altered under noninduced conditions**

Since the second copy of the $aglB$ is under control of the $\alpha$-amylase promoter, which is maltose inducible, we hypothesize that growth of MW099 ($\Delta aglB$, $saci1162::aglB$) is maltose dependent. For this reason the MW001 and the MW099 were grown in Brock medium supplemented with 0.1% maltose or 0.1% xylose (Fig. 5C). In medium containing xylose MW099 had a longer lag phase than...
MW001, but reached the same final OD (Fig. 5C). It seems that the basal transcription levels of the amyA promoter under noninduced conditions are sufficient for optimal aglB expression. In Brock medium supplemented with 0.1% maltose, the growth of the mutant as well as MW001 was reduced compared to the medium supplemented with 0.1% xylose. MW099 stopped growing at an OD$_{600}$ of 0.6, but after a lag phase of around 50 h reached the same final OD as the MW001 background strain. We believe that the 15-fold induction of the amyA promoter under induced conditions (Wagner et al. 2014), might lead to an overexpression of AglB causing the growth defect by influencing membrane organization.

**Discussion**

The OTase (Stt3p/AglB/PglB) as the key enzyme of the N-glycosylation process has to regulate and select glycosylation of sequences, thereby catalyzing the en bloc transfer of the lipid linked oligosaccharide onto a nascent protein. For this it has to interact with the translocon as well as with the subsequent protein folding process. To achieve this, higher Eukarya evolved a multimeric Otase complex, which is not only supporting but also essential for the OTase activity. Based on the lack of an N-glycosylation process in bacterial model organism *Escherichia coli* it was assumed that this co- and posttranslational modification is restricted to Eukarya. As so the discovery of a prokaryotic N-glycosylation process in Archaea as well as in a few bacterial species, depending on a single AglB or PglB Otase subunit homologous to the eukaryotic Stt3p, was unexpected (Szymanski et al. 1999; Wagner et al. 2002; Weerapan and Imperiali 2006; Maita et al. 2010).

In contrast to the essential properties of the eukaryal N-glycosylation, the prokaryotic N-glycosylation process can be abolished by the deletion of *aglB* or *pglB* (Wacker et al. 2002; Chaban et al. 2006; Abu-Qarn et al. 2007; Vandyke et al. 2009). In *C. jejuni*, the deletion of *pglB* did not lead to a drastic change of the phenotype, although more than 65 proteins with a multitude of cellular functions have been shown to be modified via N-glycosylation (Scott et al. 2011). The most important effect of the deletion of *pglB* is the nonglycosylation of the type 4 secretion system (T4SS), which affected animal colonization and invasion (Szymanski and Wren 2005). In the archaeal species *Hfx. volcanii*, *M. voltae*, and *M. maripaludis*, for which the N-glycosylation process has been studied in detail, the deletion of *aglB* does not influence growth under standard growth conditions (Chaban et al. 2006; Abu-Qarn et al. 2007; Vandyke et al. 2009). However, the deletion of *aglB* in each of these archaeal species results in an non glycosylated archaellum, as the cells appear nonarchaellated and are impaired in their motility (Chaban et al. 2006; Abu-Qarn et al. 2007; Vandyke et al. 2009; Tripepi et al. 2012). Furthermore, the depletion of the N-glycosylation process in *Hfx. volcanii* has an effect on the growth under elevated salt concentration as well as an enhanced release of the S-layer glycoproteins into the medium (Abu-Qarn et al. 2007). However, the nonessential property of *aglB* in the so far studied archaeal species is in contrast to our findings, in which we demonstrated that aglB is essential for the viability of *S. acidocaldarius*. The essentiality of aglB from *S. acidocaldarius* might reflect a stronger need for the thermostabilization of proteins by the N-glycosylation process. Indeed the number of glycosylation sites found in mesophilic and
thermophilic archaeal S-layer proteins underlines this idea (Meyer and Albers 2013). In the mesophilic and halophilic archaeon *Hfx. volcanii*, seven glycosylation sites are predicted within the S-layer amino acid sequence (Jarrell et al. 2010), of these six sites have been experimentally shown to be modified N-glycans (Sumper et al. 1990; Mengele and Sumper 1992; Abu-Qarn et al. 2007; Magidovich et al. 2010; Parente et al. 2014). In contrast, more than 30 glycosylation sites could be detected within the S-layer glycoprotein from the thermophilic *S. acidocaldarius* and other species from the *Sulfolobales*. Of the eleven N-glycosylation sites analyzed in the C-terminal part of the S-layer protein, nine were experimentally confirmed to be modified, whereas the other two sites are likely to be glycosylated (Peyfoon et al. 2010). This high amount of N-glycosylated residues, where one modification is found after an average stretch of 30–40 residues, has not been reported for any other Archaea, however, it seems that thermo(acido)philic Archaea tend to possess S-layer proteins with a remarkably high number of predicted N-glycosylation sites compared to mesophilic Archaea.

The discrepancy between the N-glycosylation frequencies in different Archaea might explain why the N-glycosylation process can be abolished in *Hfx. volcanii*, *M. maripaludis* or *M. voltae*, as these organisms show only a minor amount of glycosylation modification of the S-layer protein (Meyer and Albers 2013). Indeed possessing fully stable S-layer, as the sole cell envelope of *S. acidocaldarius*, is of great importance for the cell integrity and viability of this organism. Defects in the biosynthesis of the full-length N-glycan in the MW039 (Δagl3) and MW043 (Δagl16) deletion strains of *S. acidocaldarius* show that the growth (under high salinity) as well as the motility is strongly dependent on the N-glycan size (Meyer et al. 2011, 2013). These results demonstrated that even one missing hexose of the N-glycan has an effect on the growth and motility. These effects were increased with the reduction in the amount of the N-glycan sugars (Meyer et al. 2011, 2013).

The essentiality of the N-glycosylation process in *S. acidocaldarius* was furthermore supported by the use of the antibiotics bacitracin (Meyer and Schafer 1992) and tunicamycin (Hjort and Bernander 1999). The treatment with these antibiotics led to cell growth arrest and later cell death of *S. acidocaldarius*, as these antibiotics interfere with the initial steps of the N-glycosylation. Bacitracin blocks the release of one phosphate from dolichol pyrophosphate, thereby blocking the regeneration of dolichol phosphate, used as the N-glycan lipid carrier. Tunicamycin inhibits the initiation step of the N-glycosylation by blocking the active site of UDP-N-acetylglucosamine-1-phosphateldolichyl-phosphate GlcNAc-1-phosphotransferase (Alg7 in *Eukaria*, AlgH in Archaea). However, it should be mentioned that in contrast to the deletion of *aglB* the deletion of *aglH* homologs in *M. voltae* (Chaban et al. 2006) and *M. maripaludis* (D. VanDyke and K. F. Jarrell, unpublished data) were also unsuccessful, implying the essentiality the lipid modification with glycans, beyond the N-glycosylation.

The advantage of having now an expression system in *Sulfolobus* (Wagner et al. 2012) will lead to future experiments elucidating the catalytic mechanism of AglB, and will provide us with new insights about AglB, that is, glycosylation state, interaction partners, selectivity of substrate, and temperature activity.

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**Conflict of Interest**

None declared.

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