A Rhomboid Protease Gene Deletion Affects a Novel Oligosaccharide N-Linked to the S-layer Glycoprotein of Haloflexax volcanii*

Received for publication, January 8, 2014, and in revised form, February 21, 2014. Published, JBC Papers in Press, March 4, 2014, DOI 10.1074/jbc.M113.546531

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Background: Rhomboid proteases are ubiquitous, and their role in Archaea has not been explored.

Results: We generated a rhomboid deletion mutant that displayed a glycosylation defect.

Conclusion: Deletion of a rhomboid protease gene altered S-layer glycoprotein N-glycosylation.

Significance: This work provides structural characterization of a novel oligosaccharide bound to H. volcanii S-layer glycoprotein and relates a rhomboid protease with the protein glycosylation process.

Rhomboid proteases occur in all domains of life; however, their physiological role is not completely understood, and nothing is known of the biology of these enzymes in Archaea. One of the two rhomboid homologs of Haloflexax volcanii (RhoII) is fused to a zinc finger domain. Chromosomal deletion of rhoII was successful, indicating that this gene is not essential for this organism; however, the mutant strain (MIG1) showed reduced motility and increased sensitivity to novobiocin. Membrane preparations of MIG1 were enriched in two glycoproteins, identified as the S-layer glycoprotein and an ABC transporter component. The H. volcanii S-layer glycoprotein has been extensively used as a model to study halarchaeal protein N-glycosylation. HPLC analysis of oligosaccharides released from the S-layer glycoprotein after PNGase treatment revealed that MIG1 was enriched in species with lower retention times than those derived from the parent strain. Mass spectrometry analysis showed that the wild type glycoprotein released a novel oligosaccharide species corresponding to GlcNAc-GlcNAc(Hex)₂-SQ-Hex-SQ, in contrast to the mutant protein, which contained the shorter form GlcNAc₂(Hex)₂-SQ-Hex-SQ. A glycoproteomics approach of the wild type glycopeptide fraction revealed Asn-732 peptide fragments linked to the sulfoquinovose-containing oligosaccharide. This work describes a novel N-linked oligosaccharide containing a repeating SQ-Hex unit bound to Asn-732 of the H. volcanii S-layer glycoprotein, a position that had not been reported as glycosylated. Furthermore, this study provides the first insight on the biological role of rhomboid proteases in Archaea, suggesting a link between protein glycosylation and this protease family.

Intramembrane-cleaving proteases (I-CLiPs) hydrolyze proteins localized in lipid bilayers (1). Their activity exposes and/or releases functional domains of substrate proteins serving as a regulatory mechanism for diverse signaling pathways (2). Based on their catalytic mechanism, I-CLiPs are grouped into three classes: the S2P metalloproteases; the GxGD-type aspartyl proteases, which include presenilin/γ-secretase and signal peptide peptidases; and the rhomboid family of serine proteases (2). Rhomboid proteases (Rho) are conserved in the three domains of life and have been implicated in a variety of processes, including epidermal growth factor signaling in Droso Ball melano gente (3), mitochondrial dynamics in yeast (4, 5), and apicomplexan parasite invasion (6, 7). The relevance of Rho in the physiology of prokaryotes has been poorly investigated. The AarA rhomboid protease from the pathogenic bacterium Providencia stuartii cleaves the N-terminal extension of TatA, a membrane-bound component of the twin arginine protein translocation pathway. Processing of TatA activates the translocation process, allowing the export of an unknown quorum-sensing signal (8). In Mycobacteria, Rho null mutants display impaired biofilm formation and increased antibiotic sensitivity (9). Rho homologs are widely represented in archaeal genomes, and the predicted proteins contain the typical multispanning transmembrane domains (TMDs) and the amino acid residues involved in catalysis (Ser-His dyad), suggesting that they are probably functional enzymes (10). However, archaeal Rho remain uncharacterized, and their function has not yet been addressed.

The abbreviations used are: Rho, rhomboid protease(s); PNGase F, peptide-N-glycosidase F; TMD, transmembrane domain; qPCR, quantitative PCR; HPAEC, high performance anion exchange chromatography; PAD, pulsed amperometric detection; LID, laser-induced dissociation; SQ, sulfoquinovose (6-deoxy-6-sulfoglucose).

* This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas Grants PIP-1783 and 1548, Universidad Nacional de Mar del Plata Grant Exa 454/09 (to R. E. D. C.), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) Grant PICT-01824 (to M. I. G.), and Universidad de Buenos Aires Grant 20020100100517 (to A. C.). The Ultraflex II (Bruker) TOF/TOF mass spectrometer was supported by ANPCyT Grant PME 125.

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Archaea predominate in environments lethal to most cells, including extreme temperatures, pH values, and salinity. Haloarchaea flourish in habitats containing high salt concentrations (2–5 M NaCl). The only barrier between the cytoplasmic membrane and the harsh environment is the S-layer, a highly organized proteinaceous, two-dimensional crystalline array of one or more proteins that self-assemble around the entire cell surface. S-layer proteins are often glycosylated, and they are thought to play a critical role in the interaction of these microorganisms with the environment (11, 12). In the haloarchaeon Haloferax volcanii, the S-layer glycoprotein is an 81.7-kDa protein containing both N- and O-glycosidic bonds. O-Glycosylation of this protein occurs through binding of glucosyl (1→2) galactose disaccharides to a C-terminal threonine cluster (13, 14). Of the seven putative N-glycosidic sequons found within the S-layer glycoprotein, it was determined that Asn-370 and Asn-83 are modified by a pentasaccharide comprising two hexoses, two hexuronic acids, and a methyl ester of hexuronic acid (15, 16). It was also determined that the sequon at Asn-370 is not modified (15). The aforementioned pentasaccharide became a model to study the protein N-glycosylation process in Archaea (17), and it has also been found decorating H. volcanii flagellins (18). Interestingly, growth at different salt concentrations leads to alterations in H. volcanii S-layer glycoprotein N-glycosylation. In high salt, S-layer glycoprotein Asn-13 and Asn-83 are modified by the pentasaccharide. However, when cells were grown in low salt, substantially less pentasaccharide was detected, whereas a distinct tetrasaccharide composed of three hexoses (one of them sulfated) and a rhamnose, which is absent in cells grown at high salinity, was found at Asn-498. Thus, in response to changes in environmental salinity, H. volcanii modulates not only the N-linked glycans decorating the S-layer glycoprotein but also the sites of such posttranslational modification (19). Recently, it has been reported that two different glycosylation pathways participate in the synthesis of the tetra- and pentasaccharide (20).

To explore the biological role of Rho in Archaea, we constructed a null mutant of a zinc finger-Rho (RhoII) in the model haloarchaeon H. volcanii. The mutant evidenced reduced motility and increased sensitivity to novobiocin as well as a different electrophoretic pattern of glycoproteins compared to the parental strain. We report for the first time the presence of N-acetylgalactosamine N-linked oligosaccharides in the S-layer glycoprotein of H. volcanii and show that these sugar chains are shorter in the strain deficient in the RhoII protease. Furthermore, we provide information on the structure and composition of this novel oligosaccharide and show that it is linked to Asn-732, a putative glycosylation site where no modification had been reported so far.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**

Strains, plasmids, and primers used in this study are listed in Table 1. H. volcanii strains were grown in 18% (w/v) MGM or CA medium at 42 °C and 150 rpm. For motility assays, H. volcanii strains were stab-inoculated in 0.25% agar CA plates and grown at 42 °C for 2–3 days. Motility was determined by measuring the diameter of the swimming ring, using the ImageJ program.

*Escherichia coli* was grown in Luria-Bertani medium (LB), with ampicillin (100 µg ml⁻¹) when needed. *E. coli* was transformed by the CaCl₂ method (22). To induce the synthesis of chimeric substrates, the cultures (A₆₀₀ = 0.4–0.7) were incubated with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h.

**Cell Fractionation**

H. volcanii cultures were grown to an A₆₀₀ = 1–1.5, and cells were harvested by centrifugation (10,000 × g for 10 min, 4 °C). Cell pellets were suspended in 50 mM HCl-Tris, 2 mM NaCl (pH 7.5) and disrupted with an ultrasonic processor (3 × 30 s, 80 W). Lysates were clarified by centrifugation (17,000 × g for 20 min at 4 °C), and membranes were pelleted by centrifugation (70,000 × g for 1 h at 4 °C), washed with the same buffer, and recentrifuged for 30 min. Membrane fractions were suspended in 1× SDS-PAGE loading buffer containing 0.1% (w/v) SDS and 0.05 M DTT, incubated for 10 min at 70 °C. Samples used for oligopeptide analysis were further treated with 10 mM iodoacetamide and incubated at room temperature for 30 min in darkness.

Cell extracts of *E. coli* cells harboring recombinant plasmids that encoded chimeric Rho substrates (23) were obtained as follows. Cells were harvested by centrifugation (10,000 × g for 10 min at 4 °C), and pellets were suspended in 20 mM HCl-Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 1.5 µM pepstatin, and 1 mg ml⁻¹ lysozyme. Cells were disrupted, and lysates were clarified as described above. The supernatants were used as a source of Rho substrates.

**Protease Assay**

*E. coli* MGI655 harboring the plasmids with the heterologous substrates were used to prepare cell extracts. These preparations were incubated with H. volcanii membrane fractions in 0.2% (w/v) dodecyl maltoside, 50 mM HCl-Tris (pH 7.5), 1.2 M NaCl, and 1 mM EDTA (final volume 75 µl) at 37 °C for 16 h. After incubation, trichloroacetic acid (TCA) was added to a final concentration of 10% (v/v), and samples were incubated on ice for 30 min, centrifuged (17,000 × g, 15 min), and washed twice with cold 80% (v/v) acetone. Pellets were air-dried and suspended in Laemmli (24) sample buffer.

**SDS-PAGE and Western Blotting**

Samples were applied onto polyacrylamide gels containing 0.1% SDS. After electrophoresis, proteins were visualized with colloidal Coomassie Brilliant Blue G-250 or periodic acid-Schiff staining (25).

For Western blotting, gels were transferred to PVDF membranes and blocked with TBST buffer (25 mM Tris·HCl, pH 7.4, 0.01% (v/v) Tween 20) with 5% (w/v) skimmed milk. Membranes were incubated with 1:3000 anti-His antibody in blocking buffer, washed with TBST (3 × 10 min), and incubated with 1:10,000 alkaline phosphatase-conjugated anti-mouse IgG for 2 h. After washing (TBST, 3 × 10 min), blots were developed.
Colonies were screened by PCR, using the Hvo0727KOverify5 primer and Hvo0727KOverify3 primer, followed by sequencing of the amplicon to confirm that the chromosomal replacement event had occurred.

**Generation of a Construct Containing the Complete rhoII/endV Operon and Its Regulatory Sequences**

The chromosomal region comprising 207 bp upstream of the rhoII gene and 113 bp downstream of the endV stop codon was amplified by PCR, using genomic H. volcanii H26 DNA as a template. The amplicon (2037 bp) was cloned into the TOPO-Blunt vector (Invitrogen), digested with ApaI and HindIII, and subcloned into pTA963 previously digested with EcoRI/BamHI, and subcloned into pTA963 previously digested with EcoRI/BamHI, and subcloned into pTA963 previously digested with EcoRI/BamHI. DNA as a template. The amplicon (2037 bp) was cloned into the TOPO-Blunt vector (Invitrogen), digested with ApaI and HindIII, and subcloned into pTA963 previously digested with EcoRI/BamHI.

**Construction of an H. volcanii rhoII In-frame Knock-out Mutant**

The knock-out constructs were generated as described elsewhere (26). In brief, 800 bp flanking each end of the rhoII gene were PCR-amplified and sequentially cloned into the EcoRI/HindIII (upstream region) and the BamHI/XbaI (downstream region) sites of the haloarchaeal suicide vector pTA131. The construct described above (pMIG1) was first amplified in E. coli DH5α and then passed through E. coli GM33 (dam−) to obtain non-methylated plasmid, which was then transformed into H. volcanii H26 using the polyethylene glycol (PEG) method.9 A single homologous recombination event between one of the flanking regions on the knock-out construct and the chromosome (pop-in) was selected for by growth on CA medium, which lacks uracil. Recombinants were next grown in liquid medium comprising 207 bp upstream and 103 bp downstream of Hvo_0727 start codon and 113 bp downstream of Hvo_0727 stop codon, cloned between the Apal and BamHI sites.

**Real-time Quantitative PCR**

RNA was extracted as described previously (27), concentration was determined spectrophotometrically, and integrity was assessed by gel electrophoresis in gels. Contaminant proteins and genomic DNA were eliminated by phenol/chloroform/isoamylic alcohol (25:24:1, v/v/v) extraction and digestion with RNase A and RNase T1 DNase, respectively. The absence of contaminating DNA was assessed by PCR amplification using 7SF and 7SR primers. First strand cDNA was synthesized by incubating 2–5 µg of genomic DNA-free RNA and 0.5 µg of the indicated primer at 70 °C (5 min) and on ice (5 min). Then 0.5 µM dNTPs, 2.5 µl of 10X QPCR reaction mix with SYBR Green as the detection dye and ROX as the normalization dye. The PCR conditions consisted of a denaturation cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min, and a dissociation cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for

With 0.33 mg ml−1 nitro blue tetrazolium and 0.01 mg ml−1 5-bromo-4-chloro-3-indolyl-phosphate.
was cut out from the gel, frozen for 3 h, and washed (mixing for confidence). The protein band corresponding to the S-layer glycoprotein was cut out from the gel and washed with acetonitrile. The gel pieces were thoroughly reduced with 10 mM DTT in 50 mM NH4HCO3 at room temperature in darkness. After washing with 50 mM NaHCO3, pH 7 (30 μl), the gel pieces were rehydrated with 20 ng/μl of water and dried. The gel slices were thoroughly reduced with 10 mM DTT in 50 mM NH4HCO3, pH 7, and washed, and supernatants were removed and dried. Glycans were filtered through an Ultrafree McFilter (M, 5000), dried, resuspended in 0.1% (v/v) formic acid (20 μl), and left at room temperature for 40 min. Finally, the sample was dried and suspended in water.

**Acid Hydrolysis of the Released Oligosaccharides**

A sample of the released oligosaccharides obtained after PNGase F treatment was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 100 °C. The acid was eliminated by evaporation; the hydrolysate was dried and resuspended in water for HPAEC-PAD analysis.

**Analysis of Oligosaccharide and Monosaccharide Composition of S-layer Glycoprotein by HPAEC-PAD**

For HPAEC analysis, a DX-500 Dionex BioLC system (Dionex Corp.) with a pulse amperometric detector was used. The following columns and conditions were employed: (a) for oligosaccharides, Carbopack P-200 column equipped with a P-200 precolumn; gradient elution with 100 mM NaOH, 0–70 mM sodium acetate for 35 min; the flow rate was 0.45 ml min⁻¹; (b) for monosaccharide analysis, Carbopack P-20 column equipped with a P-20 precolumn; (i) for neutral and amino sugars, a 16 mM NaOH isocratic program was used, and (ii) for acidic sugars, a 25 mM NaOH, 80 mM NaAcO isocratic program was used; flow rate, 0.5 ml min⁻¹.

**Glycoprotein Digestion**

The protein band corresponding to the S-layer glycoprotein was cut out from the gel and washed with acetonitrile. The gel pieces were reduced with 10 mM DTT in 50 mM NH4HCO3 at 55 °C for 30 min. They were further washed with acetonitrile and alkylated with 55 mM IAA in 50 mM NH4HCO3 for 20 min at room temperature in darkness. After washing with 50 mM NH4HCO3 for 10 min and with acetonitrile for 5 min, they were dried in a SpeedVac. The gel slices were rehydrated with 20 ng μl⁻¹ trypsin (Sigma) in 40 mM NH4HCO3, 9% acetonitrile and incubated at 37 °C overnight. The peptides were extracted by sonication with 50% acetonitrile in 1% TFA, and supernatant was taken to dryness. In another case, further digestion was carried upon the addition of 20 ng μl⁻¹ Glu-C (V8) protease (Promega) in 50 mM NH4HCO3, pH 8, at 37 °C overnight. After incubation, samples were dried in a SpeedVac.

**Mass Spectrometry Analysis**

Matrices and calibrating chemicals were purchased from Sigma-Aldrich. Measurements were performed using an Ultraflex II TOF/TOF mass spectrometer equipped with a high performance solid-state laser (λ = 355 nm) and a reflector. The system is operated by the Flexcontrol version 2.4 software package (Bruker Daltonics GmbH, Bremen, Germany). Samples were irradiated with a laser power of 25–50% and measured in the linear and the reflectron modes, in positive and negative ion modes.

**Laser-induced Dissociation Tandem Mass Spectrometry (LID-MS/MS) Analysis in the MALDI-TOF/TOF-MS/MS Instrument**—The Ultraflex II MALDI-TOF/TOF MS spectrometer was used. For all experiments using the tandem time-of-flight LIFT mode, the ion source voltage was set at 8.0 kV with a precursor ion mass window of 3 Da. Precursor ions generated by LID were accelerated at 19.0 kV in the LIFT cell. The reflector voltage was set at 29.5 kV.

**Sample Preparation**—Prior to the analysis of the oligosaccharides by UV-MALDI-TOF MS, remnants of impurities were removed using a biphasic microcolumn consisting of C-18 phase and Dowex 50X-H⁺ resin. The sample was incubated on the column for 15 min and eluted with 500 μl of water and dried.

For glycomic analysis, 2,5-dihydroxybenzoic acid was used as matrix. The samples were loaded onto a ground steel plate (Bruker Daltonics GmbH) using the sandwich method. Mass spectra were the sum of 100–300 single laser shots, depending on the sample conditions.

For glycopeptide analysis, the glycopeptide mixtures obtained after protease digestion were purified by cotton HILIC SPE microtips as described elsewhere (29). The enriched glycopeptide mixtures were resuspended in 50% (v/v) acetonitrile in 1% (v/v) formic acid. The samples were loaded onto an AnchorChip target (Bruker Daltonics GmbH) as 50% mixtures with (3 μg μl⁻¹) α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% TFA.

**Spectrum Calibration**

External calibration reagents were used (commercial proteins bradykinin 1–7, M, 757.399; angiotensin I, M, 1296.685; renin substrate, M, 1758.933; and insulin β-chain, M, 3494.6506) with α-cyano-4-hydroxycinnamic acid as matrix in positive and negative ion mode and with β-cyclodextrin (cyclodextrinase, M, 1135.0) and γ cyclodextrin (cyclodextrinase, M, 1297.1) with norharmane as matrix, in positive and negative ion mode.

**RESULTS**

**Haloarchaeal Rho Display Distinctive Features and Can Process Heterologous Substrates**—To examine the occurrence of Rho in haloarchaea, a compilation of the proteases predicted in sequenced genomes was performed and manually verified.
Rholl Gene Deletion Affects Protein N-Glycosylation

**Figure 1. Haloarchaeal Rho display conserved active site residues and membrane associated Rho activity.** A, the translated amino acid sequences of Rho homologs were introduced in the TMHMM and SMART programs, and the retrieved topologies and domain architectures are schematically represented. Top, E. coli GlpG protein, representing Group 1. Middle, Hvo_1474, (Group 2). Bottom, Hvo_0727 (Group 3). Gray rectangles, TMDs; light gray rectangles, Rho domains; white circles, positions of active site serine and histidine; black ellipse, cytoplasmic zinc finger motif. The sequence of the zinc finger motif is shown at the bottom. Cyt. cytoplasm. B, cleavage of specific Rho substrates by H. volcanii solubilized membrane fractions. Cell extracts of E. coli MG1655 transformed with plasmid pKS505 (Gurken), pKS506 (LacY-TMD2), pKS507 (Spitz), or pKS508 (TatA) were incubated with (mH26) or without (−) solubilized membranes of H. volcanii H26. As a cleavage site control, cell extract of pKS508 incubated with E. coli Rosetta solubilized membranes (mEc) is shown on the right. After incubation, samples were TCA-precipitated and used in Western blotting experiments with anti-His antibody. The bands corresponding to unprocessed and processed substrates are pointed out by arrows. *, a nonspecific protein from E. coli extracts that cross-reacts with the anti-His antibody. Migration of molecular mass markers (kDa) is indicated at the left.

Using the BLAST program, available on the NCBI website. The in silico analysis showed that haloarchaea contain two or three sequences related to Rho except for the haloalkaliphilic archaeon *Natrialba magadii*, which encodes five Rho homologs. Analysis of the translated polypeptides evidenced the serine (GXSG) and histidine residues typical of the catalytic dyad of this protease family (Fig. 1A) (30) as well as the conserved asparagine and histidine residues, which have been proposed to contribute to oxyanion hole formation (31, 32). Recently, Baker and Urban (33) reported the occurrence of four primary sequence keystone regions in which both packing and hydrogen-bonding interactions are used to stabilize the structure of the *E. coli* Rho, GlpG. These regions are conserved in haloarchaeal Rho, suggesting that these enzymes are probably stabilized by the same molecular interactions as in their bacterial counterparts (not shown). The core region of bacterial Rho consists of six TMDs, whereas the eukaryotic members of this family have an additional TMD helix, either N- or C-terminal to the core six (34). Based on the SMART program (35, 36), different topologies were predicted for the haloarchaeal Rho; thus, in this work, they were classified into three groups (Fig. 1A). Group 1 is represented by the typical six-TMD proteins (HQ2329A, rrnAC2647, Nmag_2518, Hlac_0158, and NP3724A) homologous to *E. coli* GlpG. This type of Rho was not identified in *H. volcanii* and *Halobacterium* sp. Group 2 includes proteins with 8–12 TMDs (Hlac_1491, rrnAC0415, Nmag_3579, HQ1869A, Hvo_1474, NP2320A, and VNG0858C), which contain a Rho domain and extra TMDs of unknown function. Group 3 includes six TMD homologs in which the Rho domain is preceded by an N-terminal AN-1 zinc finger motif and occurs in all of the haloarchaea examined (Hvo_0727, Hlac_0599, VNG_0361, HQ1233A, rrnAC0675, Nmag_1128, and NP1156A). The *H. volcanii* Hvo_0727 zinc finger shows the typical pattern CX₉CX₉₆CX₉₂CX₄₂HX₄ₓHXC described in the conserved domain database (Fig. 1A, inset). The occurrence of the AN1 zinc finger-Rho arrangement has been reported in haloarchaeal and some methanogens (37). In this work, we expanded the search and found that this combination only occurs in members of *Euryarchaeota*, including all of the haloarchaea and *Archaeoglobus fulgidus, Archaeoglobus veneficus, Ferroglobus placidus, Methanococccoides burtonii, Methanohalophilus mahii, Methanoceela paludicola, Methanohalobiumвестигatum*, and the uncultured methanogenic archaeon RC-1. We performed a combined domain search with the SMART program (35, 36), which showed that the association of N-terminal zinc finger (B-box) domains with Rho is conserved in *Actinobacteria* and that C-terminal RBZ and DHHC zinc fingers exist in plant and fungus Rho.

To verify whether the predicted *H. volcanii* Rho were active enzymes, we measured the hydrolytic activity of solubilized membrane protein samples on chimeric protein substrates that contained the TMD of known Rho targets (23). All substrates tested, bearing the TMD of *D. melanogaster* Gurken and Spitz proteins, *E. coli* LacY-TMD2, and *P. stuartii* TatA protein, were processed in the presence of *H. volcanii* membranes (Fig. 1B). Furthermore, the products were of the same size as those generated in the presence of *E. coli* solubilized membranes, suggesting that substrate cleavage probably occurred in the same region in both organisms. Considering that the hydrolytic activity of this protease family is highly specific, this result strongly evidenced the presence of active rhomboid-like proteases in *H. volcanii*.
Construction and Phenotypic Characterization of a rhoII Deletion Mutant—To gain insight into the function of RhoII (Hvo_0727), an in-frame rhoII deletion mutant strain (denoted as MIG1) was constructed in *H. volcanii* by means of the “pop in-pop out” method (26). Gene elimination from the chromosome was confirmed by PCR using primers external to the deletion construct (Hvo0727KOverify5/H11032 and Hvo0727KOverify3/H11032), which produced amplicons of 2611 and 1708 bp in the parent (H26) and MIG1 strains, respectively (Fig. 2A, top). Additionally, absence of rhoII in MIG1 was evidenced by PCR, using primers that amplified the full-length rhoII gene (891 bp) (Fig. 2A, bottom). The rhoII sequence is 1 bp from Hvo_0726, which encodes a putative endonuclease (EndV), suggesting that these genes probably form an operon. To rule out polar effects of the rhoII deletion on the downstream gene, endV mRNA was measured by real-time qPCR. According to our results, the expression of endV was not affected by the rhoII deletion (Fig. 2B).

The rhoII gene was deleted successfully, indicating that the encoded protein is not essential for viability of *H. volcanii* under standard laboratory conditions. MIG1 was identical to the parent H26 strain with regard to colony morphology, growth rate under various conditions (MGM and minimal medium; 30, 37 and 42 °C; 1.5 and 4.8 mM NaCl), and hydrolytic activity against heterologous Rho substrates. Because *H. volcanii* encodes two Rho homologs (Hvo_0727 and Hvo_1474; Fig. 1A), the possibility that the protein product of Hvo_1474 could complement, at least partially, the rhoII deletion cannot be ruled out. However, MIG1 showed increased sensitivity to novobiocin (Fig. 2C) and a slight but reproducible reduction in motility on 0.25% CA-agar plates (Fig. 2D). Attempts to complement MIG1 with a copy of rhoII in trans were unsuccessful because no recombinant protein or mRNA could be detected when the rhoII gene was cloned in several haloarchaeal expression vectors and introduced into the mutant strain. Preliminary experiments indicated that rhoII and endV are transcribed as a bicistronic mRNA; thus, failure to detect rhoII mRNA and protein from the plasmids could be due to instability of the mRNA containing only the rhoII sequence. Therefore, we generated a PCR product that comprised both genes as well as the predicted promoter and terminator sequences and cloned it in the pTA963 vector (26) previously digested with ApaI and BamHI. This combination of restriction enzymes releases the ptaA promoter and renders a promoterless vector; therefore, the resulting plasmid (pMCF1) would express the rhoII/endV operon from its own promoter. When introduced in the MIG1 strain, this construct could successfully complement the observed...
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phenotypes (Fig. 2, C and D). No significant differences regarding novobiocin resistance or motility were observed between the parent strain harboring the empty vector and that harboring pMCF1. These results indicate that the observed differences are due to the absence of rholl.

Deficiency in Rholl Affects Glycosylation of the S-layer Glycoprotein in H. volcanii—To identify putative substrates of Rholl, we compared SDS-PAGE profiles of membrane proteins in H. volcanii H26 and M1G1. Coomassie Brilliant Blue G-250 staining evidenced two polypeptides of 190 and 98 kDa that were enriched in the M1G1 strain (Fig. 3A, left). These polypeptides were identified as the S-layer glycoprotein and a putative periplasmic substrate-binding protein of an ABC transporter (Hvo_0062), respectively, based on MALDI-TOF mass spectrometry analysis. At first glance, these two proteins did not seem to have a functional link. The putative periplasmic substrate-binding protein was predicted as a soluble polypeptide because it was unlikely that it could be a Rho substrate. Examination of the amino acid sequence of Hvo_0062 evidenced two putative N-glycosylation sites, Asn-198 and -524 (predicted with the NetNGlyc 1.0 server available on the CBS website). Consistent with this observation, periodic acid-Schiff staining confirmed that both polypeptides were glycosylated in the WT and M1G1 strains (Fig. 3A, right). Based on the similarity in electrophoretic mobility, the 98-kDa polypeptide may correspond to an unidentified glycoprotein of H. volcanii that had been previously reported by other groups (38, 39). In the parent strain, the S-layer glycoprotein showed a smeared band with apparent higher molecular masses than the protein synthesized by M1G1. The smeared band probably reflected polypeptides that migrated with lower retention times (17.784, 20.984, and 22.184 min) compared with the parent strain, supporting our hypothesis that N-glycosylation is affected in M1G1.

Structure Determination of the Novel Oligosaccharide Associated with the S-layer Glycoprotein—Considering the lack of reports on glycans bound by N-acetylglucosamine to the S-layer glycoprotein of H. volcanii and in an effort to evaluate the effect of rholl deletion on the S-layer glycoprotein glycosylation, a detailed structural characterization of the novel oligosaccharide was performed. MALDI-TOF MS analysis of the total enzymatically released oligosaccharide fraction was performed for the WT and mutant strains in the positive and negative polarity.

When the spectrum of the total enzymatically released oligosaccharide fraction from the WT was recorded using 2,5-dihydroxybenzoic acid as matrix in the negative linear mode (Fig. 3C, left), three clusters of ions were clearly observed: cluster a from m/z 650 to m/z 760, cluster b from m/z 820 to m/z 890, and cluster c, from m/z 1020 to m/z 1080. Cluster a presented a major peak at m/z 663.3 (calc. m/z 664.0202, C_{22}H_{30}N_{2}O_{18}S_{1}^{18}A) attributed to the [M − H]^{−} ion corresponding to a GlcNAc_{2}SQ trisaccharide bearing a methyl group. Furthermore, ions at m/z 678.3 (calc. m/z 678.2002) and m/z 692.3 (calc. m/z 692.0202) were attributed to the same oligosaccharide differing in the number of methyl groups (Δ14).

Regarding cluster b, it showed a major peak at m/z 840.1 (calc. m/z 839.2609, C_{30}H_{41}N_{2}O_{23}S) ascribed to a GlcNAc_{2}SQHex tetrasaccharide structure carrying two methyl groups. Accordingly, ions at m/z 853.2 and m/z 867.8 differed in the number of methyl substitutions. In addition, cluster c presented a peak at m/z 1029.2 (calc. m/z 1029.345, C_{38}H_{51}N_{2}O_{23}S^{2}X) corresponding to a GlcNAc_{2}SQHex_{2} pentasaccharide structure bearing four methyl groups. Also ions at m/z 1043.2, m/z 1057.2, and m/z 1071.4 differed in the number of methyl substituents.

The deprotonated species [M − H]^{−} corresponding to ion m/z 1029.2 was selected as precursor ion and subjected to laser-induced LID-MS/MS analysis in the MALDI-TOF/TOF-MS/MS instrument (data not shown). The main signal at m/z 875.6 (calc. m/z 875.4) was attributed to a 0.5X_{m}^{3}X_{m}^{3} cleavage of the sulfoquinovose (6-deoxy-6-sulfoglucose; SQ) unit bearing a methyl group, and signal at m/z 257.7 (calc. m/z 257.0) was diagnostic for the presence of the methyl sulfoquinovosyl unit. In addition, ion at m/z 696.9 (calc. m/z 696.4) corresponded to the loss of the non-substituted hexose unit (Δ179) from ion m/z 875.6. In accordance, the presence of the terminal non-substituted hexose was confirmed by ions at m/z 180.2 (calc. m/z 179.0) and ion at m/z 104.4 (calc. m/z 104.1, 2.2X_{m}^{2}) on the other hand, ion at m/z 897.7 (calc. m/z 897.3, 2.2X_{m}^{2}) confirmed the presence of three methyl groups in another hexose unit, one of them in position 2. A branched GlcNAc residue linked to a non-substituted hexose was confirmed by ion at m/z 363.5 (calc. m/z 364.1). When the same analysis was performed with the oligosaccharide fraction from M1G1 mutant, a similar spectrum was obtained.

In the positive ion mode (Fig. 3D), the WT strain showed a main ion at m/z 3255.2 (calc. m/z 3255.7591, C_{108}H_{182}N_{2}O_{93}S_{6}) consistent with a GlcNAc_{4}Hex_{6}(SQHex)_{2}N_{2} oligosaccharide structure bearing eight methyl groups as [M + Na]^{+}. In addition, signal at m/z 1597.5 (calc. m/z 1597.4131, C_{54}H_{91}N_{2}O_{45}S_{2}) corresponded to a GlcNAc_{6}Hex_{6}(SQHex)_{2} species bearing two methyl groups, and signal at m/z 1047.8 (calc. m/z 1047.2927, C_{36}H_{41}N_{4}O_{28}S) corresponded to a GlcNAc_{6}Hex_{3}SQ structure bearing two methyl groups. This signal correlates with ion m/z 1029.2 detected in the negative ion mode (Fig. 3C, left) differing in the number of methyl substituents. In the low molecular weight range, signal at m/z 898.9 (calc. m/z 899.2477; C_{31}H_{53}N_{2}O_{23}S) was attributed to a GlcNAc_{6}HexSQ structure bearing two methyl groups, and signal at m/z 871.9 (calc. m/z 871.2242; C_{28}H_{49}N_{2}O_{25}S) was attributed to a GlcNAc_{2}HexSQ structure carrying one methyl substituent. When MALDI-TOF/TOF-MS/MS of ion at m/z 3255.2 was performed, very few signals were observed.
could be detected (data not shown), among them \( m/z \) 2926.0 (calc. \( m/z \) 2926.7; \( C_{98}H_{165}N_2Na_3O_{83}S_5 \)) due to the \( \alpha^{\text{3}}X_{11\text{ac}} \) cleavage of the last SQ unit bearing a methyl group as described in the negative ion mode and signal at \( m/z \) 2755.8 (calc. \( m/z \) 2755.639; \( C_{90}H_{154}N_2Na_3O_{79}S_5 \)) corresponding to a GlcNAc\(_2\)Hex\(_3\)(SQHex)\(_5\) structure carrying seven methyl groups after the loss of the two acetyl groups (2 \( \times \) 42 mu). In addition, whereas ion at \( m/z \) 1415.4 (calc. \( m/z \) 1414.323; \( C_{65}H_{177}N_2Na_3O_{39}S_2 \)) was attributed to GlcNAc\(_2\)Hex(SQHex)\(_2\) bearing two methyl substituents after the loss of an acetyl group, signal at \( m/z \) 927.7 (calc. \( m/z \) 927.274;
C$_{31}$H$_{56}$N$_2$NaO$_{20}$S correlated with GlcNAc$_2$Hex(SQHex) bearing one methyl group after the loss of the two acetyl groups (data not shown). At this point, the presence of an N-linked oligosaccharide bearing at least six SQ-Hex units linked to a di-N-acetylglucosamine-dihexose backbone was confirmed.

Interestingly, the MIG1 mutant did not show the peak corresponding to GlcNAc$_2$Hex$_3$(SQHex)$_2$ that was observed in the WT in the positive ion mode, but the highest mass signal evidenced at m/z 1392.8 could be attributed to GlcNAc$_2$Hex$_3$SQ$_2$ bearing five methyl groups after the loss of two acetyl groups (2 × 42) (Fig. 3D, right). These results indicate that the MIG1 strain lacks the repeating units of SQ-Hex observed for the oligosaccharide described in this work, confirming that the MIG1 has a defect in protein glycosylation.

To determine the sugar components, the PNGase-released oligosaccharides from the parent strain were hydrolyzed with 2 n TFA and subjected to HPAEC-PAD under standard conditions for neutral and amino sugars (Fig. 4A). The analysis showed glucose (peak 1) as a main component and two minor signals coincident with glucosamine (peak 2) and mannose (peak 3). Notably, other two peaks, one of them eluting very near the void volume (R$_t$ 2.27 min), suggesting the presence of substituted monosaccharides, and the other one eluting with R$_t$ 14.1 min, corresponding to a product of partial hydrolysis (not shown), were also detected. Accordingly, when the same sample was analyzed under conditions for acidic carbohydrates (Fig. 4B), a peak corresponding to the partial hydrolysis product (R$_t$ 1.3 min) and a main unresolved peak corresponding to neutral monosaccharides (peak 4) were detected. In addition, a small signal coincident with an authentic sample of SQ was evidenced (peak 5).

The SQ-containing Oligosaccharide Is Bound to Position Asn-732 of the S-layer Glycoprotein—The S-layer glycoprotein is glycosylated at positions Asn-13, Asn-83, and Asn-498, whereas the putative glycosylation site Asn-370 is not modified. However, the possible modification of Asn-274, Asn-279, and Asn-732 has not been reported so far (19). Therefore, it was important to determine the binding site(s) of this novel oligosaccharide. For that purpose, the S-layer glycoprotein was digested with Glu-C alone or with trypsin plus Glu-C and the peptide products were examined by MALDI-TOF MS.

MALDI-TOF MS analysis in the positive ion mode of the micro-SPE-enriched fraction obtained from the WT S-layer glycoprotein after Glu-C digestion showed two major signals. Ion at m/z 3153.01 (calc. m/z 3152.1713), based on manual interpretation, was ascribed to a GlcNAc$_2$Hex$_2$NaSQ oligosaccharide bearing three methyl groups linked to the $^{745}$DTTTSDNATDTTTTDDGPTE peptide (calc. m/z 2131.8633) containing the Asn-732 glycosylation site. In accordance, signal at m/z 2154.08 (calc. m/z 2153.8633) matched with the sodiated adduct of the corresponding peptide moiety (Table 2).

When MALDI-TOF/TOF-MS/MS of the parent ion at m/z 3153 was performed (data not shown), a major signal at m/z 1308.61 (calc. m/z 1309.4044) corresponded to the DTTTSDNAT glycophosphate containing the Asn-732 (a$_{10}$ cleavage-H$_2$O-COOH) carrying a truncated diglucosamine moiety. Other peptide fragment ions, such as m/z 1883.58 (calc. m/z 1883.7625; b$_{19}$ cleavage), m/z 718.23 (calc. m/z 719.28625; x$_{-}$H$_2$O-COOH), and m/z 387.97 (calc. m/z 387.1637; z$_4$ cleavage), confirmed the glycopeptide structure.

Double digestion with trypsin plus Glu-C, followed by micro-SPE enrichment and MALDI-TOF MS analysis in the positive ion mode, provided signals corresponding to peptides containing Asn-13, Asn-83, and Asn-498 bearing the previously described penta- and tetrasaccharides (Fig. 5A and Table 2). Interestingly, another important signal at m/z 3617.00 (calc. m/z 3616.2262) was detected. This signal matched with the sodiated adduct of the Asn-732-containing peptide ($^{745}$DTTTSDNATDTTTTDDGPTE$^{745}$ peptide (calc. m/z 2131.8633) containing the Asn-732 glycosylation site. In accordance, signal at m/z 2154.08 (calc. m/z 2153.8633) matched with the sodiated adduct of the corresponding peptide moiety (Table 2).

When MALDI-TOF/TOF-MS/MS of the parent ion at m/z 3617.00 was performed (Fig. 5B), signal at m/z 3124.17 (calc. m/z 3124.15) corresponded to the peptide moiety linked to GlcNAc$_2$Hex$_4$SQNa bearing one methyl group, and signal at m/z 2848.88 (calc. m/z 2848.0549) matched with the a$_{19}$ peptide fragment linked to the same oligosaccharide. In addition, signal at m/z 2664.62 (calc. m/z 2664.0643) was attributed to the peptide moiety linked to a hydrated fragment composed of GlcNAc$_2$Hex,
and ion at m/z 2335.27 (calc. m/z 2335.8633) corresponded to the peptide moiety linked to one GlcNAc unit. Other fragment ions, such as m/z 1978.94 (calc. m/z 1978.5653), corresponding to the sodiated adduct of a peptide fragment (DNATD) linked to the proposed oligosaccharide, and m/z 376.49 (calc. m/z 376.1821), accounting for the GlcNAc-Asn unit, confirmed the novel structure.

Finally, in an attempt to detect glycopeptides of a larger molecular weight, the spectrum was acquired in the range m/z 2400–5500 (Fig. 5C). It must be noted that, although the main ions detected were the ones described above, other diagnostic signals appeared. Notably, in the high mass range region, the ion at m/z 4135.97 (calc. m/z 4136.3905) corresponded to the $^{725}_{	ext{DTTSSD}}$NATDTTDTTGDPTG$^{745}$ peptide (calc. m/z 2131.8633) linked to a GlcNAc$_2$Hex$_2$Na(SQHex)$_3$ oligosaccharide bearing five methyl groups and a water unit. In accordance, signal at m/z 4266.15 (calc. m/z 4265.4331) matched with the $^{724}_{	ext{EDTTSSD}}$NATDTTDTTGDPTG$^{745}$ peptide containing the Asn-732 site (calc. m/z 2260.9059) linked to the GlcNAc$_2$Hex$_2$Na(SQHex)$_3$ oligosaccharide bearing five methyl groups and a water unit (Table 2). On the basis of the data obtained, we were able to identify the Asn-732 as the N-glycosylation site of the novel oligosaccharide in the S-layer glycoprotein of \emph{H. volcanii}.

**DISCUSSION**

Although the structure, mechanism, and biochemical function of rhomboid proteases as intramembrane proteases have been extensively studied, the cellular roles of these enzymes as well as their natural substrates remain largely undetermined. Particularly, there are no reports on the characterization of Rho from Archaea. In this work, \textit{in silico} examination of complete genomes showed that haloarchaea encode Rho-like proteases with the canonical six-TMD architecture as well as with unique features that may include the presence of extra C-terminal segments or N-terminal AN-1 zinc fingers fused to the Rho domain (Fig. 1A). The presence of zinc finger domains fused to Rho is conserved in many euryarchaea (including all of the haloarchaea with a sequenced genome), actinomycetes, and some plant and fungus Rho. The significance of this domain combination remains elusive and is worthy of investigation. Zinc fingers usually bind one or more Zn$^{2+}$ atoms and display finger-like protrusions that can interact with target molecules. The binding specificity and the function of these domains are very diverse, and although they were first described as DNA-binding domains, they can also interact with lipids, proteins, and RNA (40). In Archaea, AN1-Zn fingers are also linked to other membrane proteases, such as transglutaminase-like thiol peptidases and zinc-dependent metallopeptidases, the chaperone DNAJ, and other TMD-containing proteins of unknown function. Based on this observation, a role in membrane-associated proteolysis and/or regulation of protein folding or stability was proposed for these zinc fingers (37).

\textit{H. volcanii} encodes two Rho homologs designated by \textit{rho1} and \textit{rho2}. Consistently, membrane fractions of this model haloarchaeon displayed hydrolytic activity against known Rho substrates (Fig. 1B). The deletion of \textit{rho} from the chromosome was successful, indicating that this protease is not essential for viability of \textit{H. volcanii} under standard laboratory conditions. The MIG1 strain did not evidence alterations in growth compared with the WT and showed no differences with respect to hydrolysis of Rho substrates (not shown). Because \textit{H. volcanii} encodes for two Rho homologs (Fig. 1A), we cannot rule out the possibility that RhoII could complement RhoII activity. However, the sensitivity to the antibiotic novobiocin was higher in MIG1 than in the WT strain (Fig. 2C). Similarly, an increased sensitivity to novobiocin has also been reported for mycobacterial Rho null mutants (9). In addition, cell motility was slightly reduced in the mutant strain (Fig. 2D). Although the observed phenotypes were mild, they were consistent and highly reproducible. Because the \textit{rho1} gene could not be expressed from plasmid vectors, to validate the observed phenotypes, a construct bearing the complete operon and its regulatory sequences was generated. Preliminary proteomics data confirmed that RhoII is expressed in the complemented strain (data not shown). Transformation of MIG1 with this construct successfully complemented the observed phenotypes. Taking into consideration that there were no alterations in \textit{endV} expression in MIG1 (Fig. 1B) and that the WT strain bearing either the empty plasmid or the plasmid containing the complete operon showed no differences, it can be concluded that the differences in novobiocin resistance and motility were due to the absence of \textit{rhoII}.

In an attempt to detect RhoII substrates, we compared the SDS-PAGE profiles of the parental and mutant strains. Protein staining evidenced two differential bands (Fig. 3A) that were identified as the S-layer glycoprotein and a periplasmic subunit of an ABC transporter. Detection of periplasmic components of ABC transporters in archaeal membrane preparations has been reported previously (41), and their presence is probably due to interaction with integral membrane subunits. Sequence analysis showed that the ABC component contained two glycosyla-

**TABLE 2**

| Peptide residues | Glycosylation site | Theoretical Peptide$^{a}$ [M+H]$^{+}$ | Observed glycopeptide (m/z) | Theoretical glycopeptide (m/z) | Glycan modification (m/z) |
|------------------|--------------------|----------------------------------------|-----------------------------|-----------------------------|----------------------------|
| 3–14             | Asn-13             | 1296.5703                              | 2184.32                     | Hex$_{(1)}$(Hex)$_{(2)}$Na(CH$_{(1)}$) |
| 65–90            | Asn-83             | 2644.3213                              | 3349.13                     | Hex$_{(2)}$(Hex)$_{(3)}$Na(CH$_{(2)}$) |
| 65–95            | Asn-83             | 3189.5546                              | 3550.50                     | Hex$_{(2)}$Na(CH$_{(2)}$) |
| 492–515          | Asn-498            | 2435.2413                              | 3146.99                     | Hex$_{(2)}$Na(CH$_{(2)}$) |
| 725–745          | Asn-732            | 2131.8633                              | 3153.01                     | Hex$_{(2)}$Na(CH$_{(2)}$) |
| 725–745          | Asn-732            | 2131.8633                              | 3152.01                     | Hex$_{(2)}$Na(CH$_{(2)}$) |
| 725–745          | Asn-732            | 2131.8633                              | 3153.01                     | Hex$_{(2)}$Na(CH$_{(2)}$) |
| 725–745          | Asn-732            | 2260.9059                              | 4266.15                     | GlcNAc$_2$Hex$_{(5)}$(CH$_{(3)}$)$_{(1)}$+H$_2$O |

$^{a}$Theoretical peptides are based on \textit{in silico} digest of the known protein sequence.

$^{b}$As [M+Na]$^{+}$.
FIGURE 5. Determination of the glycosylation site of the SQ-containing oligosaccharide. A, MALDI-TOF MS analysis in the m/z 1000–4000 range of the glycopeptides obtained by trypsin plus Glu-C digestion of the S-layer glycoprotein. B, MALDI-LID-MS/MS of ion m/z 3617.43. C, MALDI-TOF MS in the m/z 2000–5500 range of the glycopeptides analyzed in A. Filled circles with diamonds, methyl sulfoquinovose; open circles, dimethyl hexose; open squares, N-acetylglycosamine; filled circles, hexose.
protein glycosylation (20) in membrane protein that has been extensively used as a model to study observed in the protein gels. Parts. This could account for the differences in intensity, the protein more easily (43) than in their glycosylated counter-

with Coomassie Brilliant Blue G-250 because the dye can reach with reduced glycosylation tend to be stained more intensely may be affected in protein glycosylation. Moreover, proteins (probably not a Rho substrate), we hypothesized that MIG1 could be affected in protein glycosylation (20) in H. volcanii. We show, for the first time, the presence of N-linked oligosaccharides hydrolyzed by PNGase F in the S-layer glycoprotein of H. volcanii, because after the enzymatic treatment, the HPAEC-PAD profile clearly showed the presence of released oligosaccharides. Interestingly, oligosaccharides with shorter retention times accumulated in MIG1, indicating that in this strain, N-glycosylation was affected (Fig. 3B). The main ions obtained by MALDI-TOF analysis of the released material from both strains could be attributed to a novel GlcNAcHex3(SQHex)5Na2 (m/z 3255.2) for the WT and GlcNAcHex3SQNa2 for MIG1, confirming that the glycan bound to the S-layer glycoprotein was truncated in the mutant strain. Although in this work we generated a construct that complemented the other observed phenotypes, complementation of the rhoII deletion effect on glycosylation was not possible. The pMCF1 plasmid has the pyrE gene as an auxotrophic selection marker; therefore, the complemented strain had to be grown in medium lacking uracil. When H. volcanii was grown in CA medium without uracil, the material released by PNGase digestion was very scarce, preventing us from further characterizing the bound oligosaccharides.

The S-layer glycoprotein from H. volcanii has been reported to bear a pentasaccharide of known composition at positions Asn-13 and Asn-83 as well as a sulfated tetrasaccharide at position Asn-379, which is present in low salt conditions (19). However, there have been no reports on N-acetylgalactosamine N-linked long chain oligosaccharides for this protein. This prompted us to go deeper into determining the structure of the novel glycan detected in this work. By MALDI-TOF MS analysis of the released oligosaccharides in the negative ion mode, three clusters of ions attributed to the presence of a GlcNAcHexSQ structure substituted with one or two hexosyl units were detected. Differences in the degree of methyl substitution of these structures account for the detected microheterogeneity. Furthermore, LID-MS/MS spectra showed a diagnostic fragment ion corresponding to the methylsulfokinovose moiety assuring the proposed structure. In addition, MALDI-TOF MS analysis in the positive ion mode showed a GlcNAcHex3(SQHex)5 oligosaccharide. The fact that glucose was observed as the major neutral component in the HPAEC-PAD analysis after acid hydrolysis suggests a repeating SQ-Glc unit. This new structural feature, a repeating methyl-SQ-Hex disaccharide, has not been reported so far.

The N-glycosylation site of the novel oligosaccharide was also determined. MALDI-MS analysis of digested glycopeptides obtained from the S-layer glycoprotein revealed that the novel oligosaccharide is linked to the Asn-732 glycosylation site.

Fig. 6 shows a schematic representation of the H. volcanii S-layer glycosylation pattern, including the novel oligosaccharide described in this work. It has been speculated that a high degree of glycosylation may be protective against the harsh environmental conditions of S. acidocaldarius in its natural habitats (pH 2–3 and 75–80 °C) (32). In addition, an excess of negative surface charge is thought to stabilize proteins in high salt conditions, possibly via the formation of an energetically favorable protein-water-salt hydration network, as proposed to explain the highly acidic nature of the N-glycans decorating the Halobacterium salinarum S-layer glycoprotein (14, 44). Accordingly, in the S-layer glycoprotein of H. volcanii, the presence of a long N-linked oligosaccharide chain with at least six sulfokinovose-containing units would also provide a negative surface and may improve resistance against hydrolytic cleavages.

The data presented in this work represent the first study on archaeal Rho and suggest a link between these proteases and protein N-glycosylation. Determining this link will be the focus of future investigations. In addition, this work contributes to the fundamental knowledge of microbial glycobiology, describing the structure of a novel SQ-containing oligosaccharide. Moreover, our data indicate that this glycan is linked through GlcNAc to the Asn-732 site of the S-layer glycoprotein of H. volcanii, a glycosylation site that had not been studied previously. The identification of specific RhoII substrates and studies on the biosynthesis and assembly of the oligosaccharide described in this work will provide valuable insights into the N-glycosylation process and its regulation in Archaea.

Acknowledgments—We thank Dr. M. Pohlschröder (University of Pennsylvania) for plasmid pTA131, Dr. A. Large (University of Birmingham, UK) for H. volcanii H26, Dr. K. Strisovsky (Academy of Sciences, Czech Republic) for the chimeric substrates constructs and E. coli MG1655 strain, and Dr. D. Schleheck (University of Konstanz, Germany) for an authentic sample of sulfokinovose.
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