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14. ABSTRACT
Found in the harshest environments on Earth, Archaea express proteins that remain functional in the face of conditions that would otherwise lead to denaturation and aggregation. Post-translational modifications, like glycosylation, help protect archaeal proteins from such fates. Better understanding of archaeal protein glycosylation would thus provide insight into how extremophilic proteins overcome the challenges of their environments. In previous ARL-funded work, we defined a series of Agl proteins involved in the assembly and attachment of a novel pentasaccharide to select asparagine residues of the surface layer glycoprotein of the model archaeon, Haloferax

15. SUBJECT TERMS
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a. REPORT	b. ABSTRACT	c. THIS PAGE			Jerry Eichler
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Report Title

Final Report: Post-translational Modification of Extremophilic proteins: N-glycosylation in Archaea

ABSTRACT

Found in the harshest environments on Earth, Archaea express proteins that remain functional in the face of conditions that would otherwise lead to denaturation and aggregation. Post-translational modifications, like glycosylation, help protect archaeal proteins from such fates. Better understanding of archaeal protein glycosylation would thus provide insight into how extremophilic proteins overcome the challenges of their environments. In previous ARL-funded work, we defined a series of Agl proteins involved in the assembly and attachment of a novel pentasaccharide to select asparagine residues of the surface-layer glycoprotein of the model archaeon, Haloferax volcanii. In the recently ended project, we 1) identified and characterized novel Agl proteins, 2) revealed the ability of the Hfx. volcanii N-glycosylation pathway to modify non-native target proteins, 3) described components involved in the biogenesis of the dolichol phosphate that serves as glycan carrier during Hfx. volcanii N-glycosylation, 4) delineated a novel Hfx. volcanii N-glycosylation pathway recruited by cells grown at low salinity, 5) assessed the distribution of N-glycosylation pathway components across the Archaea, 6) identified two populations of the reporter Hfx. volcanii glycoprotein, the S-layer glycoprotein, one modified by a lipid anchor, the other presenting a transmembrane domain, 7) revealed the promiscuity of the archaeal oligosaccharyltransferase, AglB, 8) created the aglgenes database for cataloguing available information on the enzymes of archaeal N-glycosylation and 9) began deciphering the N-glycosylation pathways of Archaea other than Hfx. volcanii.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
07/06/2013	8.00	Jerry Eichler. Response to Jarrell and Albers: the name says it all, trends in microbiology, (11 2012): 512. doi: 10.1016/j.tim.2012.08.007
07/06/2013	14.00	J. Eichler, K. Jarrell, S. Albers. A proposal for the naming of N-glycosylation pathway components in Archaea, Glycobiology, (05 2013): 620. doi: 10.1093/glycob/cwt034
07/06/2013	12.00	Lina Kaminski, Mor N. Lurie-Weinberger, Thorsten Allers, Uri Gophna, Jerry Eichler. Phylogenetic- and genome-derived insight into the evolution of N-glycosylation in Archaea, Molecular Phylogenetics and Evolution, (08 2013): 327. doi: 10.1016/j.ympev.2013.03.024
07/06/2013	11.00	Lina Kaminski, Shai Naparstek, Lina Kandiba, Chen Cohen?Rosenzweig, Adi Arbiv, Zvia Konrad, Jerry Eichler. Add salt, add sugar: N-glycosylation in, Biochemical Society Transactions, (02 2013): 432. doi: 10.1042/BST20120142
07/06/2013	10.00	Jerry Eichler. Extreme sweetness: protein glycosylation in archaea, Nature Reviews Microbiology, (01 2013): 151. doi: 10.1038/nrmicro2957
07/06/2013	9.00	Lina Kandiba, Ziqiang Guan, Jerry Eichler. Lipid modification gives rise to two distinct Haloferax volcanii S-layer glycoprotein populations, Biochimica et Biophysica Acta (BBA) - Biomembranes, (03 2013): 938. doi: 10.1016/j.bbamem. 2012.11.023
07/10/2014	17.00	Adi Arbiv, Sophie Yurist-Doutsch, Ziqiang Guan, Jerry Eichler. AglQ Is a Novel Component of the Haloferax volcanii N-Glycosylation Pathway, PLoS ONE, (11 2013): 1. doi: 10.1371/journal.pone.0081782
07/10/2014	15.00	L. Kaminski, Z. Guan, S. Yurist-Doutsch, J. Eichler. Two Distinct N-Glycosylation Pathways Process the Haloferax volcanii S-Layer Glycoprotein upon Changes in Environmental Salinity, mBio, (11 2013): 1. doi: 10.1128/mBio.00716-13
07/10/2014	16.00	C. Cohen-Rosenzweig, Z. Guan, B. Shaanan, J. Eichler. Substrate Promiscuity: AglB, the Archaeal Oligosaccharyltransferase, Can Process a Variety of Lipid-Linked Glycans, Applied and Environmental Microbiology, (11 2013): 486. doi: 10.1128/AEM.03191-13
07/10/2014	18.00	Jerry Eichler, Adi Arbiv, Chen Cohen-Rosenzweig, Lina Kaminski, Lina Kandiba, Zvia Konrad. N-glycosylation in Haloferax volcanii: adjusting the sweetness, Frontiers in Microbiology, (2013): 1. doi: 10.3389/fmicb.2013.00403
07/10/2014	19.00	Lina Kaminski, Jerry Eichler. Haloferax volcanii N-Glycosylation: Delineating the Pathway of dTDP-rhamnose Biosynthesis, PLoS ONE, (05 2014): 1. doi: 10.1371/journal.pone.0097441
07/10/2014	20.00	K. F. Jarrell, Y. Ding, B. H. Meyer, S.-V. Albers, L. Kaminski, J. Eichler. N-Linked Glycosylation in Archaea: a Structural, Functional, and Genetic Analysis, Microbiology and Molecular Biology Reviews, (05 2014): 304. doi: 10.1128/MMBR.00052-13

07/10/2014 21.00 N. Godin, J. Eichler. aglgenes, a curated and searchable database of archaeal N-glycosylation pathway components,
Database, (06 2014): 1. doi: 10.1093/database/bau046

07/10/2014 22.00 Jerry Eichler, Julie Maupin-Furlow. Post-translation modification in Archaea: lessons from,
FEMS Microbiology Reviews, (07 2013): 583. doi: 10.1111/1574-6976.12012

07/10/2014 23.00 S. Yurist-Doutsch, J. Eichler, C. Cohen-Rosenzweig. AglS, a Novel Component of the Haloferax volcanii N-Glycosylation Pathway, Is a Dolichol Phosphate-Mannose Mannosyltransferase,
Journal of Bacteriology, (10 2012): 6909. doi: 10.1128/JB.01716-12

07/10/2014 24.00 Lina Kandiba, Jerry Eichler. Analysis of genes involved in the biosynthesis of sialic acids and other nonulosonic acid sugars in Archaea reveals a complex evolutionary history. ,
Federation European Microbiological Societies Letters, (06 2013): 110. doi:

08/07/2012 5.00 Lina Kaminski, Ziqiang Guan, Mehtap Abu-Qarn, Zvia Konrad, Jerry Eichler. AglR is required for addition of the final mannose residue of the N-linked glycan decorating the Haloferax volcanii S-layer glycoprotein ,
Biochimica et Biophysica Acta, (06 2012): 7. doi:

08/07/2012 7.00 Shai Naparstek, Ziqiang Guan, Jerry Eichler. A predicted geranylgeranyl reductase reduces the ?-position isoprene of dolichol phosphate in the halophilic archaeon, Haloferax volcanii,
Biochim. Biophys. Acta – Mol. Cell Biol. Lipids, (03 2012): 11. doi:

08/07/2012 6.00 Lina Kandiba, Olli Aitio, Jari Helin, Ziqiang Guan, Perttu Permi, Dennis Bamford, Jerry Eichler, Elina Roine. Diversity in prokaryotic glycosylation:An archaeal-derived N-linked glycan contains legionaminic acid,
Molecular Microbiology, (04 2012): 16. doi:

12/02/2014 25.00 Lina Kandiba, Jerry Eichler. Archaeal S-layer glycoproteins: post-translational modification in the face of extremes,
Frontiers in Microbiology, (11 2014): 0. doi: 10.3389/fmicb.2014.00661

TOTAL: **20**

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

2012-Molecular Biology of Archaea III. Marburg, Germany
2013-Halophiles 2013. Storrs, CT, USA
2013-Archaea: Ecology, metabolism and molecular biology. Gordon Research Conference, Lucca, Italy
2013-Proteins: From birth to death. Jerusalem, Israel
2014-Meeting of the Korean Society for Microbiology and Biotechnology. Busan, South Korea.

Number of Presentations: 5.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
03/18/2012	1.00	Naparstek S. , Guan, Z. , Eichler, J. . The predicted geranylgeranyl reductase, HVO_1799, reduces the omega position isoprene of dolichol phosphate in the halophilic archaeon, Haloferax volcanii. , Biochim. Biophys. Acta – Mol. Cell Biol. Lipids (03 2012)
03/19/2012	2.00	Lina Kandiba, Olli Aitio, Jari Helin, Perttu Permi, Dennis Bamford, Jerry Eichler , Elina Roine. Diversity in prokaryotic glycosylation: An archaeal N-linked glycan contains legionaminic acid., Molecular Microbiology (03 2012)
07/06/2013	13.00	Lina Kandiba, Jerry Eichler. Analysis of putative nonulosonic acid biosynthesis pathways in, FEMS Microbiology Letters (06 2013)
08/07/2012	3.00	Sophie Yurist-Doutsch, Jerry Eichler, Chen Cohen-Rosenzweig. AglS, a novel component of the Haloferax volcanii N-glycosylation pathway, is a dolichol phosphate-mannose mannosyltransferase, Molecular Microbiology (07 2012)
08/07/2012	4.00	Jerry Eichler, Julie Maupin-Furlow. Post-translation modification and protein degradation in Archaea: Lessons from Haloferax volcanii , FEMS Microbiology Reviews (07 2012)
TOTAL:	**5**	

Number of Manuscripts:

Books

Received		Book
TOTAL:		

Received	Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Lina Kaminski	1.00	
Lina Kandiba	1.00	
FTE Equivalent:	**2.00**	
Total Number:	**2**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Jerry Eichler	0.25	
FTE Equivalent:	**0.25**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME	
Lina Kaminski	
Shai Naparstek	
Total Number:	**2**

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attached Final Report

Technology Transfer

US Army Research Office, Award W911NF-11-1-0520
Final Report

Post-translational modification of extremophilic proteins: N-glycosylation in Archaea

Jerry Eichler, Ph.D.
Dept. of Life Sciences, Ben Gurion University, Beersheva ISRAEL

List of Illustrations and Tables

Statement of the problem studied

Found in the harshest environments on Earth, Archaea express proteins that remain functional in the face of conditions that would otherwise lead to denaturation and aggregation. Post-translational modifications, like glycosylation, help protect archaeal proteins from such fates. Better

understanding of archaeal protein glycosylation would thus provide insight into how extremophilic proteins overcome the challenges of their environments. In previous ARL-funded work, we defined a series of Agl proteins involved in the assembly and attachment of a novel pentasaccharide to select asparagine residues of the surface-layer glycoprotein of the model archaeon, *Haloferax volcanii*. In the recently ended project, we 1) identified and characterized novel Agl proteins, 2) revealed the ability of the *Hfx. volcanii* N-glycosylation pathway to modify non-native target proteins, 3) described components involved in the biogenesis of the dolichol phosphate that serves as glycan carrier during *Hfx. volcanii* N-glycosylation, 4) delineated a novel *Hfx. volcanii* N-glycosylation pathway recruited by cells grown at low salinity, 5) assessed the distribution of N-glycosylation pathway components across the Archaea, 6) identified two populations of the reporter *Hfx. volcanii* glycoprotein, the S-layer glycoprotein, one modified by a lipid anchor, the other presenting a transmembrane domain, 7) revealed the promiscuity of the archaeal oligosaccharyltransferase, AglB, 8) created the *agl*genes database for cataloguing available information on the enzymes of archaeal N-glycosylation and 9) began deciphering the N-glycosylation pathways of Archaea other than *Hfx. volcanii*.

Summary of the most important results

Published results:

Naparstek, S., Guan, Z. and Eichler, J. (2012) A predicted geranylgeranyl reductase reduces the omega position isoprene of dolichol phosphate in the halophilic archaeon, *Haloferax volcanii*. Biochim. Biophys. Acta, 1821, 923-933.

In N-glycosylation in both Eukarya and Archaea, N-linked oligosaccharides are assembled on dolichol phosphate prior to transfer of the glycan to the protein target. However, whereas only the α-position isoprene subunit is saturated in eukaryal dolichol phosphate, both the α- and ω-position isoprene subunits are reduced in the archaeal lipid. The agents responsible for dolichol phosphate saturation remain largely unknown. The present study sought to identify dolichol phosphate reductases in the halophilic archaeon, *Haloferax volcanii*. Homology-based searches recognize HVO_1799 as a geranylgeranyl reductase. Mass spectrometry revealed that cells deleted of *HVO_1799* fail to fully reduce the isoprene chains of *Hfx. volcanii* membrane phospholipids and glycolipids. Likewise, the absence of HVO_1799 led to a loss of saturation of the ω-position isoprene subunit of C_{55} and C_{60} dolichol phosphate, with the effect of *HVO_1799* deletion being more pronounced with C_{60} dolichol phosphate than with C_{55} dolichol phosphate. Glycosylation of dolichol phosphate in the deletion strain occurred preferentially on that version of the lipid saturated at both the α- and ω-position isoprene subunits.

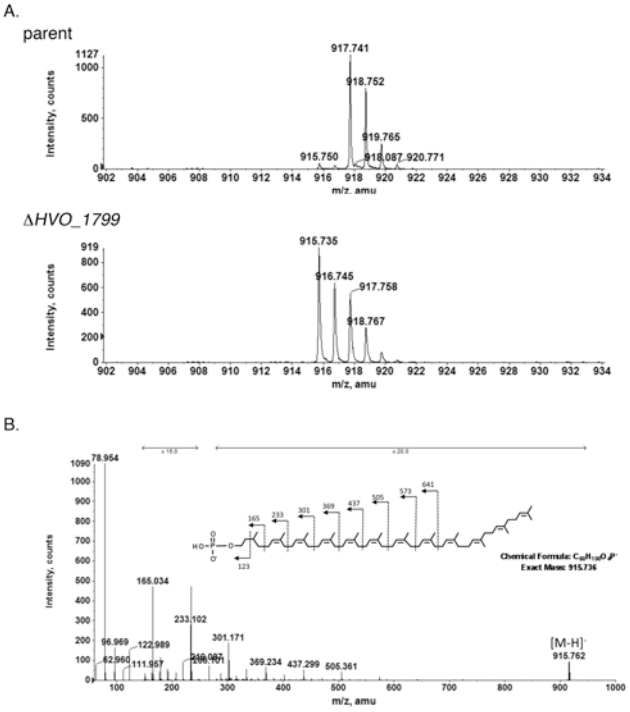


Fig 1 – Normal phase LC/ESI-MS/MS reveals that in *Hfx. volcanii* Δ*HVO_1799* cells, the ω-position isoprene of C_{60} DolP is not saturated. A. The $[M-H]^-$ ions of *Hfx. volcanii* C_{60} DolP detected at *m/z* 917.741 and 915.735 in the parent and Δ*HVO_1799* strains, respectively. B. Collision-induced dissociation MS/MS analysis of the *m/z* 915.736 $[M-H]^-$ ion peak corresponding to *Hfx. volcanii* C_{60} DolP in the *HVO_1799* deletion strain. The inset shows the fragmentation scheme and lists the chemical formula and expected mass of the starting material. The arrows marked x15.0 and x20.0 reflect magnification of ion peaks in the corresponding *m/z* region.

Kandiba L., Aitio O., Helin J., Guan, Z., Permi, P., Bamford, D., Eichler, J. and Roine, E. (2012) Diversity in prokaryotic glycosylation: An archaeal-derived N-linked glycan contains legionaminic acid. Mol. Microbiol., 84, 578-593.

VP4, the major structural protein of the haloarchaeal pleomorphic virus, HRPV-1, is glycosylated. To define the glycan structure attached to this protein, oligosaccharides released by β-elimination were analyzed by mass spectrometry and NMR spectroscopy. Such analyses showed that the major VP4-derived glycan is a pentasaccharide comprising glucose, glucuronic acid, mannose, sulphated glucuronic acid and a terminal 5-N-formyl-legionaminic acid residue. This is the first observation of legionaminic acid, a sialic acid-like sugar, in an archaeal-derived glycan structure. The importance of this residue for viral infection was demonstrated upon incubation with N-acetylneuraminic acid, a similar monosaccharide. Such treatment reduced progeny virus production by half 4 hours post-infection. LC-ESI/MS analysis confirmed the presence of pentasaccharide precursors on two different VP4-derived peptides bearing the N-glycosylation signal, NTT. The same sites modified by the native host, *Halorubrum* sp. strain PV6, were also recognized by the *Haloferax volcanii* N-glycosylation apparatus, as determined by LC-ESI/MS of heterologously expressed VP4. Here, however, the N-linked pentasaccharide was the same as shown to decorate the S-layer glycoprotein in this species. Hence, N-glycosylation of the haloarchaeal viral protein, VP4, is host-specific. These results thus present additional examples of archaeal N-glycosylation diversity and show the ability of Archaea to modify heterologously expressed proteins.

Kaminski, L., Guan, Z., Abu-Qarn, A., Konrad, Z. and Eichler, J. (2012) AglR is required for addition of the final mannose residue of the N-linked glycan decorating the *Haloferax volcanii* S-layer glycoprotein. Biochim. Biophys. Acta, 1820, 1664-1670.

Recent studies of *Haloferax volcanii* have begun to elucidate the steps of N-glycosylation in Archaea, where this universal post-translational modification remains poorly described. In *Hfx. volcanii*, a series of Agl proteins catalyze the assembly and attachment of a N-linked pentasaccharide to the S-layer glycoprotein. Although roles have been assigned to the majority of Agl proteins, others await description. A combination of bioinformatics, gene deletion, mass spectrometry and metabolic radiolabeling served to show a role for AglR in archaeal N-glycosylation at both the dolichol phosphate and reporter glycoprotein levels. The modified behavior of the S-layer glycoprotein isolated from cells lacking AglR points to an involvement of this protein in N-glycosylation. In cells lacking AglR, glycan-charged dolichol phosphate, including mannose-charged dolichol phosphate, accumulate. At the same time, the S-layer glycoprotein does not incorporate mannose, the final subunit of the N-linked pentasaccharide decorating this protein. AglR is a homologue of Wzx proteins,

annotated as flippases responsible for delivering lipid-linked O-antigen precursor oligosaccharides across the bacterial plasma membrane during lipopolysaccharide biogenesis. The effects resulting from *aglR* deletion are consistent with AglR interacting with dolichol phosphate-mannose, possibly acting as a dolichol phosphate-mannose flippase or contributing to such activity.

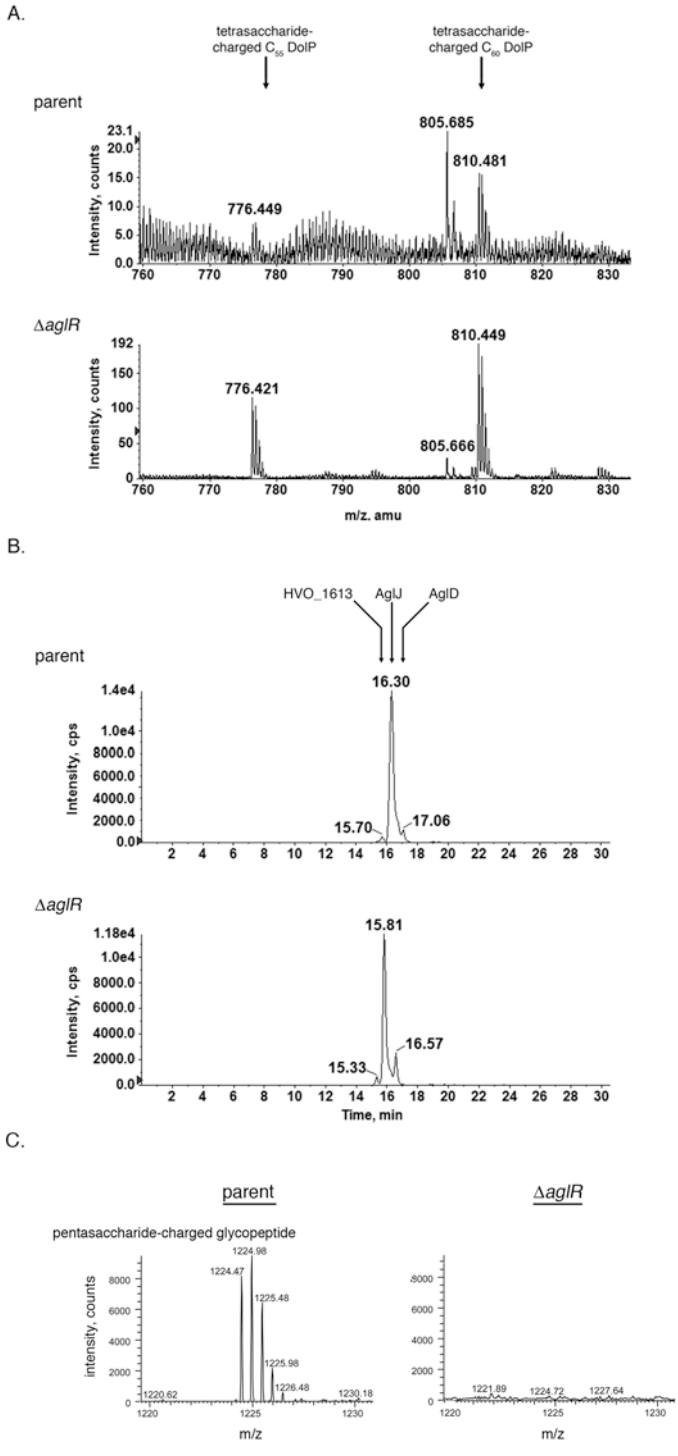


Fig 2 – Glycan-charged DolP accumulates in Δ*aglR* cells while pentasaccharide-modified S-layer glycoprotein is not generated. (A) Normal phase LC/MS/MS analysis of tetrasacchride-charged DolP from the total lipid extract of cells of the *Hfx. volcanii* parent strain (upper panels) and of Δ*aglR* cells (lower panels) was performed. Doubly charged $[M-2H]^{2-}$ ions of methyl ester of hexuronic acid-dihexuronic acid-hexose-modified C_{55} and C_{60} DolP are shown. Non-DolP-related peaks at *m/z* 805.685 serve as internal controls for changes in glycan-charged DolP peaks as a function of *aglR* deletion. Note that different y-axis scales are used in the profiles of the parent and the Δ*aglR* cells. B. EICs of the hexose-charged DolP $[M-H]^{-}$ ion at *m/z* 1079.8 from parent (upper panel) and Δ*aglR* strain cells (lower panel) are shown. The enzymes responsible for generating the three monosaccharide-charging DolP species are indicated above each peak. In Δ*aglR* cells, the AglD-generated species accumulates. C. LC-ESI/MS/MS of an Asn-13-containing *Hfx. volcanii* S-layer glycoprotein-derived pentasaccharide-modified peptide from parent (left panels) or Δ*aglR* (right panels) strain cells are shown. Above each peak, the doubly-charged $[M-2H]^{2-}$ ion species mass is indicated.

Cohen-Rosenzweig, C., Yurist-Doutsch, S. and Eichler. (2012) J. AglS, a novel component of the *Haloferax volcanii* N-glycosylation pathway, is a dolichol phosphate-mannose mannosyltransferase. J. Bacteriol., 194, 6909-6916.

In *Haloferax volcanii*, a series of Agl proteins mediates protein N-glycosylation. The genes encoding all but one of the Agl proteins are sequestered into a single gene island. The same region of the genome includes sequences also suspected but not yet verified as serving N-glycosylation roles, such as *HVO_1526*. In the following, HVO_1526, renamed AglS, is shown to be necessary for the addition of the final mannose subunit of the pentasaccharide N-linked to the S-layer glycoprotein, a convenient reporter of N-glycosylation in *Hfx. volcanii*. Relying on bioinformatics, topological analysis, gene deletion, mass spectrometry and biochemical assays, AglS was shown to act as a dolichol phosphate-mannose mannosyltransferase, mediating the transfer of mannose from dolichol phosphate to the tetrasaccharide corresponding to the first four subunits of the pentasaccharide N-linked to the S-layer glycoprotein.

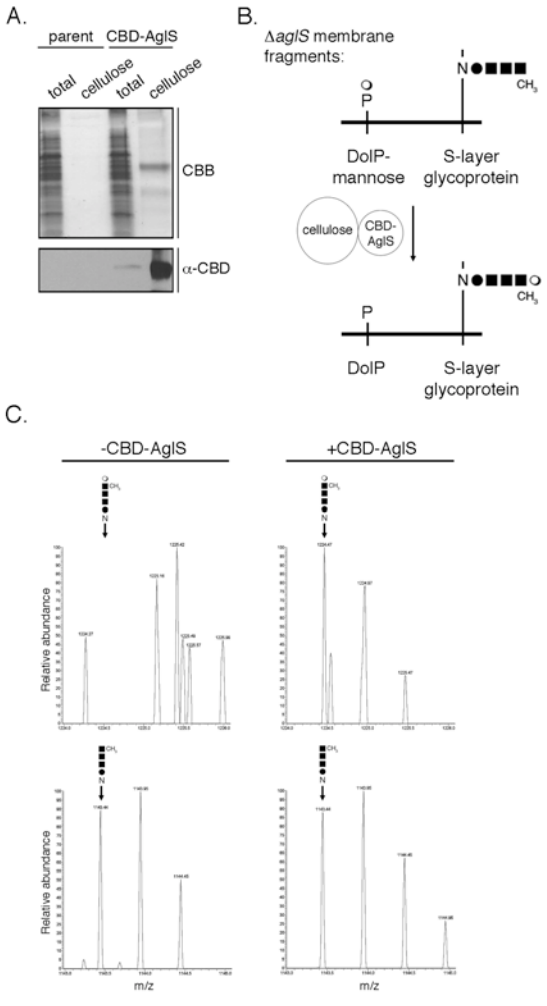


Fig 3 – AglS functions as a DolP-mannose mannosyltransferase. A. Cellulose-based purification of CBD-AglS from protein extracts of *Hfx. volcanii* parent strain cells and of cells transformed to express CBD-AglS was performed. The total protein extract and cellulose-bound proteins from each strain were separated by 10% SDS-PAGE, Coomassie-stained (CBB; upper panel) and analyzed by immunoblot using antibodies raised against the CBD moiety (lower panel). B. Schematic depiction of the *in vitro* assay of AglS function employed. Membrane fragments prepared from D*aglS* cells are combined with cellulose-bound CBD-AglS for 0-3 h at 42°C. Aliquots are drawn at 1 h intervals and LC-ESI MS is performed to determine whether AglS catalyzes the transfer of mannose from DolP-mannose to the glycan corresponding to the first four subunits of the pentasaccharide normally N-linked to the protein. C. LC-ESI MS analysis of an Asn-13-containing S-layer glycoprotein-derived peptide from Δ*aglS* cells before (-CBD-AglS column) and 3 h after (+CBD-AglS column) incubation with cellulose-bound CBD-AglS. In each column, the upper panel presents that region of the LC-ESI MS profile containing pentasaccharide-modified peptide, while the lower panel reflects that region of the profile containing tetrasaccharide-modified peptide. In each panel, the identity and position of the peak in question is indicated. The results are representative of four repeats of the experiment.

Kandiba L., Guan, Z. and Eichler, J. (2013) Lipid modification gives rise to two distinct *Haloferax volcanii* S-layer glycoprotein populations. Biochim. Biophys. Acta, 1828, 938-943.

The S-layer glycoprotein is the sole component of the protein shell surrounding *Haloferax volcanii* cells. The deduced amino acid sequence of the S-layer glycoprotein predicts the presence of a C-terminal membrane-spanning domain. However, several earlier observations, including the ability of EDTA to selectively solubilize the protein, are inconsistent with the presence of a trans-membrane sequence. In the present report, sequential solubilization of the S-layer glycoprotein by EDTA and then with detergent revealed the existence of two distinct populations of the S-layer glycoprotein. Whereas both S-layer glycoprotein populations underwent signal peptide cleavage and N-glycosylation, base hydrolysis followed by mass spectrometry revealed that a lipid, likely archaetidic acid, modified only the EDTA-solubilized version of the protein. These observations are consistent with the S-layer glycoprotein being initially synthesized as an integral membrane protein and subsequently undergoing a processing event in which the extracellular portion of the protein is separated from the membrane-spanning domain and transferred to a waiting lipid moiety.

Kaminski, L., Lurie-Weinberger M.N., Allers, T., Gophna, U. and Eichler, J. (2013) Phylogenetic- and genome-derived insight into the evolutionary history of N-glycosylation in Archaea. Mol. Phylogenet. Evol., 68, 327-339.

N-glycosylation, the covalent attachment of oligosaccharides to target protein Asn residues, is a post-translational modification that occurs in all three domains of life. In Archaea, the N-linked glycans that decorate experimentally characterized glycoproteins reveal a diversity in composition and content unequaled by their bacterial or eukaryal counterparts. At the same time, relatively little is known of archaeal N-glycosylation pathways outside of a handful of model strains. To gain insight into distribution and evolutionary history of the archaeal version of this universal protein-processing event, 168 archaeal genome sequences were scanned for the presence of *aglB*, encoding the known archaeal oligosaccharyltransferase, an enzyme key to N-glycosylation. Such analysis predicts the presence of AglB in 166 species, with some species predicted to contain multiple versions of the protein. Phylogenetic analysis reveals that the events leading to *aglB* duplication occurred at various points during archaeal evolution. In many cases, *aglB* is found as part of a cluster of putative N-glycosylation genes. The presence, arrangement and nucleotide composition of genes in *aglB*-based clusters in five species of the halophilic archaeon *Haloferax* points to lateral gene transfer as contributing to the evolution of archaeal N-glycosylation.

Kandiba, L. and Eichler, J. (2013) Analysis of genes involved in the biosynthesis of sialic acids and other nonulosonic acid sugars in Archaea reveals a complex evolutionary history. FEMS Microbiol. Lett., 345, 110-120.

Sialic acids and the other nonulosonic acid sugars, legionaminic acid and pseudaminic acid, are nine carbon-containing sugars that can be detected as components of the glycans decorating proteins and other molecules in Eukarya and Bacteria. Yet, despite the prevalence of N-glycosylation in Archaea and the variety of sugars recruited for the archaeal version of this post-translational modification, only a single report of a nonulosonic acid sugar in an archaeal N-linked glycan has appeared. Hence, to obtain a clearer picture of nonulosonic acid sugar biosynthesis capability in Archaea, 122 sequenced genomes were scanned for the presence of genes involved in the biogenesis of these sugars. The results reveal that while Archaea and Bacteria share a common route of sialic acid biosynthesis, numerous archaeal nonulosonic acid sugar biosynthesis pathway components were acquired from elsewhere via various routes. Still, the limited number of Archaea encoding components involved in the synthesis of nonulosonic acid sugars implies that such saccharides are not major components of glycans in this domain.

Kaminski, L., Guan, Z., Yurist-Doutsch, S. and Eichler, J. (2013) Two distinct N-glycosylation pathways process the *Haloferax volcanii* S-layer glycoprotein upon changes in environmental salinity. mBio, 4, e00716-13.

N-glycosylation in Archaea presents aspects of this post-translational modification not seen in either Eukarya or Bacteria. In the haloarchaeon *Haloferax volcanii*, the surface (S)-layer glycoprotein can be simultaneously modified by two different N-glycans. Asn-13 and Asn-83 are modified by a pentasaccharide, whereas Asn-498 is modified by a tetrasaccharide of distinct composition, with N-glycosylation at this position being related to environmental conditions. Specifically, N-glycosylation of Asn-498 is detected when cells were grown in the presence of 1.75 but not 3.4 M NaCl. While deletion of genes encoding components of the pentasaccharide assembly pathway had no effect on the biosynthesis of the tetrasaccharide bound to Asn-498, deletion of genes within the cluster spanning *HVO_2046*-*HVO_2061* interfered with the assembly and attachment of the Asn-498-linked tetrasaccharide. Transfer of the 'low salt' tetrasaccharide from the dolichol phosphate carrier upon which it is assembled to S-layer glycoprotein Asn-498 did not require AglB, the oligosaccharyltransferase responsible for pentasaccharide attachment to Asn-13 and Asn-83. Finally, although biogenesis of the 'low salt' tetrasaccharide is barely discernible upon growth at the elevated salinity, this glycan was readily detected under such conditions in strains deleted of pentasaccharide biosynthesis pathway genes, indicative of crosstalk between the two N-glycosylation pathways.

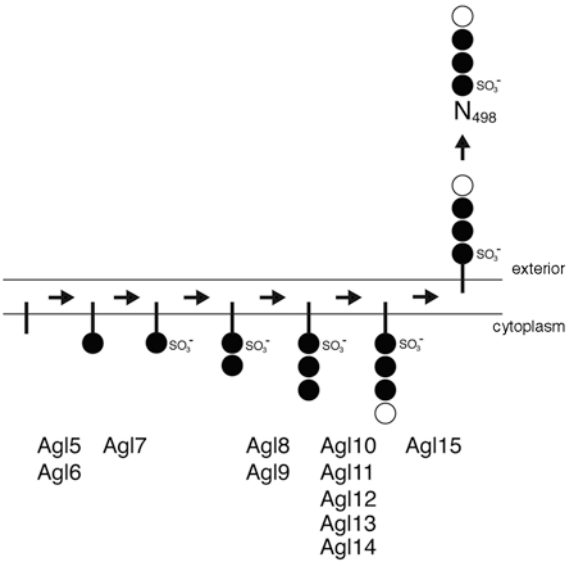


Fig 4 – Working model of the assembly of the 'low salt' tetrasaccharide decorating S-layer glycoprotein Asn-498 in *Hfx. volcanii* cells grown in 1.75 M NaCl. The sites of action of the novel Agl proteins identified in this study are depicted. In the pathway, the vertical line corresponds to DolP, the full circles to hexose and the open circle to rhamnose. The Agl proteins listed act at the cytosolic face of the plasma membrane.

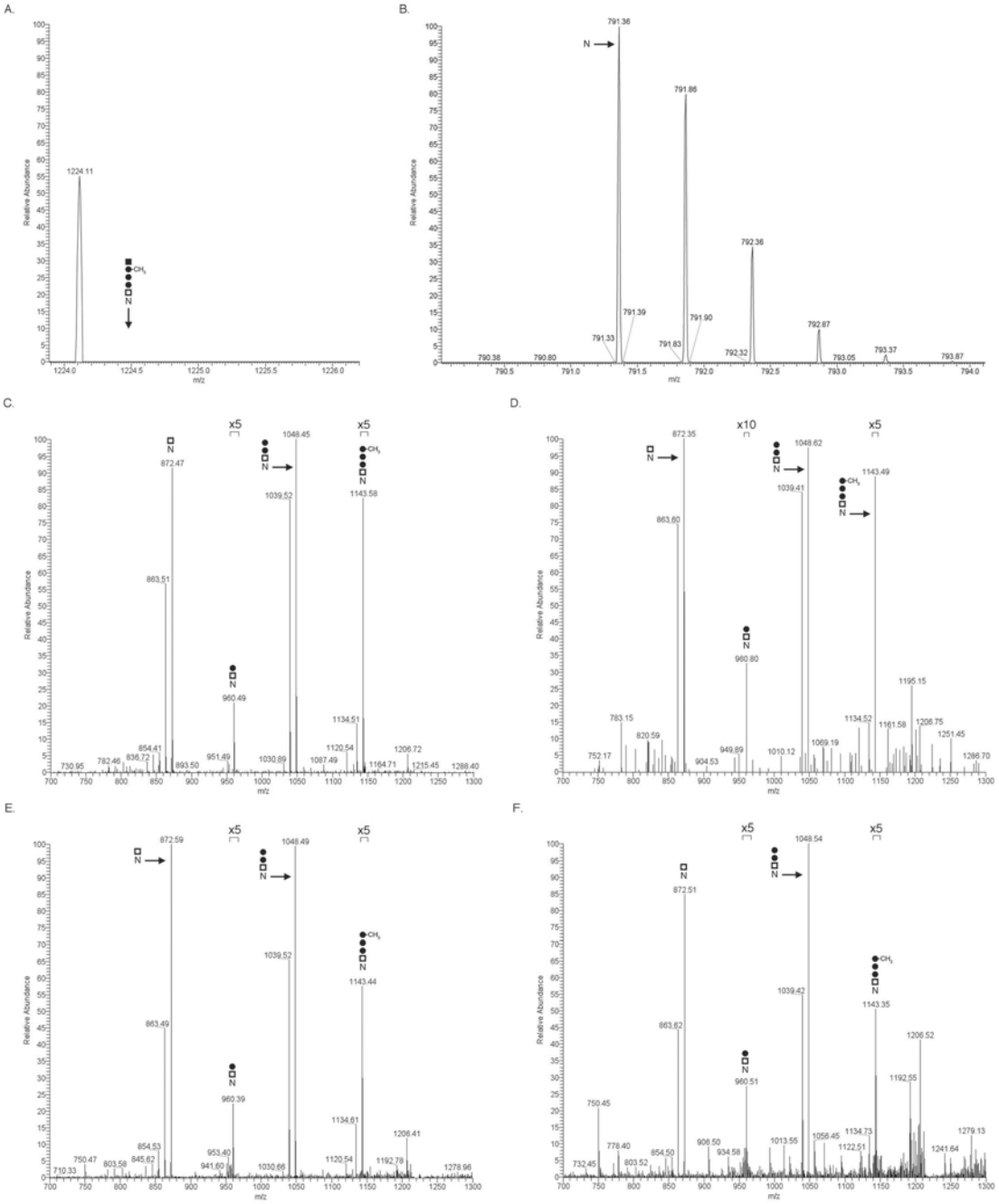
Arbiv, A., Yurist-Doutsch, S., Guan, Z. and Eichler, J. (2013) AglQ is a novel component of the *Haloferax volcanii* N-glycosylation pathway. PLoS One, 8, e81782.

N-glycosylation is a post-translational modification performed by members of all three domains of life. Studies on the halophile *Haloferax volcanii* have offered insight into the archaeal version of this universal protein-processing event. In the present study, AglQ was identified as a novel component of the pathway responsible for the assembly and addition of a pentasaccharide to select Asn residues of *Hfx. volcanii* glycoproteins, such as the S-layer glycoprotein. In cells deleted of *aglQ*, both dolichol phosphate, the lipid carrier used in *Hfx. volcanii* N-glycosylation, and modified S-layer glycoprotein Asn residues only presented the first three pentasaccharide subunits, pointing to a role for AglQ in either preparing the third sugar for attachment of the fourth pentasaccharide subunit or processing the fourth sugar prior to its addition to the lipid-linked trisaccharide. To better define the precise role of AglQ, shown to be a soluble protein, bioinformatics tools were recruited to identify sequence or structural homologs of known function. Site-directed mutagenesis experiments guided by these predictions identified residues important for AglQ function. The results obtained point to AglQ acting as an isomerase in *Hfx. volcanii* N-glycosylation.

Cohen-Rosenzweig, C., Guan, Z., Shaanan, B. and Eichler, J. (2014) Substrate promiscuity: AglB, the archaeal oligosaccharyltransferase, can process a variety of lipid-linked glycans. Appl. Environ. Microbiol., 80, 486-496.

Across evolution, N-glycosylation involves oligosaccharyltransferases that transfer lipid-linked glycans to selected Asn residues of target proteins. While these enzymes catalyze similar reactions in each domain, differences exist in terms of the chemical composition, length and degree of phosphorylation of the lipid glycan carrier, in terms of the sugar linking the glycan to the lipid carrier and in terms of the composition and structure of the transferred glycan. To gain insight into how oligosaccharyltransferases cope with such substrate diversity, the present study compared the archaeal oligosaccharyltransferase AglB from four haloarchaeal species. Accordingly, it was shown that despite processing distinct lipid-linked glycans in their native hosts, AglB from *Haloarcula marismortui*, *Halobacterium salinarum* and *Haloferax mediterranei* could readily replace their counterpart from *Haloferax volcanii* when introduced into *Hfx. volcanii* cells deleted of *aglB*. As the four enzymes show significant sequence and apparently structural homology, it would appear that the functional similarity of the four AglB proteins reflects the relaxed substrate specificity of these enzymes. Such demonstration of AglB substrate promiscuity is important not only for better understanding of N-glycosylation in Archaea and elsewhere but also for efforts aimed at transforming *Hfx. volcanii* into a glyco-engineering platform.

Fig 5 - LC-ESI MS analysis of a S-layer glycoprotein Asn-13-containing peptide from *Hfx. volcanii* Δ*aglB* cells transformed to express CBD-tagged versions of haloarchaeal AglB. A. The arrow indicates the expected position of the pentasaccharide-modified peak that is not detected in *Hfx. volcanii* Δ*aglB* cells. B. The indicated monoisotopic $[M+2H]^{2+}$ peak at *m/z* 791.36 corresponds to the non-glycosylated Asn-13-containing peptide detected in *Hfx. volcanii* Δ*aglB* cells. C-F. MS/MS analysis of a pentasaccharide-charged peptide (*m/z* 1224.48) from *Hfx. volcanii* Δ*aglB* cells transformed to express CBD-tagged *Hfx. volcanii* (C), *Hbt. salinarum* (D), *Har. marismortui* (E) and *Hfx. mediterranei* AglB (F) identified by LC-ESI MS reveals the presence of mono- (*m/z* 872), di- (*m/z* 960), tri- (*m/z* 1048) and tetrasaccharide-charged (*m/z* 1143) fragments. The region indicated by x5 and x10 reflect magnification of the ion peaks in the corresponding region of the *m/z* values on the graph. Symbols used: N, Asn-13-containing peptide; open square, hexose; full circle, hexuronic acid; full square, mannose.


A.
B.
C.
D.
E.
F.
Relative Abundance
m/z
1224.11
791.36
791.86
792.36
791.39
791.90
792.87
791.33
791.83
792.32
793.37
793.87
790.38
790.80
793.05
872.47
1048.45
1039.52
1143.58
863.51
960.49
1134.51
1120.54
1206.72
872.35
1048.62
1039.41
1143.49
863.60
960.80
1195.15
1134.52
1161.58
1206.75
1251.45
872.59
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1143.44
863.49
960.39
1134.61
1206.41
872.51
1048.54
1039.42
1143.35
863.62
960.51
1206.52
1192.55
1279.13
1241.64
x5
x10

Kaminski, L. and Eichler, J. (2014) *Haloferax volcanii* N-glycosylation: Delineating the pathway of dTDP-rhamnose biosynthesis. PLoS One, 9, e97441.

In the halophilic archaea *Haloferax volcanii*, the surface (S)-layer glycoprotein can be modified by two distinct N-linked glycans. The tetrasaccharide attached to S-layer glycoprotein Asn-498 comprises a sulfated hexose, two hexoses and a rhamnose. While Agl11-14 have been implicated in the appearance of the terminal rhamnose subunit, the precise roles of these proteins have yet to be defined. Accordingly, a series of *in vitro* assays conducted with purified Agl11-Agl14 showed these proteins to catalyze the stepwise conversion of glucose-1-phosphate to dTDP-rhamnose, the final sugar of the tetrasaccharide glycan. Specifically, Agl11 is a glucose-1-phosphate thymidylyltransferase, Agl12 is a dTDP-glucose-4,6-dehydratase and Agl13 is a dTDP-4-dehydro-6-deoxy-glucose-3,5-epimerase, while Agl14 is a dTDP-4-dehydrorhamnose reductase. Archaea thus synthesize nucleotide-activated rhamnose by a pathway similar to that employed by Bacteria and distinct from that used by Eukarya and viruses. Moreover, a bioinformatics screen identified homologues of *agl11-14* clustered in other archaeal genomes, often as part of an extended gene cluster also containing *aglB*, encoding the archaeal oligosaccharyltransferase. This points to rhamnose as being a component of N-linked glycans in Archaea other than *Hfx. volcanii*.

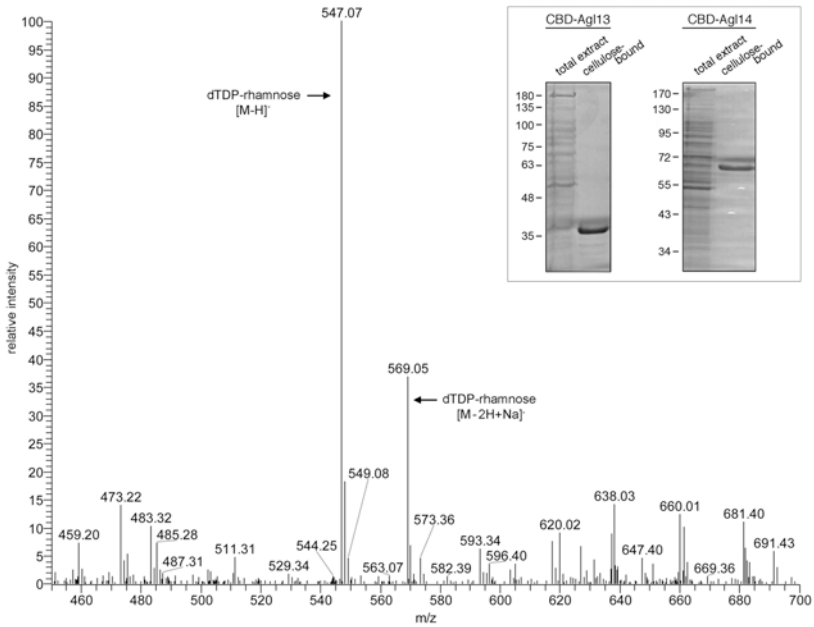

Fig 6 – Agl13 and Agl14 together convert dTDP-4-keto-6-deoxy-glucose into dTDP-rhamnose. Cellulose-bound CBD-Agl13 and CBD-Agl14 were combined with dTDP-4-keto-6-deoxy-glucose and NADPH and the soluble fraction was examined by nano-ESI/MS analysis. Peaks corresponding to dTDP-rhamnose and the sodium adduct are indicated. Inset: Purification of CBD-Agl13 and CBD-Agl14. Cell extracts and cellulose-bound protein from *Hfx. volcanii* cells transformed to express CBD-Agl13 (left) or CBD-Agl14 (right) were separated on 10% SDS-PAGE and Coomassie-stained. Protein bands corresponding to CBD-Agl13 and CBD-Agl14 are observed in each lane of cellulose-bound material. The positions of molecular weight markers are shown on the left of each gel.

Godin, N. and Eichler, J. (2014) *aglgenes*, a curated and searchable database of archaeal N-glycosylation pathway components. Database, 2014, bau046.

Whereas N-glycosylation is a post-translational modification performed across evolution, the archaeal version of this protein-processing event presents a degree of diversity not seen in either Bacteria or Eukarya. Accordingly, archaeal N-glycosylation relies on a large number of enzymes that are often species-specific or restricted to a select group of species. As such, there is a need for an organized platform upon which amassing information about *a*rchaeal *gl*ycosylation (*agl*) genes can rest. Accordingly, the *agl*genes database provides detailed descriptions of experimentally characterized archaeal N-glycosyation pathway components. For each *agl* gene, genomic information, supporting literature and relevant external links are provided at a functional intuitive web interface designed for data browsing. Routine updates ensure that novel experimental information on genes and proteins contributing to archaeal N-glycosylation is incorporated into *agl*genes in a timely manner. As such, *agl*genes represents a specialized resource for sharing validated experimental information online, providing support for workers in the field of archaeal protein glycosylation.

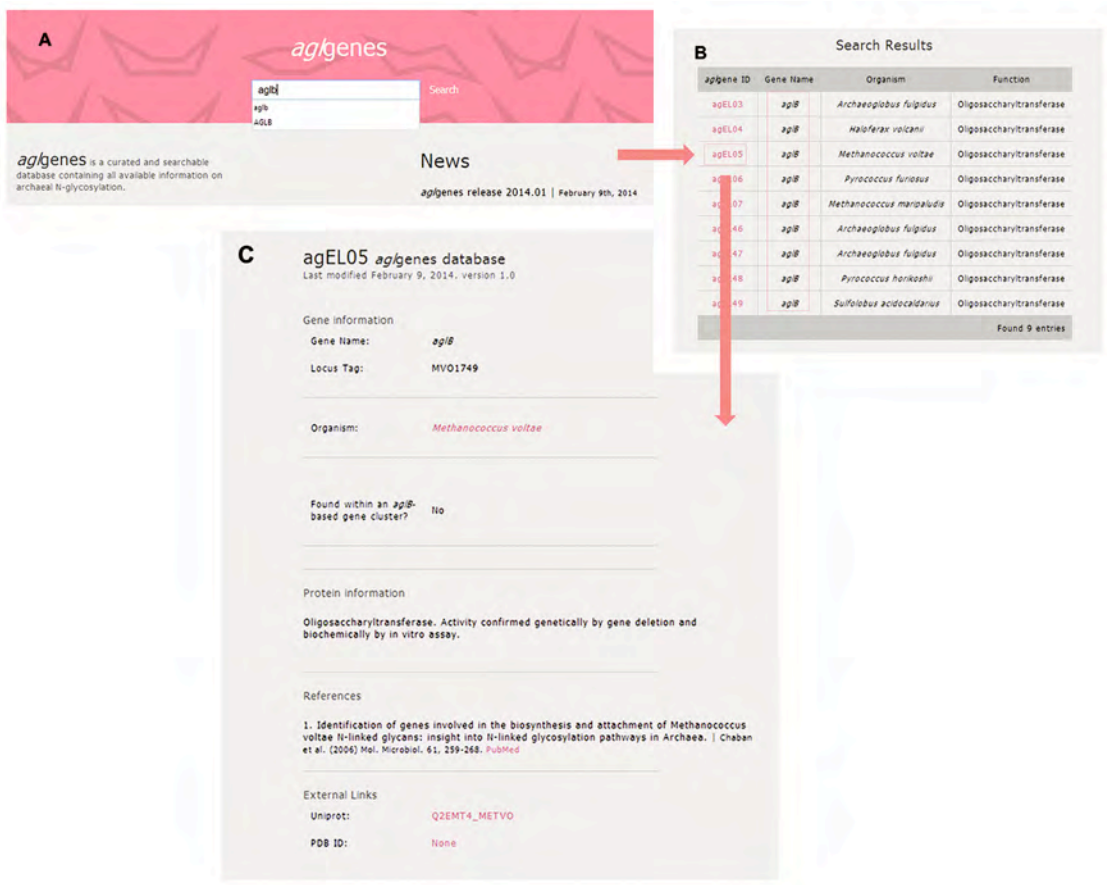

Fig 7 - Screenshots of the results of a sample *agl*genes query. A. The user inputs the string “aglb” in the search field, for example. B. The results for the query are presented in tabular format. C. After selecting the entry of interest from the list of organisms containing the gene selected in this example, detailed information for the selected *agl*genes entry is retrieved in a new web page.

Kandiba, L. and Eichler, J. (2014) Deciphering a pathway of *Halobacterium salinarum* N-glycosylation. MicrobiologyOpen, in press.

Genomic analysis points to N-glycosylation as being a common post-translational modification in Archaea. To date, however, pathways of archaeal N-glycosylation have only been described for few species. With this in mind, the similarities of N-linked glycans decorating glycoproteins in the haloarchaea *Haloferax volcanii* and *Halobacterium salinarum* directed a series of bioinformatics, genetic and biochemical experiments designed to describe that *Hbt. salinarum* pathwyay responsible for biogenesis of one of the two N-linked oligosaccharides described in this species. As in *Hfx. volcanii*, where *agl* (*a*rchaeal *gl*ycosylation) genes that encode proteins responsible for the assembly and attachment of a pentasaccharide to target protein Asn residues are clustered in the genome, *Hbt. salinarum* also contains a group of clustered homologous genes (*VNG1048G*-*VNG1068G*). Introduction of these *Hbt. salinarum* genes into *Hfx. volcanii* mutant strains deleted of the homologous sequence restored the lost activity. Moreover, transcription of the *Hbt. salinarum* genes in the native host, as well as *in vitro* biochemical confirmation of the predicted functions of several of the products of these genes provided further support for assignments made following bioinformatics and genetic experiments. Based on the results obtained in this study, the first description of an N-glycosylation pathway in *Hbt. salinarum* is offered.

		Hfx. volcanii									
		Δ*aglJ*	**Δ*aglP***	**Δ*aglQ***	**Δ*aglE***	**Δ*aglR***	**Δ*aglF***	**Δ*aglI***	**Δ*aglG***	**Δ*aglB***	**Δ*aglM***	**Δ*aglD***
Hbt. salinarum	**VNG1048G**										+	
	VNG1053G	+			+			-	-			
	VNG1054G					+						
	VNG1055G						+					
	VNG1058H			+								
	VNG1062G	+			+			-	-			
	VNG1065C		+									
	VNG1066C	+			-			+	-			
	VNG1067C	+			-			-	+			
	VNG1068G									+		
	VNG0318G											+

Table 1 – Functional replacement of *Hfx. volcanii* Agl proteins by their *Hbt. salinarum* homologues