Effects of growth conditions on archaellation and N-glycosylation in *Methanococcus maripaludis*

Yan Ding,† Zoe Lau,† Susan M. Logan,2 John F. Kelly,2 Alison Berezuk,3 Cezar M. Khursigara3 and Ken F. Jarrell1

1Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, Ontario K7L 3N6, Canada
2Human Health Therapeutics Portfolio, National Research Council, Ottawa, Ontario K1A 0R6, Canada
3Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Correspondence
Ken F. Jarrell
jarrellk@queensu.ca
John F. Kelly
john.kelly@nrc-cnrc.gc.ca

In this study, the effects of growth conditions on archaellation in *Methanococcus maripaludis* were examined. Cells were grown in a variety of media, including complex, minimal and with formate as the electron donor, with different nitrogen sources, varied salinities and at a variety of growth temperatures. Of the conditions tested, Western blot results showed that major archaellin FlaB2 levels only varied detectably as a result of growth temperature. Whilst the amount of FlaB2 was similar for cells grown at <35 °C, protein levels decreased at 38 °C and were barely detectable at 42 °C. Quantitative reverse transcription PCR experiments demonstrated that the flaB2 transcript levels were almost undetectable at 42 °C. Electron microscopy confirmed that the FlaB2 levels detected by Western blots corresponded to the state of archaellation, with cells grown at 42 °C being mostly non-archaellated. Unexpectedly, a lower apparent molecular mass for FlaB2 was observed in Western blots of cells grown at temperatures >38 °C, suggestive of a truncation in the attached N-linked tetrasaccharide at higher growth temperatures. MS analysis of archaella isolated from cells grown at 40 °C confirmed that FlaB2 was now decorated with a trisaccharide in which the third sugar was also lacking the attached threonine and acetamidino modifications found in the WT glycan.

INTRODUCTION

Archaea possess a motility apparatus, recently termed the archaellum (Jarrell & Albers, 2012), that is unique to this domain of life (Albers & Jarrell, 2015; Jarrell et al., 2013). Biochemical, structural and genetic evidence (Jarrell et al., 2013; Lassak et al., 2012a; Pohlschroder et al., 2011; Shahapure et al., 2014; Trachtenberg & Cohen-Krausz, 2006) all support a relatedness of archaella used for swimming to type IV pili, used for a kind of surface motility called twitching (Bradley, 1980; Burrows, 2012), with one key difference between the two structures being the rotation of archaella as opposed to an extension/retraction mechanism in type IV pili (Alam & Oesterhelt, 1984; Burrows, 2012; Marwan et al., 1991; Shahapure et al., 2014). A further unusual feature of archaella is the presence of N-linked glycans attached to the structural proteins (archaellins) that comprise the appendage, in contrast to the O-glycosylated type IV pilins found in certain bacterial species (Jarrell et al., 2014; Giltner et al., 2012; Meyer et al., 2013; Tripepi et al., 2012; Voisin et al., 2005; Wieland et al., 1985). In the case of *Methanococcus maripaludis*, the attached glycan has been determined to be a tetrasaccharide with the structure Sug-1,4-O-Methyl-α-L-erythrose-5-ulose-1,5-pyranose (Kelly et al., 2009). This glycan is attached at multiple sites on each of the three archaellins (FlaB1, FlaB2 and FlaB3) that form the archaellum. A large number of agl genes (archaeal glycosylation) (Chaban et al., 2006; Eichler et al., 2013) involved in the biosynthesis of the individual sugars of the N-glycan or its assembly on a dolichol phosphate lipid carrier and subsequent transfer to protein targets have been identified (Ding et al., 2013; Jones et al., 2012; Siu et al., 2015; VanDyke et al., 2009). Mutations in agl genes that result in non-glycosylated archaellins or archaellins modified with only a single sugar glycan are non-archaellated. Whilst multiple sites of N-glycosylation are found in each

†These authors contributed equally to this paper.

Abbreviations: LC, liquid chromatography; qRT, quantitative reverse transcription.
archaellin, archaella can be formed when three of the four glycosylation sites of FlaB2 are removed, but not if all four sites are eliminated (Ding et al., 2015).

Deletion and electron microscopic studies in several model archaellated species have identified a fla operon which contains most of the known genes necessary for archaellation, including typically multiple archaellins followed by several fla-associated genes (Jarrell et al., 2013; Kalmokoff & Jarrell, 1991; Ng et al., 2006; Thomas & Jarrell, 2001). The latter group includes homologues of genes involved in the assembly of type IV pili (Albers & Jarrell, 2015; Bayley & Jarrell, 1998; Peabody et al., 2003). Most of the fla operon genes are highly conserved in euryarchaeotes, such as methanogens and extreme halophiles, although a smaller subset is found in crenarchaeotes, such as Sulfolobus acidocaldarius (Szabó et al., 2007; Lassak et al., 2012b).

Archaella, by virtue of their roles in swimming and attachment to surfaces, are important organelles in the interactions of archaeal cells with their environment. Methanococcus maripaludis is considered a model archaean organism (Leigh et al., 2011) and one of the best-studied archaea with regard to its archaella (Chaban et al., 2007; Ding et al., 2015; Jarrell et al., 2011; VanDyke et al., 2009). This study reports on a variety of growth conditions and the effect they have on archaellation. Unexpectedly, these studies revealed that growth at elevated temperatures also affected the nature of the N-glycan attached to archaellins.

METHODS

Strains and growth conditions. Methanococcus maripaludis strain Mn900 (Moore & Leigh, 2005) was grown anaerobically in serum bottles containing Balch Medium III (Balch et al., 1979) under a headspace of CO2/H2 (20 : 80), unless otherwise stated. When testing for the effect of temperature on archaellation, cultures were grown in Balch Medium III with shaking (110 r.p.m.) over the range from room temperature (22 °C) to 42 °C. To test the effect of salt stress induced by NaCl, cultures were grown in Balch Medium III modified to contain either 0.3 or 5 % (w/v) NaCl, as well as the normal 2 % (w/v) NaCl. The expression of FlaB2 was also compared after growth in complex medium (Balch Medium III) or nitrogen-free minimal media supplemented with either 10 mM l-alanine or 10 mM NH4Cl (Lie & Leigh, 2002). Cells grown in minimal medium with NH4Cl but omitting CaCl2 (no calcium medium) were also examined. Cells were also cultivated in a formate medium (Costa et al., 2013). For this medium, 200 mM sodium formate with an equivalent reduction in NaCl to maintain sodium osmolarity and 200 mM MOPS buffer at pH 7 were added to McCas medium under a headspace of CO2/N2 (20 : 80). Finally, samples were also compared after growing statically or shaking at both 22 and 42 °C in Balch Medium III. In all cases, cells were transferred a minimum of three times under the various conditions before analysis.

SDS-PAGE and Western blotting. Whole-cell lysates were separated by SDS-PAGE (15 % acrylamide) (Laemmli, 1970) in a minigel system and stained with Coomassie blue G250, as described previously (Faguy et al., 1996). For Western blotting, whole-cell lysates of Methanococcus maripaludis grown under the various conditions were subjected to SDS-PAGE and then transferred to Immobilon-P membranes (Millipore) (Towbin et al., 1979). The major archaellin FlaB2 was detected with anti-FlaB2-specific chicken antibodies (IgY) (Jones et al., 2012), using horseradish peroxidase-conjugated rabbit anti-chicken IgY (Jackson ImmunoResearch) as the secondary antibody. Blots were developed using a chemiluminescent detection kit.
according to the manufacturer’s instructions (Roche Molecular Biochemicals).

**Quantitative reverse transcription (qRT)-PCR.** qRT-PCR experiments were performed to compare the levels of FlaB2 transcript under selected growth conditions. RNA template was extracted from 1 ml of an overnight culture of *Methanococcus maripaludis* Mm900 and Mm900 cells harbouring pKJ752 using an RNeasy Mini kit (Qiagen) as described by the manufacturer’s protocol with an additional DNase step (Turbo DNA-free kit; Ambion) at 37 °C for 30 min. Primers were constructed for flaB2, aglX and mmp0383 (encoding the major S-layer protein; Pohlschroeder et al., 2005) and leading to PCR products that would be 136, 102 and 149 bp in length, respectively. The forward and reverse primer sequences for flaB2, aglX and mmp0383 were: 5’-GCTGCAATAGAAGATGAGGG-3’ and 5’-GACCAGTTTACAGTTGTGTTG-3’; 5’-GATCAGATACTT- AAATTATGCAG-3’ and 5’-GATTCAACATGTTTGTGAGGG-3’; and 5’-GGTACTGAAAGCAGAGAGG-3’ and 5’-GCTAACAAT TTTACCGTCTTTAAGAG-3’, respectively. For the qRT-PCR experiments, mmp0383 was used as reference gene as levels of this S-layer protein were reported not to be affected by any nutrient limitation tested (Xia et al., 2009). In addition, PCR amplifications were performed using purified RNA that had not undergone the reverse transcription step as a control for genomic DNA contamination and with the same primer combinations with genomic DNA as template to ensure specificity of primer pairs. For these PCR experiments, the program consisted of 5 min initial denaturation at 94 °C; 30 cycles of 15 s denaturation at 94 °C, 30 s annealing at 50 °C and 1 min extension at 72 °C; and a 10 min final extension step at 72 °C. Subsequently, PCR products were examined by agarose gel electrophoresis (0.8 % agarose).

For the qRT-PCR experiments, 100 ng purified RNA from each strain was converted into a cDNA library using an iScript cDNA synthesis kit (Bio-Rad) following the manufacturer’s instructions. The abundance of the flaB2 transcript and the reference gene mmp0383 was analysed in a 20 μl qRT-PCR containing 10 nmol of each gene specific primer, 5 μl cDNA library dilution (1/125 dilution for amplification of mmp0383, and 1/25 dilution for flaB2 and aglX) and 1× EvaGreen Supermix (Bio-Rad) according to the manufacturer’s protocol. Reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Samples were subjected to an initial 5 min denaturation followed by 40 cycles of 10 s at 95 °C and 30 s at 50 °C, followed by melt curve analysis to confirm the specificity of the qRT-PCR amplicons. Triplicate and no-template control reactions were included to confirm a lack of genomic DNA contamination, and the qRT-PCR experiment was repeated three times.

**Expression of aglX in Mm900 cells.** Mm900 cells were transformed with vector pKJ752, a plasmid in which the aglX gene is cloned under the control of the nif vector in pHW40 (Jones et al., 2012), using the polyethylene glycol precipitation method (Tumbula et al., 1994). Cells transformed with the vector were subsequently grown in nitrogen-free minimal medium supplemented with puromycin (2.5 μg ml⁻¹) and either 10 mM l-alanine (nif promoter induced) or NH₄Cl (nif promoter repressed) as nitrogen source (Lie & Leigh, 2003).

**Isolation of surface structures.** Archaea and pili were isolated from cells grown at 40 °C and purified via a KBr gradient centrifugation step, as described previously (Bardy et al., 2002).

**Tryptic digestion and liquid chromatography (LC)-MS procedure.** The isolated surface structure preparation was incubated overnight at 37 °C with trypsin (Promega) at an approximate ratio of 20:1 (protein : enzyme, v/v) in 50 mM ammonium bicarbonate. The digest was then analysed by nano-LC-tandem MS (MS/MS) using a NanoAquity UPLC system (Waters) coupled to a QTOF Ultima hybrid quadrupole time-of-flight mass spectrometer (Waters). The digest were injected on an Acclaim PepPakMax100 C₁₈ µ-pre-column (5 mm × 300 μm inner diameter; Dionex/Thermo Scientific) and resolved on a 1.7 μm BEH130 C₁₈ column (100 mm × 100 μm inner diameter; Waters) using the following gradient conditions: 1–45 % acetonitrile, 0.1 % formic acid in 36 min and 45–95 % acetonitrile, 0.1 % formic acid in 2 min. The flow rate was 400 nL min⁻¹. MS/MS spectra were acquired on doubly, triply and quadruply charged ions, and searched against the National Center for Biotechnology Information nr (non-redundant) database using the Mascot search engine (Matrix Science). The spectra were searched for glycopeptide MS/MS spectra which were then interpreted by hand.

**Electron microscopy.** Transmission electron microscopy of *Methanococcus maripaludis* cells was performed as described previously (Ding et al., 2015). Briefly, cells were collected from an overnight culture by centrifugation, resuspended in 2 % (w/v) NaCl and placed on 200-mesh carbon-coated copper grids. Cells were allowed to adhere for 1 min, briefly washed with 2 % (w/v) NaCl and stained with 2 % (w/v) phosphotungstic acid, pH 7.0. Samples were viewed using a Phillips CM-10 transmission electron microscope operating at 80 kV equipped with a SIS/Olympus Morada 11-megapixel charge-coupled device camera under standard operating conditions.

**RESULTS**

**Western blot and electron microscopy analyses of *Methanococcus maripaludis* grown under different conditions.**

*Methanococcus maripaludis* was grown under a variety of conditions, and the ability of cells to produce archaellin and assemble archaella was examined by Western blotting and electron microscopy, respectively.

To investigate the effects of different environmental factors on archaellation, the major archaellin glycoprotein FlaB2 was used as a reporter protein and its production was followed by Western blot analysis of total cell lysates probed with FlaB2-specific antibody. This was performed initially for cells grown in a variety of different media. Cells were cultivated in Balch Medium III with NaCl varied from 0.3 to 2 to 5 % (w/v). The 2 % NaCl level is the normal amount found in Balch Medium III, whilst 0.3 and 5 % are reported to be the lower and upper limits that allow for growth of the organism (Whitman & Jeanthon, 2006). After at least three successive transfers, Western blot analysis revealed little difference in the amount of FlaB2 present under the three conditions (Fig. 1a). Similarly, when cells were grown in Balch Medium III as an example of a complex medium and compared with cells grown in a minimal medium with either NH₄Cl or alanine as the sole nitrogen source, no difference in the level of FlaB2 was observed in Western blots (Fig. 1b). In addition, no apparent effect on FlaB2 levels was observed when cells were grown in minimal medium with no CaCl₂ added (Fig. 1c) or when cells grown in Balch Medium III under a CO₂/H₂ headspace were compared with cells grown under CO₂/N₂ with formate as the electron donor (Fig. 1d). Electron microscopy of cells...
grown under all the above conditions revealed that all had multiple peritrichously located archaella (Fig. 1e, f).

Methanococcus maripaludis grows over the temperature range from <20 to 45 °C (Whitman & Jeanthon, 2006). In contrast to what was observed when Methanococcus maripaludis was grown in media of varying compositions, when cells were grown in Balch Medium III at various temperatures within the range of 22–42 °C, a clear difference in FlaB2 signal intensity was seen in cells grown at the upper temperatures tested (38, 40 and 42 °C) compared with the lower temperatures (Fig. 2a). Cells grown at 22, 30 and 34 °C all demonstrated similar large amounts of FlaB2. At 38 °C, a less prominent band was observed for FlaB2, and this grew even fainter at 40 °C and was essentially undetectable at 42 °C unless the blot was overexposed. When cells grown at 42 °C were subcultured back to 34 °C, the FlaB2 signal was restored to the intensity observed from cells grown continuously at 34 °C.

**Fig. 1.** Western blot and electron microscopic analysis of Methanococcus maripaludis cultivated under various growth conditions. Whole-cell lysates were examined in Western blots developed with antibodies against FlaB2 following growth of cells under various conditions. (a) Balch medium modified to contain different NaCl concentrations (0.3, 2 and 5 %). (b) Complex medium (Balch Medium III) or nitrogen-free minimal medium (MM) supplemented with either alanine or NH₄Cl. (c) Minimal medium or minimal medium without added CaCl₂ (no Ca). (d) Balch Medium III or formate medium under a headspace of CO₂/N₂. (e, f) Electron microscopy of cells grown under the same conditions used for the Western blots of (a–d). Bar, 500 nm.
peritrichous archaella. Cells grown at 38 °C had fewer archaella and cells grown at 40 °C possessed only a very few, often shorter, archaella. Cells grown at 42 °C were almost all devoid of archaella, in keeping with the extremely low production of the major archaellin structural protein FlaB2 detected in Western blots.

**qRT-PCR analysis of flaB transcript**

qRT-PCR was performed to investigate whether the temperature-dependent regulation of archaella synthesis occurred at the level of transcription. Cells grown at 34 and 42 °C were analysed. The transcript for flaB2 was targeted as it is the second gene within the fla operon, its product is a major structural protein of the archaella and the previous Western blots were developed with specific antibodies to FlaB2. Western blot analysis for FlaB2 detection was done on the same samples used for the RNA isolation to directly compare transcript levels with protein levels. Initially, PCR amplifications using primer pairs specific for flaB2 and mmp0383 with genomic DNA as template were performed to ensure primer specificity and confirm the predicted sizes of the products. Purified RNA samples that had not undergone the reverse transcription step were also subjected to PCR, confirming that they were free of genomic DNA contamination (data not shown). qRT-PCR experiments showed that flaB2 transcript levels were ~50-fold lower at 42 °C compared with 34 °C (Fig. 4).

As archaella can also be used for attachment in conjunction with pili, cells that were incubated statically at 42 °C were compared with cells grown with shaking. It was clear that cells grown at 42 °C without shaking produced increased amounts of FlaB2, although not to the levels seen in cells grown at 34 °C (Fig. 2c). The statically grown cells at

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**Fig. 2.** Western blot analysis of *Methanococcus maripaludis* grown at different temperatures. In each case, whole-cell lysates were examined in Western blots developed with antibodies against FlaB2. (a) Cells grown with shaking in Balch Medium III incubated at the temperatures indicated. (b) Cells incubated at 42 °C with shaking can be switched to 34 °C where they now synthesize FlaB2. (c) Cells grown statically at 42 °C can synthesize FlaB2.

**Fig. 3.** Electron microscopy of cells grown with shaking in Balch Medium III at various temperatures. Samples were stained with 2 % phosphotungstic acid, pH 7.0. Grids were examined in a Phillips CM-10 transmission electron microscope operating at an accelerating voltage of 80 kV. Bar, 500 nm.
42 °C, however, were not archaellated when examined by electron microscopy (data not shown), suggesting the unavailability of FlaB2 was not the sole reason for the non-archaellation state observed at 42 °C. We also examined cells grown under shaking and static conditions at 22 °C, but no apparent increase in FlaB2 or archaellation over the abundant levels already present at 22 °C was observed when cells were grown statically (data not shown).

Western blot experiments indicate a truncated N-linked glycan when cells are grown at elevated temperature

Interestingly, examination of the Western blots of the cells grown at varying temperatures revealed that not only did the intensity of the FlaB2 decrease at ≥38 °C, but the FlaB2 of cells grown at the higher temperatures migrated further in Western blots than did FlaB2 from cells grown at any of the lower temperatures (Fig. 2a). This result is typical of what is observed in Western blots of mutants carrying a deletion in genes involved in N-glycosylation (Ding et al., 2013; Jones et al., 2012; Siu et al., 2015; VanDyke et al., 2009). FlaB2 is normally modified with an N-linked tetrasaccharide attached at four locations. As the cells were shown by electron microscopy to be archaellated at 38 and 40 °C, this meant that the archaellin N-linked glycan must be at least two sugars in length, i.e. the minimum length required for cells to form archaella (VanDyke et al., 2009). To obtain an initial idea of how truncated the glycan might be in cells grown above 38 °C, lysates from cells grown at 34 and 38 °C were compared with those of ΔaglL or ΔaglA mutants (Fig. 5a). The ΔaglL and ΔaglA mutants are deleted for the gene encoding either the glycosyltransferase responsible for transferring the terminal sugar or the third sugar of the tetrasaccharide, respectively (VanDyke et al., 2009). Thus, the ΔaglL mutant has archaellins modified with a three-sugar glycan whilst the ΔaglA mutant has archaellins modified with a two-sugar glycan. The electrophoretic mobility of FlaB2 from cells grown at 38 °C aligned closely with that of the ΔaglL mutant, suggesting that archaella expressed at 38 °C may contain archaellins that bear truncated N-glycans that lack the terminal sugar and the threonine attachment found on the third sugar. The threonine modification to the third sugar only occurs after the fourth sugar has been transferred to the growing glycan and so is missing in the glycan found in the ΔaglL mutant (Ding et al., 2013; Siu et al., 2015; VanDyke et al., 2009).

MS analysis of surface appendages purified from cells grown at 40 °C

To confirm that the differences in archaellin electrophoretic mobility observed in the Western blots of cells grown at elevated growth temperature were due to truncation of the N-linked glycan, surface appendages (archaella and pili) were isolated from cells grown at 40 °C for subsequent analysis by MS. A temperature of 40 °C was chosen as the decrease in FlaB2 apparent molecular mass was observed at this temperature, whilst electron microscopy showed that the cells still produced some archaella which could be isolated for analysis. Examination of the purified preparation of surface appendages from cells grown at 40 °C by SDS-PAGE followed by Coomassie blue staining indicated mainly the presence of EpdE, the major glycoprotein subunit of Epd pili (Ng et al., 2011), but also a lesser amount of archaellin (Fig. 6). Western blot analysis confirmed that the apparent molecular mass of FlaB2 in the purified appendage sample was still at the lower molecular mass observed in the whole-cell lysates (not shown).

LC-MS/MS analysis (Fig. 7a) of the archaellin band depicted in Fig. 6 demonstrated that the glycan appears to be identical to that observed for a previous mutant deleted for aglIX. AglX is the amidotransferase needed for the generation of the acetamidino group of the third sugar of the glycan. The acetamidino modification of the third sugar is necessary for the attachment of the fourth sugar and the fourth sugar addition is needed prior to the attachment of the threonine to the third sugar. Thus, the glycan structure of the ΔaglX mutant strain is missing the terminal sugar as well as the acetamidino group and threonine attached to the third sugar. The structure of the truncated archaellin glycan in cells grown at 40 °C is depicted in Fig. 7(b).

Expression of aglIX in trans at elevated growth temperature

The LC-MS data suggested that the truncated N-linked glycan may be the result of loss of AglX, possibly because the activity of the enzyme is temperature sensitive or because the gene is not transcribed at the higher temperatures from its native promoter. Complementation of an
aglX deletion mutant has been previously accomplished with pKJ752, where a WT version of aglX is transcribed from the inducible nif promoter (Jones et al., 2012).

Mm900 cells were transformed with this plasmid, and cells were grown at both 34 and 40 °C in nitrogen-free medium supplemented with alanine to allow transcription of aglX. Cells grown in nitrogen-free medium supplemented with alanine but without pKJ752 were used as controls at both temperatures. The transcript level of aglX was analysed by qRT-PCR experiments in all cases. The levels of the aglX transcript were upregulated by 3.2-fold in WT cells grown at 40 °C compared with WT cells grown at 34 °C. At both growth temperatures, the levels of aglX transcript were increased by 1.4-fold in the cells carrying pKJ752 compared with that from the WT cells lacking the plasmid but grown in nitrogen-free medium supplemented with alanine at the same two temperatures. (c) Shifting of cells pre-grown at 34 °C to 40 °C under non-growing conditions does not result in FlaB2 of reduced electrophoretic mobility. Cells were grown at 34 °C and then the headspace was replaced with either N2 or air and cells further incubated for 2 days at 40 °C. Cells grown at 34 and 40 °C are included for comparison.

**Fig. 6.** SDS-PAGE analysis of purified surface appendages of Methanococcus maripaludis grown at 40 °C. Following electrophoresis, the gel was stained with Coomassie blue G-250. Archaellin and pilin bands are shown by arrows.

**Fig. 5.** Western blot analysis of the faster FlaB2 electrophoretic migration at higher temperature, in comparison with mutants producing FlaB2 with known glycan defects. In each case whole-cell lysates were used in Western blots developed with FlaB2-specific antibodies. (a) The electrophoretic mobility of FlaB2 from Mm900 cells grown at 38 °C is compared with Mm900 cells as well as mutants deleted for aglL and aglA all grown at 34 °C. Mm900 cells have an N-linked tetrasaccharide glycan. AglL is the glycosyltransferase for the fourth sugar and AglA is the glycosyltransferase for the third sugar. (b) Expression of AglX at 40 °C does not lead to recovery of the WT size of FlaB2. The electrophoretic mobility of FlaB2 in Mm900 cells carrying pKJ752 (expressing AglX) grown in nitrogen-free medium supplemented with alanine at either 34 or 40 °C did not change when compared with FlaB2 in Mm900 cells without the plasmid but grown in nitrogen-free medium supplemented with alanine at the same two temperatures. (c) Shifting of cells pre-grown at 34 °C to 40 °C under non-growing conditions does not result in FlaB2 of reduced electrophoretic mobility. Cells were grown at 34 °C and then the headspace was replaced with either N2 or air and cells further incubated for 2 days at 40 °C. Cells grown at 34 and 40 °C are included for comparison.
degradation of the tetrasaccharide, cells were grown at 34 °C and then switched to 40 °C under conditions where the cells would not be able to grow, i.e. where the cells were placed under a headspace of N2 or air. Under both conditions, the apparent molecular mass of FlaB2 was equal to that observed in cells grown at 34 °C, indicating that the truncated glycan did not result from a partial disintegration of the glycan at the elevated temperatures (Fig. 5c).

**DISCUSSION**

In this report, a number of growth conditions were examined to explore their effects on archaellation in *Methanococcus maripaludis*. These included a variety of medium variations as well as growth temperature. Electron microscopic examination of cells grown over a range of NaCl concentrations, in complex medium or minimal medium with NH4Cl or alanine as sole nitrogen source, or with formate as electron donor, as well as in low-calcium minimal conditions, revealed variations in the composition and length of the archaellum.

**Fig. 7.** (a) Nano-LC-MS/MS analysis of the FlaB2 tryptic glycopeptide, T 53–81, from WT *Methanococcus maripaludis* grown at 40 °C. The triply protonated (MH+3+) glycopeptide ion at m/z 1216.3 was selected for this analysis. The amino acid sequence and glycan modification of this glycopeptide are shown in the inset. The major ions arising from the fragmentation of the peptide and carbohydrate bonds are indicated in the MS/MS spectrum. This glycopeptide is modified with a trisaccharide composed of the linking GalNAc (black square) and the GlcNAc3NAcA (black circle) from the WT glycan as well as a terminal sugar that is likely to be di-N-acetyl-mannuronic acid (ManNAc3NAcA; black cross). The glycan appears to be the same as that produced by the aglX mutant strain. This trisaccharide is the predominant glycan modification on the 40 °C archaellin. (b) The full glycan of the WT strain grown at lower temperatures. The residues and modifications residues absent from the 40 °C glycan are shaded in grey.
medium all showed many peritrichously located archaella, consistent with similar levels of the major archaellin FlaB2 detected in Western blots of the corresponding cell lysates. In contrast, when cells were incubated in Balch Medium III at various temperatures, two distinct differences were noted in Western blots detecting FlaB2. First, the cells produced much reduced levels of FlaB2 as the growth temperature was increased to 38 °C, and by 42 °C, FlaB2 production had essentially ceased. This decrease in FlaB2 production was also mirrored by decreases in the flaB2 transcript amounts (qRT-PCR) and in the number of archaella observed in electron microscopic studies, where cells grown at 38–40 °C were poorly archaellated compared with cells grown at 22–34 °C and cells grown at 42 °C were non-archaellated. The lack of archaella observed when cells are grown at 42 °C is not due to the instability of the archaellar filament at this temperature, as archaella isolated from Methanococcus maripaludis are known to maintain structure up to 70 °C (Thomas & Jarrell, 2001). Second, the FlaB2 detected in Western blots of lysates of cells grown at ≥38 °C migrated faster, suggestive of a truncation in the attached N-linked glycan. MS analysis of appendages isolated from cells grown at 40 °C confirmed that the normal tetrasaccharide was indeed shortened to only a modified trisaccharide at this higher temperature.

An early study on the effects of growth medium modifications and growth temperature on archaellation in methanogens demonstrated that Methanospirillum hungatei only expressed archaella near their optimal growth temperature, even though similar archaellin levels were detected at all temperatures in Western blots (Faguy et al., 1993). Thus, the inhibition in archaella production appears to be at the post-translational level in Methanospirillum hungatei, in contrast to the current observations with Methanococcus maripaludis, which indicate a temperature effect on transcription of the archaellin flaB2. Limiting calcium levels in the medium also affected archaellation in Methanospirillum hungatei, but had no effect on archaellation in Methanococcus maripaludis.

The global responses of Methanococcus maripaludis to nutrient limitations have been reported for continuous culture at both the transcriptome and proteome levels, and complex effects on Fla proteins have been noted. Transcript levels for various archaellum synthesis genes were found to increase under H2 limitation, but decrease under leucine limitation (Hendrickson et al., 2007, 2008). In a proteome study, archaellin abundance was found to increase under phosphate limitation, but decrease under nitrogen limitation (Xia et al., 2009). Direct correlations to the current work cannot be made as these global studies did not include examination of the cells by electron microscopy to determine a direct impact on archaellation and were continuous culture studies, whilst the current data were obtained from batch cultures. Nevertheless, these studies and the current work importantly show that variations in fla transcription, Fla protein synthesis or archaellation can occur under numerous stress conditions. In the hyperthermophilic methanogen Methanococcus jannaschii, H2 limitation during growth in batch culture was first shown to affect archaellation (Mukhopadhyay et al., 2000). Under H2 excess, proteomic analysis showed that archaellins FlaB2 and FlaB3 as well as the Fla-associated proteins FlaD and FlaE were extremely low or undetectable. Importantly, electron microscopic examination of cells under both high and low H2 growth conditions in batch culture showed that cells indeed did not assemble archaella under H2 limitation. For Methanococcus maripaludis, no difference in FlaB2 synthesis or archaellation was found when cells grown under CO2/H2 were compared with cells grown under CO2/N2 with formate as electron donor.

In the Crenarchaeota, numerous factors have been shown to influence the production of archaella, with studies focused on S. solfataricus and S. acidocaldarius. In S. solfataricus, transcription of flaB (the sole archaellin) is highly induced when cells reach the stationary phase whilst little transcript is detected in the mid exponential phase. A correlation of the transcript abundance with the amount of archaella present on cells could not be done, however, as stationary-phase cells were prone to lysis. Strong induction of flaB was also observed when cells were grown under nitrogen starvation conditions (Szabó et al., 2007). In S. acidocaldarius, Western blots revealed a large increase in FlaB in stationary-phase cells compared with mid-exponential-phase cells (Lassak et al., 2012b). A variety of stresses, including increased salinity and pH as well as limiting carbon and nitrogen sources, were also tested, but only depletion of the nitrogen source tryptone led to induction of archaella expression and that was shown by qRT-PCR methodology to be at the level of transcription. Regulation of transcription of the fla operon in S. acidocaldarius has been shown to involve numerous factors, including both repressors and activators, and be coordinated with the expression of pili in such a way that conditions that favour the expression of one of the surface appendages (Aap pili or archaella) also downregulate expression of the other (Albers & Jarrell, 2015; Lassak et al., 2012b).

Aside from the effects on archaellation, our findings indicate that the archaellin N-glycan structure is also affected by growth at high temperature. In Methanococcus maripaludis, this structural change is a truncation of the glycan produced in Haloflexax volcanii, where both the structure and site of N-glycosylation in the S-layer protein vary in response to the salinity of the growth medium. In medium containing 3.4 M NaCl, Asn13 and Asn83 are modified with a pentasaccharide, whilst Asn498 is unmodified. If the medium NaCl concentration is lowered to 1.75 M, cells modify the S-layer Asn498 with a completely novel glycan, a tetrasaccharide, whilst Asn34 and Asn88 are still modified with the pentasaccharide, although at a reduced level (Kaminski et al., 2013). Whilst it is known that the archaellin FlgA1 is modified at three sites with the same pentasaccharide N-glycan that decorates the
S-layer protein (Tripepi et al., 2012), there are no data available on whether the archaellin-linked glycans vary when cells are grown in media differing in NaCl concentrations.

There are no prior reports in Archaea that show temperature-dependent changes in N-glycosylation. The finding that cells grown approaching the upper temperature limit for *Methanococcus maripaludis* produces a change in the N-glycan structure is thus unique to this study. The change in glycan structure occurs over a small increase in temperature of only 2–3 °C, coinciding with the marked decrease in flaB2 transcript levels, FlaB2 detected in Western blots and reduced number of archaella observed on the cells. The switch of the archaellin N-glycan structure is abrupt as we have not observed both WT and the modified trisaccharide glycan present at one temperature in Western blots. The truncated trisaccharide glycan detected by MS at 40 °C is identical to that previously reported in an *aglX* deletion strain. AglX plays a critical role, as the amidotransferase, in the formation of the acetamidino group found on the third sugar in the WT glycan. Both the 40 °C archaellin glycan and the glycan of the *aglX* mutant have multiple differences compared with the WT glycan; the fourth sugar is missing as is the threonine and acetamidino group attached to the third sugar. The deletion of *aglX* leads directly to the loss of the acetamidino group (Jones et al., 2012). Without this modification of the third sugar, the fourth sugar cannot be added (Jones et al., 2012). Without the fourth sugar, the final modification of the third sugar, i.e. the attachment of threonine carried out by AglU, cannot occur (Ding et al., 2013).

Mutants defective in the formation or attachment of the fourth sugar (i.e. in mutants deleted for the glycosyltransferase *aglL*) make a glycan that not only is missing the fourth sugar, but also lacks the threonine attachment to the third sugar. Notably, in such mutants the third sugar of the glycan still has the acetamidino modification (Ding et al., 2013). This lack of the acetamidino group modification of the third sugar of the glycan when cells are grown at 40 °C suggests that the defect lies in one of the genes involved in making this specific modification. Three genes have been shown to be involved in the acetamidino modification: *aglX* encoding the amidotransferase, as well as *aglY* and *aglZ*. It is thought that AglY generates NH₄ which is transferred, via a tunnel formed by AglZ, to AglX (Jones et al., 2012). The effects of deletion of *aglY* or *aglZ* are, however, only seen if cells are grown in NH₄-limited medium, which was not the case with the cells grown at 40 °C. Thus, the MS data suggest that a possible reason for the truncated glycan formed at 40 °C may be the poor transcription of *aglX* at the higher temperatures or the stability/activity of AglX at temperatures >38 °C. Attempts to reverse the truncation of the glycan observed at higher temperatures by expressing *aglX* in trans from the *nif* promoter were unsuccessful. The same plasmid had been successfully used previously to complement an *aglX* deletion strain (Jones et al., 2012), and qRT-PCR indicated that transcript levels for *aglX* were increased in cells harbouring pKJ752 expressing *aglX* at both 34 and 40 °C. Indeed, the *aglX* transcript was more abundant in WT cells at 40 °C compared with 34 °C. These data all suggest that the defect in glycan structure observed at the higher temperatures was not due to a transcription deficiency for the *aglX* operon, but more likely due to stability or inactivity of AglX at the elevated temperatures. An alternative explanation is that the glycan itself is unstable at elevated temperatures and degrades to give the modified trisaccharide. This seems unlikely, however, as Western blots of cells pre-grown at 34 °C and then shifted to 40 °C under non-growing conditions did not exhibit the electrophoretic mobility of FlaB2 with a truncated glycan.

Further studies will be needed to identify the precise cause of the decreased transcription of the *fla* operon and the N-linked glycan truncation in *Methanococcus maripaludis* grown at elevated temperatures. The observations do, however, provide an exceptionally easy way of turning on and off archaellation by growth temperature, and on a larger scale it may be envisioned that the *fla* operon promoter may be useful in vectors to regulate expression of proteins in *Methanococcus maripaludis* by a simple temperature shift from 42 to 34 °C. This may be especially useful for expression of toxic proteins.

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**REFERENCES**


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