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The Effects of Growth Conditions on Archaellation and N-Glycosylation in *Methanococcus maripaludis*

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Running title: Effects of Growth Conditions on Archaellation

ABSTRACT

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. While the amount of FlaB2 was similar for cells grown below 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 nonarchaellated. Unexpectedly, a lower apparent molecular mass for FlaB2 was observed in western blots of cells grown at temperatures beyond 38°C suggestive of a truncation in the attached N-linked tetrasaccharide at higher growth temperatures. Mass spectrometry 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 wildtype 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 and Burrows, 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-β-ManNAc3NAmA6Thr-1,4-β-GlcNAc3NAcA-1,3-β-GalNAc, where Sug is (5S)-2-acetamido-2,4-dideoxy-5-O-methyl-α-L-erythro-hexos-5-ulo-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 (*a*rchaeal *gl*ycosylation) (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 nonglycosylated archaellins or archaellins modified with only a single sugar glycan are non-archaellated. While multiple sites of N-glycosylation are found in each 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* species. Biochemical and structural work has recently unraveled the functions of several of the proteins encoded by these *fla*-associated genes, including FlaF, FlaI and FlaJ, most clearly in *Sulfolobus acidocaldarius* (Banerjee *et al*., 2012, Banerjee *et al*., 2015, Ghosh *et al*., 2011, Reindl *et al*., 2013, Shahapure *et al*., 2014). The molecular mechanism of the regulation of archaellation has been best studied in this thermoacidophile, as well. In *S. acidocaldarius,* the archaellin promoter is controlled by several factors, including repressors and activators and there is an inter-connected control of both archaella and one kind of type IV pilus, the adhesive pili (Aap pili) (Henche *et al.* 2012, Lassak *et al*., 2012b, Orell *et al*., 2013, Reimann *et al*., 2012). Recently, it was shown in *Haloferax volcanii* that the hydrophobic part of the type IV pilin signal peptide of various pilins is important for the expression of the archaella, showing an interplay in the regulation of the two surface structures in this halophile, as well (Esquivel & Pohlschroder, 2014).

While the primary role for archaella is considered to be swimming, multiple other varied functions have been reported for the structure in various diverse archaea. Archaella have been shown to be used for attachment to various abiotic surfaces, sometimes in conjunction with pili, and even in cell to cell contact with members of different species (Bellack *et al*., 2011, Jarrell *et al*., 2011, Nather *et al*., 2006, Schopf *et al*., 2008, Weiner *et al*., 2012, Zolghadr *et al*., 2010).

Oftentimes, the role in attachment is accompanied by the formation of cable-like bundles of many archaella. Data has also been presented which show a role for archaella in biofilm formation and even cell to cell communication (Shimoyama *et al*., 2009, Weiner *et al*., 2012, Zolghadr *et al*., 2010).

Surprisingly, however, studies which investigate the factors that may influence formation of archaella are limited. Early studies on methanogens revealed that *Methanospirillum hungatei* produced archaella only near its optimum growth temperatures (Faguy *et al*., 1993) . It was also shown for both *Methanococcus maripaludis* and *Methanocaldococcus jannaschii* that synthesis of archaella was not constitutive. In both organisms, archaella synthesis was shown to be induced under growth conditions where H_2 is limited (Hendrickson *et al*., 2008, Mukhopadhyay *et al*., 2000). Furthermore, proteomics analysis revealed that the expression of archaellins in *M. maripaludis* was affected by various nutritional factors, being increased when cells were phosphate-limited and decreased under conditions of nitrogen limitation (Xia *et al*., 2009). In *Sulfolobus solfataricus* and *S. acidocaldarius*, expression of the *fla* operon was induced by starvation conditions (Szabo *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. *M. maripaludis* is considered a model archaeal organism (Leigh *et al*., 2011) and one of the best studied archaea in regards 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.

MATERIALS AND METHODS

Strains and growth conditions

120 *Methanococcus maripaludis* strain Mm900 (Moore & Leigh, 2005) was grown
121 anaerobically in serum bottles containing Balch medium III (Balch *et al*., 1979) under a
122 headspace of CO_2/H_2 (20:80), unless otherwise stated. When testing for the effect of
123 temperature on archaellation, cultures were grown in Balch Medium III with shaking (110 rpm)
124 over the range from room temperature (22°C) to 42°C. To test the effect of salt stress induced
125 by NaCl, cultures were grown in Balch medium III modified to contain either 0.3% (w/v) or 5%
126 NaCl, as well as the normal 2% NaCl. The expression of FlaB2 was also compared after growth
127 in complex medium (Balch medium III) or nitrogen-free minimal media supplemented with either
128 10 mM L-alanine or 10 mM NH_4Cl (Lie & Leigh., 2002). Cells grown in minimal medium with
129 NH_4Cl but omitting $CaCl_2$ (no calcium medium) were also examined. Cells were also cultivated
130 in a formate medium (Costa *et al*., 2013). For this medium, 200 mM sodium formate with an
131 equivalent reduction in NaCl to maintain sodium osmolarity and 200 mM of
132 morpholinepropanesulfonic acid (MOPS) buffer at pH 7 were added to McCas medium under a
133 headspace of CO_2/N_2 (20:80). Finally, samples were also compared after growing statically or
134 shaking at both room temperature and at 42°C in Balch medium III. In all cases, cells were
135 transferred a minimum of three times under the various conditions before analysis.

136 **SDS-PAGE and Western blotting**

137 Whole cell lysates were separated by SDS-PAGE (15% acrylamide) (Laemmli, 1970) in
138 a minigel system and stained with Coomassie Blue G250, as previously reported (Faguy *et al*.,
139 1996).

140 For western blotting, whole cell lysates of *M. maripaludis* grown under the various
141 conditions were subjected to SDS-PAGE and then transferred to Immobilon-P membrane
142 (Millipore, Bedford, MA) (Towbin *et al*., 1979). The major archaellin FlaB2 was detected with
143 anti-FlaB2-specific chicken antibodies (IgY) (Jones *et al*., 2012), using horseradish peroxidase-
144 conjugated rabbit anti-chicken IgY (Jackson Immunoresearch Laboratories, West Grove, PA) as

the secondary antibody. Blots were developed using a chemiluminescent detection kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Laval, QC, Canada).

Quantitative Reverse Transcription PCR

Quantitative Reverse Transcription PCR (qPCR) 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 *M. maripaludis* Mm900 and Mm900 cells harbouring pKJ752 using an RNeasy Minikit (Qiagen, Inc., Mississauga, Ontario, Canada) 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; (Pohlschroder *et al*., 2005)) leading to PCR products that would be 136 bp, 102 bp and 149 bp in length, respectively. The forward and reverse primer sequences for *flaB2, aglX* and *mmp0383* were as follows: 5' GCTGCAATAGACATGAATCAGG and 5' GACCAGTTTACAGTTGTAGTGTTG; 5' GATCAGAATCCTAAATTATGCGG and 5'GATTCACCATGTTTTGTAGGG; 5' GGTACTGAAGCATACGAAGGAG and 5' GCTACAACTTTACCGTCTTTTAAGAG, respectively. For the qPCR experiments, *mmp0383* was used as reference gene since 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 sec denaturation at 94°C, 30 sec 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 qPCR experiments, 100 ng of purified RNA from each strain were converted into a cDNA library using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.) following the manufacturer's instructions. The abundance of the *flaB2* transcript and the reference gene

mmp0383 was analysed in a 20 µL qPCR reaction 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 1X SsoFast™ EvaGreen® supermix (Bio-Rad Laboratories, Inc.) 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 3 min denaturation followed by 40 cycles of 10 sec at 95°C and 30 sec at 50°C, followed by melt curve analysis to confirm the specificity of the qPCR amplicons.. Triplicate and no-template control reactions were included to confirm a lack of genomic DNA contamination, and the qPCR 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 PEG 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) and either 10 mM of L-alanine (*nif* promoter induced) or NH_4Cl (*nif* promoter repressed) as nitrogen source (Lie & Leigh, 2003).

Isolation of surface structures

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

Tryptic digestion and LC-MS procedure

The isolated surface structure preparation was incubated overnight at 37°C with trypsin (Promega, Madison, WI) at an approximate ratio of 20:1 (protein:enzyme, v/v) in 50 mM ammonium bicarbonate. The digest was then analysed by nano-liquid chromatography-tandem mass spectrometry (Nano-LC-MS/MS) using a NanoAquity UPLC system (Waters, Milford, MA)

coupled to a QTOF Ultima hybrid quadrupole time-of-flight mass spectrometer (Waters). The digests were injected onto an Acclaim PepMax100 C_{18} µ-precolumn (5 mm by 300 µm i.d.; Dionex/Thermo Scientific, Sunnyvale CA) and resolved on a 1.7- µm BEH130 C_{18} column (100 µm by 100 mm i.d.; Waters, Milford, CA) using the following gradient conditions: 1 to 45% acetonitrile (ACN), 0.1% formic acid in 36 min and 45 to 95% ACN, 0.1% formic acid in 2 min. The flow rate was 400 nL/min. MS/MS spectra were acquired on doubly, triply and quadruply charged ions and searched against the NCBInr database using the Mascot search engine (Matrix Science, Ltd., London, United Kingdom). The spectra were searched for glycopeptide MS/MS spectra which were then interpreted by hand.

Electron microscopy

Transmission electron microscopy of *M. maripaludis* cells was performed as previously described (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

M. 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.

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

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 the NaCl varied from 0.3% to 2% to 5% (w/v). The 2% NaCl level is the normal amount found in Balch medium III while 0.3% and 5% are reported to be the lower and upper limits that allow for growth of the organism (Whitman & Jeanthon, 2002, 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 (Figure 1a). Similarly, when cells were grown in Balch medium III as an example of a complex medium and compared to cells grown in a minimal medium with either NH_4Cl or alanine as the sole nitrogen source, no difference in the level of FlaB2 was observed in western blots (Figure 1b). In addition, no apparent effect on FlaB2 levels was observed when cells were grown in minimal medium with no $CaCl_2$ added (Figure 1c) or when cells grown in Balch Medium III under a CO_2/H_2 headspace were compared with cells grown under CO_2/N_2 with formate as the electron donor (Figure 1d). Electron microscopy of cells grown in all the above conditions revealed that all had multiple peritrichously located archaella (Figure 1e, f).

M. maripaludis grows over the temperature range from <20°C to 45°C (Whitman & Jeanthon, 2006). In contrast to what was observed when *M. maripaludis* was grown in media of varying compositions, when cells were grown in Balch medium III at various temperatures within the range of 22°C (room temperature) to 42°C, a clear difference in FlaB2 signal intensity was seen in cells grown at the upper temperatures tested (38°C, 40°C, and 42°C) compared to the lower temperatures (Figure 2a). Cells grown at room temperature, 30°C, 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 (Figure 2b), indicating that the observations at 42°C were not due to selection of a mutant strain that could not produce FlaB2 under any conditions. The amount of FlaB2 observed in western blots was a good indicator for how many archaella were subsequently observed by electron microscopy (Figure 3). Cells grown at room temperature up to 34°C were indistinguishable in their level of archaellation; all had numerous, 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.

qPCR analysis of *flaB* transcript.

To investigate whether the temperature-dependent regulation of archaella synthesis occurred at the level of transcription, qPCR was performed. Cells grown at 34°C and 42°C were analyzed. The transcript for *flaB2* was targeted since 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 reverse transcription step were also subjected to PCR confirming that they were free of genomic DNA contamination (data not shown). qPCR experiments showed that *flaB2* transcript levels were approximately 50 fold lower at 42°C compared to 34°C (Figure 4).

Since archaella can also be used for attachment in conjunction with pili, cells that were incubated statically at 42°C were compared to cells grown with shaking. It is 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 (Figure 2C). The statically grown cells at 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 room temperature but no apparent increase in FlaB2 or archaellation over the abundant levels already present at room temperature was observed when cells were grown statically (data not shown).

Western blot experiments indicate a truncated N-linked glycan when cells 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 and above 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 (Figure 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. Since the cells were shown by electron microscopy to be archaellated at 38°C and 40°C, this meant that the archaellin N-linked glycan must be at least two sugars in length, 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°C and 38°C were compared to those of Δ*aglL* or Δ*aglA* mutants (Figure 5a). The Δ*aglL* and Δ*aglA* 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 Δ*aglL* mutant has archaellins modified with a three sugar glycan while the Δ*aglA* 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 Δ*aglL* mutant, suggesting that archaella expressed at 38°C may contain archaellins that bear truncated N-glycans that lack the terminal

297 sugar and the threonine attachment found on the third sugar. The threonine modification to the
298 third sugar only occurs after the 4th sugar has been transferred to the growing glycan and so is
299 missing in the glycan found in the Δ*aglL* mutant (Ding *et al*., 2013, Siu *et al*., 2015, Vandyke *et*
300 *al*., 2009).

301 **Mass spectrometry analysis of surface appendages purified from cells grown at 40°C.**

302 To confirm that the differences in archaellin electrophoretic mobility observed in the
303 western blots of cells grown at elevated growth temperature were due to truncation of the N-
304 linked glycan, surface appendages (archaella and pili) were isolated from cells grown at 40°C
305 for subsequent analysis by mass spectrometry. A temperature of 40°C was chosen as the
306 decrease in FlaB2 apparent molecular mass was observed at this temperature while electron
307 microscopy showed that the cells still produced some archaella which could be isolated for
308 analysis. Examination of the purified preparation of surface appendages from cells grown at
309 40°C by SDS-PAGE followed by Coomassie blue staining indicated mainly the presence of
310 EpdE, the major glycoprotein subunit of Epd pili (Ng *et al*., 2011) but also a lesser amount of
311 archaellin (Figure 6). Western blot analysis confirmed that the apparent molecular weight of
312 FlaB2 in the purified appendage sample was still at the lower molecular weight observed in the
313 whole cell lysates (not shown).

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

322 **Expression of *aglX in trans* at elevated growth temperature.**

323 The LC-MS data suggested that the truncated N-linked glycan may be the result of loss
324 of AglX, possibly because the activity of the enzyme is temperature sensitive or because the
325 gene is not transcribed at the higher temperatures from its native promoter. Complementation of
326 an *aglX* deletion mutant has been previously accomplished with pKJ752, where a wild type
327 version of *aglX* is transcribed from the inducible *nif* promoter (Jones *et al*., 2012). Mm900 cells
328 were transformed with this plasmid and cells grown at both 34ºC and 40°C in nitrogen-free
329 medium supplemented with alanine to allow transcription of *aglX*. Cells grown in nitrogen-free
330 medium supplemented with alanine but without pKJ752 were used as controls at both
331 temperatures. The transcript level of *aglX* was analyzed by qPCR experiments in all cases. The
332 levels of the *aglX* transcript were upregulated by 3.2 fold in wildtype cells grown at 40ºC
333 compared to wildtype cells grown at 34ºC. At both growth temperatures, the levels of *aglX*
334 transcript were increased by ~1.4 fold in the cells carrying pKJ752 compared to that from the
335 wildtype cells lacking the plasmid but grown in the same medium at the same temperature (data
336 not shown), indicating that the *nif* complementation system was working in this study. When
337 FlaB2 production was examined by western blot, the electrophoretic mobility of the archaellin
338 did not shift in cells carrying pKJ752 grown under inducing conditions at 40ºC (or 34ºC). It was
339 the same as that observed in cells without pKJ752 grown in nitrogen-free medium
340 supplemented with alanine (Figure 5B), suggesting that increased transcription of *aglX* and
341 likely also increased synthesis of AglX were not able to restore the glycan defect at elevated
342 temperature. To eliminate the possibility that the truncated glycan observed at the elevated
343 temperatures was due to degradation of the tetrasaccharide, cells were grown at 34°C and then
344 switched to 40°C under conditions where the cells would not be able to grow, i.e., where the
345 cells were placed under a headspace of N_2 or air. Under both conditions, the apparent
346 molecular weight of FlaB2 was equal to that observed in cells grown at 34°C, indicating that the
347 truncated glycan did not result from a partial disintegration of the glycan at the elevated
348 temperatures (Figure 5c).

DISCUSSION

In this report, a number of growth conditions were examined to explore their effects on archaellation in *M. maripaludis*. This 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 NH_4Cl or alanine as sole N-source or with formate as electron donor, as well as in low calcium minimal 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. Firstly, 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 amount (qPCR) and in the number of archaella observed in electron microscopic studies, where cells grown at 38-40°C were poorly archaellated compared to 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 *M. maripaludis* are known to maintain structure up to 70°C (Thomas & Jarrell, 2001). Secondly, the FlaB2 detected in western blots of lysates of cells grown at 38°C and above migrated faster, suggestive of a truncation in the attached N-linked glycan. Mass spectrometry 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 posttranslational level in *Msp. hungatei*, in contrast to the current observations with *M. maripaludis* which indicate a temperature effect on transcription of the archaellin *flaB2*. Limiting calcium levels in the medium also affected archaellation in *Msp. hungatei*, but had no effect on archaellation in *M. maripaludis*.

The global responses of *M. 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 H_2 limitation but decrease under leucine limitation growth conditions (Hendrickson *et al*., 2007, Hendrickson *et al*., 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 since 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 while 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, *Mcc. jannaschii,* H_2 limitation during growth in batch culture was first shown to affect archaellation (Mukhopadhyay *et al*., 2000). Under H_2 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 H_2 growth conditions in batch culture, showed that cells indeed did not assemble archaella under H_2 limitation. For *M. maripaludis*, no difference in FlaB2 synthesis or archaellation was found when cells grown under CO_2/H_2 were compared to cells grown under CO_2/N_2 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*,

401 transcription of *flaB* (the sole archaellin) is highly induced when cells reach stationary phase
402 while little transcript is detected in mid logarithmic phase cells. A correlation of the transcript
403 abundance with the amount of archaella present on cells could not be done, however, since
404 stationary phase cells were prone to lysis. Strong induction of *flaB* was also observed when
405 cells were grown under nitrogen starvation conditions (Szabo *et al*., 2007). In *S. acidocaldarius*,
406 western blots revealed a large increase in FlaB in stationary cells compared to mid-logarithmic
407 phase (Lassak *et al*., 2012b). A variety of stresses, including increased salinity and pH as well
408 as limiting carbon and nitrogen sources, were also tested but only depletion of the nitrogen
409 source tryptone led to induction of archaella expression and that was shown by qPCR
410 methodology to be at the level of transcription. Regulation of transcription of the *fla* operon in *S.*
411 *acidocaldarius* has been shown to involve numerous factors including both repressors and
412 activators and coordinated with the expression of pili in such a way that conditions that favour
413 the expression of one of the surface appendages (Aap pili or archaella) also down-regulates
414 expression of the other (Albers & Jarrell, 2015, Lassak *et al*., 2012b).
415 Aside from the effects on archaellation, our findings indicate that the archaellin N-glycan
416 structure is also affected by growth at high temperature. In *M. maripaludis*, this structural
417 change is a truncation of the glycan produced at lower temperatures. A more extreme example
418 of glycan modification in response to growth conditions has been reported in *Hfx volcanii*. Here,
419 both the structure and site of N-glycosylation in the S-layer protein varies in response to the
420 salinity of the growth medium. In medium containing 3.4 M NaCl, Asn-13 and Asn-83 are
421 modified with a pentasaccharide while Asn-498 is unmodified. If the medium NaCl concentration
422 is lowered to 1.75 M, cells modified the S-layer Asn-498 with a completely novel glycan, a
423 tetrasaccharide, while Asn-13 and Asn-83 are still modified with the pentasaccharide, although
424 at a reduced level (Kaminski *et al*., 2013). While it is known that the archaellin FlgA1 is modified
425 at three sites with the same pentasaccharide N-glycan that decorates the S-layer protein

426 (Tripepi *et al*., 2012), there is no data available on whether the archaellin-linked glycans vary
427 when cells are grown in media differing in NaCl concentrations.
428 There are no prior reports in Archaea that show temperature-dependent changes in N-
429 glycosylation. The finding that cells grown approaching the upper temperature limit for *M.*
430 *maripaludis* results in a change in the N-glycan structure is thus unique to this study. The
431 change in glycan structure occurs over a small increase in temperature of only 2-3°C, coinciding
432 with the marked decrease in *flaB2* transcript levels, FlaB2 detected in western blots and
433 reduced number of archaella observed on the cells. The switch of the archaellin N-glycan
434 structure is abrupt as we have not observed both wildtype and the modified trisaccharide glycan
435 present at one temperature in western blots. The truncated trisaccharide glycan detected by
436 mass spectrometry at 40°C is identical to the one previously reported in an *aglX* deletion strain.
437 AglX plays a critical role, as the amidotransferase, in the formation of the acetamidino group
438 found on the third sugar in the wildtype glycan. Both the 40°C archaellin glycan and the glycan
439 of the *aglX* mutant have multiple differences compared to the wildtype glycan; the fourth sugar
440 is missing as is the threonine and acetamidino group attached to the third sugar. The deletion of
441 *aglX* leads directly to the loss of the acetamidino group (Jones *et al*., 2012). Without this third
442 sugar modification, the fourth sugar cannot be added (Jones *et al*., 2012). Without the fourth
443 sugar the final modification of the third sugar, the attachment of threonine carried out by AglU,
444 cannot occur (Ding *et al*., 2013). Mutants defective in formation or attachment of the fourth
445 sugar (i.e. in mutants deleted for the glycosyltransferase *aglL*) make a glycan that is not only
446 missing the fourth sugar but which also lacks the threonine attachment to the third sugar.
447 Notably, in such mutants the third sugar of the glycan still has the acetamidino modification
448 (Ding *et al*., 2013). This lack of the acetamidino group modification of the third sugar of the
449 glycan when cells are grown at 40°C suggest that the defect lies in one of the genes involved in
450 making this specific modification. Three genes have been shown to be involved in the
451 acetamidino modification: *aglX* encoding the amidotransferase as well as *aglY* and *aglZ*. It is

thought that AglY generates ammonia 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 NH_4-limited medium which was not the case with the cells grown at 40°C. Thus, the mass spectrometry data suggests that a possible reason for the truncated glycan formed at 40°C may be due to poor transcription of *aglX* at the higher temperatures or the stability/activity of AglX at temperatures greater than 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 qPCR indicated that transcript levels for *aglX* were increased in cells harbouring pKJ752 expressing *aglX* at both 34°C and 40°C. Indeed, the *aglX* transcript was more abundant in wildtype cells at 40°C compared to 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 *M. 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 *M. maripaludis* by a simple temperature shift from 42°C to 34°C. This may be especially useful for expression of toxic proteins.

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674

675 **Figure Legends**

676 **Figure 1.** Western blot and electron microscopic analysis of *M. maripaludis* cultivated under
677 various growth conditions. Whole cell lysates were examined in western blots developed with
678 antibodies against FlaB2 following growth of cells under the following conditions: **a**. Balch
679 medium modified to contain different NaCl concentrations (0.3, 2, 5%). **b.** Complex medium
680 (Balch medium III) or nitrogen-free minimal medium supplemented with either alanine or NH_4Cl.
681 **c**. Minimal medium or minimal medium without added $CaCl_2$. **d**. Balch medium III or formate
682 medium under a headspace of CO_2/N_2. **e and f**. Electron microscopy of cells grown under the
683 same conditions used for the western blots of **a-d**.

684 **Figure 2**. Western blot analysis of *M. maripaludis* grown at different temperatures. In each case,
685 whole cell lysates were examined in western blots developed with antibodies against FlaB2. **a.**
686 Cells grown with shaking in Balch medium III incubated at the temperatures indicated. **b**. Cells
687 incubated at 42°C with shaking can be switched to 34°C where they now synthesize FlaB2. **c**.
688 Cells grown statically at 42°C can synthesize FlaB2.

689 **Figure 3**. Electron microscopy of cells grown with shaking in Balch Medium III at various
690 temperatures. Samples were stained with 2% phosphotungstic acid, pH 7.0. Grids were
691 examined in a Hitachi 7000 electron microscope operating at an accelerating voltage of 75 kV.

692 **Figure 4**. q-PCR analysis of *flaB2* transcript at 34°C and 42°C. *mmp0383* was used as the
693 reference gene.

694 **Figure 5**. Western blot analysis of the faster FlaB2 electrophoretic migration at higher
695 temperature, in comparison to mutants producing FlaB2 with known glycan defects. In each
696 case whole cell lysates were used in western blots developed with FlaB2-specific antibodies. **a**.
697 The electrophoretic mobility of FlaB2 from Mm900 cells grown at 38°C is compared to Mm900
698 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 wildtype 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°C and 40°C did not change when compared to 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 34ºC to 40ºC under non-growing conditions does not result in FlaB2 of reduced electrophoretic mobility. Cells were grown at 34ºC and then the headspace was replaced either with N_2 or air and cells further incubated for 2 days at 40ºC. Cells grown at 34ºC and 40ºC are included for comparison.

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

Figure 7. NanoLC-MS/MS analysis of the FlaB2 tryptic glycopeptide, $T^{53\text{-}81}$, from WT of *M. maripaludis* grown at 40^0C (panel a). The triply protonated (MH_3^{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 (■) and the GlcNAc3NAcA (●) from the WT glycan as well as a terminal sugar that is likely to be di-*N*-acetyl-mannuronic acid (ManNAc3NAcA, ▬). The glycan appears to be the same as that produced by the *aglX* mutant strain. This trisaccharide is the predominant glycan modification on the 40^0C archaellin. The full glycan of the wild type strain grown are lower temperatures is shown in panel b. The residues and modifications residues absent from the 40^0C glycan are shaded in gray.

Figure 1

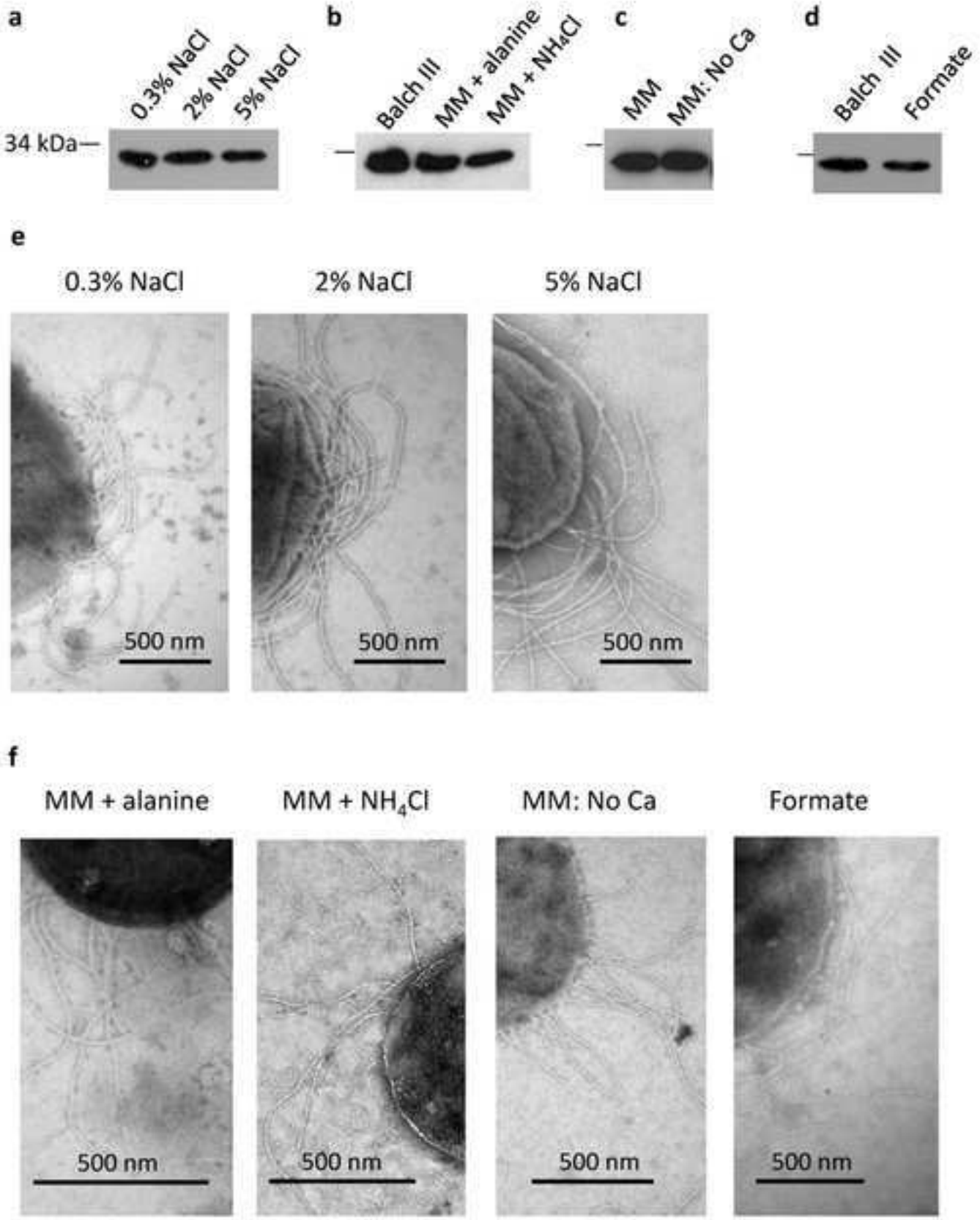

Figure 2

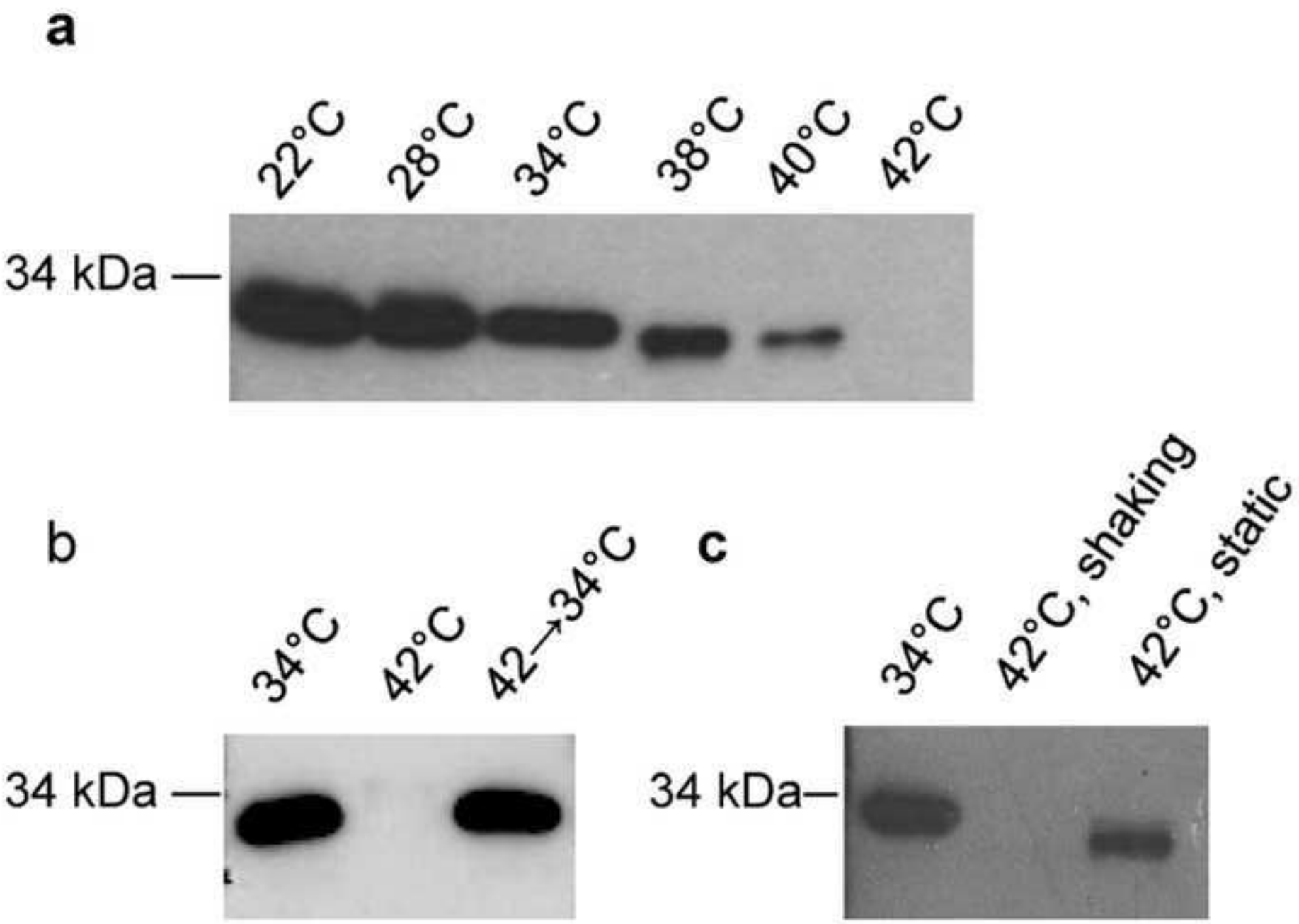

Figure 3

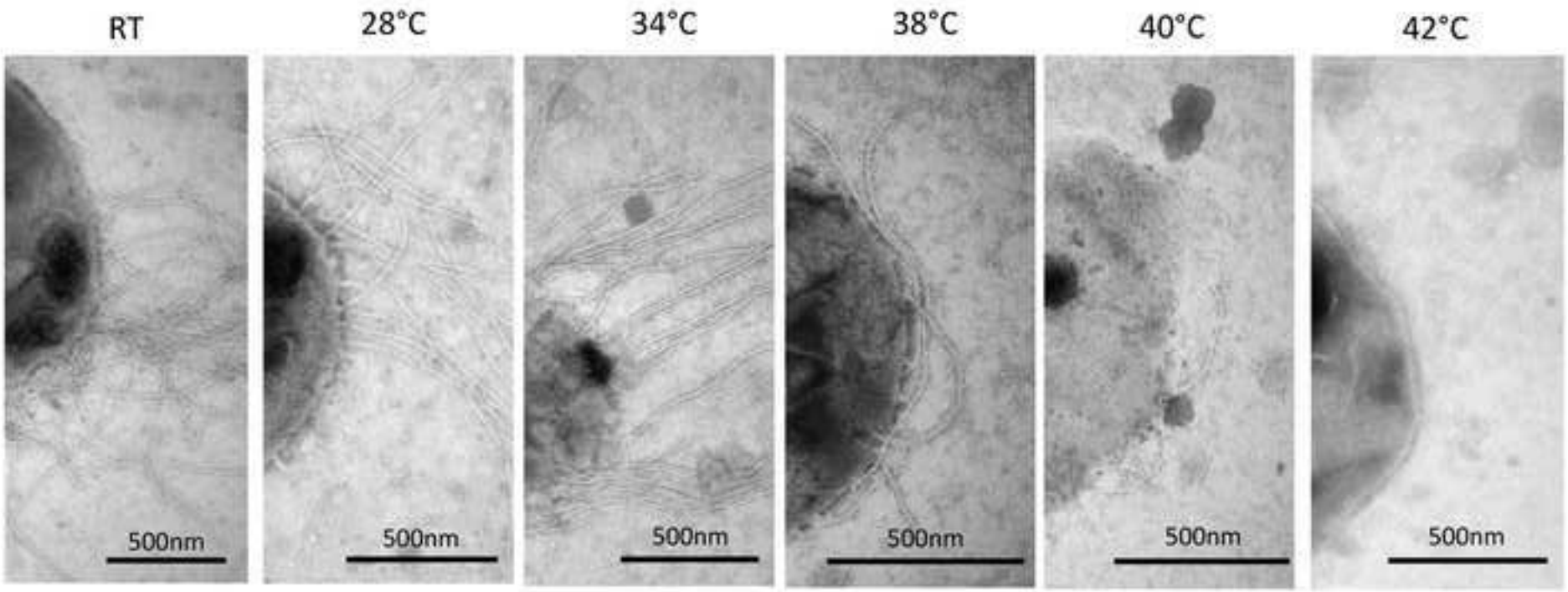

Figure 4

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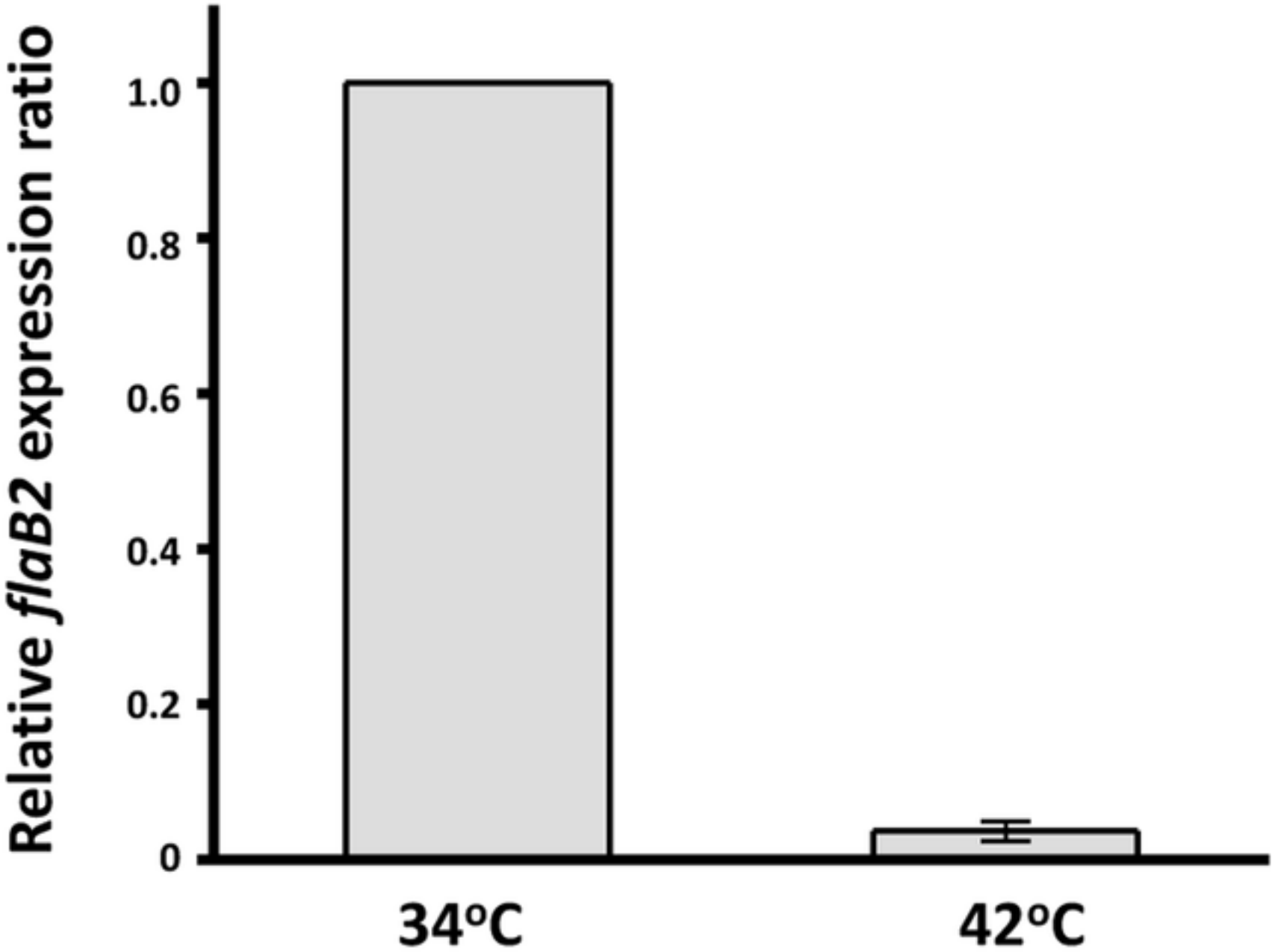

Figure 5

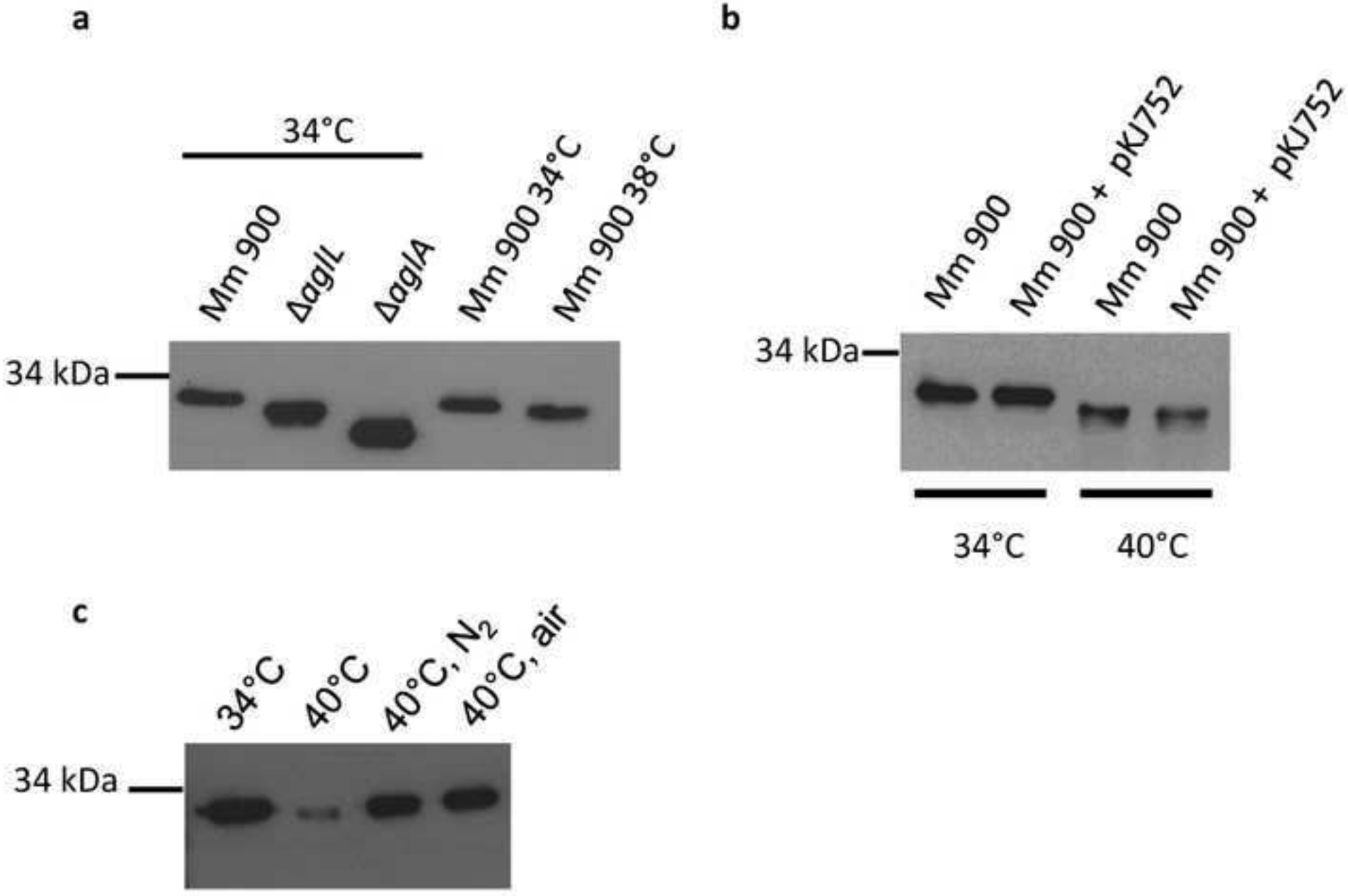

Figure 6

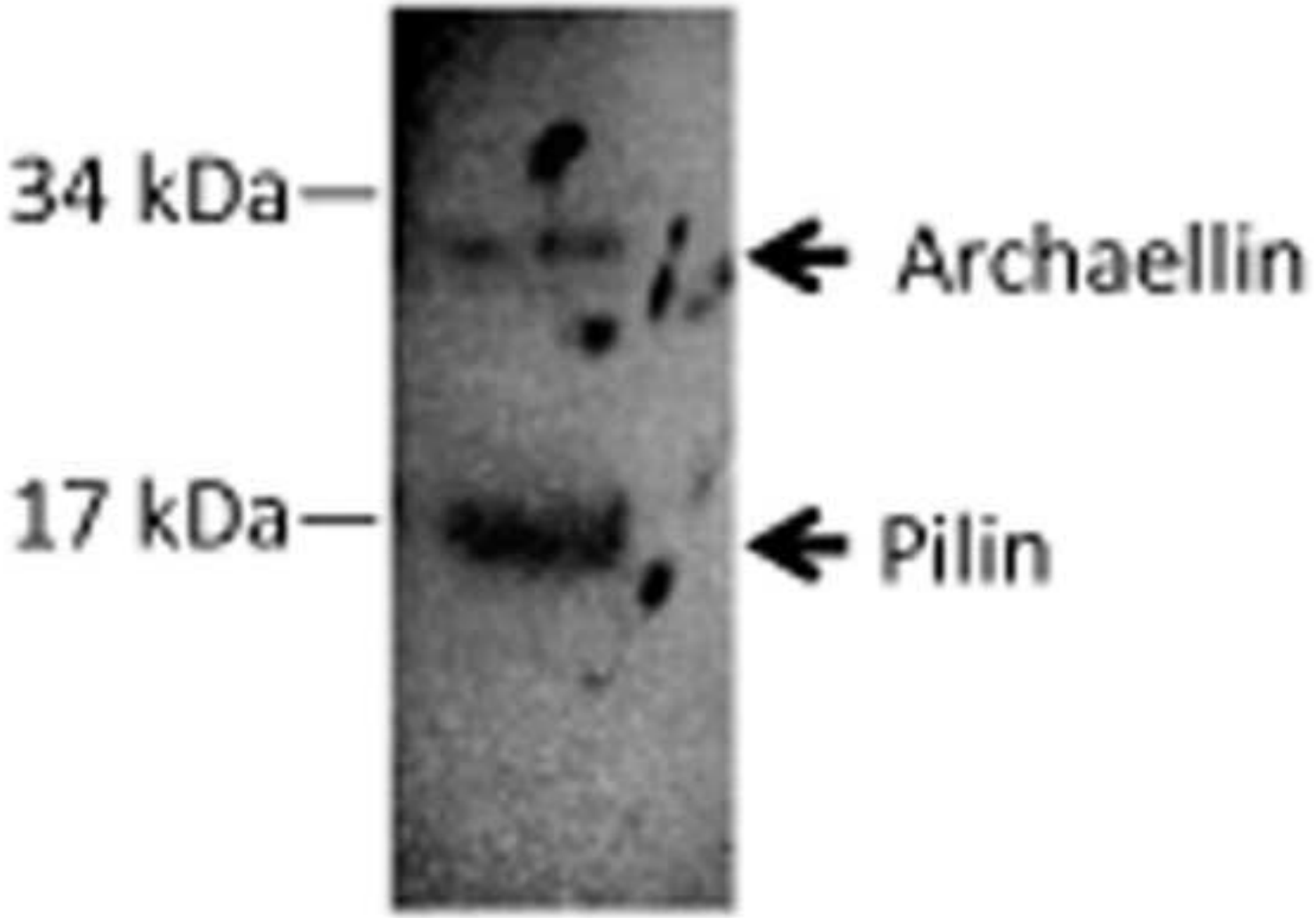

Figure 7