

2-1-2016

# Deciphering the Outer Envelope of *Thermotoga maritima*

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## Recommended Citation

Ranjit, Chaman, "Deciphering the Outer Envelope of *Thermotoga maritima*" (2016). *Master's Theses*. 869.  
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**Deciphering the Outer Envelope of *Thermotoga maritima***

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B.S., University of Pune, India 1998

M.S., Tribhuvan University, Kathmandu, Nepal 2002

A Thesis

In Partial Fulfillment of the Degree of

Master of Science

At the

University of Connecticut

2016

APPROVAL PAGE

Master of Science Thesis

**Deciphering the Outer Envelope of *Thermotoga maritima***

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University of Connecticut 2016

## **Acknowledgments**

I would like to acknowledge my major advisor, Dr. Kenneth Noll for all the time, effort and encouragement that he put in for me to complete this document and ultimately this degree. His guidance through the highs and lows of graduate school paved a path for me to move on. I thank him sincerely.

I would also like to thank my advisors; Dr. J. Peter Gogarten, Dr. David Knecht, Dr. Spencer Nyholm and Dr. Daniel Gage for sitting in the long committee meetings and guiding my work to its present state.

I am grateful for all the time Dr. Carrol Norris of the Microscopy facility spent on helping me with my confocal microscope images, and the time spent on replying to my numerous emails. I also appreciate all the help that I was given during lab meetings and presentations from the past Noll lab members, Dr. Nicholas Butzin, Dr. Amanda Petrus, Dr. Nathalie Boucher, Dr. Kristen Swithers, Kunica Asija and Daniel Bradnan. I also thank Dr. Pascal Lappiere and Matthew Fullmer for their bioinformatics expertise.

Lastly I would like to thank my family: Rohan for listening in to my numerous presentations, Sasha Maya for giggling and lighting up my day and my dear wife, Dr. Yerina Ranjit for encouraging me to go on, through our tough times and edging me forward past every hiccup that we faced.

## Table of Contents

Acknowledgments.....	iii
List of Tables And Figures.....	v
Abstract.....	vi
1.0 Introduction.....	1
1.1 The Thermotogae.....	1
1.2 Cell Envelopes of archaea and bacteria.....	2
1.2.1 Gram-positive and gram-negative bacteria.....	2
1.2.2 Archaea.....	3
1.2.3 Multicellular associations in other bacteria.....	3
1.3 Biosynthesis of the outer envelope.....	4
1.4 Outer envelope of <i>T. maritima</i> .....	5
1.5 Objective of this study.....	8
2.1 Growth medium and growth measurement.....	9
2.2 Enumeration of classes of cell types based on sizes of outer envelopes.....	10
2.3 Determination of ratios of whole cell area to cytoplasm area.....	10
2.4 Cell measurements.....	11
2.5 Gene expression.....	11
2.5.1 Sample collection.....	11
2.5.2 RNA extraction.....	12
2.5.3 cDNA synthesis.....	12
2.5.4 qPCR.....	12
2.5.5. Selection of reference and analysis genes.....	13
3.0 Results.....	15
3.1 Media design and growth curve.....	15
3.2 Qualitative analysis of changes in the cell envelope during growth.....	15
3.3 Microscopy.....	16
3.4 Gene Expression.....	18
4.0 Discussion.....	22
5.0 Tables and Figures.....	27
References.....	43

## List of Tables and Figures

<b>Table 1.</b> Coefficient of Variation (C.V.) and Stability values (M) for reference genes. ....	27
<b>Table 2.</b> P300 medium composition. ....	28
<b>Table 3.</b> Primers for genes analyzed by RT-PCR. ....	29
<b>Table 4.</b> Genes used as reference (housekeeping) genes. ....	31
<b>Figure 1. A-D</b> are phase contrast images of cells in media with various concentrations of PIPES buffer. Images are at 1000X magnification. ....	32
<b>Figure 2.</b> Schematic of the <i>T. maritima</i> cell envelope showing protein attachments to the outer envelope and peptidoglycan (4). ....	35
<b>Figure 3.</b> Growth curve of <i>T. maritima</i> cells in anaerobic P300 medium at 80°C. Dots correspond to time points of sampling. ....	36
<b>Figure 4.</b> Qualitative analysis of outer envelope distension size in cell populations over time. ....	37
<b>Figure 5.</b> Ratio of the areas of the whole cells to cytoplasm in mid-log (24.5 hours) and early stationary (ST) (59.5 hours) phases. ....	38
<b>Figure 6.</b> Average outer envelope sizes in mid-log (24.5 hours) and stationary phase (ST) (59.5 hours) ( $p < 0.001$ ). ....	39
<b>Figure 7.</b> Cytoplasm area comparison in mid-log (24.75 hours) and early stationary phase (ST) (59.5 hours) cells ( $p > 0.001$ ). ....	40
<b>Figure 8.</b> Distribution of outer envelope sizes in mid-log (24.75 hours) and early stationary phase (ST) (59.5 hours) cells. ....	41
<b>Figure 9.</b> Change in expression of genes from mid-log to stationary phase. ....	42

## Abstract

*Thermotoga maritima* is the representative member of the order Thermotogales, which is comprised of extremely thermophilic anaerobic bacteria. All members of this order are characterized by the presence of a unique outer envelope, the toga. The outer envelope appears as polar cellular distensions that sometimes are as large as the cytoplasm. The outer envelope increases in size while the cytoplasm remains the same leading to an outer envelope distension. The outer envelope distension development and increase in its size most likely occurs for storage of nutrients. Initial observations suggest that cells create larger distensions at the poles as they enter stationary phase. Preliminary data showed an increase in the number of cells with larger outer envelope distensions from the mid-log to stationary phase. Comparative measurements of cells were taken in their respective growth phases to determine cytoplasm sizes. There was a significant ( $p=4.44\text{E-}31$ ) increase in the ratio of the whole cell to the cytoplasm between the mid-log and stationary phases. There was a significant ( $p=1.25\text{E-}20$ ) increase in the area of the outer envelope and an insignificant ( $p=0.02$ ) increase in cytoplasm area between mid-log and stationary phase. This indicates a 1.69-fold increase in the outer envelope size while the cytoplasmic aspect of the cells remained the same. Furthermore, expression analysis was conducted to confirm growth phase specific gene expression. There was an increase in the expression of the (Outer membrane protein encoding genes) *ompA1*, *ompB*, *ompA2* and *ompA3* by 7.9 and 5.2, 3 and 2.2 fold respectively from mid-log to stationary phase. The beta barrel assembly machinery protein gene (*bamA*) showed only a 1.2 fold while the *csaB* was up regulated 4.4 fold between phases.

## 1.0 Introduction

### 1.1 The Thermotogae

*Thermotoga maritima* is the most studied bacterium of the phylum Thermotogae. The phylum Thermotogae is comprised of >40 species that are placed into eleven different genera (1). These organisms are anaerobic, gram-negative rods, largely comprised of mesophilic to thermophilic species that live within the range of 37°C to 90°C, and are frequently near the base of the bacterial node of the 16S ribosomal RNA Tree of Life (2). *T. maritima* cells have a doubling time of 75 minutes. They grow between pH 5.5 and 9 in NaCl concentrations of 0.25 to 3.75% with an optimum of 2.7% and an optimal temperature of 80°C. The cells are 1.5 to 11 µm long with a diameter of 0.6 µm. Cells occur singly or in pairs and exhibit a gram negative nature of staining. They are motile due to a singular polar flagellum. The cells were first isolated from geothermally heated sea floors in Italy and the Azores (3).

These bacteria represent a deep branch within the phylogenetic tree of the Bacteria, suggesting that these organisms may have retained archetypical features resembling those of primordial bacterial cells (4). All members of this phylum are characterized by the presence of an outer envelope also known as a toga (3, 5-7). The toga is a distinguishing character in this group of organisms and the name “Thermotoga” came from the presence of this feature. The outer envelope forms large balloons at the cell ends and in this way encloses a periplasmic compartment with a volume comparable to or sometimes even larger than that of the cytoplasm (4). Studying the envelope structure and encased periplasmic space may



provide clues to defining the physiology of this organism in more detail. This information will help in understanding how some of the earliest life forms survived on this planet.

## **1.2 Cell Envelopes of archaea and bacteria**

The cell envelope comprises all structures of the cell outside the cytoplasm. This includes the cytoplasmic membrane, periplasm, outer membrane and surface structures. The outer envelope is the outer membrane and other variations of the surface layer in gram-negative cells.

One of the major features unique to bacteria is the peptidoglycan cell wall. The growth and division of the cell are limited by the necessity to enlarge and divide the wall. These processes are regulated spatially and temporally by important cytoskeletal proteins MreB and FtsZ, which are distant relatives of actin and tubulin, respectively (8). The outer membrane in most organisms is a lipopolysaccharide (LPS) bilayer, different from the cytoplasmic membrane, which is a phospholipid bilayer.

### **1.2.1 Gram-positive and gram-negative bacteria**

The types of cell envelopes of most bacteria are divided into two types. Gram-negative bacteria, which are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide. Gram-positive bacteria, which lack an outer membrane but are surrounded by layers of peptidoglycan many times thicker than is found in the gram-negatives (9). The cell envelope gives bacteria their shape, provides the means by which they generate usable forms of energy for growth and division, protects the organism from host immune responses, promotes pathogenesis, is integral to the horizontal

transfer of plasmids and other mobile elements and forms the conduit through which bacteria interface with their surroundings (10).

Other bacteria that are close to the Thermotogae in the 16S rRNA gene tree have similar structures of the outer envelope. *Dictyoglomus* cells have thick outer envelopes spanning 80-100 nm with outer most layers similar to those of other proteobacteria (11). *Deinococcus* and *Thermus* have complex layered envelopes which lack LPS and have an S-layer instead. Although LPS is absent in these organisms, an unusual lipoglycan that has not been fully characterized is present (12). Proteins of the BAM (Beta barrel associated machinery) complex have been characterized noting its functionality in these organisms. The Thermotogae lack the LPS family protein synthesis machinery. Highly divergent orthologues of some of the LPS transporter system proteins have been described, which suggests a novel system of lipoprotein delivery to the outer envelope (12-14).

### **1.2.2 Archaea**

Archaea have structurally different envelopes from bacteria. Most archaeal envelopes consist of a single membrane structure enclosed by an S-layer (15). Several families of archaea have a peptidoglycan-like layer called pseudomurein, but precursors of this kind of wall are synthesized by different enzymatic pathways than peptidoglycan (8).

### **1.2.3 Multicellular associations in other bacteria**

The appearance of a toga-like structure on bacteria is an uncommon feature. There has been one account of a similar structure seen in *Roseovarius nubinhibens* (16). Though there have been instances of multiple cells residing within the same outer envelope, similar to the

phenomenon seen in *Thermus* spp and *Dictyoglomus* spp. (11), a sheath like structure is absent in single cells. Within the groups of bacteria that are closely rooted at the base of the phylogenetic tree, structurally uncommon features have been described in cell envelopes of *Aquifex*, *Deinococcus* and *Thermus*. These organisms have outer envelopes similar to those in proteobacteria with periplasmic spaces. *T. maritima* contains several hydrolyzing enzymes in the outer envelope suggesting a use for the periplasmic compartment (11). In *Dictyoglomus* spp. and *Thermus* spp., there appear to be multiple cells within the same outer envelope, and they form “rotund bodies”. The periplasmic space has been suggested to be involved in the decomposition of polysachharides (11). Rotund bodies have also been reported in *Fervidiobacterium* and *Oceanithermus* (12). As many as four cells within the same sheath have been reported in *T. maritima* cultures, too (3). It appears as if multiple cells are present surrounded by a balloon outer envelope in *T. maritima*. Multicellular (rotund) bodies were also observed in this study, but only in the stationary growth phase.

### **1.3 Biosynthesis of the outer envelope**

Understanding the biosynthesis and transport of outer membrane proteins is critical in deciphering components of the outer envelope of *T. maritima*. These outer membrane proteins are synthesized in the cytoplasm and the cytoplasmic face of the cytoplasmic membrane. Then they are translocated across the cytoplasmic membrane undergoing various modifications. The secretory (Sec) and ATP binding cassette (ABC) translocation systems are utilized for this purpose. Periplasmic chaperones deliver outer membrane proteins (OMP) to the identified assembly site of insertion. The  $\beta$ -barrel assembly

mechanism (Bam) then inserts the targeted protein into the outer membrane/outer envelope.

The *T. maritima* outer envelope is anchored to the cell wall peptidoglycan via a scaffolding transmembrane protein, outer membrane proteins OmpA1 (19). OmpA1 (previously Omp  $\alpha$ ) has been suggested to be linked to pyruvylated secondary cell wall polysaccharides (SCWP) on the peptidoglycan via a surface layer homology (SLH) domain (17). The linkage of the SCWP to the peptidoglycan remains unknown. OmpA2 is a homologue of OmpA1 found through genome sequence analysis and its sequence suggests that it contains both the hydrophobic N-terminal domain that links to the outer envelope and the SLH domain that is suggested to link OmpA1 to the SCWP (18). The outer envelope distends at the poles of the cell during later growth phases, and it appears that the OmpA1 protein localizes to the outer envelope and not to the peptidoglycan layer, at these regions (19).

In other bacteria, cytokinesis (division of the cytoplasm) and cell division are simultaneous. In the Thermotogae, cytokinesis and cell division occur separately. This process has been described in *Thermosipho globiformans* (20). Here cytokinesis occurs before the outer envelope and periplasmic space are separated between two cells. The separation of the outer envelopes does not seem to occur in the center of the spaces between the two cytoplasm, which may allow for some cells to have longer distensions on one pole than the other. Ultimately, the multiple cytokinesis occurrences before cell division within the same outer envelope allows for the formation of multicellular bodies (20).

#### **1.4 Outer envelope of *T. maritima***

The outer envelope of most gram-negative bacteria functions as a semi permeable barrier and acts as an anchor for hydrolytic enzymes. The outer envelope has not been studied very well in the Thermotogae. We do know that it also functions as a selective barrier from the outside world, and is an anchor for polysaccharide hydrolases (Fig 1.), allowing for the utilization of insoluble carbon sources (4, 21). There have only been a few studies that have examined the structural and functional components of the outer envelope.

The outer envelope lipid composition still eludes us, but we know that LPS synthesis machinery is not available in *T. maritima* (12). OmpA1 (TM0477) is a 43 kDa monomeric protein and is believed to function as an anchor to the outer envelope. It has an amino domain of 64 residues followed by a putative coiled coil segment of 300 residues and a carboxy terminal membrane-spanning segment (19). OmpB is a 42 kDa monomeric protein that is believed to function as a porin (18). Amylase A (TM1845) is a 61 kDa enzyme that contains a 25-residue putative linker sequence on the N-terminus, attached to the outer envelope through a 18-residue signal peptide, which is strongly reminiscent of a typical lipoprotein signal peptide. The linker sequence may serve as a more or less flexible, extended spacer which could result in better performance of the enzyme at the cell surface (22). Its function is to hydrolyze starch. Xylanase A (TM0061) and Xylanase B are found in the outer envelope and periplasmic cell fractions, respectively. The hydrophobic core of the signal peptide of XynA is attached to the outer envelope as depicted in Fig. 1. OmpA1 and OmpA2 are most probably present in the outer envelope (18).

The presence of various polysaccharide hydrolases at the cell surface could be to avoid loss of secreted enzymes in an extremely hot marine environment. The unique

ballooning of the outer membrane over the poles of the cells may serve to enlarge the surface area spiked with these enzymes (4).

The outer envelope extends on one or both poles of the cell and sometimes the extension by itself is larger than the cytoplasm. A study in *Thermosipho geolei* mentioned an increase in size of the periplasmic space and the outer envelope in later growth phases (6), but detailed information on this increase in this organism is not available. It was only observed that the outer envelope appears to increase in size from early log to stationary phase. In *T. globiformans*, it was observed that the large periplasm forms between newly divided cells, followed by a fission around the middle of the periplasm, which was accompanied by a sideward motion of the newly generated cell at the pole(s) (20). The cells appeared to divide by constriction, with the site not necessarily located at the center of the intercellular periplasm. A septal amorphous layer is formed after the enlargement of the periplasm, which is similar to a septal outer membrane. The formation of both septal OM and amorphous layer in the periplasm suggests that the large periplasm allows for the separate septal toga formation from cytokinesis. Because cytokinesis and septal toga formation do not occur simultaneously in *T. globiformans*, cytokinesis could occur multiple times before septal toga formation, which may be the mechanism of the production of multicellular filaments (20).

The main goal of this study is to address the issue of whether there is an increase in the outer envelope size in different growth phases and to quantify the increase over time.

## 1.5 Objective of this study

We now have a decent overview of the structure and components involved in the synthesis of the outer envelope of *T. maritima*. Though there have been observations of an increase in outer envelope distension size, there have not been any studies that confirm or quantify this increase at any particular phase of growth. This study aims to fill in that gap to confirm and quantify an increase in size between mid-log and stationary growth phases. Thus I present the aim for my study which is subdivided into four parts:

**Aim:** To determine the increase in outer envelope formation from log phase to stationary phase.

1. Demonstrate the phenomenon that has been mentioned in the literature using a semi-quantitative analysis.
2. Quantitate this change in morphology.
3. Show that the growth curve assessment is a good measure of the process: The semi-quantitative assessment (#1 above) matches the two microscopically measured time points (#2 above)
4. Demonstrate which proteins change in amount, suggesting their involvement in outer envelope formation.

## 2.0 Materials and Methods

### 2.1 Growth medium and growth measurement

*T. maritima* MSB8 grows at an optimal temperature of 80°C with a doubling time of 75 min in MMS medium (3). Cells change from rods in log phase to spheres in stationary phase. This shape change is due to the acidification of the media in the later phases of growth. The pH in the medium changed from pH 7 to pH 5 (unpublished data). Comparisons of the sizes of cells cannot be made when their shapes change, so a new growth medium was developed to maintain the rod shapes throughout all growth phases.

P300 medium (Table 2) is a modified version of the *Thermotoga* Basal (TB) medium, described by Childers *et al.* (23). TB medium was modified by eliminating yeast extract and increasing PIPES from 19.8 mM to 300 mM to stabilize the buffering capacity of the medium. An experiment was conducted to make sure the shape of the cells stayed rods and the pH of the medium did not drop. Cells shapes were observed in medium that contained 50mM to 300mM PIPES (Fig 1 A-D). It was determined that medium with 300mM PIPES was suitable for subsequent experiments. After mixing all components listed in Table 2, it was brought to a final volume of 1 L in water. The pH was adjusted to 7.5 with NaOH and steamed for 20-30 min to remove dissolved oxygen. One hundred mL was dispensed into each serum bottle and the bottles sealed in a Coy anaerobic chamber under an atmosphere of 3% H<sub>2</sub> in N<sub>2</sub>. The headspaces in the bottles were then flushed with N<sub>2</sub> for 10 min to remove H<sub>2</sub>. The bottles were autoclaved for 20 min. Before inoculation, maltose was added from a filter-sterilized, anoxic 20% (w/v) stock solution for a final concentration of 0.5% (w/v).



Five hundred microliters of culture were taken from serum bottles with a sterile 1 mL syringe. The syringe was flushed by filling it with sterile nitrogen gas before sampling. The OD 660 nm readings were taken in a Genesys 20 Thermo spectronic spectrophotometer using P300 media as a blank.

Cells were cultured anaerobically in P300 medium at 80°C. Growth was measured at 660 nm. Mid-log phase was determined to be from 0.03 to 0.3 OD<sub>660</sub> and stationary phase was determined to start around 0.4 to 0.5 OD<sub>660</sub>. Mid-log phase is defined as the optical density at the midpoint of the linear range of exponential growth. Early stationary phase is defined as the optical density at which the culture exhibits a growth rate near zero (OD<sub>660</sub> ~0.5).

## **2.2 Enumeration of classes of cell types based on sizes of outer envelopes**

Samples were taken from a growing culture at the time points indicated in Fig. 2. Cells were segregated into categories based upon the sizes of their outer envelope distentions as measured under a 100X oil immersion lens with an Olympus BX1 phase contrast microscope. At least 200 cells were observed and classified for each time point. Cells were divided into four categories: 1. very small or no visible outer envelope, 2. small outer envelope, 3. large outer envelope and 4. Very large outer envelope. Spherical bodies were visible in later phases of growth and were also counted.

## **2.3 Determination of ratios of whole cell area to cytoplasm area**

The ratios of the areas of whole cells to the areas of their respective cytoplasm were obtained from 137 cells harvested in mid-log stage and 139 cells harvested in stationary phase. The area measurements are relative size values for each image analyzed and thus

ratios were obtained for each cell. Ratios were calculated from measurements taken using a Leica confocal microscope using Leica imaging v 1.2 software.

## **2.4 Cell measurements**

203 cells from mid-log phase and 215 cells from stationary phase were harvested and measured. Cell images were captured using a Leica confocal microscope and Leica imaging v 1.2 software. FIJI (24) was used to calculate the areas of cytoplasm and whole cells. A stringent protocol for cell measurements was followed. Each cell was traced 5 times and any measurement with a standard deviation more than 5% from the mean was discarded. The mean of the remaining measurements was used in the recalculation. Any cell measurement with more than 2 readings outside the cutoff was discarded. Each image was scaled on a 1  $\mu\text{m}$  scale before areas were traced. Cytoplasm areas between the two phases and outer envelope areas were calculated. Ratios of the whole cell and the cytoplasm (Fig. 4) and average outer envelope size (Fig. 5) were obtained. The outer envelope area was calculated by subtracting the cytoplasm area from the whole area of the cell.

## **2.5 Gene expression**

### **2.5.1 Sample collection**

Three biological replicates were obtained for expression analyses. Cultures were grown in 100 mL serum bottles with P300 media. Bottles were incubated at 80°C. One mL samples of culture were taken and immediately frozen on an ethanol dry-ice bath at mid log (OD<sub>660nm</sub>: 0.05 – 0.2) and early stationary (OD<sub>660nm</sub>: 0.5-0.6) phase. Collected samples were stored at -80°C until ready for RNA extraction.

### **2.5.2 RNA extraction**

The 1 mL samples were thawed on ice for 20-30 min. While thawing, the cells were pelleted at 10000 rpm for 1 min at 4°C. The supernatant was removed and the pellet was processed according to the protocol of Zymo Research® RNA extraction kit. Samples were never treated above 4°C before RNA extraction.

### **2.5.3 cDNA synthesis**

RNA was quantified using the Qubit® protocol for nucleic acid quantification. The Promega reverse transcriptase kit was used for cDNA synthesis. RNA was treated with 3 units of DNase for 18 hours at 37°C. The cDNA and corresponding RNA was amplified by PCR to test for any DNA contamination that may have arisen from the RNA extraction protocol. Only cDNA with no amplicon in the corresponding RNA was used for the gene expression analysis.

### **2.5.4 qPCR**

Primer-BLAST (25) was used to design all primers. All primers were designed to amplify 98-103 bp amplicons with annealing temperatures between 59°C and 61°C. Primers for all genes analyzed by RT-PCR are listed in Table 3.

The expression analysis was done using BioRad Ssofast™ Evagreen® supermix on a BioRAD CFX96™ Real-time system. BioRAD CFX manager 3.1 software was used to calculate the threshold cycle (Ct) values. Expression of each gene was measured in quadruplicate and primers were tested with RNA from each phase, control DNA and a non-template control.

### 2.5.5. Selection of reference and analysis genes

All expression data were obtained using the Bio-Rad CFX manager 3.1 software. Since there were multiple plates that had to be analyzed, the plate normalization function had to be used by running the same single sample on all plates. Data for reference genes could then be analyzed. A set of four genes were chosen as candidates for reference genes that would be stably expressed in log and stationary phase (Table 3). The genes were chosen from those that have been used in the past as reference genes for expression in *T. maritima* (26, 27). These genes are not functionally related to avoid co-regulation in the tested conditions. The Bio-Rad CFX manager 3.1 software uses geNorm and NormFinder to test for stable expression (28-30). geNorm and NormFinder are algorithms that calculate the stability of gene expression between various conditions, which allows for an accurate reference value for relative expression. The model-based approach (NormFinder) ranks the candidates with minimal estimated intra- and intergroup variation, in contrast to the pairwise comparison approach of geNorm, which tends to select those genes with the highest degree of similarity of the expression profile across the sample set (28). The software provides an M value which is a measure of stability of expression between the tested conditions and a coefficient of variation (C.V.) value. The lower the M value, the higher the probability that the expression of the candidate reference gene is similar between the tested conditions. M value is the average pairwise variation of a particular gene with all other control genes, the most stable genes presenting the lowest M values. Mean CV and M values lower than 25% and 0.5, respectively, are typically observed for stably expressed reference genes in relatively homogeneous sample panels.

Literature suggests that a good candidate for a reference gene has an M value of <0.5 and a great candidate has an M value of <0.2 with at least 3 reference genes (29, 30).

Growth phase dependent marker genes were chosen for each growth phase to indicate that sampling was taken at the appropriate indicated phase of growth. TM0506 (groEL, a stress protein likely expressed during stationary phase (31), and TM1536 (pmp, a predicted membrane protein expressed during mid-log phase (32). Both genes have been shown to be up-regulated in their respective growth phases in *T. maritima* (31).

A list of genes associated with the outer envelope and its biogenesis was compiled. It included the outer membrane proteins TM0477 (OmpA1, outer membrane protein A1), TM1729 (OmpA2, outer membrane protein A2), and TM0711 (OmpA3, outer membrane protein A3), that are scaffolding proteins and TM0476 (OmpB, outer membrane protein B) a porin that is a part of the outer envelope. TM0448 (BamA, beta barrel assembly machinery protein A) was chosen as an associated outer envelope building protein. TM0682 (CsaB, polysaccharide pyruvyl transferase) was chosen because of its likely role in OmpA1 protein attachment to the peptidoglycan. The OmpA1 protein has been shown to localize on the outer envelope at the distensions of the cell, and I wanted explore to see if the polysaccharide pyruvyl transferase had any role in the increase in distension of the poles at later growth phases.

### **3.0 Results**

#### **3.1 Media design and growth curve**

*T. maritima* cells change from rods in log phase to spheres in stationary phase. Comparisons of the sizes of cells cannot be made when their shapes change, so a new growth medium was developed to maintain the rod shapes through-out all growth phases. The buffer concentration was increased to retain the shape of cells in stationary phase (Fig 1A-D). The pH did not drop below 6.5 when 300mM PIPES was in the medium allowing about 90% of the cells to remain in a rod shape. The process required a defined medium. A defined medium would allow for the altering of components according to specific studies related to the outer envelope. The P300 medium was designed for this purpose, which mainly differed from the MMS media in salt and buffer concentrations (See Materials for details). A growth curve was plotted (Fig. 3) for *T. maritima* grown in P300 medium, from which the mid-log and stationary phases were determined. Optical density (OD) values at 660 nm were noted for the various phases of growth. Mid-log phase was determined to be from 0.05 to 0.4 OD. Early stationary phase was determined to be around 0.6 OD. Samples were taken at OD values that matched mid-log and stationary phase for each of the subsequent experiments.

#### **3.2 Qualitative analysis of changes in the cell envelope during growth**

I conducted a semi qualitative analysis of the size of the outer envelope distensions and the cell membranes during different phases of growth. Cell samples were taken from time points indicated by dots in Fig. 3. Sampling was carried out from early log phase at 16.5 hours to late stationary phase at 88.5 hours. The extent of the distension of the outer envelopes of cells was assessed by viewing them under 1000X magnification using an Olympus BX1 phase

contrast microscope. Outer envelope distension sizes that were smaller than 0.3 micron were deemed not visible. The rest of the cells were separated in categories of 1.5  $\mu\text{m}$  increments. Outer envelopes from 0.31-1.8  $\mu\text{m}$  were categorized as small, 1.81-3.3  $\mu\text{m}$  as large and above 3.4  $\mu\text{m}$  as very large. The number of cells with no visible outer envelope distension decreased over time and were not observed at 46.5 hours (Fig. 4). Cells with small visible outer envelope distensions decreased from 67% of the population during log phase to 35% in the early stationary phase. In contrast, the cell population with larger outer envelopes increased from 23% to 55% during the same time frame. From hour 40 onwards, cells with extremely large outer envelopes were also observed. Rounded bodies that contained multiple cytoplasms appeared in the populations from hour 46.5 onwards and their numbers increased during later stationary phase.

These results are the first semi quantification data to support observations that have been made in the past on the increase in size of the outer envelope. There are no data that quantify the increase in outer envelope distension to date. This information is important as a foundation for the experiments that follow.

### **3.3 Microscopy**

To measure a significant change in area between growth phases, a suitable sample size had to be determined and measurements had to be taken as carefully as possible to reduce human error. In my first experiment, I harvested a minimum of 200 cells for each phase, which provided a confidence level of 95% and margin of error of 5.69%.

First, I measured the increase in the ratio of the areas of the whole cell (the area enclosed by the outer envelope) to the cytoplasm (the area enclosed by the cytoplasmic membrane)

in the two growth phases. The ratio for the log phase cells was  $1.49 \pm 0.32$  and stationary phase cells was  $1.81 \pm 0.33$  with  $p=4.44\text{E-}31$ . (Fig. 5). There was a significant ( $p<0.001$ ) increase in the ratio of the whole cell to the cytoplasm between the mid-log and stationary phases. This observation is consistent with reports that the distension of the outer envelope is more apparent in stationary phase cells (3). There are three possibilities for this increase between the two phases: (i) the outer envelope increases and the cytoplasm shrinks, (ii) the outer envelope stays the same and the cytoplasm shrinks, and (iii) the outer envelope increases and the cytoplasm remains the same. To determine which possibility is correct, the sizes of both the whole cell (outer envelope enclosed area) and cytoplasm for both phases needed to be compared.

I measured the sizes of the whole cells and cytoplasm and defined the size of the outer envelope (size of the periplasm) as the difference between the whole cell and cytoplasm areas in cells from both phases. This is the area of the outer envelope that extends beyond the poles of the cell and is the part of the envelope that is observed in more cells as cultures approach the stationary growth phase. I used a minimum of 200 cells for each phase, which provided a confidence level of 95% and a margin of error of 6.9%. The stringent protocol (see Methods) used to obtain area values diminished the effect of human error during measurement.

Measurements of 200 cells from log phase and 215 cells from stationary phase were taken to calculate the differences between the whole cell and cytoplasm areas. The outer envelope area for the mid-log phase cells was  $1.13 \pm 0.68 \mu\text{m}^2$  and for stationary phase cells was  $1.91 \pm 0.92 \mu\text{m}^2$  with  $p=1.25\text{E-}20$  (Fig. 6). The mean area for the cytoplasm was  $2.20 \pm 0.90 \mu\text{m}^2$  for mid-log phase and  $2.40 \pm 0.87 \mu\text{m}^2$  for stationary phase with  $p=0.02$  (Fig. 7). There



was a significant ( $p < 0.001$ ) increase in the area of the outer envelope and an insignificant ( $p > 0.001$ ) increase in cytoplasm area between mid-log and stationary phase (33). This indicates a 1.69-fold increase in the outer envelope size while the cytoplasmic aspect of the cells remained the same. This shows that the third possibility explains the increase in visible outer envelope distensions as cultures age, the outer envelope increases in cells while the cytoplasm remains the same size.

To compare data gathered by measuring cells with the previous qualitative measure of changes in cell shape (Fig. 4), cells with different measured outer envelope sizes were separated into categories using criteria similar to those used for outer envelope categorization depicted in Fig. 4. Outer envelope sizes that were smaller than  $0.3 \mu\text{m}$  were deemed not visible. The rest of the cells were separated in categories of  $1.5 \mu\text{m}$  increments. Outer envelopes from  $0.31\text{--}1.8 \mu\text{m}$  were categorized as small,  $1.81\text{--}3.3 \mu\text{m}$  as large and above  $3.4 \mu\text{m}$  as very large. Rounded bodies were not included in the cell size measurement. The results showed a pattern very similar to that as seen in the qualitative observations for log phase cells at 28.5 hours and early stationary phase cells at 55.5 hours (Figs. 4 & 8) indicating that the qualitative observations provided an accurate measure of the changes in the extent of toga distension throughout the growth of a culture.

### **3.4 Gene Expression**

To gather data in support of this hypothesis, I analyzed gene expression patterns between the two growth phases using genes that encode proteins that are present in the outer envelope and are likely involved in its formation. Genes previously shown to be up regulated at mid-log and stationary phases were used as growth phase indicators. The continued synthesis of the outer membrane as cells enter stationary phase could be the

result of up regulation of genes related to the outer envelope. Real time expression analysis was used to measure the change in expression of the target genes.

To eliminate non-biological variation, gene expression analysis involving qRT-PCR requires stringent normalization strategies. Among the several approaches available, the use of reference genes is currently the preferred method of normalization (29). The use of reference genes which have stable expression between conditions is critical for gene expression analysis (34). To identify those genes a list of candidate genes is compiled and then their stable expression is verified (28). The 4 control genes that were selected (*gap*, *recA*, *rpsB* and *eno*) have previously been used as control genes in *T. maritima* expression analyses (26, 27). The gene *recA* has also been shown to be expressed similarly between log and stationary phase in *Staphylococcus pseudintermedius* (35). Two methods (geNorm and NormFinder), were used to calculate the gene expression stability values which were compiled by the BioRads CFX manager software.

The number of genes used for reference gene calculation is a tradeoff between practical considerations and accuracy of the measurement (29). A minimum of 3 reference genes are recommended for analysis. Since my panel of test genes (Table 2) was small (only 6 genes; 4 structural protein coding genes and 2 outer envelope associated genes), only 4 reference genes (Table 1) were tested for stable expression between log and stationary phase. The use of at least three stably expressed genes is recommended for calculation of the normalization factor (NF)(29). An M value is obtained using the algorithms in geNorm. An arbitrary cut off M value of 0.5 and C.V. of 25% is recommended for the pairwise variation calculations to choose the appropriate gene sets using geNorm (29). A value of 0.5 is considered a good candidate, while values around 0.2 and below are very good candidates. The *gap*, *eno*, and

*rpsB* genes were the best candidates for the experiments (Table 1). *recA* had an M value greater than 1, and C.V. value greater than 25%, thus was not used in the normalization.

As markers of the growth phase, two genes (*pmp* and *groEL*) shown to be highly expressed in log phase and stationary phase respectively in *T. maritima* (36), were chosen as markers of the growth phase. As predicted, *groEL* was more highly expressed during stationary phase (3.5 fold increase from mid-log phase), and *pmp* (TM1536, a predicted membrane protein) was more highly expressed in logarithmic phase (0.35 fold increase in stationary phase) (Fig. 9). The survival protein gene *surE*, was also chosen as a marker for stationary phase cells (37). Results indicated that there was a 3.9 fold increase from mid-log to stationary phase for this gene. Thus the genes used as markers for the respective growth phases were up regulated at their predicted phases validating the time point/ phase of growth that the cells were in at time of sampling.

There was a 7.9 and 5.2 fold increase in the expression of the *ompA1* and *ompB* genes indicating that these genes were more active during the stationary phase when the outer membrane increased in size compared to the mid-log phase (Fig. 9). *ompA2* and *ompA3* are homologs to *ompA1* and are suggested to encode structural proteins in the outer envelope. The fold change in expression of *ompA2* was 3 fold suggesting a possible role in outer envelope structure development during stationary phase. The change in expression of *ompA3* between mid-log and stationary phase was only 2.2 fold, indicating a possible role of this protein in the outer envelope construction during stationary phase. The beta barrel assembly machinery protein gene (*bamA*) showed only a 1.2 fold increase in expression during stationary phase. The BamA protein is involved in the insertion of beta barrel proteins into the outer envelope and it was assumed that there would be an increase in expression

during stationary phase when the outer envelope increased in size. This suggests that the amount of protein being produced in stationary phase is sufficient for the requirement of the insertion of OmpB proteins. *csaB* was up regulated 4.4 fold in stationary phase. This gene responsible for encoding pyruvyl transferase which is necessary for pyruvylation of polysaccharides on the SCWP for attachment to the SLH domain on the Omp proteins.

Thus most of the genes that were chosen to represent proteins on the outer membrane appear to increase in expression in stationary phase as compared to mid-log phase. These results are consistent with the finding that the outer envelope continues to grow into stationary phase.

## 4.0 Discussion

The outer envelope of *T. maritima* plays an important role in protection because it is the outer barrier of the cell. It also is an anchor for hydrolytic enzymes, allowing for the utilization of complex polysaccharides. Until recently, the reason of having an extended periplasmic space was not known. Now it is suggested to be used for storage and hydrolyzation of polysaccharides (20). Currently there is limited information on the composition of the outer envelope in *T. maritima* and even less understanding of the increase in size of its distensions at the cell poles. Outer membrane protein Omp $\alpha$  (OmpA1 in *T. maritima*) is a structural protein that has been suggested to link the outer envelope to the peptidoglycan on the inner cell wall in a manner like that of similar proteins in other bacteria (17). S-Layer homology (SLH) domains on outer membrane proteins are used by different organisms to bind to pyruvylated secondary cell wall polymers (SCWP) linked to the peptidoglycan. *T. maritima* OmpA1 contains one SLH domain and this may be its link to the peptidoglycan via pyruvylated SCWP (17, 38). I have not confirmed the presence of a SCWP on the peptidoglycan in *T. maritima* and whether this is the mechanism of linkage to the outer membrane protein. This may be an appropriate study for the future. In this study, I examined the expression of the polysaccharide pyruvyl transferase gene *csaB*. This gene encodes the protein that is involved in the addition of the pyruvyl moiety to the SCWP on the peptidoglycan. This addition is essential in the linkage of the peptidoglycan to the outer membrane protein. It appears that there was a significant increase in expression of this gene between the mid-log and stationary growth phases (~5 fold

increase during stationary phase). This suggests that there may be an increase in outer membrane attachments to the peptidoglycan with an increase in the amount of outer membrane proteins produced as the outer envelope increases in size. As the outer envelope increases in size with an increase in the number of OmpA1 proteins, the linking to the inner wall via the pyruvylated SCWP seems to increase. This occurrence was unexpected as with the enlargement of the outer envelope, I expected that there would be a decrease in the linkages of the outer envelope to the peptidoglycan. And an increase in gene expression does not always mean increase in protein production at the cellular level. So assessing the amount of protein produced at the different growth phases and studying the nature of linkages at the poles of the cell would help answer this anomaly. The nature of linkage may play an important role in the formation of bulges at the poles, and ultimately (rounding up) formation of rotund bodies in late stationary phase.

Rounded or rotund bodies are seen in other relatives of the Thermotogae. The *Dictyoglomus* rotund bodies are formed by the end of the logarithmic growth phase, especially when glucose or polysaccharides (starch, xylan) are supplemented to the growth medium. The formation of multicellular like bodies can be explained by the partial detachment of the protoplast from the outer envelope during cell division. When the outer envelope is partially detached from the protoplast, mechanical forces generated by protoplast elongation may drive cell rearrangement of daughter cells inside the compartment. During the following rounds of cell division, the overall shape of the compartment changes forming the rotund bodies(11).

The knowledge of synthesis of the outer envelope in different bacteria (closely and distantly related) will help in understanding the same process in *T. maritima*. In various studies with gram positive organisms, newly synthesized S-layer subunits were seen to be incorporated as helical bands, mainly at the septal regions, as perpendicular bands with respect to the longitudinal axis of the cell and also shown to happen at the site of division, including the accumulation of an excess of S-layer material at the site of division that can detach from the cell (39-41). In *Bacillus anthracis*, two S-proteins (Sap and EA1) are present simultaneously at the cell surface in different ratios depending on the growth phase. At the late exponential growth phase, Sap covers the entire surface of the bacterium, whereas EA1 is limited to defined spots. The opposite situation is observed in the stationary phase (42). It would be interesting to see the arrangement of the different OmpA proteins of *T. maritima*. Though there is an up-regulation of all the OmpA proteins in stationary phase, it would be interesting to see if there was localization of particular homologues at different regions of the cell.

In *Thermus spp.* one of the most abundant proteins of the outer envelope is SlpA, a protein that forms a hexagonal array over the whole cell, which is the building block of the S-layer. SlpA anchors the envelope to the cell wall-associated polysaccharides through a single N-terminal SLH (S-layer homology) domain. (43). The few analyses carried out so far point to two main models for the incorporation of S-proteins, either localized at the septal regions of the cells as in most gram-positive bacilli, or a diffuse one in which the new subunits are incorporated all over the cell surface. In gram positives, the S-layer binds to a thick, relatively inert cell wall, so it is likely that its

synthesis should concentrate at the regions of cell wall growth, whereas in gram negatives the S-layer binds to a theoretically more dynamic structure, such as the LPS layer of the outer membrane. The extension of the outer envelope of *Thermus thermophilus* takes place at a central region of the cell (44).

*T. maritima* probably has a similar mechanism of outer envelope synthesis as that of *Th. thermophilus*. Furthermore, it may have a similar mechanism of subsequent cytokinesis and cell division as in *T. globiformans*, to form prominent outer envelope extensions at the poles of the cell, and ultimately also forming rounded bodies.

Literature suggests that organism size may vary according to nutrient availability in the media and is largely due to its developmental stage (45). Enlarged periplasmic spaces may be suitable for the cells to scavenge nutrients from the environment and store them. The presence of a larger outer envelope or periplasmic space may suggest a scavenging state of the organism. It would also be interesting to see if the sizes of the outer envelopes varied with the different nutrients available in the environment.

Here I have demonstrated that the distensions of the outer envelope including an increased periplasmic space are larger at later growth phases, using a semi-quantitative analysis on a growth curve assessment (Fig 3 and 4). This validates the phenomenon that has been mentioned in the literature. This information of the increased space also allows for specific studies to further detail the composition of the outer envelope. Then I quantified the increase in size of the outer envelope microscopically by first measuring the increase in ratio of the whole cell to the cytoplasmic aspect (Fig 5). There was an increase in ratio between the growth phases indicating an increase in the outer envelope size. Secondly, I measured the increase



in the outer envelope extensions by subtracting cytoplasm areas from whole cell areas. The results showed a statistically significant increase over growth phase (Fig 6). The semi-quantitative growth curve assessment matched the microscopy evaluation of outer envelope size change, validating the semi-quantitative approach (Fig 4 and 8). This meant that the time points other than those taken at mid-log and stationary phase were most likely properly represented in the chart (Fig 4). Finally gene expression analysis was conducted on genes that coded for proteins that were involved in the formation of the outer envelope. All the structural genes were up regulated during stationary phase, again validating the growth curve assessment and microscopy results (Fig 9). These results complete the objectives that I set out to achieve and determine that there is an increase in outer envelope formation from log phase to stationary phase.

## 5.0 Tables and Figures

**Table 1. Coefficient of Variation (C.V.) and Stability values (M) for reference genes. Coefficient of Variation (C.V.) and Stability values (M) for reference genes. A C.V. of <25% and M value of <0.5 represents stable expression between test phases. The genes *gap*, *eno* and *rpsB* were used for analysis.**

<b>Gene</b>	<b>C.V.</b>	<b>M Value</b>
<i>gap</i>	0.0056	0.1361
<i>eno</i>	0.0970	0.2102
<i>rpsB</i>	0.0914	0.198
<i>recA</i>	0.5403	1.0948

**Table 2. P300 medium composition.**

Component	Amount per Liter
PIPES (1.5 Na salt)	90 g
NaCl	20 g
KCl	2 g
MgSO <sub>4</sub> • 7H <sub>2</sub> O	0.5 g
NH <sub>4</sub> Cl	0.25 g
CaCl <sub>2</sub> • 2H <sub>2</sub> O	50 mg
K <sub>2</sub> HPO <sub>4</sub>	50 mg
Na <sub>2</sub> WO <sub>4</sub> • 2H <sub>2</sub> O (3.3 mg/ml)	1 mL
Cysteine • HCl	0.5 g
Resazurin (0.1%)	1 mL
Vitamins and trace minerals	As in Childers et al. 1992 (23)

**Table 3. Primers for genes analyzed by RT-PCR.**

<b>Locus</b>	<b>Primer name and sequence</b>	<b>Gene annotation/ Description</b>
TM0477 (ompA1)	ompA1 F - GGCTGCGAGTTTTTCGTTCA  ompA1 R - CAACATCGGGCTCAAACCTTGG	Outer membrane protein  A1
TM1729 (ompA2)	ompA2 F - TGTCGAGAAAAACAACACCGC  ompA2 R - CTTGCCGTCTTCTGCTGAGT	Outer membrane protein  A2
TM0711 (ompA3)	ompA3 F - CCACGATCGCTCTCGATGAA  ompA3 R - GCCGCGGAGGTAATTTTCGAG	Outer membrane protein  A3
TM0476 (ompB)	ompB F - ATAGGCGCTTGTGGCATTGT  ompB R - GCTGTTGCTCTGAAACTCGG	Outer membrane protein  B

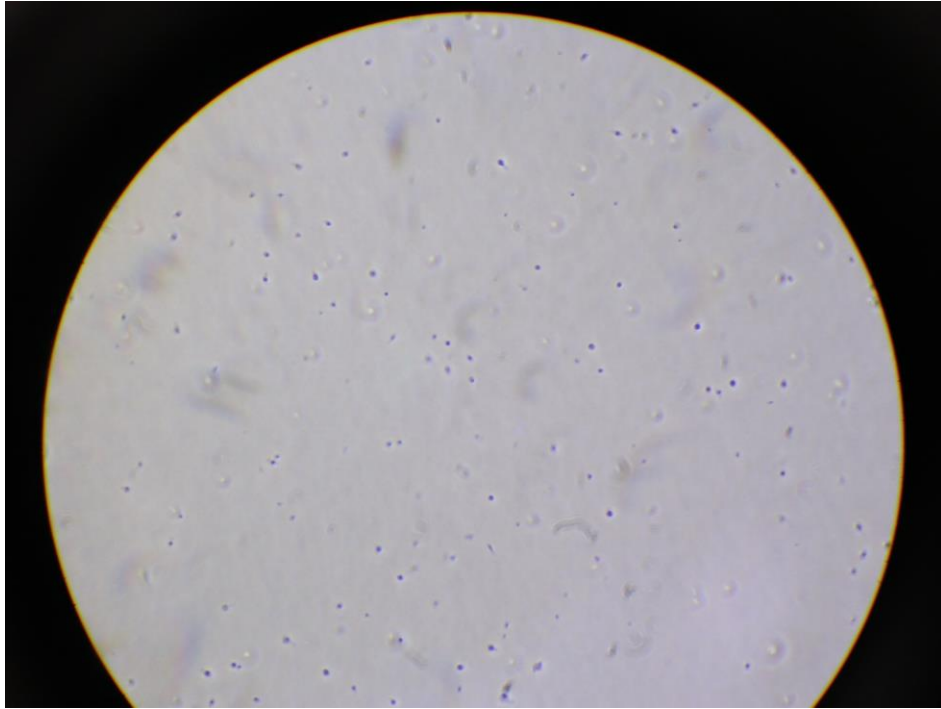
TM0448 (bamA)	bamA F - CCTGCTGTTCTGAAGGTAGGA bamA R - CAGACCGTACGTGGTGGAAA	Beta barrel assembly machinery protein A
TM0682 (csaB)	csaB F - TGTGTTCTTCCTGAAATCTCCGA csaB R - CCCTGGAAAAGCCGTCGATA	Polysaccharide pyruvyl transferase

**Table 4. Genes used as reference (housekeeping) genes.**

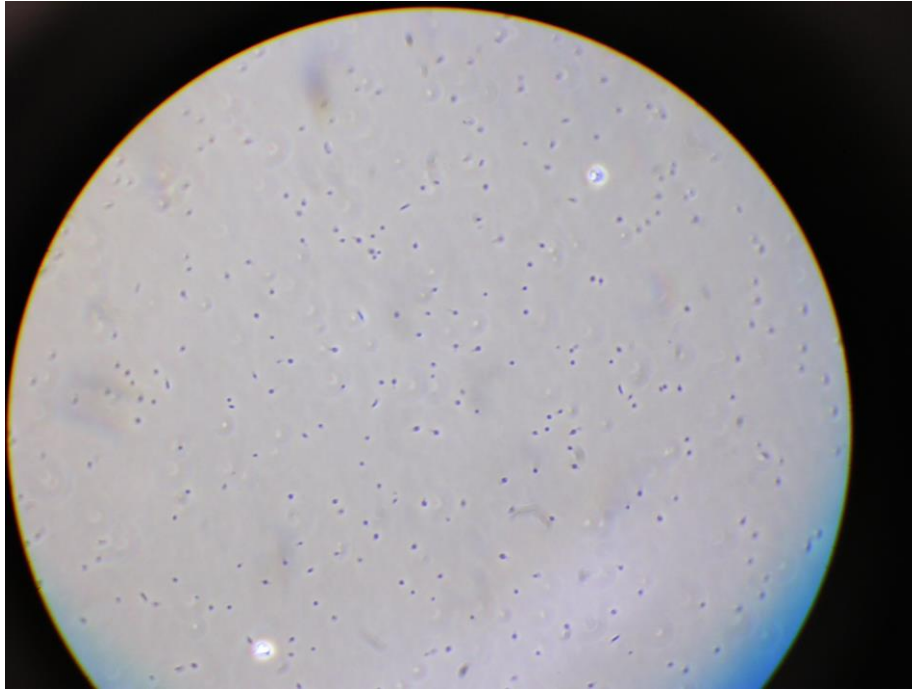
Locus	Gene annotation	Annotated function
TM0688 ( <i>gap</i> )	TM_0688 QRT F – TGCCCTGGAAGGACCTCGGT TM_0688 QRT R – CTTGGCGGGCGCTGTGATGA	glyceraldehyde 3 phosphate dehydrogenase
TM1859 ( <i>recA</i> )	TM1859 QRT F – GGCACGTCTCATGTCGCAGG TM1859 QRT R – TTGTGGTTTCCGGGCTCCCA	DNA repair protein
TM0762 ( <i>rpsB</i> )	TM0762 QRT F – TGGAACCCGAAGATGGCTCCCT TM0762 QRT R – TCGCACCTTCGCTGGCTTTCT	30S ribosomal protein S2
TM0877 ( <i>eno</i> )	TM0877 Q F – ACGGTGGACAGCACGCAGAC TM0877 Q R – TCCGCTCCTGCCCTCAAAGC	enolase

**Figure 1. A-D are phase contrast images of cells in media with various concentrations of PIPES buffer. Images are at 1000X magnification.**

**Figure 1.A. Cells grown in medium containing 50mM PIPES had a pH of 5.57 and rod shaped cell population of <1%**

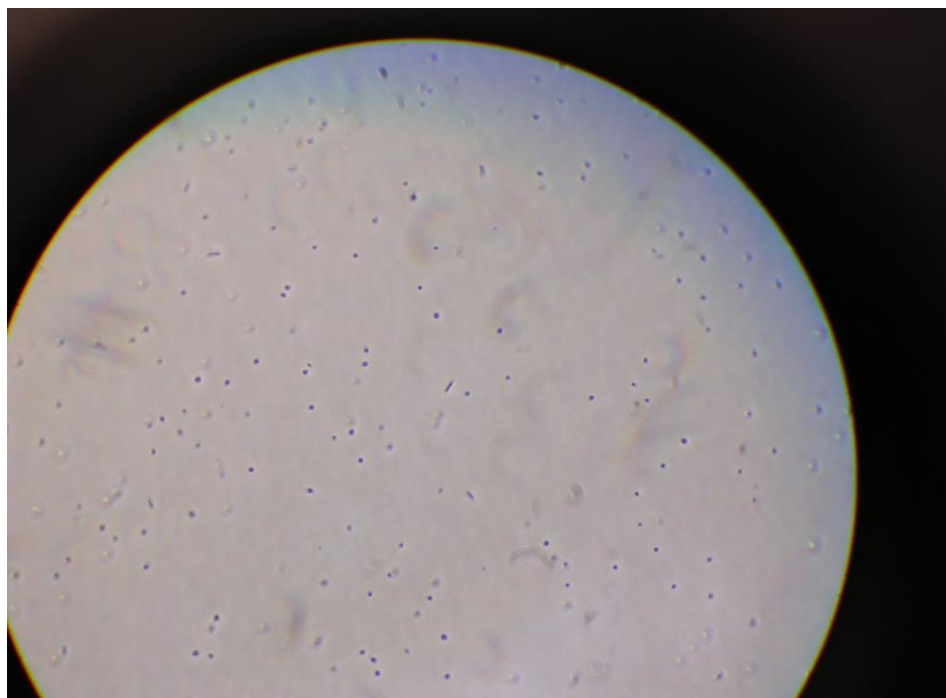


**Figure 1. B. Cells grown in medium containing 100mM PIPES had a pH of 5.91 and rod shaped cell population of ~5%**

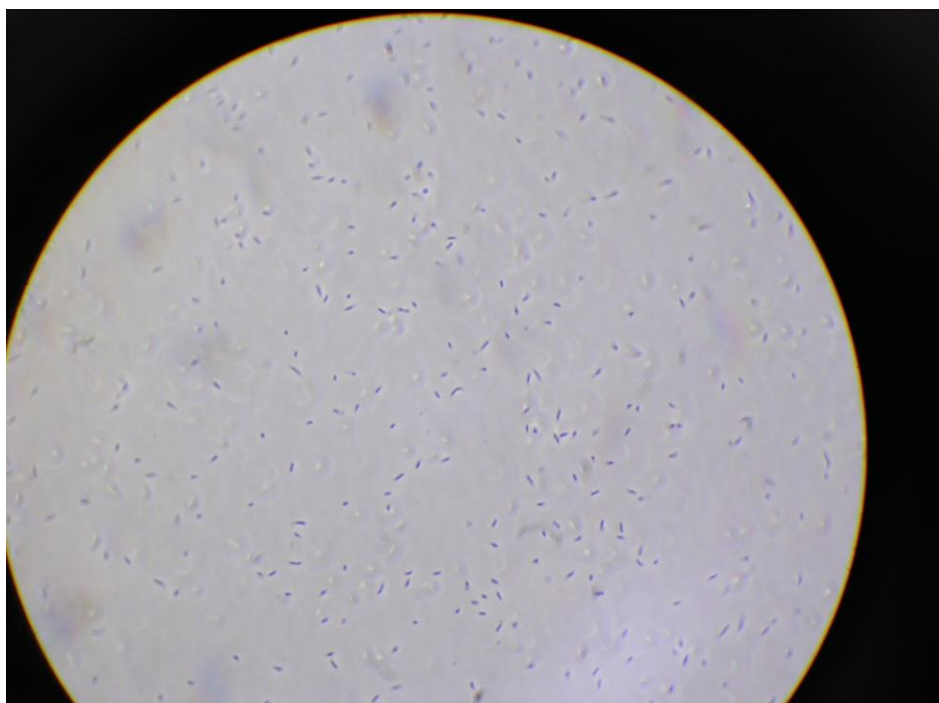




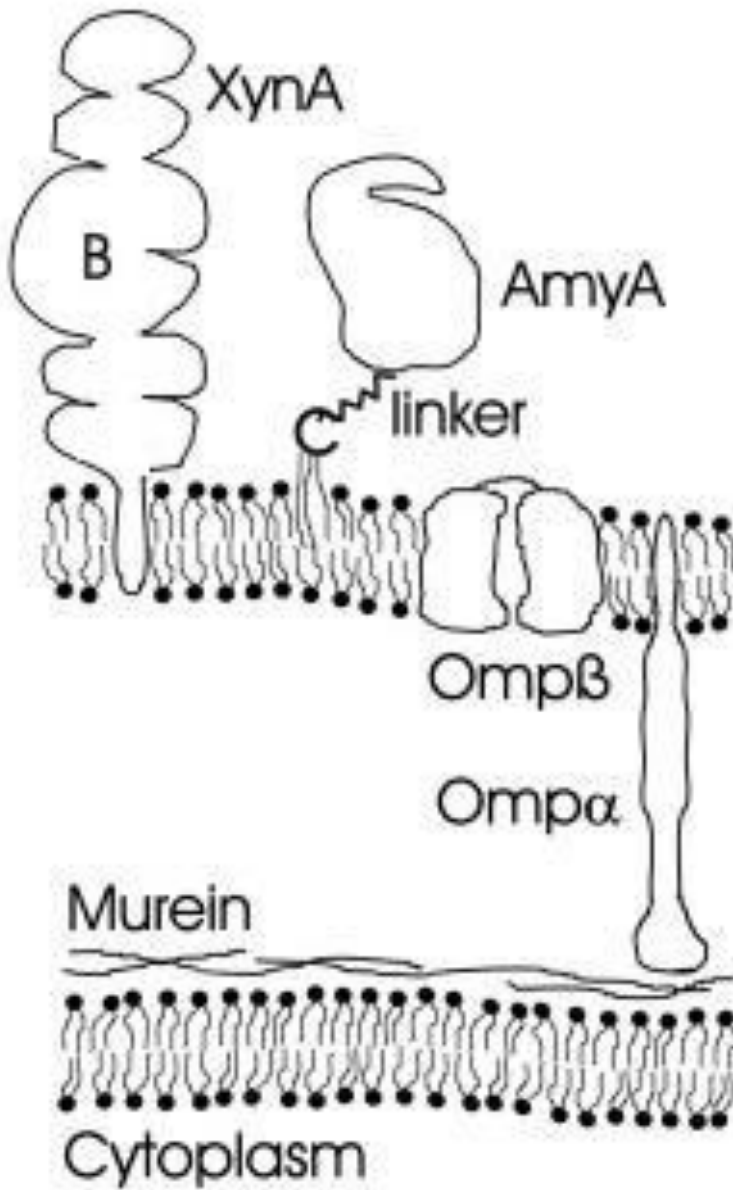
**Figure 1. C. Cells grown in medium containing 200mM PIPES had a pH of 6.31 and rod shaped cell population of ~10%**



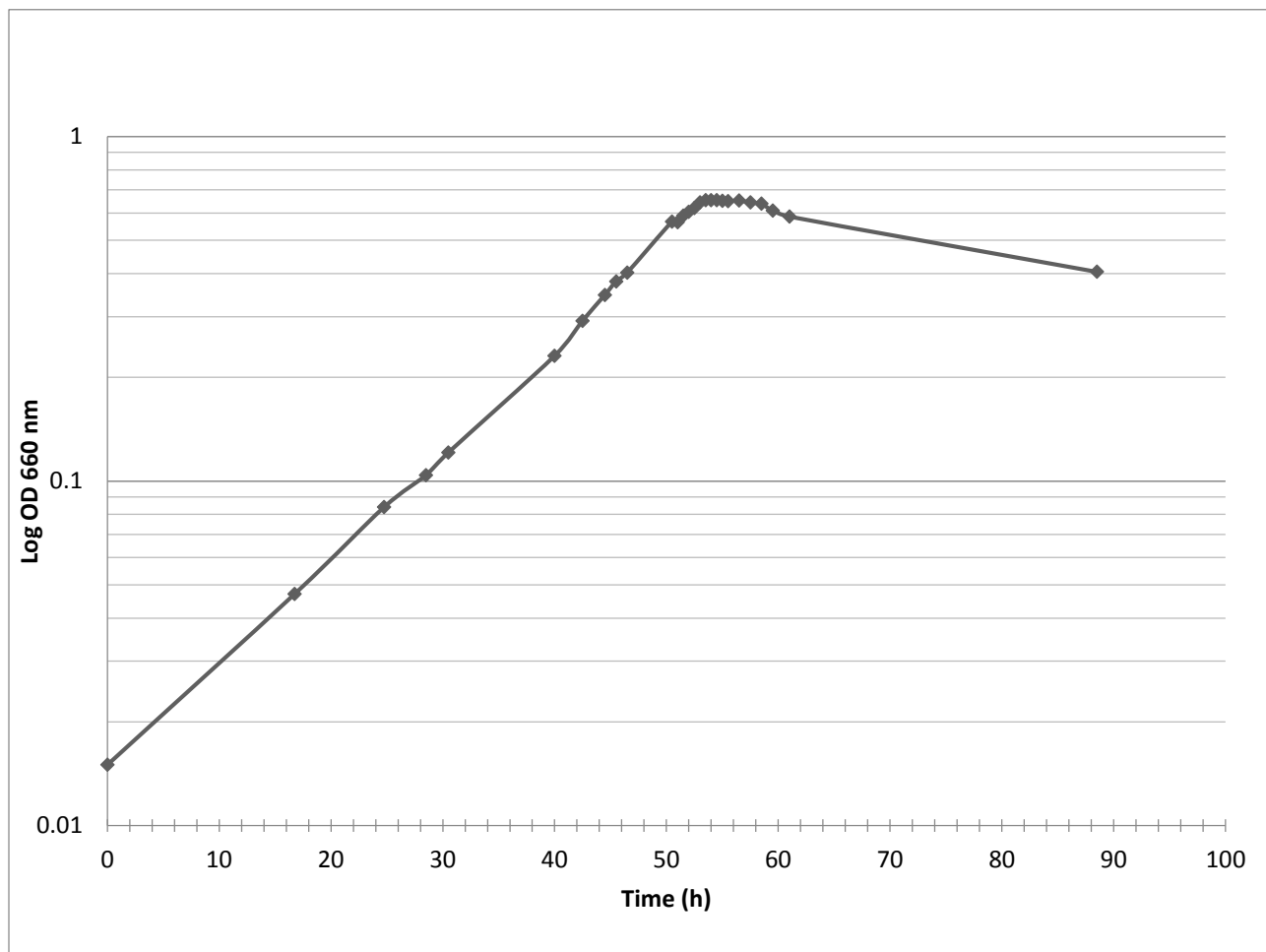
**Figure 1. D. Cells grown in medium containing 300mM PIPES had a pH of 6.54 and rod shaped cell population of ~90%**



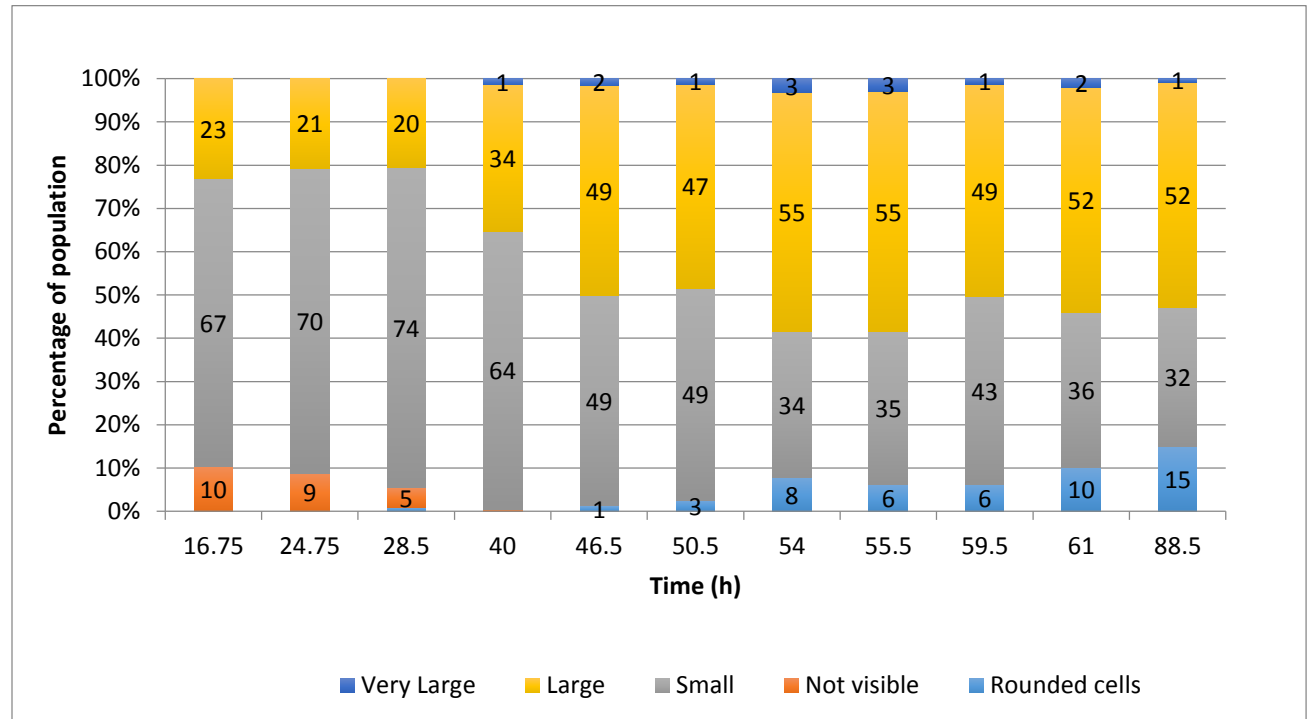
**Figure 2. Schematic of the *T. maritima* cell envelope showing protein attachments to the outer envelope and peptidoglycan (4).**



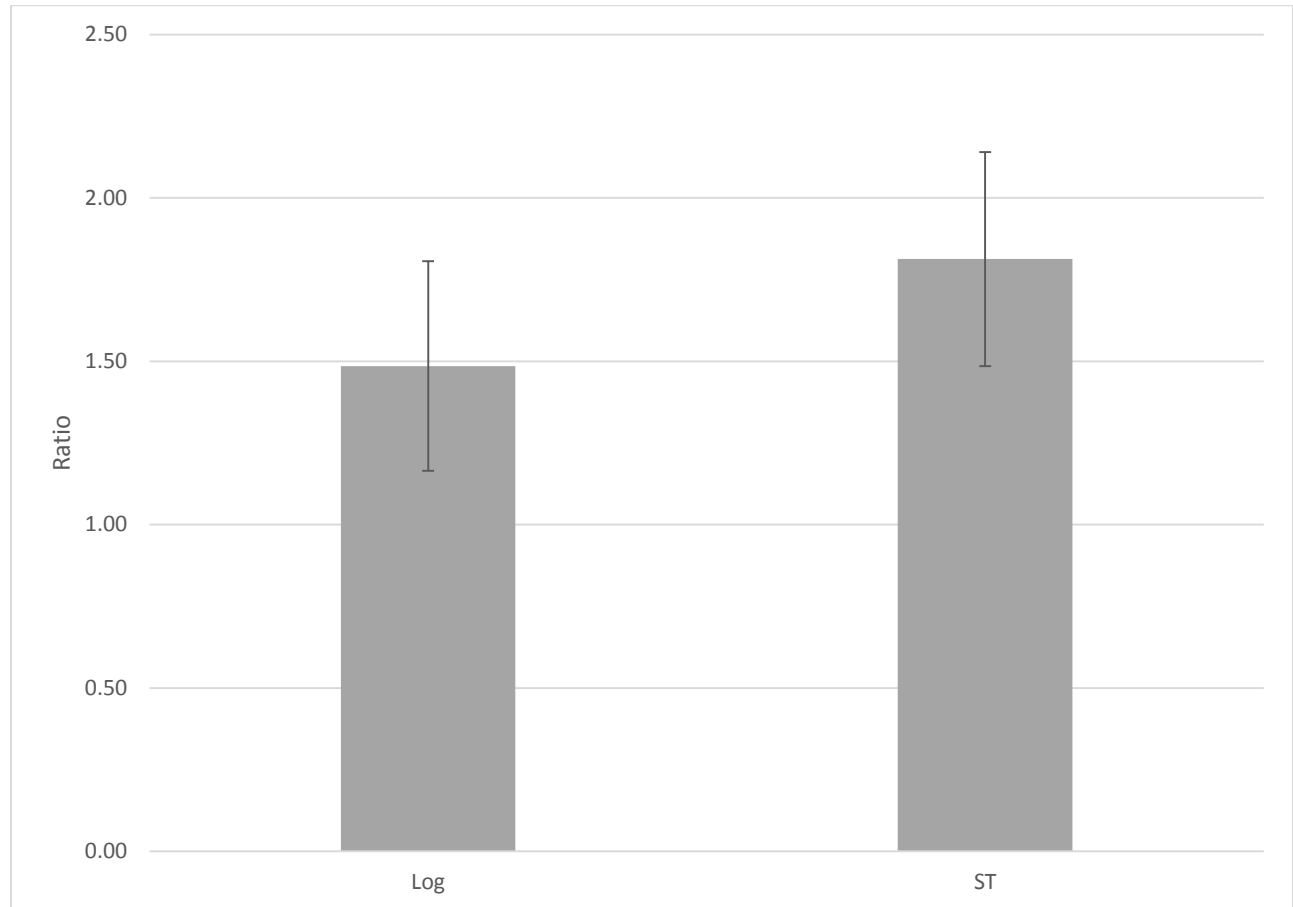
**Figure 3. Growth curve of *T. maritima* cells in anaerobic P300 medium at 80°C. Dots correspond to time points of sampling.**



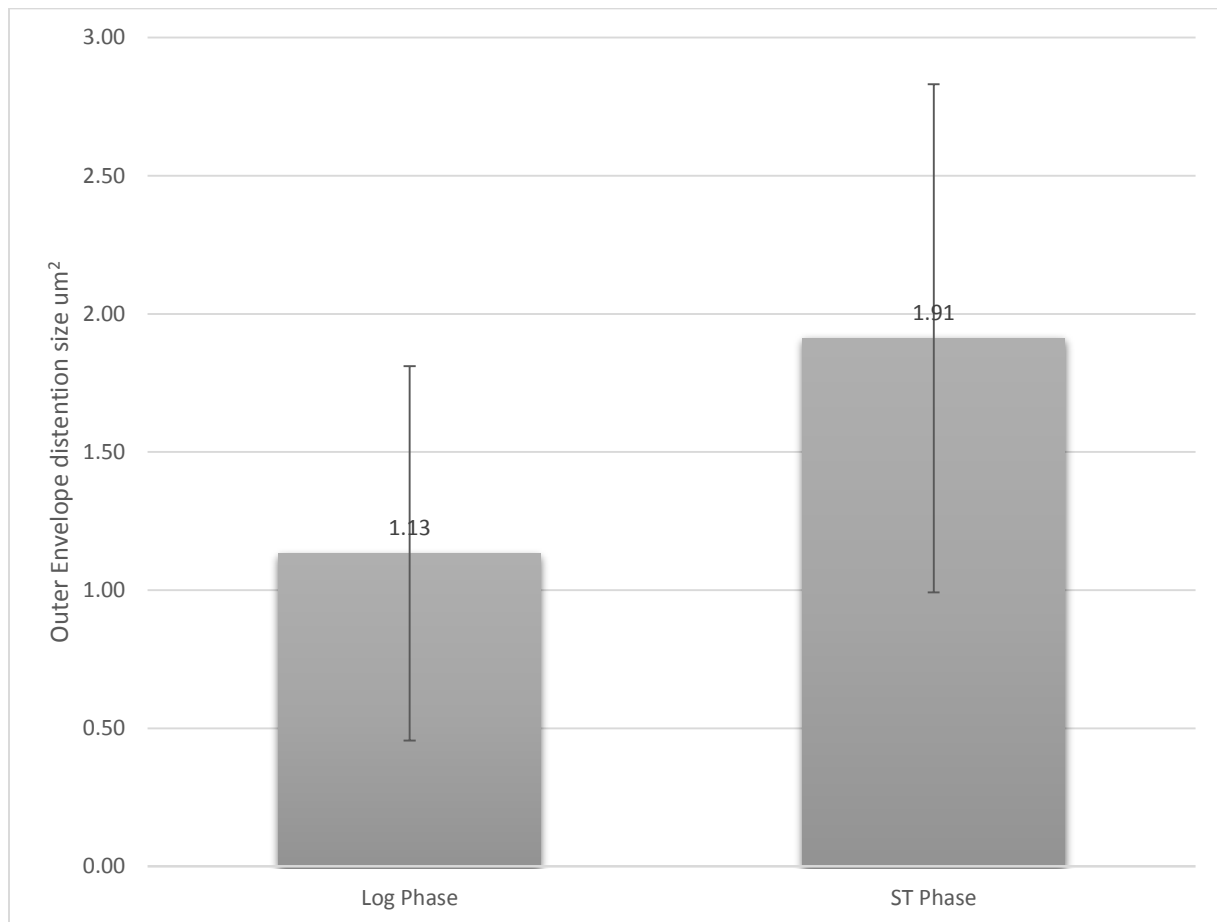
**Figure 4. Qualitative analysis of outer envelope distension size in cell populations over time.**



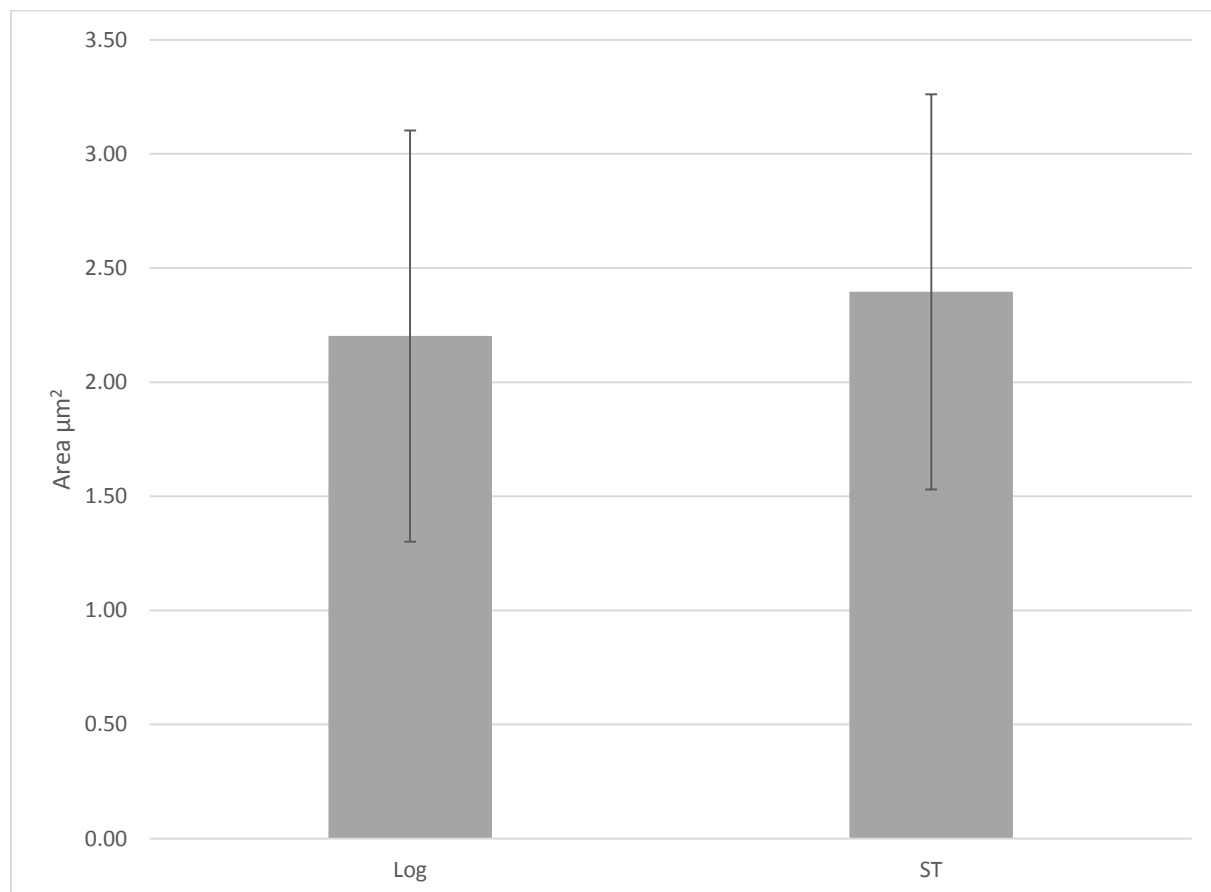
**Figure 5. Ratio of the areas of the whole cells to cytoplasms in mid-log (24.5 hours) and early stationary (ST) (59.5 hours) phases.**



**Figure 6. Average outer envelope sizes in mid-log (24.5 hours) and stationary phase (ST) (59.5 hours) ( $p < 0.001$ ).**

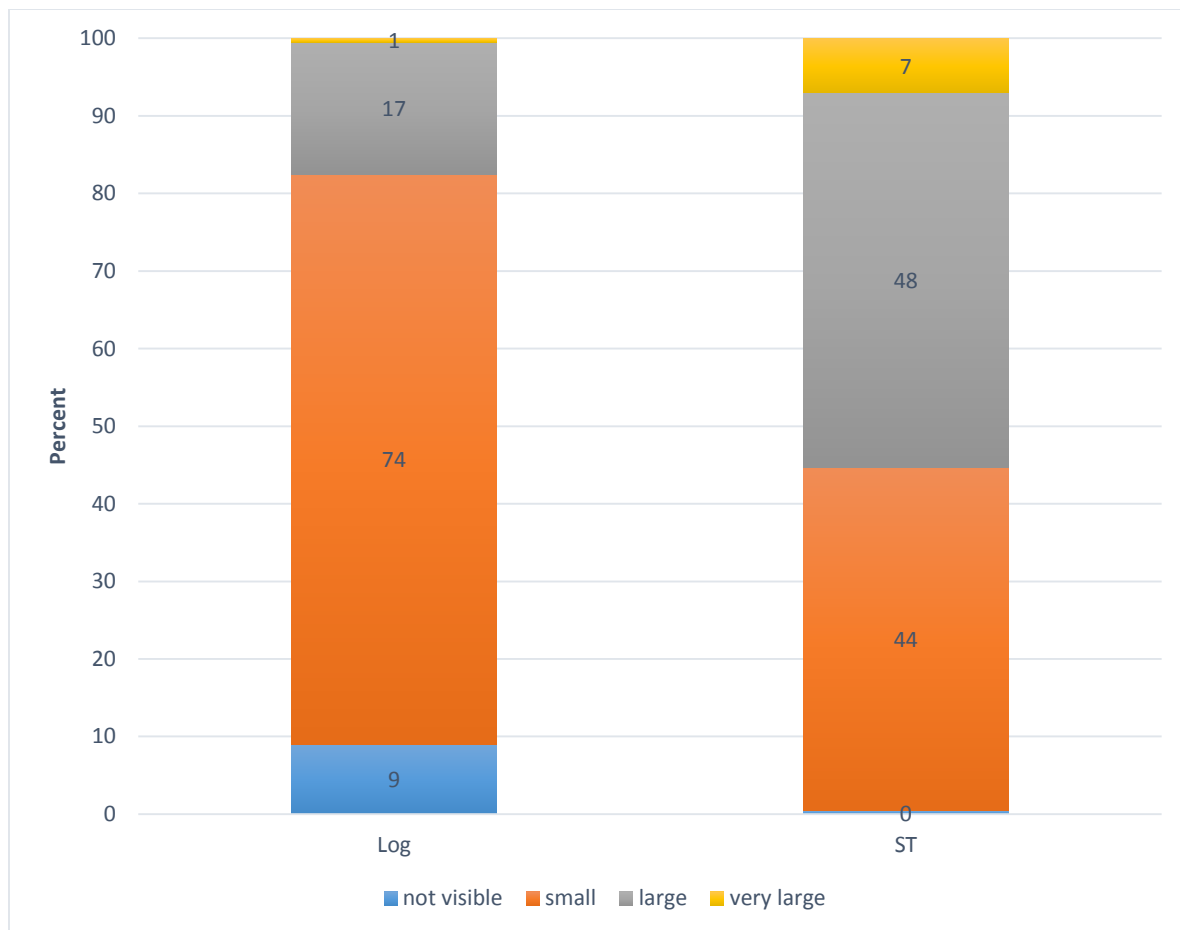


**Figure 7. Cytoplasm area comparison in mid-log (24.75 hours) and early stationary phase (ST) (59.5 hours) cells ( $p>0.001$ ).**



**Figure 8. Distribution of outer envelope sizes in mid-log (24.75 hours) and early stationary phase (ST) (59.5 hours) cells.**

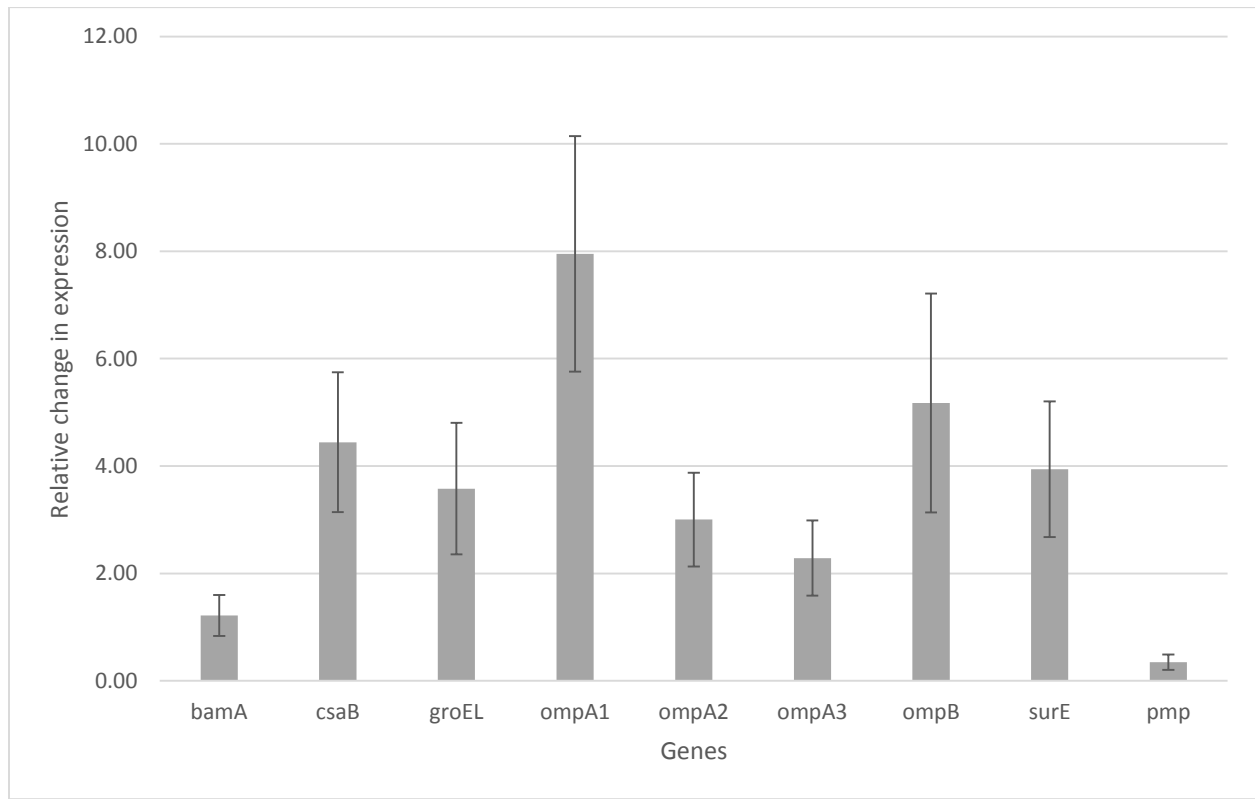
**Distribution of outer envelope sizes in mid-log (24.75 hours) and early stationary phase (ST) (59.5 hours) cells. The cells were separated in categories of 1.5  $\mu\text{m}$  increments. Outer envelopes from 0.31-1.8  $\mu\text{m}$  were categorized as small, 1.81-3.3  $\mu\text{m}$  as large and above 3.4  $\mu\text{m}$  as very large.**





**Figure 9. Change in expression of genes from mid-log to stationary phase.**

**Change in expression of genes from mid-log to stationary phase. Beta barrel assembly machinery A (bamA), Polysaccharide pyruvyl transferase (csaB), Heat shock protein (groEL), Outer membrane protein A1 (ompA1), Outer membrane protein A2 (ompA2), Outer membrane protein A3 (ompA3), Outer membrane protein B (ompB), Stationary phase survival protein (surE), and periplasmic membrane protein (pmp) Error bars are standard error of the mean.**



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