4-2013

Characterization of a DedA FamilyTemperature Sensitive Mutant

Angelica R. Simmons

Characterization of a DedA FamilyTemperature Sensitive Mutant

By Angelica R. Simmons

Undergraduate Honors Thesis under the direction of

Dr. William T. Doerrler

Submitted to the LSU Honors College in partial fulfillment of the

Upper Division Honors Program.

April, 2013

Louisiana State University &

Agricultural and Mechanical College

Baton Rouge, Louisiana

ACKNOWLEDGEMENTS:

I would like to thank God for the incredible opportunities that He has provided me with during my time at Louisiana State University. I would like to thank my Louisiana Science, Technology, Engineering and Mathematics (LA-STEM) Research Scholars program especially Dr. Isiah Warner and Melissa Crawford. Without this scholarship, I would not have had funding to partake in this research. Dr. Doerrler accepted me into his laboratory as a freshman and worked patiently with me. I would like to thank him for taking me into his laboratory, having faith in me throughout this process and always making me as a necessary part of his team. I also owe gratitude to former members of the Doerrler laboratory, Lisa Boughner, Ph.D. and Rakesh Sikdar, Ph.D., for aiding me in various ways. I greatly appreciate all the time and advice given by my thesis committee members, Dr. Brenda Nixon and Dr. Greg Pettis. Lastly, my family and friends have been critical components of my support system. I would like to thank them for listening ears, kind words and sincere prayers.

TABLE OF CONTENTS:

LIST OF FIGURES:

LIST OF TABLES:

ABSTRACT:

The functions of two inner membrane proteins of *E. coli*, YqjA and YghB, which are members of the conserved and essential DedA family, remain unknown. BC202, a strain with deletions of both *yqjA* and *yghB* display altered phenotypes including cell division defects, an altered lipid composition and failure to grow at 42 °C. YqjA and YghB share 61% amino acid identity and are individually nonessential due to redundant functions. In addition, the E. coli genome carries six additional genes (eight total) belonging to the DedA membrane protein family. We have created a mutant with nonpolar deletions of all DedA genes that contains only a plasmid copy of YghB. This strain, ARS100 is indistinguishable from the parent strain as long as inducing agent IPTG is supplied in the growth media. A temperature sensitive variant was isolated that grows normally at 30 °C and at elevated temperatures, 42 °C, displays phenotypes that result from the loss of all DedA family function. Characterizing this mutant will serve a vital role in determining the function of the DedA family. ARS 102 contains five missense mutations in YghB. ARS 102 displays cell division defects even at 30 °C and lyses and dies after a few minutes at 42 °C. ARS 102 exhibits a lower membrane potential at 42ºC. Further studies include evaluating cellular lysis and the regulation of envelope stress response pathways.

INTRODUCTION:

Antibiotics are used to treat several types of bacterial infections. Antibiotic resistance causes the limited effectiveness of these drugs. This resistance is seen heavily in gram-negative bacteria because of the presence of their outer membrane. Many of these bacteria encode for efflux pumps that are capable of keeping drug concentrations within the cell minimal. Because bacteria are so efficient at combating antibiotics, new drugs are constantly in demand. Better understanding the role of the DedA family of proteins could lead to a new antibiotic target.

Escherichia coli is a Gram-negative bacteria that serves as a model organism for many important human pathogens. Its envelope consists of both an inner (cytoplasmic) and an outer membrane. The inner membrane houses a plethora of integral proteins performing an assortment of tasks necessary for development and maintenance of the cell. Its genome encodes for proteins belonging to an ancient and highly conserved integral membrane protein family named DedA. Members of this large protein family are found not only in bacteria but also in archaea and eukaryotes as well. To date there are over 3,000 DedA protein family members documented in the NCBI database (Khafizov et al., 2010) (Doerrler et al., 2013). Several DedA proteins may be encoded within one genome. E. coli contains eight different DedA family genes (Blattner et al., 1997) within its genome including *yqjA, yghB, yabI, dedA, yqaA, ydjx, yohD,* and *ydjZ*.

Although there are a multitude of DedA family proteins, little is understood of the function of these proteins. Within the E. coli DedA family, YqjA and YghB share 61% amino acid identity. A mutant with deletions of *yqjA* and *yghB*, named BC202, displays cell division defects at all temperatures and does not grow at elevated temperatures

(Thompkins et al., 2008). BC202 has an inefficient twin arginine transport (Tat) pathway that results in secretion of periplasmic amidases to be reduced (Sikdar and Doerrler, 2010). Neither YqjA nor YghB are directly involved in the export of these proteins (Lee et al. 2006).

While E. coli contains eight DedA family genes, the Lyme disease pathogen *Borrelia burgdorferi* contains only one (Frazer et al., 1997). The lack of redundancy has been taken advantage of to find that the gene encoding for the DedA homolog (*bb0250*) is essential. Introduction of this gene to BC202 has been shown to rescue the mutant from temperature sensitivity and cell division defects (Liang et al., 2010). The effects of *bbo250* essentiality remain independent of the inefficiency of the Tat pathway being that no Tat components (A/E, B or C) are found in *B. burgdorferi* (Dilks et al., 2003). Also, while ΔTat mutants display the familiar chaining phenotype characteristic of cell division defects, they have not been found to exhibit temperature sensitivity (Stanley et al.,2001 and Ize et al., 2003). These results suggest that DedA proteins carry out functions that are independent of the Tat pathway.

Recently we have shown that BC202 activates multiple envelope stress response pathways including Cpx, Psp, Bae and Rcs. However, activation of these pathways is not the cause of the cell division or growth defects of BC202. The membrane potential is also lowered in BC202 as measured using a fluorescent dye called JC-1. Growth and cell division defects of BC202 can be corrected by growth on media of pH 6.0 or by overexpression of MdfA, a Na^+/ K^+-H^+ antiporter. These results suggest that YqjA and YghB are a novel family of membrane transporters required to maintain the proton motive force (Sikdar et al., 2013).

We have shown that the DedA genes are collectively essential in *E. coli* meaning that the loss of all eight genes results in cell death. Thus, the essentiality of DedA family has been demonstrated in two species, *B. burgdorferi* (Liang et al., 2010) and *E. coli* (Boughner and Doerrler, 2012). The collective essentiality of the DedA family in *E. coli* was demonstrated by isolation of a mutant with nonpolar deletions of all DedA genes. Such a mutant could only be isolated in the presence of a plasmid that expresses one *dedA* gene under control of an inducible promoter. An example of such a mutant is BAL801 which harbors deletions of all DedA family genes and expresses the *dedA* gene from an arabinose inducible promoter. BAL801 slowly dies when grown in the absence of arabinose (Boughner and Doerrler, 2012).

It normally takes 5-6 hours to observe the phenotypic effects of the depletion of the plasmid encoded DedA gene in strain BAL801 described above when the cells are grown in the presence of repressor glucose (Boughner and Doerrler 2012). When cells are grown in glucose, it takes time to dilute the existing proteins present. Such long time periods can complicate analysis of mutant phenotypes. A similar problem is seen in MsbA depletion experiments. MsbA is an essential antigen-binding cassette (ABC) transporter in *E. coli*. In attempts to determine whether MsbA serves a role in lipid trafficking, cells were depleted of the Msb*A* proteins, which took approximately 5 hours to occur (Zhou et al., 1998). The key to determining the role of this protein came after isolation of a temperature sensitive mutant (WD2) with one substitution mutation (Alanine270→Threonine) (Doerrler et al. 2001). WD2 stops growing within one hour following shift to the nonpermissive growth temperature and was found to accumulate lipids in the inner leaflet of the inner membrane (Doerrler et al., 2004). For similar

reasons, it would be highly advantageous to isolate a quickly inactivating, temperature sensitive DedA family mutant.

We have isolated a quickly inactivating temperature sensitive mutant to study the direct effects of the essentiality of the DedA family. We have named this mutant ARS102. These cells overcome possible complications associated with the time it takes to dilute YghB out of cells when grown in the presence of glucose. The cells harbor nonpolar deletions of all eight DedA family genes and only contain YghB harboring several missense mutations that cause cells to undergo growth arrest upon shift to the nonpermissive temperature of 42ºC. In order to introduce mutations into our plasmids, we used a low fidelity DNA polymerase to amplify *yghB*. The cloned mutant YghB genes were introduced into BAL701. BAL701 contains only *yghB* on its chromosome with all other DedA genes deleted (Boughner and Doerrler 2012). BAL701 is the parent strain referred to above. The YghB gene was deleted from the transformant (in the presence of inducing agent arabinose) and the resultant transductants were screened for temperature sensitivity. Plasmids from temperature sensitive mutants were then isolated and sequenced. ARS100 contains a wildtype *yghB* and serves as a control in our experiments, while ARS102 contains *yghB* with five missense mutations (proline49→serine, glutamine89→ leucine, phenylalanine118→leucine, leucine159→phenylalanine, leucine175→phenylalanine). ARS102 grows at 30ºC but not at 42ºC and displays cell division defects at all temperatures. When grown in liquid media, growth arrest occurs after 60 minutes at 42ºC. We also have preliminary data displaying nucleoid alterations in ARS102 as well as a lowered membrane potential, characteristics that are also observed in other DedA mutants (Boughner and Doerrler,

unpublished observations; Sikdar et al., 2013). By having a temperature sensitive *yghB* mutant, we may be able to elucidate the exact role of the DedA protein family.

MATERIALS AND METHODS:

Materials. All materials were purchased from New England BioLabs, Sigma-Aldrich, VWR International, Qiagen or BD.

Mutant Generation. Genemorph random mutagenesis kit containing Mutazyme, a low fidelity DNA polymerase, was purchased from Agilent Technologies and used to amplify *yghB* using primers 5' GCAAGCTTGGGGAAAATCGTCAGGCGTTACAG-3' (HindIII site underlined) and GCTCTAGAAAGGA**G**ATATACATATGGCTGTTATTCAAGATATCATCGCTG-3' (XbaI site underlined) according to manufacturer's instructions. The amplified mutant *yghB* genes were digested with XbaI and HindIII, cloned into pWSK29 and introduced into BAL701. Transformants were transduced with P_1 lysates containing ΔyghB::Kan^R to delete the chromosomal *yghB*. Transductants were screened for temperature sensitivity at 42ºC. Plasmids from temperature sensitive mutants were isolated and sequenced at the LSU Department of Biological Sciences Genomics Facility.

Sequencing. Because of low copy number, PCR product was sequenced instead of sequencing directly from the plasmid. The YghB gene was amplified using high fidelity Phusion polymerase. Wildtype and mutated *yghB* sequences may be found in the appendix.

Growth on Solid Media. Cells were grown in grown in ampicillin (100 µg /mL) and IPTG (0.2 mM) overnight. One hundred milliliters of cell culture was plated onto each ampicillin plate using standard plating protocol. Plates were incubated at 30ºC and

42ºC. If temperature sensitivity was observed, a single colony was streaked onto another plate and allowed to incubate at the previously stated temperatures.

Growth in Liquid Media. Cells of ARS 100 and ARS 102 were grown in ampicillin (100 µg /mL) and IPTG (0.2 mM) overnight. Cells were diluted 1:100 into fresh media containing appropriate supplements and allowed to grow until OD_{600} reached 0.5-1.3. Cells were diluted into pre-warmed (42ºC) based upon starting concentrations and treated media. Cells were grown in a water bath shaking at 225 rpm. OD_{600} readings were taken every ten minutes and recorded until growth arrest occurred in ARS102.

Microscopy. All microscopy was taken using a Leica DM-RXA2 deconvolution microscope. Images were recorded with the 100X lens, 1.3-numerical-aperature oil immersion objective lens using Slidebook software. Cells of specific time points were centrifuged and resuspended to a final concentration of OD_{600} of 1 in LB and 10 µL was placed on a slide of 1% agarose for imaging.

Membrane Potential using Deconvolution Microscopy. Determination of the membrane potential found by altering an existing protocol that uses the JC-1 red/green dye assay (Jovanovic et al.,2006) (Engl et al., 2009). A stock JC-1 (Life Technologies) in 5 mg/mL in dimethyl sulfoxide (DMSO) was prepared and kept at -20ºC until use. 4.5µL was diluted into 16 µL followed by adding 900µL of permealization buffer consisting of 10mM Tris, pH 7.5, 1mM EDTA, and 10mM glucose in a stepwise fashion to create a working solution. Cells were prepared using previously mentioned protocol. Cells were collected and resuspended in 100µL of working solution of JC-1. These cells were then incubated at 30ºC in the dark. 10 µL was placed on a slide of 1% agarose for

imaging. Green fluorescent protein (GFP) and tetramethyl rhodamine isocynate (TRITC) filters were used to record 530nm (green) and 590nm (red) emissions. Slidebook software was used to find ratios of green to red fluorescence from 100 cell units (Sikdar et al., 2013).

Nucleoid Appearance using Deconvolution Microscopy. Overnight cultures were diluted (1:100) into LB with appropriate additives. Cells were grown until reaching an OD_{600} 0.5-1.3 then diluted again according to initial dilution. This dilution of cells was placed into prewarmed supplemented media. At appropriate time points, cells were resuspended into fresh LB to reach an OD_{600} of 2.0. For DAPI (4', 6 diamidino-2-phenylindole; 100µg ml^{-1}) staining, 2µL of DAPI was mixed with 20µL of cells. 10µL of the DAPI/cell mixture was placed on a slide coated with agarose.

RESULTS:

ARS102 contains plasmid encoded *yghB* with five missense mutations resulting in temperature sensitive growth. We used Mutazyme to introduce mutations in *yghB* during PCR followed by transformation of PCR product into BAL701 (Boughner and Doerrler 2012) and transduction of Δ*yghB*::Kan^R to generate ARS100. To generate ARS100, everything was done as previously described for ARS102 excluding the use of Mutazyme. ARS100 has no chromosomal DedA family gene but only a wildtype plasmid copy of *yghB*. ARS100 grows at all temperatures and displays normal cell division. ARS102 contains five missense mutations. These mutations occur at residue locations 49, 89, 118, 159 and 175 (Table 1). The first two mutations, proline to serine and glutamine to leucine, result in amino acid changes from nonpolar to polar and polar to nonpolar, respectively. Following the generation of our mutants, we screened for temperature sensitivity. ARS102 grows at 30ºC but does not grow at 42ºC (Figure 1).

Residue Location	**ARS100 (Wildtype)**	**ARS102 (*yghB* Mutant)**
49	Proline	Serine
89	Glutamine	Leucine
118	Phenylalanine	Leucine
159	Leucine	Phenylalanine
175	Leucine	Phenylalanine

Table 1- ARS102 contains a mutant plasmid copy of *yghB* with five missense mutations at residue locations 49, 89, 118, 159 and 175. ARS 100 contains a *yghB* plasmid copy with no mutations.

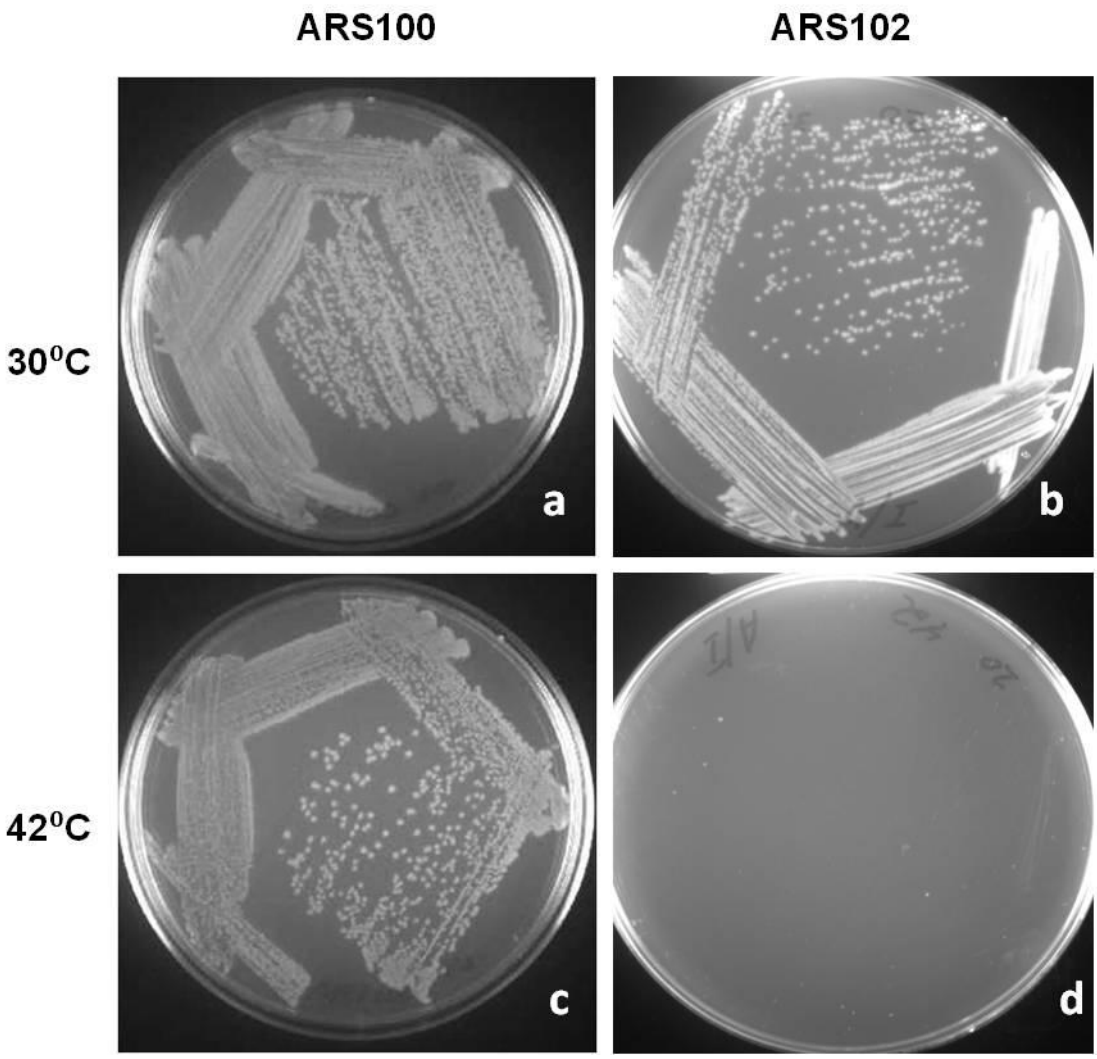

Figure 1- Cells were grown in grown in ampicillin (100 µg /mL) and IPTG (0.1 mM for ARS100 and 0.2 mM for ARS102) and then streaked on properly treated plates. Plated cells were placed at 30ºC (a and b) and at 42ºC (b and d) overnight.

ARS102 displays cell division defects at all temperatures. ARS102 does not properly complete cell division when grown at 30ºC (Figure 2a-2e), or at elevated temperatures (Figure 2f- 2j). The cell division defects appear very similar to those present in BC202 (Thompkins et al., 2008). Comparing the appearance of cells at the first timepoint, which is immediately before cells were transferred to 42ºC, there is a distinct difference of cellular morphology between ARS100 and ARS102. Interestingly, after 45 minutes at 42ºC, a small percentage of ARS100 mutants display a chaining phenotype indicative of cell division defects.

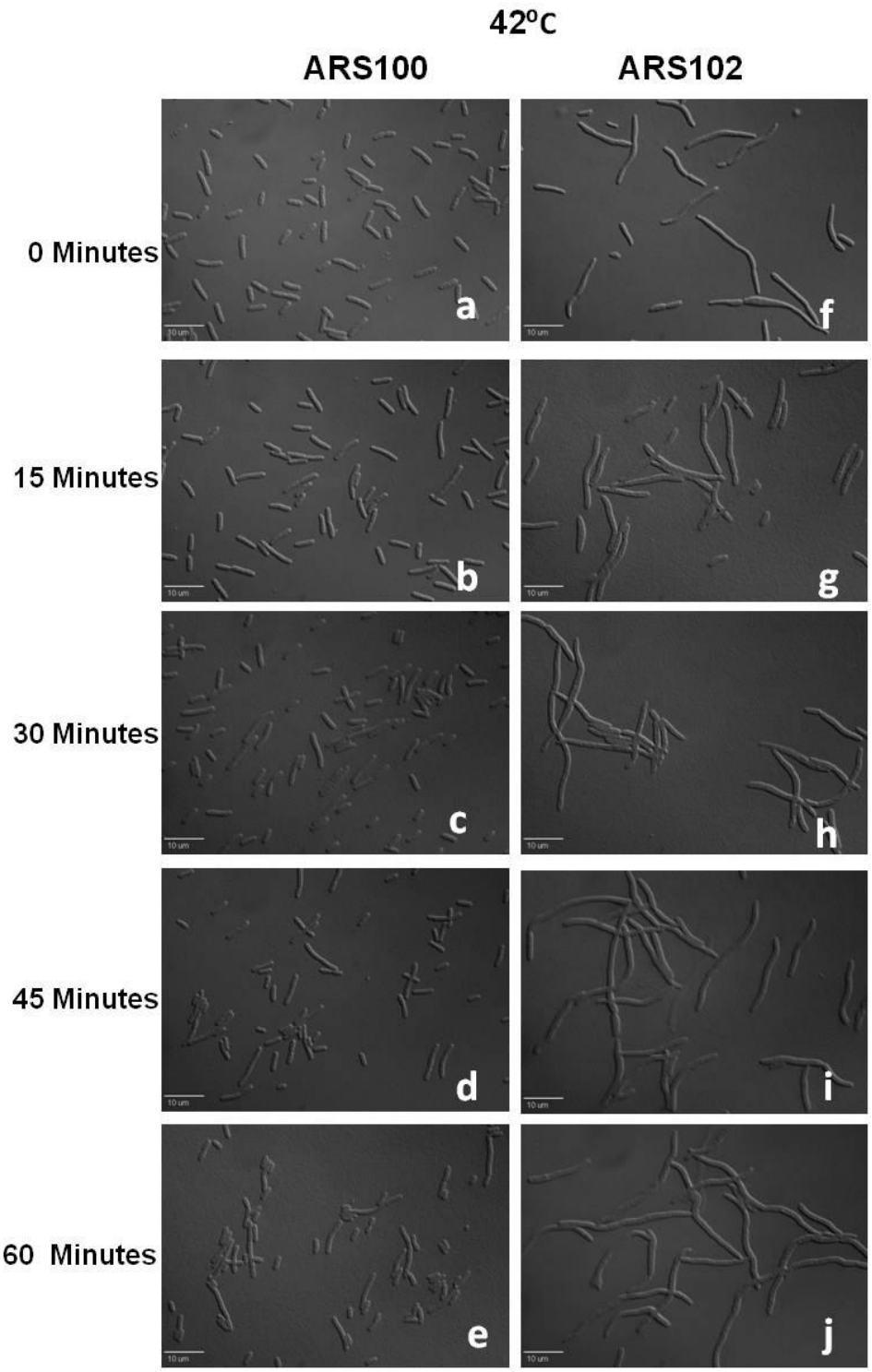

Figure 2- Overnight cultures were diluted 1:100 into fresh media supplemented with ampicillin (100 µg /mL) and IPTG (0.2 mM). Cells were grown in grown at 30ºC until reaching an OD_{600} 0.5-1.3. Cells were diluted based upon starting concentration (usually 1:5) and transferred into pre-warmed (42ºC) supplemented media. Microscopic images were taken for samples at 15 minute intervals of ARS100 (a-e) and ARS102 (f-j) for 1 hour with a Leica RXA2 deconvolution microscope.

ARS102 is a quickly inactivating mutant. After approximately 65 minutes of growth at 42ºC, ARS102 goes into growth arrest (Figure 3). Even before growth arrest occurs in ARS102 it appears to be less healthy than ARS100, taking longer to grow in the same conditions. ARS100 continues to grow exponentially at elevated temperatures.

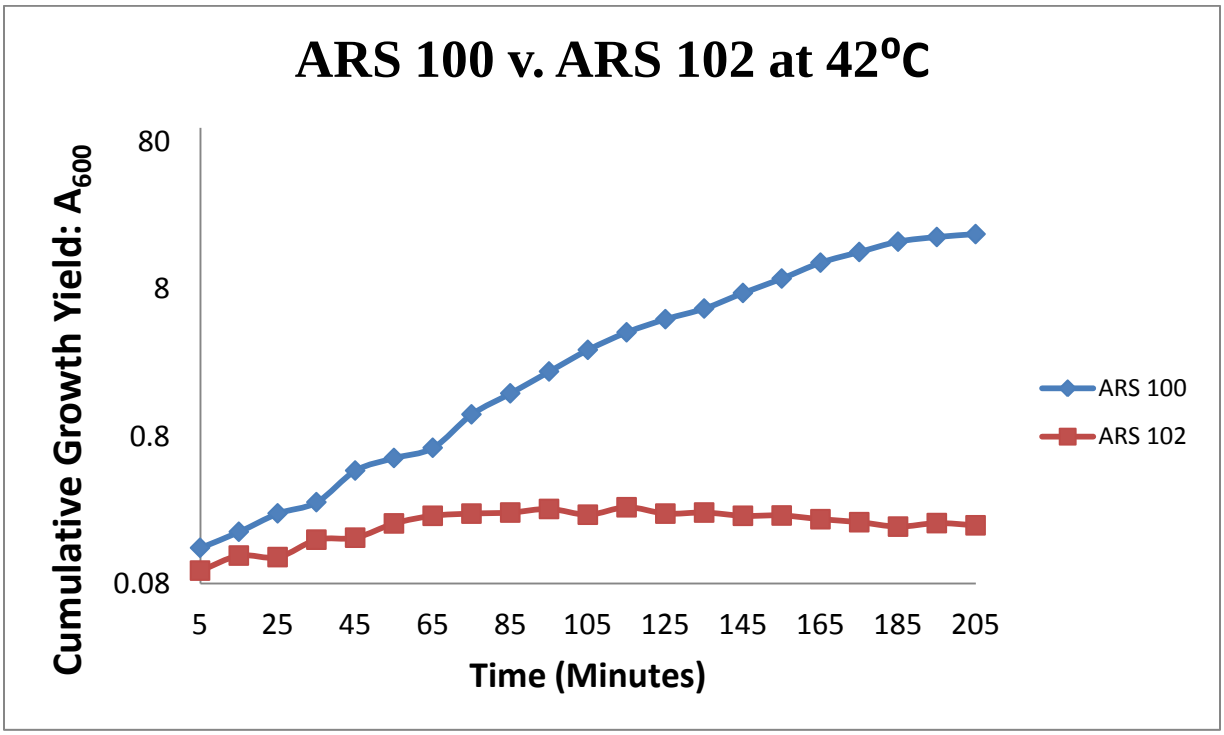

Figure 3- Overnight cultures were diluted 1:100 into fresh media supplemented with ampicillin (100 µg /mL) and IPTG (0.2 mM). Cells were grown in grown at 30ºC until reaching an OD_{600} 0.5-1.3. Cells were diluted based upon starting concentration (usually 1:5) and transferred into pre-warmed (42ºC) supplemented media. Optical density readings were taken at 10 minute intervals until growth arrest was observed in ARS102.

The nucleoid of ARS102 is much less organized than that of ARS100. After 60 minutes of growth at 42ºC, the proper organization of DNA is lacking in ARS102 cells (Figure 4d). Cellular DNA organization of ARS100 appears seems to be efficient for daughter cells to obtain equivalent amounts of DNA (Figure 4b). At this point, cells of ARS102 are not dividing but if they were to, they would have a difficult time processing identical daughter cells.

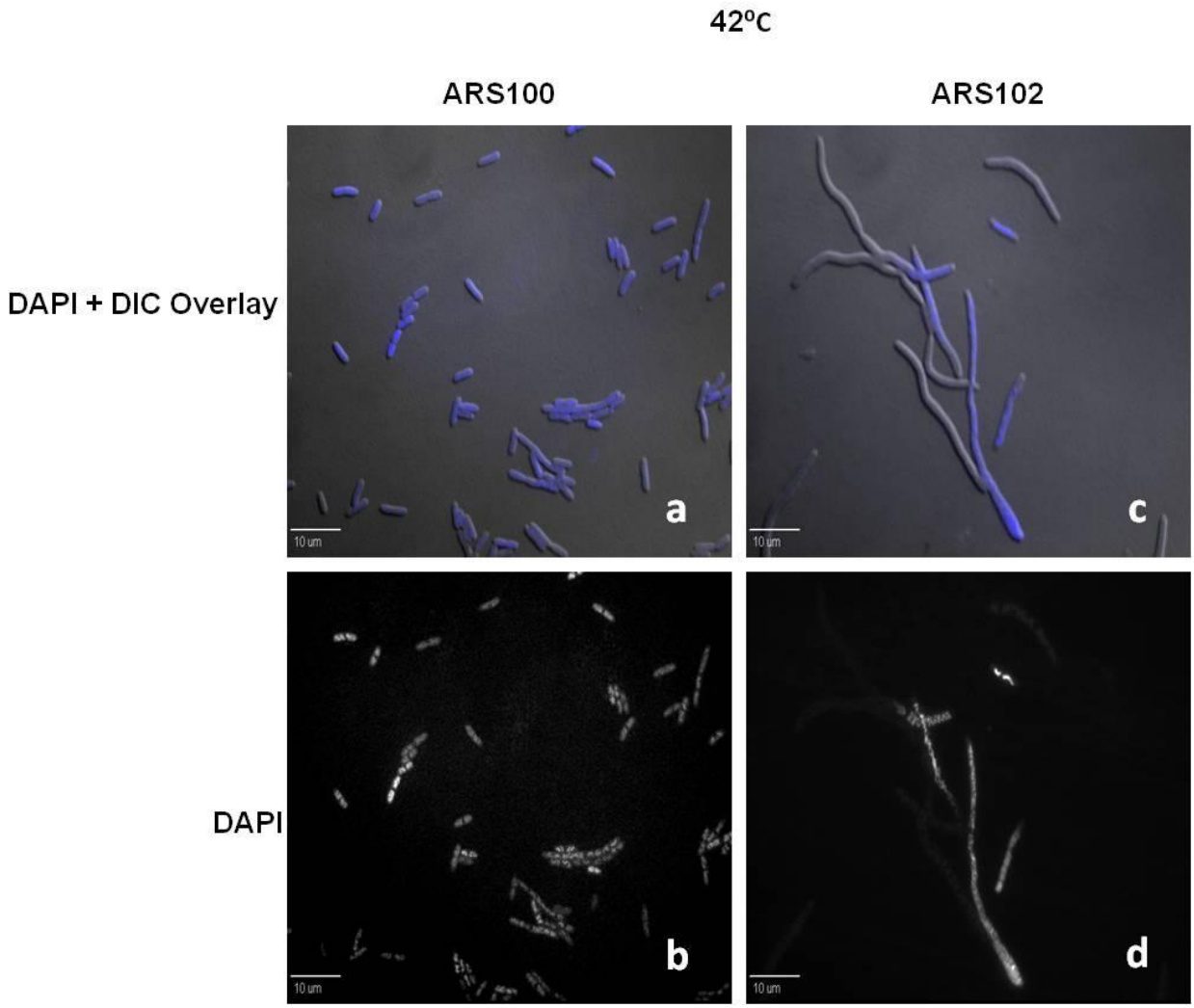

Figure 4- Overnight cultures were diluted 1:100 into fresh media supplemented with ampicillin (100 µg /mL) and IPTG (0.2 mM). Cells were grown in grown at 30ºC until reaching an OD_{600} 0.5-1.3. Cells were diluted based upon starting concentration (usually 1:5) and transferred into pre-warmed (42ºC) supplemented media. Cells were imaged with a Leica RXA2 deconvolution microscope. DAPI staining signifies DNA (a and c). The DAPI with DIC overlay is used to show nucleoid alterations with the outlining cells (b and d).

ARS102 exhibits a lower membrane potential. The membrane potential in bacteria exists as a cell being more negative inside than it is outside. This membrane potential as well as a transmembrane pH difference results in the proton motive force. Since ARS102 has a lower membrane potential (Figure 5), it also has a lowered proton motive force. Many cellular processes are dependent on the proton motive force. Inefficiency of the proton motive force has been shown to induce a variety of envelope stress pathways in other *E. coli* mutants (Sikdar et al., 2013).

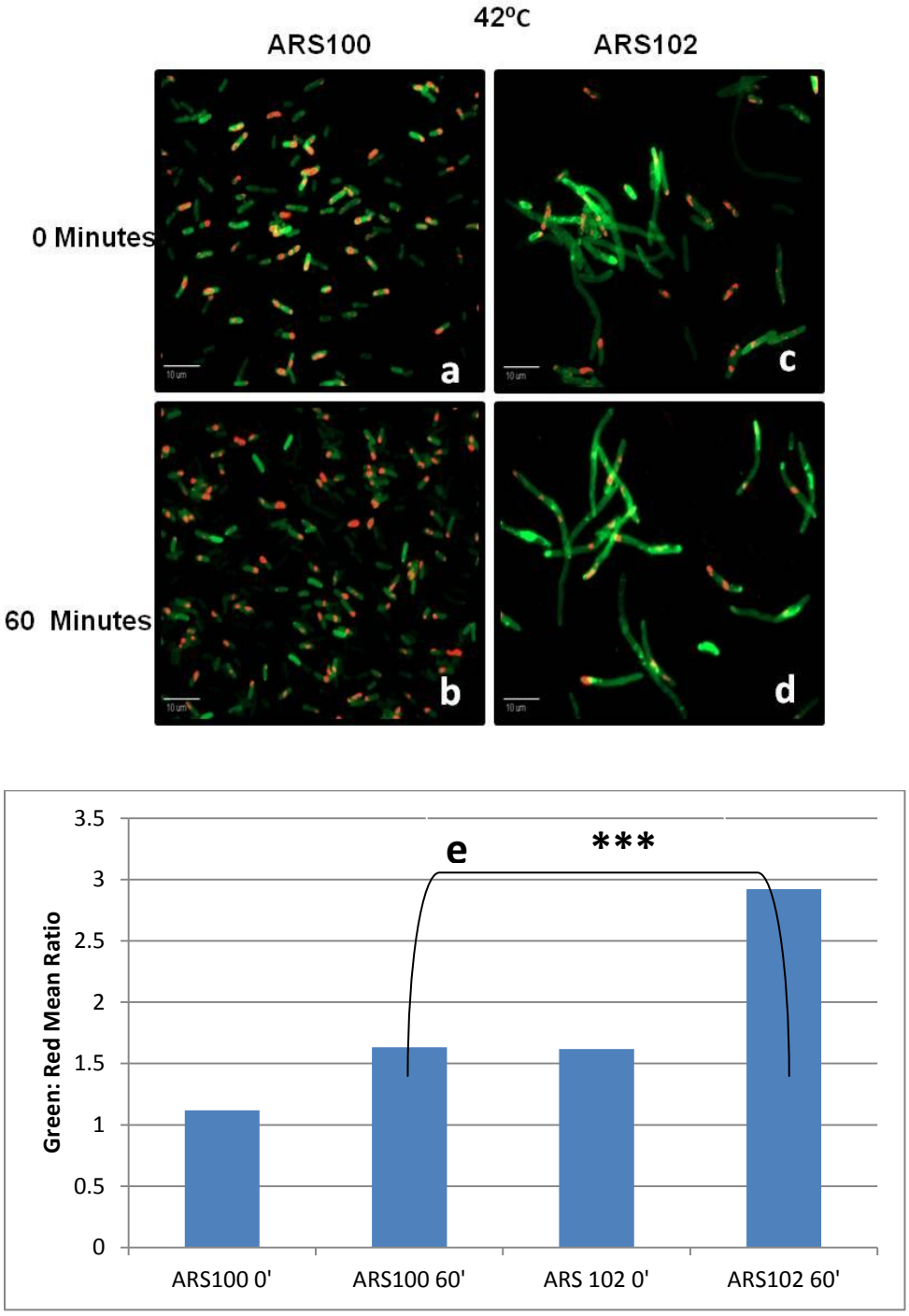

Figure 5- Overnight cultures were diluted 1:100 into fresh media supplemented with ampicillin (100 µg /mL) and IPTG (0.2 mM). Cells were grown in grown at 30ºC until reaching an OD_{600} 0.5-1.3. Cells were diluted based upon starting concentration (usually 1:5) and transferred into pre-warmed (42ºC) supplemented media. Cells were resuspended into a working solution of JC-1 dye assay and incubated in the dark for 30 minutes. Wavelength emissions (530nm and 560nm) were recorded using GFP and TRITC, respectively. Higher proportion of green fluorescence denotes lower membrane potential. Each bar represents the green:red mean ratio. Statistical analysis was found using unpaired t-test, ***,$p<0.0001$.

DISCUSSION:

The DedA protein family in *E. coli* is composed of eight members and the function of all remains unknown. This protein family is highly conserved and is present throughout all domains of life. To aid in elucidating the function of these proteins, we have isolated a quickly inactivating mutant with all chromosomal DedA genes deleted for. Our mutants are supported by a plasmid copy of one of the DedA genes, *yghB.* ARS100 contains wildtype *yghB* on a plasmid, and ARS102 contains a mutant variant of *yghB* on a plasmid. We have found that ARS102 contains five missense mutants in *yghB* (Table 1). These mutations result in the following phenotypes: temperature sensitivity (Figure 1 and 3), cell division defects at all temperatures (Figure 2), nucleoid alterations (Figure 4), and lowered membrane potential (Figure 5).

We wanted to isolate a quickly inactivating mutant to determine first hand effects of the absence of all DedA genes. Mutants containing only one DedA gene on an inducible plasmid have been found to take 5-6 hours to enter growth arrest when grown in the presence of the repressor glucose (Boughner and Doerrler, 2012). With this long frame time, it is possible that indirect phenotypes may occur for reasons other than the deletion of all DedA genes. This same problem has been seen when the function of MsbA. The role of this protein in lipid trafficking was not determined until isolation of a quickly inactivating temperature sensitive mutant (Doerrler et al., 2001).

The structure of a protein is highly important. Without specific shape, a protein may lose proper function. Mutations from polar to nonpolar amino acids or vice versa may result in more harmful effects than mutations that result in polar to polar amino acid changes or nonpolar to nonpolar mutations (Volkenstein, 1965). One mutation seen in

ARS102 results in a proline (nonpolar) to serine (polar) amino acid change. According to the predicted topology of YghB, this mutation occurs in the transmembrane domain. Proline is associated with kinks in α-helices so it is expected that this mutation may result in noticeable phenotypes. Another mutation is a glutamine to leucine, which is a polar to nonpolar amino change that occurs in the cytoplasmic loop. The final three missense mutations result in phenylalanine to leucine and leucine to phenylalanine mutations. Aromatic amino acids have been shown to be important in activation of proteins and contain necessary structural features (Regier et al., 1993).

ARS102 lack proper organization of cellular DNA. Two of the mutations in this strain are in the cytoplasmic loop of the protein. Our lab has found that the cytoplasmic loop of purified DedA (one of the proteins within the DedA family) binds to DNA using a DNA cellulose column (Boughner et al., unpublished). If the cytoplasmic loop is not functioning properly, disorganization of the nucleoid is possible since it is not binding to DNA appropriately. BAL801 displays an increased concentration of DNA when grown in the presence of the repressor glucose but not when grown in the presence of the inducer arabinose (Boughner, unpublished). It is possible that the DedA family plays a role in maintaining proper amounts and organization of genomic material within the cell.

This mutant has a lowered membrane potential that is reflective of that of BC202 (Sikdar et al., 2013). We know that BC202 causes activation of several envelope stress response pathways including Cpx, Psp, Bae and Rcs. It is possible that several envelope stress response pathways may also be activated in this strain as well since pathways have been shown to be induced in the presence of a lowered membrane potential.

After 45 minutes of growth, some of ARS100 cells start to display slight cell division defects. This observation suggests that plasmid support of *yghB* is not as efficient as the chromosomal gene or that *yghB* cannot completely substitute for loss of other DedA family genes. However; the plasmid support of *yghB* allows for viability at elevated temperatures, proper DNA organization, and sustained membrane potential. It appears that the phenotype of cell division defects remain completely distinct from the other phenotypes.

CONCLUSION:

ARS102 is a quickly inactivating temperature sensitive mutant that displays cell division defects at all temperatures. This mutant contains five missense mutations at various locations in the YghB protein. Other phenotypes include a disorganized nucleoid and a lowered membrane potential. We are one step closer to elucidating the function of YghB and one step closer to determining the potentiality of the DedA family as a target for new antibiotics. Further studies include site mutagenesis of one or multiple amino acids at a time to pinpoint cause of phenotypic change. We may also further characterize ARS102 by determining if induction of envelope stress pathways occurs and by repeating microscopy using DAPI and JC-1 dyes.

REFERENCES:

Blattner, F. R., G. Plunkett, 3rd, et al. (1997). "The complete genome sequence of Escherichia coli K-12." Science **277**(5331): 1453-1462.

Boughner, L. A. and W. T. Doerrler (2012). "Multiple deletions reveal the essentiality of the DedA membrane protein family in Escherichia coli." Microbiology **158**(Pt 5): 1162-1171.

Dilks, K., R. W. Rose, et al. (2003). "Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey." J Bacteriol **185**(4): 1478-1483.

Doerrler, W. T., H. S. Gibbons, et al. (2004). "MsbA-dependent translocation of lipids across the inner membrane of Escherichia coli." J Biol Chem **279**(43): 45102-45109.

Doerrler, W. T., M. C. Reedy, et al. (2001). "An Escherichia coli mutant defective in lipid export." J Biol Chem **276**(15): 11461-11464.

Doerrler, W. T., R. Sikdar, et al. (2013). "New functions for the ancient DedA membrane protein family." J Bacteriol **195**(1): 3-11.

Engl, C., G. Jovanovic, et al. (2009). "In vivo localizations of membrane stress controllers PspA and PspG in Escherichia coli." Mol Microbiol **73**(3): 382-396.

Fraser, C. M., S. Casjens, et al. (1997). "Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi." Nature **390**(6660): 580-586.

Ize, B., N. R. Stanley, et al. (2003). "Role of the Escherichia coli Tat pathway in outer membrane integrity." Mol Microbiol **48**(5): 1183-1193.

Jovanovic, G., L. J. Lloyd, et al. (2006). "Induction and function of the phage shock protein extracytoplasmic stress response in Escherichia coli." J Biol Chem **281**(30): 21147-21161.

Khafizov, K., R. Staritzbichler, et al. (2010). "A study of the evolution of inverted-topology repeats from LeuT-fold transporters using AlignMe." Biochemistry **49**(50): 10702-10713.

Lee, P. A., D. Tullman-Ercek, et al. (2006). "The bacterial twin-arginine translocation pathway." Annu Rev Microbiol **60**: 373-395.

Liang, F. T., Q. Xu, et al. (2010). "BB0250 of Borrelia burgdorferi is a conserved and essential inner membrane protein required for cell division." J Bacteriol **192**(23): 6105-6115.

Regier, J. L., F. Shen, et al. (1993). "Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator." Proc Natl Acad Sci U S A **90**(3): 883-887.

Sikdar, R. and W. T. Doerrler (2010). "Inefficient Tat-dependent export of periplasmic amidases in an Escherichia coli strain with mutations in two DedA family genes." J Bacteriol **192**(3): 807-818.

Sikdar, R., A. R. Simmons, et al. (2013). "Multiple envelope stress response pathways are activated in an Escherichia coli strain with mutations in two members of the DedA membrane protein family." J Bacteriol **195**(1): 12-24.

Stanley, N. R., K. Findlay, et al. (2001). "Escherichia coli strains blocked in Tat-dependent protein export exhibit pleiotropic defects in the cell envelope." J Bacteriol **183**(1): 139-144.

Thompkins, K., B. Chattopadhyay, et al. (2008). "Temperature sensitivity and cell division defects in an Escherichia coli strain with mutations in yghB and yqjA, encoding related and conserved inner membrane proteins." J Bacteriol **190**(13): 4489-4500.

Volkenstein, M. V. (1965). "Coding of polar and non-polar amino-acids." Nature **207**(994): 294-295.

Zhou, Z., K. A. White, et al. (1998). "Function of Escherichia coli MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis." J Biol Chem **273**(20): 12466-12475.

APPENDIX:

yghB in ARS100

ATGGCTGTTATTCAAGATATCATCGCTGCGCTCTGGCAACACGACTTTGCCGCGCT
GGCGGATCCTCATATTGTTAGCGTTGTTTACTTTGTCATGTTTGCCACGCTGTTTTT
AGAAAACGGCCTGCTGCCCGCCTCATTTTTGCCAGGCGACAGCTTGTTGATACTG
GCAGGCGCATTGATTGCCCAGGGGGTTATGGATTTTCTGCCTACGATTGCGATTCT
GACCGCCGCAGCAAGTCTGGGCTGCTGGCTAAGTTATATTCAGGGGCGCTGGTTA
GGGAATACCAAAACGGTGAAAGGCTGGCTGGCACAGCTTCCTGCTAAATATCACC
AGCGCGCCACCTGCATGTTTGACCGCCACGGTCTGCTGGCGCTGCTGGCTGGAC
GTTTTCTTGCATTTGTCCGTACGCTGCTGCCAACCATGGCGGGAATTTCCGGTCTG
CCAAACCGCCGCTTCCAGTTTTTCAACTGGTTAAGTGGATTGCTGTGGGTCAGCGT
GGTAACCAGTTTTGGCTATGCCTTAAGTATGATTCCGTTCGTTAAACGCCATGAAG
ATCAGGTAATGACGTTCCTGATGATCCTGCCAATTGCCTTGTTAACCGCTGGCTTG
TTAGGCACGCTGTTTGTGGTGATTAAAAAAAAATACTGTAACGCCTGA

yghB in ARS102 (with mutations)

ATGGCTGTTATTCAAGATATCATCGCTGCGCTCTGGCAACACGACTTTGCCGCGCT
GGCGGATCCTCATATTGTTAGCGTTGTTTACTTTGTCATGTTTGCCACGCTGTTTTT
AGAAAACGGCCTGCTGCCCGCCTCATTTTTGTCAGGCGACAGCTTGTTGATACTGG
CAGGCGCATTGATTGCCCAGGGGGTTATGGATTTTCTGCCTACGATTGCGATTCTG
ACCGCCGCAGCAAGTCTGGGCTGCTGGCTAAGTTATATTCTGGGGCGCTGGTTAG
GGAATACCAAAACGGTGAAAGGCTGGCTGGCACAGCTTCCTGCTAAATATCACCA
GCGCGCCACCTGCATGTTAGACCGCCACGGTCTGCTGGCGCTGCTGGCTGGACG
TTTTCTTGCATTTGTCCGTACGCTGCTGCCAACCATGGCGGGAATTTCCGGTCTGC
CAAACCGCCGCTTCCAGTTTTTCAACTGGTTTAGTGGATTGCTGTGGGTCAGCGTG
GTAACCAGTTTTGGCTATGCCTTTAGTATGATTCCGTTCGTTAAACGCCATGAAGAT
CAGGTAATGACGTTCCTGATGATCCTGCCAATTGCCTTGTTAACCGCTGGCTTGTT
AGGCACGCTGTTTGTGGTGATTAAAAAAAAATACTGTAACGCCGGA

Primers used for sequencing of mutated *yghB*

FP_pWSK29	CCAGTCACGACGTTGTAAAAC
RP_pWSK29	GTTGTGTGGAATTGTGAGCG