

Comparative Assessment of Growth and Gene Regulation between 4 Serotypes of *Streptococcus pneumoniae* in Broth and Cell Culture

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How to cite this paper: Seabolt, B., Brady, J. and Speshock, J. (2018) Comparative Assessment of Growth and Gene Regulation between 4 Serotypes of *Streptococcus pneumoniae* in Broth and Cell Culture. *Advances in Microbiology*, 8, 763-777. <https://doi.org/10.4236/aim.2018.810050>

Received: May 7, 2018

Accepted: October 5, 2018

Published: October 8, 2018

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Abstract

Streptococcus pneumoniae is a medically important pathogen capable of causing human infections of pneumonia, bacteremia, otitis media, and meningitis. Although there are vaccinations available, infections with *S. pneumoniae* still remains a global problem. *S. pneumoniae* is a highly adaptable bacterial species with numerous serotypes based on capsular polysaccharides. The different serotypes vary in their ability to colonize and causing pathology. Here we compared the regulation of five different virulence factors from four common serotypes of *S. pneumoniae* that vary in their carriage, morbidity, and mortality rates in the human population using two different *in vitro* methods, broth and cell culture. We determine that there is variation of virulence factor gene regulation within a serotype using two different culture methods, and variation between the serotypes in the same culture condition. The regulation of genes appeared to have a correlation with the ability of the various serotypes to grow in broth culture, adhere to cultured lung cells, and invade the cultured lung cells, as serotypes that shared similar regulation of virulence factors tended to behave similarly in culture. Many studies with *S. pneumoniae* rely on the use of one selected serotype, but since there is a wide variation in the growth and regulatory mechanisms of these bacteria. As demonstrated here, future studies should utilize more strains in models before concluding mechanisms of pathobiology.

Keywords

Pneumococcus, Serotypes, Gene Expression, Regulation

1. Introduction

Streptococcus pneumoniae is a transient, opportunistic pathogen found in the upper respiratory tracts of children and adults. *S. pneumoniae* can result in serious pathology, but is rare in healthy individuals and tends to cause morbidity and mortality in mainly those who are under two years of age, over 60 years of age, or are otherwise immunocompromised [1] [2]. However, there are still nearly 1 million cases of severe pneumococcal disease annually in the United States [3] even with available pneumococcal vaccinations [4]. The global mortality resulting from this bacterial species is substantially higher, especially in those countries with poor access to the pneumococcal vaccines [5].

There are more than 90 serotypes of *S. pneumoniae* classified by the structure of their polysaccharide capsule [6]. Disease severity and carriage rate are highly impacted by serotype, and several serotypes have been more highly implicated in causing majority of the severe pneumococcal diseases such as meningitis and bacteremia [7]. While it is known that host immune responses play a significant role in the severity of pneumococcal disease, it is also hypothesized that the expression of virulence genes in *S. pneumoniae* will contribute to colonization and pathology. A gene expression study was performed to assess the variation in expression between four different *S. pneumoniae* serotypes using the first component of the capsule operon (*cpsA*), and the virulence factors choline-binding proteins (*cbpA*), autolysin (*lytA*), pneumolysin (*ply*), and hemolysin (*spxB*). The pneumococcal capsule is made up of a repeating polysaccharide sequence unique to each serotype, yet the arrangement of genes within the capsule loci, designated *cpsA-D*, is conserved [8]. CbpA, is a highly-conserved adhesin that allows *S. pneumoniae* to translocate across nasopharyngeal epithelial cells and invade the circulatory system [9]. The pneumococcal autolysin (*lytA*) hydrolyzes the peptidoglycan cell wall to allow for the release of the pneumolysin [10]. The pneumolysin is a pore-forming toxin expressed by the *ply* gene in virtually all pneumococcal serotypes. At high concentrations, pneumolysin creates holes in cholesterol-containing cell membranes; at lower, sub-lytic concentrations it has a host of pro-inflammatory effects which aid in bacterial dissemination [11] [12]. SpxB is involved in the reduction of O₂ to H₂O₂ [13], and decreased activity of *spxB* leads to increased capsule production [14]. *S. pneumoniae* uses pyruvate oxidase as a way to measure the oxygen level of the immediate environment and regulates the level of capsule production based on the oxygen levels [14].

This study compared the growth and gene expression between two invasive serotypes (3 and 9 V) to two less pathogenic serotypes (5 and 19 F) *in vitro* to compare the expression of the virulence factors described above. A broth culture was used to induce expression, as well as a human lung epithelial cell line (A549) to examine differences in gene expression impacted by the presence of eukaryotic host cells. The differential gene regulation was then compared to the growth phenotypes of the strains in broth culture as well as the ability to adhere or invade A549 cells.

2. Materials and Methods

2.1. Bacterial Strains

Strains 6303 (serotype 3), 6305 (serotype 5), 700671 (serotype 9 v), and 49619 (serotype 19 F) were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). The cultures were grown by inoculating Trypticase Soy Broth (TSB; Becton Dickinson, Franklin Lakes, NJ) and incubating at 37°C overnight. The cultures were frozen in a 25% glycerol solution at –80°C until needed.

2.2. Growth Curves in Broth

Each serotype (3, 5, 9 v, and 19 F) was grown by adding 200 microliters (µL) of overnight culture to 10 milliliters (mL) of trypticase soy broth (TSB) and incubated at 37°C for 8 hours to compare growth rates of the different serotypes. At every hour the optical density (OD) was measured via spectrophotometry using a Beckman DU530 spectrophotometer (Beckman Coulter, Brea, CA) for each replicate (n = 4) at a wavelength of 600 nm.

2.3. RNA Isolation from Broth Cultures

Ten mL of TSB was inoculated with 10 µL of culture for each of the four serotypes and was incubated at 37°C overnight. The following morning 10 mL of fresh TSB for each serotype was inoculated with 200 µL and was grown at 37°C to mid-logarithmic phase (OD = 0.250). When log phase was reached, the incubation was stopped and the cells were centrifuged at 4000× g for 5 minutes to obtain a cell pellet (Eppendorf 5810R, Hamburg, Germany). The supernatant was discarded and the cells were re-suspended in 350 µL tri-reagent (Sigma Aldrich, St. Louis, MO). The samples were vortexed for approximately two minutes, and then allowed to sit at room temperature for five minutes. Then the samples were vortexed for two additional minutes which was followed by a one minute centrifugation at 11,500× g (Eppendorf 5810R, Hamburg, Germany). The supernatant was transferred to a new tube and 350 µL of 100% ethanol was added. The contents were transferred to an RNA mini spin column (Enzymax LLC, Lexington, KY) and centrifuged for one minute at 1000× g. The resulting flow-through was discarded and 700 µL of high stringency wash buffer (2 M GTC; 1% v/v SDS; 55% ethanol) was added to the column. The sample was again centrifuged for one minute at 1000× g and the flow-through was discarded. Next, 700 µL of low stringency wash buffer (80% ethanol) was added, followed by one minute of centrifuging at 1000× g. The flow-through was discarded, and the spin column was dried by centrifuging for one minute at 1000× g. Finally, 50 µL of RNAase-free water was added to the column and was spun for thirty seconds at 1000× g. The resulting flow-through, containing the RNA, was then stored at –80°C.

2.4. Cell Culture

A549 human lung epithelial cells were obtained from the ATCC (CRM-CCL-185,

Manassas, VA). The cells were cultured in RPMI-1640 media (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin and incubated at 37°C in 5% CO₂. To create a monolayer on the bottom of a six-well plate for the adherence and invasion assays, the cells were washed two times with phosphate-buffered saline (PBS) and detached from the flasks using 0.25% trypsin-EDTA (VWR, Radnor, PA). The suspended cells were seeded into a six-well plate at a density of 1 × 10⁶ cells/well with 3 mL of the supplemented RPMI per well and were incubated for 24 hours at 37°C in 5% CO₂.

2.5. Adhesion and Invasion Assay

A culture of each serotype isolated at mid-logarithmic phase was added to 20 mL PBS supplemented with magnesium and calcium (PBS+/+), and 3 mL of these inocula were added to 6 wells for each serotype (multiplicity of infection (MOI) = 10). The plates were incubated for one hour at 37°C and 5% CO₂ and then the bacterial suspensions were removed, followed by two washes with PBS. Gentamicin Sulfate (Teknova, Hollister, CA) was added to three of the six wells in 100 µg/mL concentration. Because gentamicin is unable to diffuse across or be transported across the eukaryotic plasma membrane, it killed only extracellular bacteria, leaving those which had successfully entered the epithelial cells alive. Three wells per serotype examined the ability of the bacteria to invade the cells, while the remaining three wells examined adherence + invasion. After allowing the plates to incubate at room temperature for approximately ten minutes, the gentamicin was removed, and the plates were washed twice with PBS. Sterile water was added to all six wells to lyse the epithelial cells and release all intracellular bacteria. The bacteria were plated in triplicate on trypticase soy agar (TSA) with 5% sheep blood (BD, Franklin Lakes, NJ) and left to incubate overnight at 37°C and 5% CO₂. The number of colony-forming units (CFUs) was counted the following day.

2.6. RNA Isolation from Cell Culture

For cell culture RNA extraction, following the one hour incubation with the bacterial cultures as stated above, the cells were washed two times with PBS, but instead of the addition of gentamicin, 350 µL of tri-reagent was added. Following the addition of the tri-reagent to the cell wells, the RNA was isolated from the cells as described above for the broth assay.

2.7. Quantitative Reverse Transcriptase PCR (qRT-PCR)

The mRNA obtained from the broth cultures and the cell cultures were quantified using the Qubit HS RNA kit and Qubit fluorometer (Invitrogen, Carlsbad, CA). 500 ng of RNA was used as a template for synthesis of cDNA using the qScriptTM cDNA SuperMix reagents and protocol (Quanta Biosciences, Beverly, MA). Real-time polymerase chain reaction (RT-PCR) reactions were set-up using the PerfeCTa qPCR reagent according to the manufacturer's protocol

(Quanta Biosciences, Beverly, MA) using the primers listed in **Table 1**, and were cycled and analyzed using a Roche Light Cycler 480 (Basel, Switzerland). An initial 10 minute incubation at 50°C allowed for the conversion of the RNA to complementary DNA using reverse transcriptase, which was then followed by the following cycles for DNA amplification: 1 minute enature at 95°C; 45 cycles of 10 seconds at 95°C, 30 seconds at 60°C with a quantification fluorescence read, and 30 seconds at 72°C; followed by a melt curve of 10 seconds at 95°C, 30 seconds at 40°C with continuous fluorescence readings to 95°C; and a final cool down to 40°C. qRT-PCR was used to determine the level of mRNA present for 6 genes: *cpsA*, *cbpA*, *ply*, *lytA*, and *spbx*, and the 16S *rRNA* gene, which served as an internal control. The data from the serotype 19 F was used for normalization purposes to establish a comparative fold change between serotypes using the delta-delta Ct methodsince this serotype was the slowest growing in culture, low in binding efficiency to the cultured lung cells, and had lowest gene expression in most assessments [15]. Melt analysis of the qRT-PCR amplicons was used to confirm PCR specificity.

2.8. Statistical Analysis

Graphing and statistical analysis was performed using GraphPad Prism 7 (version 7.02). To assess statistical significance between different serotypes a Kruskal-Wallis one-way ANOVA was used. In cases in which there was significant difference, a post-hoc analysis was performed using the Tukey Honestly Significant Difference method.

3. Results

3.1. Comparison of Bacterial Growth

Serotypes 5, 9 v, and 19 F behave similarly in trypticase soy broth, and gradually increase over an 8 hour period (**Figure 1**). Serotype 3, however, has an earlier and more abrupt exponential phase and enters the stationary phase by 5 hours (**Figure 1**). Serotype 3 reached the mid-logarithmic phase point used for RNA isolation ($OD_{600} = 0.250$) by 3 hours, whereas the other 3 serotypes took a little over 5 hours to reach this point.

Table 1. Primer sequences for qRT-PCR. The primers were created by using PubMed database along with Primer3Plus software.

Gene	Forward Primer	Reverse Primer
<i>CpsA</i>	ACGCAACTGACGAGTGTGAC	GATCGCGACACCGAACTAAT
<i>CbpA</i>	CTTTCACATCGGACTCAGCA	CTTCCAAGCTCATCCCTGAA
<i>SpxB</i>	ATTCGGCGGCTCAATCGGGG	CAGCACGGCAGGCTTCGTCA
<i>Ply</i>	GCTTATGGGCGCCAAGTCTA	CAAAGCTTCAAAAGCAGCCTC
<i>LytA</i>	ACGCAATCTAGCAGATGAAGCA	TCGTGCGTTTTAATCCAGCT
16S <i>rRNA</i>	CTGCGTTGTATTAGCTAGTTGGTG	TCCGTCCATTGCCGAAGATTC

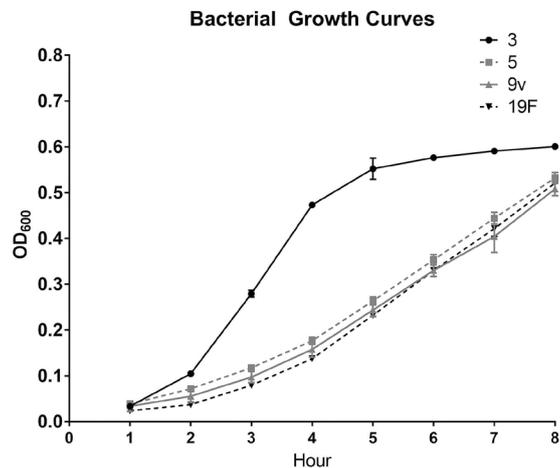


Figure 1. Bacterial growth curves in TSB. Ten milliliters (mL) of trypticase soy broth (TSB) was inoculated with 200 microliters (μL) of overnight culture ($n = 4$) for each serotype. The bacteria were grown at 37°C and every hour for 8 hours 1 mL of culture was read by spectrophotometry to determine the absorbance at an optical density of 600 nm.

3.2. Comparison of Gene Expression in Broth Culture

19 F was selected as a base-line for the gene expression data since the replicates were very close in this serotype, and it had lower gene expression for nearly every gene tested. The 9 v expression of the autolysin gene (*lytA*) was very close in expression to the 19 F, but an increase in expression was observed with the serotypes 3 and 5, with a 5.5 and 7.8-fold increase, respectively (**Figure 2(a)**). Only the serotype 5 increase was significant due to the variation observed in the serotype 3 expression (**Figure 2(a)**). An increase in pneumolysin gene (*ply*) gene expression was observed in all of the serotypes over 19 F, with a 9-fold, 3.6-fold, and 2.6-fold increase in serotypes 3, 5, and 9 v, respectively (**Figure 2(b)**), but only the serotype 3 increase was statistically significant. Choline binding protein gene (*cbpA*) expression was very similar in levels between most samples, but a few outliers in the 9 v serotype did result in a significant difference in expression between this serotype and serotype 3 (**Figure 2(c)**). The genes for hydrogen peroxide production (*spxB*) and capsule production (*cpsA*) had very little variation between samples (**Figure 2(d)** and **Figure 2(e)**).

3.3. Adherence and Invasion of A549 Cells

Gentamicin sulfate does not cross eukaryotic cell membranes, and thus only kills extracellular bacteria. The addition of gentamicin allows for the determination of the ability of pneumococcal cells to invade A549 lung epithelial cells. Without gentamicin, the cell counts will include both adherent and internalized cells. Approximately twice as much bacteria were present in the gentamicin negative culture for serotype 9 v, suggesting that about half (~46%) of the bacteria successfully invaded the epithelial cells (**Figure 3**). 19 F also had a little over half of the culture residing inside of the cells (58%) (**Figure 3**). Although 9 v colonized

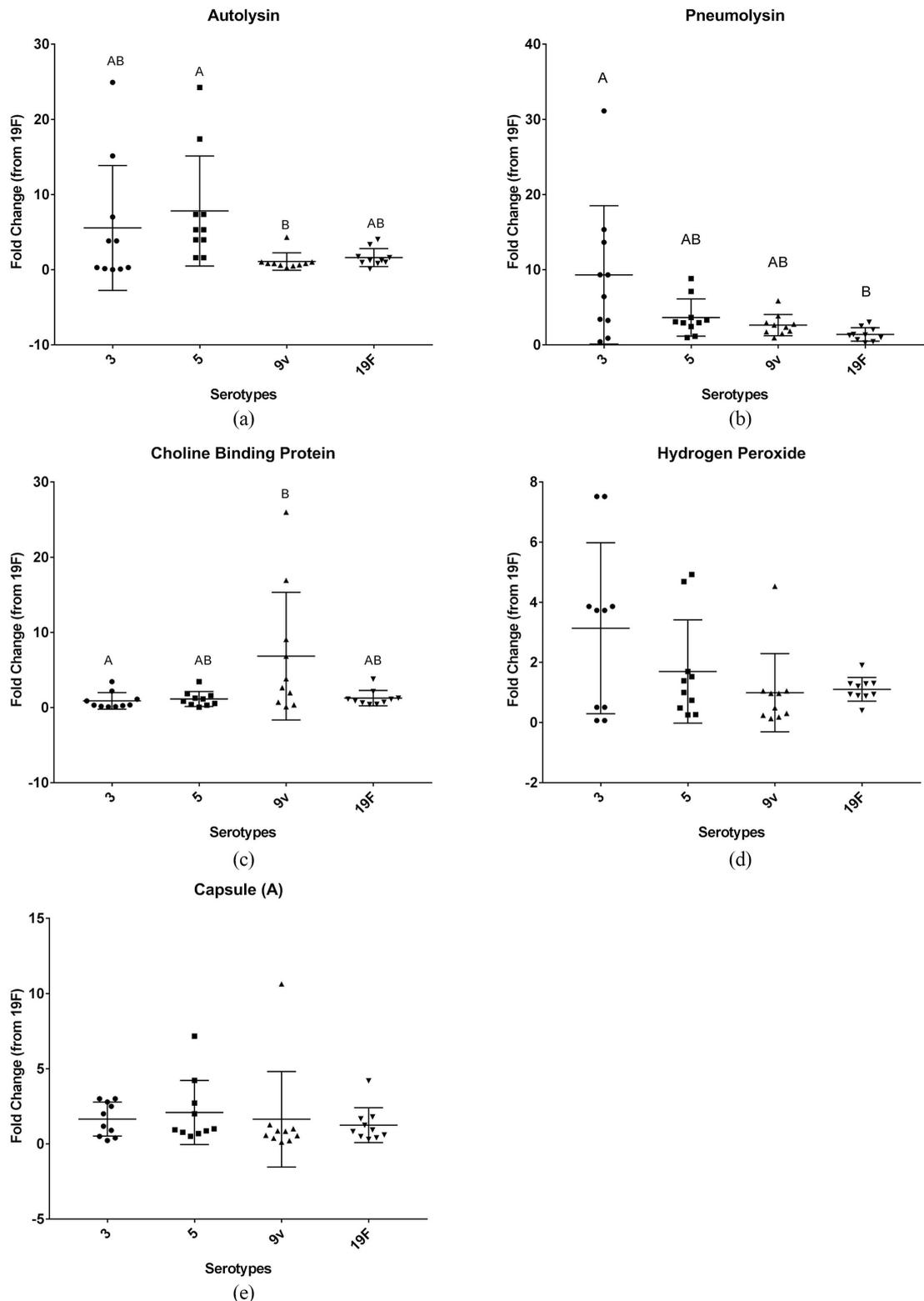


Figure 2. Bacterial gene expression following growth in TSB. The four serotypes of *S. pneumoniae* were grown to mid-logarithmic phase in TSB, and following RNA isolation, gene expression was assessed and compared for the autolysin (a), pneumolysin (b), choline binding protein (c), hydrogen peroxide production (d), and the first gene of the capsule operon (E) (n = 10). Statistical differences are denoted with differing letters when $p < 0.05$ as determined via ANOVA.

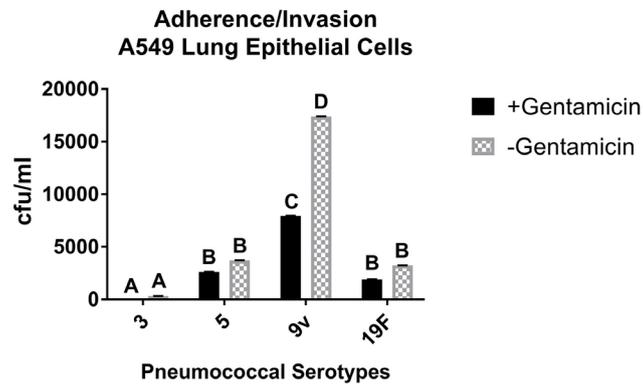


Figure 3. Pneumococcal adherence and invasion in lung epithelial cells. The four *S. pneumoniae* serotypes were grown to mid-logarithmic phase and added to A549 lung epithelial cells at an MOI of 10. Following a 1 hour incubation, gentamicin was added to half of the wells ($n = 4$) to kill extracellular bacteria. The wells with and without gentamicin addition were compared among the four serotypes. Different letters indicate a significant change with $p < 0.05$ as determined via ANOVA.

the epithelial cells slightly better, serotype 5 showed a slightly better ability to invade (~70%) (Figure 3). Serotype 3 colonized the lung cells very poorly and was not capable of cell invasion (Figure 3).

3.4. Gene Expression in *S. pneumoniae*-Infected A549 Cells

Although a little more variation was observed in the expression by serotype 3, there was no significant change in expression of the autolysin between the four serotypes (Figure 4(a)). Expression of the pneumolysin was significantly increased in the serotypes 3 and 5 over 19 F when cultured with A549 cells, but expression was not significantly higher than the serotype 9 v (Figure 4(b)). Serotypes 3 and 5 also showed similar expression of the choline binding protein A, and both had significantly greater expression than 9 v and 19 F (Figure 4(c)). There was no significant change in hydrogen peroxide gene expression (Figure 4(d)), and the capsule expression was only significantly upregulated in the serotype 5 bacteria when exposed to A549 cells (Figure 4(e)).

3.5. Comparison of Gene Expression between Broth and Cells

When using the broth critical point values for determining the fold change of cell culture gene expression, it was concluded for the most part the pneumococci gene expression in cell culture is lower than what it is in broth culture (Figure 5). Expression of the autolysin, pneumolysin, and choline binding protein A genes were all downregulated in cell culture when compared to broth culture by all four serotypes of *Streptococcus pneumoniae* used in this study (Figures 5(a)-(c)). The hydrogen peroxide gene expression was downregulated in cell culture for all serotypes except for 5, which had a lot of variation within the cell culture data (Figure 5(d)), and expression of the first gene of the capsule operon was downregulated in serotypes 5 and 19 F (Figure 5(e)).

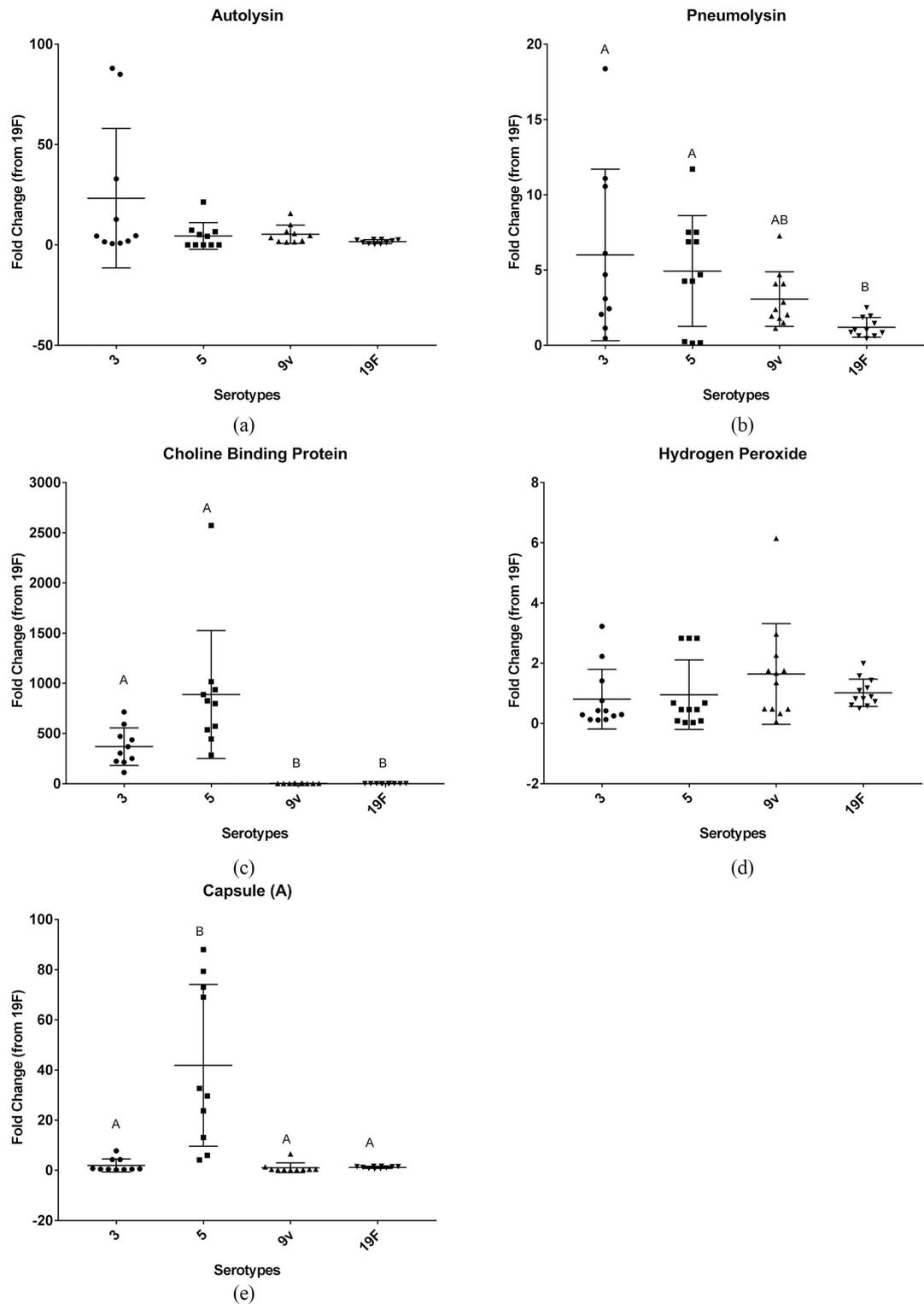


Figure 4. Bacterial gene expression following 1 hour exposure to lung epithelial cells. The four serotypes of *S. pneumoniae* were grown to mid-logarithmic phase in TSB, and added to A549 cells for 1 hour at an MOI of 10. Following incubation, RNA was isolated and gene expression was assessed and compared for the autolysin (a), pneumolysin (b), choline binding protein (c), hydrogen peroxide production (d), and the first gene of the capsule operon (e) ($n = 10$). Statistical differences are denoted with differing letters when $p < 0.05$ as determined via ANOVA.

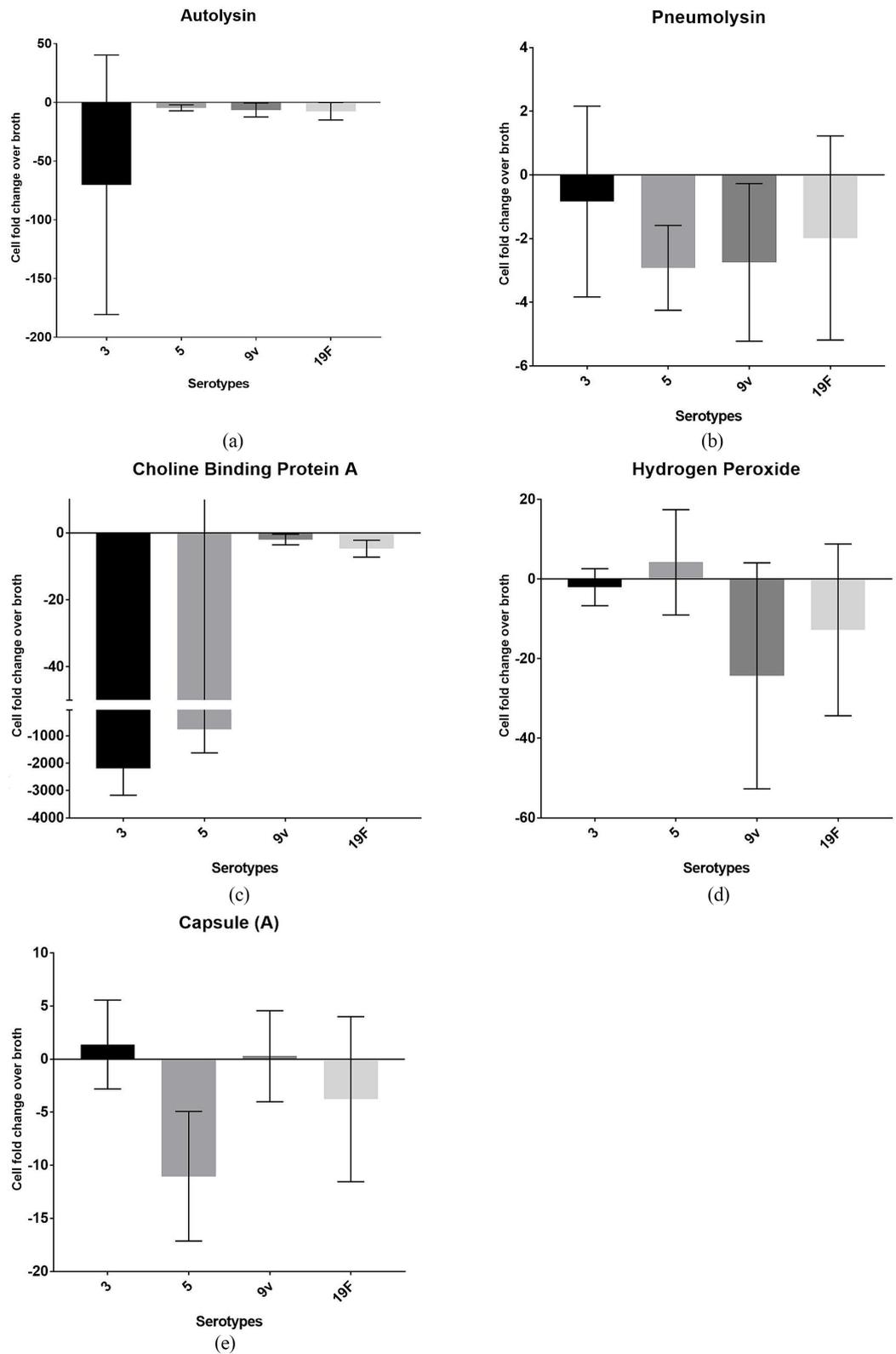


Figure 5. Gene expression comparison of cell culture versus broth culture. Using the delta-delta Ct method and the broth critical threshold (Ct) values as a control, the data was re-analyzed to show a comparison of gene expression between the normalized Ct values obtained from *S. pneumoniae* isolates exposed to cell cultures versus those only grown in broth culture.

4. Discussion

Serotypes 5, 9 v, and 19 F grew at a similar rate in broth culture (**Figure 1**), and with the exception of an upregulated autolysin expression in serotype 5, their gene expression was very similar too (**Figure 2**). Serotype 3 had a more rapid entrance into exponential phase (**Figure 1**), and demonstrated slightly upregulated autolysin and pneumolysin expression compared to the other serotypes (**Figure 2**), but for the most part, any of these serotypes could serve as a representative serotype for pneumococcal gene expression in broth culture.

In the lung epithelial cultures using A549 cells, again serotypes 5, 9 v, and 19 F behaved quite similarly in both adherence and invasion of A549 cells (**Figure 3**), while serotype 3 did not adhere as well or invade at all (**Figure 3**). Since serotype 3 produced far more choline binding protein than serotypes 9 v and 19 F (**Figure 4(c)**), it was surprising to see that it did not colonize as well to lung epithelial cells since A549 cells have choline in their phospholipid bilayer [16], especially since it is known to cause invasive infections in humans [17]. Therefore it is hypothesized that the capsule may have impacted the ability of these four serotypes to colonize lung epithelial cells, and not the binding proteins. Although three of the four serotypes produced similar levels of *cpsA* mRNA, with only serotype 5 being upregulated in the lung cell culture (**Figure 4(e)**), the composition of the capsule likely impacted colonization and invasion potential, and the regulation of the capsule may be different *in vivo* than in transformed cell lines.

There are 90 characterized serotypes of pneumococcal capsule, and several ungroupable structures, most of which are anionic, and thus repel from cells [18]. The serotypes vary in their ability to cause disease. Serotype 3 is frequently associated with invasive pneumococcal disease (IPD) in the elderly, and has a high risk of mortality [18]. It however has been shown to have a poor carriage rate in humans [19]. The metabolic cost of producing the very thick capsule associated with serotype 3 pneumococci has been suggested to impact its presence in the normal microflora [19]. Serotype 5 has a low carriage rate, but has a high association with IPD, although not as fatal [19]. Serotype 9 v has a high carriage, but low mortality rate, while 19 F has a high carriage and high mortality rate, especially in children [19].

There was a lot of variation in the autolysin and pneumolysin expression from the serotype 3 pneumococci when exposed to A549 cells, which could reflect those cell cultures that had higher colonization rates (**Figure 4(a)** and **Figure 4(b)**). Serotype 3 had higher average expression of these two virulence factors, although only the pneumolysin was significant due to high levels of variation (**Figure 4(a)** and **Figure 4(b)**). Serotype 5 had significantly increased expression of pneumolysin over 19 F, while 9 v had slightly elevated expression (**Figure 4(b)**), which is surprising since 9 v has a lower mortality rate than 19 F [20]. The expression levels for the gene that regulates hydrogen peroxide production had no variation among the strains in cells or broth (**Figure 2(d)** and **Figure 4(d)**) suggesting that this virulence factor is produced by a variety of strains, or is only

essential *in vivo*.

Since the pathobiology of *S. pneumoniae* differs greatly between various serotypes, there have been many attempts to better understand differential gene regulation between the various serogroups [21] [22] [23]. However, a majority of these attempts utilize bacteria grown in broth culture, and typically only one serotype [21] [22] [23]. A lot of the research performed still relies on Avery's D39 serotype 2 strain [22] [23] because of its ease of transformation and virulence in mice [24]. However, serotype 2 is rare in humans and not associated with IPD clinical cases [25] [26]. It is important to understand how other strains interact with human cells to cause colonization and infection, especially for the more common and more pathogenic serotypes. We have demonstrated here that the serotypes can differ in their ability to regulate various genes (**Figure 2** and **Figure 4**), and that host cells can greatly influence the regulation of gene expression by *S. pneumoniae* (**Figure 5**). We examined frequent colonizers of humans (5 and 19 F) with two that have been highly implicated in adult IPD cases (3 and 9 v), although the epidemiological data for *S. pneumoniae* infection does vary dramatically depending on the source country [24] [25] [26], and invasive strains have been isolated for all four serogroups [26], suggesting variation within the serogroups. For being more frequently implicated in invasive diseases, serotype 9 v behaved similarly to 19 F in both broth and cell culture not only in gene expression, but also in growth and adhesion and invasion abilities (**Figures 1-4**). Serotype 5 grew similarly in broth and had similar gene expression to 9 v and 19 F when grown in broth (**Figures 1-2**), but differed more when grown in cell culture. It adhered and invaded at a similar level (**Figure 3**), but serotype 5 had a lot of variability in gene expression when exposed to A549 cells, and had significant upregulations of choline binding proteins and capsule expression compared with 9 v and 19 F (**Figure 4**), suggesting that for this particular serotype, these virulence factors may be important for cell colonization. Serotype 3 differed greatly in its ability to grow in broth culture (**Figure 1** and **Figure 3**), and had increased pneumolysin and autolysin activity (**Figure 2** and **Figure 4**) compared to the other serotypes, which likely contributes to its frequency of IPD.

Although there are many more serotypes and virulence factors that can be studied with the pneumococcus, it is demonstrated here that studying gene regulation with this bacterial species can be challenging due to the diversity and variability of the species. Various strains behave differently from one another in the same medium, and can vary dramatically when observed in different culture conditions. Host cell factors impact the way that the pneumococci regulate their genes, and surprisingly most bacterial genes that we examined here were down-regulated in the presence of the host cells as compared to growth in broth (**Figure 5**). It was hypothesized that we would see the exact opposite effect since all the genes that were included in this assay are known virulence factors for *S. pneumoniae*, and thus we thought that the presence of host cells would increase the expression, and not decrease it. This again reiterates the need to study bacte-

ria in a more natural system since understanding gene regulation is most important when the bacteria are in their natural setting.

Acknowledgements

We would like to thank the Offices of Student Research and Creative Activities and Faculty Research of Tarleton State University for funding this project. All biosafety protocols were approved by the Institutional Biosafety Committee at Tarleton State University, and were strictly adhered to for the pneumococci and human cell line.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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