8-14-2015

The Impact of Pyruvate Oxidase (SpxB) on the Release of the Toxin Pneumolysin in Streptococcus Pneumoniae

Joseph Colby Bryant

The impact of pyruvate oxidase (SpxB) on the release of the toxin pneumolysin in *Streptococcus pneumoniae*

By

Joseph Bryant

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

August 2015

The impact of pyruvate oxidase (SpxB) on the release of the toxin pneumolysin in *Streptococcus pneumoniae*

By

Joseph Bryant

Approved:

_____

Justin A. Thornton
(Major Professor)

_____

Heather R. Jordan
(Committee Member)

_____

Keun Seok Seo
(Committee Member)

_____

Mark E. Welch
(Graduate Coordinator)

_____

R. Gregory Dunaway
Professor and Dean
College of Arts & Sciences

Name: Joseph Bryant

Date of Degree: August 14, 2015

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Justin Thornton

Title of Study: The impact of pyruvate oxidase (SpxB) on the release of the toxin pneumolysin in *Streptococcus pneumoniae*

Pages in Study: 50

Candidate for Degree of: Master of Science

Streptococcus pneumoniae (pneumococcus) is a major human pathogen and commensal organism of the nasopharynx. A major virulence factor of the pneumococcus is the cholesterol dependent, pore forming cytolysin pneumolysin. This toxin acts extracellularly, but the mechanism of release has not been well elucidated. Despite being a catalase negative organism, the pneumococcus produces up to millimolar concentrations of hydrogen peroxide through the activity of pyruvate oxidase. In all strains analyzed, deletion of the pyruvate oxidase gene yielded a significant reduction in the amount of PLY observed in the supernatant via western blot. A single strain, WU2 was also observed to have a significant ($p<.05$) reduction in the amount of PLY observed in the supernatant when treated with extracellular catalase. Furthermore, a significant correlation between hydrogen peroxide production and PLY release was observed in a panel of 15 clinical isolates.

DEDICATION

I would like to dedicate this work to my parents, Myra and Johnny Bryant and my fiancée Jessica Swinea. Thank you for your love and support.

ACKNOWLEDGEMENTS

I would like to thank my committee members without whom this work would not have been possible. I would like to first give my sincere thanks to Dr. Justin A. Thornton, my major professor, for his mentorship and guidance. I would also like to give my appreciation to the other members of my committee, Dr. Heather R. Jordan and Dr. Keun Seok Seo. Your input and aid for my work was greatly appreciated

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

1.1 Streptococcus pneumoniae and disease burden

Streptococcus pneumoniae is a gram-positive, catalase negative major human pathogen worldwide, as it is the causative agent of otitis media, bacteremia, septicemia, pneumonia, and meningitis (1). Additionally, this pathogen is the leading cause of death for children under the age of five worldwide (2, 3). Diseases caused by pneumococcus, such as bacterial pneumonia, also significantly impact immunocompromised populations including the elderly (4). Pneumococcal disease carries a significant economic burden due to the high costs associated with antibiotic resistance, estimated to be responsible for 233 million dollars in healthcare expenses and is further magnified if parental visits to pediatricians are considered, as pneumococcus is the most common etiological agent of acute otitis media (5). For instance, healthcare costs from acute otitis media in the United States have been estimated to range from approximately 3 billion dollars to potentially as high as 6 billion dollars (6, 7). Pneumococcus has also been observed to have a synergistic effect upon coinfection with the influenza virus, as viral infection increases bacterial counts, indicating that a significant portion of morbidity and mortality resulting from influenza infection could be attributable to pneumococcal disease (8, 9).

Despite this major impact on human health, however, the organism is a common commensal organism found in the nasopharynx, with carriage rates of up to 70%,

depending on the population (10). The typical process of disease progression involves transfer of bacteria to the lower respiratory system from the nasopharynx, thereby leading to pneumonia. From there, the organism can enter the bloodstream causing bacteremia and eventually cross the blood brain barrier to cause meningitis (2, 3, 11).

The pneumococcus possesses an arsenal of virulence factors capable of enhancing its pathogenicity in the host. The pneumococcus has over 90 different serotypes of anti-phagocytic polysaccharide capsules that allow it to thwart the innate immune system, with differences in strain virulence based on capsule type (12, 13). These capsule types vary in composition and size, accounting for a great deal of variability among strains producing different capsule serotypes (14). Additionally, the pneumococcus possesses a number of surface proteins, including PspA, neuraminidase, hyaluronidase, that are capable of triggering an immune response from the host (15–18).

Currently, there are two types of vaccines commercially available for the prevention of pneumococcal disease: 1) a conjugated polysaccharide vaccine conferring immunity to 7-13 capsule types, approved for children and infants; 2) a capsular polysaccharide vaccine conferring immunity to 23 different strains that imparts immunity in adults (19–21). While the introduction of these vaccines has led to a reduction in the amount of pneumococcal disease observed in at-risk groups, serotype replacement is increasing (22–25).

1.2 Pneumolysin

Streptococcus pneumoniae possesses a number of virulence factors that augment its pathogenicity. Among these virulence factors is the 53-kDa cholesterol-dependent pore-forming cytolysin (CDC), pneumolysin (PLY) (26). PLY is found to be a major

virulence factor, being diagnostic in the cerebrospinal fluid for meningitis, reducing ciliary beat frequency in the lungs, and attenuating the virulence of the organism *in vivo* when deleted from the chromosome (27–30). Strain-based variations in the amount of PLY produced by pneumococcus have been identified in the literature as well (31). Additionally, PLY has been found to activate the classical pathway of complement and interact with Toll Like Receptor 4 (TLR4) (27, 29). Production of PLY has also been observed to be significant for pneumococcal biofilm formation, an important step in colonization of the nasopharynx, with *ply*- mutants less able to form biofilms than wild type (28, 32, 33).

Toxoid versions of PLY have been proposed as potential protein-based vaccine antigen candidates to replace the current capsular vaccines with one that would ideally be universal for a conserved domain of the protein (34–36). The mechanism through which PLY forms pores is well elucidated, with the toxin binding to cholesterol in the cell membrane, followed by oligomerization to form a pre-pore complex on the surface of the eukaryotic cell. After which a pore approximately 260 angstroms in diameter is formed, allowing for loss of intracellular proteins, ions and other molecules from the cell (37–39).

Pneumolysin is the only known cholesterol-dependent cytolysin that lacks a N-terminal secretion sequence for release into the extracellular space via the Sec-dependent pathway of protein secretion. Additionally, it lacks any sequence for any other known secretion pathway (26, 40). Other members of the CDC family, such as listeriolysin O in *Listeria monocytogenes* and perfringolysin O in *Clostridium perfringens* have been demonstrated to be released via the Sec-dependent pathway (41–43). Interestingly, it has been published that when the Sec-dependent export signal is added back to PLY in the

pneumococcus, it is incapable of being secreted, indicating that PLY may actually be incompatible with the secretion pathway (44). In spite of this lack of a secretion signal, the protein is still found in the extracellular space (26, 28). A number of mechanisms through which PLY can be released to the extracellular space have been identified. The major mechanism for PLY liberation is by autolysis via the activity of the autolytic cell wall amidase *lytA* (45). Through this mechanism, the cell wall is broken down and intracellular products are released to the extracellular space. Another mechanism that has been identified is the fratricide of other pneumococci induced by competence, or the natural uptake of genetic material by the pneumococcus (46). Fratricide releases PLY due to the killing of non-competent pneumococci by competent pneumococci resulting in uptake of the released DNA. This process of killing also releases PLY to the extracellular space. Interestingly however, is that the release of PLY has been observed in mutants that lack the *lytA* gene (47). This indicates that some aspect of PLY release is not attributable to the activity of *lytA,* as PLY is still seen in the extracellular space of mutants lacking that gene, but the specific mechanism related to this release has not been identified. Additionally, association of active PLY with the cell wall of pneumococcus that is unrelated to the activity of *lytA* has also been demonstrated in a panel of strains (48).

1.3 Pneumococcal hydrogen peroxide

Although *Streptococcus pneumoniae* is a catalase negative organism, it is still observed to produce up to millimolar concentrations of hydrogen peroxide (49). Hydrogen peroxide is produced through the aerobic metabolism of pyruvate via the enzyme pyruvate oxidase (SpxB) converting pyruvate to lactate, producing CO_2 and

H_2O_2 as metabolic byproduct (49). This metabolism only occurs in an aerobic environment (50, 51). While this hydrogen peroxide produced by the pneumococcus is toxic to the bacterial cell at elevated concentrations, it is still required for the organism to exhibit virulence *in vivo* and it is found to confer an advantage to survival in colonization by the pneumococcus (51, 52). Pneumococcal H_2O_2 has a significant impact on the host, as it is capable of causing neuronal cell death during meningitis, necrosis and apoptosis of neutrophils, and necrosis and apoptosis of alveolar cells (52–54, 59). The production of hydrogen peroxide also aids the pneumococcus in competing with other organisms in the relatively oxygen-rich nasopharynx. It has been demonstrated that pneumococcal H_2O_2 confers an advantage when considering competition in the nasopharynx with other inhabitants such as *Haemophilus influenza* and *Staphylococcus aureus* that are more sensitive to stress from H_2O_2 (55, 56). Additional work indicates that Δ*spxB* mutants of pneumococcus are outcompeted by *spxB*+ pneumococci in an *in vivo* rat model, lending further evidence to pyruvate oxidase conferring a competitive advantage as pneumococcal H_2O_2 was lethal to the competing Δ*spxB* mutant (57). Usage of pyruvate oxidase also appears to confer protection from killing by H_2O_2 making the organism more resistant to killing than other inhabitants of the nasophayrnx (49, 56, 57).

Of note is the fact that pyruvate oxidase is not the sole pathway through which hydrogen peroxide can be produced in the pneumococcus. The organism can also produce hydrogen peroxide through the activity of lactate oxidase (LctO) (49, 58). This pathway of hydrogen peroxide production has been studied less than the better-known pathway involving pyruvate oxidase. During glucose metabolism, up to 75% of the pyruvate that is processed by the organism is converted into lactate. In this environment,

lactate oxidase is very important for converting lactate back to pyruvate for continued ATP production when pyruvate production via glycolysis has slowed due to reductions in the amount of glucose available to the organism (49). However, a great deal of the H_2O_2 produced by the pneumococcus can likely be attributed to the activity of pyruvate oxidase (49, 50, 52).

1.4 References

1. **Bogaert, D, R de Groot, and P. Hermans**. 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. The Lancet infectious diseases.

2. **Kabra, S. K., R. Lodha, and R. M. Pandey**. 2010. Antibiotics for community-acquired pneumonia in children. The Cochrane database of systematic reviews CD004874.

3. **O'Brien, KL, LJ Wolfson, JP Watt, and E Henkle**. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. The Lancet.

4. **Sousa, D, Justo, I & Dominguez, A.** Community-acquired pneumonia in immunocompromised older patients: incidence, causative organisms and outcome. Clinical Microbiology (2013). doi:10.1111/j.1469-0691.2012.03765.x

5. **Reynolds, C. A., J. A. Finkelstein, T. G. Ray, M. R. Moore, and S. S. Huang**. 2014. Attributable healthcare utilization and cost of pneumonia due to drug-resistant streptococcus pneumonia: a cost analysis. Antimicrobial resistance and infection control. Springer **3**:1–8.

6. **Gates, G. A.** 1996. Cost-effectiveness considerations in otitis media treatment. Otolaryngol Head Neck Surg **114**:525–30.

7. **Marcy, S.** Management of acute otitis media. The Pediatric Infectious Disease Journal 22, 673-674 (2003).

8. **Smith, A. et al.** Kinetics of Coinfection with Influenza A Virus and Streptococcus pneumoniae. PLoS Pathogens 9, e1003238 (2013).

9. **Short, K., Habets, M., Hermans, P. & Diavatopoulos, D.** Interactions between Streptococcus pneumoniae and influenza virus: a mutually beneficial relationship?. Future Microbiology 7, 609-624 (2012).

10. **Waroux, O., S. Flasche, D. Prieto-Merino, and J. W. Edmunds**. 2014. Age-Dependent Prevalence of Nasopharyngeal Carriage of Streptococcus pneumoniae before Conjugate Vaccine Introduction: A Prediction Model Based on a Meta-Analysis. PloS one. Public Library of Science **9**:e86136.

11. **Mook-Kanamori, B., Geldhoff, M., van der Poll, T. & van de Beek, D.** Pathogenesis and Pathophysiology of Pneumococcal Meningitis. Clinical Microbiology Reviews 24, 557-591 (2011).

12. **Epstein, F., Tuomanen, E., Austrian, R. & Masure, H**. Pathogenesis of Pneumococcal Infection. New England Journal of Medicine 332, 1280-1284 (1995).

13. **Kelly, T., J. P. Dillard, and J. Yother**. 1994. Effect of genetic switching of capsular type on virulence of Streptococcus pneumoniae. Infection and immunity **62**:1813–9.

14. **Austrian, R**. 1981. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. Review of Infectious Diseases.

15. **Briles, D., Yother, J. & McDaniel, L.** Role of Pneumococcal Surface Protein A in the Virulence of Streptococcus pneumoniae. Clinical Infectious Diseases 10, S372-S374 (1988).

16. **Berry, AM, RA Lock, SM Thomas, and DP Rajan**. 1994. Cloning and nucleotide sequence of the Streptococcus pneumoniae hyaluronidase gene and purification of the enzyme from recombinant Escherichia coli. Infection and immunity.

17. **Camara, M, GJ Boulnois, and PW Andrew**. 1994. A neuraminidase from Streptococcus pneumoniae has the features of a surface protein. Infection and immunity.

18. **Quin, LR, QC Moore, and LS McDaniel**. 2007. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. Infection and immunity.

19. **Smith, K, JJ Muther, AL Duke, E McKee, and NY Zheng**. 2013. Fully human monoclonal antibodies from antibody secreting cells after vaccination with Pneumovax® 23 are serotype specific and facilitate opsonophagocytosis. Immunobiology.

20. **Sucher, A., Chahine, E., Nelson, M. & Sucher, B**. Prevnar 13, the New 13-Valent Pneumococcal Conjugate Vaccine. Annals of Pharmacotherapy 45, 1516-1524 (2011).

21. **Isaacman, DJ, MA Fletcher, B Fritzell, and V Ciuryla**. 2007. Indirect effects associated with widespread vaccination of infants with heptavalent pneumococcal conjugate vaccine (PCV7; Prevnar). Vaccine.

22. **Singleton, R. et al.** Invasive Pneumococcal Disease Caused by Nonvaccine Serotypes Among Alaska Native Children With High Levels of 7-Valent Pneumococcal Conjugate Vaccine Coverage. *JAMA* 297, 1784 (2007).

23. **Brueggemann, AB, R Pai, DW Crook, and B Beall**. 2007. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS pathogens.

24. **Group, S.** 1998. Randomised trial of 23-valent pneumococcal capsular polysaccharide vaccine in prevention of pneumonia in middle-aged and elderly people. The Lancet.

25. **Huss, A., Scott, P., Stuck, A., Trotter, C. & Egger, M.** Efficacy of pneumococcal vaccination in adults: a meta-analysis. *Canadian Medical Association Journal* 180, 48-58 (2009).

26. **Mitchell, T. J., and C. E. Dalziel**. 2014. The biology of pneumolysin. Subcell. Biochem. **80**:145–60.

27. **Mitchell, T., Andrew, P., Saunders, F., Smith, A. & Boulnois, G.** Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Molecular Microbiology* 5, 1883-1888 (1991).

28. **Marriott, H., Mitchell, T. & Dockrell, D.** Pneumolysin: A Double-Edged Sword During the Host-Pathogen Interaction. *CMM* 8, 497-509 (2008).

29. **Srivastava, A., P. Henneke, A. Visintin, S. C. Morse, V. Martin, C. Watkins, J. C. Paton, M. R. Wessels, D. T. Golenbock, and R. Malley**. 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. Infection and immunity **73**:6479–87.

30. **Hirst, R. A., A. Kadioglu, C. O'callaghan, and P. W. Andrew**. 2004. The role of pneumolysin in pneumococcal pneumonia and meningitis. Clin. Exp. Immunol. **138**:195–201.

31. **Benton, K. A., J. C. Paton, and D. E. Briles**. 1997. Differences in virulence for mice among Streptococcus pneumoniae strains of capsular types 2, 3, 4, 5, and 6 are not attributable to differences in pneumolysin production. Infection and immunity. Am Soc Microbiol **65**:1237–1244.

32. **Shak, JR, HP Ludewick, KE Howery, F Sakai, and H Yi**. 2013. Novel role for the Streptococcus pneumoniae toxin pneumolysin in the assembly of biofilms. MBio.

33. **Rubins, J. et al.** Pneumolysin in pneumococcal adherence and colonization. *Microbial Pathogenesis* 25, 337-342 (1998).

34. **Briles, DE, SK Hollingshead, GS Nabors, and JC Paton**. 2000. The potential for using protein vaccines to protect against otitis media caused by Streptococcus pneumoniae. Vaccine.

35. **Douce, G, K Ross, G Cowan, J Ma, and TJ Mitchell**. 2010. Novel mucosal vaccines generated by genetic conjugation of heterologous proteins to pneumolysin (PLY) from Streptococcus pneumoniae. Vaccine.

36. **Kirkham, L. et al.** Construction and Immunological Characterization of a Novel Nontoxic Protective Pneumolysin Mutant for Use in Future Pneumococcal Vaccines. Infection and immunity 74, 586-593 (2005).

37. **Tilley, SJ, and HR Saibil**. 2006. The mechanism of pore formation by bacterial toxins. Current opinion in structural biology.

38. **Tilley, SJ, EV Orlova, R. Gilbert, and PW Andrew**. 2005. Structural basis of pore formation by the bacterial toxin pneumolysin. Cell.

39. **Gonzalez, M., Bischofberger, M., Pernot, L., van der Goot, F. & Frêche, B.** Bacterial pore-forming toxins: The (w)hole story?. *Cellular and Molecular Life Sciences* 65, 493-507 (2007).

40. **Walker, JA, RL Allen, and P Falmagne**. 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of Streptococcus pneumoniae. Infection and immunity

41. **Alonzo, F., and N. E. Freitag**. 2010. Listeria monocytogenes PrsA2 is required for virulence factor secretion and bacterial viability within the host cell cytosol. Infection and immunity. **78**:4944–57.

42. **Schnupf, P., J. Zhou, A. Varshavsky, and D. A. Portnoy**. 2007. Listeriolysin O secreted by Listeria monocytogenes into the host cell cytosol is degraded by the N-end rule pathway. Infection and immunity. **75**:5135–47.

43. **Tweten, R. K.** 1989. Cloning and expression in Escherichia coli of the perfringolysin O (theta-toxin) gene from Clostridium perfringens and characterization of the gene product. Infection and immunity **56**:3228–34.

44. **Price, K. E., N. G. Greene, and A. Camilli**. 2012. Export requirements of pneumolysin in Streptococcus pneumoniae. J. Bacteriol. **194**:3651–60.

45. **Martner, A., C. Dahlgren, J. C. Paton, and A. E. Wold**. 2008. Pneumolysin released during Streptococcus pneumoniae autolysis is a potent activator of intracellular oxygen radical production in neutrophils. Infection and immunity **76**:4079–87.

46. **Claverys, JP, B Martin, and LS Håvarstein**. 2007. Competence- induced fratricide in streptococci. Molecular microbiology.

47. **Balachandran, P., S. K. Hollingshead, J. C. Paton, and D. E. Briles**. 2001. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. Journal of bacteriology **183**:3108–16.

48. **Price, KE, and A Camilli**. 2009. Pneumolysin localizes to the cell wall of Streptococcus pneumoniae. Journal of bacteriology.

49. **Taniai, H., K. Iida, M. Seki, M. Saito, S. Shiota, H. Nakayama, and S. Yoshida**. 2008. Concerted action of lactate oxidase and pyruvate oxidase in aerobic growth of Streptococcus pneumoniae: role of lactate as an energy source. J. Bacteriol. **190**:3572–9.

50. **Spellerberg, B., D. R. Cundell, J. Sandros, B. J. Pearce, I. Idanpaan-Heikkila, C. Rosenow, and H. R. Masure**. 1996. Pyruvate oxidase, as a determinant of virulence in Streptococcus pneumoniae. Mol. Microbiol. **19**:803–13.

51. **Pesakhov, S. et al.** Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of Streptococcus pneumoniae. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1768, 590-597 (2007).

52. **Duane, P. G., J. B. Rubins, H. R. Weisel, and E. N. Janoff**. 1993. Identification of hydrogen peroxide as a Streptococcus pneumoniae toxin for rat alveolar epithelial cells. Infection and immunity **61**:4392–7.

53. **Braun, J. S., J. E. Sublett, D. Freyer, T. J. Mitchell, J. L. Cleveland, E. I. Tuomanen, and J. R. Weber**. 2002. Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. J. Clin. Invest. **109**:19–27.

54. **Zysk, G., L. Bejo, B. K. Schneider-Wald, R. Nau, and H. Heinz**. 2000. Induction of necrosis and apoptosis of neutrophil granulocytes by Streptococcus pneumoniae. Clinical and experimental immunology **122**:61–6.

55. **Pericone, C. D., K. Overweg, P. W. Hermans, and J. N. Weiser**. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infect. Immun. **68**:3990–7.

56. **Regev-Yochay, G., K. Trzcinski, C. M. Thompson, R. Malley, and M. Lipsitch**. 2006. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. J. Bacteriol. **188**:4996–5001.

57. **Regev-Yochay, G., K. Trzcinski, C. M. Thompson, M. Lipsitch, and R. Malley**. 2007. SpxB is a suicide gene of Streptococcus pneumoniae and confers a selective advantage in an in vivo competitive colonization model. J. Bacteriol. **189**:6532–9.

58. **UDAKA, S., J. KOUKOL, and B. VENNESLAND**. 1959. Lactic oxidase of Pneumococcus. Journal of bacteriology **78**:714–25.

59. **Rai, P. et al.** Streptococcus pneumoniae secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells. Proc Natl Acad Sci USA 201424144 (2015). doi:10.1073/pnas.1424144112

CHAPTER II

DETERMINATION OF THE EXTENT TO WHICH PYRUVATE OXIDASE IMPACTS PNEUMOLYSIN RELEASE

2.1 Introduction

Streptococcus pneumoniae is a commensal organism of the nasopharynx that causes a number of major human diseases such as sinusitis, otitis media, bacteremia, meningitis, septicemia, and bacterial pneumonia (1, 2). As a major human pathogen, it has a significant impact upon public health and the economy, causing 3 billion to 6 billion dollars in health care costs as well as lost work from doctors visits related to otitis media alone (3, 4). Pneumococcus possesses a host of virulence factors including a variety of pneumococcal surface proteins such as PspA, neuraminidase and an anti-phagocytic polysaccharide capsule (5–7). This chapter will focus on two virulence factors in particular, the toxin pneumolysin (PLY) and the protein pyruvate oxidase (SpxB).

One of the primary virulence factors of the pneumococcus is PLY, a 53-kDa member of the cholesterol dependent cytolysin (CDC) family of pore forming toxins (8). Other members of this family of proteins have been demonstrated to be secreted via the Sec-dependent pathway in other gram-positive organisms like listeriolysin O in *Listeria monocytogenes* and perfringolysin O in *Clostridium perfringens*. However, it is interesting to note that this mechanism of transport for PLY does not occur in the

pneumococcus as it lacks the N-terminal secretion signal required for Sec-dependent secretion (9–11). Interestingly, however, PLY is still observed in the supernatant of actively growing cells and exerts its toxic functions extracellularly (8, 12). Furthermore, differences between strains have been observed for the production of PLY *in vitro* (23). The method of secretion of this toxin has been difficult to characterize, although a number of mechanisms have been identified in the literature. Initially, it was thought that pneumococcal autolysis through the activity of *lytA*, an autolytic cell wall amidase that breaks down the cell wall was essential for releasing PLY into the surrounding space (13, 14). However, release of PLY into the supernatant is still observed in pneumococci lacking the gene *lytA*, indicating that the bacterium possesses additional mechanisms for PLY release (16). A number of other mechanisms have since been identified. One of which is fratricide of other pneumococci by competent organisms, as pneumococcus is a naturally competent organism (15). In this process, pneumococci that have become competent release products that are toxic to non-competent cells. The death of these cells leads to the release of PLY.

Another major virulence factor of the pneumococcus is the enzyme pyruvate oxidase, encoded by the *spxB* gene (17). Although pneumococcus is a catalase negative organism, it is capable of producing up to millimolar concentrations of hydrogen peroxide (H_2O_2) as a byproduct when it performs glycolysis reactions in an aerobic environment, making its production oxygen-dependent, as the organism is a facultative anaerobe (18). While this H_2O_2 is toxic to the pneumococcus in higher concentrations, SpxB appears to confer an advantage to the organism in resistance to H_2O_2 killing and *in vivo* virulence, due to greater ATP depletion in *spxB* negative mutants (19, 20).

Additionally, pyruvate oxidase gives the pneumococcus a competitive advantage against other organisms when colonizing the nasopharynx as it has a higher tolerance for H_2O_2 than other inhabitants like *H. influenzae* or *S. aureus* (21, 22). In addition to toxicity to other inhabitants of the nasopharynx, pneumococcal H_2O_2 has been observed to have deleterious effects on host cells as well. Published work, along with unpublished work from our lab indicates that pneumococcal H_2O_2 causes DNA damage to host cells (32). Additionally, it causes damage to alveolar cells and host immune cells (24, 25). Despite the lethal effect of H_2O_2 on the pneumococcus, a link between pyruvate oxidase activity and PLY release has not been reported. Based on the lethal impact of H_2O_2 on the pneumococcus, we decided to investigate the impact of H_2O_2 production upon PLY release.

In this study, we identified a significant ($p<0.005$) reduction in the amount of PLY released into the supernatant of isogenic Δ*spxB* mutants of strains WU2 and AW267, considered high PLY releasing strains. This relationship was also seen with strains T4 ($p<0.005$), and T4R ($p<0.05$) with both demonstrating a significant reduction in the amount of PLY observed when *spxB* is removed. Additionally, a significant ($p<0.05$) correlation between H_2O_2 production and PLY release was observed in a panel of clinical isolates obtained from St. Jude's Children's Research Hospital. It was demonstrated by qRT-PCR that the reduction in PLY was due to differences in release, and could not be attributed to differences in transcription of *ply* when *spxB* is removed. Additionally, it was demonstrated that removal of exogenous hydrogen peroxide by the use of catalase, an H_2O_2 degrading enzyme, led to a significant reduction in PLY released from WU2. Furthermore, we tested the hypothesis that H_2O_2 causes PLY release by

causing a reduction in membrane potential, making the cellular membrane leaky, with preliminary data using the fluorometric dye, JC-1, indicating that this may be the case. Finally, we confirmed that the reduction in PLY release is not attributable to differences in autolysis between the mutant and wild type.

2.2 Materials and Methods

2.2.1 Strains used

The wild-type strains used for this study were high PLY-releasing strains AW267 and WU2, and low PLY-releasing strains T4 and T4R (unencapsulated mutant of T4). Isogenic Δ*spxB* mutants were generated in each of these strains by replacing the gene with an erythromycin cassette for selection purposes. Where indicated, these strains were treated with 10µg of catalase to neutralize extracellular H_2O_2. Clinical significance was investigated through the use of a panel of 21 clinical isolates obtained from St. Jude's Children's Research Hospital.

2.2.2 Growth of strains

Bacterial strains were grown to a mid-log phase (OD_{600} 0.5) in Todd Hewitt media supplemented with 0.5% yeast extract (THY) in a 37˚C water bath in 5 mL cultures. Upon reaching the desired OD, 1 mL of bacterial culture was centrifuged for 5 minutes at 15,000 rpm. After centrifugation, the supernatant was removed and 500µL was filtered over a 0.2µm polyethersulfone (PES) (Celltreat) filter to remove any remaining bacteria not pelleted during centrifugation. After obtaining supernatants, H_2O_2 concentrations were calculated using a colorimetric peroxide detection assay (Pierce).

2.2.3 Treatment with hydrogen peroxide

T4RΔ*spxB* was supplemented with exogenous H_2O_2 (500µM) for one hour on ice. Serial dilution plate counts were made before and after treatment to ensure that concentrations were not lethal to the organism.

2.2.4 Western blot method

After obtaining bacterial supernatants, PLY was analyzed through the use of SDS-PAGE western blotting. Four microliters of 5X Laemmi buffer was added to 20µL of bacterial supernatant and heated at 99˚C for 5 minutes in a thermocycler. After boiling the protein for 10 mins, 20µL of denatured sample was loaded onto a 10% SDS-PAGE gel. The gel was allowed to elute via electrophoresis until the dye front had entirely eluted (BioRad). BR Spectra (Thermo Scientific) ladder was used to determine protein size on the gel. The proteins were then transferred to a PVDF membrane (Millipore) utilizing the semi-dry transfer method for 35 minutes. Following this transfer of proteins, the membrane was blocked in Tris Buffered Saline+0.1% Tween (TTBS) with 5% milk for 30 minutes while agitating. Following the blocking step, the blot was probed overnight at 4˚C with 1:200 rabbit anti-PLY polyclonal serum diluted in 5% milk + TTBS. Following overnight probing of the membrane, it was then washed in TTBS 4 times for 5 minutes each while agitating. Following the wash of the membrane, the blot was then probed with a secondary goat anti-rabbit IgG horseradish peroxidase-conjugated antibody at a dilution of 1:2500 (BioRad) for 1 hour at room temperature while agitating. Following probing with the secondary antibody, it was then washed with TTBS 4 times, 5 minutes each while agitating. The blot was developed using enhanced chemiluminescent substrate (ECL) (Pierce) for 1 minute. Following the application of the substrate, the blot

was exposed to radiography film for 15 seconds and developed. The density of the PLY band was determined using ImageJ software (NIH) to make a relative determination of PLY release to the supernatant.

2.2.5 Dot blot method

The amount of PLY released from clinical supernatants, was quantitated via dot blot. A PVDF membrane was placed in a 96-well vacuum dot blotting apparatus (BioRad) and the wells were washed with 100µL of phosphate buffered saline (PBS) twice. A known amount of recombinant PLY at a concentration of 1.2 mg/mL was diluted down to 500 ng/mL and then serially diluted 1:2 into PBS 8 times to yield a standard curve that could be used to interpolate protein concentrations from supernatants. Forty microliters of each protein standard was applied to a PVDF membrane in a vacuum dot blotting apparatus (BioRad). One-hundred microliters of clinical supernatant was applied to the membrane as well. The samples were allowed to filter via gravity on the membrane for 45 minutes at room temperature without any application of vacuum. After the gravity filtration of the clinical proteins, the vacuum was then applied gently. After vacuum filtration of the proteins, the blot was again washed with 100µL of PBS per well. Following the PBS wash of the membrane, it was then blocked with 5% milk in TTBS for 30 minutes while agitating. After blocking the membrane, 10 mL of 1:200 rabbit anti PLY polyclonal serum was applied to the blot overnight at 4˚C. After probing with the primary, the blot was then washed 4 times 5 minutes each using TTBS while agitating. Following the wash of the membrane, 10 mL of 1:2500 goat anti rabbit IgG antibody in 5% milk+TTBS was applied to the blot for 1 hour at room temperature while agitating. Following probing the membrane with the secondary antibody, the blot was again washed

4 times 5 minutes each using TTBS. After washing the blot, ECL substrate was applied to the blot for 1 minute. After applying the substrate, the blot was exposed to film for 15 seconds. The density of the dots was determined using ImageJ software. The density of dots of a known concentration used to generate a regression that could be used to interpolate the unknown clinical protein concentrations. These interpolated values were normalized to clinical bacterial counts per 100,000 cells. This data was graphed versus H_2O_2 produced per 100,000 cells.

2.2.6 Determination of *ply* fold change

Pneumococcal strains AW267, T4R, WU2, or their isogenic Δ*spxB* mutants were grown to an OD of 0.5 in THY. Where indicated, upon reaching OD 0.5, T4RΔ*spxB* was treated with 500μM hydrogen peroxide for 1 hour on ice, with a control treatment getting no hydrogen peroxide. This concentration was used as it was found to be non-lethal to the organism (Appendix 2). Upon reaching this optical density, 2 mL of bacterial culture was added to 4 mL of RNAprotect (Qiagen) and incubated at room temperature for 5 minutes. Following this incubation period, 2 mL of this suspension was pelleted at 13,000 rpm for 5 minutes. After centrifugation, the supernatant was removed and the pellet was then resuspended in 1 mL of cold RNAse free PBS. After resuspending, the bacteria were once again pelleted for 5 minutes at 13,000 rpm, the supernatant was removed, and the bacteria were resuspended in 400 μL of RLT lysis buffer (Qiagen). After resuspention of the bacteria, the cells were then sonicated 3 times for 15 seconds on ice. After sonication, the sonicated bacteria were added to bead-beating tubes along with 500 μL of zirconium beads, 700 μL of RLT buffer was also added. The tubes were sealed with parafilm, and the tubes were loaded into a bead-beating apparatus (BioSpec). The tubes were bead

beaten for 2 minutes twice, after which the samples were then centrifuged shortly on a tabletop centrifuge. 700µL of this supernatant was then passed over a QIAshredder homogenization column (Qiagen) at 13,000 rpm for 30 seconds. 100% ethanol was added to the homogenized sample. Following this, RNA was extracted from the samples utilizing the RNeasy RNA purification column with a RNAse free DNAse treatment step to remove contaminating DNA (Qiagen). The RNA was eluted from the column in 20µL of RNAse free water. RNA values were obtained via fluorometric analysis utilizing a Qubit (Life Technologies) and contaminating DNA was detected utilizing the same apparatus. After which, 50 ng of bacterial RNA were then treated with Maxima reverse transcriptase enzyme to generate cDNA from the RNA. Quantitative RT-PCR was used with this cDNA as template following a 1:100 dilution of the cDNA. using a 96 well real-time PCR thermocycler (Applied Biosystems). Fold change of the *ply* gene was determined using the $\Delta\Delta C_T$ method, using *gyrA* as an internal housekeeping gene. Fold change in the isogenic *spxB* mutant was compared against the wild type in that strain.

2.2.7 Quantitation of pneumococcal membrane potential

Pneumococcal strains T4R, and AW267 and isogenic Δ*spxB* mutants were grown to a mid log phase of growth (OD_{600} 0.5) in Todd Hewitt media with 0.5% yeast extract. One milliliter of bacterial culture was centrifuged 5 minutes at 15,000 rpm, and resuspended in 500µL of PBS supplemented with 2% sterile glucose. The bacteria were then incubated 15 minutes at 37˚C. In order to examine the change in pneumococcal membrane potential upon interaction with H_2O_2 the JC-1 mitochondrial membrane potential dye was utilized.

These glucose-supplemented cells were treated with 5µL of JC-1 and vortexed. One-hundred microliters of the dyed cells were added to 96 well plates. These cells were then brought to a total volume of 200µL by adding 100µL of 500 µM, 1 mM, and 2 mM hydrogen peroxide in PBS, yielding half the concentration indicated. Additionally, 0.1% sodium deoxycholate was used at a final concentration of 0.05% to act as a positive control for membrane depolarization. One-hundred microliters of PBS was added to the non-treated cells to half the concentration of glucose. Fluorescence of these cells was measured at 485 nm to indicate strong membrane potential, and 530 nm to indicate weak membrane potential. A ratiometric analysis was performed by calculating the ratio of strong membrane potential to weak membrane potential over the course of 3 hours at 37˚C.

2.2.8 Assessment of the impact of autolysis upon release

Pneumococcal strain T4R and its isogenic Δ*spxB* mutant were grown to a mid log phase of growth (OD_{600} 0.5) in 3 mL of THY. Upon reaching the desired OD, Triton X-100 was added to the media in a concentration of 0.1%. Upon adding the Triton, the OD of the cultures was recorded every 5 minutes for 20 minutes or until the OD of the culture reached zero. Additionally, a second chemical lysis assay was performed utilizing 0.05% sodium deoxycholate in the same conditions as the above assay.

Additionally, a fluorometric quantitation of the amount of DNA observed in the supernatant of T4R and T4RΔ*spxB* was performed utilizing a Qbit fluorometer in order to utilize an alternate method to ascertain any differences between autolysis between the mutant and wild type, 2 µL of bacterial supernatant was assayed for DNA content.

2.2.9 Statistical analysis

A Student's t test was performed to determine statistical significance of differences. A P-value less than 0.05 was considered statistically significant. For the clinical data, a linear regression was performed to determine if a correlation existed between H_2O_2 produced and PLY released. A P value less than 0.05 was considered statistically significant.

2.3 Results and Discussion

2.3.1 Impact of *spxB* deletion on PLY release

The deletion of *spxB* from the chromosome caused a significant ($p<0.005$) reduction in the amount of PLY released into the supernatant in two of the 4 mutant strains, when compared to their wild-type counterparts, AW267 (Fig 2.1A), WU2 (Fig 2.1B), and T4 (Fig 2.1C). Additionally, a significant ($p<0.05$) reduction in PLY release was also observed in *spxB*- strains of T4R (Fig 2.1D). This indicates that pyruvate oxidase plays a role in the release of PLY in these strains. H_2O_2 values were quantified for each of the strains and their isogenic mutants (Fig 2.2). These results lends evidence to the fact that this mechanism of release appears to hold true for a wide range of strains, which seems to concur with previous findings that there are strain related differences for PLY production (13).

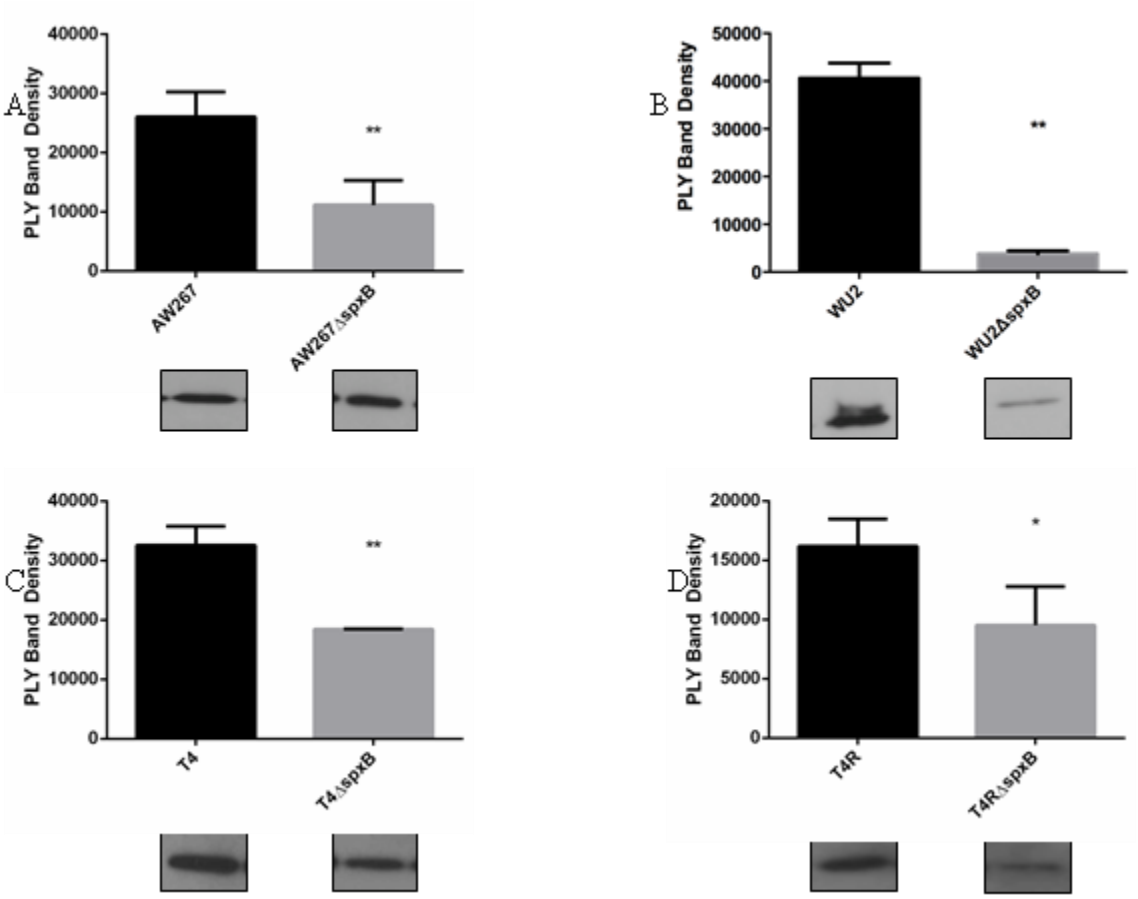

Figure 2.1 PLY band densities for wild type and Δ*spxB* mutants

Supernatants were obtained from all four strains, AW267, WU2, T4, and T4R along with their respective isogenic Δ*spxB* mutants. The band density of their PLY band was quantitated using ImageJ following western blotting of the sterile supernatant. Significance of $p<0.05$ is represented by a single asterisk (*), significance of $p<0.005$ is represented by two asterisks (**). Each figure represents at least 3 replicate experiments, plus and minus the standard deviation. Representative PLY bands are shown beneath each figure.

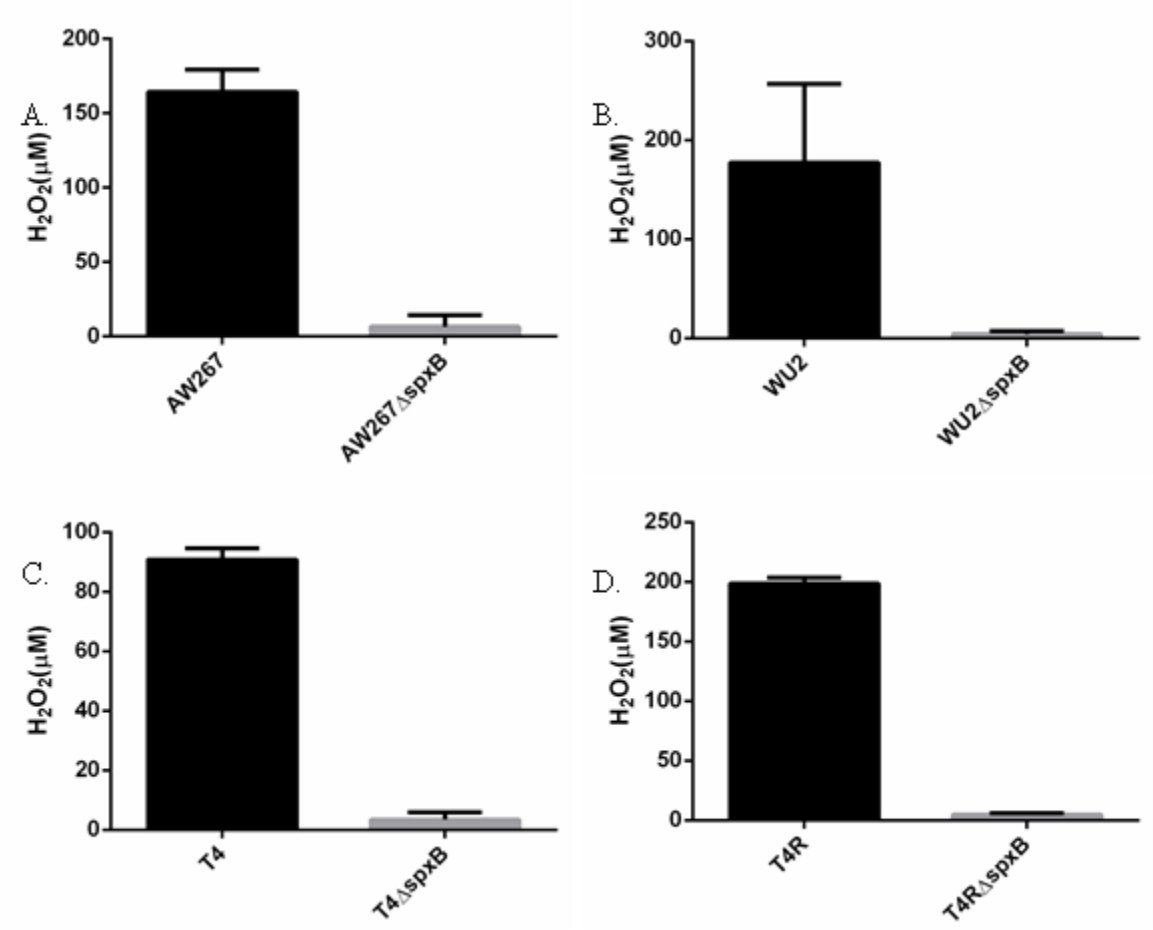

Figure 2.2 H_2O_2 quantification for wild type and Δ*spxB* mutants

Supernatants obtained from AW267, WU2, T4, T4R, and their respective Δ*spxB* mutants were assessed for H_2O_2 content via colorimetric assay. Data shown is the average of three independent experiments plus and minus the standard deviation.

2.3.2 Correlation of H_2O_2 production and PLY release

In order to investigate the clinical relevance of this H_2O_2 dependent PLY release, we assessed PLY release and H_2O_2 production in a panel of 15 clinical isolates. These data were normalized to cell count in order to account for the potential variation in cell density at the OD of 0.5. Upon performing a linear regression on the data obtained from a panel of 15 clinical isolates, a significant correlation ($p<0.05$, $r^2=.3167$) between PLY release and H_2O_2 production per 100,000 bacterial cells was observed (Fig 2.3). Previous

data collection of unfiltered bacterial supernatants indicated a more significant correlation between H_2O_2 production and PLY release (Appendix 1). This potentially indicates that cell wall association played some role in the correlation, as it was more statistically significant when bacterial cells were present in the supernatant (26).

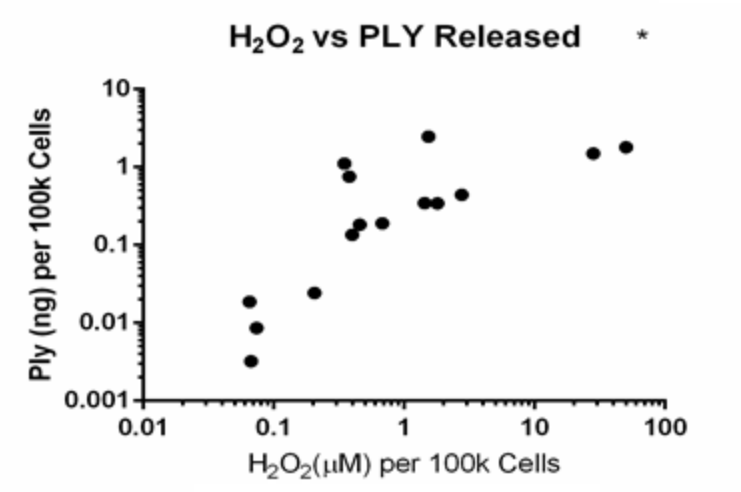

Figure 2.3 Correlation of H_2O_2 vs PLY released in clinical isolates.

An analysis of sterile supernatants of 15 clinical isolates was performed. Hydrogen peroxide was quantitated for each strain and normalized to cell counts per 100,000 cells. The amount of PLY in the supernatant was quantitated utilizing dot blotting and also normalized per 100,000 cells. A significant ($p<0.05$) correlation ($r^2=.3167$) was observed between H_2O_2 production and PLY release. ($n=15$, $p<0.05$, $r^2=.3167$)

2.3.3 Effects of SpxB on *ply* gene expression.

One potential explanation for the reduction in PLY release is that production of H_2O_2 could increase *ply* gene expression, increasing the amount of PLY observed in the supernatant of the wild type. In order to investigate this possibility, we quantitated *ply* gene expression for wild type and *spxB*- strains using qRT-PCR. Finally, we investigated the possibility that exogenous H_2O_2 could have some impact on *ply* expression in an *spxB*- strain using the same method. The results from the qRT-PCR of WU2 (Fig 2.4A), AW267 (Fig 2.4B), and T4R (Fig 2.5) indicate that in every case, the Δ*spxB* is seen to have upregulation of transcription of *ply*. In AW267Δ*spxB*, *ply* transcription of *ply* 2.83 fold, for WU2Δ*spxB* was upregulated 1.96, and in T4RΔ*spxB ply* expression was found to be upregulated 2.3 fold. This lends evidence to the hypothesis that the reduction observed in Figure 2.1 is not attributable to reduction of transcription when *spxB* is removed from the chromosome. Furthermore, upon treatment with 500μM H_2O_2 for 1 hour on ice, T4RΔ*spxB* (Fig 2.5) exhibited a 1.45 fold upregulation in the *ply* gene versus T4RΔ*spxB* with no H_2O_2 treatment. These data not only demonstrate that hydrogen peroxide is not contributing to increased *ply* gene expression, but also that PLY production isn't associated with a metabolic byproduct of SpxB. It appears, if anything, SpxB activity decreases *ply* expression. Previous literature indicates that environmental conditions have no significant impact upon PLY transcription when transcription of *ply* is investigated in various environments like the blood, lungs and nasopharynx (27).

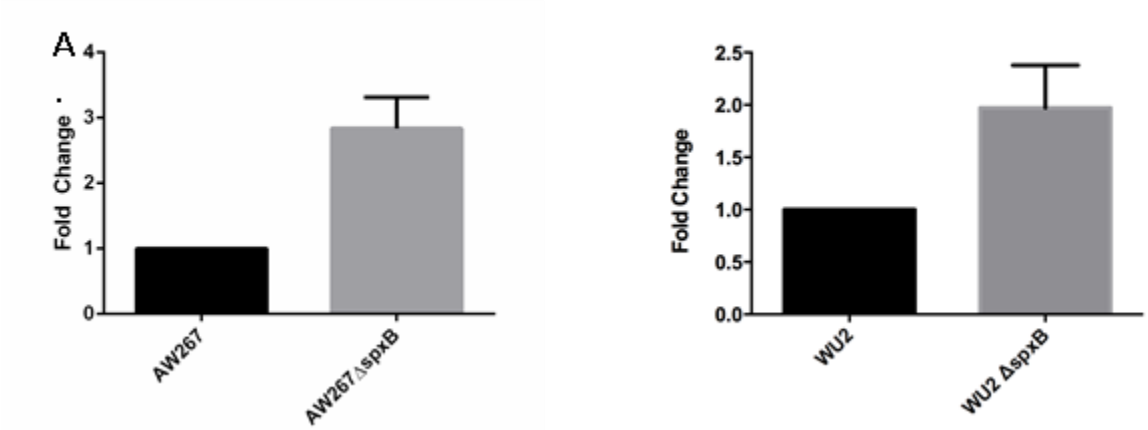

Figure 2.4 Fold change of *ply* gene expression of WU2 and AW267 compared to respective mutants.

Gene expression in WU2 and its isogenic *spxB* mutant (Fig 2.4A) and AW267 and its isogenic *spxB* mutant (Fig 2.4B) was measured via $\Delta\Delta C_T$. Deletion of *spxB* in WU2 demonstrates a 1.96 fold upregulation of *ply*. Upregulation was also seen in AW267Δ*spxB* where a 2.83 fold upregulation was observed. Data shown is an average of 3 experiments

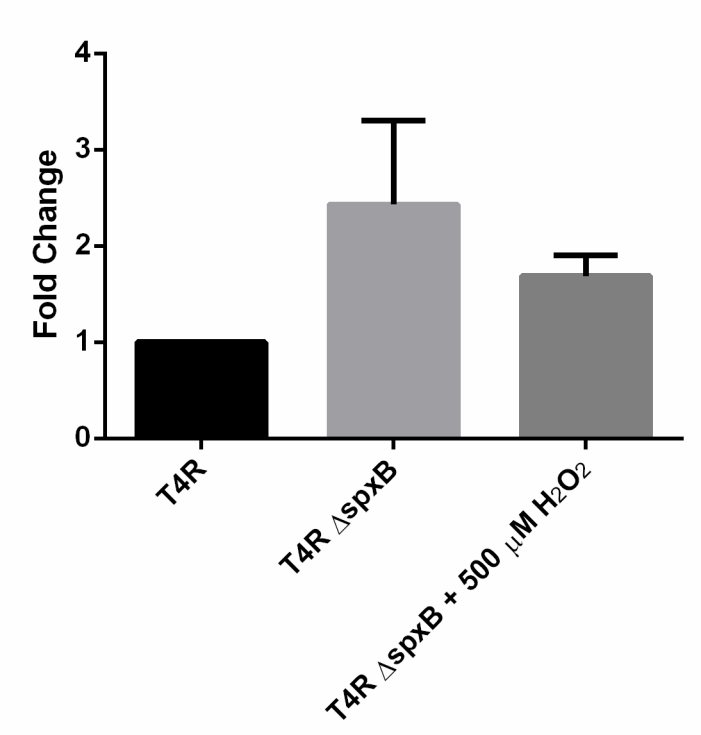

Figure 2.5 Fold change of *ply* in following exogenous H_2O_2 exposure.

Fold change in *ply* expression for T4RΔ*spxB* was measured via $\Delta\Delta C_T$ following treatment with 500μM H_2O_2 additionally, data for untreated T4RΔ*spxB* is shown. A 2.3 fold upregulation versus the wild type was observed in the untreated mutant, along with a 1.45 fold upregulation in the treated mutant versus wild type. Data shown is an average of 3 experiments.

2.3.4 Analysis of extracellular hydrogen peroxide

In order to ascertain the impact of extracellular H_2O_2 versus intracellular H_2O_2 a twofold approach was taken. In order to remove extracellular H_2O_2 from the wild type pneumococcus, catalase, a H_2O_2 degrading enzyme was used. Removal of intracellular hydrogen peroxide was achieved through the deletion of *spxB*. Upon analyzing these supernatants via western blot, an interesting effect was seen. A significant ($p<0.05$) negative impact upon PLY release in WU2 versus WU2 supplemented with catalase (Fig 2.6A) was observed. However, despite the removal of *spxB* attenuating the release of

PLY in all other strains, addition of catalase failed to achieve a significant difference between wild type and the wild type supplemented with catalase (Fig 2.6B). H_2O_2 values for each of the strains and conditions was determined via colorimetric assay (Fig 2.7). This could indicate that the strain dependent difference as described above, however it appears that H_2O_2 acts more extracellularly in WU2. This relationship was further investigated by supplementing T4RΔ*spxB* with 500μM H_2O_2 on ice for one hour. However there was no significant difference between the amount of PLY quantified in the supernatant by dot blot in the treated samples versus the untreated samples (Fig 2.8). This further indicates that H_2O_2 may have an intracellular impact or that the amount of time that the H_2O_2 was applied was insufficient to cause release.

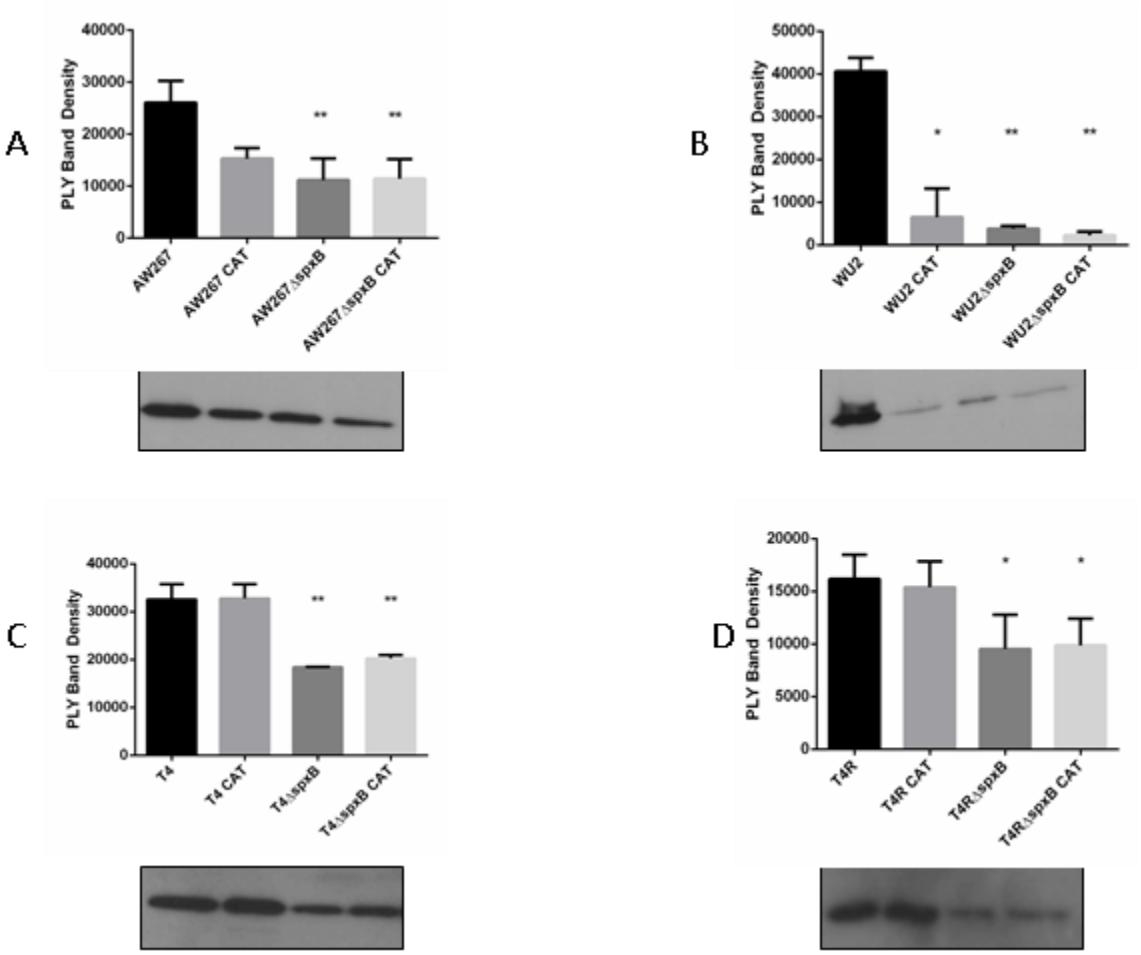

Figure 2.6 Impact of catalase treatment upon PLY band density in wild type and Δ*spxB* mutants

PLY band densities for strains treated with 10 µg of catalase was determined using densitometry of western blots of sterile supernatants. Significance of $p<0.05$ is noted by a single asterisk (*), significance of $p<0.005$ is demonstrated by two asterisks (**). Data shown is from 3 replicate experiments. Representative PLY bands are shown below the figures.

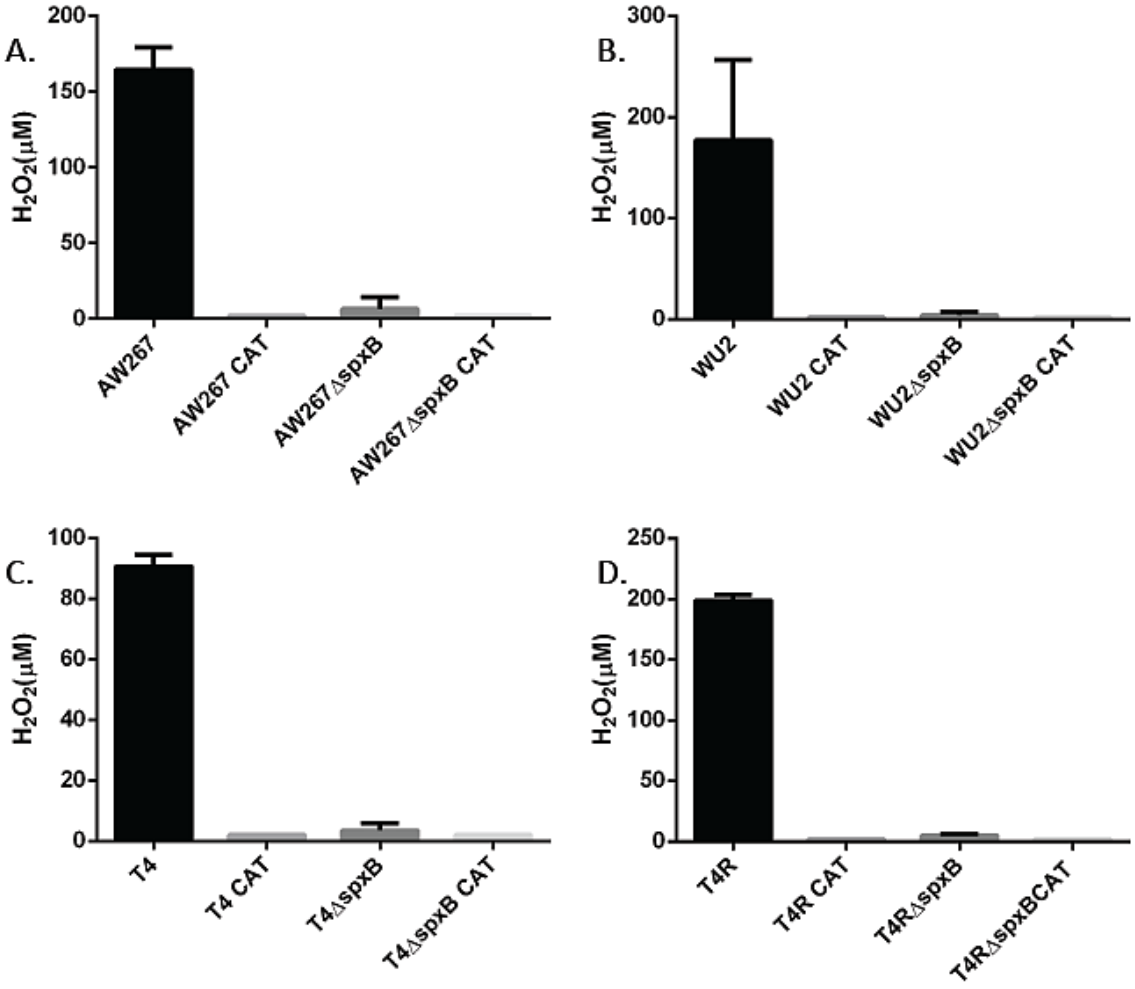

Figure 2.7 H_2O_2 quantitation of wild type and mutants supplemented with catalase

H_2O_2 values for each strain's wild type and Δ*spxB* mutant with and without 10µg of catalase added was assessed via colorimetric assay. Each figure represents three independent trials, plus and minus the standard deviation.

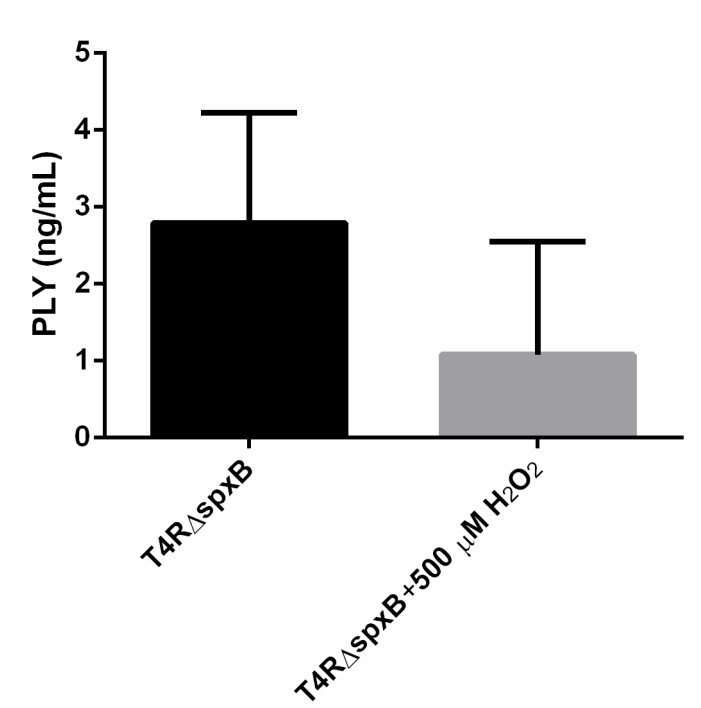

Figure 2.8 PLY quantitation following exogenous treatment with H_2O_2

T4RΔ*spxB* was grown to an OD of 0.5, placed on ice for 1 hour and either treated with 500μM H_2O_2 or no treatment. The amount of PLY in the supernatant was quantitated via dot blot. Analysis of this data indicated no significant impact from incubating T4RΔ*spxB* with H_2O_2 Data shown is from 3 duplicate experiments.

2.3.5 Assessment of the impact of autolysis

To determine if differences in autolysis could be causing the reduction in the amount of PLY observed in the supernatant, three assays were performed. A direct assay, using 0.1% Triton X-100 or 0.5% sodium deoxycholate to chemically lyse the cells, and an indirect assay to quantitate the amount of DNA found in sterile filtered supernatants. Chemical lysis of T4R and T4RΔ*spxB* indicated that there was no difference in autolysis between the mutant and wild-type (2.9) (Appendix 6). Furthermore, quantitation of the amount of DNA found in the supernatant also shows no difference between T4R and T4RΔ*spxB* as well (Appendix 6). This indicates that higher levels of autolysis in the wild

type strain does not appear to be a good explanation for the increased levels of PLY in the supernatant of the wild type versus the mutant. This is also supported by the fact that the bacterial counts between mutant and wild type are not significantly different at OD 0.5 (Appendix 4).

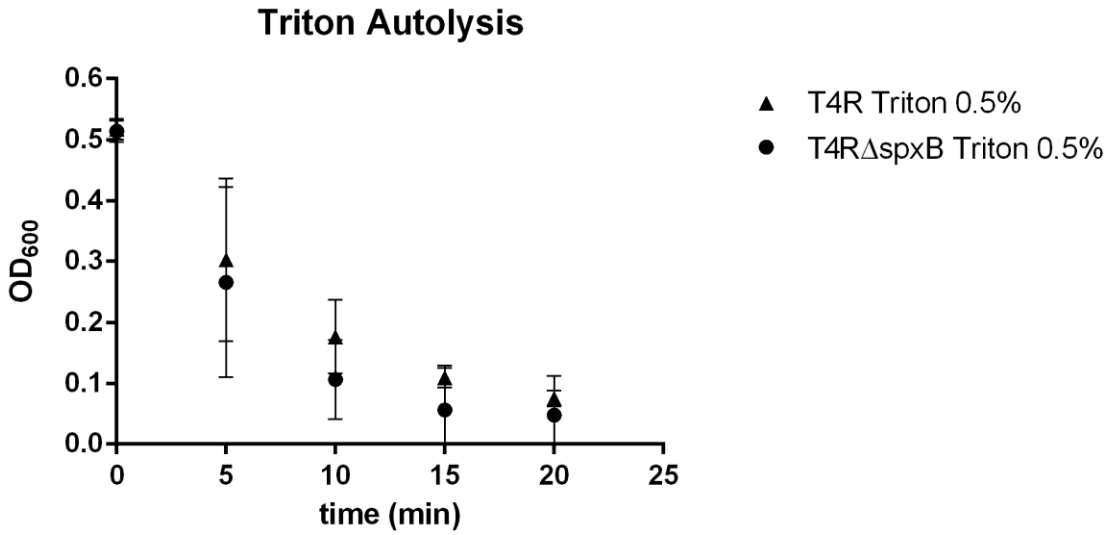

Figure 2.9 Difference in autolysis between T4R and T4RΔ*spxB*

Assessment of the lysis of T4R and T4RΔ*spxB* over 20 minutes at OD 0.5 shows no significant difference between the mutant and wild type with respect to autolysis. Data shown is from 3 duplicate experiments.

2.4 Conclusion

The data presented in this chapter makes a strong case for the production of hydrogen peroxide by pyruvate oxidase activity allowing for the release of PLY *in vitro*. Initially to investigate the impact of pyruvate oxidase on PLY release, we examined a panel of wild type pneumococcal strains, WU2, AW267, T4, and T4R and their isogenic Δ*spxB* mutants to determine the extent to which H_2O_2 impact PLY release. Removal of pyruvate oxidase in strains WU2, AW267, and T4 causes a significant ($p<0.005$)

reduction in the amount of PLY detected in the supernatant by western blot. Additionally, this trend is seen in T4R, with a significant ($P<0.05$) reduction observed when *spxB* is deleted. This indicates that the effect appears to be general across strains, lending strong evidence to support the hypothesis that *spxB* activity leads to PLY release.

The case for H_2O_2 causing PLY release is further strengthened by the presentation of a statistically significant correlation ($p<.05$) that exists between H_2O_2 production and PLY release in a panel of 15 clinical isolates. This indicates that this relationship appears to hold true in a clinically relevant sampling of pneumococcus and could relate to the strain differences in PLY production and H_2O_2 production. Previous data indicated a more statistically significant correlation when bacterial supernatants were unfiltered, this could potentially indicate a role for the association of pneumolysin with the cell wall of the pneumococcus leading to a deleterious impact on the correlation (26). However, this data supports the data shown for the removal of *spxB*.

Finally, qRT-PCR data indicates that the reduction in PLY that has been observed in strains WU2 and AW267 is not attributable to the reduction in the transcription of the *ply* gene when *spxB* is deleted from the chromosome. This appears to concur with previous data showing that the location of pneumococcal growth causes no significant difference in the transcription of *ply in vivo* (27). This could indicate that *spxB* is acting as an oxygen dependent switch for the release of PLY, for example with the organism releasing more PLY in areas where high amounts of oxygen are available for the organism, like the nasopharynx, where PLY could aid in colonization via biofilm formation (30).

Removal of extracellular H_2O_2 via catalase only appears to significantly reduce the amount of PLY released by the pneumococcus in the strain WU2. This effect does not appear for T4, T4R, or AW267. This seems to indicate that the location of the action of H_2O_2 could vary in a strain dependent manner. In order to investigate if pneumococcal H_2O_2 was leading to a loss of membrane potential, and thus leakiness that could be responsible for the release of PLY, we utilized the JC-1 ratiometric dye. However, preliminary data from the JC-1 ratiometric potential assay (Appendix 3) appears to indicate that perhaps the 1 hour time of application of H_2O_2 was insufficient to achieve the membrane depolarization that could be attributable to release. The data found in this preliminary JC-1 assay appears to indicate that wild-type strains undergo a loss of membrane potential that is not seen in the Δ*spxB* mutants, indicating that pneumococcal H_2O_2 could be the cause. However, these findings still need to be verified by a secondary method like $DiBAC_3(4)$, another potentiometric dye, and Propidium iodide, a cell permeability dye. Future investigation could likely increase the concentration of H_2O_2 supplemented to the bacteria as the non-lethality of the H_2O_2 was confirmed via serial dilution plate counts before and after supplementation with H_2O_2 (Appendix 2).

Finally, it appears that differences in autolysis are not a likely explanation for the increased amount of PLY found in the supernatant for the wild type. No difference in the rate of autolysis was observed when T4R and T4RΔ*spxB* was treated with 0.1% Triton X-100. Additionally, no difference was observed in the amount of DNA observed in the supernatant, which we expect would only be detected due to autolysis.

These findings are interesting since PLY is not the only protein that is excreted by the pneumococcus without a transport sequence. The literature has previously indicated

that both enolase and GAPDH/SDH are proteins that can be exported to the exterior of the pneumococcus without any discernable N-terminal secretion signal (28, 29). It would be interesting to investigate if the potential mechanism that has been elucidated in this investigation would apply to the secretion of these proteins as well. As it appears that the mechanism is conserved across a variety of pneumococcal strains. Furthermore, the ubiquity of this method of release would be interesting to investigate in other organisms that produce H_2O_2 as a potentially generalized mechanism of protein release. Finally, considering that pyruvate oxidase would be more utilized in an aerobic environment such as the nasopharynx, considering the importance of biofilm formation for colonization of the nasopharynx, and the positive relationship of PLY on the formation of biofilms, this could potentially be a way for the pneumococcus to control the amount of PLY that is released in an oxygen dependent manner (30, 31).

2.5 References

1. O'Brien, K. L., L. J. Wolfson, J. P. Watt, E. Henkle, M. Deloria-Knoll, N. McCall, E. Lee, K. Mulholland, O. S. Levine, and T. Cherian. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 374:893–902.

2. Musher, DM. 1992. Infections caused by Streptococcus pneumoniae: clinical spectrum, pathogenesis, immunity, and treatment. Clinical infectious diseases.

3. Gates, G. A. 1996. Cost-effectiveness considerations in otitis media treatment. Otolaryngol Head Neck Surg 114:525–30.

4. Marcy, S. Management of acute otitis media. *The Pediatric Infectious Disease Journal* 22, 673-674 (2003).

5. Mitchell, AM, and TJ Mitchell. 2010. Streptococcus pneumoniae: virulence factors and variation. Clinical Microbiology and Infection. Wiley Online Library 16:411–418.

6. Quin, LR, QC Moore, and LS McDaniel. 2007. Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. Infection and immunity.

7. Camara, M, GJ Boulnois, and PW Andrew. 1994. A neuraminidase from Streptococcus pneumoniae has the features of a surface protein. Infection and immunity

8. Mitchell, T. J., and C. E. Dalziel. 2014. The biology of pneumolysin. Subcell. Biochem. 80:145–60.

9. Schnupf, P., J. Zhou, A. Varshavsky, and D. A. Portnoy. 2007. Listeriolysin O secreted by Listeria monocytogenes into the host cell cytosol is degraded by the N-end rule pathway. Infection and immunity 75:5135–47.

10. Tweten, R. K. 1989. Cloning and expression in Escherichia coli of the perfringolysin O (theta-toxin) gene from Clostridium perfringens and characterization of the gene product. Infection and immunity 56:3228–34.

11. Boulnois, G., Paton, J., Mitchell, T. & Andrew, P. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of Streptococcus pneumoniae. *Molecular Microbiology* 5, 2611-2616 (1991).

12. Marriott, H., Mitchell, T. & Dockrell, D. Pneumolysin: A Double-Edged Sword During the Host-Pathogen Interaction. *CMM* 8, 497-509 (2008).

13. Benton, KA, JC Paton, and DE Briles. 1997. Differences in virulence for mice among Streptococcus pneumoniae strains of capsular types 2, 3, 4, 5, and 6 are not attributable to differences in pneumolysin. Infection and immunity. Apr; 65(4): 1237–1244. (1997).

14. 18.Paton, J., Andrew, P., Boulnois, G. & Mitchell, T. Molecular Analysis of The Pathogenicity of Streptococcus Pneumoniae: The Role of Pneumococcal Proteins. *Annu. Rev. Microbiol.* 47, 89-115 (1993).

15. Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois. 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of Streptococcus pneumoniae. Infection and immunity 55:1184–9.

16. Claverys, JP, B Martin, and LS Håvarstein. 2007. Competence- induced fratricide in streptococci. Molecular microbiology.

17. Balachandran, P., S. K. Hollingshead, J. C. Paton, and D. E. Briles. 2001. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. Journal of bacteriology 183:3108–16.

18. Spellerberg, B., D. R. Cundell, J. Sandros, B. J. Pearce, I. Idanpaan-Heikkila, C. Rosenow, and H. R. Masure. 1996. Pyruvate oxidase, as a determinant of virulence in Streptococcus pneumoniae. Mol. Microbiol. 19:803–13.

19. Taniai, H., K. Iida, M. Seki, M. Saito, S. Shiota, H. Nakayama, and S. Yoshida. 2008. Concerted action of lactate oxidase and pyruvate oxidase in aerobic growth of Streptococcus pneumoniae: role of lactate as an energy source. J. Bacteriol. 190:3572–9.

20. Regev-Yochay, G., K. Trzcinski, C. M. Thompson, M. Lipsitch, and R. Malley. 2007. SpxB is a suicide gene of Streptococcus pneumoniae and confers a selective advantage in an in vivo competitive colonization model. J. Bacteriol. 189:6532–9.

21. Pericone, C. D., S. Park, J. A. Imlay, and J. N. Weiser. 2003. Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. J. Bacteriol. 185:6815–25.

22. Pericone, C. D., K. Overweg, P. W. Hermans, and J. N. Weiser. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infection and immunity 68:3990–7.

23. Regev-Yochay, G., K. Trzcinski, C. M. Thompson, R. Malley, and M. Lipsitch. 2006. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. J. Bacteriol. 188:4996–5001.

24. Duane, P. G., J. B. Rubins, H. R. Weisel, and E. N. Janoff. 1993. Identification of hydrogen peroxide as a Streptococcus pneumoniae toxin for rat alveolar epithelial cells. Infection and immunity 61:4392–7.

25. Zysk, G., L. Bejo, B. K. Schneider-Wald, R. Nau, and H. Heinz. 2000. Induction of necrosis and apoptosis of neutrophil granulocytes by Streptococcus pneumoniae. Clinical and experimental immunology 122:61–6.

26. Price, KE, and A Camilli. 2009. Pneumolysin localizes to the cell wall of Streptococcus pneumoniae. Journal of bacteriology.

27. LeMessurier, K. S., A. D. Ogunniyi, and J. C. Paton. 2006. Differential expression of key pneumococcal virulence genes in vivo. Microbiology (Reading, Engl.) 152:305–11.

28. Pancholi, V, and VA Fischetti. 1998. α-Enolase, a novel strong plasmin (ogen) binding protein on the surface of pathogenic streptococci. Journal of Biological Chemistry.

29. Jin, H., S. Agarwal, S. Agarwal, and V. Pancholi. 2011. Surface export of GAPDH/SDH, a glycolytic enzyme, is essential for Streptococcus pyogenes virulence. MBio 2:e00068–11.

30. Shak, JR, HP Ludewick, KE Howery, F Sakai, and H Yi. 2013. Novel role for the Streptococcus pneumoniae toxin pneumolysin in the assembly of biofilms. MBio.

31. Kadioglu, A., J. Weiser, J. Paton, and P. Andrew. 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nature Reviews Microbiology.

32. Rai, P. et al. Streptococcus pneumoniae secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells. Proc Natl Acad Sci USA 201424144 (2015). doi:10.1073/pnas.1424144112

CHAPTER III

CONCLUSION

The pneumococcus is a major human pathogen causing significant morbidity and mortality worldwide (1). It is the causative agent of a number of invasive human diseases such as bacteremia, septicemia, otitis media, meningitis, and pneumonia (1, 2). Despite this major impact on human health, however, pneumococcus is carried as a commensal organism in the nasopharynx in up to 70% of individuals, depending on the population (3). A better understanding of how the organism is able to utilize the myriad of virulence factors that it is capable of expressing is important to developing future treatments for pneumococcal disease, and reducing its burden on health care systems worldwide.

The literature review presented in the first chapter of this thesis examined the role that *Streptococcus pneumoniae* plays in human health as a major causative agent of disease when the immune system becomes compromised (4). An important aspect of the pneumococcus' ability to cause disease is its wide range of virulence factors that it possesses. Our work focused on two virulence factors in particular, pyruvate oxidase and pneumolysin (PLY). Pyruvate oxidase is an enzyme that allows for the aerobic metabolism of pyruvate in order to phosphorylate ADP to produce ATP (5). Through the activity of this enzyme, the pneumococcus is capable of producing up to millimolar concentrations of H_2O_2. This is significant as the organism is catalase negative and thus has limited capacity to neutralize the H_2O_2 that is produced as a byproduct (6). However,

expression of pyruvate oxidase is seen to confer a strong competitive advantage to the pneumococcus in the nasopharynx, allowing it to outcompete other potential inhabitants by exploiting their sensitivity to hydrogen peroxide (7, 8). Additionally, removal of pyruvate oxidase appears to attenuate virulence in mouse models and confer resistance to killing by H_2O_2 (9, 10). Pneumococcal H_2O_2 also acts as a potent virulence factor for host cells, causing necrosis, apoptosis, and DNA damage (15, 16).

In addition to pyruvate oxidase, the pneumococcus possesses another virulence factor, the 53-kDa cholesterol dependent pore forming cytolysin, pneumolysin (11). While this toxin acts on host cells extracellularly, it is not well characterized how this protein is secreted from the bacteria (12–14). A number of mechanisms have been observed, such as autolysis via *lytA* releasing intracellular products through breaking down the cell wall, competence induced fratricide of non-competent cells, and other release that is not attributable to the activity of *lytA* (17–19). Based on the autolytic effect of pyruvate oxidase activity, we investigated a potential link between H_2O_2 production and PLY release in pneumococcus.

Chapter 2 summarizes our findings determining the impact of pyruvate oxidase on the release of PLY in the pneumococcus. Initially, the impact of the deletion of *spxB* from the chromosome on the release of PLY to the extracellular space was investigated. Removal of *spxB* was found to greatly attenuate the amount of H_2O_2 found in the supernatant. Strains WU2, AW267, and T4 with deletion of *spxB,* resulted in a significant ($p<0.005$) reduction in the amount of PLY detected via western blot. Additionally, a significant ($p<0.05$) reduction was also observed in T4R, indicating that this mechanism of release could be general and likely is not specific to only a few strains. Also, deletion

of *spxB* greatly reduced the amount of H_2O_2 produced *in vitro.* After this finding, the relevance of this effect was investigated in a panel of 15 clinical isolates obtained from St. Jude's Children's Hospital. Results showed that there was a significant correlation ($p<0.05$, $r^2=.3167$) between the amount of PLY released (quantitated via dot blot) and H_2O_2 produced when normalized to bacterial counts. This further supports the evidence obtained through the deletion of the *spxB* gene. Analysis of the transcription of the *ply* gene when *spxB* was deleted in AW267, WU2, and T4R indicated that in each case, transcription of the *ply* gene was upregulated (WU2Δ*spxB* = 1.96 fold, AW267Δ*spxB* = 2.83 fold, and T4RΔ*spxB* = 2.3 fold). This confirmed that the reduction in PLY release observed upon the deletion of the *spxB* gene was not due to altered expression of Ply but was indeed due to abrogated PLY release into the extracellular space. Additionally, supplementation of 500μM H_2O_2 to T4RΔ*spxB* for 1 hour resulted in a 1.45 fold upregulation of the *ply* gene, indicating that exogenously supplemented H_2O_2 had no significant impact upon *ply* transcription.

In order to ascertain if H_2O_2 acts extracellularly, we treated wild type strains with catalase and found a significant reduction in the amount of PLY released ($p<0.05$) in the strain WU2. However, this effect was not observed in any other strain, indicating that the mechanism by which H_2O_2 acts could potentially be variable. Finally the impact of H_2O_2 upon the membrane potential of the pneumococcus was investigated utilizing the JC-1 ratiometric membrane potential dye. This study was conducted in order to determine whether pneumococcal hydrogen peroxide could be reducing membrane potential, leading to the cell becoming leaky with release of intracellular components. The results from the preliminary assay suggest that T4R experiences a drop in membrane potential

after 1 hour of growth that was not seen in T4RΔ*spxB*. Additionally, a similar loss of membrane potential was observed in T4RΔ*spxB* treated with 1 mM H_2O_2 at the 1 hour time point as well. While this preliminary data indicates that pneumococcal H_2O_2 could be causing the membrane to become leaky, the findings need to be confirmed with a second assay utilizing $DiBAC_3(4)$, another potentiometric dye, and Propidium Iodide, a cell impermeable dye to determine if the findings are accurate. Finally, autolysis was ruled out as a cause for the difference in release between the mutant and wild type as no discernable difference in autolysis was found in T4R and T4RΔ*spxB*.

In this work, a novel mechanism of transport for intracellular proteins to the extracellular space in *Streptococcus pneumoniae* has been identified. Future directions would determine whether *spxB* complementation of mutant strains would restore the capacity to release PLY. This has currently been achieved in strains T4RΔ*spxB* and T4Δ*spxB* through ligating the SpxB gene to the PNE-1 pneumococcal shuttle vector. However, analysis of the nature of the complemented strain's PLY release has not yet been analyzed. Further research on this mechanism could also lead to insights into mechanisms that block PLY release in the pneumococcus and attenuate virulence, leading to new treatments for pneumococcal disease. Additionally, this mechanism should be of interest for other proteins that are secreted by the pneumococcus but lack secretion signals, such as enolase and GAPDH/SDH, as it would be interesting to investigate how the impairing H_2O_2 expression impacts the secretion of these proteins. (20, 21) Even more significant would be the application of this mechanism of transport to other H_2O_2 producing bacteria, to determine whether this could potentially have applications in other organisms.

3.1 References

1. O'Brien, K. L., L. J. Wolfson, J. P. Watt, E. Henkle, M. Deloria-Knoll, N. McCall, E. Lee, K. Mulholland, O. S. Levine, and T. Cherian. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 374:893–902.

2. Musher, DM. 1992. Infections caused by Streptococcus pneumoniae: clinical spectrum, pathogenesis, immunity, and treatment. Clinical infectious diseases.

3. Waroux, O., S. Flasche, D. Prieto-Merino, and J. W. Edmunds. 2014. Age-Dependent Prevalence of Nasopharyngeal Carriage of Streptococcus pneumoniae before Conjugate Vaccine Introduction: A Prediction Model Based on a Meta-Analysis. PloS one. Public Library of Science 9:e86136.

4. Sousa, D. et al. Community-acquired pneumonia in immunocompromised older patients: incidence, causative organisms and outcome. *Clinical Microbiology and Infection* 19, 187-192 (2013).

5. Taniai, H., K. Iida, M. Seki, M. Saito, S. Shiota, H. Nakayama, and S. Yoshida. 2008. Concerted action of lactate oxidase and pyruvate oxidase in aerobic growth of Streptococcus pneumoniae: role of lactate as an energy source. J. Bacteriol. 190:3572–9.

6. McLeod, JW, and J Gordon. 1922. Production of hydrogen peroxide by bacteria. Biochemical Journal.

7. Regev-Yochay, G., K. Trzcinski, C. M. Thompson, R. Malley, and M. Lipsitch. 2006. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. J. Bacteriol. 188:4996–5001.

8. Pericone, C. D., K. Overweg, P. W. Hermans, and J. N. Weiser. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infect. Immun. 68:3990–7.

9. Regev-Yochay, G., K. Trzcinski, C. M. Thompson, M. Lipsitch, and R. Malley. 2007. SpxB is a suicide gene of Streptococcus pneumoniae and confers a selective advantage in an in vivo competitive colonization model. J. Bacteriol. 189:6532–9.

10. Pericone, C. D., S. Park, J. A. Imlay, and J. N. Weiser. 2003. Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. J. Bacteriol. 185:6815–25.

11. Zysk, G., L. Bejo, B. K. Schneider-Wald, R. Nau, and H. Heinz. 2000. Induction of necrosis and apoptosis of neutrophil granulocytes by Streptococcus pneumoniae. Clin. Exp. Immunol. 122:61–6.

12. Duane, P. G., J. B. Rubins, H. R. Weisel, and E. N. Janoff. 1993. Identification of hydrogen peroxide as a Streptococcus pneumoniae toxin for rat alveolar epithelial cells. Infect. Immun. 61:4392–7.

13. Mitchell, AM, and TJ Mitchell. 2010. Streptococcus pneumoniae: virulence factors and variation. Clinical Microbiology and Infection. Wiley Online Library 16:411–418.

14. Braun, J. S., J. E. Sublett, D. Freyer, T. J. Mitchell, J. L. Cleveland, E. I. Tuomanen, and J. R. Weber. 2002. Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. J. Clin. Invest. 109:19–27.

15. Mitchell, T. J., and C. E. Dalziel. 2014. The biology of pneumolysin. Subcell. Biochem. 80:145–60.

16. Martner, A., C. Dahlgren, J. C. Paton, and A. E. Wold. 2008. Pneumolysin released during Streptococcus pneumoniae autolysis is a potent activator of intracellular oxygen radical production in neutrophils. Infection and immunity. 76:4079–87.

17. Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois. 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of Streptococcus pneumoniae. Infection and immunity. 55:1184–9.

18. Claverys, JP, B Martin, and LS Håvarstein. 2007. Competence- induced fratricide in streptococci. Molecular microbiology.

19. Balachandran, P., S. K. Hollingshead, J. C. Paton, and D. E. Briles. 2001. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. J. Bacteriol. 183:3108–16.

20. Jin, H., S. Agarwal, S. Agarwal, and V. Pancholi. 2011. Surface export of GAPDH/SDH, a glycolytic enzyme, is essential for Streptococcus pyogenes virulence. MBio 2:e00068–11.

21. Pancholi, V, and VA Fischetti. 1998. α-Enolase, a novel strong plasmin (ogen) binding protein on the surface of pathogenic streptococci. Journal of Biological Chemistry.

APPENDIX A

SUPPLEMENTAL DATA

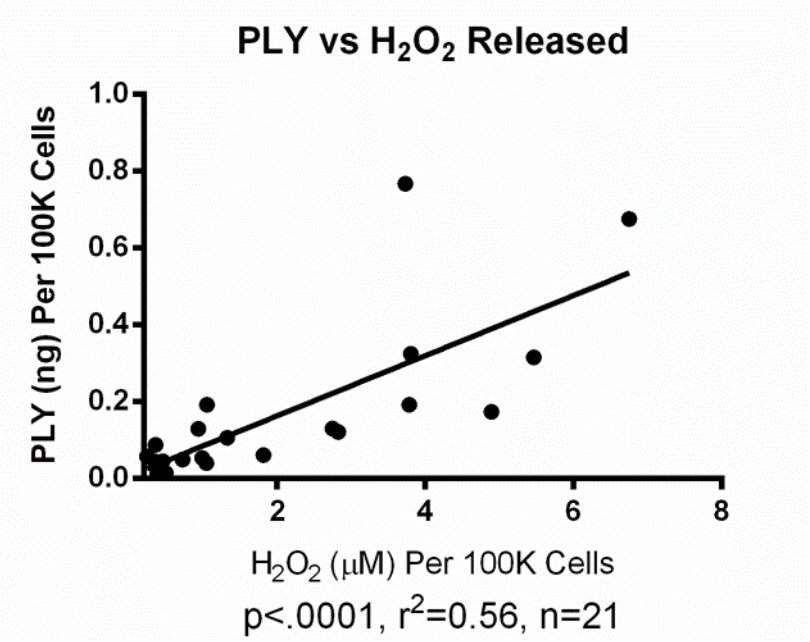

Figure A.1 Correlation of H_2O_2 produced and PLY released in unfiltered clinical supernatants

This figure represents previously collected clinical data, wherein a stronger correlation was observed with unfiltered supernatants. This graph represents a significant correlation ($p<0.0001$, $r^2=0.56$, $n=21$) between H_2O_2 per 100,000 cells, and PLY per 100,000 cells.

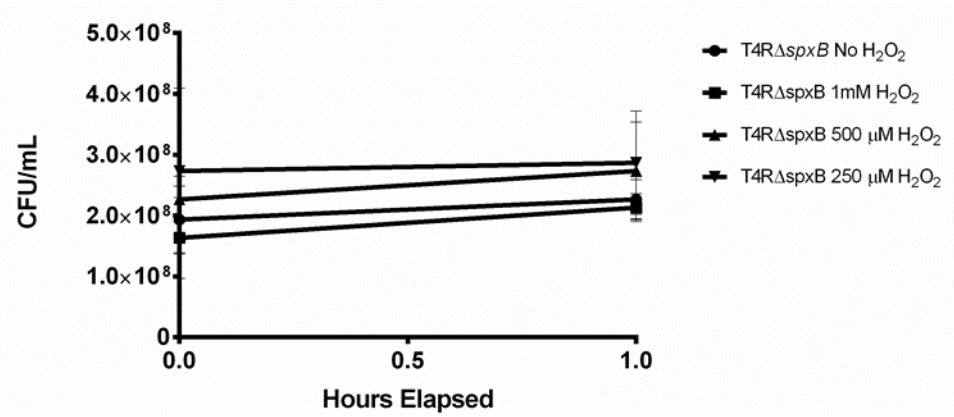

Figure A.2 Cell counts following H_2O_2 treatment

Plate counts of T4RΔ*spxB* were performed before and after incubation with H_2O_2 at the specified concentrations for 1 hour on ice. No significant difference was observed between any concentration of H_2O_2 and the untreated wild type.

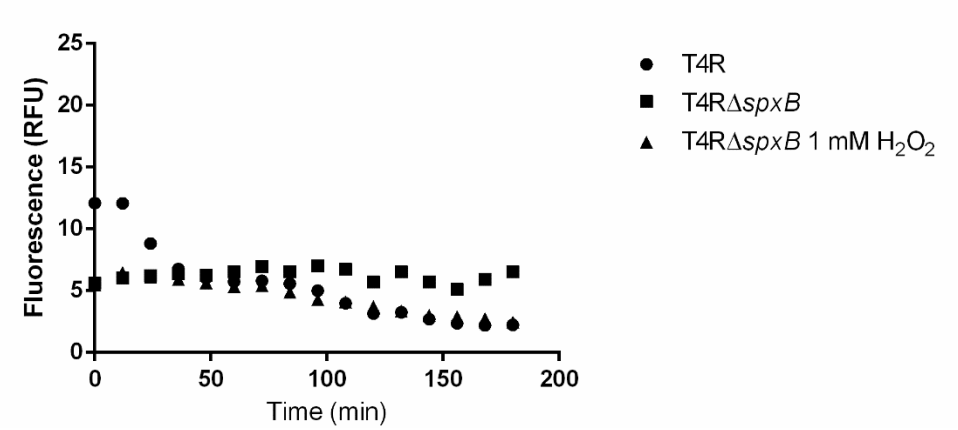

Figure A.3 JC-1 Ratiometric Analysis of T4R

A ratiometric analysis was used as an attempt to assess membrane potential. This data appears to suggest a difference in membrane permeability between the mutant when compared to the wild type and the mutant supplemented with catalase.

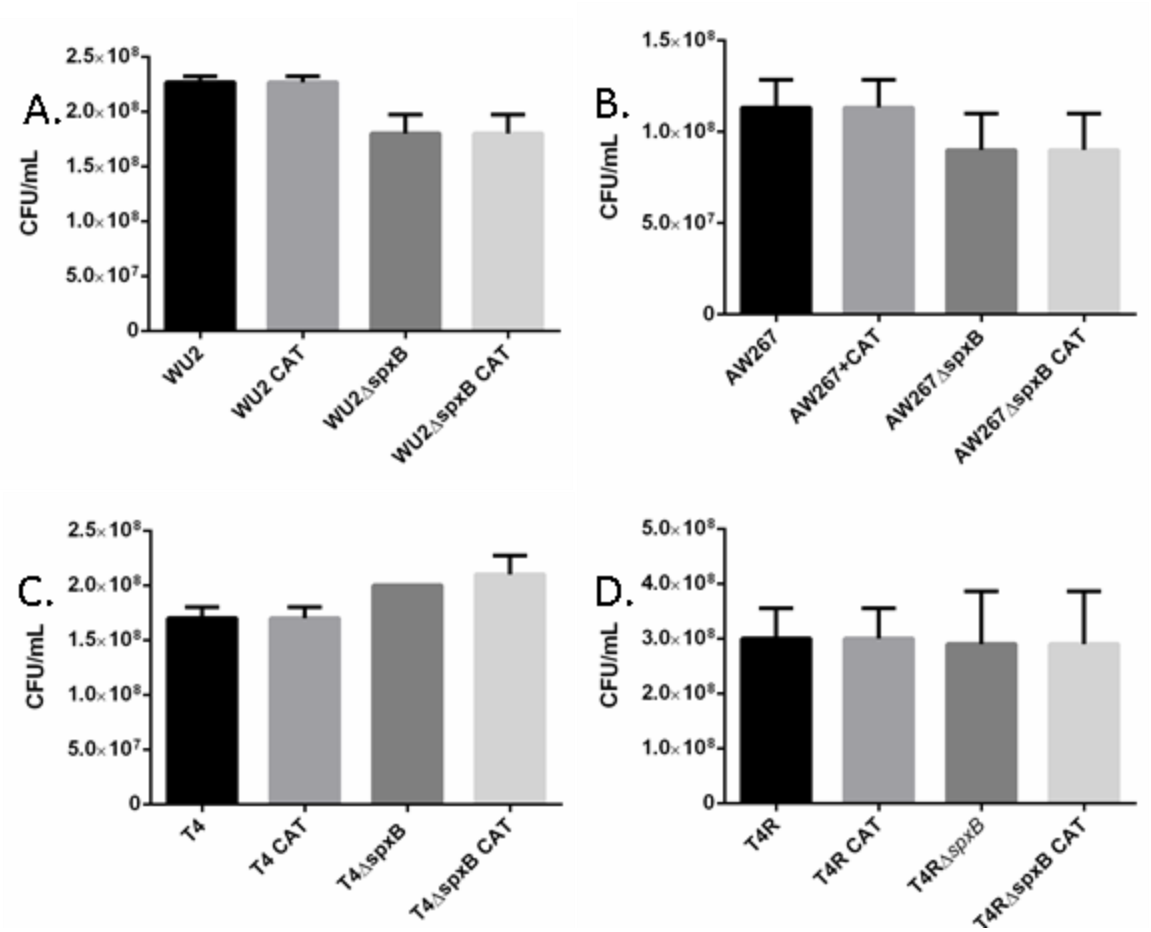

Figure A.4 Bacterial counts

Bacterial counts at 0.5 are shown for AW267 (Appendix 4A), WU2 (Appendix 4B), T4 (Appendix 4C), and T4R (Appendix 4D) with their respective Δ*spxB* mutants with and without 10µg of catalase added. Bacteria were serially diluted to determine original concentration. This data indicates that there is no significant difference in bacterial counts for each of the strains investigated. The data shown is an average of 3 experiments each plus and minus the standard deviation for each data set.

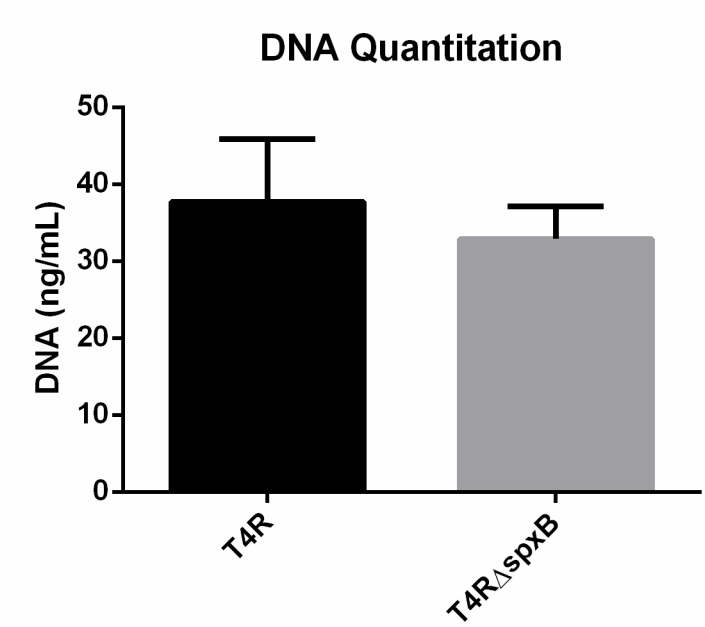

Figure A.5 DNA quantitation

The fluorometric quantitation of the DNA contained in the sterile supernatant of T4R and T4RΔ*spxB* at OD 0.5. No significant difference was observed between the two, indicating no significant difference in autolysis. This figure represents 3 indpendent experiments, plus and minus the standard deviation of the average value.

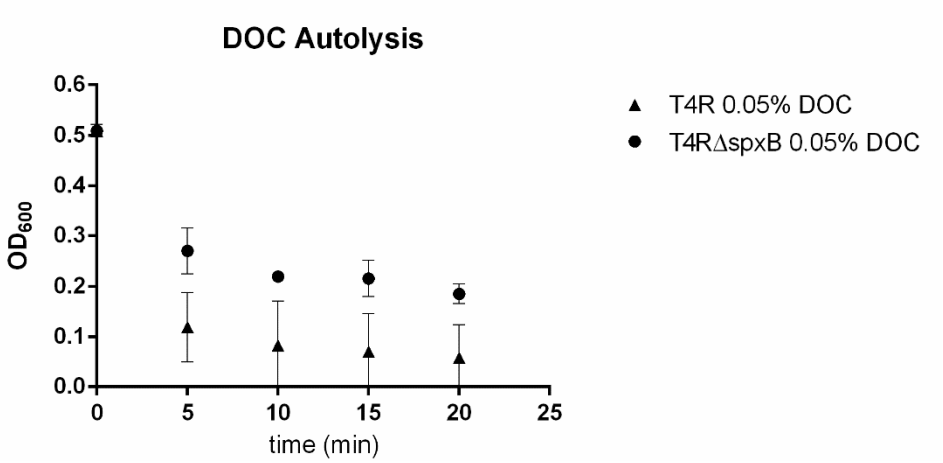

Figure A.6 Assessment of autolysis by DOC

Quantitation of the difference of autolysis in T4R and T4RΔ*spxB* following supplementation with 0.05% deoxycholate at OD 0.5 in THY media.