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Antibiotic Tolerance and Heteroresistance: Associated Fitness Costs and Potential in Evading Antibiotic Killing

Abstract

Streptococcus pneumoniae is a prominent human pathogen that causes both invasive and non-invasive diseases, such as otitis media, pneumonia, meningitis, and bacteremia. Although it is frequently an asymptomatic colonizer of the human nasopharynx, *S. pneumoniae* is a major cause of morbidity and mortality in the immune compromised population, young children, and the elderly. Up until the 1970s, *S. pneumoniae* was susceptible to almost all antibiotics. Since then, this pathogen has gained resistance to a variety of antibiotic treatments, including beta-lactams, macrolides, and fluoroquinolones.

In the first chapter, we focused on fluoroquinolone resistance in *S. pneumoniae*. Fluoroquinolones are one of the most frequently prescribed antibiotics, yet fluoroquinolone resistance in *S. pneumoniae* is still rare compared to other antibiotics resistance, such as beta-lactams. In this study, we investigated the mechanism(s) underlying this intriguing case by assessing the efficiency and fitness costs of horizontal transfer of fluoroquinolone resistance determinants. We hypothesized that the fitness tradeoffs incurred by resistance determinants would define the likelihood of such resistance to emerge in a clinical setting. Clinically relevant fluoroquinolone resistance requires both on-target mutations in topoisomerase IV *parC* and DNA gyrase *gyrA*. The wild-type *S. pneumoniae* TIGR4 was not readily transformed with single mutations in *gyrA* or *parC*; however, it was readily transformed with double on-target mutations in *gyrA* and *parC*. Compared to the wild type, the single on-target mutants were attenuated, whereas the double on-target mutant was virulent. This suggests that clinically relevant, high-level fluoroquinolone resistance requires the combination of several on-target mutations, which could be acquired via horizontal transfer. The combination of the extremely low probability of acquiring two or more mutations simultaneously from different target genes and the deleterious fitness tradeoffs imposed by individual on-target mutations in *gyrA* or *parC* likely result in the infrequent prevalence of fluoroquinolone resistance in *S. pneumoniae*. Through in vitro serial passaging, we identified a novel mutation (N291D) in the efflux pump *patA* that facilitated the acquisition of the on-target mutations in *parC* and *gyrA* via horizontal transfer with minimal fitness tradeoffs. We also modeled the evolution of fluoroquinolone resistance in a murine host and identified mutation(s) that arose and fixated during in vivo passaging. Interestingly, the experimentally-evolved isolates from the in vivo passaging study did not encode on-target mutations for fluoroquinolone resistance and instead displayed tolerance, which potentially facilitated the subsequent acquisition of fluoroquinolone resistance.

In the next chapter, we investigated how fitness tradeoffs and horizontal transfer play a role in the emergence and spread of another mainstay of treatment of pneumococcal infection, beta-lactams—specifically, penicillin, which inhibit wall synthesis. We found that recombination with related viridans species via horizontal transfer may be preferable to de novo on-target mutations in penicillin-binding proteins in *S. pneumoniae* to acquire resistance more rapidly without initially losing in vivo fitness. Initial recombinants retained virulence in vivo and could readily acquire higher resistance via subsequent transformation. The final recombinants displayed tolerance to penicillin, having reduced kill kinetics compared to the wild type. This suggests that *S. pneumoniae* might have minimized fitness tradeoffs by developing tolerance via horizontal transfer with related viridans group streptococci, which would serve as a stepping stone for subsequent development of resistance.

In the next study, we explored an underlying mechanism of antibiotic tolerance in *S. pneumoniae*. In our model of the evolution of antibiotic resistance, *rny* that encodes ribonuclease Y (RNase Y) was a mutational hotspot across multiple antibiotics. The *rny* knockout mutant was fully virulent, indicating that deletion of this gene imposed minimal to no fitness tradeoffs. Disruptions in RNA degradation resulted in tolerance to several classes of antibiotics and reduced antibiotic treatment efficacy in vivo.

In the final chapter, we investigated whether other phenomena that allow bacteria to withstand antibiotic killing, such as heteroresistance, can affect antibiotic treatment outcomes clinically. We found that vancomycin heteroresistance is associated with treatment failure and poor outcomes in coagulase-negative staphylococci (CoNS) from pediatric leukemia patients.

Taken together, this dissertation provides insights into strategies of *S. pneumoniae* for striking a balance between maximizing resistance potential while minimizing fitness tradeoffs, thereby potentially contributing to the development of more-effective antibiotics for treatment of pneumococcal disease. It also provided insights into the association between heteroresistance in CoNS and clinical outcomes.

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UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTORAL DISSERTATION

**Antibiotic Tolerance and Heteroresistance:
Associated Fitness Costs and Potential in Evading
Antibiotic Killing**

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Tina H. Dao

Advisor:

Jason W. Rosch, PhD

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The University of Tennessee*

in

*Biomedical Sciences: Microbiology, Immunology, and Biochemistry
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DEDICATION

To my late father Hong Dao and my mother Nga Pham for their unconditional love,
sacrifice, and unwavering support.

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As I reflect on my research journey thus far, I feel very grateful and fortunate to have been surrounded by the talented and supportive group of mentors, professors, lab mates, family, and friends. I probably would not be able to fulfill this journey without the following individuals. First and foremost, I want to express my sincere appreciation to my PI, Jason Rosch PhD, who had relentlessly guided and encouraged me since I first started my research journey. I came to the Rosch laboratory as a rising sophomore in college without much prior research skills and experience. I have grown up as a scientist in the Rosch laboratory. I always admire his talents, brilliant ideas, and patience.

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ABSTRACT

Streptococcus pneumoniae is a prominent human pathogen that causes both invasive and non-invasive diseases, such as otitis media, pneumonia, meningitis, and bacteremia. Although it is frequently an asymptomatic colonizer of the human nasopharynx, *S. pneumoniae* is a major cause of morbidity and mortality in the immune compromised population, young children, and the elderly. Up until the 1970s, *S. pneumoniae* was susceptible to almost all antibiotics. Since then, this pathogen has gained resistance to a variety of antibiotic treatments, including beta-lactams, macrolides, and fluoroquinolones.

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In the next chapter, we investigated how fitness tradeoffs and horizontal transfer play a role in the emergence and spread of another mainstay of treatment of pneumococcal infection, beta-lactams- specifically, penicillin, which inhibit wall synthesis. We found that recombination with related viridans species via horizontal transfer may be preferable to *de novo* on-target mutations in penicillin-binding proteins in *S. pneumoniae* to acquire resistance more rapidly without initially losing *in vivo* fitness.

Initial recombinants retained virulence *in vivo* and could readily acquire higher resistance via subsequent transformation. The final recombinants displayed tolerance to penicillin, having reduced kill kinetics compared to the wild type. This suggests that *S. pneumoniae* might have minimized fitness tradeoffs by developing tolerance via horizontal transfer with related viridans group streptococci, which would serve as a stepping stone for subsequent development of resistance.

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In the final chapter, we investigated whether other phenomena that allow bacteria to withstand antibiotic killing, such as heteroresistance, can affect antibiotic treatment outcomes clinically. We found that vancomycin heteroresistance is associated with treatment failure and poor outcomes in coagulase-negative staphylococci (CoNS) from pediatric leukemia patients.

Taken together, this dissertation provides insights into strategies of *S. pneumoniae* for striking a balance between maximizing resistance potential while minimizing fitness tradeoffs, thereby potentially contributing to the development of more-effective antibiotics for treatment of pneumococcal disease. It also provided insights into the association between heteroresistance in CoNS and clinical outcomes.

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LIST OF ABBREVIATIONS

BCA	Bicinchoninic Acid Assay
CFU	Colony Forming Unit
CLSI	Clinical Laboratory and Standards Institute
CoNS	Coagulase-Negative Staphylococci
CSP	Competence-Stimulating Peptide
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
qT-PCR	Real-time Polymerase Chain Reaction
<i>rny</i>	RNase Y gene
TIGR4	wild-type <i>S. pneumoniae</i> serotype 4 (The Institute for Genomic Research)

CHAPTER 1. INTRODUCTION

Streptococcus pneumoniae Epidemiology

Streptococcus pneumoniae was first identified in 1881 and recognized as the causative agent of lobar pneumoniae in the late 1880s [1, 2]. It is a major human pathogen, especially in the pediatric (children under 2 years), geriatric, and immunocompromised populations worldwide (>65 years) [3]. Other high-risk groups include those with chronic lung disease, diabetes, asthma, smoking, alcohol abuse, solid or hematologic malignancies, and sickle cell disease [4]. Although *S. pneumoniae* is usually an asymptomatic member of the nasopharynx microbiota, it has the remarkable ability to invade different human organs, causing mild to severe infection, such as otitis media, pneumonia, meningitis, bacteremia, and sepsis [5].

S. pneumoniae Microbiology

S. pneumoniae is a non-motile, alpha-hemolytic, Gram-positive encapsulated bacterium. The bacteria appear as lancet-shaped diplococci and are identified in the laboratory by their sensitivity to optochin and bile-mediated lysis (deoxycholate) [6]. The whole genome of *S. pneumoniae* TIGR4 (serotype 4) from a patient with meningitis was sequenced and released by the Institute for Genetic Research in 1997. Since then, other clinical strains have also been sequenced. Significant genetic diversity (up to 20%) in DNA composition among clinical strains have been observed [7, 8]. Whole genome sequence analysis indicates that *S. pneumoniae* lacks genes required for a complete tricarboxylic acid cycle and the respiratory electron transport chain [9]. Instead, *S. pneumoniae* generates energy from fermentation and metabolism of a wide range of carbohydrates and glycans [10]. This ability enables the bacteria to adapt and survive in different host niches that contain distinct prevailing carbohydrates.

Transformation

Naturally competent for genetic transformation, *S. pneumoniae* can uptake and recombine exogenous DNA into its genome for genetic variability and advantageous traits. Horizontal transfer via transformation is a main driver of adaptation and antibiotic resistance in this species. This phenomenon of transformation was first studied *in vivo* by Frederick Griffith [11], fueling the discovery of DNA as the hereditary material [12]. Transformation in *S. pneumoniae* is activated by a competence stimulating peptide (CSP), which is a pheromone that competent bacterial cells produce to initiate the uptake and homologous recombination of exogenous DNA. *S. pneumoniae* utilizes this transformation machinery to acquire antibiotic resistance determinants and other advantageous genes from within species and closely related species coinhabiting similar host niches, such as *S. mitis* and *S. oralis* [13-15]. For example, it has been shown that *S.*

pneumoniae and *S. mitis* exchange distinct fluoroquinolone or beta-lactams resistance-conferring regions in penicillin-binding proteins and DNA gyrase [16-18].

Current Antibiotic Treatment for Pneumococcal Infection

For mild cases of pneumococcal infection in which oral antibiotics are warranted, amoxicillin, second- or third-generation cephalosporins, or oral levofloxacin, if the strain is susceptible, are recommended [19]. For mild to moderate pediatric pneumococcal pneumonia caused by penicillin-sensitive strains, amoxicillin is the first-line therapy. Alternatives include third-generation cephalosporins, including ceftriaxone or cefotaxime, for patient populations with widespread penicillin resistance. For community-acquired pneumonia in adults, amoxicillin, which is still a drug of choice if the strain is susceptible, macrolides (azithromycin, erythromycin, or clarithromycin), or a respiratory fluoroquinolone (moxifloxacin, gemifloxacin, or levofloxacin) are recommended for mild cases; in severe cases, requiring hospitalization, ceftriaxone or cefotaxime are first line agents, followed by vancomycin for life threatening infection [20]. To treat pneumococcal meningitis caused by susceptible strains, ceftriaxone is the first line of therapy. In intermediate to resistant cases, high dose ceftriaxone or cefotaxime is supplemented with vancomycin.

Antibiotic Resistance in *S. pneumoniae*

For decades, common clinically used antibiotics such as beta- lactams, macrolides, and fluoroquinolones have been the standard treatment for pneumococcal infections. However, there has been a worldwide increase in the number of cases of *S. pneumoniae* resistant to multiple classes of antibiotics, including fluoroquinolones (levofloxacin, ciprofloxacin, and gemifloxacin), folate inhibitors (trimethoprim and sulfamethoxazole), cell-wall synthesis inhibitors (penicillin), and protein synthesis inhibitors (azithromycin, doxycycline, tetracycline, and minocycline [21]. According to the CDC, in 2019, 30% of about 30,000 cases of invasive pneumococcal diseases resulted from *S. pneumoniae* that was resistant to at least one antibiotic [22]. In the United States, approximately 20% to 40% of pneumococcal isolates are resistant to macrolides [21, 23]; approximately 1.4% and 2% are intermediate and resistant to beta-lactams, respectively [24]; and only 1% to 2% are resistant to fluoroquinolones. Antibiotic resistance has complicated treatment for pneumococcal infections and often results in treatment failure in cases that otherwise would have been effectively treated with commonly used antibiotics [25, 26]. The rapid emergence of antibacterial resistance poses a major threat to global public health by rendering standard treatments for pneumococcal infection less effective and more costly.

Beta-Lactams Resistance

Over the past few decades, beta-lactams (such as penicillins, cephalosporins, and carbapenems) have been the first-line treatment of pneumococcal infection. However, global widespread resistance to beta-lactams have complicated pneumococcal infection treatment since the late 1970s [27, 28]. Beta-lactam antibiotics induce bacterial lysis and loss of viability by binding to penicillin-binding proteins, thereby inhibiting cell wall synthesis [29]. Resistance of *S. pneumoniae* to beta-lactams occurs when alterations in the penicillin-binding proteins reduce their binding affinity to the antibiotics [30, 31]. Beta-lactam resistance in *S. pneumoniae* is most often conferred by mosaic variants of the target enzymes—penicillin-binding proteins. The mosaic variants often result from homologous recombination during horizontal transfer within species and/or from related oral streptococcal species, such as *S. mitis* and *S. oralis* [32].

Fluoroquinolone Resistance

Unlike the widespread beta-lactam resistance, fluoroquinolone (FQ) resistance in *S. pneumoniae* is still very low. Only 1 to 2% of pneumococcal isolates in the United States are resistant to fluoroquinolones [33, 34]. The highest prevalence of fluoroquinolone resistance (4-13%) was reported in Croatia and Hong Kong [35, 36]. Fluoroquinolones (such as ciprofloxacin, levofloxacin, and moxifloxacin) inhibit topoisomerase IV (*ParC* and *ParE*) and *gyrA*se (*GyrA*), which are responsible for unwinding supercoiled DNA, thereby halting DNA replication and causing cell death [37]. Mutations in the on-target genes *parC* and/or *gyrA* confer resistance to fluoroquinolone resistance in *S. pneumoniae*. Acquisition of fluoroquinolone resistance occurs in a stepwise fashion thru acquisition of antibiotic resistance determinants or thru adaptation via selection of beneficial *de novo* mutations [38]. It is quite concerning that clinical isolates classified as susceptible might have already harbored single point mutations in either *parC* or *gyrA*, which could acquire subsequent mutations to gain high-level resistance and disseminate.

On-Target versus Off-Target Mutations

There are multiple pathways underlying resistance against an antibiotic. Antibiotic resistance mutations can be classified into two categories: on-target and off-target mutations. Off-target mutations are mutations that occur outside of the primary target(s) of the antibiotic. They are not the direct or intended target(s) of a particular antibiotic but can confer some level of resistance. During an antibiotic exposure, bacteria can activate pathways that are not directly targeted by that antibiotic to survive. Such pathways include stress response, metabolic regulation, transcriptional regulation, efflux / influx regulation, and genes involved in metabolism, transport, and regulation of gene expression [39-41]. For example, fluoroquinolones, such as ciprofloxacin and levofloxacin, inhibit gyrase A and topoisomerase IV activities. Mutations in the genes encoding gyrase A or topoisomerase IV are considered the on-target mutations for

fluoroquinolone resistance, whereas mutations in an efflux pump (for example, *patA*) are considered off-target mutations.

Antibiotic Tolerance and Heteroresistance

Often overshadowed by the prominent phenomenon of antibiotic resistance, tolerance and heteroresistance are among the highly possible culprits of unexplained antibiotic treatment failure and recalcitrant infections [42, 43]. Tolerance refers to the ability of the bacteria to withstand the lethal actions of bactericidal antibiotics without significant increases in the minimum inhibitory concentration (MIC) [44]. While antibiotic resistance is readily detected by traditional clinical MIC assays and thus has been extensively studied and characterized, tolerance and heteroresistance are often overlooked and undetected. Currently, there is no gold standard for identifying antibiotic tolerant strains. Although antibiotic kill kinetics (or time-kill curves) have been conducted to detect antibiotic tolerance, those assays are often laborious and challenging [45]. Despite these obstacles, antibiotic tolerance has been reported in both Gram-negative and Gram-positive bacteria and served as a potential avenue for the subsequent development of high-level resistance.

Heteroresistance has also been reported in multiple pathogens- such as *S. aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*- against a variety classes of antibiotics, such as beta-lactams, vancomycin, aminoglycosides [46, 47]. Heteroresistance is a phenomenon when the majority of the population is susceptible to an antibiotic, while a small proportion of bacterial cells demonstrates resistance to that antibiotic [43]. The resistant subpopulation occurs at a very low frequency, about 1 in 10^5 cells. They are often undetected by clinical testing and usually misclassified as susceptible. In contrast, persistence describes a population in which a small subpopulation (from 0.001% to 1%) is tolerant to an antibiotic [48, 49]. Similar to tolerance, there is no standardized clinical testing method to detect heteroresistance or persistence. The gold standard method to detect heteroresistance is population analysis profile [50]; however, it is extremely laborious and thus not feasible for clinical use. The presence of the resistant subpopulation contributes to reduced efficacy of antibiotics as these bacteria are not effectively eradicated and can potentially expand upon subsequent antibiotic exposure [43, 51, 52]. Emergence of heteroresistance also poses a significant threat to the healthcare community since it potentially precedes antibiotic resistance [53].

Fitness Tradeoff

Bacterial fitness refers to the ability of the bacteria to adapt and thrive in an environment. Antibiotic resistance conferred by on- and/or off-target mutation(s) is often accompanied with fitness costs [54, 55]. While those mutations enable the bacteria to survive under antibiotic pressure, they often render the target enzymes suboptimal in the absence of antibiotics. Therefore, those mutations can reduce bacterial fitness, which manifests as reduced growth rates, virulence, and colonization. Mutations that impart

little to no compromise in fitness costs to the bacteria have a higher chance of surviving and persisting in the population [54, 56].

Hypothesis and Objectives

In this study, we investigated the impact of fitness tradeoffs on the spread and emergence of antibiotic resistance in *S. pneumoniae*. In chapters 2, 3 and 4, we tested our hypothesis that the emergence and spread of fluoroquinolones and beta-lactams resistance determinants via horizontal transfer depends on the extent of fitness costs associated with those determinants. To survive and disseminate, resistant bacteria must strike a balance between maximizing resistance potential and preserving fitness. We also studied other phenomena that contribute to the emergence and spread of antibiotic resistance in *S. pneumoniae* and treatment failure. In chapter 6, we studied tolerance in *S. pneumoniae* based on our hypothesis that tolerance likely occurs as a stepping stone to the development of antibiotic resistance to circumvent high fitness costs of on-target mutations. Finally, in chapter 5, we investigated whether vancomycin heteroresistance is associated with poor clinical outcomes in bloodstream infections caused by coagulase-negative staphylococci.

CHAPTER 2. FITNESS CONSTRAINTS HINDER HORIZONTAL TRANSFER OF FLUOROQUINOLINE RESISTANCE DETERMINANTS IN *STREPTOCOCCUS PNEUMONIAE*

Introduction

S. pneumoniae is a major cause of morbidity and mortality particularly in young children and elderly populations [3]. Annually, about half a million deaths among children younger than the age of 5, the immunocompromised, and the elderly result from pneumococcal disease worldwide [57]. Although *S. pneumoniae* usually colonizes the upper respiratory tract asymptotically, it can become pathogenic especially when host defenses are weakened or compromised, causing mild to invasive diseases, such as sinusitis, acute otitis media, pneumonia, bacteremia, and meningitis [3, 5]. For decades, common clinically used antibiotics such as beta-lactams, macrolides, and fluoroquinolones have been the standard treatment for pneumococcal infections [58]. Antibiotic resistance has complicated treatment for pneumococcal infections and often results in treatment failure in cases that otherwise would have been effectively treated with commonly used antibiotics. There has been a worldwide increase in the number of cases of *S. pneumoniae* resistant to multiple classes of antibiotics, including fluoroquinolones (levofloxacin, ciprofloxacin, and gemifloxacin), folate inhibitors (trimethoprim and sulfamethoxazole), cell-wall synthesis inhibitors (penicillin), and protein synthesis inhibitors (azithromycin, doxycycline, tetracycline, and minocycline) [21]. According to the CDC, in 2015, 30% of about 30,000 cases of invasive pneumococcal disease resulted from *S. pneumoniae* that was resistant to more than one antibiotic [24]. In the United States, approximately 20% to 40% of pneumococcal isolates are resistant to macrolides [22, 23] and 13% to 30% are resistant to beta-lactams [23, 59-61]. Unlike macrolide and beta-lactam resistance, there have been paradoxical observations in the case of fluoroquinolone resistance. Globally, fluoroquinolones are one of the most frequently prescribed antibiotics [33]. Despite a high usage rate, only limited cases of highly fluoroquinolone-resistant pneumococcal isolates have been reported [25, 26]. The prevalence of fluoroquinolone resistance remains low regardless of the frequent usage of fluoroquinolone in Asia and Europe, Spain, ranging from 0.6% to 9.8% (**Table 2-1**). In North America, surveillance studies showed that fluoroquinolone resistance is still uncommon, approximately <2% (**Table 2-1**).

Ciprofloxacin was the first fluoroquinolone prescribed to treat lower respiratory tract infections [62]. Currently, ciprofloxacin is not the first-line treatment for pneumococcal infection due to its limited potency against *S. pneumoniae* and potential for rapid emergence of resistance [35, 63-66]. A newer fluoroquinolone, levofloxacin, which has greater potency against *S. pneumoniae*, is one of the current standard treatments for community-acquired pneumococcal pneumoniae [67]. Acquisition of levofloxacin resistance in *S. pneumoniae* has been reported to occur rapidly during antibiotic therapy [68]. High-level fluoroquinolone resistance requires alterations in the quinolone resistance-determining regions in the on-target genes, topoisomerase IV (*parC*)

Table 2-1. Prevalence of fluoroquinolone resistance from 1988 to 2015

Fluoroquinolone Usage (Prescriptions per 1000 Persons)	Fluoroquinolone Prevalence	Periods	Region	Ref
37.5	1.2% isolates first step mutation in <i>parC</i> 6.7% efflux phenotype	2002-2006	Germany	[69]
Not available	0.2% resistant to levofloxacin	2004-2005 to 2014-2015	Germany	[70]
Ciprofloxacin 38.2 to 59.4	1.8% resistant to ciprofloxacin	1988-2006	Canada	[25, 71, 72]
Respiratory fluoroquinolone 5.3 to 27.4	1.2% non-susceptible to levofloxacin 1.1% non-susceptible to moxifloxacin	1988-2006	Canada	[25, 71, 72]
Not available	0% in 1993 to 1.7% in 1997 and 1998	1988 to 1997	Canada	[64]
Not available	1.5%	1993 to 1994	Canada	[69]
	2.9%	1997 to 1998		
Not available	<2% resistant to levofloxacin	2007-2016	Canada	[25, 73]
39 to 106	≤ 2% resistant to levofloxacin	1995-2002	USA	[33, 34]
	≤ 1% resistant to moxifloxacin	2008		
Not available	4-13% resistant to fluoroquinolones (ciprofloxacin)	2000	Croatia and Hong Kong	[36]
Not available	4% resistant to newer fluoroquinolones (levofloxacin, moxifloxacin)	2009 to 2010	Asia	[74]
Not available	7.1%	1998-1999	Spain	[75]
Not available	1.64 % resistant to ciprofloxacin, 0% to other fluoroquinolones	2000-2002	Hungary	[76]

and/or DNA gyrase (*gyrA*), that decrease the drug binding affinity of the target enzymes [77]. Both topoisomerase IV and DNA gyrase are responsible for relieving chromosomal supercoiling and DNA torsion during DNA replication and transcription [78]. Topoisomerase IV relaxes positive supercoiling and decatenates interlocked daughter chromosomes to complete DNA replication [79]. Complementary to the role of topoisomerase IV, DNA gyrase induces negative supercoiling or relaxes positive supercoils.

In addition to the high usage rate of fluoroquinolones, *S. pneumoniae* is naturally competent. The bacteria can naturally transform and acquire resistance determinants via horizontal transfer [80]. The emergence and spread of fluoroquinolone resistance in *S. pneumoniae* result from *de novo* mutations or horizontal transfer of resistance determinants [38]. Horizontal transfer can accelerate the spread of antibiotic resistance at a more rapid rate than *de novo* mutations, which occur at very low frequencies (10^{-8} to 10^{-7} per generation) [81]. However, fluoroquinolone resistance is still extremely low, and the reason underlying the low prevalence of fluoroquinolone resistance remains unknown. We hypothesized that there may be barriers to acquiring fluoroquinolone-resistant mutations and/or to fixating those mutations in the population. One such barrier could be high fitness costs associated with those mutations. In the absence of antibiotics, mutations with high fitness costs would diminish the ability of the bacteria to survive and replicate in the host, where the bacteria are under selective pressure from the immune system [55]. In this paper, we demonstrated that horizontal transfer of on-target fluoroquinolone resistance determinants imposed deleterious fitness costs in *S. pneumoniae* and that high-level fluoroquinolone resistance required a specific combination of on-target mutations. The high fitness costs associated with the on-target fluoroquinolone resistance determinants and the requirement for a select combination of mutations likely contribute to the paucity of fluoroquinolone-resistant strains of *S. pneumoniae* worldwide despite continued high-level fluoroquinolone usage clinically.

Methods

Media and growth conditions

For this study, *S. pneumoniae* was either grown in C+Y, a chemically-defined semi-synthetic casein liquid media supplemented with 0.5% yeast extract, ThyB media (Todd Hewitt Broth + 0.2% yeast extract from Sigma Aldrich), or on blood agar plates containing tryptic soy agar (EMD Chemicals, New Jersey) and 3% defibrinated sheep blood. Cultures of *S. pneumoniae* were inoculated from overnight streaked blood agar plates and then incubated at 37°C in 5% CO₂. In addition to the respective fluoroquinolone for selection of transformants, all blood agar plates were supplemented with neomycin at 20 µg/mL to select for *S. pneumoniae*. *S. pneumoniae* is naturally resistant to neomycin. Strains used in this study and their respective ciprofloxacin and levofloxacin MICs (µg/mL) are listed in **Table 2-2**.

Table 2-2. Strains used in this study and respective MICs

Strains	Description	MIC (µg/mL)		Source
		Ciprofloxacin	Levofloxacin	
TIGR4	TIGR4 wild type, serotype 4	4	1	www.tigr.org
CDC001	Serotype 9V	4	1	CDC
D39	D39 wild type, serotype 2	4	1	
Tn-seq mutant library	TIGR4	4	1	Generated by Amy Iverson This study
S79Y <i>parC</i>	Single point mutation S79Y in topoisomerase IV <i>parC</i>	8	2	This study
S81F <i>gyrA</i>	Single point mutation S81F in <i>gyrA</i>	8	2	This study
S79Y <i>parC</i> & S81F <i>gyrA</i>	Double point mutations S79Y in <i>parC</i> and S81F in <i>gyrA</i>	16	4	This study
S79Y <i>parC</i> & D435N <i>gyrB</i>	Double point mutations S79Y in <i>parC</i> and D435N in <i>gyrB</i>	8	2	This study

MIC determination via E-tests

The wild-type TIGR4, single point mutants S79Y *parC* and S81F *gyrA*, and double mutants S79Y *parC* & S81F *gyrA* and S79Y *parC* & D435N *gyrB* strains were grown on blood agar plates overnight, inoculated into ThyB, and incubated 37°C with 5% CO₂ until reaching mid-logarithmic phase at OD₆₂₀ ~0.4. Then, 100 µL of the bacterial culture were spread onto blood agar plates. A levofloxacin E-test strip (bioMérieux) was added onto the center of each plate. Minimum inhibitory concentration MIC (µg/mL) was determined to be the concentration at which the symmetrical inhibition ellipse edge intersects the E-test strips on the plate [82].

Genomic DNA extraction

To extract genomic DNA for transformation and whole genome sequencing in this study, the aqueous / organic extraction method using phenol chloroform was performed. TIGR4 and mutant strains were grown in ThyB to late logarithmic phase at OD₆₂₀ ~0.6 and pelleted at 6000 x g for 10 minutes followed by resuspension in 1 mL 1x PBS containing 50 µL 10% DOC, 50 µL 10% SDS, and 10 µL of 10mg/mL Proteinase K (Sigma). The mixture was incubated for lysis until clear (approximately 5 minutes) at 37°C, mixed with 500 µL phenol:chloroform:isoamyl alcohol (Sigma), transferred to phase-lock tubes (Quantabio), and centrifuged at maximum speed for the separation of aqueous and organic phases. Then 500 µL chloroform: isoamyl alcohol was mixed with the aqueous phase and centrifuged as above. For DNA precipitation, the aqueous phase was transferred to a new Eppendorf tube containing 100% ethanol. Precipitated DNA was washed with ice cold 70% ethanol, pelleted, dried, and resuspended in nuclease-free water.

Transformation

Cultures of *S. pneumoniae* were inoculated in C+Y media and incubated at 37°C with 5% CO₂ until OD₆₂₀~0.07 to 0.1. Then, 5 µg of gDNA and 3 µL of CSP₂ were added to 1 mL of the bacterial cultures. Three different types of DNA were used for transformation experiments: 2 kb PCR fragments encoding select point mutations, genomic DNA of fluoroquinolone resistant *S. pneumoniae*, or genomic DNA of *S. viridans* clinical isolates. The genomic DNA from both species was extracted using the aqueous / organic extraction protocol as described previously. After addition of DNA and CSP, the cultures were incubated for 3 hours at 37°C with 5% CO₂, followed by plating of 100 µL of the transformation mixture 5 blood agar plates containing select antibiotics. Colony-forming units (CFUs/mL) were enumerated at 24 and/or 48 hours of incubation. Transformation efficiency was calculated as the ratio of transformant colonies (CFUs/mL) on select antibiotic plates to the total number of colonies (CFUs/mL) on blood plates without the antibiotic.

***In vitro* passaging**

TIGR4 was diluted 1:100 fresh ThyB containing increasing subinhibitory concentrations of either ciprofloxacin or levofloxacin and incubated until reaching exponential phase (OD₆₂₀~0.6). Then bacteria stocks in glycerol were stored at -80°C before serving as an inoculum for the next passage. Each subsequent passage contained progressively increasing 2-fold dilutions of the respective antibiotic. The passaging continued for 30 days. Bacterial culture of the last passage at the highest concentration of ciprofloxacin or levofloxacin was spread on blood agar plates, and three colonies were selected for identification of mutations that arose at the end of passaging with either levofloxacin or ciprofloxacin via whole genome sequencing.

Mouse experiments

To determine the virulence of the mutants in terms of fitness costs *in vivo*, 6-week-old female BALB/c mice (Jackson laboratory) were intranasally infected with the wild-type TIGR4 or the mutant strains (10⁶ CFUs in 100 µL). At 24 hours post challenge, 2 µL of nasal samples and 5 µL of blood samples were collected via nasal lavage and tail bleeding, respectively. The nasal and blood samples were then serially diluted and spotted on blood agar plates for enumeration of bacterial burden. Plates were incubated overnight at 37°C and 5% CO₂. Mice were closely monitored for disease progression for 10 days and euthanized via CO₂ asphyxiation. Bacterial titers (CFUs/mL) were compared using non-parametric Mann-Whitney t-test in Prism 7.

Ethics statement

All experiments involving animals were performed with prior approval of and in accordance with guidelines of the St. Jude Institutional Animal Care and Use Committee. The St. Jude laboratory animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in Guide for the Care and Use of Laboratory Animals.

RNA extraction, rRNA depletion, and RNA-Seq

Wild-type TIGR4, S79Y *parC*, S81F *gyrA*, and double mutants S79Y *parC* & S81F *gyrA*, and S79Y *parC* & D435N *gyrB* were grown in C+Y until mid-exponential phase (OD₆₂₀ ~0.4) and pelleted via centrifugation at 6,000 x g for 10 minutes. The supernatant was discarded, and RNA was extracted from the pellets using the Qiagen RNeasy kit. Extra DNA removal steps were conducted to eliminate DNA from the RNA samples, following the protocol from DNase Inactivation Reagent from DNA Free DNA removal kit (Thermo AM1906). To remove rRNA from the extracted RNA samples, rRNA depletion was conducted based on the protocol developed by Culviner *et al.* [83].

Library preparation for RNA sequencing was completed following the protocol of NEBNext Ultra II RNA Library Prep kit for Illumina (E7770, E7775).

***In vivo* passaging**

To model the evolution of fluoroquinolone resistance *in vivo*, three groups of 7-week-old immunocompetent BALB/c mice were intranasally infected with 100 μ L containing 10^6 CFUs of TIGR4. Six hours post-infection, mice were administered a sublethal dose of levofloxacin intraperitoneally. The initial dosage of levofloxacin (25 mg/kg) was utilized as it eliminated 90% to 99% of the bacterial population (data not shown). After 15 passages, the levofloxacin dosage was doubled in each subsequent passage. For each passage, twelve hours after levofloxacin treatment, bacteria were collected from lungs of infected mice via dissection. The lungs were homogenized, then bacteria in lung homogenates were collected via centrifugation at 300 x g for 5 minutes, plated on blood agar plates and grown at 37°C with 5% CO₂ for 12 hours, collected from the agar plates, and frozen in aliquots containing glycerol. Frozen aliquots from each passage served as the inoculum to infect the next group of mice for the subsequent, respective lineage, and the remaining aliquots were used for genomic DNA extraction.

Whole genome sequencing

Genomic DNA samples were sent to Hartwell Center for Bioinformatics and Biotechnology whole genome sequencing. Sequence libraries were prepared according to Illumina HiSeq protocol. The reads were assembled in Breseq and compared to the TIGR4 reference genome (AE005672.3) for identification of mutations resulting from recombination events and *de novo* point mutations.

Antibiotic kill curves

The wild-type TIGR4, single- and double-point mutants were grown in ThyB to early-exponential phase OD₆₂₀ ~0.2. The bacteria were exposed to progressively increasing concentrations of ciprofloxacin, levofloxacin, and moxifloxacin, ranging from 1x to 4x MIC of the wild type. Before and after antibiotic exposure, bacterial CFUs were serially diluted and spotted onto blood agar plates. Plates were incubated overnight at 37°C with 5% CO₂.

H₂O₂ production measurement

The evolved isolates and TIGR4 were grown for 7 hours in ThyB until mid-exponential phase (OD₆₂₀ ~0.4), centrifuged, and resuspended in 1x PBS. Bacterial supernatant was collected for the Amplex Red Hydrogen Peroxide/Peroxidase assay (ThermoFisher Scientific A22188), then 50 μ L Amplex Red reagent was added to 50 μ L

of the supernatant in each microplate well. The absorbance value (OD₅₆₂) was measured by using a BioTek plate reader. Amount of H₂O₂ of each strain was calculated and normalized to its amount of total cellular protein, which was determined via a bicinchonic acid (BCA) protein assay.

Results

Fluoroquinolone resistance in *S. pneumoniae* did not occur readily via *de novo* mutations

Although the prevalence of fluoroquinolone resistance remains relatively low compared to other antibiotics, it still occurs clinically (**Table 2-1**). Cases of levofloxacin resistance in *S. pneumoniae* arising during therapy and resultant treatment failure have been reported [68]. Thus, we aimed to gain insights into the emergence and spread of fluoroquinolone resistance by modeling the evolution of fluoroquinolone resistance and identifying mutations favored by fluoroquinolone pressure and/or the host's immune response. First, we attempted to identify mutations that arose under antibiotic pressure solely via *in vitro* passaging with either ciprofloxacin or levofloxacin. After 30 passages, TIGR4 did not develop significant resistance to ciprofloxacin as it only became resistant to 8 µg/mL (2x MIC) (**Figure 2-1A**), which is markedly lower than clinically relevant MICs. Levofloxacin resistance was not observed after an extensive period of *in vitro* passaging (**Figure 2-1B**). Three independent colonies isolated from the *in vitro* passaging population under ciprofloxacin pressure were sequenced. Whole genome sequencing data demonstrated that those three colonies encode double point mutations in the on-target genes *parC* and *gyrA*, D83N and S81F, respectively. The *in vitro* passaging isolates under levofloxacin pressure were not sequenced because an MIC higher than that of TIGR4 was not reached. The TIGR4 strain developed slight resistance to ciprofloxacin as a result of double mutations in the on-target enzymes.

We also modeled the evolution of fluoroquinolone resistance under the host's immune response. To search for mutations associated with fluoroquinolone resistance *in vivo*, we developed a murine challenge model for studying the evolution of fluoroquinolone resistance under both levofloxacin and immune pressure (**Figure 2-2A**). The wild-type TIGR4 underwent 30 passages in three independent lineages of immunocompetent BALB/c mice under increasing levofloxacin pressure (**Figure 2-2B**). Whole genome sequencing analysis of each experimentally-evolved isolate collected from the final passage indicated the absence of on-target mutations in either *gyrA* or *parC*. We measured the levofloxacin MIC of each isolate using E-tests. Surprisingly, MICs of the isolates (1.5 µg/mL) were relatively similar to that of the parental, wild-type strain (1 µg/mL) (**Table 2-3**). No marked change in levofloxacin MIC observed in the experimentally-evolved isolate suggests that the isolates did not become resistant to the antibiotic.

Figure 2-1. *S. pneumoniae* demonstrated *de novo* resistance *in vitro*, while developed tolerance *in vivo*

A and B) *in vitro* passaging. *S. pneumoniae* was exposed to gradually increasing concentrations of both ciprofloxacin and levofloxacin, respectively for 30 days.

Figure 2-2. *In vivo* passaging with levofloxacin

A) Schematic diagram of *in vivo* passaging workflow. Seven-week-old BALB/c mice were intranasally infected TIGR4. Six hours post-infection, mice were administered a sublethal dose of levofloxacin intraperitoneally. Twelve hours after levofloxacin treatment, bacteria were collected from lungs of infected mice. Bacteria from the lungs were spread plated on blood agar plates for 12 hours, and frozen in aliquots containing glycerol. Frozen aliquots from each passage served as the inoculum to infect the next group of mice for the subsequent, respective lineage, and the remaining aliquots were used for genomic DNA extraction. B) *in vivo* passaging. Bacteria burden was enumerated after each passage under levofloxacin pressure.

Table 2-3. MIC of evolved isolates from *in vivo* passaging

Strains	MIC (µg/mL)
TIGR4	1
L1, G29, N0*	1.5
L2, G29, N0	1.5
L3, G29, N0	1.5

*L1, G29, N0 denotes bacterial lineage 1, generation (passage 29), non-depleted mice, respectively.

Extremely low transformation efficiency suggests infrequent horizontal acquisition of fluoroquinolone resistance determinants in *S. pneumoniae*

Besides *de novo* mutations, fluoroquinolone resistance in *S. pneumoniae* can occur via horizontal transfer. Horizontal transfer (specifically transformation) promotes the rapid spread of resistance in *S. pneumoniae* [84]. However, the low prevalence of fluoroquinolone-resistant clinical isolates despite high usage suggests that horizontal transfer of fluoroquinolone resistance determinants might not occur as readily as expected. To determine the transformation frequency of horizontal transfer of fluoroquinolone on-target resistance determinants in *S. pneumoniae*, we transformed the wild-type TIGR4 with PCR fragments encoding a single mutation in either on-target genes, S81F in *gyrA* or S79Y in *parC* and selected transformant colonies on 1x MIC of ciprofloxacin (**Figure 2-3A-I**) or 1x MIC of levofloxacin (**Figure 2-3J-R**). The on-target mutations were selected for the transformation experiments because they are often screened for and highly prevalent in fluoroquinolone-resistant clinical isolates [85-89]. As shown in **Figure 2-3A** and **J**, transformation with single on-target point mutations with the wild-type TIGR4 did not readily occur. When transformation did occur, the transformation efficiency was very low ranging from 10^{-8} to 10^{-5} CFU/mL per 5 μ g DNA of PCR fragments encoding either S81F in *gyrA* or S79Y in *parC*. Based on previous studies on competence of *S. pneumoniae*, the transformation efficiency ranges from 10^{-8} to 10^{-7} per nucleotide [81]. For our positive control, the wild-type TIGR4 received genomic DNA of a group of *S. pneumoniae* mutants that have transposon insertions in non-essential genes in *S. pneumoniae* (Tn-seq library) and the spectinomycin-resistance cassette used for transformants selection. This positive control was expected to have a much higher chance of transformation and would demonstrate that the bacteria were competent during transformation. As expected, the transformation efficiency of TIGR4 with the Tn-seq library was consistent at 10^4 CFUs/mL per 5 μ g DNA, suggesting that the bacteria were competent during the transformation experiments. Therefore, the low transformation efficiency observed with on-target mutations for fluoroquinolone resistance was not due to lack of the bacterial competence, but likely due to the deleterious fitness costs associated with those mutations (**Figure 2-3A** and **J**). The negative control contained no DNA to ensure that fluoroquinolone resistance was conferred by the incorporation of the single mutation in *parC* or *gyrA* from the added PCR fragments and not by encoding spontaneous mutations in those genes. No spontaneous mutants were observed in our negative control (**Figure 2-3A** and **J**). To determine whether this low transformation efficiency is serotype-specific, we also transformed other *S. pneumoniae* serotypes that are observed in the clinical setting, D39 serotype 2 (**Figure 2-3D** and **M**), and CDC001 serotype 9V, and observed similar transformation efficiencies (Figures 2-3 G and P). These data indicate that there may be barriers to horizontal transfer for fluoroquinolone resistance as single on-target point mutations alone were insufficient to transform TIGR4, CDC001 (serotype 9V), or D39 (serotype 2) strains to acquire fluoroquinolone resistance. Thus, low transformation frequency occurred in multiple serotypes of *S. pneumoniae*.

Figure 2-3. Low transformation efficiency for FQ resistance determinants was observed in TIGR4, D39, and CDC001 serotypes

Single-point mutation recombinants included S79Y *parC* or S81F *gyrA*. Tn-seq library served as positive control for *S. pneumoniae* baseline for competency. *S. pneumoniae* clinical isolates include isolates 188, 68, 66, 93, 112, 114, 176, and 182. *S. viridans* clinical isolates include isolates 1-20. A-I. Transformants were selected on 1x MIC ciprofloxacin. A, D, and G) Transformation efficiency of TIGR4, D39, and CDC001 (serotype 9V), respectively, with PCR fragments encoding on-target mutations in *parC* and/or *gyrA*. B, E, and H. Transformation efficiency of TIGR4, D39, and CDC001 (serotype 9V), respectively, with genomic DNA of fluoroquinolone-resistant pneumococcal clinical isolates. C, F, and I) Transformation efficiency of TIGR4, D39, and CDC001 (serotype 9V), respectively, with genomic DNA of fluoroquinolone-resistant *S. viridans* clinical isolates. J-R) Transformants were selected on 1x MIC levofloxacin. Similar formats as A through I figures regarding recipient strains and DNA donors. Transformation with genomic DNA of Tn-seq library served as a positive control for competence. Transformation efficiency was calculated as the ratio of the number of transformants (CFUs/mL) selected on either ciprofloxacin or levofloxacin to the number of total bacteria (CFUs/mL).

Compensatory mutations in the genome have been shown to ameliorate fitness costs imparted by on-target mutations, thereby facilitating the acquisition as well as allowing them to fixate in the population [90, 91]. We sought to examine whether the fluoroquinolone resistance determinants along with potential compensatory mutations in the genome of fluoroquinolones resistant *S. pneumoniae* clinical isolates could increase the transformation efficiency and facilitate the acquisition of fluoroquinolone resistance. Even with potential compensatory mutations in the genomic DNA of the resistant *S. pneumoniae* isolates, the transformation efficiency of TIGR4, D39, and CDC001 remained extremely low (**Figure 2-3B, E, H, K, N, and Q**). Transformation with genomic DNA of the resistant intraspecies isolates that encoded double point mutations in both *parC* and *gyrA* occurred more often than those with genomic DNA that encoded only single point mutation in either *parC* or *gyrA*. Another major reservoir of antibiotic resistance determinants for *S. pneumoniae* was viridans group streptococcal clinical isolates [92]. We attempted to determine the transformation efficiency of *S. pneumoniae* with the interspecies genomic DNA from resistant viridans group clinical isolates. Most of the fluoroquinolone-resistant viridans group streptococcal clinical isolates, which we obtained from the clinical microbiology laboratory at SJCRH, encoded double point mutations in both *gyrA* and *parC* except isolate #5 having triple point mutations in *gyrA*, *gyrB*, and *parC*. Transformation with genomic DNA of fluoroquinolone-resistant viridans group streptococci still rarely occurred. The TIGR4 strain was transformed with isolates 16, 17, and 18, which encoded double point mutations in *parC* and *gyrA* at 10^{-7} per 5 μ g / DNA (**Figures 2-3C and L**). No transformants were observed from the transformation with D39 and CDC001 strains (**Figures 2-3F, I, O, and R**). Taken together, infrequent transformation efficiencies with both intra- and inter-species genomic DNA encoding on-target mutations for fluoroquinolone resistance and potential compensatory mutations corroborate that there are barriers to acquisition of fluoroquinolone resistance determinants via horizontal transfer in *S. pneumoniae*.

Individual fluoroquinolone resistance mutations impose severe fitness defects *in vitro* that are likely alleviated upon secondary mutation

A key factor that determines whether a resistant strain will survive and disseminate in the environment or in the host is the fitness cost associated with the mutation(s) [91]. In *S. pneumoniae*, fitness refers to the ability of the bacteria to colonize, replicate, invade different bodily organs, and disseminate to other hosts [93]. As previously mentioned, the extremely low transformation efficiency of *S. pneumoniae* with only on-target mutations for fluoroquinolones resistance suggest that those mutations likely impose deleterious fitness costs. Without antibiotic pressure, mutations that confer antibiotic resistance but carry high fitness costs are less likely to persist in the population [54, 56]. Therefore, we hypothesized that mutations likely to emerge in clinical settings are those with resistance potential and the least fitness tradeoffs during *in vitro* growth as well as *in vivo* challenge. To test this hypothesis, we generated single- and double-point mutants via transformation with a PCR fragment encoding the desired mutation(s). We successfully generated single point mutants S79Y *parC* and S81F *gyrA*, and double point mutants S79Y *parC* & S81F *gyrA*, and S79Y *parC* & D435N *gyrB* via

transformation with PCR fragments encoding specific mutations and confirmed the presence of those mutations without any other on-target mutations via whole genome sequencing. During the transformation, the single point mutants received one type of PCR fragments encoding either S79Y *parC* or S81F *gyrA* mutation, whereas the double point mutants S79Y *parC* & S81F *gyrA* received a mixture of two types of PCR fragments (each type encoded either of the mutations). These mutations have been shown to confer high-level resistance to fluoroquinolones and circulate in the clinical setting [72, 94-96]. The encoded mutations decreased the susceptibility of the bacteria to ciprofloxacin and levofloxacin. The MICs of ciprofloxacin and levofloxacin of TIGR4 were 4 and 2 µg/mL, respectively (**Table 2-2**). While single point mutants S79Y *parC* and S81F *gyrA* and the double point mutant S79Y *parC* & D435N *gyrB* were able to grow on blood agar plates supplemented with 4 µg/mL ciprofloxacin or 2 µg/mL levofloxacin, the double point mutant S79Y *parC* and S81F *gyrA* was able to grow at 8 µg/mL ciprofloxacin and 4 µg/mL levofloxacin. We then compared the growth rates of the single point mutants S79Y *parC* and S81F *gyrA*, and double point mutants S79Y *parC* & S81F *gyrA*, and S79Y *parC* & D435N *gyrB* in the absence of antibiotic pressure. The single on-target mutants S79Y *parC* and S81F *gyrA* demonstrated marked delay in growth compared to the wild-type TIGR4 (**Figure 2-4A**). Interestingly, the double mutant S79Y *parC* & S81F *gyrA* had similar growth rate to that of the wild type, while the double mutant S79Y *parC* & D435N *gyrB* had the similar growth pattern to that of the S79Y *parC* mutant (**Figure 2-4A**).

Next, we determined the fitness of the single and double mutants by determining their virulence in murine models. For our pneumococcal virulence model, we intranasally infected 6-week-old BALB/c mice with high-volume inoculum (10^6 CFUs in 100 µL) containing the susceptible, parental TIGR4 strain or the single on-target mutations in *gyrA* and/or *parC* or the double mutants. At 24 hours post challenge, we collected nasal and blood samples via nasal lavage and tail bleeding, respectively. Compared to the wild-type TIGR4, the single mutants with a point mutation in either *gyrA* or *parC* displayed attenuated virulence. Unlike the single mutant in *gyrA*, the single mutant in *parC* was able to colonize the nasopharynx as effectively as the wild type (**Figures 2-4B** and **C**). Although there was 1 log decrease in bacterial burden compared to the wild type, the double point mutant S79Y *parC* and S81F *gyrA* still adequately colonized the nasopharynx (**Figures 2-4B** and **C**). Both the single mutants S79Y *parC* and S81F *gyrA* were not detected in the bloodstream, indicating that they were not able to disseminate and/or survive in the bloodstream to cause invasive disease (**Figures 2-4D** and **E**). In addition to measuring bacterial burden in the nasopharynx and blood, we monitored infected mice for survival for 10 days. Mice infected with the single point mutants S79Y *parC* and S81F *gyrA* and the double point mutant S79Y *parC* & D435N *gyrB* showed a significant increase in survival compared to those infected with TIGR4 (**Figures 2-4F -I**). Loss of virulence of the single point mutants and the double points mutant S79Y *parC* & D435N *gyrB* was consistent with their undetectable bacterial burden in the blood. However, mice infected with the double point mutant S79Y *parC* & S81F *gyrA* initially had reduced mortality but eventually succumbed to infection compared to mice infected with the wild type (**Figure 2-4H**). The *in vitro* growth and virulence phenotype of the double point mutant S79Y *parC* & D435N *gyrB* resembled that of the single *parC*

Figure 2-4. Fitness defect was demonstrated *in vitro* and *in vivo* (blood) with single-point mutation recombinants of *parC* and *gyrA*, and *in vivo* (nasal swish) with single and double point mutants

The recombinants' CFUs/mL were compared to that of the respective wild-type strains via the non-parametric Mann-Whitney t-test. *p < 0.05, **p < 0.01, ****p < 0.001. Bars represent SEM. A) Growth curves *in vitro* in semi-chemically defined media (C+Y). B and C) Nasal lavage 24 and 48 hours p.i., respectively. D and E) Blood titers 24 and 48 hours p.i, respectively. F) Survival curves of TIGR4 and S79Y *parC* mutant. G) Survival curves of TIGR4 and S81F *gyrA* mutant. H) Survival curves of TIGR4 and S79Y *parC* & S81F *gyrA* double point mutant. I) Survival curves of TIGR4 and S79Y *parC* & D435N *gyrB* double point mutant

mutant, suggesting that the fitness costs and transcriptional changes were mediated mainly by the *parC* mutation (**Figure 2-4**). Here, the single on-target mutations in either *gyrA* or *parC* imposed deleterious fitness costs, while double mutations in both target enzymes maintained virulence similar to the wild type. These results suggest that the acquisition of mutations in only one of the on-target enzymes (*gyrA* or *parC*) is associated with detrimental fitness costs manifested in the attenuation of *S. pneumoniae* TIGR4. Detrimental fitness costs imposed by the individual on-target mutations in *parC* and *gyrA* likely attribute to the limited transformation efficiencies with fluoroquinolone resistance determinants. Acquisition of those on-target mutations might have successfully occurred, but transformants could not survive and propagate due to the fitness costs. Therefore, deleterious fitness costs represent an important barrier to the acquisition of fluoroquinolone resistance via horizontal transfer in *S. pneumoniae*.

Combination of levofloxacin and host pressure induced tolerance phenotype rather than high-level resistance

The absence of on-target mutations for fluoroquinolone resistance and resistance development in the experimentally evolved isolates led us to examine whether the isolates displayed tolerance phenotype. Although there is still no clear standardized measure for tolerance as for resistance, one method to determine tolerance is by conducting kill curves. Tolerant strains, by definition, can survive transient exposure to high antibiotic concentrations that are otherwise lethal, thereby having slower kill kinetics than susceptible strains [44]. The experimentally-evolved isolates were eliminated more slowly than the wild type at lethal concentrations of levofloxacin (1x and 2x MICs) (**Figures 2-5A and B**). This confirmed that the experimentally-evolved isolates developed tolerance instead of resistance to levofloxacin under the antibiotic and the host's immune pressure. We next tested whether the experimentally-evolved isolates also conferred cross-tolerance to other clinically-relevant fluoroquinolones, such as ciprofloxacin and moxifloxacin. Compared to the wild-type TIGR4, the isolates exhibited similar killing kinetics in both ciprofloxacin and moxifloxacin (**Figures 2- 5C, D, E, and F**). This indicates that the isolates did not confer cross-resistance to other fluoroquinolones and that their tolerance phenotype was specific only to the antibiotic in which they were previously passaged.

From the whole genome sequencing data of the passaged isolates, we identified mutations that most likely conferred tolerance as those that arose in more than one independent lineage, were absent prior to *in vivo* passaging, and fixated in the bacterial population at high frequencies. Mutations in isochorismatase arose in three independent lineages during *in vivo* passaging with levofloxacin, starting in passage 8-10 and remaining in the population (**Figure 2-6**). Examples of those mutations are A44D, A4D, and H172D (**Figure 2-6 and Appendix**). Mutations in pyruvate oxidase (M1T, Q456*) and chlorohydrolase (Q17*, F19V) occurred in subsequent passages and remained in the population until the final passage in two lineages (**Figure 2-6**). The missense mutation in the start codon M1T and the nonsense mutation Q456* in the pyruvate oxidase gene would very likely result in a non-functional and truncated protein product of this gene.

Figure 2-5. Experimentally-evolved isolates of *S. pneumoniae* exhibited tolerant phenotype to levofloxacin

A and B) Kill curves conducted with 1x MIC and 2x MIC of levofloxacin, respectively. T4 316 (blue) is the wild type. T4 316 L1-L3, G29, Nd (red) represent 3 independent evolved lineages in immunocompetent mice. C and D) Kill curves with ciprofloxacin at 1x MIC and 2x. E and F) moxifloxacin at 4x and 8x MIC respectively.

Figure 2-6. Muller plots representing relative abundance of selected mutations arose in multiple lineages during *in vivo* passaging with levofloxacin
A) Lineage 1. B) Lineage 2. C) Lineage 3.

Those mutations in isochorismatase, pyruvate oxidase, and chlorohydrolase are off-target mutations. The absence of on-target mutations in GyrA and ParC likely explains the lack of fluoroquinolone resistance observed in the experimentally-evolved isolates. Although tolerance has been shown to rapidly lead to resistance [97], the tolerant experimentally-evolved isolates did not increase transformation efficiency. The result suggests that in our case, tolerance did not facilitate horizontal transfer of fluoroquinolone resistance determinants (data not shown). Perhaps, the off-target mutations were more advantageous than the on-target mutations by allowing the bacteria to transiently evade levofloxacin and immune clearance without compromising bacterial fitness.

The evolved isolates from *in vivo* passaging produced significantly lower production of H₂O₂

Besides the on-target inhibitions, fluoroquinolones have been shown to exert redox stress on bacteria [98]. Dwyer *et. al.* demonstrated that norfloxacin (a first-class fluoroquinolone and an inhibitor of Gyrase A) increased the generation of hydroxyl radicals via superoxide generation in *E. coli* [39]. Hence, in the presence of fluoroquinolone, the bacteria are under redox stress from two major sources: their own H₂O₂ product and fluoroquinolone-induced radicals. The nonsense and missense mutations in the pyruvate oxidase identified from whole genome sequencing analysis led us to assess H₂O₂ production among the evolved isolates and if altered production of H₂O₂ could contribute to the tolerance phenotype observed in the isolates. Using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit, we measured H₂O₂ production in each strain. The $\Delta spxB\Delta lctO$ double knockout served as a negative control because it has been shown to produce very little H₂O₂ [99]. Compared to TIGR4, all three evolved isolates from the *in vivo* passaging produced significantly less H₂O₂ (**Figure 2-7**). This phenotype is likely attributed to the nonsense and missense mutations, suggesting the lack of pyruvate oxidase activity among these evolved isolates. As lower H₂O₂ production lessens the redox stress burden, it is possible that the bacteria are now able to manage fluoroquinolone-derived redox stress more effectively, thereby withstanding the antibiotic killing via tolerance.

Striking similarities were observed in the transcriptional profiles in the double point mutant S79Y *parC* & S81F *gyrA* and in the wild-type TIGR4

To understand the mechanism(s) underlying the attenuation of the single on-target point mutants in *parC* and *gyrA*, we next sought to ascertain differences in the transcriptional profiles of those single- and double-point mutants compared to the wild-type TIGR4 in *S. pneumoniae* in the absence of fluoroquinolones by utilizing RNA-seq. The S81F *gyrA* mutant upregulated multiple enzymes and transporters responsible for the metabolism of different monosaccharides, such as glucose, fructose, and mannose, approximately 2.7 to 5.7 log₂ fold change (**Table 2-4**). Alpha, alpha-phosphotrehalase was downregulated at -5.83 log₂ fold change. This enzyme participates in starch and

Figure 2-7. The evolved isolates produced minimal H₂O₂

H₂O₂ production was measured using the Amplex Red kit. $\Delta spxB \Delta lctO$ double knockout served as the negative control. Amount of H₂O₂ was normalized to amount of protein each respective strain produced. The amount of protein produced was determined via BCA assay. **p-value ≤ 0.01 .

Table 2-4. Significant gene expression of the S81F *gyrA* point mutant vs TIGR4

Annotation	Log₂ Fold Change
Rhamnulokinase	5.79
L-fuculose-phosphate aldolase	5.08
Fucose isomerase	4.51
YhcH/YjgK/YiaL family protein	4.34
PTS sugar transporter subunit IIA	4.27
Sugar ABC transporter permease	4.23
PTS sugar transporter subunit IIB	4.01
Sugar ABC transporter substrate-binding protein	3.69
N-acetylmannosamine-6-phosphate 2-epimerase	3.60
PTS mannose/fructose/sorbose/N-acetylgalactosamine transporter subunit IIC	3.57
PTS mannose/fructose/sorbose transporter family subunit IID	3.42
LacI family transcriptional regulator	3.34
Glycoside hydrolase family 32 protein	3.25
PTS beta-glucoside transporter subunit IIBCA	3.04
Iron-containing alcohol dehydrogenase	2.92
Glycoside hydrolase family 95 protein	2.92
Hypothetical protein	2.81
Sugar ABC transporter permease	2.72
PadR family transcriptional regulator	2.70
PTS transporter subunit EIIC	2.59
Carbohydrate ABC transporter permease	2.51
Sugar ABC transporter permease	-2.69
Extracellular solute-binding protein	-2.69
UDP-glucose--hexose-1-phosphate uridylyltransferase	-2.77
Carbohydrate ABC transporter permease	-2.90
Sucrose phosphorylase	-3.22
PTS system trehalose-specific EIIBC component	-3.48
PI-1 pilus system sortase SrtC-3	-4.57
PI-1 pilus system sortase SrtC-2	-4.69
PI-1 pilus transcriptional regulator RlrA, transcriptional regulator (positive regulator)	-4.75
PI-1 pilus system sortase SrtC-1	-4.96
Alpha, alpha-phosphotrehalase	-5.09
RrgC pilus, LPXTG cell wall anchor domain-containing protein	-5.74
RrgB pilus, isopeptide-forming domain-containing fimbrial protein	-5.83
RrgA pilus, VWA domain-containing protein	-6.07

sucrose metabolism (**Table 2-4**). Furthermore, downregulation of UDP-glucose-hexose-1-phosphate uridylyltransferase, which is an enzyme involved in nucleotide sugars metabolism, was observed (**Table 2-4**). Those sugar metabolic enzymes also participate in the nucleotide synthesis pathway. In contrast to the S81F *gyrA* mutant, the S79Y *parC* mutant did not significantly alter its metabolic transcriptional response. A marked change was the downregulation of its competence-stimulating peptide *comC* and ATP-dependent Clp protease ATP-binding subunit at -2.72 and -4.01 log₂ fold change (**Table 2-5**). Isoleucine-tRNA ligase was upregulated at 2.97 log₂ fold change (**Table 2-5**). Similar to the transcriptional profile of the S79Y *parC* mutant, the profile of S79Y *parC* & D435N *gyrB* mutant showed downregulation of Clp protease ATP-binding subunit. Molecular chaperones DnaJ and DnaK were also downregulated at -2.54 and -3.13 log₂ fold change (**Table 2-6**). Again, these results suggest that the transcriptional changes and decreased virulence observed in the double point mutant S79Y *parC* & D435N *gyrB* are primarily due the S79Y mutation in *parC* and not from D435N mutation in *gyrB*. The transcriptional profile of the double point mutant S79Y *parC* & S81F *gyrA* almost resembled that of the wild type, except the downregulation of 4 genes, Gfo/Idh/MocA family oxidoreductase, sugar ABC transporter substrate-binding protein, neuraminidase NanB, and YhcH/YjgK/YiaL family protein (**Table 2-7**). There were not many significant alterations in the transcriptional profiles of the double mutant compared to that of the wild type. The double point mutant S79Y *parC* & S81F *gyrA* restored virulence to the level of the wild type. This could explain the similarities in their virulence and common prevalence in clinical setting. It also suggests that clinically relevant, high-level fluoroquinolone likely requires the specific combination of on-target mutations in ParC and GyrA.

Discussion

In this study, we aimed to investigate the discrepancy between high usage rate of fluoroquinolones and the low resistance rate of this class of antibiotic. We found that the single- and double-point mutants in the target enzymes, topoisomerase IV *parC* and DNA gyrase *gyrA*, generated via transformation exhibited comparable MICs to those of clinical isolates. However, there were severe fitness costs associated with the acquisition of fluoroquinolone resistance determinants in *S. pneumoniae* via horizontal transfer unless double point mutations in GyrA and ParC were acquired simultaneously (**Figure 2-3A, D, G, J, M, and P**). The fate of a resistance mutation in the bacterial population depends on the balance of the resistance potential that the mutation confers versus the fitness costs of maintaining that mutation. Mutations in on-target enzymes that circumvent the antibiotic's mechanism of actions often disrupt important cellular processes, such as cell wall synthesis, protein synthesis, and DNA replication, in which *parC* and *gyrA* participate. Clinical studies report that high-level fluoroquinolone resistant *S. pneumoniae* isolates often harbor double point mutations in ParC and GyrA [100-102]. The presence of the on-target double point mutant in clinical settings is consistent with our finding that the double point mutant exhibited high resistance potential with low fitness costs, while maintaining virulence level similar to that of the wild type [103]. Rozen and colleagues also reported that the second mutations in either

Table 2-5. Significant gene expression of the S79Y *parC* point mutant

Annotation	Log ₂ Fold Change
LPXTG cell wall anchor domain-containing protein	3.05
Isoleucine--tRNA ligase	2.97
Isopeptide-forming domain-containing fimbrial protein	2.92
VWA domain-containing protein	2.55
Competence-stimulating peptide ComC	-2.72
ATP-dependent Clp protease ATP-binding subunit	-4.01

Table 2-6. Significant gene expression of S79Y *parC* & D435N *gyrB* double point mutant vs TIGR4

Annotation	Log ₂ Fold Change
Isoleucine--tRNA ligase	2.74
PI-1 pilus tip adhesin RrgA, VWA domain-containing protein	2.54
PTS system mannose/fructose/N-acetylgalactosamine-transporter	-2.54
Molecular chaperone DnaK	-2.54
IS200/IS605-like element ISSpn6 family transposase	-2.61
ATP-dependent Clp protease ATP-binding subunit	-3.08
Molecular chaperone DnaJ	-3.13
tRNA	-4.15

Table 2-7. Significant gene expression of S79Y *parC* & S81F *gyrA* vs TIGR4

Annotation	Log ₂ Fold Change
Gfo/Idh/MocA family oxidoreductase	-2.72
Sugar ABC transporter substrate-binding protein	-3.55
Neuraminidase NanB	-3.69
YhcH/YjgK/YiaL family protein	-4.26

ParC or GyrA not only increased resistance potential but also lessened the fitness costs associated with fluoroquinolone resistance mutation(s) [104]. The high fitness costs imparted by the on-target mutations that confer fluoroquinolone resistance partly explain the low prevalence of fluoroquinolone resistance. The transformation frequency in *S. pneumoniae* is approximately 10^{-7} to 10^{-8} per nucleotide [81]; therefore, the frequency of acquiring both on-target mutations is tremendously reduced to approximately 10^{-14} per nucleotide. Therefore, the infrequent fluoroquinolone resistance is likely attributed to the combination of extremely low rate of acquisition of both double point mutations in *parC* and *gyrA* via horizontal transfer and the fitness costs that limit the dissemination of single point mutants. These results indicate that successful acquisition and dissemination of fluoroquinolone resistance in *S. pneumoniae* requires a balance between resistance potential and fitness costs.

We next focused on elucidating the underlying mechanism associated with the deleterious fitness costs imparted by the on-target mutations for fluoroquinolone resistance by examining the transcriptional profiles of the mutants in the absence of fluoroquinolones (**Tables 2-4 to 2-7**). Downregulation of alpha, alpha-phosphotrehalase and UDP-glucose-hexose-1-phosphate uridylyltransferase responsible for nucleotide and sugar metabolism in the single point mutant S81F *gyrA* suggests that once *gyrA* is mutated, nucleotides metabolism for DNA replication and downstream protein synthesis must be reduced to maintain homeostasis. Nucleotides are generally energetically costly and must be conserved efficiently [105, 106]. As DNA replication is disrupted due to the mutation in *gyrA*, nucleotides could accumulate, and excess in nucleotides could then inhibit DNA replication and protein synthesis as a negative feedback mechanism. Inhibited DNA replication likely results in reduced growth rates, causing the bacteria to be eliminated more rapidly by the host's immune responses, hence their attenuation in virulence. Furthermore, transcriptional changes in carbohydrates metabolism incurred by the on-target mutations likely limit survival and dissemination of *S. pneumoniae* to different host's niches. Based on the RNA-seq analysis, sugar metabolism was one of the transcriptional responses altered due to the on-target mutations for fluoroquinolone resistance. Most transcriptional alterations in sugar metabolism were detected in the single point mutant S81F *gyrA* (**Table 2-4**). Without a fully functional tricarboxylic (TCA) cycle and a respiratory electron transport chain, *S. pneumoniae* relies solely on fermentation to produce energy by oxidizing carbohydrates [9]. As the bacteria translocate from the nasopharynx to different host's niches, they likely encounter different types of carbohydrates specific to that niche. For example, in inflamed tissues and blood, glucose is the predominant carbohydrate, whereas in the nasopharynx, free sugars are limited, but glycans serve as the source of sugars [107, 108]. In order to effectively disseminate, *S. pneumoniae* must be able to utilize different sources of carbohydrates. Therefore, loss of ability to replicate and adapt to different carbohydrates might be associated with reduced colonization as well as complete attenuation in the bloodstream.

To understand the mechanisms underlying the development of fluoroquinolone resistance *in vivo*, we identified mutations that arose under levofloxacin pressure. During extensive *in vivo* passaging, in which the bacteria were under selective pressure from

both levofloxacin and the host's immune response, resistance did not occur; instead, tolerance developed (**Figures 2-5A and B**). Tolerance was likely induced by the host's immune clearance and antibiotic pressure. Whole genome sequencing data provided insights into the genomic changes that likely confer levofloxacin tolerance. The majority of the mutations arose in metabolic genes. Actively dividing bacterial processes are targeted and inhibited by antibiotics [109-111]. Previous studies have shown that tolerant bacteria inhibit their metabolism and growth to evade antibiotic killing [112]. On-target mutations for resistance can impart deleterious costs, but mutations for tolerance allow the bacteria to survive in the host. Tolerance has been shown as a prerequisite pathway for development of subsequent high-level resistance [97]. Zhang and colleagues demonstrated that activated PpnN (YgdH) in *E. coli* participated in the inhibition of nucleotides synthesis and increase in degradation to confer tolerance to ciprofloxacin and ofloxacin (a fluoroquinolone) [113]. PpnN is a nucleosidase playing a role in the metabolism of purine nucleotides; therefore, it is not a direct target of fluoroquinolones. However, not only does PpnP confer tolerance to fluoroquinolones, but it also offers fitness advantages during stringent responses to stresses [113]. Therefore, metabolic changes manifested from the transcriptional changes in those metabolic genes observed in the experimentally-evolved isolates likely result in antibiotic tolerance.

Taken together, we demonstrated that fitness tradeoffs associated with the individual on-target mutations in *gyrA* and *parC* likely restricted the development of fluoroquinolone resistance in *S. pneumoniae*. However, the combination of double point mutations in *gyrA* and *parC* not only restored the fitness of the bacteria but also conferred a significant increase in fluoroquinolone resistance level. The bacteria developed tolerance in order to evade antibiotic killing while circumventing the deleterious fitness tradeoffs. Although it seems quite difficult for fluoroquinolone resistance in *S. pneumoniae* to develop, antibiotic stewardship guidelines should be followed to minimize the emergence and spread of fluoroquinolone resistance in the future due to the transition from undetected tolerance to resistance. By predicting which mutations have the potential to facilitate the acquisition of high-level resistance and become clinically important, we will be able improve surveillance, tailor treatment regimens, and provide insights into the development of more-effective antibiotics.

CHAPTER 3. N291D MUTATION IN *PATA* FACILITATES HORIZONTAL TRANSFER OF HIGH-LEVEL FLUOROQUINOLONE RESISTANCE DETERMINANTS

Introduction

Streptococcus pneumoniae is an opportunistic Gram-positive pathogen and a major cause of hospitalization and mortality in young children and the elderly [3]. *S. pneumoniae* causes mild to severe infections, ranging from otitis media, pneumonia, sepsis, to meningitis. Previously, pneumococcal infections were effectively treated with commonly prescribed antibiotics, such as beta-lactams, fluoroquinolones, and macrolides [58]. However, the rapid emergence of antibiotic resistance among *S. pneumoniae* isolates has increased treatment failure rates, posing a serious public health problem worldwide [21]. Antibiotic resistance in *S. pneumoniae*, including fluoroquinolone resistance, has spread globally via horizontal transfer of resistance mutations and the emergence of *de novo* mutations. Fluoroquinolones inhibit DNA gyrase and topoisomerase IV [37]. The mutations in the main target enzymes GyrA and ParC are on-target mutations that confer resistance against fluoroquinolones, such as ciprofloxacin and levofloxacin [114]. On-target point mutations S81F in GyrA and S79Y in ParC have been found in clinical isolates collected from different areas worldwide, such as in Canada between 1993 to 1998 [25, 71, 72]. Mutations that occur in genes that are not directly targeted by the antibiotics, such as genes involved in metabolism, transport, and regulation of gene expression, are classified as off-target mutations. Current CLSI guidelines (Clinical & Laboratory Standards Institute) often fail to detect low-level resistance against fluoroquinolones that fall into the non-susceptible range and do not screen for off-target mutations. Examples of off-target mutations are ones that mediate the overexpression of *patA* and *patB*, which encode ATP-dependent efflux proteins clinically relevant for low-level fluoroquinolone resistance in *S. pneumoniae* [115]. One efflux pump responsible for low-level fluoroquinolone resistance in *S. pneumoniae* is PmrA [116].

Generally, horizontal transfer (specifically transformation) promotes the rapid spread of resistance in *S. pneumoniae*. However, the low prevalence of fluoroquinolone resistant clinical isolates despite high usage suggests that horizontal transfer of fluoroquinolone resistance determinants might not occur as readily as previously thought [117, 118]. Fluoroquinolone resistance in *S. pneumoniae* is a fascinating case study as isolates that are resistant to low-level fluoroquinolones sometimes do not have mutations in the on-target enzymes, topoisomerase IV ParC and DNA gyrase GyrA, suggesting that there are off-target mutations that contribute to the acquisition and/or exert resistance [119]. Pan *et al.* showed that mutants that are resistant to low levels of the fluoroquinolone ciprofloxacin do not require any variations of mutations in the on-target genes [120-122]. Other studies also demonstrated that low-level resistance against fluoroquinolones in *S. pneumoniae* does not require on-target mutations, which if subsequently acquired would confer high-level resistance [123]. Off-target mutation(s) play a role in conferring and/or facilitating the acquisition of mutations in the antibiotic targets to drive high-level

resistance and that the fitness tradeoffs incurred by both the off-target facilitator and the on-target resistance mechanisms will determine whether the mutation will emerge in the clinic. Whether resistance mutation(s) remain in the bacterial population partly depends on the balance of the fitness advantage that the mutation confers versus the potential fitness costs of maintaining the mutation. Therefore, we hypothesized that horizontal transfer of antibiotic resistance determinants in *S. pneumoniae* likely requires off-target mutations besides the on-target mutations.

Clinically, only mutations in the upstream region of *patA* have been reported and shown to upregulate the expression of this efflux pump [115, 124]. In this study, we showed that the N291D mutation in *patA* slightly increased the transformation efficiency of both the *gyrA* and *parC* mutations that confer high-level resistance for ciprofloxacin. As such, we concluded that the mutation in *patA* likely facilitated the subsequent acquisition of on-target mutations, thereby serving as a facilitating mutation of high-level resistance mechanisms. The off-target mutation N291D in *patA* has never been reported. The findings of this study will provide insights into the underlying mechanisms that facilitate the acquisition of high-level antibiotic resistance in *S. pneumoniae*.

Methods

Growth conditions

Streptococcus pneumoniae (wild-type TIGR4) and TIGR4-derivative point mutants were incubated on tryptic soy agar (TSA EMD Chemicals) plates containing 20 µg/mL neomycin and 3% sheep blood, in semi-chemically defined C+Y, or in nutrient-rich media ThyB supplemented with 5% CO₂. The purpose of neomycin supplementation was to select for *S. pneumoniae*. Bacterial cultures were inoculated from frozen glycerol stocks or from bacterial plates incubated overnight. Strains used are listed in **Table 3-1**.

In vitro passaging

To determine whether the mutation N291D in PatA accelerated the acquisition of fluoroquinolone resistance, TIGR4 and the point mutants were serially passaged in ThyB supplemented initially with subinhibitory, increasing concentrations of ciprofloxacin or levofloxacin. Once the bacteria reached exponential phase OD₆₂₀ ~0.6, they were collected, stored in glycerol at -80°C, and used to inoculate the next passage. The bacteria were exposed to progressively increasing 2-fold dilutions of respective antibiotic in subsequent rounds of passaging of the antibiotic. The passaging continued for 12 days.

Table 3-1. Strains used in this study

Strains	Description
TIGR4	Wild-type <i>S. pneumoniae</i> , serotype 4
$\Delta hexA$	<i>hexA</i> knockout
N291D <i>patA</i>	Single point mutant N291D point mutation in <i>patA</i> efflux pump
S79Y <i>parC</i> & N291D <i>patA</i>	Double point mutant: point mutation S79Y in <i>parC</i> & point mutation N291D in <i>patA</i>
S81F <i>gyrA</i> & N291D <i>patA</i>	Double point mutant: point mutation S81F in <i>gyrA</i> & point mutation N291D in <i>patA</i>

Mutant generation and transformation

The N291D *patA* point mutant was generated via transformation of TIGR4 the genomic DNA of the *in vitro* evolved isolates resistant to the highest concentration of ciprofloxacin. The point mutant was confirmed via whole genome sequencing. TIGR4 and the N291D *patA* mutant were incubated in C+Y media to OD₆₂₀~0.07 to 0.1. Then, the bacteria received stimulating peptide 2 (CSP-2) [125] and 5 µg of 2 kb PCR fragments encoding either S79Y in topoisomerase IV *parC* or S81F in *gyrA* or 5 µg of genomic DNA of fluoroquinolone resistant *S. pneumoniae* or viridans clinical isolates. For selection of transformants, the bacteria were incubated for 3 hours at 37°C supplemented with 5% CO₂, and 100 to 200 µL of the transformation mixture were spread on blood agar plates supplemented with 1 to 2x MIC of ciprofloxacin or levofloxacin and incubated overnight at 37°C and 5% CO₂. Transformation efficiency was calculated as the ratio of CFUs/mL of the transformants selected on ciprofloxacin- or levofloxacin-containing plates to the total number of bacteria grown on blood agar plates. Single- and double-point mutants were generated via transformation. For the double point mutants, the N291D *patA* were successively transformed with the fluoroquinolone resistance determinants S79Y *parC* or S81F *gyrA*. Individual colonies were selected for whole genome sequencing to confirm the presence of point mutations in *parC* and *gyrA*.

qRT-PCR analysis

RNA extraction and qRT-PCR were conducted based on a protocol adapted by Echlin *et al.* [126]. Briefly, TIGR4 and the N291D *patA* mutant were grown in C+Y until OD₆₂₀ ~0.4 and exposed to the antibiotics for 30 minutes to 1 hour, and RNA of each strain was extracted using the QiaShredder, RNeasy Kit (Qiagen), and DNA-free kit (Thermo Scientific). Then cDNA synthesis was performed using Superscript III First Strand Synthesis kit (Invitrogen), followed by qRT-PCR with SYBR Green. Fold change of *patA* was calculated as $2^{-\Delta\Delta CT}$.

Fluoroquinolones compound accumulation experiments

S. pneumoniae TIGR4 and N291D *patA* were inoculated into 500 mL of ThyB from bacteria grown on blood agar plates overnight. Cultures were grown at 37°C until mid-log OD₆₀₀ (0.6-0.8). The bacteria were pelleted at 4,500 x g for 10 minutes at 4°C, and the supernatant was discarded. The pellets were re-suspended in 20 ml of PBS and pelleted as before, and the supernatant was discarded. This process was repeated a total of two times. The pellets were resuspended in 16.6mL of fresh 1x PBS and aliquoted into 1.5 mL Eppendorf tubes (1 mL each). The number of colony-forming units (CFUs) was determined by a calibration curve. The OD₆₀₀ of TIGR4 and the mutant were 1.190 and 1.197, respectively. Compound was added to the 1 mL aliquots for a final concentration of 0.25 mM (25 µL of 10 mM stock or DMSO). Samples were incubated and shaken at 37°C for 10 minutes. After incubation, 800 µL of the cultures were carefully layered on 700 µL of silicone oil (AR20, cooled to -78°C). Each pellet was dissolved in 200 µL of

HPLC grade water and lysed via beat beating in which approximately 100 μ L of bead were added to the cells and then subject to 3x 45 second beating cycles. Compounds were extracted by adding 200 μ L of HPLC grade methanol. The lysates were pelleted at 13,000 r.c.f. for 2 minutes at room temperature and the supernatant was collected (150 μ L). Extracts were allowed to sit at room temperature for an hour to allow for additional lysate precipitation to occur. This remaining debris was removed by centrifuging at 20,000 r.c.f. for 10 minutes at room temperature. Lysates were spun through a 0.22 μ M filter prior to LC-MS/MS analysis. Bacteria were pelleted through the oil by centrifuging at 13,000 r.c.f. for 2 minutes at 4°C (supernatant remains above the oil); the supernatant and oil were then removed by pipetting. Cell pellets were flash frozen and stored at -80°C until lysis. LC-MS/MS analysis of accumulated compounds were conducted following our standard small molecule accumulation protocols. MS/MS methods were optimized for each individual compound.

Virulence studies

To examine virulence of TIGR4 and the point mutants, bacteria were grown in C+Y to logarithmic phase OD₆₂₀ ~0.8 and frozen in glycerol stocks at -80°C. Prior to the infection challenge, the enumerated glycerol stocks were thawed, washed, resuspended in 1x PBS, and diluted to the appropriate concentration for infection. Anesthetized, seven-week-old female BALB/c mice from Jackson Laboratory were intranasally infected with 30 μ L containing 1×10^7 CFUs of TIGR4 or the mutants. After the infection, the infected mice were monitored for disease progression, including lethargy, conjunctivitis, meningitis, pneumonia, and bacteremia. At 24 hours post-infection, blood samples, lungs, and nasal passage were collected via tail-bleeding and dissection, respectively. Bacterial burden was enumerated by serial dilution in 1x PBS and plating onto blood agar. Bacterial titers (CFUs/mL) were compared using the non-parametric Mann-Whitney t-test. Mouse survival curves were analyzed using the Kaplan-Meier survival curves and Mantel-Cox log rank test.

Results

N291D mutation is specific for increasing ciprofloxacin efflux, but not levofloxacin

In this study, we sought to identify mutations that can facilitate the acquisition of on-target mutations with high-level resistance by generating many possible mutations involved in fluoroquinolone resistance via *in vitro* passaging with ciprofloxacin and identifying mutations via whole genome sequencing (**Figure 3-1**). The goal of including Δ hexA in the *in vitro* passaging experiment was to maximize the number of mutations that potentially confer resistance to ciprofloxacin. Deletion of *hexA* results in defects in the mismatch repair system, thereby increasing the mutational frequency [127]. Compared to TIGR4, Δ hexA became resistant to ciprofloxacin at a significantly higher

Figure 3-1. *In vitro* serial passaging of TIGR4 and $\Delta hexA$
TIGR4 and $\Delta hexA$ were serially passaged in increasing 2-fold dilutions of ciprofloxacin. Ciprofloxacin MIC of TIGR4 is 4 $\mu\text{g/mL}$.

concentration (64 µg/mL), which is equal to the MIC (minimum inhibitory concentration) of clinical resistant isolates (**Figure 3-1**) [128]. Because extraneous mutations not related to ciprofloxacin resistance likely occurred in $\Delta hexA$, we transformed TIGR4 with the genomic DNA of the most resistant, evolved $\Delta hexA$ isolate and selected transformants on increasing concentrations of ciprofloxacin to identify the mutation(s) underlying ciprofloxacin resistance. On-target point mutations S81F in *gyrA* and S79Y in *parC* were identified in several evolved isolates. Those on-target mutations have been found in clinical isolates collected from different areas worldwide [102, 103, 129]. One off-target mutation of interest was N291D in the efflux pump *patA*. Clinically, mutations in the upstream region of *patA* have been reported and shown to upregulate the expression of this efflux pump; however, this particular mutation has never been reported. We next sought to characterize the point mutation N291D in *patA*. The N291D mutation did not induce overexpression of *patA* both in the absence and presence of antibiotics, such as ciprofloxacin, levofloxacin, and cefepime (**Figure 3-2A**).

We hypothesized that the mutation could enhance the capacity of the efflux pump to pump out fluoroquinolones, such as ciprofloxacin and/or levofloxacin. To test this hypothesis, we measured and compared the intracellular concentration of ciprofloxacin and levofloxacin of TIGR4 and the N291D mutant under the antibiotic pressure via IM-MS. Trimethoprim served as a non-efflux control compound for both strains. Overall, the intracellular concentration of ciprofloxacin in the N291D mutant decreased 2.62-fold compared to that of TIGR4 (**Figure 3-2B**). Only minor reduction in the accumulation of levofloxacin was observed in the N291D mutant (**Figure 3-2B**). The mutation did not affect trimethoprim accumulation (**Figure 3-2B**). These results indicate that the N291D mutation in *patA* arose as a result of the unique adaptation to effectively export ciprofloxacin.

N291D mutation in *patA* accelerated the acquisition of on-target mutations in *gyrA* and *parC* for fluoroquinolone resistance in *S. pneumoniae*

The mutation in *patA* conferred low level resistance to ciprofloxacin (1xMIC) and potentially could serve as the first step that allows for the acquisition of clinically relevant resistance. Next, we sought to investigate whether the N291D *patA* point mutant could facilitate the acquisition of high-level fluoroquinolone resistance via horizontal transfer. We transformed the wild-type TIGR4 and the N291D *patA* point mutant with 2 kb PCR fragments encoding a single mutation in either on-target genes *gyrA* or *parC* and selected transformant colonies on at least 1xMIC of ciprofloxacin or 1xMIC or levofloxacin. The negative control contained no DNA to ensure that fluoroquinolone resistance was conferred by incorporation of the single mutation in *parC* or *gyrA* from the added PCR fragments and not by spontaneous mutations. In contrast, the positive control (Tn-seq library) received genomic DNA of a group of mutants that have transposon insertions in non-essential genes in *S. pneumoniae*. This control should have a much higher chance of transformation and would demonstrate that the bacteria were competent during transformation. The similar transformation efficiencies of the wild-type TIGR4 and the N291D *patA* mutant with genomic DNA of Tn-seq mutants library in the positive

Figure 3-2. Characterization of N291D in *patA*

A) qT-PCR results demonstrated that N291D did not induce overexpression of *patA*. B) N291D *patA* mutant had increased ciprofloxacin efflux but not levofloxacin. Intracellular concentration of ciprofloxacin and levofloxacin measured via ICP-MS. Trimethoprim was a non-efflux susceptible comparator. **p-value ≤ 0.01 . This experiment was conducted by Stephanie Reeve, PhD from Lee laboratory at St. Jude.

control showed that the mutation in *patA* does not increase the bacterial competence. As shown in (**Figure 3-3A** and **C**), transformation with single on-target point mutations with TIGR4 did not readily occur as the previous studies showed.

When transformation did occur, the transformation efficiency was very low at about 10^{-8} to 10^{-6} per 5 μg DNA (**Figure 3-3A** and **C**). This result indicates that there may be barriers to horizontal transfer for fluoroquinolone resistance as single on-target point mutations alone were insufficient to transform the wild type to reach high level of resistance. Once acquired the single on-target mutations, TIGR4 became resistant to 4 $\mu\text{g/mL}$ of ciprofloxacin (1xMIC). In contrast, the transformation efficiency of the N291D *patA* point mutant with the PCR fragments was in the range of 10^{-8} to 10^{-3} per 5 μg DNA (**Figure 3-3B** and **D**). The subsequent acquisition of the N291D *patA* point mutant with the point mutations in *parC* and *gyrA* increased the level of resistance to ciprofloxacin from 4 $\mu\text{g/mL}$ to 16 $\mu\text{g/mL}$, 1x to 4x clinical MICs, respectively (**Table 3-1**). In the presence of ciprofloxacin, the N291D *patA* point mutant slightly increased the transformation efficiency with genomic DNA of fluoroquinolone-resistant pneumococcal isolates (**Figure 3-3E** and **F**). However, no increase in transformation efficiency was observed in the presence of levofloxacin, which is consistent with our previous finding that the N291D mutation was specific for ciprofloxacin efflux. These results suggest that the point mutation N291D in *patA* potentially facilitated the acquisition of primary target point mutations in *gyrA* and *parC* via horizontal transfer.

Another strategy for *S. pneumoniae* to acquire fluoroquinolone resistance is gaining *de novo* mutations. Therefore, we also determined if the N291D mutant developed fluoroquinolone resistance more quickly than the wild type by conducting *in vitro* passaging, in which the bacteria were exposed to progressively increasing concentrations of either levofloxacin or ciprofloxacin. During *in vitro* serial passaging, the N291D *patA* mutant displayed resistance against ciprofloxacin at earlier rounds of passaging than the wild type (**Figure 3-4A**). Consistent with our previous finding that the N291D mutation was specific for exporting ciprofloxacin, the mutant did not facilitate levofloxacin resistance development (**Figure 3-4B**). Taken together, these data suggest that the N291D mutation could potentially serve as a stepping stone for subsequent high-level resistance via both horizontal transfer and *de novo* mutations, which are two mechanisms underlying fluoroquinolone resistance in *S. pneumoniae*.

N291D mutation imparted no fitness costs in *S. pneumoniae*

Previous results demonstrated that the N291D mutation potentially increased the acquisition of fluoroquinolone resistance via horizontal transfer and *de novo* mutation. We next ascertained if the N291D mutation imposed deleterious fitness costs by characterizing the growth phenotype *in vitro* and virulence in the host of the point mutation. The extent of the fitness costs associated with the mutation N291D determines the mutant's potential to disseminate and facilitate the subsequent acquisition of high-level resistance. The single on-target point mutants S79Y *parC* and S81Y *gyrA* displayed

Figure 3-3. N291D *patA* mutant likely facilitated the acquisition of ciprofloxacin via horizontal transfer

A and B) Transformation efficiency of TIGR4 and the N291D *patA* mutant with PCR fragments encoding point mutations in *parC* and *gyrA*. Transformants were selected on blood agar plates containing 1x MIC ciprofloxacin. C and D) Transformation efficiency of TIGR4 and the N291D *patA* mutant with PCR fragments encoding point mutations in *parC* and *gyrA*. Transformants were selected on blood agar plates containing 1x MIC levofloxacin. E and F) Transformation efficiency of TIGR4 and the N291D *patA* mutant genomic DNA from resistant pneumococcal clinical isolates. Transformants were selected on blood agar plates containing 1x MIC ciprofloxacin. G and H) Transformation efficiency of TIGR4 and the N291D *patA* mutant with genomic DNA from resistant pneumococcal clinical isolates. Transformants were selected on blood agar plates containing 1x MIC levofloxacin. Transformation efficiency is the ratio of number of transformants selected on either ciprofloxacin or levofloxacin. Note: Transformation efficiency of TIGR4 with ciprofloxacin and levofloxacin serve as the reference and has been shown in previous study in Chapter 2.

Figure 3-4. The N291D *patA* mutant acquired *de novo* mutation(s) for ciprofloxacin resistance via more rapidly than the wild type

A) *in vitro* serial passaging with the N291D *patA* mutant and TIGR4 under ciprofloxacin pressure. B). *in vitro* serial passaging with the N291D *patA* mutant and TIGR4 under levofloxacin pressure.

noticeable growth delays during *in vitro* growth curve in nutrient-rich C+Y (Figure 3-5A). Intriguingly, the N291D *patA* mutant showed similar growth rate to that of TIGR4 (Figure 3-5A). The double point mutants N291D *patA* & S79Y *gyrA* and N291D *patA* & S79Y *parC* grew similarly to TIGR4 until late logarithmic phase followed by delayed lysis (Figure 3-5A). The N291D *patA* & S81F *gyrA* also grew similarly but lysed more quickly than TIGR4 (Figure 3-5A). To examine the fitness costs *in vivo*, we intranasally infected 6-week-old BALB/c mice with high-volume inoculum (100 μ L with 10^6 CFUs) containing the on-target and/or facilitating mutations or the susceptible, parental TIGR4 strain. At 24-hour post challenge, we collected nasal and blood samples via nasal lavage and tail bleeding, respectively. Compared to TIGR4, the single mutants having a point mutation in either *gyrA* or *parC* were generally attenuated (Figures 3-5B to E). Unlike the single mutant in *gyrA*, the single mutant in *parC* was able to colonize the nasal passage as effectively as the wild type. The single mutant in *patA* was able to colonize and was as virulent as the wild type (Figures 3-5B and C). Interestingly, the double mutants N291D *patA* & S79Y *parC* and N291D *patA* & S81F *gyrA* colonized more effectively than the single on-target mutants S81F *gyrA* or S79Y *parC*. This suggests that the mutation in *patA* reduced the fitness costs of the single on-target mutants' that impairs colonization, thereby improving the ability of the single on-target mutants to colonize and possibly increasing the chances of the bacteria to acquire higher levels of resistance. Both the single on-target mutants in *gyrA* or *parC* were not detected in the bloodstream, indicating that they were not able to disseminate and/or survive in the bloodstream to cause invasive disease (Figures 3-5D and E). The N291D mutation in *patA* was able to ameliorate the fitness costs of the on-target *parC* mutation in terms of invasiveness (Figures 3-5D and E). Both the single on-target mutants in *gyrA* or *parC* were not detected in the bloodstream, indicating that they were not able to disseminate and/or survive in the bloodstream to cause invasive disease. These results suggest that the horizontal acquisition of mutations in only one of the on-target enzymes (*gyrA* or *parC*) acquired via transformation is associated with high fitness costs, thus rendering the susceptible phenotypes *in vivo*. However, once facilitating mutation(s) without considerable fitness costs emerges, they could promote further acquisition of on-target mutations to drive clinically significant resistance without loss of fitness.

Discussion

In this study, we attempted to identify potential off-target mutation(s) that not only conferred low-level fluoroquinolone resistance but also accelerated subsequent acquisition of high-level fluoroquinolone resistance in *S. pneumoniae*. We specifically characterized a novel mutation N291D in the efflux pump *patA* in regard to its contribution to fluoroquinolone resistance in *S. pneumoniae*. The transformation efficiency results indicated that the mutation N291D likely served as a facilitator mutation that promoted the acquisition of on-target mutations and that, together, they conferred high level of fluoroquinolone resistance. This mutation in the efflux pump likely decreased the intracellular concentration of the antibiotic, which offered the bacteria a survival advantage that then led to the accumulation of more mutations

Figure 3-5. The N291D *patA* mutant had negligible effects on *in vitro* growth rate and virulence in an intranasal murine infection model

Six-week old BALB/c mice were infected intranasally 100 μ L containing 1×10^6 CFUs.

A) *in vitro* growth curves. B) Nasal lavage 24 hours post-infection. C) 48 hours post-infection. D) Blood titers 24 post-infection. E) Blood titers 48 post-infection. **p-values ≤ 0.01 ; ***p-values ≤ 0.001 ; ****p-values ≤ 0.0001 .

subjected to selection for fluoroquinolone resistance. Furthermore, the N291D did not impart any fitness costs *in vivo*, thereby enabling the bacteria to disseminate in clinical setting and enhancing the likelihood of subsequent acquisition of fluoroquinolone resistance development via horizontal transfer and/or *de novo* mutations. However, our finding that the high specificity of the mutation for ciprofloxacin instead of levofloxacin is consistent with that of Avrain *et al.* that PatA and PatB efflux pumps do not transport levofloxacin [130]. Taken together, the N291D mutation in *patA* once in clinical setting circulating likely poses a dangerous impetus for the emergence and spread of fluoroquinolone resistance by having minimal fitness costs, enhanced likelihood for the bacteria to obtain subsequent mutation for high-level resistance and remaining undetectable by current standard clinical testing.

CHAPTER 4. INTERSPECIES RECOMBINATION EVENTS CONFER POTENTIAL VIRULENCE AND FITNESS ADVANTAGES OVER *DE NOVO* MUTATIONS IN BETA-LACTAM RESISTANT *STREPTOCOCCUS PNEUMONIAE*

Introduction

Streptococcus pneumoniae remains a major human pathogen causing pneumonia, otitis media, bacteremia and meningitis in both children and adults. Globally, an estimated 300,000 deaths in children were attributed to pneumococcal infection in 2015, despite the introduction and use of pneumococcal conjugate vaccines [57]. Additionally, pneumococcal resistance to antibiotics in the United States has not appreciably decreased over the past few years, with resistance estimates hovering, for example, between 2% - 2.4% for penicillin over the past 5 years [131-134]. In 2019 alone, drug-resistant *S. pneumoniae* was responsible for an estimated 900,000 infections and 3,600 deaths and has been designated as a serious threat by the Center for Disease Control [135]. Moreover, β -lactam antibiotics are among the most commonly utilized drugs to treat pneumococcal infection, and while there is ongoing debate on the clinical impact on mortality and illness severity with β -lactam resistant *S. pneumoniae* [136, 137], it remains important to understand the mechanisms by which resistance can arise.

S. pneumoniae is a naturally competent pathogen, able to readily uptake genomic material from neighboring pneumococcal cells and closely related streptococcal species [30, 138, 139]. Consequently, the pneumococcus can utilize this ability to gain resistance via alterations in the genes encoding drug targets, such as the penicillin-binding proteins (PBPs) [30, 140, 141]. Importantly, previous work had identified recombination events, as opposed to spontaneous single nucleotide polymorphisms, as the major driver of rapid resistance acquisition [142]. Indeed, commensal streptococcal species resistant to penicillin and other β -lactams have been identified as reservoirs with which *S. pneumoniae* can recombine [143, 144]. However, genomic recombination in response to drug selective pressure can often result in virulence and fitness defects in exchange for drug-resistance [145, 146]. Therefore, understanding the consequences of these resistance-acquiring recombinations and the determinants driving this selection are critical to studying antibiotic resistance.

Here, we propose that steep fitness trade-offs associated with *de novo* mutations restrict their emergence in pneumococcal populations in response β -lactam exposure and that horizontal transfer of resistance genes via interspecies recombination may be preferable due to reduced fitness tradeoffs. Using β -lactam-resistant recombinant pneumococcal strains, we observed enhanced virulence *in vivo* with initial recombinant strains compared to antibiotic-evolved resistant pneumococcal strains possessing *de novo* point mutations. While *in vitro* characterization revealed only modest differences in adherence to mammalian cells, invasive potential, macrophage phagocytosis, and affinity for penicillin-binding proteins between strains, recombinant strains demonstrating initial small MIC increases were comparably virulent to wild type parent strains and exhibited

roughly equivalent bacterial burden within the blood, lung tissue, and colonization in the nasal passages. Sequencing of strains revealed fairly sizeable regions of apparent recombination, including a conserved pattern of recombination around the PBP2X locus in all recombinant strains tested. These findings indicate that horizontal gene transfer, as opposed to *de novo* mutation, is easily achievable and grants decreased susceptibility to β -lactams without a significant *in vivo* cost in *S. pneumoniae*.

Methods

Strains and growth conditions

Streptococcus pneumoniae was inoculated from frozen glycerol stocks onto tryptic soy agar (TSA Milipore Sigma) plates containing 20 μ g/mL neomycin and 3% defibrinated sheep blood and then incubated overnight at 37°C with 5% CO₂. Because *S. pneumoniae* is naturally resistant to neomycin, it was added to prevent contamination of other bacterial species. Liquid cultures using semi-defined media C+Y or ThyB (Todd Hewitt Broth BD 249240) and 2% yeast extract (BD 212750) were inoculated with the bacteria grown on the blood agar plate [147].

Epsilometric MIC determination

Bacterial strains were inoculated into C+Y from overnight growth on agar plates and incubated at 37°C with 5% CO₂ until reaching mid-logarithmic phase at OD₆₂₀ ~0.4. Then, 100 μ L of the bacterial culture were spread plated on blood agar plates supplemented with neomycin before E-test strips (bioMérieux) were added onto the center of the plates. Plates were incubated overnight at 37°C with 5% CO₂. MIC was determined in terms of μ g/mL where the symmetrical inhibition ellipse edge intersected the E-test strips on the plate. The recombinant strains were tested with either penicillin or cefepime as representative β -lactam antibiotics.

Recombinant pneumococcal strain generation and transformation

TIGR4 and D39 were grown in C+Y media to OD₆₂₀ ~ 0.07 to 0.1. Genomic DNA from penicillin- or cefepime-resistant *S. pneumoniae* or *S. viridans* strains and the competence stimulating peptide 2 or 1 (CSP-1 or 2) were added to TIGR4 or D39 cultures, respectively [125]. Transformation with genomic DNA of the Tn-seq library mutants served as the positive control as a measure of *S. pneumoniae*'s competence during each experiment. The Tn-seq library mutants represented a group of mutants that had transposon insertions in non-essential genes in *S. pneumoniae*. The bacteria were incubated at 37°C with 5% CO₂ for 3 hours, and the transformants were selected on blood agar plates supplemented with appropriate concentration of penicillin or cefepime, 3% sheep blood, and 20 μ g/mL of neomycin after overnight incubation at 37°C and 5% CO₂.

Transformation efficiency was calculated as the ratio of the number of transformant colonies (CFUs/mL) selected on penicillin- or cefepime-containing plates to the total number of bacteria on neomycin-containing plates.

***In vitro* experimental evolution**

TIGR4 was used as parental strain in antibiotic evolution experiments. Four replicate populations were grown in fresh ThyB with an increasing concentration of penicillin, or vancomycin for antibiotic adaptation populations. Four replicate populations were serially passaged in ThyB as controls to identify background adaptations in antibiotic adaptation experiments. When populations were adapted to their antibiotic environment (ThyB supplemented with 3x MIC of the respective antibiotic), a single colony was picked from each experiment and checked for its adaptive phenotype by growth curve experiments. Whole genome sequence was performed for each population and clones.

***In vivo* virulence determination**

To examine the bacteria's virulence, the wild-type TIGR4 and the derivative, transformant mutants were grown in C+Y to logarithmic phase $OD_{620} \sim 0.8$ and frozen as glycerol stocks at -80°C . Prior to infection, the glycerol stocks were thawed, washed, resuspended in 1x PBS, and enumerated to confirm the correct CFUs infection dose. Anesthetized seven-week-old female BALB/c mice from Jackson Laboratory were infected with the wild type and the mutants via low-volume intranasal instillation of 30 μL containing 1×10^7 CFUs. After the infection, mice were monitored for survival and disease progression, including lethargy, conjunctivitis, meningitis, pneumonia, and bacteremia. Blood samples, lungs, and nasal passage were collected at 24 post-infection via tail-bleeding (5 μL of collected blood was diluted into 1x PBS containing heparin) and dissection, respectively. Bacterial burden was enumerated by serially diluting into 1x PBS and plating onto blood agar, and bacterial titers were compared using the non-parametric Mann-Whitney t-test. Mouse survival curves were analyzed using the Mantel-Cox log rank test.

Lung harvest and histology

From each infected mouse, the left lung was collected for enumeration of bacterial burden, while the right lung was sent to SJCRH histology / pathology department for evaluation of inflammation severity. Briefly, right lobes were perfused and fixed with 10% formalin. Sections were stained with hematoxylin-eosin for evaluation under light microscopy. A scoring system was developed to quantify and compare histopathology of lungs infected by the recombinants to their respective wild-type strains. Criteria for the scoring system included vascular injury, leukocytes in lungs, collapsed alveoli, pulmonary edema. The scoring system was graded on severity and

extent of injuries, ranging from 0 = within normal limits, 1 = minimal (rare or inconspicuous lesions), 2 = mild (multifocal or small, focal, or widely separated, but conspicuous lesions), 3 = moderate (multifocal, prominent lesions), 4 = marked (extensive to coalescing lesions or areas of inflammation with some loss of structure), or 5 = severe (diffuse lesion with effacement of normal structure). Cytokines were also collected and analyzed for inflammatory signals, including GM-CSF, IL-1 β , IL-6, IL-10, IL-12 (p40), and TNF- α , using the Milliplex® MAP Kit (EMD Millipore Catalog# MCYTOMAG-70K). Briefly, serum or homogenized lung tissue supernatant was diluted and assayed against a standard curve using magnetic beads coated with cytokine-specific antibodies. Streptavidin-phycoerythrin was incubated with samples for detection and median fluorescence intensity (MFI) was read via Luminex® 200™. MFI output was normalized based on dilution and standard curve and converted to cytokine concentration in each sample.

***In vivo* nasal colonization determination**

To enumerate bacterial burden in the nasopharynx of the infected mice at 24 hours post-challenge, the nasal passage composed of nasal cavity, nasopharynx, paranasal sinus was isolated, mashed on a cell strainer, and washed with 750 μ L 1x PBS. The mixture was then serially diluted and spotted on blood agar plates prior to incubation at 37°C supplemented with 5% CO₂ overnight.

BOCILLIN FL-penicillin-binding protein affinity assay

From overnight cultures on blood agar plates, the bacterial strains were inoculated into C+Y and grown to OD₆₂₀ ~0.4. Then 1 mL of each bacterial culture was pelleted, washed with 100 μ L of 1x PBS, and resuspended in 100 μ L of 1x PBS containing 0.5 μ g of BOCILLIN FL penicillin (ThermoFisher Scientific). After being incubated for 30 minutes at 37°C, the bacteria were pelleted at 6,500 x g for 10 minutes and washed with 100 μ L of 1x PBS to remove unbound BOCILLIN FL penicillin. Each bacterial pellet was resuspended in 100 μ L of 1x PBS, TritonX100 0.1%, and 1x complete mini protease inhibitor tablet before lysis at 37°C for 30 minutes and freezing overnight at -20°C. BCA assay was performed to determine the concentration of proteins in each pellet. The pellets were boiled for 3 minutes at 100°C, then 10 μ g of protein per sample was resuspended with 1 x LDS loading buffer without reducing agent and loaded onto an SDS-PAGE gel (4-12%). Penicillin-binding protein bands were visualized using ChemiDoc with Pro-Q Emerald 488 settings and quantified using Image Lab software.

Adhesion and invasion assays

A modified version of the adhesion and invasion protocol developed by Orihuela and colleagues was followed [148]. Briefly, A549 lungs epithelial cells (ATCC) were cultivated in 24-well-tissue culture-treated plates (Costar, Corning) until having 90-95%

confluent monolayers numbering roughly 4×10^5 cells/mL. After being activated at 37°C with TNF- α (10 ng/mL) in F12K media (ATCC) supplemented with 10% FBS and 20 μ g/mL gentamicin, cells were washed twice with 1x PBS and then infected with 1 mL of $\sim 10^7$ bacteria at mid-logarithmic phase OD₆₂₀ ~ 0.4 , which yields the MOI of $\sim 25:1$. Once bacteria and cells were incubated for 30 minutes at 37°C, the supernatant was serially diluted and plated onto blood agar to determine the number of non-adherent bacteria. Cells with adhered bacteria were washed twice with 1x PBS and detached with 100 μ L of 0.1% Trypsin-ETDA (ThermoFisher) in 1x PBS for 5 minutes at 37°C. Cells were resuspended in 900 μ L 1x PBS. To determine the number of adherent bacteria, the cell suspension was serially diluted and plated onto blood agar. Ratios of adherent bacteria to total bacteria number for each strain were calculated. Total bacteria number was calculated by adding adherent bacteria and non-adherent bacteria. Each bacterial strain was represented in three biological replicates in independent experiments.

For the invasion assay, A549 cells were seeded, activated, and infected as in the adhesion assay except the following. After bacterial infection, cells were incubated at 37°C with 5% CO₂ for 2 hours and washed twice with 1x PBS. To eliminate extracellular bacteria, cells were incubated with 1 mL of infection media, which contained F12 media, 1:100 Pen/Strep solution, and 200 μ L gentamicin), for 1 hour at 37°C 5% CO₂ before washing 3 times with 1x PBS. Then, 100 μ L of 0.1% Trypsin-EDTA in 1x PBS was added to each well to detach cells, and the suspension was incubated for 5 minutes at 37°C. Cells were lysed by incubating with 200 μ L of 0.025% ice cold Triton x100 in water for 8 minutes at 37°C. Finally, to determine the number of intracellular bacteria, cells were resuspended completely by pipetting, and 200 μ L of the suspension was spread onto blood agar plates. Ratios of intracellular bacteria to total number of bacteria were compared those of TIGR4. Total number of bacteria is equal to the sum of intracellular bacteria and extracellular bacteria, which were determined by plating the supernatant immediately after the 3-hour incubation post-infection with bacteria. Each bacterial strain was assayed in three biological replicates conducted in independent experiments.

Macrophage phagocytosis assay

J774A.1 murine macrophages were subcultured in DMEM (Dulbecco's Modified Eagle Medium, ThermoFisher) supplemented with FBS (fetal bovine serum, 10% vol/vol, Sigma), 50 mg/mL streptomycin and 50 units/mL penicillin (Sigma). Once reaching at 70-80% confluent monolayers in 24-well plates, cells were washed 3 times with 1x PBS and resuspended in DMEM containing 3% bovine serum albumin (BSA, Sigma) without antibiotics. Then, cells were activated with fresh murine serum prior to infection with 100 μ L of bacteria at OD₆₂₀ ~ 0.1 , which corresponds to a multiplicity of infection of 50 bacteria to 1 macrophage). Cells were incubated with bacteria at 37°C for 30 minutes or 1 hour, and then washed followed by incubation for 1 minute with DMEM containing 100 μ g/mL gentamicin. The washing and incubation process was repeated 3 times. Finally, the macrophages were lysed using 0.025% Triton, serially diluted, and plated onto blood agar plates to enumerate viable engulfed, intracellular bacteria.

Time-kill assay

The WT and derivative, transformant, mutant strains were grown in ThyB to early logarithmic phase $OD_{620} \sim 0.2$. The bacteria were exposed to respective 10x MIC of either penicillin or cefepime. Bacterial CFUs were tittered and plated onto neomycin-containing blood agar plates prior to antibiotic exposure and hourly for 4 hours post-exposure.

Genomic DNA extraction

Genomic DNA of the WT and the derivative, transformant strains were extracted via the aqueous / organic extraction method using phenol chloroform. Briefly, the bacteria were grown in C+Y to late logarithmic phase at $OD_{620} \sim 0.6$ and were pelleted at $6000 \times g$ for 10 minutes followed by lysis in 1 mL 1x PBS containing 50 μ L 10% DOC, 50 μ L 10% SDS, and 10 μ L of 10 mg/mL Proteinase K (Sigma). The suspension was incubated until clear (approximately 5 minutes) at 37°C, mixed with 500 μ L phenol: chloroform: isoamyl alcohol (Sigma), transferred to phase-lock tubes (Quantabio), and centrifuged at maximum speed for the separation of aqueous and organic phases. Then 500 μ L chloroform: isoamyl alcohol was added to, mixed with the aqueous phase, and centrifuged. To precipitate DNA, the aqueous phase was transferred to a new Eppendorf tube containing 100% ethanol. Precipitated DNA was washed with chilled 70% ethanol, pelleted, dried, and resuspended in nuclease-free water.

Whole genome sequencing and analysis

Extracted genomic DNA from the recombinants were sent to Hartwell Center for whole genome sequencing. Sequence libraries were barcoded using the Nextera kit (Illumina), and run on the Illumina HiSeq2000 platform as previously described [149]. The reads were mapped and compared to the TIGR4 reference genome for analyzing mutations resulting from recombination events.

Results

Early recombinant pneumococcal strains exhibit MICs comparable to experimentally-evolved isolates

Table 4-1 displays the minimum inhibitory concentrations of the β -lactam antibiotics penicillin and cefepime for generated strains, parents, and donor strains used in this study. Recombinant β -lactam resistant pneumococcal strains were generated via transformation with genomic DNA (gDNA) from clinical isolates of resistant viridans group streptococci (VGS). Second- and third-round recombinants, so named because they are the result of successive transformations of the previous “round” of successful transformants, were subsequently created by repeating transformation an additional one

Table 4-1. MICs of wild-type pneumococcal strains, their derivative, recombinant mutants, viridans group streptococcal donor strains, and experimentally-evolved pneumococcal populations

Strain	ID	Description	Penicillin MIC ($\mu\text{g/mL}$)
T4	T4	Parent	0.008
T4R1P	T4+SV5	1 st recombinant	0.032
T4R2P.1	T4+SV5+SV3	2 nd recombinant	0.047
T4R3P.1	T4+SV5+SV3+SV11	3 rd recombinant	0.19
T4R2P.2	T4+SV5+SV8	2 nd recombinant	0.047
T4R3P.2	T4+SV5+SV8+SV4	3 rd recombinant	0.38
D39	D39	Parent	0.008
D39R1P	D39trSV2A Pen	1 st recombinant	0.023
D39R2P	D39Sv2AtrSV5A Pen	2 nd recombinant	0.047
D39R1C	D39trSV2A Cefep	1 st recombinant	0.023
D39R1C	D39SV2AtrSV5A Cefep	2 nd recombinant	0.016
T4 316	T4 316	Parent	0.012
T4EV	TvO VanR Pop1	Experimentally-evolved population	0.023
T4EP	TvO PenR Pop1	Experimentally-evolved population	0.023
SV2	SV2	VGS clinical isolate	0.75
SV3	SV3	VGS clinical isolate	1.5
SV4	SV4	VGS clinical isolate	2
SV5	SV5	VGS clinical isolate	4
SV8	SV8	VGS clinical isolate	>32

or two times, respectively, in the presence of VGS gDNA with colony selection on penicillin or cefepime blood agar plates. In contrast, β -lactam resistant point mutant populations were experimentally evolved via *in vitro* continual passaging of the initial parent strain T4 316 in sub-inhibitory concentrations of antibiotics. When comparing the MICs to penicillin of the recombinant isolates versus the experimentally-evolved populations, first recombinants (T4R1P and D39R1P) possessed similar penicillin susceptibilities as the experimentally-evolved isolates T4EP and T4EV. Both the penicillin-evolved pneumococci population T4EP and the vancomycin-evolved population T4EV were selected as comparators due to comparable increases in penicillin MIC as the initial recombinant strains. As expected with subsequent transformations, MICs to penicillin and cefepime increased in the second- and third-round transformation strains. Though recombinant isolates were screened on single antibiotics (either penicillin or cefepime), many strains, particularly, the later (second- and third-round) recombinant series showed decreased susceptibility to both agents, suggesting that chromosomal recombinations at common β -lactam targets may be involved.

Inter- and intraspecies recombinants exhibited similar transformation efficiencies

gDNA of 20 clinical VGS strains were isolated for use in generating recombinant β -lactam resistant *S. pneumoniae*. Of these, the gDNA of ten VGS strains yielded successful transformants when co-incubated with either wildtype D39 (serotype 2) or TIGR4 (T4, serotype 4). gDNA from known resistant pneumococcal isolates was also transformed into the parent strains as comparators. The transformation efficiencies for the interspecies recombinants varied between 10^{-6} and 10^{-8} per 5 μ g of gDNA, which was comparable to the transformation efficiencies of generating intraspecies recombinants (**Figure 4-1** and **4-2**). Subsequent transformations using first-round transformants also did not alter transformation efficiency when generating second- and third-round recombinants. Overall, our results suggest that for VGS strains whose DNA was readily uptaken by *S. pneumoniae*, the transformation efficiency was close to that of intraspecies recombination under the same conditions. However, the success of DNA uptake and recombination is likely strain- and condition-dependent.

First recombinant pneumococci are not attenuated *in vivo* compared to wild-type strains

In order to compare *in vivo* fitness and other phenotypic traits between β -lactam resistant strains, we challenged anesthetized, 7-week-old BALB/c mice with intranasal inoculums of either recombinant pneumococci or the penicillin and vancomycin experimentally-evolved populations and monitored survival and enumerated bacterial burden in the blood 24 hours post-infection (**Figure 4-3**). Early recombinant pneumococcal strains – i.e. the first recombinants T4R1P, D39R1P, and D39R1C exhibited blood titers equivalent or greater to that of the parent strains T4 and D39. A similar observation was made with regard to overall mouse survival in which early

Figure 4-1. Transformation efficiencies of parental TIGR4 with genomic DNA of viridans group streptococci

Transformation efficiency was calculated as the ratio of the number of recombinant CFUs selected on penicillin-containing blood agar plates to the total number of CFUs. A) First round transformation efficiencies. B and C) second round transformation efficiencies. D and E) and third round transformation efficiencies of TIGR4 parental strain. Positive control transformation efficiencies were generated from Tn-Seq library genomic DNA.

Figure 4-2. Transformation efficiencies of parental D39 with genomic DNA of viridans group streptococci

Transformation efficiency was calculated as the ratio of the number of recombinant CFUs selected on either penicillin or cefepime-containing blood agar plates to the total number of CFUs. First round transformation efficiencies in D39 on either B) penicillin or C) cefepime and second round transformation efficiencies screened on D) penicillin or E) cefepime are displayed for VGS donor gDNA as well as Tn-Seq library gDNA positive controls. Transformation efficiencies for resistant pneumococcal genomic DNA was also performed as a comparator.

Figure 4-3. Blood titers and mouse survival after intranasal challenge with recombinant versus experimentally-evolved pneumococci

Seven-week old BALB/c mice were intranasally infected with 10^6 CFUs of either A and B) TIGR4 and its derived recombinant strains. C and D) D39 and its derived recombinant strains. E and F) T4 316 and its derived experimentally-evolved populations in groups of five. Bacterial blood titers were obtained via tail-vein bleed 24 hours post-infection. Mouse survival was followed out to 10 days post-infection.

recombinant strains displayed virulence equal to or greater than that of the wildtype parent strains. In contrast, the experimentally evolved isolates were highly attenuated, exhibiting significantly lower blood titers compared to the T4 316 (serotype 4) parent isolate and greatly reduced 10-day mortality. Second and third recombinants in general also showed attenuated virulence in the mouse model, with the exception of the second-round recombinant D39R2C, which behaved similarly to the first recombinant parent. Based on our observations, early recombinants do not initially possess attenuated traits *in vivo*, and attenuation was not observed until *S. pneumoniae* underwent further recombination via horizontal gene transfer. Furthermore, the levels of attenuation observed in the later recombinant pneumococcal strains was comparable to the decreased virulence seen in mice challenged with the penicillin and vancomycin experimentally-evolved isolates. These data initially suggest that early recombinants can possess decreased susceptibility to β -lactam agents without significant defects to *in vivo* fitness.

***In vitro* phenotypes of recombinant versus experimentally-evolved pneumococcal strains were not significant**

To further assess the phenotypes of the resistant recombinant strains in comparison to the experimentally-evolved pneumococcal isolates, strains were incubated with A549 human alveolar basal epithelial cells to compare strain capacity for invasion and adhesion (**Figures 4-4 A and B**). While some changes in the ability of the recombinant strains to adhere to cells were observed, overall results were inconsistent between any of the tested strains that might explain differences in attenuation seen *in vivo* between the second and third recombinants compared to the first recombinant series. For example, while the second- and third-round recombinants T4R2P.1, T4R3P.1, T4R2P.2, and T4R3P.2 showed elevated adhesion to alveolar epithelial cells compared to TIGR4, the same could not be said of the second-round recombinants D39R2P and D39R2C, which were not significantly different from D39 (**Figure 4-4A**). Similarly, quantification of intracellular pneumococcal cells revealed differences inconsistent between recombinants and the wildtype, suggesting that changes in invasion were not primarily responsible for fitness differences seen *in vivo* (**Figure 4-4B**). Additionally, neither experimentally-evolved strain showed significantly different adherence compared to T4 316 (**Figure 4-4A**).

We next assessed whether differences in macrophage phagocytosis existed between recombinant strains, their parents, and experimentally-evolved isolates. For this, J774A.1 murine macrophages were co-incubated with respective pneumococcal strains at an approximate MOI of 50. In comparison with each respective strain's parent, D39R1C and T4EV demonstrated significantly higher macrophage phagocytosis, while D39R1P exhibited significantly lower phagocytosis (**Figure 4-4C**). All other strains did not significantly differ from their respective parent, as measured by the proportion of intracellular *S. pneumoniae* cells present inside lysed macrophages versus total pneumococcal cells present overall. The measure of intracellular *S. pneumoniae* may indicate a significant alteration in the ability of the pneumococcal cells to survive intracellularly after phagocytosis and/or the amount/ability of macrophages to ingest the

Figure 4-4. Adhesion and invasion and macrophage phagocytosis for recombinant pneumococcal strains versus parental strains and experimentally-evolved populations

A) Adhesion assays using A549 murine lung epithelial cells were performed to examine the adhesion of TIGR4 and D39 and derivative first, second, and third recombinant strains, T4R2P.1, T4R3P.1, T4R2P.2, and T4R3P.2, respectively, and experimentally-evolved strains and their WT T4 316. Adhesion ratio was calculated as the ratio of the number of adhered CFUs to the total number of CFUs, which was the sum of adherent and non-adherent CFUs. B) Invasion assays using A549 murine lung epithelial cells were performed to examine the invasion of TIGR4 and D39 and derivative first and third recombinant strains, T4R2P.1, T4R3P.1, T4R2P.2, and T4R3P.2, to A549 cells. Invasion ratio was calculated as the ratio of the number of adhered CFUs to the total number of CFUs at time of plating. The total bacterial number expressed as CFUs was the sum of invaded (engulfed) and remaining bacteria in the supernatant. In both the adhesion and invasion assays, each strain was measured in three biological replicates. C) Enhanced macrophage phagocytosis observed with D39-derived first recombinant D39R1C and T4E compared to their parent strains. The recombinants and their respective WT strains were incubated with activated murine macrophage cells J774A.1 at MOI 50. Macrophage killing assay were conducted in three biological replicates. The recombinants and experimentally-evolved. Strains were compared to their respective parent strains using unpaired t-tests.

A **B** **C**

invading pneumococcus. However, here we saw no strong trends of recombination versus *de novo* mutation leading to consistent changes in killing by macrophage.

In order to determine alterations in PBP-binding affinity of the recombinant strains and the experimentally-evolved isolates, BOCILLIN FL, a fluorescent penicillin derivative, was added to actively dividing bacteria during mid-logarithmic phase, and whole-cell protein was purified and quantified. A standard concentration of 10 µg was loaded and run via gel electrophoresis which allowed for the visualization of fluorescent PBP bands, in which band intensity would correlate with PBP abundance and BOCILLIN FL-binding affinity. Quantification of band intensity revealed no large changes in PBP-binding between any strain and its parent (**Figure 4-5**). Overall, many of the recombinant strains showed very slight decreases in band intensity for many, if not all, of the six visualized PBPs, perhaps suggesting a slight decrease in the PBPs' affinity to BOCILLIN FL compared to the parent strain. However, our data indicate that the differences in BOCILLIN FL-PBP binding affinities between recombinant strains was also modest, and therefore not likely wholly responsible for differences in virulence and fitness seen between recombinant strains.

Initial recombination with resistant determinants to β -lactams, such as penicillin and cefepime, induced tolerance to those antibiotics

Due to their subtle increases in MICs to penicillin and cefepime, we next sought to investigate whether the recombinants displayed tolerance phenotypes by examining their kill kinetics in the presence of antibiotics. The Tupelo strain, a prototypical tolerant pneumococcal strain, served as a reference strain and positive control for tolerance experiments [150]. Upon exposure to lethal concentrations of penicillin or cefepime (respective 10x MIC), both TIGR4-derived and D39-derived third-recombinants exhibited a more gradual decline in bacterial CFUs than that of the susceptible wild type. The kill kinetics of the third-round, penicillin-screened recombinants also more closely resembled that of the tolerant Tupelo strain, indicating that these recombinants displayed tolerance to otherwise lethal concentrations of the antibiotics (**Figure 4-6**). In contrast, the first- and second-round recombinants in TIGR44 and D39 backgrounds as well as the experimentally-evolved isolates T4EP and T4EV showed tolerance to neither penicillin nor cefepime (**Figure 4-6**).

First recombinants resulted in similar bacterial burden in the blood and lungs and similar nasal colonization as wildtype during murine intranasal infection

Initial experiments testing the recombinants' virulence in our murine model indicated that the first-round recombinants in both TIGR4 and D39 backgrounds were not attenuated in terms of bacterial burden in the bloodstream and mouse survival (**Figure 4-3**). To further characterize the effects of early recombination in pneumococcal pathogenesis, we examined the first compared to the less β -lactam susceptible third

Figure 4-5. BOCILLIN FL-binding assay in recombinant strains, parental strains, and experimentally-evolved pneumococci

The A) T4-derived recombinants, B) D39-derived recombinants, and C) the experimentally-evolved pneumococci were transiently exposed to fluorescent, PBP-binding BOCILLIN FL, and fluorescent band intensity for 6 PBPs were visualized and quantified. Difference in BOCILLIN FL affinity for PBPs compared to the parent is expressed as log-fold change in band intensity.

Figure 4-6. Bacterial time-kill curves with penicillin and cefepime

Half-hour bacterial titers were taken after exposure to 10x MIC of either penicillin for A) T4-derived strains, B) D39-derived strains, and C) T4 316-derived strains or cefepime for (D) D39-derived strains and E) T4 316-derived strains. Bacterial growth is represented as a percentage of the bacterial CFU/mL taken at time=0 prior to addition of antibiotic. The prototypical tolerant *S. pneumoniae* strains, Tupelo, and the $\Delta lytA$ mutants in T4 and D39 were included as reference strains for tolerance phenotype.

recombinants' virulence in different host's niches, including nasal passages, blood, and lungs. At 24 hours post-infection, mice infected with TIGR4-derived first recombinants had relatively equivalent bacterial burden in the blood, nasal passages, and lungs compared to mice infected with T4 (**Figure 4-7**). In contrast, mice infected with T4-derived third recombinants had significantly lower bacterial burden in the blood and nasal passages, $p < 0.001$ and $p < 0.05$, respectively. Displaying the same trend although not statistically significant, mice infected with D39-derived first- and third-round recombinants had relatively lower bacterial burden in all three host's niches compared to mice infected with D39. Compared to the initial virulence experiment, these results recapitulated that mutations from initial recombination did not diminish *S. pneumoniae*'s ability to colonize and invade the host, whereas mutations resulting from subsequent recombinations, and consequently the further decreased antibiotic susceptibility, may decrease the bacteria's virulence.

Pulmonary histopathological studies revealed heightened inflammation in T4-derived, but not D39-derived recombinants

With the lung tissues collected at 24 hours post-infection, we examined the signs of inflammatory response and extent of lung injuries from infection with the recombinant mutants and their respective wild-type strains, including vasodilation of blood vessels (arterioles, arteries, veins), neutrophil migration from the bloodstream into the pulmonary air space, vascular injuries, leukocytes in lungs, collapsed alveoli, and pulmonary edema. Cumulative pathological scoring of the right lobar sections of the five mice in each infected group revealed slightly higher severity of interstitial inflammation, alveolar involvement, and presence of alveolar neutrophils in T4R1P (25, 36, and 40, respectively) and T4R3P.1 (32, 28, and 34, respectively) compared to T4 (12, 21, and 20, respectively). By contrast, the D39-derivative recombinants D39R1P and D39R2P did not have subjectively different scoring compared to D39. Heat-killed versions of T4, T4R1P, D39, and D39R1P completely lacked any evidence of infection or inflammation, suggesting that dead cells and cell fragments were not responsible for damage in the lung (**Figure 4-8**).

Inflammatory cytokine profiling revealed no major differences in blood or lung tissue after intranasal infection

During acute inflammation of lung tissues caused by pneumococcal infection, neutrophils that infiltrate the lungs and migrate into the pulmonary airspace induce the expression of regulatory inflammatory cytokines, such as GM-CSF, IL-1 β , IL-6, IL-10, IL-12 (p40), and TNF- α [151]. There were few significant differences in the expression of cytokines responsible for the acute inflammatory phase collected and analyzed from mice infected with first or third recombinants compared to their parental strains (**Figure 4-9**). Indeed, only expression of IL-6 and TNF- α in the serum of T4R3P.1-infected mice compared to those of T4 infected mice were statistically decreased ($p = 0.0079$). Consistent with the pulmonary histopathological findings, most differences

Figure 4-7. Bacterial burden in the blood, lungs, and nose 24-hours post-intranasal challenge

Pneumococcal titers from either (A) chest cavity blood, (B) lung tissue, or (C) nasal passages were obtained in each case for T4 or D39, their respective first recombinants, and the last recombinant exhibiting the highest MIC (T4R3P.1 and D39R1P, respectively). Titers were obtained 24 hours after intranasal infection with each pneumococcal strain. N=5 mice per infected group.

Figure 4-8. Pulmonary histopathology of murine lungs infected with recombinant mutants vs. the respective wildtype strains, TIGR4 or D39

Representative images of right lobar sections 24 hours post-infection are shown at 10X magnification for A) heat-killed T4, B) T4, C) heat-killed D39, D) D39, E) heat-killed T4R1P, F) T4R1P, G) heat-killed D39R1P, H) D39R1P, I) T4R3P.1, and J) D39R2P strains used in the respective infected mice (n=5 for non-heat-killed strains and n=3 for heat-killed strains).

Figure 4-9. Cytokine profiles in collected serum and lung tissue in a murine intranasal infection model 24 hours post-infection
 Concentrations in either the serum or lung tissue of infected mice for A) GM-CSF, B) IL-1 β , C) IL-6, D) IL-10, E) IL-12 (p40), and F) TNF- α are shown here for T4, D39 and recombinant pneumococcal strains T4R1P, T4R3P.1, D39R1P, and D39R2P as well as their heat-killed counterparts. Results are based on five mice (biological replicates) per non-heat-killed strain with serum and lung samples run in technical duplicate. Results for heat-killed strains were done in biological triplicates. **p-values ≤ 0.01 .

in cytokines induced by recombinants compared to their parent strains were slight. Overall, the degree of inflammation and inflammatory markers as a result of infection with recombinant strains closely resembled that of the parent strains, indicating that the recombinants' abilities to cause inflammation and tissue injury was not attenuated.

Recombinant pneumococcal isolates showed large, regional genomic changes

When examining their genome sequence against their parent strains, recombinant pneumococcal isolates showed several large SNP-rich regions, likely indicating sections of the genome where recombination with the exogenous VGS DNA took place (**Figure 4-10A and B**). For example, in the first recombinant series T4R1P, a segment of roughly 4 kb containing multiple genes, including the β -lactam target PBP2X, is densely populated with SNPs. Presumably this region underwent recombination with the VGS DNA from isolate SV5, in which antibiotic pressure selected for recombinant PBP2X. As one might expect, recombinant PBP2X was conserved in successive recombinations screened on penicillin, and accumulated additional SNPs occurring after each additional transformation. Indeed, changes in the ~300,000 bp region containing the PBP2X locus was prominent and conserved in every recombinant isolate sequenced (**Figures 4-10A and B**). The third series recombinants T4R3P.1 and T4R3P.2, both derived from T4R1P and possessing recombinant PBP2X, also both integrated recombinant changes in PBP2B, despite the fact that the donor DNA used to generate the recombinants was sourced from two different VGS isolates.

Similar broad changes to PBP2X and PBP2B were seen in D39 recombinants. The first recombinant screened on cefepime, D39R1C, for example, possessed several mutations in PBP2X after a single transformation spanning a roughly 260 bp section of the gene. A much larger recombination occurred after a successive transformation with SV5 VGS gDNA as the changes to the PBP2X gene in D39R2C spanned more than 2 kb (**Figure 4-10C and D**). By contrast, D39R1P and D39R2P were first and second recombinants, respectively, utilizing the same donor gDNA as D39R1C and D39R2C, except screened using penicillin. Here, an initial large 2kb recombination within PBP2X was seen in the first recombinant, while a >1kb segment in PBP2B was changed after re-transformation (**Figure 4-10C and D**). Thus, while the genes targeted during recombination under β -lactam selective pressure were often the same, the magnitude and scope of these changes could vary considerably.

Additionally, we observed variable changes in non-PBP regions of the genome for each recombinant. Both isolated polymorphisms and SNP-heavy regions of recombination were present in all recombinant series. Notably, T4R1P and all of its derivative strains possessed a recombinant MurM gene, involved in the production of pneumococcal branched-stem peptides and PBP-mediated β -lactam resistance [152]. Also within this series of recombinant isolates was the presence of a 5 kb recombinatorial region encompassing, among others, genes encoding LuxS, which helps in the regulation of virulence and fitness genes during biofilm formation, ClpB, a heat-shock and stress-

Figure 4-10. Genome mapping and frequency of SNPs in recombinant vs. parental strain

Maps of genomic changes present in A) the T4 parent and recombinant series T4R1P, T4R2P.1, and T4R3P.1 and B) the T4 parent and recombinant series T4R1P, T4R2P.2, and T4R3P.3 compared to the reference strain TIGR4 (NCBI Reference Sequence: NC_003028.3) as well as C) the D39 parent and recombinant series D39R1C and D39R2C and D) the D39 parent and recombinant series D39R1P and D39R2P compared to the reference strain D39 (NCBI Reference Sequence: NC_008533.2). Strains are denoted by differently colored rings. SNPs are illustrated by darkened lines with frequency denoted by length of the line across the width of the ring (i.e. SNPs conserved within 100% of the sequenced population span the width of each respective ring). Position along the pneumococcal genome is circumscribed on the outer edge of each figure.

response protein, and DexB, a highly conserved glucosidase known to flank one side of the pneumococcal capsular polysaccharide locus. No other PBP-associated genes were localized in this region, suggesting that one of more of these gene changes may be advantageous for the pneumococcal cell with regard to β -lactam exposure. These changes come largely in contrast with the experimentally-evolved isolates, which possessed few conserved (100% of the sequenced population) point mutations. Decreased susceptibility for T4EP likely stems from a single Y586S amino acid change in PBP2X. Other changes included single point mutations in the cell wall biosynthesis component, MurE, the capsular polysaccharide biosynthesis gene, Cps4E, a DHH subfamily 1 protein, and an ATP-binding cassette transporter. Likewise, T4EV possessed two conserved mutations, again in Cps4E and in the cell division-associated gene FtsW. In both of these isolates, many of these genes have been linked either as resistance determinants or virulence factors in *S. pneumoniae* [153-155]. However, it is unknown whether these genomic changes would be solely responsible for the *in vivo* attenuation observed and increased MICs to the β -lactams.

Discussion

In this study, we have generated recombinant pneumococcal isolates with decreasing susceptibilities to β -lactam antibiotics. In turn, these isolates were tested and shown to have variable fitness in intranasal mouse infection models, where bacterial blood and lung tissue titers, nasal colonization, and overall lethality tended to be inversely related with serial recombination and increased MIC. The *in vitro* experimentally-evolved pneumococcal isolates similarly demonstrated *in vivo* attenuation to an extent similar to the second- and third-round recombinants. However, all first-round recombinants exhibited *in vivo* fitness similar to the wild-type parent strains despite slightly elevated MICs to the β -lactams.

We identified few significant changes to virulence phenotypes in the recombinant isolates compared to wildtype. Furthermore, many differences measured *in vitro* did not appear to translate *in vivo*. For example, the second and third recombinants, T4R2P.1, T4R2P.2, T4R3P.1, and T4R3P.2 all exhibited increased adhesion to A549 cells *in vitro*, which suggests possible advantages to colonization. However, nasal colonization titers with T4R3P.1 in mice actually showed decreased colonization 24 hours post-infection compared to T4 316. Similarly, the scoring of lung histology comparing the resistant recombinant pneumococcal strains suggested that there was a subjective increase in inflammation with recombinant *S. pneumoniae* strains compared to wildtype, despite lack of significance in inflammatory cytokine profiling of the lungs and blood. Consistent with the alterations in PBP2X found in all resistant pneumococcal recombinants, a slight decrease in BOCILLIN FL-binding affinity was observed for PBP2X in all recombinants compared to the parent isolate. Our results appear to indicate that changes to any one virulence trait studied here were relatively minor and that perhaps the cumulative effect of these deficits results in the overall attenuation seen *in vivo* with some of the recombinant strains.

While the evolution of antibiotic resistance is very often accompanied by a tradeoff in fitness, growth, and/or virulence, the underlying cost associated with resistance development is highly variable and dependent on the genetic mechanisms driving resistance [56, 146]. Altered β -lactam susceptibility has been tied to differences in virulence and transmission in pneumococcal isolates [156, 157] and specifically the acquisition of resistant PBP alleles has been shown to come at the expense of fitness determinants in competitive nasal colonization models [145]. Our findings demonstrated that resistant strains evolved via different mechanisms, i.e. horizontal transfer versus *de novo* mutation, can have rather dissimilar *in vivo* fitness despite exhibiting similar susceptibilities. The attenuation to invasion without any significant change in colonization seen *in vivo* with the *de novo* mutant strains indicates that resistance acquired by horizontal transfer may be preferable from an evolutionary standpoint. Mechanistically, it is possible that horizontal transfer allows for a hugely diverse exchange of genetic material among isolates, which under drug pressure might engender selection for low-cost resistance traits or accompanying compensatory genetic changes.

In all cases of β -lactam induced resistance within the isolates we examined, we saw changes to the penicillin-binding protein PBP2X, and in many cases, especially in later recombinants, alterations in PBP2B. Some mutations in PBP1A and PBP2X have been identified as compensatory mutations that may restore fitness to *S. pneumoniae*, stabilizing resistance mutations by allowing their dissemination in the face of competition with wildtype strains [158]. Within our isolates, upwards of 200 amino acid changes were found in PBP2X of many of the recombinant series, which contrasts with the experimentally evolved penicillin-resistant T4EP population, possessing only a single amino acid substitution in the central transpeptidase domain of PBP2X. Despite the relative lack of PBP changes, T4EP was greatly attenuated *in vivo*. The recombinant pneumococcal isolates, on the other hand, began showing attenuated *in vivo* characteristics only after larger increases in MIC in the later recombinations, unsurprisingly when many of the isolates began showing large changes in PBP2B. Thus, it appears that the changes to PBP2X in the first recombinant isolates had a net-neutral or net-minimal cost to fitness *in vivo*, with possible compensatory changes conferred by substitutions within PBP2X itself, or from other alterations in the recombinant genome. Several large recombination regions in resistant isolates also involved genes not directly known to be associated with β -lactam resistance. For example, T4R1P possessed genomic recombinations involving LuxA, DexB, and ClpB, which are themselves not known to be directly involved in β -lactam resistance. These alterations, and in particular the changes that occurred in the tolerant third recombinant strains, may be of interest for investigating determinants and tolerance and resistance. However, the broad scale of change seen within many of these genes poses a significant hurdle to identifying the contributions of any one individual mutation.

Here we have shown evidence that *S. pneumoniae*'s innate competence provides for greater genetic diversification and adaptability compared to *de novo* mutation. This advantage has important implications not only for the pneumococcus, but also non-pneumococcal streptococci. In part, this flexibility afforded by genetic recombination via horizontal gene transfer may explain the lack of β -lactam resistance observed in non-

competent species of streptococcus, such as *S. pyogenes* [159]. A reliance on *de novo* mutation without accompanying plasticity to allow compensatory changes could explain the constraints in PBP variability seen in *S. pyogenes*, for example [160]. By contrast, the genomic plasticity of *S. pneumoniae* allows greater diversity, selection, and adaptation in low-fitness environments [161, 162], such as in the presence of antimicrobial agents. Based on this and our current data, we propose that the genetic variability via recombination grants a considerable advantage, not only in developing antibiotic resistance, but also in simultaneous adaptation to the host-response.

CHAPTER 5. TOLERANCE INDUCED BY DISRUPTION IN RNA DEGRADATION EVADES FITNESS TRADEOFFS, WHILE POTENTIALLY FACILIATING THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE

Introduction

Streptococcus pneumoniae is a leading cause of mild to severe infections, especially in the pediatric and geriatric populations [3], and the burden of pneumococcal disease is further complicated by the rampant spread of antibiotic resistance worldwide. Currently, a variety of routine screening methods almost exclusively detects antibiotic resistance in clinical isolates [163], while overlooking other bacterial phenomena that can result in treatment failure, such as antibiotic tolerance [164]. Despite these obstacles, tolerant strains are consistently identified amongst both Gram-positive and Gram-negative clinical isolates.

Antibiotic tolerance was first described in *S. pneumoniae* in 1970 [165]. The capacity of a bacterium to evade the bactericidal action of antibiotics without discernible shifts in resistance is known as antibiotic tolerance [44]. Initially described over 40 years ago, antibiotic tolerance results in the bacterial population having much reduced susceptibility to the lethal action of antibiotics. While antibiotic resistance is readily measured by alterations in minimum inhibitory concentration, tolerance typically manifests as delayed kill kinetics in the absence of discernible shifts in MIC. Identifying antibiotic tolerant strains requires measurement of antibiotic kill kinetics, which entails periodic sampling of bacterial counts in antibiotic-containing media to measure reduction in bacteria over time, thereby making their classification much more laborious and challenging. Whereas the basis for antibiotic resistance is well understood, the mechanisms underlying antibiotic tolerance remain markedly less characterized despite recent studies that have provided insight into the various underlying mechanisms. Often overshadowed by the prominence of antibiotic resistance, antibiotic tolerance can result in antibiotic treatment failure and recalcitrant bacterial infections, and it is thought to be a potential avenue for the subsequent development of high-level resistance [97, 166, 167].

Antibiotic tolerance is classified into phenotypic tolerance and genotypic tolerance. Under environmental changes such as starvation stress, lowering pH, or presence of autolytic enzymes' inhibitors, almost all bacteria become resistant to cell wall inhibitors, i.e. beta-lactams [168, 169]. In such nutrients deprived conditions, the bacteria can activate the stringent response by inhibiting essential cellular processes, such as the synthesis of cell wall peptidoglycan, proteins, and DNA during replication [170]. As non-growing or slow-growing bacteria decrease their cellular activities, for example cell wall synthesis, they exhibit reduced death and lysis and evade bactericidal action of beta-lactams [169].

Genotypic tolerance results from genetic mutations involved in the lytic pathway. In *S. pneumoniae*, absence or inactivity of the autolysin *lytA* leads to loss of lysis and reduced killing [165]. It was shown that this reduced killing was due to inhibition of

autolytic enzymes, such as the major autolysin LytA in *S. pneumoniae* [171, 172]. LytA is a cell wall hydrolase that is constitutively expressed during the entire cell cycle but induces lysis only during stationary phase [165]. Tolerant pneumococcal isolates showed lytic defects [173] and mutations disrupting autolytic pathway, including the two-component regulatory system (VncS-R), the heat-shock protein (ClpC), the zinc-metalloprotease (ZmpB), and the ABC transporters (Psa and Pst) [174-178].

Infection resolution via bacterial clearance does not rely solely on antibiotics as the immune system is also required to completely eradicate an infection. Therefore, having an impaired immune system increases not only those individuals' susceptibility to infection but also their chance of developing chronic infections. Without a fully functional immune system, bacterial clearance in the immunocompromised host relies on the efficacy of antibacterial treatment to successfully eradicate the infection. In many cases, conventional dosing of first-line antibiotics is appropriate for treating bacterial infections in immunocompromised patients. However, in some cases, infections causing mild or moderate disease in immunocompetent patients can be difficult to treat or even fatal in an immunocompromised host. Adding to the difficulty of determining the correct treatment for immunocompromised patients, *in vitro* antibacterial susceptibility tests do not always accurately predict *in vivo* treatment outcome because the dynamics between the pathogen, infection, and antibiotics are considered. This is of particular importance to high-risk individuals with impaired immune function as successful treatment does not have the added benefit of synergistic clearance by the host immune system thus allowing for pathogen outgrowth following cessation of antibiotic exposure. While such mechanisms of evading antibiotic eradication are likely to be operative regardless of host immune status, these high-risk populations remain of a particularly heightened concern.

In this study, we aimed to identify mutations and study mechanisms that enable *S. pneumoniae* to overcome antibiotic treatment in our *in vivo* model of the evolutionary pathway of antibiotic resistance and resultant treatment failure in murine hosts with different immune status. We focused on exploring disruption of RNA degradation as a mechanism underlying antibiotic tolerance in *S. pneumoniae* that could potentially accelerate the emergence and spread of antibiotic resistance. RNA degradation has only been studied extensively in *E. coli*, *B. subtilis*, and *S. aureus*. One of the essential ribonucleases in bacterial RNA degradosome complex is RNase Y. It is a degradosome scaffold protein for mRNA degradosome assembly composed of RNA helicase CshA and PNPase [179-184]. Within the RNA degradosome, RNase Y interacts with other components, such as CshA, which is a RNA helicase: ATP-dependent RNA helicase. Previous studies indicated a surprising association between RNases and antibiotic resistance and tolerance. *M. tuberculosis* clinical isolates resistant to isoniazid harbored nonsense and insertion and/or deletion mutations in RNase J [185, 186]. In addition, *M. leprae* clinical isolates also contained polymorphisms in RNase D and RNase J that were favored during antibiotic stress [187]. However, the role of RNA degradation in antibiotic resistance or tolerance has not been explored. Understanding how RNA degradation plays a role in the conferring antibiotic tolerance, thereby acting as a precursor in the development of antibiotic resistance will provide insight into curtailing antibiotic resistance in *S. pneumoniae*.

Methods

Growth conditions

The wild-type *S. pneumoniae* TIGR4 and the deletion mutants were grown on tryptic soy agar (TSA, EMD Chemicals) supplemented with 3% of defibrinated sheep blood and 20 µg/mL of neomycin, in Todd Hewitt Broth (ThyB), or in semi-defined media (C+Y) [188]. *S. pneumoniae* produces neomycin, and is thus resistant to the antibiotic [189]. Neomycin was added to blood agar plates to prevent contamination of other bacterial species. Bacteria from frozen stocks were inoculated onto blood agar plates and incubated overnight at 37°C supplemented with 5% CO₂. Then, the bacteria from the blood agar plates were inoculated into liquid cultures and incubated at 37°C supplemented with 5% CO₂.

Mutant generation

Deletion mutants (Δsp_1739 , Δsp_1790 , and $\Delta sp_1739 \Delta sp_1790$) in TIGR4 316 background were generated via overlap extension PCR [190]. Briefly, 1 kb upstream and downstream regions of the target gene were amplified via PCR using the following [SP_1739 up Fwd, SP_1739 up ERM rvs, SP_1739 down ERM fwd, SP_1739 down rvs, SP_1790 Up Fwd, SP_1790 up SPEC Rvs, SP_1790 down SPEC Fwd, SP_1790 down Rvs]. The upstream and downstream regions were then spliced with an erythromycin- or a spectinomycin-resistance cassette. The final PCR product was transformed into TIGR4 316 at OD~0.07 to 0.1 with competence-stimulating peptide 2 (CSP-2), and the knockout mutants Δsp_1739 and Δsp_1790 were selected on blood agar plates containing either 1 µg/mL erythromycin or 200 µg/mL spectinomycin, respectively. For the double knockout mutant $\Delta sp_1739 \Delta sp_1790$, deletion of Δsp_1790 was performed in the Δsp_1790 background. To confirm the deletion mutants, PCR was conducted using the following primers to verify the presence of the antibiotic cassette and absence of the targeted gene. Primers used and strains generated are listed in **Table 5-1**.

Transformation

TIGR4 and the knockout mutants were grown in C+Y media to OD₆₂₀~0.07 to 0.1 before they were exposed to fluoroquinolone resistance determinants and competence stimulating peptide 2 or 1 (CSP-1 or 2). Fluoroquinolone resistance determinants contained 5 µg of 2 kb PCR fragments encoding either S79Y in topoisomerase IV *parC* or S81F in *gyrA* or 5 µg of genomic DNA of fluoroquinolone resistant *S. pneumoniae* or Viridans clinical isolates. The transformation mixture was incubated for 3 hours at 37°C supplemented with 5% CO₂. To select for transformants, 100 to 200 µL of the transformation mixture were spread on blood agar plates supplemented with 1 to 2x MIC of ciprofloxacin or levofloxacin. The plates were incubated overnight at 37°C and 5% CO₂. Transformation efficiency was calculated as the ratio of CFU/mL of the

Table 5-1. Strains and primers used in this study

Strains/Primers	Description/Sequence
TIGR4 316	Wild-type, pathogenic <i>S. pneumoniae</i> , serotype 4, from Tim van Opijnen laboratory at Boston College
Δsp_{1739}	RNAse Y knockout
Δsp_{1790}	RNA helicase knockout
$\Delta sp_{1739}\Delta sp_{1790}$	RNAse Y & RNA helicase double knockout
sp_{1739} up Fwd	CTTCGATACGGCGACGCAC
sp_{1739} up ERM rvs	GAGTCGCTTTTGTAAATTTGGATAAGAAAGAGCAG TTGAAAAATTACTG
sp_{1739} down ERM fwd	GTTTGCTTCTAAGTCTTATTTCCGATTTCATGTTTT TACCTCATTTTATTG
sp_{1739} down rvs	GAACCTTGGGATGCTATGACCC
sp_{1790} Up Fwd	GGCATAGCGCTTTTACCTCCTG
sp_{1790} up SPEC Rvs	GTATTCACGAACGAAAATCGATAGCAGTCCTTTCTA ACGTGGATTTG
sp_{1790} down SPEC Fwd	GAAAACAATAAACCCCTTGCATATGAATCCTTTTCAA AAAATTGCACTTTCC
sp_{1790} down Rvs	GTCCTTACTATGACTTAGCTAGACAAC

transformants grown on ciprofloxacin- or levofloxacin-containing plates to the total number of bacteria.

Genomic DNA extraction

The aqueous / organic extraction method using phenol chloroform was conducted to extract genomic DNA for transformation and whole genome sequencing. TIGR4 and the knockout mutants were grown in ThyB to late logarithmic phase at OD₆₂₀ ~0.6 and were pelleted at 6000 x g for 10 minutes followed. Bacterial pellets were resuspended in 1 mL 1x PBS containing 50 µL 10% DOC, 50 µl 10% SDS, and 10 µL of 10mg/mL Proteinase K (Sigma). The resuspended mixture was incubated for lysis until clear (approximately 5 minutes) at 37°C, mixed with 500 µL phenol: chloroform: isoamyl alcohol (Sigma), transferred to phase-lock tubes (Quantabio), and centrifuged at maximum speed for the separation of aqueous and organic phases. The aqueous phase was mixed with 500 µL chloroform: isoamyl alcohol and centrifuged for 5 minutes at maximum speed. To precipitate genomic DNA, the aqueous phase was transferred to a new Eppendorf tube containing 100% ethanol. Finally, precipitated DNA was washed with ice cold 70% ethanol, pelleted, air dried at room temperature, and resuspended in nuclease-free water.

***In vivo* passaging**

For this study, we modeled the evolution of fluoroquinolone resistance *in vivo* with different clinically relevant antibiotics, such as levofloxacin, cefepime, and linezolid. Three groups of 7-week-old immunocompetent BALB/c mice were intranasally instilled with 100 µL containing 10⁶ CFUs of TIGR4. Six hours post-infection, mice were intraperitoneally administered a sublethal dose of the antibiotic. Initial dosages of each antibiotic: 100 µL of levofloxacin (25 mg/kg), cefepime (1 mg/kg), vancomycin (0.25 mg/kg) was determined in preliminary studies. The initial dosages eliminated 90% to 99% of the bacterial population and were selected to impose sufficient antibiotic pressure while not completely eradicating the entire bacterial population (data not shown). After 15 passages, the antibiotic dosage was increased to 2x in each subsequent passage. Twelve hours post-antibiotic treatment, lungs and blood from chest cavity of infected mice were harvested. To collect bacteria from the infected lungs, the lungs were homogenized and centrifuged at 300 x g for 5 minutes. The resultant supernatant was spread plated on blood agar plates for 12 hours, and frozen in aliquots containing glycerol. The blood was mixed with 1x PBS containing heparin or EDTA and serially diluted before spotted on blood agar plates to enumerate bacterial burden after each passage. Bacteria collected from each passage was frozen using 80% glycerol in triplicates. For subsequent passage, frozen bacterial aliquots from previous passage of each respective lineage served as the inoculum to infect the next group of mice. The other aliquots were used for genomic DNA extraction and whole genome sequencing.

MIC determination

To measure the MIC of TIGR4 316 and the knockout mutants, bacteria were grown on blood agar plates overnight at 37°C with 5% CO₂. The bacteria grown overnight were used to inoculate into ThyB and incubated at 37°C with 5% CO₂ until they grew to mid-logarithmic phase at OD₆₂₀ ~0.4. Then, 100 µL of the bacterial culture were spread plated on blood agar plates. An E-test strip (bioMerieux) containing increasing concentrations of an antibiotic was carefully placed onto the center of the plates, which were then incubated overnight at 37°C with 5% CO₂. MIC (µg/mL) of each strain was determined at the concentration where the symmetrical inhibition ellipse edge intersects the E-test strips on the plate.

***In vitro* growth curves**

The wild-type TIGR4 and mutants were grown overnight on blood agar plates and diluted 1:100 in C+Y in triplicates. Bacterial growth was measured as OD₆₂₀ every 30 minutes by a microplate reader.

Time-kill curves

Kill curves were conducted to examine whether the experimentally-evolved isolates and the knockout mutants displayed tolerance phenotype to different classes of antibiotics. TIGR4 and the knockout mutants were grown in ThyB at 37°C supplemented with 5% CO₂ until reaching mid-logarithmic phase at OD₆₂₀ ~0.2. Bacteria were exposed to increasing concentrations of different classes of antibiotics, ranging from TIGR4's 1x to 4x MIC, for 4 hours. Hourly after antibiotic exposure, bacteria were serially diluted and spotted on blood agar plates, which were incubated overnight at 37°C and 5% CO₂.

Whole genome sequencing

Sequence libraries were prepared according to Illumina HiSeq protocol. To identify mutations that rose during *in vivo* passaging, the reads were assembled using Breseq and compared to the TIGR4 reference genome (AE005672.3).

Virulence and competitive index experiments

For the bacterial virulence study, TIGR4 and the mutants were grown in C+Y to logarithmic phase OD₆₂₀ ~0.8 and frozen in glycerol stocks at -80°C. Bacteria in each glycerol stock were enumerated. Immediately before infecting the mice, the glycerol stocks were thawed, washed, resuspended in 1x PBS, and diluted for appropriate infecting inoculum concentration. Anesthetized seven-week-old female BALB/c mice from Jackson Laboratory were infected with the TIGR4 or the mutants via low-volume

intranasal instillation of 30 μL containing 1×10^7 CFUs. Mice were monitored for disease progression, including lethargy, conjunctivitis, meningitis, pneumonia, and bacteremia post-infection. Blood samples, lungs, and nasal passage were collected at 24 post-infection via tail-bleeding (5 μL of collected blood was diluted into 1x PBS containing heparin) and dissection, respectively. For the competitive index experiment, TIGR4 and the Δsp_1739 mutant were equally mixed in the infection inoculum. Seven-week-old anesthetized BALB/c mice were intranasally instilled 100 μL of the inoculum containing 1×10^6 CFUs. Sixteen hours post-infection, the mice were peritoneally administered levofloxacin, vancomycin, or cefepime. Blood and lungs were collected at 6 hours post-treatment. Bacterial burden was enumerated by serially diluting into 1x PBS and plating onto blood agar, and bacterial titers were compared using the non-parametric Mann-Whitney t-test. Mouse survival curves were analyzed using the Kaplan Meier survival curves and Mantel-Cox log rank test.

Antibiotic treatment experiment

To ascertain the effect of disruption in RNA degradation on antibiotic efficacy, seven-week-old anesthetized BALB/c mice were intraperitoneally infected with 100 μL containing 105 CFU/mL of either TIGR4 or the knockout mutants. Mice were monitored closely for signs of infection. Sixteen-hour post-infection, blood was extracted via tail bleeding to enumerate bacterial burden prior to antibiotic treatment. Mice were then intraperitoneally administered 100 μL of levofloxacin (25 mg/kg), cefepime (1 mg/kg), or (0.25 mg/kg) vancomycin. Two hours after antibiotic treatment, blood was extracted via tail bleeding every 2 hours up to 6 hours. Pre- and post-treatment bacterial burden in each mouse in either TIGR4-infected or the mutant-infected group was compared using Wilcoxon matched-pairs signed rank test. Kaplan-Meier survival curves were compared using the log rank test.

Ethics statement

All experiments involving animals were performed with prior approval of and in accordance with guidelines of the St. Jude Institutional Animal Care and Use Committee. The St. Jude laboratory animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in Guide for the Care and Use of Laboratory Animals.

Results

***Sp_1739* served as a mutational hot spots for point mutations during pressure by multiple antibiotics and different immune states during *in vivo* passaging**

For this study, we developed an *in vivo* serial passaging model to investigate underlying mechanism(s) leading to the development of antibiotic resistance in both immunocompetent and immunocompromised mice. We completed almost 30 rounds of passaging with a variety of clinically relevant antibiotics. Escalating dosages of antibiotics did not eradicate bacterial burden during the infection, resulting in treatment failure (**Figure 5-1**); however, MICs of the experimentally-evolved isolates were comparable to that of the wild type (data not shown). Without a marked increase in MICs of the experimentally-evolved isolates, those isolates were predicted to display tolerance against the antibiotics to overcome antibiotic killing. Indeed, the isolates that underwent *in vivo* passaging with levofloxacin displayed reduced kill kinetics during time-kill curves with levofloxacin (**Figure 5-2**). Time-kill curves of the experimentally-evolved isolates with other antibiotics will be conducted in the near future to determine if the isolates display cross tolerance to other classes of antibiotics.

We next analyzed the whole genome sequences of the experimentally-evolved isolates to identify mutation(s) that arose after each round of passaging and likely contributed to conferring tolerance. Currently, only whole genome sequencing results of experimentally-evolved isolates under cefepime, linezolid, and levofloxacin pressure were available for analysis. The whole genome sequence of each evolved isolate was compared to that of the parental TIGR4. We discovered that in multiple independent murine lineages under the pressure of those different antibiotics, there were numerous missense and nonsense mutations along the *rny* gene (*sp_1739*) or RNase Y (**Tables 5-2**). The mutations started to arise and remained fixated in the population. There were also a few mutations in other ribonucleases J, ribonuclease HIII, and ribonuclease R. Intriguingly, those mutations were clustered in the genes responsible for RNA degradation.

Deletion of *sp_1739* does not decrease antibiotic susceptibility compared to the wild-type TIGR4

We previously observed that the RNase Y gene served as the mutational hotspot during *in vivo* passaging with different classes of antibiotics. Multiple mutations occurring along the *rny* gene likely disrupted RNA degradation. To determine whether disruption of RNA degradation affects antibiotic susceptibility of *S. pneumoniae*, we generated deletion mutants of *rny* (*sp_1739*) and a putative RNA helicase *cshA* (*sp_1790*), which are expectedly major components of the RNA degradosome complex of *S. pneumoniae*. Previous work demonstrated that RNase Y is responsible for the degradation of a significant amount of mRNA transcripts in *B. subtilis*. Depletion of RNase Y resulted in remarkable accumulation of those transcripts [183].

Figure 5-1. *In vivo* passaging of TIGR4 with different antibiotics and host immune status

A) Cefepime, neutrophil-depleted. B) Cefepime, macrophage-depleted. C) Cefepime, non-depleted. D) Levofloxacin, neutrophil-depleted. E) Levofloxacin, macrophage-depleted. F) Levofloxacin, non-depleted. G) Linezolid, neutrophil-depleted. H) Linezolid, macrophage-depleted. I) Linezolid, non-depleted.

Figure 5-2. Experimentally-evolved isolates of *S. pneumoniae* exhibited tolerant phenotype to levofloxacin

A and B) Kill curves conducted with 1x MIC and 2x MIC of levofloxacin, respectively. T4 316 (blue) is the wild type. T4 316 L1-L3, G29, Nd (red) represent 3 independent evolved lineages in immunocompetent mice.

Table 5-2. Summary of mutation occurrence in cefepime *in vivo* passaging

Antibiotic	Macrophage-depleted			Neutrophil-depleted			Non-depleted		
	L1	L2	L3	L1	L2	L3	L1	L2	L3
Cefepime	✓	✓	✓		✓	✓	✓	✓	
Levofloxacin		✓	✓		✓	✓	✓	✓	
Linezolid	✓		✓		✓	✓		✓	

*L1, 2, and 3 denotes lineage 1, 2, and 3.

We also generated a double knockout of both *sp_1739* and *sp_1790* to maximally disassemble the RNA degradosome complex. Using E-test strips, we measured the mutants' MIC of levofloxacin, cefepime, and linezolid, the antibiotics to which the experimentally-evolved isolates were exposed during *in vivo* passaging. Surprisingly, levofloxacin, cefepime, and linezolid MICs of the single and double knockout mutants were similar to that of the wild type (**Table 5-3**). No change in the mutants' MIC to those antibiotics suggest that the resulting disruption in RNA degradation did not alter their susceptibility to the antibiotics and did not induce antibiotic resistance in *S. pneumoniae*.

Disruption in RNA degradation induced tolerance in *S. pneumoniae* to multiple classes of clinically relevant antibiotics

Since the single and double knockout mutants were not resistant to the antibiotics, we next searched for other bacterial survival strategies that enabled the bacteria to overcome antibiotic treatment. One of the bacterial survival strategies is tolerance. Tolerant bacteria can withstand antibiotic killing without having an increase in MIC [44]. To determine whether the mutants were tolerant to the antibiotics, we conducted time-kill curve experiments and examined their kill kinetics as well as bacterial survival hourly following antibiotic exposure. Once TIGR4 and the single and double knockout mutants were actively dividing during the mid-logarithmic phase, they were exposed to lethal concentrations of a variety of bactericidal antibiotics in different classes. Compared to TIGR4, the single knockouts Δsp_1739 and Δsp_1790 showed reduced killing when exposed for 4 hours to bactericidal antibiotics, including beta-lactams (cefepime), glycopeptides (vancomycin), RNA synthesis inhibitors (rifampin), fluoroquinolones (ciprofloxacin), but not protein synthesis inhibitors (kanamycin) (**Figure 5-3**). The double knockout mutant $\Delta sp_1739\Delta sp_1790$ displayed a similar trend to the single knockout strain, having less killing to those antibiotics compared to the wild type (**Figure 5-3**). Reduced kill kinetics observed in the mutants indicate that the deletion of the RNA degradation conferred tolerance against several classes of antibiotics. Furthermore, defects in the RNA degradation machinery caused by multiple mutations occurring in the mutational hotspot *rny* likely contributed to the tolerance phenotype that the experimentally-evolved isolates developed during *in vivo* passaging.

Deletion of *sp_1739* and/or *sp_1790* did not significantly attenuate *S. pneumoniae* virulence while displaying slight survival advantages in response to antibiotic treatment

Defects in RNA degradation conferred antibiotic tolerance. We next aimed to characterize the mutants' phenotypes both *in vitro* and *in vivo*. In the absence of antibiotics, the Δsp_1739 , Δsp_1790 , and $\Delta sp_1739\Delta sp_1790$ mutants displayed overall similar growth rates *in vitro* (**Figure 5-4**). We next investigated whether defects in RNA degradation impart any fitness costs by examining the Δsp_1739 mutant's virulence. We either intranasally or intraperitoneally infected 7-week-old BALB/c mice with TIGR4 316 or the Δsp_1739 mutant and enumerated bacterial burden in the blood.

Table 5-3. MIC of TIGR4 316 and the knockout mutants

Strains	Antibiotic ($\mu\text{g/mL}$)		
	Cefepime	Linezolid	Levofloxacin
TIGR4 316	0.032	3	2
Δsp_{1739}	0.032	1.5	1.5
Δsp_{1790}	0.032	3	3
$\Delta sp_{1739}\Delta sp_{1790}$	0.032	1.5	1.5

Figure 5-3. Disruption in mRNA degradation conferred tolerance to multiple classes of antibiotics except protein synthesis inhibitors

Time-kill assays were conducted with different antibiotics as indicated at 1x MIC cefepime, 1x MIC linezolid, 1x MIC ciprofloxacin, 1x MIC vancomycin, 1x MIC rifampin, and 1x MIC kanamycin. Bacterial number (CFUs/mL) were enumerated 4 hours after antibiotic exposure during exponential phase. Bacterial survival was calculated as the ratio of CFUs/mL 4 hours post-exposure to the antibiotic to CFUs/mL pre-exposure.

Figure 5-4. *In vitro* growth curves of RNase Y mutants and TIGR4

The mutants and wild type were grown in the absence of antibiotics, and growth was recorded by OD every 30 minutes. This experiment was conducted by Juance Ortiz-Marquez PhD in the Opijnen laboratory at Boston College.

Due to resource constraints, only the Δsp_1739 mutant was tested in this virulence experiment because mutations occurred frequently in *sp_1739* during the *in vivo* passaging and also is known to be the major endoribonuclease for mRNA decay [183, 191, 192]. In the low-volume intranasal route of infection, the Δsp_1739 mutant was almost fully virulent compared to the wild-type (**Figure 5-5**). Although there was approximately 1-log decrease in bacterial burden of Δsp_1739 mutant in the nasopharynx (**Figure 5-5A**), all of the mutants were able to disseminate into the bloodstream as effectively as the wild type (**Figure 5-5B**). This suggests that the disruption in RNA degradation did not attenuate the virulence of *S. pneumoniae*. A competitive index between the wild type and the Δsp_1739 mutant revealed that the mutant outcompeted the wild type under vancomycin, cefepime, and levofloxacin treatment (**Figure 5-6**). This finding was more apparent in the lungs than in the blood (**Figure 5-6**). These results are consistent with our previous finding that the experimentally-evolved isolates encoding many mutations in the *rny* gene remained virulent while exhibiting antibiotic tolerance. Taken together, these results suggest that *S. pneumoniae* likely developed tolerance to survive under temporary antibiotic pressure without losing bacterial fitness, *i.e.* virulence potential.

Discussion

In this study, we aimed to identify mutations(s) and underlying mechanisms of antibiotic failure in both immunocompetent and immunocompromised hosts. We demonstrated that antibiotic tolerance developed instead of antibiotic resistance under antibiotic pressure independent of the host's immune pressure during our *in vivo* serial passaging with the initial set of antibiotics levofloxacin, cefepime, and linezolid (**Figure 5-2**). The development of tolerance among the isolates was consistent with previous studies that showed that cyclic antibiotic treatments promotes tolerance [97, 193-195]. During each round of the *in vivo* serial passaging, the antibiotics were administered only once at 7 hours post-infection instead of shorter intervals or continuous infusion. This presented as transient antibiotic exposure that allows an incomplete eradication of bacteria population and prolonged bacterial survival under antibiotic pressure, thus promoting the development of tolerance. After we characterized the isolates' tolerance phenotype, we analyzed whole genome sequences of the experimentally evolved isolates following each round of passaging to identify tolerance-associated mutation(s). Mutations arising during the *in vivo* serial passaging occurred most frequently in *rny*, which encodes the ribonuclease RNase Y. Deletion mutants of RNase Y and another essential player of the RNA degradosome complex in *S. pneumoniae* also resulted in tolerance and imparted little to no fitness costs (**Figure 5-5** and **5-6**). Therefore, tolerance was likely attributed to the resultant defects in RNA degradation. The *rny* knockout mutant exhibited tolerance to a variety classes of antibiotics, except the protein synthesis inhibitors, such as kanamycin (**Figure 5-3**). Defects in RNA degradation increased intracellular RNA concentration, which likely upregulates the downstream protein synthesis to decrease the RNA levels and make nucleotides available for other cellular processes.

Figure 5-5. Disruption RNA degradation decreased *S. pneumoniae* virulence in the nose but not in the blood

Seven-week-old mice were intranasally infected, and nasal and blood samples were harvested 24 hours post-infection A) Nasal titers. B) Blood titers. *p-value ≤ 0.05.

Figure 5-6. Δsp_{1739} mutant showed increased survival under vancomycin, cefepime, and levofloxacin treatment during competitive index (CI) experiment
Mice were infected intranasally with high-volume inoculum containing equally mixed TIGR4 and Δsp_{1739} . Sixteen-hour post-infection, infected mice were then peritoneally administered levofloxacin, cefepime, or vancomycin at dosages that eliminated approximately 90% of the bacteria. Blood and lungs were harvested via dissection, and bacteria were collected via centrifugation and serially tittered. Competitive index of the Δsp_{1739} and the wild type was calculated as the ratio of the CFUs/mL of the mutant to that of TIGR4.

Tolerance might have been the bacterial survival strategy withstanding the transient exposure to the antibiotic while minimizing fitness costs of defects induced by on-target mutations. In stress conditions, it would be beneficial for bacteria to maintain only specific sets of mRNA to focus on translation of genes essential for overcoming that particular stressor, therefore mRNA degradation would be essential for the rapid adaption to environmental changes in order to shift responses specific for that stressor. Thus, a critical role of mRNA degradation is replenishing ribonucleotides for synthesis of essential transcripts specific for that particular stressor.

Tolerance induced by disruption of RNase Y and/or RNA helicase facilitated the acquisition of antibiotic resistance via *de novo* mutations. Tolerance prolongs bacterial survival, potentially enabling replication after completion of antibiotic treatment. With higher numbers of bacterial survival during antibiotic exposure, more mutations that occur during subsequent replication may be deemed favorable and select for antibiotic resistance. However, no increase in transformation efficiency was observed with fluoroquinolones. Although, we have not tested transformation efficiency of the experimentally-evolved isolates with other antibiotics, we hypothesized that fitness costs might have been increased due to simultaneous disruption of RNA degradation and DNA replication. DNA replication is likely inhibited by accumulated RNA through negative feedback mechanisms. Fluoroquinolone resistance mutations in *gyrA* and *parC* have been shown to interrupt DNA replication [196], therefore compounding the inhibition of DNA replication previously due to disrupted RNA degradation. As part of our ongoing and future research, we plan to generate point mutants in the *rny* gene to characterize their individual contributions to the isolates' tolerance phenotype. The most frequently occurring mutations will be selected to generate the point mutants as they show the greatest potential to confer tolerance and minimize fitness costs. Different combinations of those mutations will also be generated because some may act as compensatory mutations restoring bacterial fitness, rather than conferring tolerance.

CHAPTER 6. VANCOMYCIN HETERORESISTANCE AND CLINICAL OUTCOMES IN COAGULASE-NEGATIVE STAPHYLOCOCCAL BLOODSTREAM INFECTIONS¹

Introduction

Vancomycin is the definitive therapy for many serious gram-positive infections; however, although most such infections remain susceptible to vancomycin, therapeutic failure is common. One potential explanation for the poor outcomes is heteroresistance, that is, the presence of a small subpopulation of resistant organisms recalcitrant to antibiotic therapy. This phenomenon has been well-described in some bacteria, including *Enterobacteriaceae* and *Staphylococcus aureus* [46, 198].

Heteroresistance does appear to be associated with poor treatment outcomes in methicillin-resistant *S. aureus* infections [51, 198]; however, the clinical significance of heteroresistance in coagulase-negative staphylococci (CoNS) is unknown [199-205]. CoNS are gram-positive bacteria that colonize human skin and mucosa. They are a frequent etiology of central line-associated bloodstream infections (CLABSI), especially in immunocompromised patients [206-208]. Although often thought of as benign, CoNS CLABSI in vulnerable populations can cause prolonged hospitalization and serious illness, including organ dysfunction and neurocognitive damage [209, 210]. These bacteria typically appear susceptible to vancomycin, which is recommended as first-line therapy; however, antibiotic treatment failure or relapse is common [211, 212].

The possibility that heteroresistance contributes to poor clinical response in CoNS infections has not been evaluated. Clinical susceptibility testing, which uses an inoculum of approximately 10^6 CFU/mL, typically cannot detect a resistant subpopulation at a frequency of 10^{-6} or lower. Since the genetic basis for vancomycin heteroresistance remains unknown, molecular testing is unavailable. Therefore, vancomycin heteroresistance might be present in CoNS causing bloodstream infections in immunocompromised patients and might impair the clinical response to vancomycin therapy. We undertook a retrospective study to evaluate the frequency, risk factors, and clinical significance of vancomycin heteroresistance in CoNS CLABSI in pediatric patients undergoing treatment for leukemia at St. Jude Children's Research Hospital (St. Jude).

¹ Reprinted from final submission with permission. Tina H. Dao, Ramzi Alsallaq, Joshua B. Parsons, Jose Ferrolino, Randall T. Hayden, Jeffrey E. Rubnitz, Iftekhar M. Rafiqullah, D. Ashley Robinson, Elisa B. Margolis, Jason W. Rosch, Joshua Wolf. *Antimicrobial Agents and Chemotherapy*. Oct 2020, 64 (11) e00944-20; <http://doi.org/10.1128/AAC.00944-20> [197].

Methods

Clinical data

This was a retrospective cohort study comprising all patients who developed CoNS CLABSI during treatment for leukemia at St. Jude between January 1, 2010, and March 28, 2016. The study was approved by the St. Jude institutional review board (approval XPD16-036). Participants were identified from the electronic medical record, and data on demographics, malignancy, vancomycin exposure, and infection outcomes were abstracted from the electronic medical record. Episodes were excluded if the isolate was not available for testing, was not identified as CoNS on sequencing, or was not successfully tested for heteroresistance.

Bacterial isolates

CoNS isolates were obtained from the clinical microbiology laboratory, where they were stored for quality assurance purposes in glycerol at -80°C until tested. A prototypical vancomycin-heteroresistant *S. aureus* strain (Mu3) was obtained from ATCC (Manassas, VA) for use as a positive control. All isolates were classified as susceptible to vancomycin by conventional testing (with VITEK 2.); MIC range, <0.5 – $2\text{ }\mu\text{g/mL}$) [213].

Evaluation of vancomycin heteroresistance

Population analysis profiling (PAP) was used to identify vancomycin heteroresistance in clinical isolates. PAP was performed in accordance with standard published techniques for detecting hetero–vancomycin-intermediate *S. aureus* (hVISA) [50, 214]. Each isolate was grown in 10 mL of tryptic soy broth (BD Sciences, catalog no. 211825) overnight at 37°C . Of the 74 available clinical isolates, 30 were analyzed in 2016 and 44 were analyzed in 2017. The latter set of isolates required the addition of 0.2% yeast extract (BD Biosciences, catalog no. 212750) for growth. Overnight stationary-phase cultures were serially diluted 1:100 in $1\times$ PBS to give dilutions ranging from 10^{-2} to 10^{-8} , and 100 μL of each dilution was spread on brain heart infusion (BHI) agar plates (made from Bacto Brain Heart Infusion and Bacto agar from BD Biosciences) containing 0, 1, 2, 4, 6, or 8 $\mu\text{g/mL}$ of vancomycin (vancomycin hydrochloride, Sigma-Aldrich, St. Louis, MO). Each strain at each dilution was inoculated onto duplicate plates for each antibiotic concentration. After a 48-h incubation at 37°C , colonies were enumerated to estimate the bacterial survival, expressed as colony-forming units per milliliter (CFU/mL), at each vancomycin concentration. The average number of colonies on duplicate plates was used to calculate the CFU/mL for each vancomycin concentration; CFU/mL was plotted as a function of vancomycin concentration, and the area under the curve (AUC) was calculated using GraphPad Prism 7. The AUC for each clinical isolate was then compared by a simple ratio to the average AUC for the

heteroresistant reference strain Mu3, obtained using the identical technique, and if the AUC ratio was 0.9 or greater, the isolate was classified as heteroresistant [50].

Genomic sequencing

Each clinical isolate was inoculated in TSB and grown overnight at 37°C for DNA extraction. The entire overnight culture of each isolate was pelleted at 6000 RPM for 10 min, and the pellets were resuspended in TSM solution (50 mM Tris, pH 7.5, 0.5 M sucrose, 10 mM MgCl₂) before the addition of lysostaphin at 2 mg/mL. The mixture was then incubated at 37°C for 10 min. Once the bacterial cells had lysed, DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. DNA libraries for next-generation sequencing were prepared using a Nextera DNA Flex Library Kit (Illumina, San Diego, CA) and sequenced on an Illumina Hi-Seq 2000 sequencing system.

Sequencing and typing

Sequence reads for 74 clinical strains and Mu3 were adapter trimmed (minimum length 15 bp) and then quality trimmed (minimum base quality 12) by using BBMap (<https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmap-guide/>). Sequences were assembled *de novo* by using SPAdes v3.11.1. Sequence types (STs) from species-specific multilocus sequence typing (MLST) schemes were extracted from the assemblies by using mlst v2.8 (<https://github.com/tseemann/mlst>). For *Staphylococcus epidermidis* strains with typeable STs, genetic clusters were assigned according to the method of Tolo *et al.*[215]. Sequences were aligned by the Burrows–Wheeler Alignment tool bwa v0.7.17, using *S. epidermidis* DAR1907 (GenBank accession no. CP013943.1) as a reference strain. Sequences were coordinate sorted by using Picard v1.141 and realigned around short insertion–deletion polymorphisms (indels) with Genome Analysis Toolkit (GATK) v2.8-1. Variants were called by GATK and filtered for a minimum base quality of 30 and the presence of the variant in all strains. The functional effects of the variants were determined using SnpEff v4.

Statistical analysis

The first available episode for each participant was used to evaluate associations between preceding vancomycin exposure and heteroresistance. Preceding vancomycin exposure was calculated as the number of days in the preceding 60 days on which intravenous vancomycin was administered. The Mann–Whitney U test was used to evaluate the significance of differences between groups. An unadjusted P-value of less than 0.05 was considered to indicate statistical significance.

To evaluate associations between heteroresistance and clinical outcomes, the first episode for each participant was again selected, but episodes that were probably the result

of contaminants, defined as episodes with single positive blood cultures and at least 1 simultaneously collected negative blood culture, were excluded. Treatment failure was defined as death during the CLABSI episode or recurrence of BSI with the same organism within 180 days; poor clinical response was defined as persistent positive blood cultures collected at least 1 day after the initiation of vancomycin or treatment failure as defined above. Poor clinical response was designed to be a more sensitive measure of decreased initial response to vancomycin therapy, as death and recurrence are relatively uncommon in this cohort.

The log-rank test was used to compare the cumulative incidence of each clinical outcome with heteroresistant and non-heteroresistant bacteria. Cases were censored at unrelated death, completion of cancer therapy, permanent discharge from the institution, 180 days after onset of BSI, or completion of the study period. Potential confounders were each evaluated for their association with outcome and were included in stratified survival analyses, pooled over all strata, if the P value was 0.1 or less. Statistical analyses were performed with SPSS (IBM SPSS Statistics for Windows, Version 26.0: IBM Corporation, Armonk, NY)

Results

Incidence of heteroresistance

A total of 74 isolates from 65 study participants were available; 7 participants had 2 episodes of CoNS CLABSI and 1 had 3 episodes. Two of the 65 participants were excluded because their isolates were not identified as CoNS following sequencing, and 1 was excluded because the heteroresistance testing was unsuccessful due to poor *in vitro* growth. The characteristics of the 62 included participants are presented in **Table 6-1**. Analysis of the first isolates from all participants revealed that 24 (39%) were classified as heteroresistant, suggesting that heteroresistance is common in CoNS isolated from bloodstream infections in the pediatric leukemia population.

Genetic characterization of bacterial isolates

Most isolates were identified as *S. epidermidis* by sequencing (**Table 6-2**). All heteroresistant isolates were *S. epidermidis* ($P = 0.005$), but they represented several sequence types. The most common sequence type was ST2, a well-known hospital pathogen [216], and all *S. epidermidis* isolates were classified as members of genetic clusters associated with hospital sources (GC1, GC5, and GC6) [215, 217]. There was no evidence of clonal outbreaks, but all heteroresistant isolates were *S. epidermidis*, with no heteroresistance being observed in either *S. haemolyticus* or *S. hominus*.

We next looked for mutations in genes known to confer vancomycin resistance. Mutations in *rpoB*, specifically D471E and I527M, are associated with resistance to

Table 6-1. Characteristics of evaluable participants (n = 62)

Characteristic	Non-heteroresistant (n = 38)		Heteroresistant (n = 24)		All participants (n = 62)	
	n	(%)	n	(%)	n	(%)
Age in years, mean (SD)	10.6	(6.2)	7.5	(6.3)	9.4	(6.4)
Sex						
Female	12	(31.6%)	14	(58.3%)	26	(41.9%)
Male	26	(68.4%)	10	(41.7%)	36	(58.1%)
CVC type						
Tunneled	14	(36.8%)	16	(66.7%)	30	(48.4%)
CVC						
Port	15	(39.5%)	4	(16.7%)	19	(30.6%)
PICC	2	(5.3%)	0	(0%)	2	(3.2%)
Other	7	(18.4%)	4	(16.7%)	11	(17.7%)
Leukemia type						
ALL	26	(68.4%)	13	(54.2%)	39	(62.9%)
AML	12	(31.6%)	11	(45.8%)	23	(37.1%)
History of HCT	7	(18.4%)	6	(25%)	13	(21%)
Race						
White	28	(73.7%)	16	(66.7%)	44	(71%)
Black	7	(18.4%)	4	(16.7%)	11	(17.7%)
Other	3	(7.9%)	4	(16.7%)	7	(11.3%)

SD, standard deviation; CVC, central venous catheter; PICC, peripherally inserted central catheter; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; HCT, hematopoietic cell transplantation

Table 6-2. Species and multilocus sequence type for all first included CoNS isolates (n = 62)

Species/Sequence Type	Non-heteroresistant (n = 38)		Heteroresistant (n = 24)	
	n	(%)	n	(%)
<i>Staphylococcus epidermidis</i>	28	53.8	24	46.2
ST2	7	58.3	5	41.7
ST5	3	33.3	6	66.7
ST16	2	100	0	0
ST22	0	0	1	100
ST23	0	0	1	100
ST57	0	0	1	100
ST73	0	0	1	100
ST83	3	60	2	40
ST89	1	100	0	0
ST173	0	0	1	100
ST218	1	50	1	50
ST291	0	0	1	100
NT	11	73.3	4	26.7
<i>Staphylococcus haemolyticus</i>	4	100	0	0
ST1	1	100	0	0
ST3	1	100	0	0
NT	2	100	0	0
<i>Staphylococcus hominis</i>	6	100	0	0
ST18	2	100	0	0
NT	4	100	0	0
Total	38	(61.3%)	24	(38.7%)

ST, sequence type; %, percentage of species/sequence type; NT, non-typeable

rifampicin and vancomycin in strains of *S. epidermidis* of ST2 and ST23 lineages [216]. Mutations in *rpoB* were identified in 8 *S. epidermidis* strains, of which 3 were heteroresistant. One strain of ST23 had dual resistance alleles in *rpoB* (E471, M527); it was resistant to rifampicin (MIC > 32) and heteroresistant to vancomycin. This suggests that mutations in *rpoB* are neither necessary nor sufficient for vancomycin heteroresistance in the studied strains.

Vancomycin exposure

We next tested whether vancomycin exposure before infection increased the risk of a heteroresistant organism being recovered. In the 60 days before infection, participants with heteroresistant CoNS had received more days of vancomycin therapy ($P = 0.11$) and more days of vancomycin prophylaxis ($P = 0.026$) than had participants without heteroresistant CoNS (**Figure 6-1**). These data suggest that prolonged exposure to intravenous vancomycin at the subtherapeutic doses used for prophylaxis may contribute to the emergence or selection of heteroresistant bacterial populations.

Effect of heteroresistance on clinical outcomes

We evaluated the effect of vancomycin heteroresistance on the clinical response to vancomycin treatment. Only 40 episodes were included in this analysis; the other 22 were excluded as being possibly attributable to blood culture contamination (see exclusion criteria in methods). Seven of these 40 participants (18%) experienced treatment failure and 15 (38%) experienced poor clinical response. Of 6 participants with relapse of infection, sequence typing was available for 3 pairs of isolates; in all cases, the sequence type of the initial and subsequent isolates were identical. Participants with heteroresistant infections had higher cumulative incidences of treatment failure ($P = 0.012$) (**Figure 6-2**) and poor clinical response ($P = 0.001$) (**Figure 6-3**) when compared to participants without heteroresistant infections. Of the other variables evaluated, only vancomycin MIC and age of the participant were considered to be potential confounders. Vancomycin MIC was significantly associated with poor clinical response ($P = 0.037$) but not with treatment failure ($P = 0.669$). Age group was also significantly associated with poor clinical response ($P = 0.023$), but its apparent association with treatment failure was not statistically significant ($P = 0.054$). Variables that were not associated with treatment failure or poor clinical response were CVC type ($P = 0.727$ and $P = 0.377$, respectively), leukemia type ($P = 0.476$ and $P = 0.939$, respectively), sex ($P = 0.800$ and $P = 0.601$, respectively), and self-reported race ($P = 0.802$ and $P = 0.964$, respectively). Therefore, vancomycin MIC and participant age were included in stratified analyses. These analyses showed that poor clinical response and treatment failure remained associated with heteroresistance after controlling for vancomycin MIC ($P = 0.008$ and $P = 0.021$, respectively) and for age group ($P = 0.014$ and $P = 0.12$, respectively). An additional subgroup analysis restricted to *S. epidermidis* isolates also showed that heteroresistance was significantly associated with treatment failure ($P = 0.028$) and poor clinical response ($P = 0.006$).

Figure 6-1. Vancomycin exposure in the 60 days before infection for heteroresistant and non-heteroresistant isolates (N = 62)

Box plots showing the differences between heteroresistant and non-heteroresistant infections with respect to vancomycin exposure ($P = 0.11$) and prophylactic vancomycin exposure ($P = 0.026$). The boxes represent the 25th to 75th percentiles, the whiskers extend up to 1.5 times the box height, and the circles and stars represent outliers and extreme outliers, respectively.

Figure 6-2. Cumulative incidence of treatment failure in patients with CoNS bloodstream infection (N = 40)

Figure 6-3. Cumulative incidence of poor clinical response in patients with CoNS bloodstream infection (N = 40)

Discussion

In this study, vancomycin heteroresistance was common in CoNS isolates from CLABSI in children with leukemia. Data on the incidence of heteroresistance in CoNS are limited, but the high rate found in this study is similar to that reported for various CoNS species in various disease contexts in previous studies [199-205, 218]. Clonal spread of heteroresistant CoNS has been described previously, especially in neonatal intensive care units [204, 219]. However, in this study, there was no single genetic marker for heteroresistance and a wide range of sequence types was involved. This suggests that the phenomenon of heteroresistance likely emerged on multiple occasions and by different mechanisms. Gene amplification may be a key genetic mechanism of heteroresistance in gram-negative bacteria [220]. However, heteroresistance to vancomycin may differ from heteroresistance to other antibiotics; because gram-positive organisms commonly have high MICs within the susceptible range (the result of “MIC creep”) [221], heteroresistance might emerge from minor changes in these near-resistant subpopulations, rather than from the dramatic increases in MIC seen in gram-negative species. The study has some important limitations. The retrospective nature of the data collection means that some information might have been missed or misclassified or some confounding variables excluded. Additional variables, such as CVC-removal and concomitant antibiotic therapy, were not included in the analysis, but were not expected to vary by heteroresistance status. The finding of poorer clinical outcomes in patients with heteroresistant infections might also be attributed to host factors, linkage disequilibrium, or another factor that affects heteroresistance testing. Because the method of detecting heteroresistance is not standardized, alternative laboratory approaches might yield different results and interpretations. Using a sensitive but non-standard definition of poor clinical response improved our ability to evaluate the effect of heteroresistance on clinical outcomes, but the clinical relevance of these outcomes is uncertain. A major challenge to incorporating heteroresistance measurement into clinical practice and diagnosis is the absence of a widely accepted clinical test for the heteroresistance phenotype. In cases of infection for which rapid diagnosis and susceptibility testing are paramount, the time- and resource-intensive methodology used in this study is likely to be impractical.

The study also has several strengths that support our findings. We were able to connect information obtained from laboratory experiments to clinical data for a large cohort of patients with CoNS bloodstream infection, which enabled us to demonstrate the differences in clinical outcomes associated with heteroresistance. The 180-day duration of follow-up was appropriate because relapse of CLABSI after that time is uncommon [222, 223]. Including isolates collected during the same time period and from the same population helped to exclude confounding by secular or cohort effects.

Similar to other mechanisms of resistance, prior exposure to antibiotics increased the risk of heteroresistance for this study population [224, 225]. Intriguingly, exposure to intravenous vancomycin administered as prophylaxis appeared to be more strongly associated with risk of heteroresistance than were other intravenous exposures. Subtherapeutic antibiotic levels over an extended period may provide strong selective

pressure by encouraging the emergence of heteroresistance or helping to maintain transient heteroresistance that emerges as a result of unstable gene amplifications or mutations [226]. The immunocompromised state of the study participants might also have contributed to the evolution of heteroresistance by reducing the rate of clearance of heteroresistant bacteria [227]. Concerns about contributing to the rise of antibiotic resistance have limited the implementation of glycopeptide prophylaxis [228, 229]. A previous study found that vancomycin prophylaxis increased the risk of vancomycin-resistant enterococcal (VRE) infections [229]; therefore, the finding that vancomycin prophylaxis also appears to contribute to heteroresistance in CoNS is important.

Modeling of heteroresistance in mammalian models has suggested that it can contribute to antibiotic treatment failure in patients with *S. aureus*, *Enterobacter* spp., or *Klebsiella* spp. infections [51, 52, 230]. Moreover, vancomycin heteroresistance in *S. aureus* infections appeared to contribute to treatment failure in multiple studies [231, 232]. However, little information is available about the effect of heteroresistance on outcomes in CoNS infections. One case-control study including CoNS and *S. aureus* found increased mortality in patients with heteroresistant infections [205], and a recent case report described a neonate with a heteroresistant *Staphylococcus capitis* infection that responded poorly to treatment with vancomycin [233]. To our knowledge, this cohort study is the first to show that the clinical response to vancomycin is demonstrably worse in infections caused by heteroresistant CoNS.

CHAPTER 7. CONCLUSION

In conclusion, as we have seen in chapters 2, 3 and 4, fitness tradeoffs present a major barrier to the acquisition of fluoroquinolone and beta-lactam resistance via horizontal transfer in *S. pneumoniae*. To fight against antibiotics, the bacteria must maximize resistance potential without compromising their fitness.

In chapter 2, *S. pneumoniae* does not readily acquire on-target fluoroquinolone resistance determinants from resistant pneumococcal and viridans clinical isolates via horizontal transfer. The low prevalence of fluoroquinolone resistance is likely partly attributed to high fitness costs associated with on-target mutations in *gyrA* and *parC*. Another possible contributing factor is that high-level resistance to fluoroquinolones require the contribution of multiple on-target mutations, which could be simultaneously acquired via horizontal transfer. In chapter 3, a potential off-target mutation N291D in the *patA* efflux could potentially facilitate the acquisition of subsequent high-level fluoroquinolone resistance.

In chapter 4, similar to fluoroquinolone resistance, horizontal transfer can facilitate the development beta-lactams resistance, but with a cost. Early recombinants may retain virulence *in vivo* and achieve similar levels of β -lactam resistance with experimentally evolved penicillin-resistant populations. Recombination with related species may be preferable in *S. pneumoniae* to develop tolerance and more rapidly achieve subsequent resistance without initially sacrificing *in vivo* fitness.

In chapter 5, in *S. pneumoniae*, disruptions in RNA degradation also resulted in antibiotic tolerance that likely reduces antibiotic efficacy. Tolerance is likely a strategy for *S. pneumoniae* to withstand antibiotic killing while minimizing fitness tradeoffs and preparing for the subsequent acquisition of resistance. And in chapter 6, we found that heteroresistance in CoNS is associated with poor clinical outcomes and treatment failure in pediatric patients with leukemia at St. Jude Children's Research.

The findings of this dissertation will provide insights into the underlying mechanisms that facilitate the acquisition of high-level antibiotic resistance and reduce antibiotic efficacy. Understanding those mechanisms will be critical for the development of more-effective antibiotics and surveillance.

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APPENDIX. LIST OF MUTATIONS FROM WHOLE GENOME SEQUENCING ANALYSIS OF *IN VIVO* PASSAGING EVOLVED ISOLATES WITH LEVOFLOXACIN

[illegible]

VITA

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